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Adrien Thurotte

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THÈSE DE DOCTORAT
DE L'UNIVERSITÉ PARIS-SACLAY
PRÉPARÉE L'UNIVERSITÉ PARIS-SUD

Ecole doctorale n°567
Sciences du végétal : du gène à l'écosystème
Spécialité de doctorat : Biologie

par

M. ADRIEN THUROTTE

Étude des domaines fonctionnels impliqués dans l'interaction
entre la protéine cyanobactérienne photoprotective Orange
Carotenoid Protein et ses partenaires

Thèse présentée et soutenue à l'i2BC, le 30 Octobre 2015.

Composition du Jury :

| | | | |
|------|-------------------------|---|-----------------------|
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| M. | JEAN-FRANÇOIS HUMBERT | Directeur de recherche ENS (Paris) | (Rapporteur) |
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EXCLUSIF!

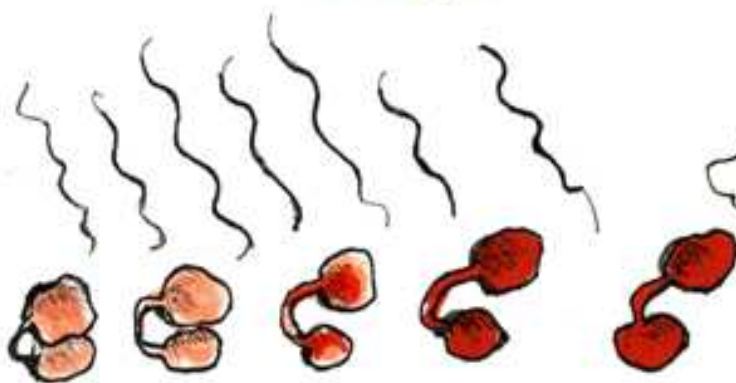
NOUVEAU SCANDALE ENVIRONNEMENTAL



DES CYANOBACTÉRIES
ÉQUIPÉES D'UN
DISPOSITIF SECRET
QUI



**GASPILLE
L'ÉNERGIE!**



QUAND IL EST ÉCLAIRÉ D'UNE
CERTAINE FAÇON !!!



!!! CE DISPOSITIF DIABOLIQUE
SABOTE LES CAPTEURS SOLAIRES



L'OPÉRATION EST
D'UNE DISCRÉTION
TOTALE !!!

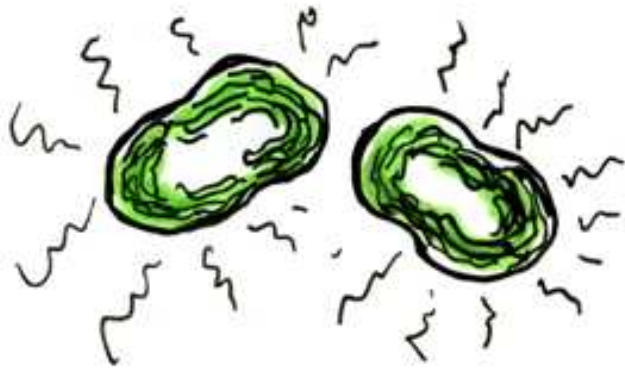
!!! HORMIS
L'ENVOI D'UN
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L'AFFAIRE AURAIT PU RESTER
IGNORÉE DE TOUS, SANS LA
SAGACITÉ DE CHERCHEURS OPÉRANT
DANS UNE MYSTÉRIEUSE BASE SECRÈTE



**CHEF! C'EST PAS
CROYABLE! LE CAROTÉNOÏDE
A BOUGÉ DE 12 ANGSTROMS!**

TOUT ÇA POUR QUE CES PIMBÊCHES
DE CYANOBACTÉRIES PUISSENT SE
PAVANER SANS RISQUE AU SOLEIL



VOUS GÊNEZ PAS!



FAITES BOUILLIR L'EAU
PENDANT QUE VOUS Y ÊTES!

ET EN PLUS ELLES
PUENT L'OXYGÈNE!

ADRIEN THUROTTE

vous révélera les dessous de
ce scandale lors de sa
SOUTENANCE DE THÈSE

«Etude des domaines fonctionnels impliqués dans
l'interaction entre la protéine cyanobactérienne
photoprotectrice Orange Carotenoid Protein et ses partenaires»

LE 30 OCTOBRE 2015
14 HEURES

Salle de Conférences Georges Prévost
INSTITUT DES SCIENCES VÉGÉTALES - BÂTIMENT 23
CNRS Gif-sur-Yvette

la soutenance sera suivie d'un POT, bien entendu

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L'AUTOMNE

On voit tout le temps en automne
Quelque chose qui vous étonne
C'est une branche tout à coup
Qui s'effeuille dans votre cou.

C'est un petit arbre tout rouge
Un, d'une autre couleur encor'
Et puis partout des feuilles d'or
Qui tombent sans que rien ne bouge.

LUCIE DELARUE-MARDRUS

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ABBREVIATIONS

APC : Allophycocyanin
ApcD, ApcF, ApcE : Emetteurs terminaux des PBs
ATP : Adenosine triphosphate
ARN : Acide Ribo Nucléique
ADN : Acide Desoxyribo Nucléique
Chla : Chlorophylle a
CrtO : β -carotene ketolase
CrtR : β -carotene hydroxylase
Cytb6f : Cytochrome b6f
ECN : Echinenone
ERO : Espèce Réactive de l'Oxygène
Fd : Ferredoxin
Flv : Flavodiiron protein
FRP : Fluorescence Recovery Protein
hECN : hydroxy-echinenone
LHC : Light Harvesting Complex
M : Molar
Mb : Mega-base
NADP : Nicotinamide Adenine Dinucleotide Phosphate
OCP : Orange Carotenoid Protein
ORF : Open Reading Frame
PAM : Pulse Amplitude Modulation
PB : Phycobilisome
PC : Phycocyanin
PCB : Phycocyanobilin
PCC : Pasteur Core Collection
PC : Plastocyanin
PDB : Protein Data Bank
PQ/PQH2 : Plastoquinone/plastoquinol
psbA2 : Photosystem II D1 protein
PSI/PSII : Photosystem I/photosystem II
PTOX : Plastid Terminal Oxidase
QA/QB : Quinone A et B
RCP : Red Carotenoid Protein
ROS : Reactice Oxygen Species
WT : Wild-type

INTRODUCTION

1.1 PRÉAMBULE

Les couleurs qui nous entourent sont captivantes et oniriques. Une couleur transforme la manière que nous avons de percevoir visuellement un objet en absorbant une partie du rayonnement incident. Les êtres vivants utilisent des pigments, molécules colorées, pour assurer des fonctionnalités très diverses. La rhodopsine permet aux animaux dotés d'un organe visuel de voir le monde qui les entoure. Les anthocyanes colorent les cerises de manière très appétissante. La mélanine protège les cellules humaines des rayonnements ultra-violet. La chlorophylle est une molécule clef du processus de photosynthèse.

Les premiers organismes à photosynthèse oxygénique sont les ancêtres des cyanobactéries. La photosynthèse leur a permis de capturer la lumière visible pour la transformer en énergie chimique^[1]. Au cours de ce processus, l'énergie provenant de la lumière du soleil permet d'extraire des électrons des molécules d'eau afin de réduire le CO_2 en carbohydrates riches en énergie, relâchant en parallèle des molécules de dioxygène.

Avant l'apparition de la photosynthèse, les organismes utilisaient le H_2S , le NH_3 , des acides organiques ou le Fe^{2+} comme donneur d'électron ou d'hydrogène. Ces composés sont présents en faible quantité comparative-ment à l'eau qui abonde sur terre. Les organismes à photosynthèse oxygénique, qui utilisent une ressource abondante grâce à un métabolisme inédit, ont alors connu une croissance exponentielle. L'augmentation de la concentration en dioxygène atmosphérique (burst oxydatif) lié à leur métabolisme il y a 2,4 à 2,2 milliards d'années^[2] changera la donne énergétique. En effet, la respiration aérobie fournit vingt fois plus d'énergie que la respiration anaérobie^[3], permettant l'apparition d'organismes eucaryotes multicellulaires.

La lignée verte ne comprend pas seulement les cyanobactéries, mais également tous les organismes eucaryotes photosynthétiques qui nous entourent : plantes, mousses, algues... Les organismes eucaryotes photosynthétiques partagent un organite spécialisé dans la capture de la lumière, le chloroplaste. Ce dernier est dérivé d'une cyanobactérie ancestrale qui a été intégrée au sein d'une cellule eucaryote au cours d'un événement nommé endosymbiose^[4]. Pour qu'une endosymbiose perdure dans le temps, il faut que les deux partenaires trouvent des avantages réciproques. Les avantages qui ont permis cette vie en concubinage restent en discussion. En effet, les produits de la photosynthèse sont stockés par les cyanobactéries sous forme ADP-glucose alors que l'hôte eucaryote dispose d'une machine enzymatique adaptée à l'UDP-glucose, non compatible avec l'ADP-glucose. Selon Steven Ball^[5], la présence d'un troisième partenaire bactérien impliqué dans ce qui serait "un ménage à trois" apportant les enzymes complémentaires permettant des échanges métaboliques entre hôte et symbiote a été nécessaire. Cependant la question reste ouverte car des analyses phylogénétiques viennent d'être publiées contredisant cette théorie^[6].

1.1.1 Les cyanobactéries

Les cyanobactéries représentent l'un des plus gros phyla chez les procaryotes. Ces organismes ont un rôle central dans l'équilibre écologique de la planète car ils représentent 30% de la production de CO_2 . Elles ont colonisé

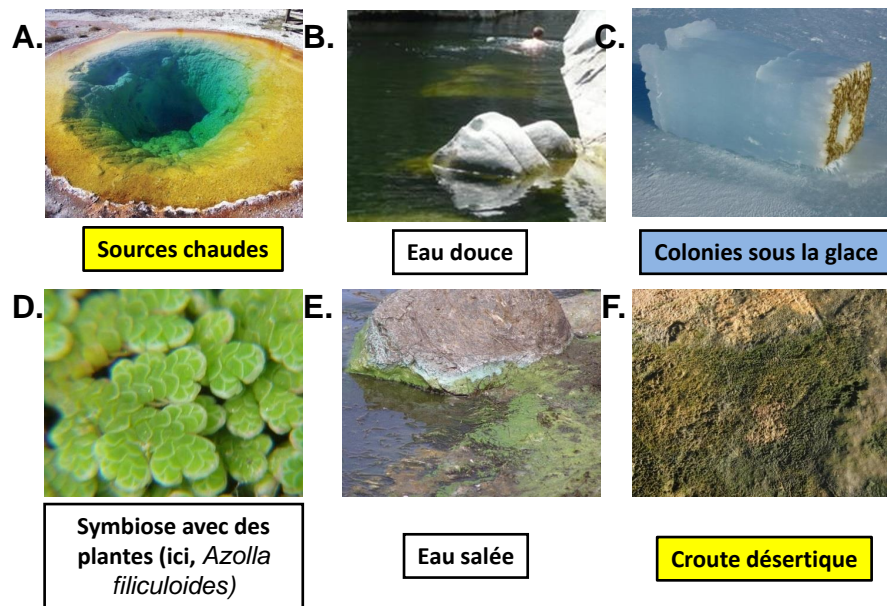


Figure 1.1 – Exemple de milieux de vie des cyanobactéries
 (A) Source chaude du parc de Yellowstone aux Etats Unis – domaine public (B) Communauté d’organismes photosynthétiques dans une rivière du parc national des Cévennes - CC-BY-SA Magali Thurotte (C) Colonie d’organismes photosynthétiques vivant plusieurs dizaines de centimètres sous la glace - Lars Chresten Lund Hansen (D) *Azolla filiculoides* - GFDL, Kurt Stüber (E) Cyanobactéries (probablement *Microcystis aeruginosa*) échouées sur un rocher à l’est du lagon Rodeon, Marin County, Californie – CC-BY-SA Mary Cousins (F) Croute désertique contenant notamment des cyanobactéries (CC-BY-SA, Aleksey Nagovitsyn)

les environnements extrêmes (Figure 1.1), des lacs volcaniques^[7] aux glaces de l’antarctique^[8].

Certaines espèces de cyanobactéries peuvent vivre en symbiose avec un champignon, formant le lichen^[9], ou avec des plantes supérieures, comme l’*Azolla*^[10]. Dans ce dernier cas, la cyanobactérie *Anabaena azollae* est capable de fixer l’azote atmosphérique en azote assimilable par la plante.

D’autres espèces sont connues pour leur capacité à proliférer jusqu’à former des films à la surface de l’eau, appelés blooms ou efflorescence 1.2. Un tel évènement est favorisé par l’élévation de la température de l’eau, sa stagnation et sa richesse en nutriments. Dans les années à venir, les efflorescences seront de plus en plus fréquentes car le réchauffement global de la température se répercutera sur la température des masses d’eau^[11] et l’agriculture intensive utilise une quantité important d’engrais (comme l’azote et le phosphate) qui sont lessivés et transportés vers les lacs et les rivières. Outre l’augmentation de la turbidité et des odeurs émanant des zones d’efflorescences, un problème sanitaire est présent car certaines espèces de cyanobactéries produisent des toxines pouvant rendre l’eau irritante ou toxique^[12]. L’impact économique de tels problèmes est jugé sévère dans des régions misant une part de leur intérêt touristique sur les lacs et les rivières.

Cependant, une forte concentration en cyanobactéries ne produisant pas de toxines peut être une aubaine. Depuis des temps anciens, des peuples comme les aztecs récoltent et consomment certaines cyanobactéries, comme la spiruline (*Arthrospira platensis*).

Aujourd’hui encore, l’homme continue ce procédé comme par exemple au Tchad, où l’éthnie des Kanembu continuent de récolter la spiruline qui

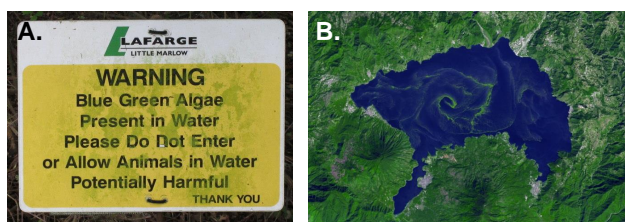


Figure 1.2 – Panneau d’avertissement et image d’une efflorescence cyanobactérienne (A) Panneau de mise en garde de la dangerosité potentielle de l’eau du lac Spade Oak, à côté de la ville de Little Marlow en Angleterre (BY-SA David Hawgood) (B) Image d’une efflorescence cyanobactérienne vu par satellite au lac Atitlan (Guatemala). D’après les journaux locaux, l’efflorescence est apparue car les eaux usées rejetées dans le lac sont riches en nitrates. De plus, l’agriculture environnante utilise une quantité importante d’engrais. Les précipitations lessivent les sols et entraînent les engrais vers le lac. Ce phénomène est accentué par le relief en forme de cuvette formé par les montagnes et volcans entourant le lac. - Domaine public, NASA Earth Observatory

pullule dans les eaux chaudes (35-37°C) du lac Chad Basin sous un soleil généreux (Figure 1.3).

La spiruline est ensuite séchée au soleil puis découpée afin d’être facilement transportée, stockée et vendue sur des marchés locaux^[13]. Sa production et son conditionnement sont également industrialisés dans de nombreux pays (Amérique du Sud et Asie en particulier) en tant que complément alimentaire. En effet, la spiruline présente une haute teneur en protéines, minéraux (fer, calcium, sodium et magnésium), et antioxydants et une faible teneur en calories et cholestérol. Ses propriétés nutritionnelles combinées à sa facilité de culture font de l’utilisation de la spiruline comme complément nutritionnel est une piste jugée crédible par les médecins pour lutter contre la malnutrition^[14].

Les énergies fossiles mondiales assurent à l’Homme encore quelques dizaines d’années d’autonomie énergétique (40 ans pour le pétrole, 50 ans pour l’uranium, 70 ans pour le gaz naturel, 230 ans pour le charbon)^[15]. Cependant, le problème ne se situe pas tant au niveau des ressources mais plutôt au niveau des émissions de gaz à effets de serre tels que le CO₂^[11]. Les cyanobactéries sont un espoir dans la recherche de sources d’énergies alternatives aux énergies fossiles car le bilan carbone est proche de zéro. La maîtrise des outils de biologie moléculaire chez la cyanobactérie modèle *Synechocystis* permet de changer son métabolisme pour répondre à des besoins spécifiques, par exemple une sur-production de dihydrogène^[16], d’éthanol^[17] ou de lipides pouvant être transformés en biodiesel^[18].

Par ailleurs, le métabolisme des cyanobactéries peut être modifié pour que ces organismes produisent des composés à forte valeur ajoutée tels que le sucrose, 2,3-Butanediol (utilisé dans les résines et comme solvant de certains colorants), l’acide lactique (additif alimentaire E270), mannitol (additif alimentaire E421) ou le glycérol (utilisé en pharmacie et cosmétique)^[19].

1.1.2 Classification, physiologie et génétique

Le groupe des cyanobactéries est un embranchement monophylétique incluant au moins 7500 espèces. La classification morphologique des cyanobactéries distingue 5 sections (Rippka et al., 1979). Les deux premières sont unicellulaires, les trois dernières sont constituées par des cyanobactéries formant des colonies filamenteuses ou ramifiées. Les espèces de la section IV

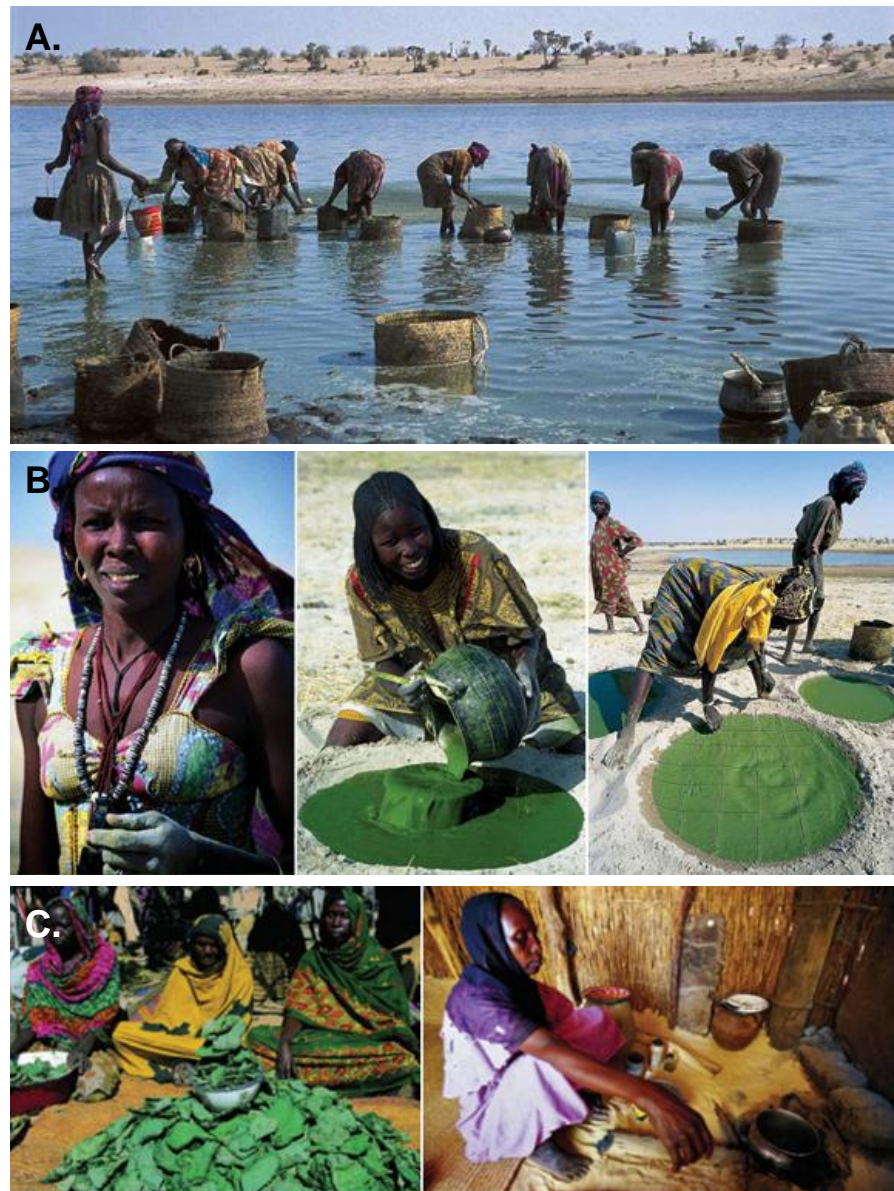


Figure 1.3 – Collecte, séchage et vente de la spiruline
Femmes Kanenbu (A) collectant la spiruline au bord du lac Boudou Andja, (B) séchant la spiruline au soleil et (C) vendant la spiruline séchée sur un marché local.^[13]

possèdent des cellules spécialisées, les hétérocystes, capables de fixer l’azote atmosphérique. Récemment, les avancées technologiques du séquençage à haut débit ont permis d’établir des classifications moléculaires reposant sur les similitudes de séquences de l’ARN 16S de la petite sous-unité du ribosome.

La cyanobactérie *Synechocystis* PCC6803 (Pasteur Culture Collection) est l’organisme modèle étudié dans nombreux laboratoires incluant le laboratoire où j’ai effectué ma thèse. Elle a été isolée d’un lac d’eau douce en Californie. La cellule (Figure 1.4) est entourée d’une enveloppe composée d’une membrane plasmique, d’une couche de peptidoglycane et d’une membrane externe typique d’une bactérie gram négative.

L’espace cellulaire est le plus souvent distingué en deux zones, une périphérique contenant des membranes de thylakoïdes, et une centrale contenant les chromosomes circulaires, les plasmides et les carboxyzomes^[20].

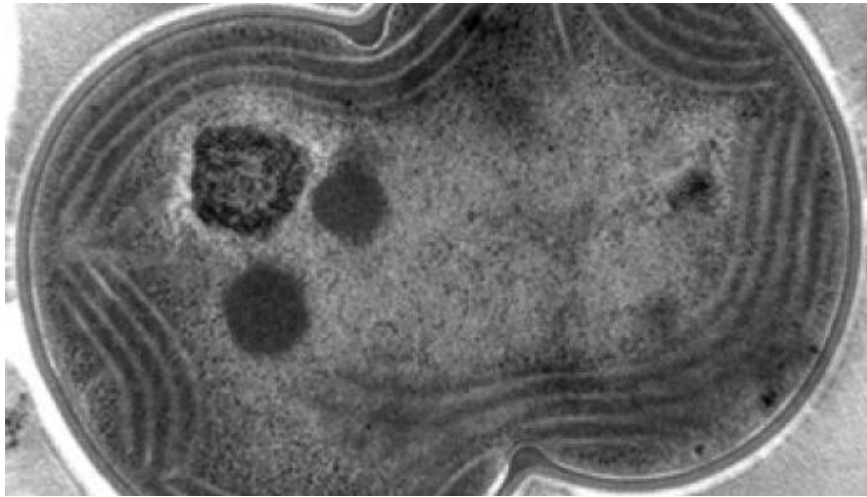


Figure 1.4 – Cellule de *Synechocystis* observée par microscopie électronique - Copyright : Robert Roberson

Les thylakoïdes sont le siège de la photosynthèse oxygénique et de la respiration. Ils forment des vésicules concentriques le plus souvent situées le long de la membrane interne des cyanobactéries. Les antennes cyanobactériennes collectrices de lumière, les phycobilisomes sont des complexes extramembranaires solubles de taille importante (7-15MDa). Ils sont situés sur la face externe et séparent les membranes externes de deux thylakoïdes différents d'une distance constante de 50 nm correspondant à leur hauteur^[21].

Les carboxyzomes sont des compartiments hexagonaux protéiques peu perméables aux gaz. Ils contiennent les enzymes RubisCO (ribulose 1,5-bisphosphate carboxylase-oxygenase) et CA (Carboxic anhydrase) et leur assurent un environnement pauvre en O_2 propice à leur activité. La RubisCO est une enzyme du cycle de Calvin qui permet la carboxylation du ribulose-1,5-bisphosphate (RuBP). Cependant, elle peut également réagir avec le dioxygène pour former un produit secondaire qui sera recyclé au cours de la photo-respiration. Pour un fonctionnement optimal, la RubisCO doit se trouver dans un environnement faible en O_2 et à forte concentration en CO_2 . Cet environnement est créé au sein du carboxyzome par la déshydratation de bicarbonate (HCO_3^-) formant du CO_2 ^[22,23].

En termes de génétique, le génome de 3,5 Mbp de *Synechocystis* a été le premier génome cyanobactérien séquencé^[24]. La maîtrise de la recombinaison homologe explique que *Synechocystis* se soit imposé comme organisme modèle. Depuis lors, les nouvelles données de séquence annotées sont recensées au sein de plusieurs bases de données, (Cyanobase, kazusa.or.jp/cyano et Joint Genome Institute, jgi.doe.gov par exemple). Le développement des puces à ARN a permis l'élaboration de nombreux profils d'expression ARN dans de diverses conditions de stress, dont certaines données sont rassemblées dans une plateforme libre d'accès nommée CyanoExpress^[25]. En 2010, une carte des sites d'initiation de la transcription est publiée^[26]. Elle met en évidence le rôle certainement sous-estimé des ARN anti-sens dans la régulation des gènes chez *Synechocystis*. Il existe des ARN anti-sens pour 27% des gènes et ces derniers représentent 65% des ARN totaux. Le fonctionnement des certains ARN anti-sens est connu depuis 2006, comme IsiR qui permet de créer un délai dans la traduction de la protéine IsiA^[27].

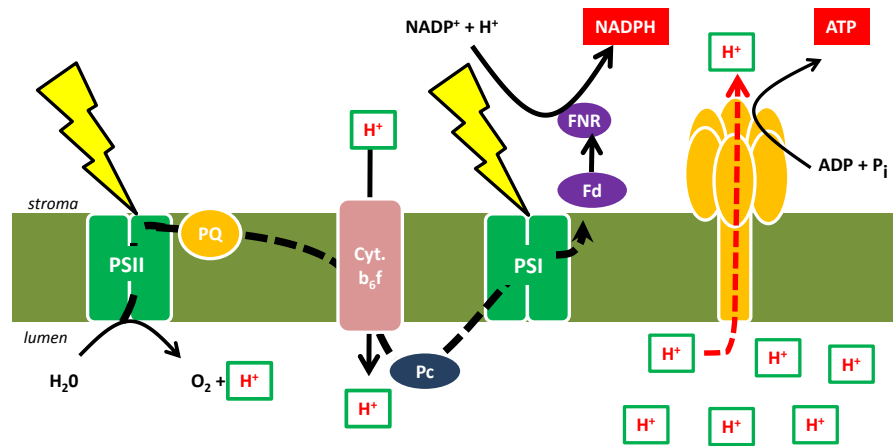


Figure 1.5 – Représentation schématique de la chaîne de transfert d'électrons photosynthétique.

Les électrons (e^-) proviennent de l'oxydation de molécules d'eau (H_2O). L'oxydation de deux molécules d'eau libère 4 électrons qui réduiront chacun à tour de rôle la tyrosine Z du PSII. De plus, 4 protons H^+ sont libérés dans le lumen, contribuant au gradient de protons trans-thylakoidien. Ce gradient de protons permettra la synthèse d'ATP par l'ATPase, qui utilise le gradient de protons comme moteur. Les électrons seront ensuite transportés jusqu'au PSI via le cytochrome b_6f . Une seconde séparation de charge photo-induite au PSI permet la réduction de la FNR (ferredoxine-NADP+ reductase) par la Fd (ferredoxin), qui pourra à son tour réduire le NADPH. Le NADPH ainsi que l'ATP sont indispensables au cycle de fixation du CO_2 ou cycle de Calvin-Benson.

1.1.3 La photosynthèse

La photosynthèse désigne le processus permettant aux organismes photosynthétiques de synthétiser de la matière organique en exploitant l'énergie lumineuse. Au cours des milliards d'années d'évolution depuis l'endosymbiose à l'origine des chloroplastes, les complexes photosynthétiques (PSII, PSI et cytochrome b_6f) ont été très conservés, démontrant qu'ils sont la réponse optimale à la transformation de l'énergie lumineuse en énergie chimique.

L'énergie lumineuse est capturée par des antennes collectrices de lumière qui la canalisent vers les centres réactionnels du photosystème I et du photosystème II. Ces antennes peuvent être internes (CP43/CP47) ou externes (les phycobilisomes, chez les cyanobactéries). Le PSII contient 35 chlorophylles^[28] tandis que le PSI en contient 96^[29].

L'énergie collectée permet d'alimenter la chaîne de transfert d'électrons photosynthétique qui convertit l'énergie lumineuse en force motrice (gradient de protons H^+ de part et d'autre de la membrane des thylakoïdes) et pouvoir réducteur (NADPH Nicotinamide Adénine Dinucléotide Phosphate). Le gradient de protons permet à la F-ATPase de synthétiser de l'ATP (Adénosine Tri Phosphate), molécule mobile à haut pouvoir énergétique. Ensemble, l'ATP et le NADPH fournissent notamment l'énergie pour que le CO_2 soit incorporé par la RubisCO au ribulose 1,5 biphosphate. Ce composé est un intermédiaire du cycle de Calvin-Benson qui servira à synthétiser des sucres, molécules de réserve énergétique.

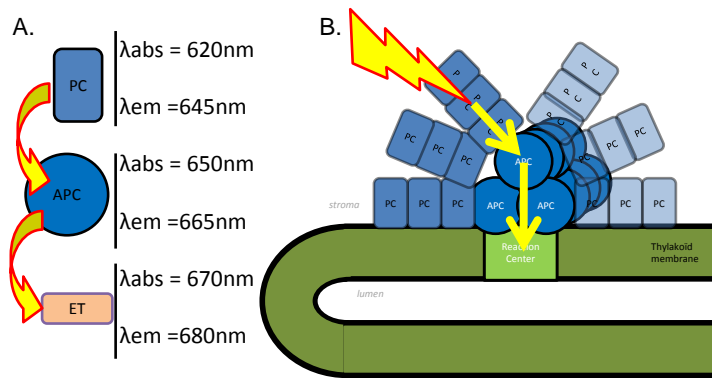


Figure 1.6 – Le PBs est l’antenne collectrice de lumière chez les cyanobactéries.
 (A) Longueur d’onde du maximum d’absorption et du maximum d’émission de la phycocyanine, de l’allophycocyanine et des émetteurs terminaux.
 (B) Le PBs est constitué de bras de phycocyanine (PC) qui rayonnent autour d’un cœur d’allophycocyanine (APC)

Les complexes protéiques majeurs de la chaîne de transfert d’électrons sont les deux photosystèmes (PSI et PSII) et le cytochrome *b6f* (Figure 1.5)

Le centre réactionnel du photosystème II (PSII) est composé d’un hétérodimère de protéines D1 et D2 et du cyt *b559*. L’hétérodimère D1/D2 lie quatre chlorophylles qui forment le centre réactionnel (P680) qui participe à la séparation de charge donnant un électron à la phéophytine. L’électron est ensuite transféré à la quinone A (QA) puis à la quinone B (QB). Cette dernière doit être deux fois réduite avant de quitter son site d’attachement sous forme de plastoquinol (PQH_2). La chlorophylle $P680^+$ regagne un électron en réduisant la tyrosine Z. Le complexe d’oxydation de l’eau (OEC), formé par quatre atomes de manganèse et un de calcium, permettra à son tour de réduire la tyrosine Z. A chaque cycle, la tyrosine Z est réduite quatre fois et deux molécules d’eau sont oxydées, libérant quatre protons H^+ dans le lumen du thylakoïde.

Le second complexe membranaire est le cytochrome *b6f*. Il a pour rôle d’oxyder le pool de plastoquinones. Cette oxydation permet la libération de deux protons H^+ dans le lumen du thylakoïde et la réduction du transporteur d’électrons soluble, la plastocyanine.

Le troisième complexe est le photosystème I qui utilise l’énergie lumineuse pour transférer un électron de la plastocyanine localisée dans le lumen du thylakoïde à la ferrédoxine du côté stromal. L’électron est donné par la paire "spéciale" de chlorophylle (P700) à l’état excité à la phylloquinone, puis successivement à trois clusters fer-souffre pour finalement réduire la ferrédoxine. Du côté donneur, la chlorophylle spéciale P700 est réduite par la plastocyanine.

La FNR (Ferrédoxine NADP Réductase) est localisée du côté donneur du PSI. Elle réduit le NADP en NADPH en utilisant la ferrédoxine réduite par la séparation de charge au niveau du PSI.

Les phycobilisomes

Chez les cyanobactéries, la capture de l’énergie lumineuse est assurée en majorité par des macrostructures protéines-pigments de taille importante (7 à 15 mDa), les phycobilisomes (PBs, pour revue^[30-35]). Les PBs extramembranaires et solubles sont localisés du côté cytoplasmique de la membrane des thylakoïdes. Ils capturent la lumière entre 500 nm et 670 nm grâce à de protéines-pigments appelés phycobiliprotéines qui lient de manière co-

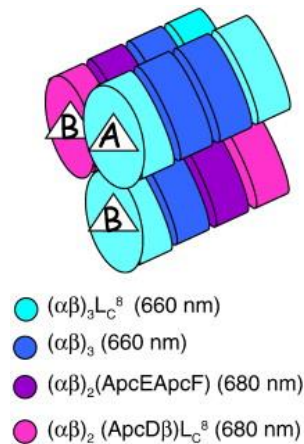


Figure 1.7 – Représentation schématique du cœur des PBs de *Synechocystis*. Chacun des trois cylindres du cœur est composé de 4 disques. Chaque disque est composé d'un assemblage de trois hétérodimères. Le rôle des sous-unités spéciales ApcD, ApcE et ApcF est décrit dans le texte. Extrait de Jallet et al., 2013^[37].

valente des chromophores, les bilines. Les bilines sont des chaînes tétrapyrroliques ouvertes capables d'absorber la lumière (pour revue^[36]).

Les phycobilisomes sont typiquement composés d'un cœur d'allophycocyanine et de projections radiales, également appelées bras dont la composition peut varier selon les espèces. Chez *Synechocystis*, les bras sont composés de phycocyanine alors que les liaisons protéines-pigments phycoérythrine ou phycoérythrocyanine. Cette diversité de pigments permet aux phycobilisomes certaines souches d'absorber une plus large partie du spectre.

Les pigments des bras des phycobilisomes absorbent dans des longueurs d'ondes plus élevées que ceux du cœur. Ainsi, le cœur des phycobilisomes va piéger l'énergie qui ne peut pas remonter vers les bras des phycobilisomes, et le transfert est directionnel des bras, vers le cœur (Figure 1.6). Le cœur des phycobilisomes est composé de 2 à 5 macrostructures appelées cylindres formés par des protéines-pigments (allophycocyanine).

Chaque cylindre est constitué par 4 disques (sauf quand le cœur comporte 5 cylindres, dans ce cas, deux cylindres ne comportent que 2 disques), et chaque disque est lui-même formé par 3 hétérodimères de la protéine-pigment allophycocyanine (ApcA-ApcB) dont le maximum d'émission est à $\lambda = 660 \text{ nm}$ (Figure 1.7).

Le cylindre sommital ne comporte que des trimères ApcA-ApcB. Les deux cylindres inférieurs contiennent chacun deux émetteurs terminaux. Les émetteurs terminaux sont des protéines-pigments spéciales dont le maximum d'émission est à 680 nm, assurant au phycobilisome un couplage maximal avec la bande d'absorption du PSII ($\lambda = 680 \text{ nm}$). L'émetteur terminal ApcD remplace une sous-unité ApcA, et les émetteurs terminaux ApcE et ApcF forment un hétérodimère qui remplace un hétérodimère $\alpha\beta$. En plus de son rôle d'émetteur terminal, ApcE est également impliqué dans la structure du cœur du PB. La séquence protéique d'ApcE est constituée d'une région liant une biline (PBP), et de plusieurs régions répétées de liaison aux cylindres du cœur (REP).

Le nombre de domaines REP (de 2 à 4) détermine le nombre de cylindres du cœur (de 2 à 5). Les résultats qui seront présentés au cours de chapitre 1 ont été obtenus avec des phycobilisomes dont l'architecture du cœur diffère.

Arthrospira platensis et *Synechocystis*^[38] ont 3 cylindres, *Anabaena variabilis*^{[39][40]} à 5 cylindres et *Synechococcus elongatus*^[33] en possède 2. Les autres linkers qui assurent la structure des phycobilisomes ne sont pas colorés. Il

s'agit de CpcC (extension des bras), CpcD (coiffe l'extrémité des bras), CpcG (relie les bras au coeur) et L_c8 (structure du coeur)^[41]. Les PBs sont associés aux thylakoïdes. Cependant, le mécanisme d'association n'est pas élucidé. Les PBs ne possèdent pas de domaines transmembranaires et ils pourraient être maintenus à la surface des membranes par des liaisons faibles avec les têtes polaires des lipides^[42]. Il est suggéré qu'une protubérance qui serait formée par ApcE et ApcF soit l'empreinte d'une cavité du côté donneur du PSII^[32]. Les PBs peuvent aussi transférer l'énergie au PSI^[43], et former de super complexes PBs-PSI-PSII^[44].

Des expériences de FRAP (Fluorescence Recovery After Photobleaching) rapportent que les PBs sont très mobiles^[45,46]. Cependant, si la mobilité des PBs est démontrée, ces articles ne permettent pas de dire si la mobilité n'intervient que dans le cadre de la destruction des chromophore intervenant lors la mise en place de leur protocole expérimental, ou bien si les PBs sont constamment mobiles dans des conditions normales de croissance.

1.2 STRESS LUMINEUX ET PHOTOPROTECTION

Les PBs sont des antennes collectrices très efficaces qui permettent de capturer une part importante de la lumière incidente. Cependant, la collecte d'une quantité d'énergie supérieure à la capacité d'assimilation au cours du métabolisme crée un stress oxydant.

Un tel stress peut se produire lorsqu'une cellule est soumise à des conditions de lumière changeantes : réapparition du soleil radieux après le passage d'un nuage ou masqué par un l'ombre d'un arbre agité par le vent. Les biofilms cyanobactériens sont également des structures dynamiques dans lesquelles les cellules sont alternativement soumises à la lumière directe à la surface du biofilm puis ombragées par d'autres cellules^[47].

1.2.1 *Stress oxydant*

En tant qu'organismes phototrophes, les cyanobactéries ont à faire face au stress oxydant généré au cours de la photosynthèse en plus de celui généré au cours de la respiration. La photosynthèse produit du pouvoir réducteur sous forme de NADPH qui sert par exemple à alimenter le cycle de fixation du carbone ou cycle de Calvin Benson. L'énergie est également stockée sous forme de gradient de protons nécessaire à la production d'ATP, molécule à haut pouvoir énergétique utilisé dans de nombreux processus cellulaires. L'état redox de la cellule doit être finement régulé afin de disposer d'une quantité suffisante de pouvoir réducteur sans pour autant trop réduire les transporteurs d'électrons car de dangereuses réactions secondaires avec l'oxygène pourraient survenir^[49-51].

Le stress oxydant photosynthétique dont il est question dans ce chapitre désigne les dommages induits par les espèces réactives de l'oxygène (ROS, Reactive Oxygen Species ou ERO, Espèces Réactives de l'Oxygène en Français) générées au cours de la photosynthèse, l'oxygène singulet (¹O₂), le H₂O₂ et l'anion superoxyde ($\cdot\text{O}_2^-$). Elles peuvent être générées au niveau du PSII, du PSI, et des phycobilisomes^[48]. Deux autres formes réduites peuvent être générées au cours par exemple des réactions de détoxification de l'oxygène singulet et de l'anion superoxyde, ce sont le peroxyde d'hydrogène (H₂O₂), le radical hydroxyl ($\cdot\text{OH}$). Les ROS sont hautement réactifs et peuvent endommager des composants cellulaires. L'oxygène singulet peut réagir avec les protéines, les pigments et les lipides. L'anion superoxyde et le radical hydroxyle possèdent un électron non apparié qui les rendent très réactifs avec les biomolécules. Le H₂O₂ est l'espèce la moins réactive,

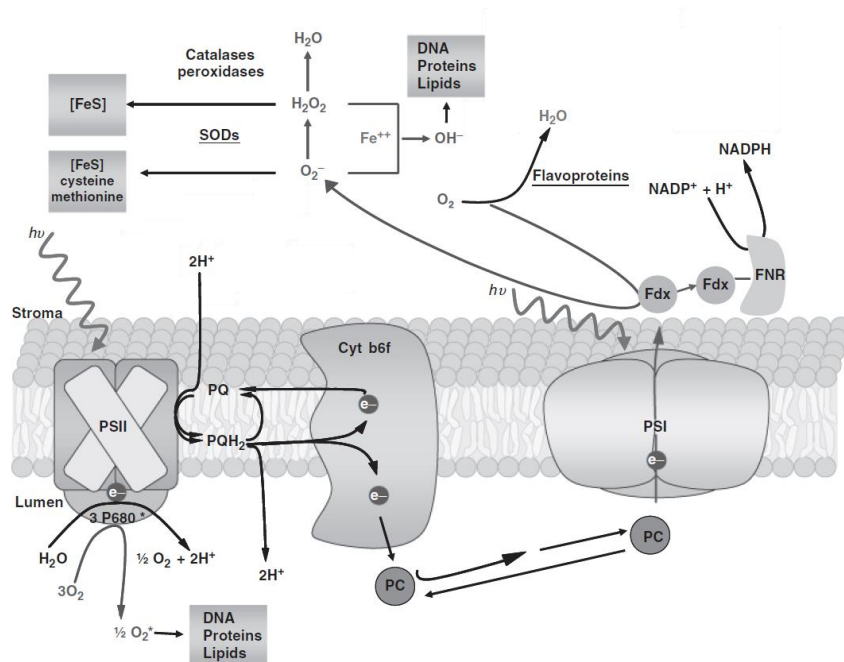


Figure 1.8 – Production des espèces réactives de l’oxygène et leur cible.

Les espèces réactives de l’oxygène sont formées à partir de l’O₂. Au niveau du PSII, l’oxygène singulet est produit par transfert d’énergie entre des chlorophylles excitées et l’oxygène. Dans le PSI, la réduction du dioxygène par les électrons provenant du PSII génèrent le radical anion superoxyde. Le radical anion superoxyde est déprotoné en H₂O₂ et O₂ par la superoxyde dismutase, et le H₂O₂ est réduit en H₂O par les catalases et les peroxydases. Les flavoprotéines de type A peuvent réduire l’O₂ en eau en utilisant le pouvoir réducteur au niveau du PSI. Extrait de Latifi et al., 2009^[48]

mais elle peut former le très réactif radical hydroxyl au cours de la réaction de Fenton.

Au niveau du PSII, c’est l’oxygène singulet qui est l’ERO majoritairement générée^[52]. La durée de vie de l’état excité de la chlorophylle (jusqu’à quelques nanosecondes) permet la création d’un potentiel électrochimique par une séparation de charge. Si la séparation de charge entre chlorophylle et phéophytine se prolonge dans le temps lorsque les quinones Q_A et Q_B sont réduites, alors la recombinaison de charge peut créer un état triplet de la chlorophylle, qui en réagissant avec l’oxygène forme le singulet oxygène. Aussi sous forte intensité lumineuse, les chlorophylles des photosystèmes peuvent se retrouver en état triplet et réagir avec des molécules d’oxygène.

Au PSI, dans des conditions de lumière intense, le dioxygène peut être utilisé comme accepteur primaire d’électrons du côté stromal du PSI, et former ainsi des anions superoxyde.

Une équipe a montré en 2007 que de l’oxygène singulet et de l’anion superoxyde peuvent être produits au niveau des phycobilisomes^[53]. Ces deux ERO peuvent endommager les phycobiliprotéines. Le rôle dans la signalisation des espèces réactives de l’oxygène, et plus particulièrement le H₂O₂, est bien décrit chez les plantes^[54] et *E.coli*^[55]. Les données disponibles chez les cyanobactéries sont moins abondantes. On peut néanmoins citer l’importance des Histidines Kinases His16, His33, His 34 et His41 qui seraient impliqués dans la détection et la signalisation du H₂O₂^[56].

1.2.2 Mécanismes de photoprotection

Les cyanobactéries ont développés plusieurs stratégies afin de survivre aux stress lumineux. L'échappement est une stratégie communément utilisée par les organismes mobiles. Si la cyanobactérie modèle *Synechocystis* est dotée d'un pili^[57] lui permettant de se déplacer, sa force ne lui permet certainement pas d'aller à l'inverse des mouvements de courants aquatiques^[58]. Ne pouvant échapper au stress, les cyanobactéries peuvent utiliser plusieurs stratégies pour, *in fine*, réduire les ROS présents dans la cellule : diminuer l'énergie capturée, utiliser différemment cette énergie ou transformer les ROS en molécules chimiques inoffensives.

Régulation de la capture de lumière

Une excitation préférentielle du PSI a pour effet d'oxyder le pool de plastoquinones, alors qu'une excitation préférentielle du PSII a pour effet de le réduire. Le mécanisme dit de transition d'état détermine la part d'énergie qui sera dirigée vers le PSI ou le PSII. La transition d'état peut impliquer un mouvement des PBs^[59] et ou un mouvement des photosystèmes qui induisent une redirection du flux d'énergie des phycobilisomes aux PSII ou PSI ou du PSII vers le PSI selon la théorie du spillover^[60]. La régulation de la transition d'état dépend de l'état redox de la cellule : la réduction du pool de plastoquinones induit une transition vers l'état 2 dans lequel l'énergie absorbée est préférentiellement transférée au PSI.

Afin de diminuer l'énergie arrivant aux centres réactionnels, les cyanobactéries peuvent soit désassembler, soit modifier la composition de leurs antennes. Le désassemblage des PBs implique deux processus. Le premier est la dégradation des phycobiliprotéines par l'oxygène singulet et l'anion superoxyde, tous deux générés au sein de ces mêmes antennes sous forte intensité lumineuse^[53]. Le désassemblage peut également être enzymatique. La protéine Nbla induit le désassemblage des phycobilisomes^[61] et est un marqueur des protéines à protéolyser^[62,63]. L'hétérodimère de protéines Nbla1-2 (*SSL0452* et *SSL0453*) interagit avec ClpC, une protéine chaperonne partenaire d'une protéase^[64]. Les deux gènes *SSL0452* et *SSL0453* sont sur-exprimés en condition d'absence des sources de nitrates, à l'obscurité^[65] et en condition de stress oxydant^[66]. Ils sont réprimés dans un environnement riche en CO₂^[67].

Valves électroniques

Lors d'un stress lumineux, les transporteurs d'électrons photosynthétiques sont excessivement réduits. Il existe des valves à électrons qui permettent d'oxyder les transporteurs d'électrons. Chez les cyanobactéries, les flavodiïrons (Flv) en sont un exemple. Flv1 et Flv3 forment un hétérodimère qui transfère des électrons du côté accepteur du PSI à l'oxygène sans formation de ROS et réduisent l'O₂ en H₂O^[68]. Flv2 et Flv4 prennent des électrons du côté accepteur du PSII et les transmettent à un accepteur aujourd'hui inconnu^[69]. Certaines cyanobactéries possèdent une hydrogénase bidirectionnelle (Hox) capable de réduire des protons pour former du dihydrogène en utilisant les électrons au niveau du côté accepteur du PSI.

Transfert cyclique

L'activation de voies de transfert d'électrons cycliques autour du PSI est un autre moyen pour les cyanobactéries de contrôler leur état redox. Le transfert cyclique ne fait pas intervenir le PSII, ainsi, il n'y a pas de réduction d'H₂O. Seul le gradient de protons augmente, et donc la quantité

d'ATP synthétisée. Il existe au moins deux voies de transfert cyclique d'électrons qui permettent de transférer des électrons de la ferrédoxine (ou FNR) au pool de plastoquinones (ou cyt b_6f). La première implique la protéine PGR5^[70] et la seconde le complexe NDH-1 (NADH déshydrogénase)^[71].

Systèmes antioxydants enzymatiques

Les ROS générées sont pris en charge par une machinerie de détoxification composée d'enzymes et d'antioxydants non enzymatiques. Les systèmes antioxydants enzymatiques permettent de rendre inoffensif le radical superoxyde en le transformant successivement en H_2O_2 (Super Oxide Dismutase) puis en H_2O (catalases et peroxidases) (review^[48]). Afin de prévenir l'apparition de ROS en diminuant l' O_2 disponible pour leurs formations, les flavoprotéines cyanobactériennes transforment l' O_2 en H_2O ^[68].

Système antioxydants non enzymatiques

Le système antioxydant enzymatique est complété par un système antioxydant non enzymatique dont les trois principaux acteurs sont le glutathion, les caroténoïdes et l' α tocophérol.

Le glutathion est un tripeptide (γ -Glu-Cys-Gly) abondant chez la plupart des organismes vivants. Chez les cyanobactéries, sa concentration cellulaire est de 0,1 à 10 mM. Il est présent sous deux formes, réduites (GSH) et oxydées (GSSG). La forme réduite peut réagir avec une molécule de H_2O_2 et former du H_2O inoffensif. Chez certains organismes, le glutathion est un cofacteur de la glutathion peroxidase qui catalyse la transformation du H_2O_2 en H_2O ^[72].

Les caroténoïdes sont des molécules hydrophobes synthétisées au sein des membranes plasmiques. *In vivo*, ils peuvent rester au coeur des membranes plasmiques et thylakoides dont ils peuvent modifier les paramètres de fluidité ou bien être intégrés au sein de caroténo-protéines. Les cyanobactéries peuvent synthétiser une grande diversité de caroténoïdes tels que les kéto-caroténoïdes ou les caroténoïdes glycosylés (les voies de synthèse seront plus amplement discutées dans le chapitre 2).

Les caroténoïdes sont classifiés en trois groupes selon l'hydrophobicité de leurs deux extrémités : (1) n'affectent pas la fluidité membranaire (echinénone, avec une tête polaire) (2) augmentent la fluidité membranaire (β -carotène, sans tête polaire) (3) rigidifient la membrane (xanthophylls avec deux têtes polaires)^[73]. Le froid diminuant la fluidité membranaire, les enzymes impliquées dans leurs voies métaboliques et spécialement dans la saturation des chaînes hydrocarbonées sont finement régulées selon la température^[73]. Les caroténoïdes peuvent être intégrés au sein de protéines, nommées caroténo-protéines. Ces dernières peuvent être hydrophobes et intégrées dans les membranes ou hydrophiles et solubles. Elles ont suscité un regain d'intérêt au cours de la dernière décennie, et une place importante leur est désormais attribuée dans la régulation de la photosynthèse.

Les caroténoïdes sont des pigments ($\lambda=400\text{nm}$ à 550nm) hydrophobes capables de dissiper l'énergie de la chlorophylle à l'état excité sous forme de chaleur ou de réagir directement avec l'oxygène singulet. Les caroténoïdes, présents dans les membranes des thylakoïdes, sont indispensables à la stabilisation du PSI, du PSII et du cytochrome b_6f . Au sein des photosystèmes, ils peuvent contribuer à la capture de la lumière, au transfert d'énergie et à la dissipation de l'énergie (revue par^[73]). Les caroténoïdes ont également un rôle d'écran protecteur car ils peuvent capturer une partie du rayonnement lumineux et le dissiper sous forme de chaleur.

L' α tocophérol, ou vitamine E, est un lipide soluble synthétisé uniquement par les organismes photosynthétiques. L' α tocophérol est impliqué dans la

détoxication de l'oxygène singulet au niveau du PSII et dans la protection des lipides contre la peroxydation. Contrairement aux caroténoïdes, après son action, l'atocopherol est converti en hydroperoxyde. Cette conversion est réversible par l'action de l'ascorbate^[74].

L'OCP (Orange Carotenoid Protein), dont le rôle dans la photoprotection sera décrit plus bas, est également un système antioxydant non enzymatique car le caroténoïde intégré au sein de l'OCP est capable de détoxifier l'oxygène singulet.

Dissipation thermique de l'énergie

Les cyanobactéries possèdent au moins trois systèmes de caroténo-protéines ou chlorophyll-caroténo-protéines photoprotecteurs utilisant les propriétés de dissipation thermique d'énergie propre aux caroténoïdes.

Le premier agit au niveau des antennes collectrices et permet de diminuer l'énergie transmise aux centres réactionnels. Il implique la protéine orange à caroténoïde (OCP) qui est activée par une forte lumière bleue-verte. La forme active de l'OCP se fixe au niveau des PBs. Le mécanisme sera décrit plus en détail dans les paragraphes à venir. Les cyanobactéries accumulent également des protéines HLIPs (High Light Inducible Polypeptides)^[75,76] qui lient des molécules de chlorophylle a ainsi que des caroténoïdes^[77]. Ce sont des protéines transmembranaires localisées au niveau du PSII^[78]. Enfin, la protéine IsiA est une protéine photoprotectrice liant des chlorophylles et des caroténoïdes. Elle s'associe soit sous forme d'anneaux autour du PSI où elle fonctionne comme une antenne pour le PSI^[79], soit sous forme d'anneaux vides qui ont une fonction photoprotectrice de dissipation d'énergie^[80].

1.3 LA CAROTÉNO-PROTÉINE OCP (ORANGE CAROTENOID PROTEIN)

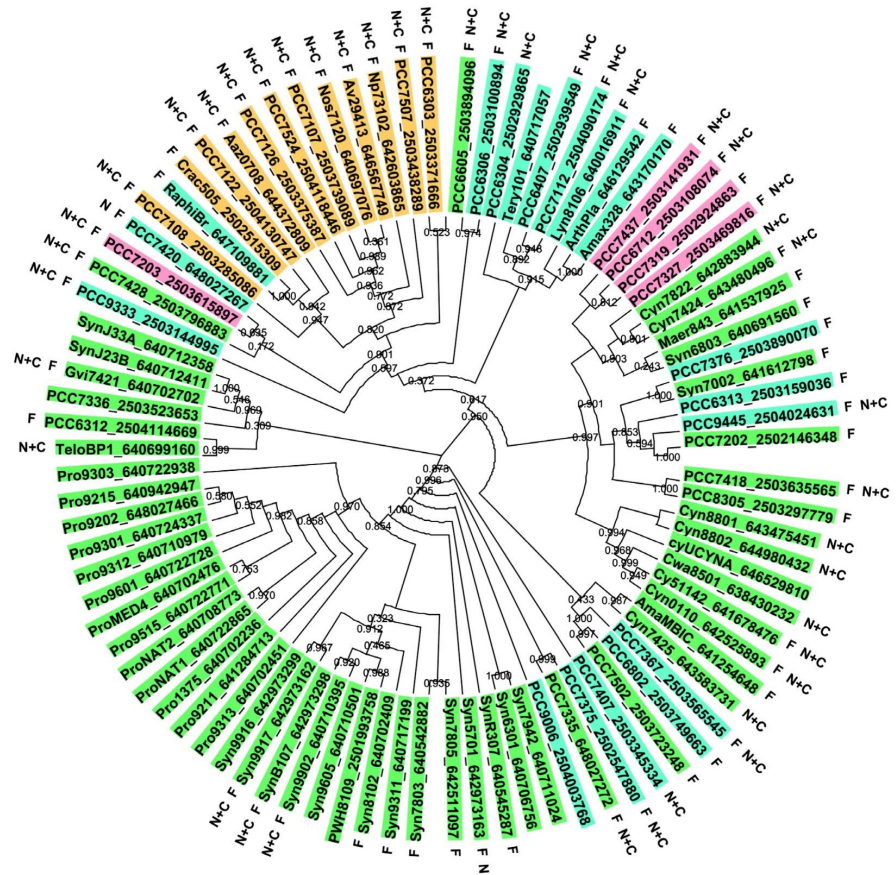
Chez les cyanobactéries, une forte intensité de lumière active le mécanisme de photoprotection impliquant l'OCP. Ce mécanisme réversible à faible lumière permet de diminuer l'énergie arrivant aux centres réactionnels. L'OCP est à la fois le photosenseur et l'acteur. La protéine photoactivée se fixe au coeur des PBs et augmente la part d'énergie dissipée sous forme de chaleur^[81]. Une seconde protéine, la FRP (Fluorescence Recovery Protein) permet de détacher l'OCP des PBs, et donc d'augmenter la part d'énergie transmise aux centres réactionnels.

1.3.1 Régulation de l'expression

Ce mécanisme de photoprotection est présent dans la majorité des cyanobactéries. Les *prochlorococcus* marines, qui ne possèdent pas de phyco-bilisomes, en sont dépourvues. Aucune relation ne peut être actuellement déterminée entre niche écologique, mode de vie unicellulaire ou pluricellulaire et présence ou absence d'OCP^[82] (Figure 1.9).

Chez la plupart des cyanobactéries d'eau douce qui en sont pourvues, les deux gènes codants respectivement pour l'OCP et la FRP, (*SLR1963* et *SLR1964*, chez *Synechocystis* 6803), sont situés derrière l'autre sur le chromosome. En revanche, chez les cyanobactéries marines, un gène codant pour une β -carotène kétolase est présent entre les deux (revues^[84-86]). Les gènes *SLR1963* et *SLR1964* possèdent chacun un promoteur^[26] et une structure forte de terminaison est présente entre les deux séquences codantes^[87].

Ainsi, les deux séquences codantes ne forment vraisemblablement pas un opéron. Cette hypothèse est étayée par la publication de la carte des sites d'initiation de la transcription chez *Synechocystis*^[26] : il existe des transcrits OCP et des transcrits FRP présentant chacun une extrémité 5' protégée par



Gvi7421, *Gloeobacter violaceus* PCC 7421; Cwa8501, *Crocospaera watsonii* WH 8501; PCC7202, *Cyanobacterium stanieri* PCC 7202; PCC7502, *Synechococcus* sp. PCC 7502; PCC7001, *Cyanobium* sp. PCC 7001; PCC6307, *Cyanobium gracile* PCC 6307; Cy51142, *Cyanosphaera* sp. ATCC 51142; Cyn0110, *Cyanosphaera* sp. CCY 0110; Cyn7424, *Cyanosphaera* sp. PCC 7424; Cyn7425, *Cyanosphaera* sp. PCC 7425; Cyn7822, *Cyanosphaera* sp. PCC 7822; Cyn8801, *Cyanosphaera* sp. PCC 8801; Cyn8802, *Cyanosphaera* sp. PCC 8802; PCC8305, *Dactylococcopsis salina* PCC 8305; PCC7418, *Halothece* sp. PCC 7418; Maer843, *Microcystis aeruginosa* NIES-843; Syn6301, *Synechococcus elongatus* PCC 6301; Syn7942, *Synechococcus elongatus* PCC 7942; Syn107, *Synechococcus* sp. BL 107; Syn9311, *Synechococcus* sp. CC9311; Syn9605, *Synechococcus* sp. CC9605; Syn9902, *Synechococcus* sp. CC9902; Syn123B, *Synechococcus* sp. JA-2-3Ba(2-13); SynJ33A, *Synechococcus* sp. JA-3-3Ab; Syn7002, *Synechococcus* sp. PCC 7002; PCC7335, *Synechococcus* sp. PCC 7335; SynR307, *Synechococcus* sp. RCC307; Syn9916, *Synechococcus* sp. RS9916; Syn9917, *Synechococcus* sp. RS9917; Syn5701, *Synechococcus* sp. WH 5701; Syn7803, *Synechococcus* sp. WH 7803; Syn7805, *Synechococcus* sp. WH 7805; Syn8102, *Synechococcus* sp. WH 8102; PWH8109, *Synechococcus* sp. WH 8109; PCC7336, *Synechococcus* sp. PCC 7336; Syn6803, *Synechocystis* sp. PCC 6803; TeloBP1, *Thermosynechococcus elongatus* BP-1; cyUCYNA, *Cyanobacterium* UCYNA; PC10605, *Cyanobacterium* sp. PCC 10605; PCC7428, *Gloeocapsa* sp. PCC 7428; PCC7126, *Microchaete* sp. PCC 7126; Aaz0708, *Anabaena azolae* 0708; Av29413, *Anabaena variabilis* ATCC 29413; PCC7122, *Anabaena cylindrica* PCC 7122; PCC7108, *Anabaena* sp. PCC 7108; Crac505, *Cylindrospermopsis raciborskii* CS-505; Nsp9414, *Nodularia spumigena* CCY9414; Np73102, *Nostoc punctiforme* PCC 73102; Nos7120, *Nostoc* sp. PCC 7120; PCC7107, *Nostoc* sp. PCC 7107; RaphiBr, *Raphidiopsis brooki* D9; PCC6303, *Calothrix* sp. PCC 6303; PCC7507, *Calothrix* sp. PCC 7507; Amax328, *Arthrospira maxima* CS-328; ArthPiA, *Arthrospira platensis* str. Paraca; PCC9333, *Crinium epissammum* PCC 9333; PCC7407, *Gelidium* sp. PCC 7407; PCC6306, *Leptolyngbya* sp. PCC 6306; PCC7375, *Leptolyngbya* sp. PCC 7375; PCC7376, *Leptolyngbya* sp. PCC 7376; Lyn8106, *Lynbya* sp. PCC 8106; PCC7420, *Microcoleus chthonoplastes* PCC 7420; PCC6304, *Oscillatoria acuminata* PCC 6304; PCC6407, *Oscillatoria* sp. PCC 6407; PCC7112, *Oscillatoria* sp. PCC 7112; PCC6802, *Pseudanabaena* sp. PCC 6802; PCC7367, *Pseudanabaena* sp. PCC 7367; PCC6313, *Spirulina major* PCC 6313; Tery101, *Trichodesmium erythraeum* IMS101; PCC6712, *Chroococcoidopsis* sp. PCC 6712; PCC7203, *Chroococcoidopsis thermalis* PCC 7203; PCC7319, *Pleurocapsa* sp. PCC 7319; PCC7327, *Pleurocapsa* sp. PCC 7327; PCC7437, *Stania cyanosphaera* PCC 7437; Pro9601, *Prochlorococcus marinus* AS9601; Pro9202, *Prochlorococcus marinus* str. MIT 9202; Pro9211, *Prochlorococcus marinus* MIT 9211; Pro9215, *Prochlorococcus marinus* MIT 9215; Pro9301, *Prochlorococcus marinus* MIT 9301; Pro9303, *Prochlorococcus marinus* MIT 9303; Pro9312, *Prochlorococcus marinus* MIT 9312; Pro9313, *Prochlorococcus marinus* MIT 9313; Pro9515, *Prochlorococcus marinus* MIT 9515; ProNAT1, *Prochlorococcus marinus* NATL1A; ProNAT2, *Prochlorococcus marinus* NATL2A; Pro1375, *Prochlorococcus marinus* CCMP1375; ProMEDA, *Prochlorococcus marinus* pastoris CCMP1986; AmaMBIC, *Acaryochloris marina* MBIC11017; uncyPop, uncultivated cyanobacterium (Poplar biomass community MLE1-like); PCC6805, *Chamaesiphon* sp. PCC 6805; PCC9006, *Prochlorothrix hollandica* PCC 9006; PCC9445, *Spirulina* sp. PCC 9445; PCC6312, *Synechococcus* sp. PCC 6312; PCC7524, *Nostoc* sp. PCC 7524

Figure 1.9 – Distribution du gène de l’OCP parmi les cyanobactéries. L’identifiant des organismes est surligné par section (section I en vert, section II en rouge, section III en bleu et section 4 en jaune). F = full-length OCP, N, C désignent des gènes dont la séquence correspond au domaine N- ou C- terminal de l’OCP, respectivement. Extrait de Kirilovsky and Kerfeld, 2012 [83].

une extrémité tri-phosphate et correspondant donc à une unité de transcription.

Les données recueillies au sein de la base de données CyanoExpress^[25] montrent que l'abondance des transcrits slr1963 et slr1964 ne varient pas de la même manière lors de stress, confirmant la présence de promoteurs spécifiques. Le transcript OCP est plus abondant au cours des premières dizaines de minutes d'un stress lumineux^[88], d'un stress salin^[89,89], d'une exposition aux UV^[90]. La FRP est réprimée lors d'une carence en carbone supérieure à 3h^[91] et surexprimée lors d'une carence en fer^[92].

1.3.2 Structure tertiaire

En 1981, Holt et Krogmann sont les premiers à isoler l'OCP chez les cyanobactéries *Spirulina maxima* (*Arthrospira platensis*), *Aphanizomenon flos-aquae* et *Microcystis aeruginosa*^[93]. En 1997, Wu et Krogmann identifient le gène *SLR1963* comme codant pour l'OCP chez *Synechocystis* et caractérisent la protéine d'un point de vue biochimique^[94]. La structure de l'OCP a été résolue en 2003 par l'équipe de Cheryl Kerfeld^[95].

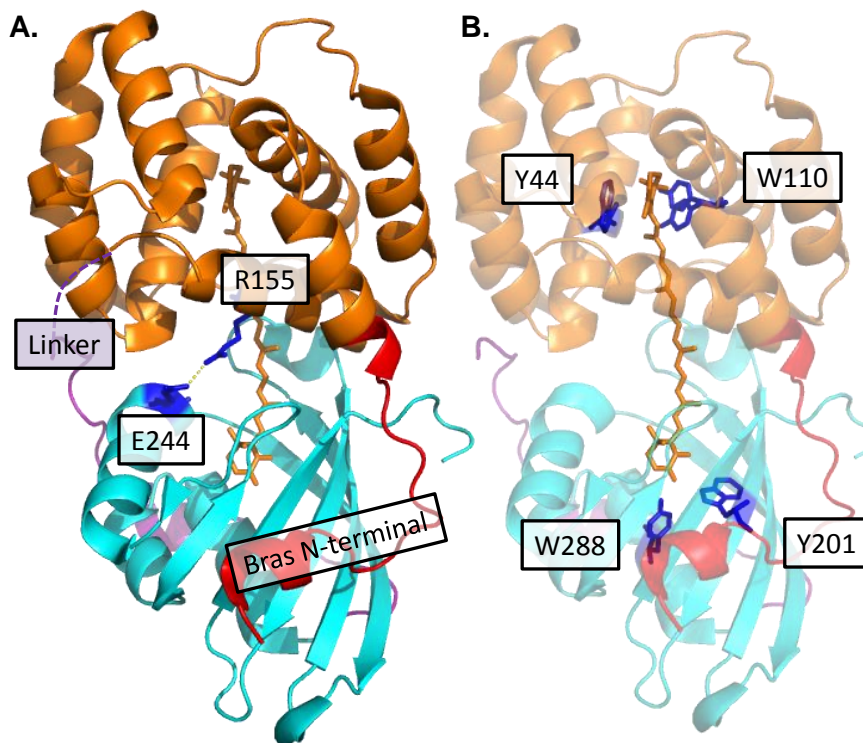


Figure 1.10 – Structure tridimensionnelle de l'OCP

Représentation de la structure tridimensionnelle de l'OCP. Le domaine C-terminal est coloré en cyan, le N-terminal en orange, la région linker en violet et le caroténoïde est représenté par des bâtonnets oranges. (A) Les résidus R155 et E244 sont représentés en bâtonnets bleus. (B) Les 4 résidus Y44, W110, Y201 et W288 sont représentés en bâtonnets bleus.

L'OCP (figure 1.10) est une protéine soluble de masse 35 kD constituée d'un domaine N-terminal riche en hélice α et d'un domaine C-terminal composé d'hélices α et de feuillets β . Ces deux domaines sont reliés par un linker. L'extrémité N-terminale de la protéine forme un appendice qui chevauche le domaine C-terminal.

Un caroténoïde, le 3'hydroxyechinenone (hECN) est lié de manière non covalente. Il est en conformation trans. Les résidus Trp288 et Tyr201 forment

une liaison hydrogène avec le groupe carbonyle dans l'3hECN. Sans ce groupe carbonyle, la protéine n'est plus photo-active^[96]. Le caroténoïde est stabilisé au niveau du domaine N-terminal par les résidus Y44 et W110. Le caroténoïde lié par l'OCP, le 3hECN est de couleur jaune ($\lambda_{\text{max}} = 450$ nm) en solution (Polivka et al., 2005). La structure tertiaire de l'OCP induit une distorsion du caroténoïde créant un déplacement bathochromique du spectre d'absorption du caroténoïde qui présente deux pics à $\lambda = 467$ nm et $\lambda = 496$ nm et une épaulement à $\lambda = 440$ nm.

1.3.3 Photoactivation

Une lumière bleue verte intense convertit la protéine orange en une forme rouge dont le spectre d'absorption présente un seul maximum à $\lambda_{\text{max}} = 510$ nm. La conversion de la forme orange à la forme rouge se fait à la lumière, et dépend de l'intensité de lumière bleue verte. La reconversion de la forme rouge à la forme orange à l'obscurité est fortement dépendante de la température. La photoactivation implique des changements conformationnels de la protéine et du caroténoïde^[97]. Les données FTIR (Spectroscopie infrarouge à transformée de Fourier) révèlent des changements dans la structure secondaire de l'OCP lors du passage à la lumière. Il y a une perte d'hélice α et compaction des feuillets β ^[97]. Contrairement au processus de photo-isomérisation des protéines photosensibles à rhodopsines comme la ASR (*Anabaena* sensory rhodopsin), ces changements conformationnels n'impliquent pas d'isomérisation du chromophore.

Deux articles récents permettent de mieux comprendre les changements conformationnels sous-jacents à la photoactivation. Le premier démontre que l'arginine 155 est indispensable à l'activité de l'OCP^[98]. Cette arginine est localisée à l'interface entre le domaine N-terminal et C-terminal. Elle appartient au domaine N-terminal et forme un pont salin avec le glutamate 244 qui fait partie du domaine C-terminal. Si ce pont salin n'existe plus, alors la protéine reste sous forme active. Ces données ont conduit le laboratoire à poser l'hypothèse que les deux domaines N-terminal et C-terminal pourraient bouger réciproquement afin de rendre accessible l'interface entre les deux domaines accessibles.

1.3.4 L'OCP, une protéine photoprotectrice

Seule l'OCP rouge, photoactivée, peut s'attacher au cœur des phycobilisomes^[81] (voir la représentation à l'échelle de l'OCP à côté d'un PBs figure 1.11). L'OCP quenche l'énergie d'une molécule d'APC émettant à 660 nm du cœur du phycobilisome^[99,100], et ne requiert pas la présence des émetteurs terminaux ApcD, ApcE, ou ApcF^[101].

Le mécanisme de quenching de l'APC660 par le caroténoïde reste en discussion. Il pourrait reposer sur le transfert de l'énergie d'excitation^[102,103], de transfert de charge^[99,104] ou bien d'interaction excitoniques^[105] entre une biline et un caroténoïde. La durée de vie de l'état excité du caroténoïde dans l'OCP rouge est très bref (3,2 ps). Ce délai est de trois ordres de grandeur en dessous de la durée d'excitation des bilines, faisant de l'OCP un très bon quencher de l'énergie^[106].

1.3.5 La Fluorescence Recovery Protein (FRP)

La FRP, ou Fluorescence Recovery Protein, est indispensable pour que les PBs retrouvent leur capacité de capture de lumière, et donc leur niveau initial de fluorescence^[86]. C'est une protéine soluble de masse 13 kDa codée par le gène slr1964. *In vivo*, une cyanobactérie dépourvue de FRP ne re-

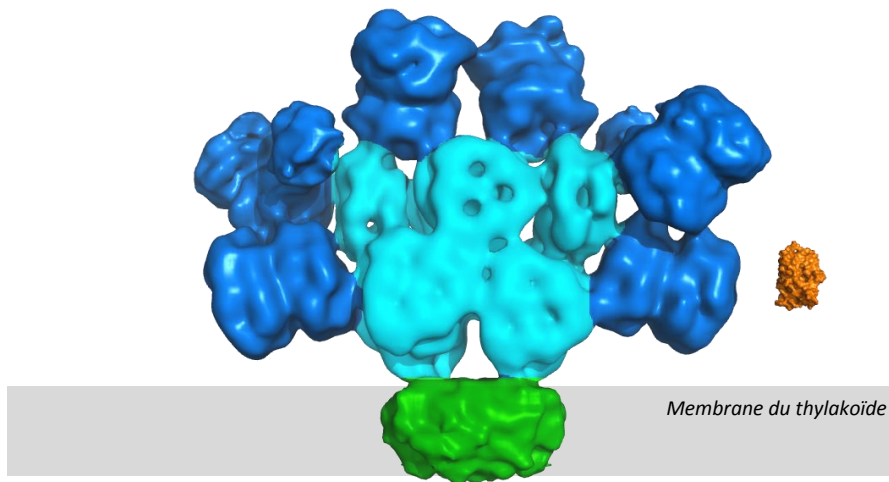


Figure 1.11 – Représentation de la structure tridimensionnelle des PBs et de l'OCP côte à côte

Phycobilisome (cœur en cyan et bras en bleu marine), OCP (en orange) et PSI (en vert). Les structures représentées sont extraites de la base de données PDB pour l'OCP (1ALL) et EmDataBank (EMD-2822) pour la carte de densité du phycobilisome d'*Anabaena variabilis*.

trouve pas son niveau initial de fluorescence^[86]. En revanche, si la FRP est surexprimée, le quenching de fluorescence n'est plus induit^[87]. Cependant, la question de savoir si la FRP permet seulement d'accélérer la reconversion de l'OCP rouge en OCP orange, ou bien aide l'OCP à se détacher des phycobilisomes en plus de catalyser sa reconversion en forme orange reste ouverte. La FRP est active à la lumière et à l'obscurité. Ainsi n'existerait pas de forme inactive de la FRP : la protéine serait active constitutivement. La quantité de quenching de fluorescence *in vivo* dépend donc de plusieurs paramètres : la quantité de lumière qui converti l'OCP orange en OCP rouge, le ratio OCP : PBS, la stabilité de l'OCP rouge (température) ainsi que le ratio OCP : FRP. La quantité de quenching de fluorescence est donc dépendante de la concentration en OCP, et du rapport entre concentration en OCP et concentration en FRP.

1.4 PROBLÉMATIQUE DE LA THÈSE

La figure 1.12 présente le modèle de photoprotection par l'OCP au début de ma thèse en 2012. L'OCP est photoactivée par la lumière bleue verte. Ce processus implique l'ouverture de la protéine. La forme ouverte peut alors s'attacher au coeur des phycobilisome et augmenter la part d'énergie dissipée sous forme de chaleur. La FRP permet de retourner à l'état initial du système. Au cours de mon travail de thèse, je me suis intéressé à mieux caractériser au niveau moléculaire les trois étapes suivantes du mécanisme de photoprotection (étapes numérotées sur la figure 1.12) :

Photoactivation (étape 1)

Afin de comprendre les changements structuraux sous-jacents à la photoactivation, la structure du domaine N-terminal de l'OCP a été résolue. Nous avons démontré que le caroténoïde se déplace de 12Å au sein de l'OCP lors de la photoactivation. J'ai construit des OCP modifiées afin d'empêcher le mouvement du caroténoïde au sein de l'OCP et de démontrer que ce mouvement a bien lieu lors de la photoactivation. En outre, j'ai étudié par une approche de mutation ponctuelle une structure spécifique, le bras N-terminal.

Attachement de l'OCP aux phycobilisomes (étape 2)

Il existe trois architectures pour le coeur des PBs qui peuvent contenir 2, 3 ou 4 cylindres d'allophycocyanine. Une corrélation entre force d'attachement de l'OCP et architecture pourrait nous informer sur la manière dont l'OCP se fixe aux PBs. J'ai purifié des PBs présentant les 3 architectures et caractérisé l'activité OCP pour chacun d'entre eux.

Interaction entre OCP et FRP (étape 3)

Les données structurales de la FRP étaient jusqu'alors manquantes. Je rapporte la résolution de la structure et l'identification du site actif de la FRP qui nous ont permis de prédire *in silico* le site d'attachement putatif de la FRP sur le domaine C-terminal de l'OCP. Le site actif de la FRP a été validé par une approche de mutagenèse faite dans notre laboratoire par Adjélé Wilson et moi-même. Ces données ont permis d'établir un modèle d'interaction entre OCP et FRP. Des résidus de l'OCP candidats pour interagir avec la FRP ont été modifiés par mutagenèse.

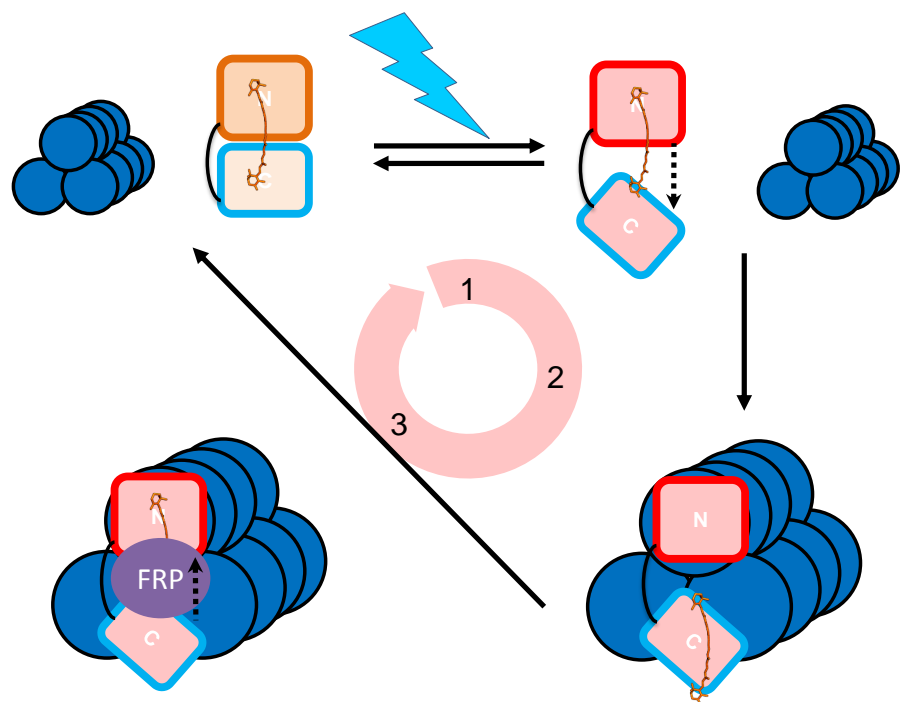


Figure 1.12 – Schéma du mécanisme de photoprotection par l'OCP. L'OCP est photoconvertie par la lumière bleue-verte (1). L'OCP rouge active est ouverte et peut s'attacher aux phycobilisomes (2). La FRP intervient pour accélérer la reconversion de l'OCP sous forme orange (3).

SPÉCIFICITÉS DES OCPs : INTERACTION AVEC DIFFÉRENTS PBS

(COLLABORATION AVEC DENIS JALLET)

L'OCP s'attache au cœur des PBs^[81] qui sont composés par plusieurs cylindres composés de trimers d'allophycocyanine. Il existe 3 types d'architectures du cœur des PBs hemi-discoïdaux. Il y a toujours deux cylindres à la base. Au-dessus, il a un ou trois cylindres. Les cyanobactéries *Synechocystis* et *Arthrospira platensis* possèdent trois cylindres, *Synechococcus elongatus* deux et *Anabaena variabilis* cinq. Parmi ces souches, *Synechocystis*, *A. platensis* et *A. variabilis* possèdent le mécanisme de photoprotection par l'OCP. *S. elongatus* ne possède pas le gène codant pour l'OCP et en est donc dépourvue.

Dans cet article, nous répondons à deux questions. D'une part, nous avons cherché à savoir si l'OCP de *Synechocystis* était capable de s'attacher aux PBs quelle que soit leur architecture. D'autre part, nous voulions savoir si deux OCPs extraites de deux espèces de cyanobactéries possédant la même architecture du cœur des PBs possédaient les mêmes caractéristiques en termes de photoconversion et de spécificité. Ma contribution à ce travail a été la purification des PBs et leur caractérisation (gels, absorbance, fluorescence, quenching de Fluorescence) *in vitro* et la réalisation des tests préliminaires d'activité des OCPs.

2.0.1 Résumé de l'article Jallet et al, 2013

Les OCPs comportant un HisTag de *Synechocystis* et de *A. platensis* ont été surexprimées chez la cyanobactérie *Synechocystis* et purifiées sur une colonne d'affinité nickel (la surexpression de protéines chez *A. platensis* n'est pas décrite à ce jour). Les phycobilisomes de *S. elongatus*, *Synechocystis*, *A. platensis* et *A. variabilis* ont été purifiés sur gradient de sucrose. Nous avons réalisé des cinétiques de photoconversion, et utilisé le système de reconstitution *in vitro* du mécanisme de photoprotection^[81] pour mesurer l'activité des deux OCPs en présence des différents PBs.

Le suivi de la cinétique de photoconversion des OCPs révèle que l'OCP d'*A. platensis* est photo-convertie plus vite que l'OCP de *Synechocystis* (figure 6 de l'article). Lors de la reconstitution *in vitro* du mécanisme de photoprotection en présence des PBs isolés des 4 souches de cyanobactéries, l'OCP de *Synechocystis* induit une large diminution de la fluorescence de ses propres PBs (90%), une faible diminution de la fluorescence de ceux d'*A. variabilis* (20%), et aucune diminution de fluorescence avec les autres PBs. (figure 4 de l'article). En revanche, l'OCP de *A. platensis* dans les mêmes conditions est capable d'induire un quenching de fluorescence plus important que la fluorescence des PBs de toutes les souches (Figure 7c). Par ailleurs, l'attachement le plus fort est obtenu pour les deux OCPs avec les PBs de *Synechocystis*. Le système de reconstitution *in vitro* a été développé en utilisant une concentration en phosphate de 0.8 M. L'utilisation d'un tampon plus concentré (1.4 M) augmente la force d'attachement d'OCP attachée. A 1.4 M de phosphate, les deux OCPs sont capable d'induire une quenching de fluorescence de tous les PBs, qui reste cependant plus important avec l'OCP de *A. platensis*. Une fois que l'OCP y est attachée, elle peut quencher la fluorescence des différents PBs testés.

Le tampon phosphate maintient les PBs assemblés, mais favorise également les interactions entre protéines

Conclusion

Dans cet article, nous avons montré que, malgré leur très forte homologie, les OCPs de *Synechocystis* et *A. platensis* ne présentent pas la même photoactivité ni la même affinité pour les PBs. De plus, l'affinité de chacune des deux OCPs pour les PBs des différentes souches n'est pas corrélée avec l'architecture du cœur des PBs. Par ailleurs, si les PBs de *S. elongatus* peuvent être quenchés par l'OCP, cela signifie que le cylindre supérieur n'est pas requis pour la fixation de l'OCP. Les deux cylindres inférieurs sont donc suffisants pour fixer l'OCP.

Il ne semble donc pas y avoir eu de phénomène de co-évolution entre architecture des PBs et structure tertiaire de l'OCP. Le site de fixation de l'OCP au sein des PBs doit être une structure très conservée car l'OCP peut interagir avec les PBs extraits d'une souche dépourvue d'OCP.

Des résultats complémentaires à cet article sont présentés à la fin du chapitre page 55.

Specificity of the Cyanobacterial Orange Carotenoid Protein - Influences of Orange Carotenoid Protein and Phycobilisome Structures

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Abstract

Cyanobacteria have developed a photoprotective mechanism that decreases the energy arriving at the reaction centers by increasing thermal energy dissipation at the level of the phycobilisome (PB), the extramembranous light-harvesting antenna. This mechanism is triggered by the photoactive Orange Carotenoid Protein (OCP), which acts both as the photosensor and the energy quencher. The OCP binds the core of the PB. The structure of this core differs in diverse cyanobacterial strains. Here, using two isolated OCPs and four classes of PBs, we demonstrated that differences exist between OCPs related to PB binding, photoactivity, and carotenoid binding. *Synechocystis* PCC 6803 (hereafter *Synechocystis*) OCP, but not *Arthrospira platensis* PCC 7345 (hereafter *Arthrospira*) OCP, can attach echinenone in addition to hydroxyechinenone. *Arthrospira* OCP binds more strongly than *Synechocystis* OCP to all types of PBs. *Synechocystis* OCP can strongly bind only its own PB in 0.8 M potassium phosphate. However, if the *Synechocystis* OCP binds to the PB at very high phosphate concentrations (approximately 1.4 M), it is able to quench the fluorescence of any type of PB, even those isolated from strains that lack the OCP-mediated photoprotective mechanism. Thus, the determining step for the induction of photoprotection is the binding of the OCP to PBs. Our results also indicated that the structure of PBs, at least *in vitro*, significantly influences OCP binding and the stabilization of OCP-PB complexes. Finally, the fact that the OCP induced large fluorescence quenching even in the two-cylinder core of *Synechococcus elongatus* PBs strongly suggested that OCP binds to one of the basal allophycocyanin cylinders.

Introduction

The cyanobacterial Orange Carotenoid Protein (OCP) is a photoactive soluble protein of 35 kD that binds a ketocarotenoid, 3'-hydroxyechinenone (hECN). It is present in the majority of phycobilisome (PB)-containing cyanobacterial strains^[82,83]. The PBs are light-harvesting extramembrane complexes formed by a core from which rods radiate. The core and rods are constituted of water-soluble blue and red phycobiliproteins, which covalently attach bilins (for review^[30,33,34,107,108]). The OCP was first described by Holt and Krogmann^[93], and its structure was determined in 2003^[95]. However, its function was discovered only in 2006^[109] and its photoactivity in 2008^[97]. The OCP is essential in a photoprotective mechanism that decreases the energy arriving at the reaction centers under high irradiance. Strong light induces thermal dissipation of the energy absorbed by the PBs, resulting in a decrease of PB fluorescence emission and of energy transfer from the PBs to the reaction centers^[109]. This process, which is light intensity dependent, is induced by blue or green light but not by orange or red light^[109,110]. The absorption of strong blue-green light by the OCP induces changes in the conformation of the carotenoid, converting the inactive orange dark form (OCP_o) into an active red form (OCP_r ;^[97]). In OCP_o , the hECN is in an all-trans-configuration^[95,111]. In OCP_r , the apparent conjugation length of the carotenoid increases, resulting in a less distorted, more planar structure^[97]. Fourier transform infrared spectra showed that conformational changes in the protein are also induced^[97] that are essential for the induction of the photoprotective mechanism. Only OCP_r is able to bind to the core of PBs and to induce thermal energy

dissipation^[81,96,97,112]. Since the photoactivation of the OCP has a very low quantum yield (0.03;^[97]), the concentration of activated protein is zero in darkness and very low under low-light conditions^[97,112]. Thus, the photoprotective mechanism functions only under high-light conditions.

The crystal structures of the *Arthrospira maxima* OCP and of the *Synechocystis* PCC 6803 (hereafter *Synechocystis*) OCP were solved in 2003 and 2010, respectively^[95,113]. These structures, assumed to correspond to the dark OCP_o form, are essentially identical. The OCP consists of an all- α -helical N-terminal domain (residues 1-165), unique to cyanobacteria, and an α -helical/ β -sheet C-terminal domain that is a member of the Nuclear Transport Factor2 superfamily (residues 191-320; *Synechocystis* numbering). Both domains are joined by a linker (residues 166-190; *Synechocystis* numbering) that appears to be flexible. The hECN molecule spans the N- and C-terminal domains of the protein, with its carbonyl end embedded in and hydrogen bonded to two absolutely conserved residues (Tyr-201 and Trp-288) in the C-terminal domain. The carotenoid is almost entirely buried; only 3.4% of the 3' hECN is solvent exposed^[95]. *Synechocystis* OCP can also bind with high-affinity echinenone (ECN) and zeaxanthin. While the ECN OCP is photoactive, the zeaxanthin OCP is photoinactive^[96], indicating the importance of the carotenoid carbonyl group for photoactivity. The largest interface through which the two domains interact and through which the carotenoid passes is stabilized by a small number of hydrogen bonds, including one formed between Arg-155 and Glu-244^[113]. This salt bridge stabilizes the closed structure of OCP_o . Upon illumination, protein conformational changes cause the breakage of this bond and the opening of the protein^[98]. Arg-155, which becomes more exposed upon the separation of the two domains, is essential for the OCP_r binding to the PBs^[98].

After exposure to high irradiance, when the light intensity decreases, recovery of full antenna capacity and fluorescence requires another protein, the Fluorescence Recovery Protein (FRP;^[86]). The active form of this soluble 13-kD protein is a dimer^[114]. It interacts with the OCP_r C-terminal domain^[86,114]. This accelerates the red-to-orange OCP conversion and helps the OCP to detach from the PB^[81,86].

Genes encoding the full-length OCP are found in the vast majority of cyanobacteria but not in all; 90 of 127 genomes recently surveyed contain at least one gene for a full-length OCP^[82]. The genomes of *Synechococcus elongatus* and *Thermosynechococcus elongatus*, two cyanobacterial strains used as model organisms in photosynthesis and stress studies, do not contain a full-length ocp gene. These strains also lack FRP and β -carotene ketolase (involved in ketocarotenoid synthesis). As a consequence, these strains lack the OCP-related photoprotective mechanism and are more sensitive to fluctuating light intensities^[115].

The core of the hemidiscoidal PBs of *Synechocystis*, the model organism routinely used for the study of the OCP-related photoprotective mechanism, consists of three cylinders, each one formed by four trimers of allophycocyanin (APC; Fig. 2.1; for review^[30,34,107,116,117]). The APC trimers are predominantly assembled from a two-subunit heterodimer, α APC- β APC, which binds two phycocyanobilins, one in each subunit. Of the 12 total APC trimers in the PB core, eight are trimers of α APC- β APC. These trimers have a maximal emission at 660 nm (APC660). The upper cylinder contains only APC660 trimers. In contrast, each basal cylinder contains only two APC660 trimers. Each basal cylinder also contains the following: (1) a trimer in which one α APC subunit is replaced by a special α APC-like subunit called ApcD, and (2) a trimer in which one β -subunit is replaced by ApcF, a β APC-like subunit, and one α -subunit is replaced by the N-terminal domain of ApcE, an α APC-like domain (Fig. 2.1). The trimers containing one or two of these special subunits have a maximal emission at 680 nm (APC680). In each cylinder, the two external trimers are stabilized by an 8.7-kD linker protein.

The C-terminal part of *Synechocystis* ApcE contains three interconnected repeated domains of about 120 residues (called Rep domains) that are similar to the conserved domains of rod linkers. Each Rep domain interacts with an APC trimer situated in different cylinders, which stabilizes the core of PB^[118-121]. The ApcE protein also determines the number of APC cylinders that form the PB core^[122,123]. Indeed, there are PBs containing only the two basal cylinders, as in *S. elongatus* (ex *S. elongatus* PCC 7942) and *Synechococcus* PCC 6301. In these strains, the approximately 72-kD ApcE possesses only two Rep domains. There also exist pentacylindrical cores in which, in addition to the three cylinders existing in *Synechocystis* PBs, there are two other

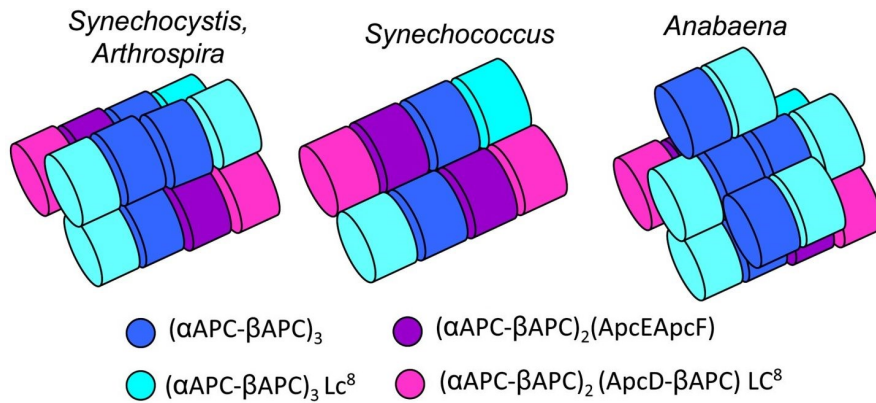


Figure 2.1 – Schematic orthogonal projections of the various PB cores. In the PBs containing three or five cylinders, the top complete cylinder is formed by four $\alpha\text{APC}-\beta\text{APC}$ trimers emitting at 660 nm. Each of the basal cylinders of three types of PBs contains two $\alpha\text{APC}-\beta\text{APC}$ trimers emitting at 660 nm and two trimers emitting at 683 nm. In one of them, one αAPC is replaced by ApcD, and in the other one, $\alpha\text{APC}-\beta\text{APC}$ is replaced by the dimer ApcF-ApcE. In the five cylinder PBs, two additional semicylinders formed by two $\alpha\text{APC}-\beta\text{APC}$ trimers are present. In all the cylinders, the two external trimers include an 8.7-kD linker protein (ApcC).

cylinders, each formed by two APC660 trimers, for example in *Anabaena variabilis*, *Anabaena* PCC 7120, and *Mastigocladus laminosus*^[124,125]. In the pentacylindrical PBs, ApcE (approximately 125 kD) contains four Rep domains^[123]. Finally, ApcE is also involved in the interaction between the PB and the thylakoids.

The bicylindric and tricylindric cores are surrounded by six rods formed generally by three hexamers of the blue phycocyanin (PC) or two PC hexamers and one hexamer containing phycoerythrin or phycoerythrin cyanonin. The rods and the hexamers are stabilized by nonchromophorylated linker proteins. A linker protein, LRC also stabilizes the binding of the rods to the core. The pentacylindric PBs can contain up to eight rods. The quantity and length of rods and the presence of phycoerythrin or phycoerythrocyanin at the periphery of the rods depends on environmental conditions like light intensity or quality^[124,126].

The OCP probably binds to one of the APC660 trimers^[99-101], and the presence of the rods stabilizes this binding to *Synechocystis* PBs^[81]. The different structures of PBs in other strains could affect the binding of the OCP. Thus, we undertook a study about the relationship between the structure of PBs and OCP binding in preparation for introducing the OCP-related photoprotective mechanism into *S. elongatus* using *Synechocystis* genes. In this study, we used the *in vitro* reconstitution system developed by Gwizdala et al.^[81] with three different types of isolated PBs: *Arthrospira platensis* PCC 7345 (hereafter *Arthrospira*) PBs, having a tricylindrical core like *Synechocystis* PBs; *Anabaena variabilis* (hereafter *Anabaena*) PBs, having a pentacylindrical core; and *S. elongatus* PCC 7942 (hereafter *Synechococcus*) PBs, having a bicylindrical core. We also used two different OCPs, the *Synechocystis* OCP and the *Arthrospira* OCP. Each OCP was isolated from mutant *Synechocystis* cells overexpressing one or the other ocp gene with a C-terminal His tag.

Results

PB Isolation and Characterization

To isolate PBs *Synechocystis*, *Arthrospira*, *Anabaena*, and *Synechococcus* cells were broken in a highly concentrated potassium phosphate buffer (0.8-1 M). Triton X-100 was then used to solubilize membranes and release PBs in the aqueous phase. This phase was collected and deposited on Suc gradients. After ultracentrifugation, fully assembled PBs concentrated in a well-defined dark blue band at the bottom 0.75 M Suc layer (for details, see “Materials and Methods”).

The protein composition of the isolated PBs was analyzed by SDS-PAGE. The major phycobiliproteins, α/β -subunits of PC or APC, appeared as intense bands in the

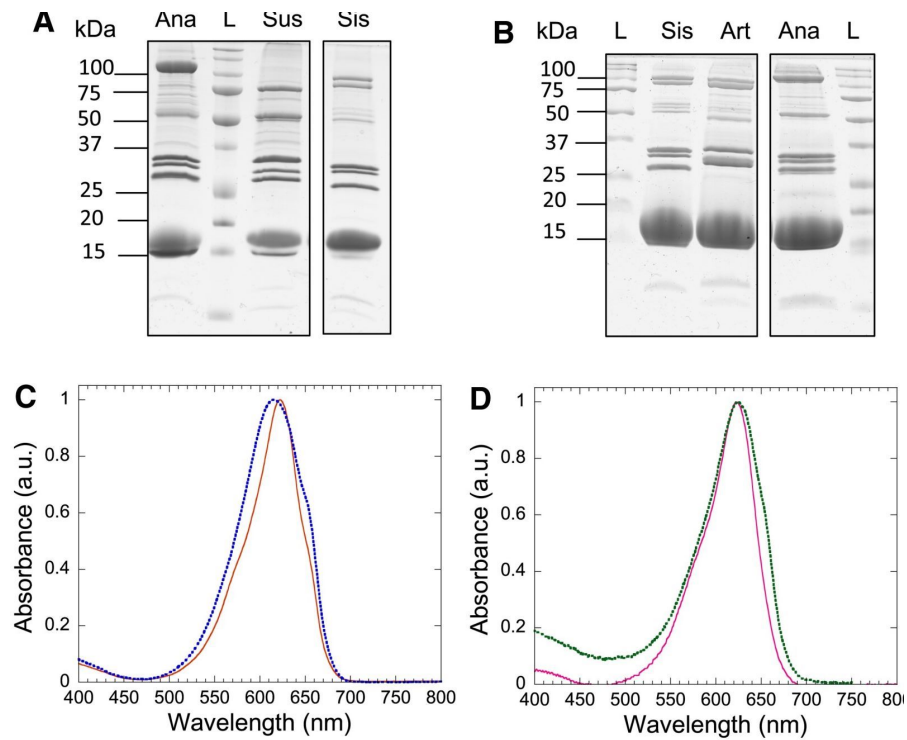


Figure 2.2 – Composition analysis of the various PBs. A, Polypeptide composition of the isolated *Anabaena* PBs (Ana), *Synechococcus* PBs (Sus), and *Synechocystis* PBs (Sis). L, Ladder. B, Polypeptide composition of the isolated *Synechocystis* PBs, *Arthrospira* PBs (Art), and *Anabaena* PBs. C and D, Room-temperature absorption spectra of the PBs isolated from *Synechocystis* (solid orange line) and *Arthrospira* (dashed blue line; C) or *Synechococcus* (solid pink line) and *Anabaena* (dashed green line; D). Spectra are normalized at the maximum of absorbance around 620 nm. a.u., Absorbance units.

region between 16 and 20 kD (Fig. 2.2, A and B). Bands for ApcD and ApcF completely overlapped them. The rod-to-core and rod linkers (LRC and LR) were distributed from 27 to 35 kD (Fig. 2.2, A and B). The molecular mass of these proteins differed slightly in the four strains, as described previously in the literature^[127–130]. ApcE had a higher molecular mass, between 75 and 125 kD, depending on PB architecture. In *Anabaena*, where PB cores contain five APC cylinders, the molecular mass of ApcE was 120 kD. The molecular mass of ApcE was only 95 kD in PBs containing three APC cylinders (*Synechocystis* and *Arthrospira*) and 75 kD in PBs containing two APC cylinders (*Synechococcus*; Fig. 2.2, A and B). The small rod (10 kD) and core (8.7 kD) linkers were barely detected on our SDS-PAGE gel.

Absorption spectra were recorded to obtain further insights into the relative quantities of PC and APC in the isolated PBs. PC, which is the most abundant phycobiliprotein in the PB, has a maximum of A₆₂₀ for *Synechocystis*, *Synechococcus*, and *Anabaena* PBs (Fig. 2.2, C and D). The absorbance band was larger and the maximum shifted to 615 nm in *Arthrospira* PBs (Fig. 2.2C), owing to the fact that some PC α -subunits bind phycobiliviolin instead of phycocyanobilin^[131]. A more or less pronounced shoulder could be seen at 650 nm, related to APC absorbance. The PC-to-APC ratio was estimated by fitting the observed spectra to a combination of PC and APC absorbance spectra. As expected, the PC-to-APC ratio was higher in *Synechococcus* PBs (approximately 4.6) and lower in *Anabaena* PBs (approximately 1.3) than in *Synechocystis* PBs (approximately 3). Such differences correlate well with the PB core architectures, *Synechococcus* containing less and *Anabaena* containing more APC cylinders than *Synechocystis*. *Arthrospira* PBs seemed to contain less PC than *Synechocystis* PBs. The ratio of PC to APC in *Arthrospira* PBs was approximately 2, as already described^[129]. In *Anabaena* and *Arthrospira* PBs, the rods seemed to contain only two PC hexamers, as observed previously^[128,129].

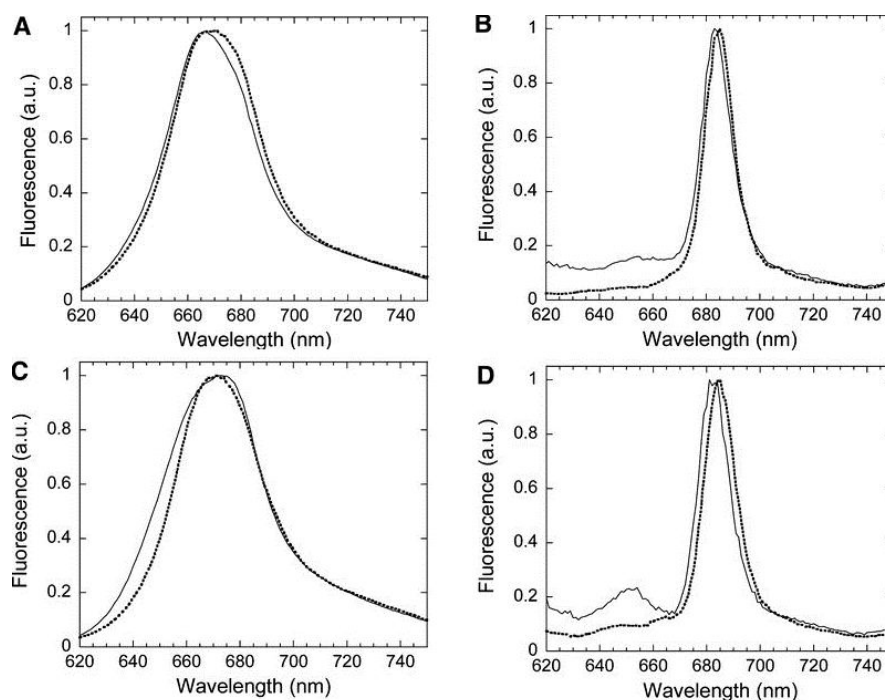


Figure 2.3 – Room temperature (A and C) and 77 K (B and D) fluorescence emission spectra of the isolated PBs. A and B, *Synechocystis* PBs (solid lines) are compared with *Arthrospira* PBs (dashed lines). C and D, *Synechococcus* PBs (solid lines) are compared with *Anabaena* PBs (dashed lines). Spectra are normalized to the maximum of emission. Excitation was at 590 nm. a.u., Absorbance units.

Fluorescence emission spectra were used to confirm functional energy transfer in the PBs. In intact PBs at room temperature, excitation flows from PC to APC, which in turn equilibrates with terminal emitters (ApcD, ApcF, and ApcE). That results in a fluorescence peak with maximum at around 670 nm (at room temperature) when the PC is preferentially excited (excitation light at 590 nm; Fig. 2.3, A and C). At 77 K, energy back flow becomes less probable, so excitation arriving to the terminal emitters gets trapped. A fluorescence peak appeared at 683 to 684 nm for *Arthrospira*, *Anabaena*, and *Synechocystis* PBs (Fig. 2.3, B and D), which was slightly blue shifted in *Synechococcus* PBs (681 nm; Fig. 2.3D). Disconnected PC resulted in a small band at 645 nm, especially in *Synechococcus* PBs (Fig. 2.3, C and D). Altogether, these data indicated that the isolated PBs were well assembled and functionally connected.

Quenching in Vitro Using *Synechocystis* OCP

To test whether *Synechocystis* OCP can interact with the different PBs and trigger their fluorescence quenching, an *in vitro* reconstitution system was employed^[81]. *Synechocystis* OCP was isolated from a *Synechocystis* mutant strain overexpressing the ocp gene and lacking the β -carotene hydroxylase (CrtR). In this strain, the OCP binds only ECN (not zeaxanthin or 3' hECN). The ECN-binding OCP isolated from the Δ CrtR strain is photoactive and induces a large blue light-induced fluorescence quenching in cells^[96,132].

Reconstitution experiments require a minimum of 0.8 m phosphate to maintain PB integrity. However, it was already demonstrated that such high phosphate concentrations hinder OCP photoactivation^[81]. In the experiments described here, the OCP was converted to the red form before being mixed with the PB solution. OCP conversion from its orange/inactive form (OCP_0) to its red/active one (OCP_r) was triggered with strong white light in Tris-HCl buffer at 4°C. OCP_r was then added to a PB solution in 0.8 m potassium phosphate buffer under continuous blue-green illumination (900 μ mol photons m⁻² s⁻¹), and the fluorescence quenching was followed using a pulse amplitude modulated (PAM) fluorometer^[81]. After *Synechocystis* OCP_r addition, the *Synechocystis* PB fluorescence decreased strongly and rapidly (95% in 50 s;

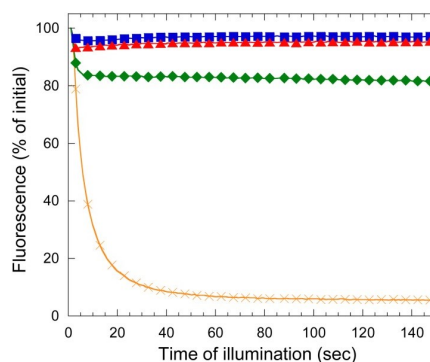


Figure 2.4 – Fluorescence quenching induced by *Synechocystis* OCP *in vitro*. The PBs ($0.012 \mu\text{m}$) were illuminated with blue-green light ($900 \mu\text{mol m}^{-2} \text{s}^{-1}$) in the presence of an excess of preconverted *Synechocystis* OCP_r ($0.48 \mu\text{m}$; 40 per PB) at 23°C and 0.8 m potassium phosphate. Fluorescence decrease was measured using a PAM fluorometer for *Synechocystis* PBs (orange crosses), *Arthrospira* PBs (blue squares), *Synechococcus* PBs (red triangles), and *Anabaena* PBs (green diamonds).

Fig. 2.4). Almost no effect was observed for the other PBs. For *Anabaena* PBs, only a slight quenching (20%) could be seen, which became even smaller for *Synechococcus* and *Arthrospira* PBs (approximately 3%; Fig. 2.4). This indicates that *Synechocystis* OCP interacts poorly with PBs from other strains or that it cannot trigger their fluorescence quenching under conditions in which it strongly interacts with *Synechocystis* PBs (0.8 m phosphate).

Arthrospira OCP Purification

The next question addressed was whether all OCPs are specific to their cognate PBs. Since *Arthrospira* OCP is also well characterized and its structure is known, we selected it to test for OCP-PB specificity. No method is known that allows introducing modifications in the *Arthrospira* genome, precluding the production of His-tagged OCP in *Arthrospira* cells. Thus, a region containing the *Arthrospira* ocp and frp genes was cloned into the pPSBA2 plasmid^[133]. A sequence encoding for six His residues was added at the 3' end of the *Arthrospira* ocp gene, which was under the control of the strong psbA2 promoter. *Synechocystis* wild-type and ΔCrtR cells were then transformed using the resulting plasmid. In the mutants obtained, the endogenous *Synechocystis* ocp gene (slr1963) was interrupted by introduction of a spectinomycin/streptomycin resistance cassette. This last step led to the oApOCPWT and oApOCP ΔCrtR *Synechocystis* mutants, producing *Arthrospira* OCP. Additional details are described in “Materials and Methods.”

Western-blot analysis of cell extracts using a primary antibody directed against *A. maxima* OCP permitted estimation of the OCP content in mutant and wild-type *Synechocystis* cells (Fig. 2.5A). The western blot revealed a 35-kD band corresponding to the OCP. The band was much stronger in oApOCPWT cells than in wild-type *Synechocystis* cells, indicating a large accumulation of *Arthrospira* OCP in this mutant. By contrast, almost no OCP was detected in the oApOCP ΔCrtR strain. For comparison, it is shown that in oSynOCP ΔCrtR (a strain overaccumulating *Synechocystis* OCP), the *Synechocystis* OCP content was largely higher than in ΔCrtR . Thus, *Arthrospira* OCP is unable to accumulate in a strain lacking hECN, while *Synechocystis* OCP is not affected by the lack of this carotenoid^[132].

Arthrospira OCP was purified from oApOCPWT cells using a protocol similar to that developed for *Synechocystis* OCP isolation^[97] (see “Materials and Methods”). In the dark, the absorbance spectrum of the isolated *Arthrospira* OCP showed peaks at 467 and 496 nm as well as a shoulder around 440 nm (Fig. 2.5B). This spectrum is identical to those already published for *A. maxima*^[93,111] and *Synechocystis* OCP^[97]. After illumination, OCP_o converted to its red form. Its absorbance spectrum matched the one of *Synechocystis* OCP_r (Fig. 2.5B). The carotenoid content of the isolated *Arthrospira* OCP was analyzed by HPLC. hECN was detected in 81% of OCPs, while the 19% remaining OCPs bound ECN (Supplemental Fig. S1).

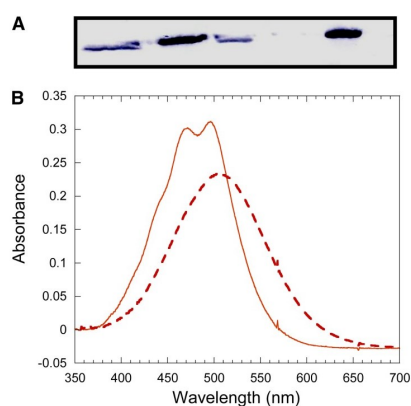


Figure 2.5 – Isolation of *Arthrospira* OCP. A, Immunoblot detection using a primary antibody directed against *Arthrospira* OCP on whole cell extracts of wild-type (lane 1), oApOCPWT (lane 2), Δ CrtR (lane 3), oApOCP Δ CrtR (lane 4), and oSynOCP Δ CrtR (lane 5) cells. Three micrograms of chlorophyll was deposited per well. B, Absorbance spectra of *Arthrospira* OCP_o (orange solid line) and OCP_t (red dashed line) isolated from oApOCPWT cells.

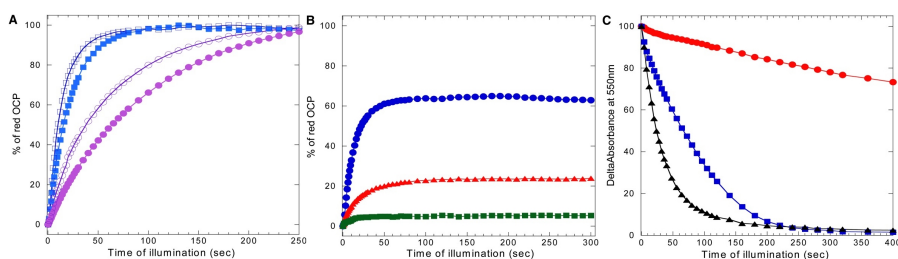


Figure 2.6 – Light-driven photoconversion and dark recovery of *Arthrospira* OCP. *Arthrospira* OCP_o (1.8 μ m) was illuminated using strong white light (5,000 μ mol m⁻² s⁻¹), and its A₅₅₀ was recorded over time. A, In 40 mM Tris-HCl, pH 8, *Synechocystis* OCP (closed symbols) and *Arthrospira* OCP (open symbols) were compared at 23°C (squares) or 9°C (circles). Data were normalized to the final percentage of the red form in each condition. B, *Arthrospira* OCP in 40 mM Tris-HCl (blue circles), 0.8 m potassium phosphate (red triangles), or 1.4 m potassium phosphate (green squares) during its photoconversion at 23°C. Data were normalized to the final percentage of the red form at 9°C. C, After *Arthrospira* OCP photoconversion, the light source was turned off, and A₅₅₀ evolution was followed at 9°C with (blue squares) or without (red circles) FRP addition (one per two OCPs). Recovery was also followed at 23°C (black triangles). Data were normalized to the initial A₅₅₀.

Arthrospira OCP photoactivation kinetics were then studied. OCP_t accumulation under illumination results in an increase of the 550-nm absorbance (Fig. 2.5B) that can be monitored over time. Figure 2.6A compares the photoactivation kinetics of hECN-*Arthrospira* and ECNtextit*Synechocystis* OCPs at 9°C and 23°C. At both temperatures, *Arthrospira* OCP_o converted faster to OCP_t ($t_{1/2}$ approximately 37 and 10 s, respectively) than *Synechocystis* OCP_o ($t_{1/2}$ approximately 62 and 14 s, respectively). In addition, with recovery to the orange form being nonnegligible at 23°C, only 62.9% of the *Arthrospira* OCP and 54.5% of the *Synechocystis* OCP were in the red form at equilibrium. A similar difference in the kinetics of photoconversion was observed when ECN-*Arthrospira* OCP was compared with the ECNtextit*Synechocystis* OCP (Supplemental Fig. S2), thus indicating that the differences in kinetics were not due to differences in the bound carotenoid. These experiments were made in 40 mM Tris-HCl and did not mimic the buffer conditions applied for quenching reconstitution (i.e. high phosphate concentration;^[81]). Indeed, high phosphate concentration also affected the accumulation of *Arthrospira* OCP_t, as in the case of *Synechocystis* OCP_t (^[81]). Only 24% of *Arthrospira* OCP was converted to the red form at 0.8 m phosphate and 5.3% at 1.4 m phosphate at 23°C (Fig. 2.6B).

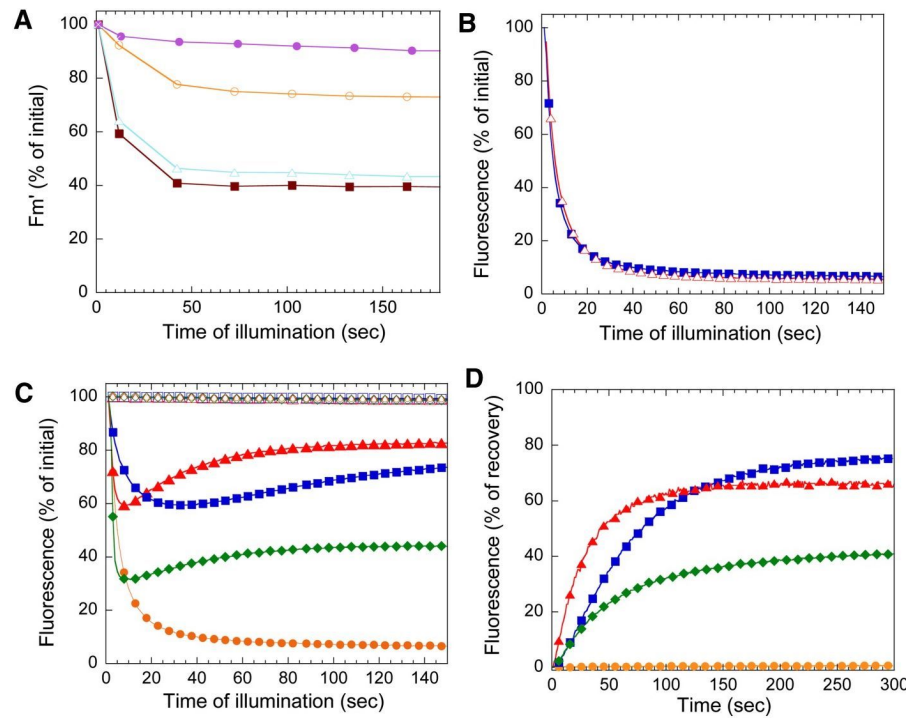


Figure 2.7 – Fluorescence quenching induced by *Arthrospira* OCP *in vitro* and *in vivo*. A, Fluorescence quenching triggered by strong blue-green light ($1,400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) in *Synechocystis* wild-type (orange open circles), oSynOCPΔCrtR (cyan open triangles), oApOCPWT (brown closed squares), and oApOCPΔCrtR cells (purple closed circles) cells at 33°C . F_m' , Maximum PSII fluorescence in the light-adapted state. B, Isolated *Synechocystis* PBs were illuminated with blue-green light ($900 \mu\text{mol m}^{-2} \text{s}^{-1}$) in the presence of an excess of preconverted *Synechocystis* OCP_r (orange triangles) or *Arthrospira* OCP_r (blue squares; $0.48 \mu\text{m}$; 40 per PB) at 0.8 m potassium phosphate, 23°C . C, Fluorescence quenching induced by strong blue-green light ($900 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 0.8 m potassium phosphate in the absence (open symbols) or the presence (closed symbols) of an excess of *Arthrospira* OCP_r in *Synechocystis* PBs (orange circles), *Arthrospira* PBs (blue squares), *Synechococcus* PBs (red triangles), and *Anabaena* PBs (green diamonds). D, Fluorescence recovery in darkness of “quenched” PBs. The light was turned off after 300 s of illumination. Symbols are as in C.

Recovery from OCP_r to OCP_o was also studied (Fig. 2.6C). At 9°C , in 40 mM Tris-HCl, *Arthrospira* OCP_r recovers very slowly, while at 23°C , it reverts to OCP_o rapidly ($t_{1/2}$ approximately 22 s), similar to *Synechocystis* OCP ([97]). Addition of the FRP from *Synechocystis* largely accelerated the conversion of OCP_r to OCP_o at 9°C ($t_{1/2}$ from 15 min to 60 s; Fig. 2.6C), indicating that *Arthrospira* OCP is able to interact with *Synechocystis* FRP.

Quenching *in Vitro* Using *Arthrospira* OCP

The decrease of fluorescence yield induced by strong blue-green light in wild-type, oApOCPWT, oApOCPΔCrtR, and oSynOCPΔCrtR *Synechocystis* cells is compared in Figure 2.7A. As expected, due to the low concentration of the OCP in the oApOCPΔCrtR strain (Fig. 2.5A), almost no fluorescence quenching was observed in this strain. In contrast, in the oApOCPWT strain, strong blue-green light induced a huge fluorescence quenching as in the oSynOCPΔCrtR strain (60% drop in about 50 s). *In vitro*, when *Synechocystis* PBs were illuminated in the presence of *Arthrospira* OCP_r at 0.8 m phosphate, *Arthrospira* OCP rapidly quenched almost all PB fluorescence with kinetics similar to that of *Synechocystis* OCP (Fig. 2.7B). Thus, *Arthrospira* OCP is able to interact with *Synechocystis* PBs *in vivo* and *in vitro* and to quench their fluorescence.

Figure 2.7C reveals that *Arthrospira* OCP_r was also able to induce a large quenching of *Synechococcus*, *Arthrospira*, and *Anabaena* PB fluorescence in 0.8 m potassium

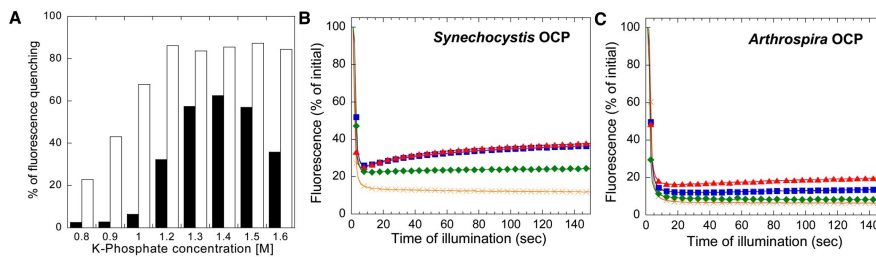


Figure 2.8 – Effect of potassium (K) phosphate concentration on PB fluorescence quenching. PBs ($0.012 \mu\text{m}$) were illuminated with blue-green light ($900 \mu\text{mol m}^{-2} \text{s}^{-1}$) in the presence of preconverted OCP_r ($0.48 \mu\text{m}$; 40 per PB) at 23°C . A, Percentage of *Arthrospira* PB fluorescence quenching reached after 5 min of illumination in the presence of *Synechocystis* OCP_r (black bars) or *Arthrospira* OCP_r (white bars) in increasing potassium phosphate concentrations from 0.8 to 1.6 m. B, Fluorescence decrease induced by *Synechocystis* OCP_r in *Synechocystis* PBs (orange crosses), *Arthrospira* PBs (blue squares), *Synechococcus* PBs (red triangles), and *Anabaena* PBs (green diamonds) at 1.4 m potassium phosphate. C, Fluorescence decrease induced by *Arthrospira* OCP_r in *Synechocystis* PBs (orange crosses), *Arthrospira* PBs (blue squares), *Synechococcus* PBs (red triangles), or *Anabaena* PBs (green diamonds) at 1.4 m potassium phosphate.

phosphate buffer; in contrast, *Synechocystis* OCP_r was inactive (Fig. 2.4). Illumination of PBs in the absence of OCP did not induce any fluorescence quenching (Fig. 2.7C). In *Anabaena* PBs, a fast and large-magnitude fluorescence decrease occurred (68% decrease in 10 s) followed by a slow regain phase related to dislodging of the OCP from the PB and partial reversion of OCP_r to OCP_0 in the solution (9-300 s; final quenching, 57.2%). Similar profiles were observed with *Synechococcus* PBs (40.7% decrease after 8 s, 17.7% at 300 s) and *Arthrospira* PBs (40.8% after 34 s, 22.75% at 300 s). Thus, *Arthrospira* OCP seems to be less specific than *Synechocystis* OCP. Moreover, it seems to interact more strongly with *Synechocystis* and *Anabaena* PBs than with *Arthrospira* PBs. This was also illustrated by the faster fluorescence recovery in the dark for *Synechococcus* and *Arthrospira* PBs than for *Anabaena* and *Synechocystis* PBs (Fig. 2.7D).

The differences between *Arthrospira* and *Synechocystis* OCP are not due to the fact that one attaches hECN and the other ECN. Results similar to that shown in Figure 2.4 were obtained when hECN-*Synechocystis* OCP, isolated from wild-type *Synechocystis* cells, was used in the *in vitro* quenching experiments (Supplemental Fig. S3A). A similar observation was made when employing ECN-*Arthrospira* OCP isolated from oApOCP Δ CrtR instead of the 3' hECN binding OCP from oApOCPWT cells (Supplemental Fig. S3B). The observed differences in PB binding must be due to differences in the protein, not the pigment, of the two OCPs.

Effect of Increasing Phosphate Concentration on the Induction of Fluorescence Quenching

Increasing phosphate concentration strengthens the *Synechocystis* OCP binding to *Synechocystis* whole PBs and PB cores^[81]. The OCP is unable to induce fluorescence quenching of isolated cores of PBs at 0.5 m phosphate, but it induces a large quenching at 0.8 m phosphate^[81]. Although at 0.5 m phosphate, OCP is able to induce the total quenching of whole *Synechocystis* PBs, the rate of quenching increases with the concentration of phosphate (Supplemental Fig. S4). We tested if *Synechocystis* OCP is able to bind to other PBs at concentrations higher than 0.8 m phosphate. Figure 2.8A shows the percentage of fluorescence quenching induced for *Arthrospira* PBs in potassium phosphate buffers from 0.8 to 1.6 m. An optimum appeared at 1.4 m for *Arthrospira* PBs, where fluorescence yield dropped by 62.5% after 300 s (instead of 2.5% at 0.8 m). Figure 2.8B compares the fluorescence quenching induced by *Synechocystis* OCP_r in *Arthrospira*, *Synechococcus*, and *Anabaena* PBs at 1.4 m phosphate. At this concentration, *Synechocystis* OCP induced a large fluorescence quenching in *Anabaena* PBs (75.1%), *Arthrospira* PBs (60.9%), and *Synechococcus* PBs (59.3%). Nevertheless, the fluorescence quenching induced was still smaller than in *Synechocystis*

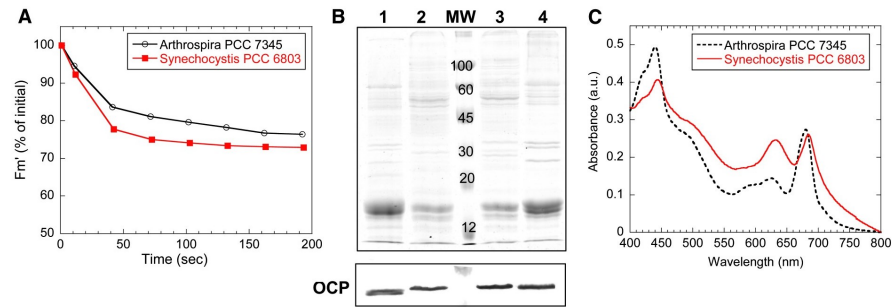


Figure 2.9 – Comparison of OCP-related fluorescence quenching in *Arthrospira* and *Synechocystis* cells. A, Fluorescence quenching triggered by strong blue-green light ($1,400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) in *Synechocystis* wild-type (red closed squares) and *Arthrospira* (black open circles) cells at 33°C . F_m' , Maximum PSII fluorescence in the light-adapted state. B, Coomassie blue-stained gel electrophoresis (top) and immunoblot detection (bottom) of the OCP protein in *Synechocystis* wild-type cells (lane 1), *Arthrospira* cells (lane 2), and membrane-PB fractions (prepared as described by Wilson et al. [2006]) obtained from *Arthrospira* (lane 3) or *Synechocystis* (lane 4). Each lane contains $2 \mu\text{g}$ of chlorophyll. C, Room temperature absorbance spectra of *Synechocystis* wild-type (solid red line) and *Arthrospira* (dashed black line) cells. Cells were diluted to $3 \mu\text{g}$ chlorophyll mL^{-1} . a.u., Absorbance units.

PBs (88.6% ; Fig. 2.8B), suggesting that *Synechocystis* OCP is still more specific for *Synechocystis* PBs even at 1.4 m phosphate. Note that for *Arthrospira* and *Synechococcus* PBs, a minimum fluorescence yield was reached after about 15 s , followed by a slow recovery even under illumination. Part of the OCP_i reverted spontaneously to OCP_o , and the binding strength to these PBs was not sufficient to compensate. Changing buffer concentrations showed that *Synechocystis* OCP is able to trigger fluorescence quenching in any PB when the binding is sufficiently strong. Increasing phosphate concentration also strongly influenced *Arthrospira* PB fluorescence quenching by *Arthrospira* OCP (Fig. 2.8, A and C). Between 0.8 and 1.2 m phosphate, a maximum fluorescence quenching was observed after 15 to 35 s , followed by a partial fluorescence recovery as shown in Figure 2.7C. At higher phosphate concentrations, the fluorescence quenching increased to 85.5% and the recovery phase almost disappeared, suggesting an irreversible OCP binding. At 1.4 m phosphate, *Arthrospira* OCP_i induced 94% , 78.5% , and 92% fluorescence in *Synechocystis* PBs, *Synechococcus* PBs, and *Anabaena* PBs, respectively (Fig. 2.8C).

Comparison of Blue Light-Induced Fluorescence Quenching in *Arthrospira* and *Synechocystis* in Vivo

Figure 2.9A shows that strong blue-green light induced a slightly faster and larger fluorescence quenching in *Synechocystis* cells than in *Arthrospira* cells. This can be ascribed to a lower concentration of OCP in *Arthrospira* cells or to a weaker interaction between the OCP and *Arthrospira* PBs *in vivo*. We first tested the quantity of OCP present in *Arthrospira* and *Synechocystis* cells and membrane-bound PB complexes isolated from both strains. It was already shown that all the OCP present in the cells is attached to these complexes (^[109]). Western-blot analysis showed that OCP is present in similar concentrations in both strains (Fig. 2.9B), suggesting a weaker OCP interaction or a less effective induction of fluorescence quenching in *Arthrospira* PBs. On the other hand, absorbance spectra of whole cells presented a much larger chlorophyll (absorbance at 680 nm)-to-phycoobiliprotein (absorbance at $615\text{-}655 \text{ nm}$) ratio in *Arthrospira* cells than in *Synechocystis* cells (Fig. 2.9C). This indicated a lower PBs concentration in *Arthrospira* and, as a consequence, a higher OCP-to-PB ratio. This is also suggested by SDS-PAGE, in which the bands in the $15\text{-}20\text{-kD}$ region were stronger in *Synechocystis* than in *Arthrospira* cells and membrane-bound PB complexes (Fig. 2.9B). Since the ratio of OCP to PBs is higher in *Arthrospira* cells than in *Synechocystis* cells, a larger fluorescence quenching was expected in *Arthrospira* cells. This was not observed.

Discussion

This study was aimed at determining whether the OCP isolated from a given cyanobacterial strain can bind to PBs from a different strain, including those that lack the OCP, and can induce their fluorescence quenching. Using an *in vitro* reconstitution system, the combinations between various OCP,s (from *Synechocystis* and *Arthrospira*) and PBs (from *Synechocystis*, *Arthrospira*, *Synechococcus*, and *Anabaena*) were tested. The results obtained demonstrated that different OCPs are not equivalent in their capacity to induce PB fluorescence quenching or in their specificity. At 0.8 mM phosphate, *Synechocystis* OCP induced a large fluorescence quenching only in *Synechocystis* PBs (Fig. 2.4), and *Arthrospira* OCP was able to induce a rather large quenching in all types of PBs (Fig. 2.7C). At higher phosphate concentrations, *Synechocystis* OCP was able to induce fluorescence quenching of all PBs; however, the amplitude of quenching was always smaller than that induced by the *Arthrospira* OCP (Fig. 2.8A). It was previously shown that PB fluorescence quenching and OCP binding to PBs are correlated and that the absence of fluorescence quenching is due to a lack of binding between OCP and PBs [81]. Thus, the results described in this work strongly suggest that *Arthrospira* OCP binds more strongly than *Synechocystis* OCP to all tested PBs. However, each strain's OCP is able to quench the fluorescence of all PBs once bound to it.

Influence of OCP Primary Structures on Carotenoid Binding

The other distinctive difference between the two OCPs resides in their capacity to bind ECN instead of hECN; *Synechocystis* OCP binds both carotenoids, but *Arthrospira* OCP cannot stabilize the ECN binding. The crystal structures of *Synechocystis* OCP_o (1.65 Å resolution; [113]) and of *A. maxima* OCP_o (2.1 Å resolution; [95]) are known. The latter is 100% identical in primary structure to the *Arthrospira* OCP used in this study. The carotenoid structure and the secondary and tertiary protein structures of *Synechocystis* and *Arthrospira* OCPs are nearly identical [113]. The primary structures of the two OCPs investigated in this study are 83% identical. By analyzing the amino acids that differ between *Arthrospira* and *Synechocystis* OCPs, we can develop hypotheses about the underlying basis of the differences observed in carotenoid binding and in their ability to induce fluorescence quenching (probably due to changes in PB binding specificity) in these two OCPs.

In wild-type cells, both *Synechocystis* and *Arthrospira* OCPs bind hECN. When the *Synechocystis* ocp gene is overexpressed in a *Synechocystis* mutant lacking zeaxanthin and hECN, cells contain a high concentration of OCP binding only ECN [96,113]. In contrast, overexpression of the *Arthrospira* ocp gene in the same *Synechocystis* mutant results in cells in which almost no *Arthrospira* OCP is accumulated (Fig. 2.5A). Likewise, in a wild-type background, the carotenoid preferentially bound differs: *Synechocystis* OCP isolated from the overexpressing strain contained more ECN (50%-82% depending on the preparation) than hECN (11%-32%; [96,113]).

This is probably related to the higher concentration of ECN than that of hECN in cells. In contrast, *Arthrospira* OCP overexpressed in wild-type *Synechocystis* cells binds preferentially hECN over ECN (81% versus 19%; Supplemental Fig. S1), strongly suggesting that *Arthrospira* OCP is not able (or is less able) to stably bind ECN. The interaction of the carotenoid hydroxyl group with the protein seems to be essential for the stabilization of carotenoid binding in *Arthrospira* OCP. We have demonstrated that in *Synechocystis* OCP, the interaction of Tyr-44 and Trp-110 with the hydroxyl ring of the carotenoid is important for photoactivity [113]. In addition, the interaction between Trp-110 and the carotenoid seems to be important to stabilize ECN binding. When Tyr-44 was replaced by Ser, the OCP was present in high concentrations in the overexpressing Y44S-OCP cells and still bound 70% ECN, despite increased solvent accessibility of the carotenoid [113]. However, when Trp-110 was replaced by Ser or Phe, the mutated OCPs bound only 20% of ECN and the OCP concentration in the cells was reduced [113]. The replacement of Trp by Phe did not affect photoactivity but destabilized ECN binding, indicating that the interaction between Trp-110 and the carotenoid is essential for carotenoid binding. Due to the high sequence identity among amino acids forming the carotenoid-binding pocket and their similar orientation in the structures, it is difficult to explain why *Arthrospira* OCP cannot stably bind ECN. We can hypothesize that small changes in the

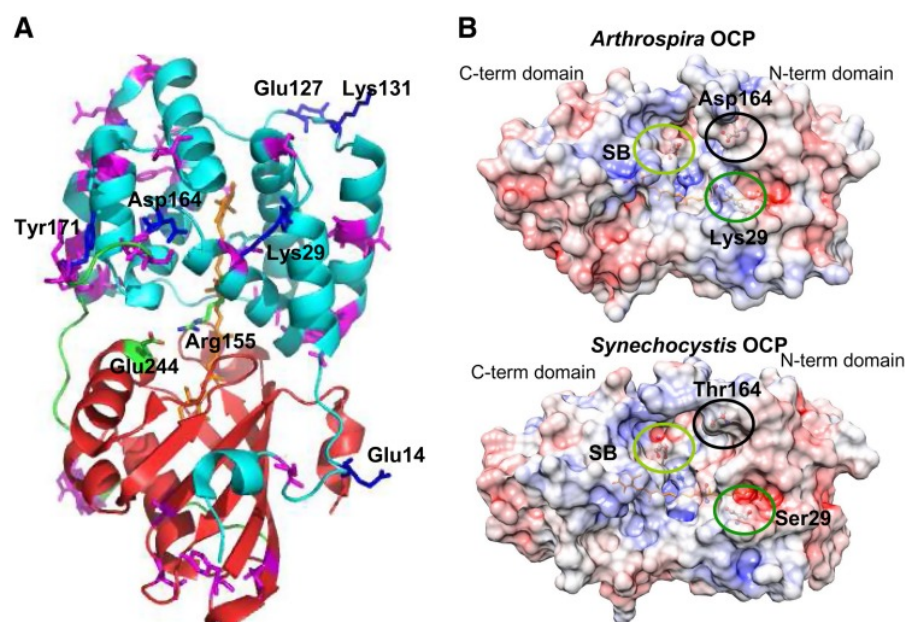


Figure 2.10 – Structural differences between *Arthrospira* OCP and *Synechocystis* OCP. A, Overview of the *A. maxima* OCP structure. Teal, N-terminal domain; green, linker region; red, C-terminal domain. 3' hECN is shown in orange. The amino acids changing between *Arthrospira* OCP and *Synechocystis* OCP are represented using purple sticks, and blue sticks are used for the ones bearing charges (and for Tyr-171). B, Electrostatic surface maps of *Arthrospira* and *Synechocystis* OCPs. For details, see “Materials and Methods.” SB, Salt bridge between Arg-155 and Glu-244(246).

positions of amino acid side chains could disturb the interaction between Trp-110 and the carotenoid ring. In the *Arthrospira* OCP structure, a conserved water molecule (H_2O 452) is hydrogen bonded directly to the 3'-OH group of 3' hECN^[95]. It is possible that this bond is partially responsible for the stabilization of 3' hECN in *Arthrospira* OCP. We cannot discard that subtle differences in solvent accessibility and the number of water molecules in the carotenoid pocket could also destabilize the $\pi-\pi$ interactions between the carotenoid ring and Trp-110 or other hydrophobic carotenoid-protein interactions, rendering the ECN binding more or less favorable. Finally, nothing is known about the mechanism of carotenoid binding to the OCP, and it is possible that nonconserved amino acids outside the carotenoid-binding pocket of *OCP_o* could be involved in this process.

Comparison of OCP-PB Interactions of Both OCPs

No significant differences in photoconversion kinetics or fluorescence quenching kinetics and amplitude were observed for hECN-OCP versus ECN-OCP from *Arthrospira* (Supplemental Figs. S2 and S3). Thus, the differences observed in photoactivity and fluorescence quenching properties of ECN-OCP from *Synechocystis* versus hECN-OCP from *Arthrospira* most likely result from differences in protein structures between both OCPs. Many amino acid substitutions between *Synechocystis* OCP and *Arthrospira* OCP are localized on the outer surface of the N-terminal domain: several neutral amino acids in the N-terminal domain of *Synechocystis* OCP are replaced by charged amino acids in *Arthrospira* OCP (Fig. 2.10A). These substitutions include N14E, S29K, Q72R, R112E, A127E, Q130K, and T164D. If the OCP-PB interaction has an important electrostatic component, the additional charged residues could strengthen the interactions between *Arthrospira* OCP and PBs. In addition, the replacement of a negative charge in *Synechocystis* OCP (Asp-115) by a positive charge in *Arthrospira* OCP (Arg-115) could also affect electrostatic interactions with the PBs. Differences in OCP binding strength to PBs are probably the cause of differences in fluorescence quenching, as demonstrated previously^[81].

The OCP's binding to the PB is known to involve a direct interaction between OCP's Arg-155 residue and the APC protein^[98]. Electrostatic surface plots of the re-

gion around Arg-155 of *Arthrospira* and *Synechocystis* OCPs are shown in Figure 2.9B. In each structure, Arg-155 (which forms a salt bridge with Glu-244/246 in the non-quenching form of the OCP) is buried near the carotenoid chromophore in a large, centralized surface depression at the interface of the C-terminal and N-terminal domains of the protein. In this interface, most amino acids are identical or conservatively substituted (i.e. Asp-146 versus Glu-146 or Lys-231 versus Arg-233). However, we note two nonconservative substitutions at the entrance to this surface depression : Ser-29 and Thr-164 (*Synechocystis*) to Lys-29 and Asp-164 (*Arthrospira*). While residue 29 is not highly conserved among OCP orthologs^[113], the positively charged Lys-29 of *Arthrospira* adds an additional positive charge in the surface depression leading to Arg-155. If the PB-OCP interaction involving Arg-155 is electrostatic in nature, the addition of a positive charge in this region could potentially enhance the binding interaction. The Thr-164 residue of *Synechocystis* is likewise notable, since residue 164 is highly conserved across orthologs^[113] and is most often negatively charged (as is the case with Asp-164 in *Arthrospira*). Given its proximity to Arg-155, the nature of its nonconservative substitution versus Asp-164 of *Arthrospira*, and its deviation from the majority of OCP sequences, we speculate that Thr-164 might be at least partly responsible for the unique specificity of *Synechocystis* OCP to *Synechocystis* PBs.

Finally, the flexible linker region between the C- and N-terminal domains could also lead to differences in activity of both OCPs if this region is involved in light-induced protein conformational changes or PB binding. The flexible linker is the least conserved region of the primary structure across OCP orthologs^[113] and possesses 59.5% identity in amino acid sequences between the OCPs investigated in this work. It is shorter in *Synechocystis* OCP, lacking two amino acids (Ser-170 and Tyr-171), which could restrict the opening of the protein in the red form and subsequently affect the binding to APC trimers. These residue-specific hypotheses await further testing. Additional interpretation is hindered by the fact that no structural data are yet available concerning OCP, or OCP-PB complexes.

Influence of PB Structure on the OCP-PB Interaction

In addition to differences in amino acid sequence between the *Arthrospira* and *Synechocystis* OCP, the PB structures also influenced the amplitude and kinetics of fluorescence quenching : a given OCP reacted differently with PBs isolated from different cyanobacterial strains. This is particularly true in 0.8 m phosphate, insufficient for a full stabilization of the OCP-PB complex when PBs other than *Synechocystis* PBs were used. In the past, this concentration of phosphate was considered optimal for *in vitro* fluorescence quenching induction^[81]. Here, our results clearly demonstrated that *Synechocystis* PBs are quenched much more efficiently by both *Synechocystis* and *Arthrospira* OCPs than any other PB (Figs. 4 and 8). It could be assumed that OCP and PBs from the same cyanobacterial strain, having evolved concomitantly, developed a high affinity toward each other for an optimized response to high light. The fact that *Arthrospira* PBs are only poorly quenched by *Arthrospira* OCP (Fig. 2.7C) raises important questions. One possibility is that the *Arthrospira* PBs used here could have been partially disassembled or could have lost some components required for OCP binding during isolation. However, absorption spectra combined with 77 K fluorescence emission spectra indicated that they were assembled and functionally connected PBs (Figs. 2 and 3). No striking variations compared with *Synechocystis* PBs appeared in terms of peptide composition, as revealed by SDS-PAGE (Fig. 2.2A). Nevertheless, Tian et al. (2011, 2012) showed that although the mechanism inducing fluorescence quenching was identical *in vivo* and *in vitro* and that the amounts of quenching were similar, the rate of quenching was slightly slower in the *in vitro* system than *in vivo*. This indicated that even in the case of *Synechocystis*, slight variations in PB structure, not detectable in fluorescence spectra, could affect OCP binding. In the case of *Arthrospira* PBs, perhaps changes in the core of the PB are induced that affect OCP binding but not energy transfer. A second interpretation can be formulated in which *Synechocystis* PBs are simply more prone to quenching than *Arthrospira* PBs even *in vivo*. The reasons could lie in different overall structures between the PBs or more specific changes in the primary structure of one or more PB proteins. We showed that a similar concentration of OCP induced slightly slower and smaller fluorescence quenching in *Arthrospira* cells than in *Synechocystis* cells and that a larger OCP-to-PB ratio in *Arthrospira* cells did not lead to larger fluorescence quenching.

Although our results suggest that *Arthrospira* PBs have a lower affinity for OCP than *Synechocystis* PBs *in vivo*, the effect seems to be much less pronounced than *in vitro*, since the difference in the amplitude of fluorescence quenching was small between both strains.

Electron microscopy on isolated complexes showed that *Synechocystis*, *Arthrospira*, *Synechococcus*, and *Anabaena* PBs have a common hemidiscoidal organization with PC rods radiating from an APC core (for review^[30,34]). The number of APC cylinders differs from strain to strain. This could be a factor influencing OCP-PB interactions, as OCP binds to APC upon photoactivation^[99-101]. However, our results did not confirm the hypothesis of a greater OCP binding in PBs with more APC trimers. *Synechococcus* PBs have only two APC cylinders but behaved like *Arthrospira* PBs (with three cylinders) in terms of fluorescence quenching *in vitro* (Figs. 4, 7, and 8). The missing upper cylinder, containing α/β -subunits of APC plus a small linker polypeptide, seems not to be required for OCP attachment. *Anabaena* PBs were generally less quenched than *Synechocystis* PBs. Thus, the two extra APC cylinders present in these PBs seem not to result in an increased affinity for OCP. Our results strengthen the proposal that the OCP binds to a basal APC cylinder.

Many isolated phycobiliproteins in their trimeric or hexameric aggregation states have been crystallized and their x-ray structures determined (for review^[30]). However, a global picture of fully assembled PBs is still lacking. For example, it is not known how PC rods and APC cores are biochemically connected. PC rods stabilize OCP binding to PBs^[81], and species-specific changes of this connection could lead to different affinities for the OCP. It was recently shown that a more or less pronounced cavity exists between the APC trimers^[134]. This could influence PC rod binding or OCP attachment, but a systematic crystallographic study is needed for verification. We have already observed that OCP is able to induce less fluorescence quenching when mixed with "old" PB preparations or reconstituted PBs, in which energy transfer to the terminal emitters was slightly decreased, suggesting a weaker connection between APC trimers (D. Jallet and D. Kirilovsky, unpublished data). Thus, the interaction between APC trimers seems to be important for OCP binding stabilization. This interaction could be modified in the PBs of the different cyanobacteria strains at least in the *in vitro* isolated preparations.

Finally, the OCP is thought to interact with α/β APC subunits emitting at 660 nm^[99-101] or with the ApcE core membrane linker^[135] or both^[136]. α APCs and β APCs are extremely well conserved, particularly within the group of cyanobacteria employed for this study (between 77% and 90% identity in primary structures compared with *Synechocystis*). ApcE is less conserved^[123], especially in the N-terminal phycobiliprotein part (60%-64% identity in primary structures to *Synechocystis* within the group employed here), because it contains a quite variable loop region of unknown function. This could also influence OCP attachment. Once again, the lack of structural data on OCP₇-PB complexes makes any further interpretation merely speculative.

Conclusion

This study results in four important conclusions about the relationship between OCP and PBs. (1) Structurally distinct OCPs from different strains exhibit unique PB interaction properties. *Synechocystis* OCP manifests a high specificity for its own PBs. *Arthrospira* OCP has a stronger affinity for all PBs relative to that of *Synechocystis* OCP and, therefore, appears to be less specific. Our results suggest a role for electrostatics in the interaction. (2) The structure of the PBs, probably the interactions between APC trimers forming the core cylinders, could have a big influence on OCP binding and its stabilization. (3) The upper APC cylinder is not necessary for OCP interaction with PBs, strongly suggesting that OCP binds to one of the basal APC cylinders. (4) Once OCP binds to the PB, it is able to quench the fluorescence of any type of PBs, even those isolated from strains lacking OCP. This is true for both OCPs investigated here. We also conclude that *Arthrospira* OCP would be a better candidate to introduce the photoprotective mechanism in *S. elongatus* and *T. elongatus* cells. These two cyanobacterial strains lack the OCP and are more sensitive to high-light conditions^[115].

Materials and methods

Culture Conditions

Synechocystis PCC 6803, *Synechococcus elongatus* PCC 7942, and *Anabaena variabilis* cells were grown photoautotrophically in a modified BG11 medium containing double amounts of sodium nitrate^[137]. Cells were kept in a rotary shaker (120 rpm) at 30°C, under CO₂ enrichment, illuminated by fluorescent white lamps giving a total intensity of about 90 μmol photons m⁻² s⁻¹. The *Arthrospira platensis* PCC 7345 culture conditions were identical, except for temperature. They were grown at 23°C. Cells were maintained in their logarithmic phase of growth.

oApOCP Plasmid Construction

A 1.7-kb DNA region containing the *ocp* and *frp* genes was amplified by PCR using genomic DNA of *Arthrospira* as a template. Two synthesized oligonucleotides containing the sequences for creating NdeI and HpaI restriction sites were employed for that purpose: *F_AP_OFNdeI* (5'-GACTTCCATATGCCATTACCATTCAGTCTGGC-3') and *R_AP_OFHpaI* (5'-GTAAGCGTTAACAGTCCAACACTCAACCCGC-3'). The boldface nucleotides correspond to the restriction sites and to the nucleotides coding the histidines.

The resulting PCR product was cloned into pPSBA2^[133] into the NdeI and HpaI restriction sites of the plasmid. Nucleotides encoding for 6His were added on the 3' side of OCP by site-directed mutagenesis (Quickchange XL kit; Stratagene) using the mutagenic oligonucleotides *F_AP_OFHis* (5'-CACCACCACCACCACCCTAGAA-TAGAGTTCACCTAGAAATTATATAGG-3') and *R_AP_OFHis* (5'-GTGGTGGTGGTGGTGGTGGCGCACCAAGTTCAACAACCTCTTTGG-3'). A 1.3-kb kanamycin resistance cassette was finally inserted in the unique HpaI site, situated 48 bp downstream of the FRP stop codon.

Transformation, Selection, and Genetic Analysis of Mutants

The oApOCP plasmid construct was used to transform wild-type and ΔCrtR *Synechocystis* cells (lacking the β-carotene hydroxylase and unable to produce zeaxanthin or 3' hECN), giving the oApOCPWT and oApOCPΔCrtR mutants, respectively. Selection was made at 33°C, under dim light (30 μmol photons m⁻² s⁻¹), on plates containing 40 μg mL⁻¹ kanamycin. The endogenous *slr1963* gene was then interrupted using the ΔOCP plasmid construct bearing a spectinomycin/streptomycin resistance cassette^[109]. To confirm the complete segregation of the different mutants, PCR analysis and specific digestions by restriction enzymes were performed.

Isolation of PBs

The protocol used derives from that described by Ajlani et al. (1995). After reaching optical density at 800 nm = 1, cyanobacteria cells were harvested through centrifugation at 54,00g, 23°C, for 6 min. They were washed twice using 0.8 m potassium phosphate buffer, pH 7.5, and their chlorophyll concentrations were then determined and adjusted to 1 mg mL⁻¹. Protease inhibitors were added (1 mM EDTA, 1 mM caproic acid, and 1 mM phenylmethylsulfonyl fluoride) as well as DNase (50 μg mL⁻¹) prior to breaking. *Synechococcus*, *Anabaena*, and *Arthrospira* cells were broken using a French press system (800 p.s.i.). *Synechocystis* cells were broken through vortexing in the presence of glass beads (diameter, 200 μm). The unbroken cells were removed by centrifugation at 2,000g, 23°C, for 5 min. The supernatant was incubated in the presence of 2% (v/v) Triton X-100 under dim stirring, at 23°C, during 2 h. For *Synechocystis*, *Arthrospira*, and *Synechococcus* PBs, the Triton X-100 phase and debris were removed by centrifugation at 20,000g, 23°C, for 20 min, and the dark blue supernatant was directly loaded onto a discontinuous Suc gradient. For *Anabaena* PBs, the Triton X-100-treated supernatant was centrifuged at 86,000g, 23°C, for 1 h. The dark-green supernatant was collected, and PBs were precipitated through centrifugation at 130,000g, 23°C, for 1 h. The dark blue pellet was resuspended using 1 m potassium phosphate buffer, pH 7.5, and deposited onto a Suc gradient. The Suc gradient for isolation of all PBs contained 0.25, 0.5, 0.75, and 1.5 m Suc layers in 1 m (final) potassium phosphate buffer, pH 7.5. The gradient was spun at 150,000g,

23°C, for 12 h. The different layers were collected, and their absorbance spectra were recorded.

Calculation of PB Concentrations

The calculation of PB concentrations was based on absorbance spectra. For *Synechocystis* PBs, 95% of the A620 comes from the absorption of PC^[138]. The extinction coefficient of a PC hexamer is 2,370 mm⁻¹ cm⁻¹^[116]. We estimated that the extinction coefficient of *Synechocystis* PBs containing 18 hexamers of PC (six rods with three PC hexamers each) is 42,660 mm⁻¹ cm⁻¹. We estimated that PC contributes to 85% of the A620 in *Arthrospira* PBs and *Anabaena* PBs and to 95% in *Synechococcus* PBs (Supplemental Fig. S1). Taking into account the different architectures, this leads to extinction coefficients of 39,390 mm⁻¹ cm⁻¹ for *Arthrospira* PBs, 30,004 mm⁻¹ cm⁻¹ for *Synechococcus* PBs, and 52,520 mm⁻¹ cm⁻¹ for *Anabaena* PBs.

Purification of the *Arthrospira* and *Synechocystis* OCP

His-tagged *Arthrospira* OCP was purified from oApOCPWT or oApOCPΔCrtR *Synechocystis* mutants and *Synechocystis* OCP from oSynOCPΔCrtR as described^[97] using a Ni-ProBond resin column and a Whatman DE-52 column. The isolated OCP was dialyzed against 40 mM Tris-HCl, pH 8.0, and frozen at -80°C.

Protein Separation and Immunoblot Analysis

Proteins were analyzed by SDS-PAGE on 12% polyacrylamide/2 M urea gels in a Tris-MES system^[139]. PB samples were concentrated by precipitation with 10% (v/v) TCA prior to loading (equal quantities in each lane). For whole cell extracts, 3 μg of chlorophyll was deposited per well. The gels were stained by Coomassie Brilliant Blue. The OCP protein was detected using a polyclonal antibody directed against *Arthrospira maxima* OCP.

Absorbance Measurements

The orange-to-red OCP conversion (and red-to-orange recovery) was monitored in a Specord S600 spectrophotometer (Analytikjena) and triggered using strong white light (5,000 μmol m⁻² s⁻¹). PB absorbance spectra were recorded in a Uvikon XL spectrophotometer (Secomam) at 23°C. Fluorescence Measurements

PAM Fluorometer

Fluorescence yield quenching was monitored using a pulse amplitude fluorometer (101/102/103-PAM; Walz). Measurements were made in 1-cm-pathlength stirred cuvettes. Experiments carried out on whole cells were performed at a chlorophyll concentration of 3 μg mL⁻¹ at 33°C. In vitro reconstitutions were handled with a PB concentration of 0.012 μM in potassium phosphate buffer (pH 7.5) concentrations ranging from 0.8 to 1.6 M at 23°C. Fluorescence quenching was induced *in vivo* by 1,400 μmol m⁻² s⁻¹ blue-green light (halogen white light filtered by a Corion cutoff 550-nm filter; 400-550 nm). In vitro blue-green light of 900 μmol m⁻² s⁻¹ was used for quenching.

Emission Spectra

Fluorescence emission spectra were monitored in a CARY Eclipse spectrophotometer (Varian). For studies at room temperature, samples were placed in a 1-cm stirred cuvette. For 77 K measurements, samples were collected in Pasteur pipettes and then frozen by immersion in liquid nitrogen. Excitation was made at 590 nm.

OCP Structural Modeling and Analysis

Prior to the calculation of electrostatic surface potentials, missing residues in *Synechocystis* OCP were modeled using Coot (Emsley and Cowtan, 2004), which allowed for the placement of previously missing portions of the flexible linker and the side chain of Thr-164 in this structure. Electrostatic surface potentials of chain A from

3MG1 and 1M98 were calculated using the Adaptive Poisson-Boltzmann Solver plugin implemented in the PyMOL Molecular Graphics System (version 1.6; Schrödinger). Supplemental Data

Acknowledgments

We thank Sandrine Cot and Markus Sutter for technical assistance, Adjélé Wilson for technical assistance in OCP preparation and discussions and Dr. Ghada Ajlani for fruitful discussions.

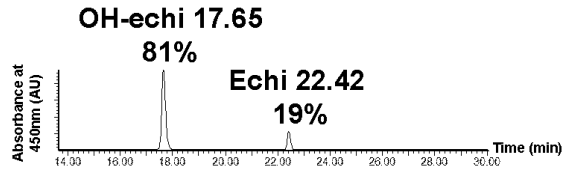


Fig.S1. HPLC analysis of carotenoids isolated from purified *Arthrospira* OCP (oApOCPWT mutant cells)

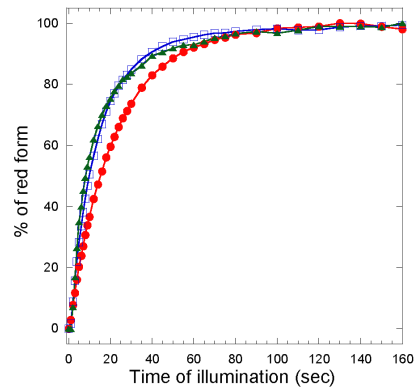


Figure S2. Photoactivation kinetics of the 3^hECN or ECN binding *Arthrospira* OCP

OCP⁰ (1.8 μ M) was illuminated using strong white light (5000 μ mol.m⁻².s⁻¹). The accumulation of OCP^r was followed by measuring ΔA_{550nm} over time in TrisHCl 40mM pH 8, at 23°C; 3^hECN-binding *Arthrospira* OCP isolated from oApOCPWT cells (blue squares), ECN-binding *Arthrospira* OCP isolated from oApOCP Δ CrtR cell (green triangles) and ECN-binding *Synechocystis* OCP isolated from oScOCP Δ CrtR cells (red circles). Data are normalized to the maximal ΔA_{550nm} corresponding to 100% OCP^r

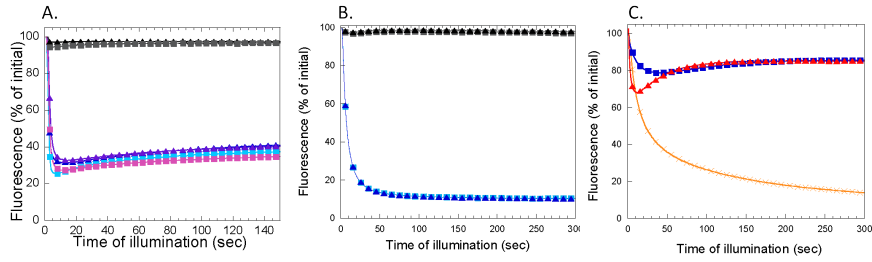


Figure S3. Effect of ECN or 3'hECN binding on phycobilisome fluorescence quenching induced by *Synechocystis* and *Arthrospira* OCP in vitro.

The isolated PBs were illuminated with blue-green light ($900\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) in the presence of an excess of preconverted OCP^R at 23°C.

A) Fluorescence quenching induced by adding 40 3'hECN binding *Synechocystis* OCP^R (isolated from WT, tagged cells; triangles) or 40 ECN binding *Synechocystis* OCP^R (isolated from oOCP6803ΔCrtR; squares) under strong blue-green illumination ($900\mu\text{molphotons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) to *Synechococcus* PBs at 0.8 MK-Phosphate (Black/grey curves) and 1.4M K-Phosphate (blue curves) or to *Arthrospira* PBs at 1.4M K-Phosphate (purple/pink curves).

B) Fluorescence quenching induced by adding 40 3'hECN (triangles) or ECN (squares)-binding *Synechocystis* OCP^R, at 0.8M K-Phosphate under strong blue-green illumination, to *Arthrospira* PBs (black/grey curves) or *Synechocystis* PBs (blue curves)

C) Fluorescence quenching induced by adding 20 ECN binding *Arthrospira* OCP^R (isolated from oApOCPΔCrtR cells) per *Arthrospira* PB (blue squares), *Synechococcus* PB (red triangles) and *Synechocystis* PB (orange crosses) in 0.8 M K-Phosphate. A ratio of 20 OCP per PB instead of 40 OCP per PB was used because of the extremely small amounts of *Arthrospira* OCP retrieved from the oApOCPΔCrtR cells. Due to the small quantities of ECN-*Arthrospira* OCP isolated from the ΔCrtR strain and the hECN-*Synechocystis* OCP isolated from WT cells we were unable to test all the different PBs.

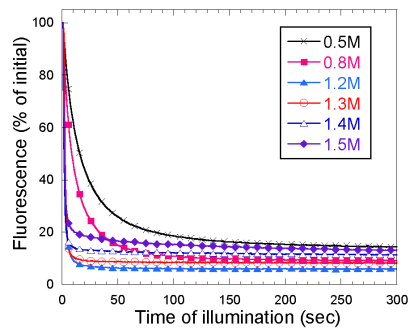


Figure S4. Effect of K-Phosphate concentration on *Synechocystis* PBs fluorescence quenching by *Synechocystis* OCP^R

Synechocystis PBs ($0.012\mu\text{M}$) were illuminated with blue-green light ($900\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) in the presence of pre-converted *Synechocystis* OCP^R ($0.48\mu\text{M}$; 40 per PB) at 23°C using various K-Phosphate buffer concentrations: 0.5M black crosses, 0.8M pink closed squares, 1.2M light blue closed triangles, 1.3M red open circles, 1.4M deep blue open triangles and 1.5M violet closed diamonds.

2.0.2 Résultats complémentaires à l'article Jallet et al., 2013

Nous avons observé dans l'article précédent que l'OCP de *A. platensis* est photoconvertie plus rapidement que l'OCP de *Synechocystis*. De plus, elle est moins spécifique et s'attache plus fort aux différents PBs que l'OCP de *Synechocystis*. La séquence protéique des deux OCPs sont très proches (83% d'identité de séquence protéique).

L'une des principales différences entre les OCP de *Synechocystis* et de *A. platensis* est la longueur de la région linker qui relie les domaines N- et C-terminaux. Ce linker est plus court chez *Synechocystis* (résidus 170 et 171 absents). Aussi, afin de savoir si cette différence de taille de linker est responsable des différences entre OCP de *Synechocystis* et *A. platensis*, j'ai modifié l'OCP de *Synechocystis* pour la doter d'un linker aussi long que celui de *A. platensis*. J'ai ensuite testé la photoactivité, et le quenching de fluorescence des PBs induit par cette OCP modifiée.

Résultats

J'ai créé par mutagenèse ponctuelle (primers utilisés en annexe 1) et isolé chez *E. coli* (voir le protocole d'expression et de purification présenté dans l'article Bourcier et al., 2015^[140]) une version modifiée de l'OCP de *Synechocystis* dont le linker est allongé avec les deux acides aminés présents dans l'OCP de *A. platensis*.

Afin de tester la photoactivité, j'ai effectué des cinétiques de photoconversion en suivant l'absorbance à 550 nm (longueur d'onde à laquelle les changements sont les plus importants au cours de la photoactivation) au cours du temps à 18°C. Une fois illuminée durant 5 minutes, l'extinction de la lumière permet de suivre la cinétique de retour à la forme orange. La cinétique de photoactivation et la cinétique de retour à la forme orange étant identiques entre OCP de *Synechocystis* au linker allongé et le WT, je conclus que la taille du linker n'influence pas la photoactivation de la protéine.

La reconstitution *in vitro* du mécanisme de photoprotection permet de suivre l'activité de quenching de fluorescence de l'OCP activée. Brièvement, l'OCP est dans un premier temps photoconvertie puis ajoutée à une solution de PBs en suspension et maintenue rouge par une forte lumière bleue-verte. La fluorescence des PBs est suivie au cours du temps. Après ajout d'OCP, on observe une diminution de la fluorescence des PBs (figure 2.11). La diminution de fluorescence induite par l'OCP de *Synechocystis* au linker plus long est plus lente et moins importante que l'OCP WT. La lumière est ensuite éteinte, et la cinétique de retour de la fluorescence mesurée. Le retour de la fluorescence est plus rapide avec cette OCP modifiée, traduisant une plus faible affinité pour les PBs que l'OCP WT.

Conclusion

Mon hypothèse à l'origine de ces expériences était que la différence de longueur du linker entre celui de *Synechocystis* et celui de *A. platensis* pouvait expliquer que l'OCP de *A. platensis* se photo-converti plus vite, et son affinité pour les PBs est plus forte. Cependant, les résultats montrent que la vitesse de photoconversion est identique et son affinité pour les PBs est plus faible. En conclusion, la différence de taille du linker n'est pas suffisante pour expliquer les différences entre les deux OCPs.

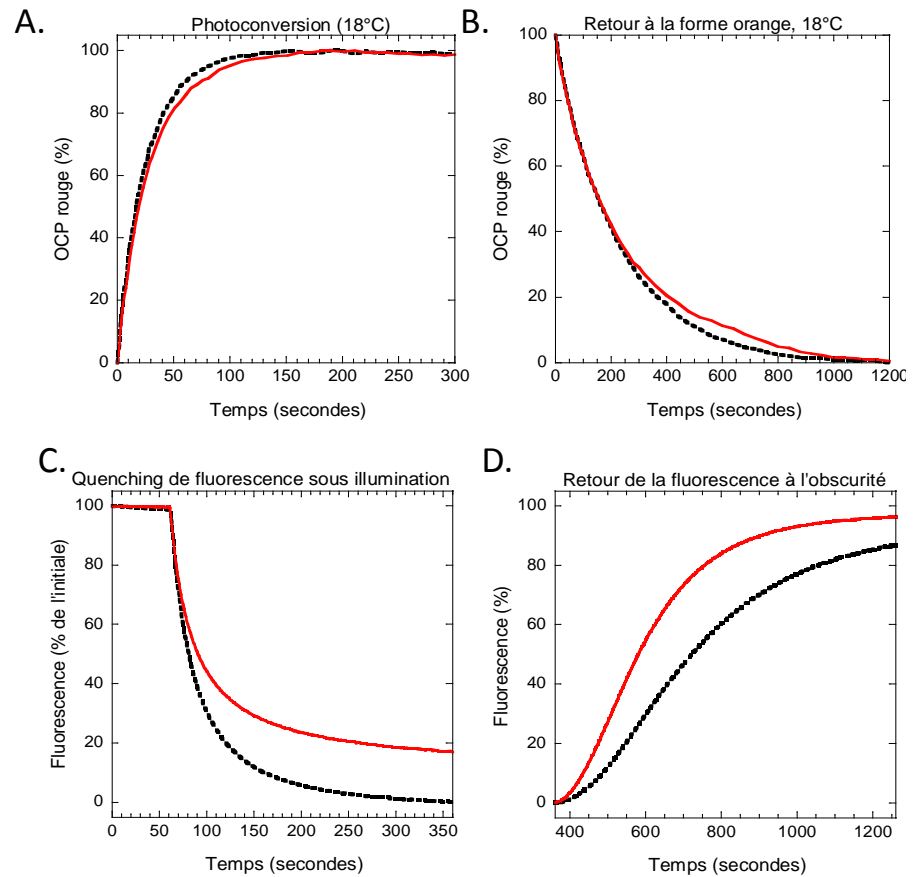


Figure 2.11 – Caractérisation de l'OCP de *Synechocystis* dont le linker est allongé de 2 acides aminés

(A) Cinétique de photoactivation par une forte lumière blanche ($5000 \mu\text{moles de photons} * m^{-2} * sec^{-1}$) et (B) cinétique de retour à la forme orange à l'obscurité des OCP modifiées dont le linker est allongé (rouge) et WT (noir en pointillé) à 18°C. (C) Quenching de fluorescences des PBs à 23°C induit par les OCP modifiée dont le linker est allongé (rouge) et WT (noir en pointillé) pré-illuminés 5 minutes par une forte lumière blanche ($5000 \mu\text{moles de photons} * m^{-2} * sec^{-1}$) à 4°C. Les PBs sont illuminés avec une forte lumière bleu-verte puis l'OCP rouge est ajoutée à 60 secondes d'illumination. Ratio 40 OCP par 1 PBs, tampon K-phosphate 0,5M. (D) Après 5 minutes d'illumination, la lumière est éteinte et la récupération de la fluorescence est suivi au cours du temps.

COLLABORATION AVEC CÉLINE BOURCIER

Le travail de mise au point de la production d'OCP chez *E. coli* a été réalisé au laboratoire et breveté par Céline Bourcier (financement CIFRE). L'entreprise partenaire, Phycosource, s'intéresse aux propriétés antioxydantes de l'OCP en vue de l'utiliser en cosmétique et pharmacologie. Le rôle antioxydant de l'OCP a été démontré au laboratoire^[141]. L'OCP a donc un double rôle : quencher l'excès d'énergie au niveau des antennes et quencher l'oxygène singulet. Jusqu'alors, au laboratoire, l'OCP était surexprimée chez la cyanobactérie *Synechocystis*. La séquence codante de l'OCP (originelle ou modifiée) a été clonée à la place du gène très exprimé *psbA2*. Cependant, la quantité d'OCP purifiée, si elle est suffisante pour l'usage au laboratoire, reste insuffisante pour un usage industriel (3 semaines sont nécessaires pour obtenir 40 mg de protéines à partir de 30 L de cyanobactéries). L'organisme de production est *Escherichia coli* dont les rendements de production sont jugés bons et dont la culture en bioréacteurs est parfaitement maîtrisée par l'industrie.

Le choix du caroténoïde lié s'est porté sur l'échinénone. L'OCP isolée chez le mutant de *Synechocystis* Δ CrtR, qui ne synthétise plus de 3'hECN, lie majoritairement l'échinénone. Elle présente les mêmes propriétés physiques et fonctionnelles que l'OCP-WT liant le 3'hECN^[96]. Par ailleurs, l'enzyme CrtR permet non seulement la synthèse de 3'hECN mais également la synthèse de Zéaxanthine depuis le β carotène. L'OCP peut lier ce caroténoïde, cependant, l'OCP-Zéaxanthine n'est pas photoactive et n'induit pas de quenching de fluorescence.

J'ai contribué à cet article en mesurant d'activité des OCP modifiées avec le système de reconstitution *in vitro* de quenching de fluorescence des PBs.

3.0.1 Résumé de l'article Bourcier de Carbon et al, 2015

Jusqu'alors, les caroténo-protéines étaient obtenues par intégration *in vitro* d'un caroténoïde dans une apo-protéine (protéine sans caroténoïde) produite par un organisme tierce. Dans cet article, nous présentons un système de production d'holo-OCP (protéine avec caroténoïde) par *E. coli*. Ce système de production implique 3 plasmides dans lesquels sont clonés les gènes codant pour les enzymes de la voie de biosynthèse de l'échinénone et de l'OCP (figure 2 de l'article) :

- pAC- β contient les enzymes (*crtE*, *crtI*, *crtY* et *crtB*) impliquées dans la voie de biosynthèse du β -carotène à partir d'isopentylpyrophosphate
- pBAC contient l'enzyme *crtO* (ou *crtW*) qui catalyse la transformation du β -carotène en échinénone (ou canthaxanthine) sous le contrôle du promoteur inductible (arabinose) *araC*
- pCDF contient la séquence codante de l'OCP (de *Synechocystis*, *A. variabilis* ou *A. platensis*) sous le contrôle du promoteur inductible (IPTG) T7

L'induction séquentielle des deux plasmides par l'arabinose, puis l'IPTG permet de débiter la synthèse d'apo-OCP seulement lorsque les membranes sont déjà riches en caroténoïde (échinénone ou cantaxanthine). Une induction du promoteur IPTG trop précoce crée une accumulation d'apo-OCP. Un HisTag est ajouté à la protéine, soit au début (N-ter), soit à la fin (C-ter) de la séquence codante.

Le meilleur rendement de production (holo-OCP par litre de culture) est obtenu avec l'OCP dont le tag est placé en N-terminal (holo-OCP >95%). Quand le tag est placé en C-terminale, la quantité de holo-OCP obtenue est moindre (25-40%). L'ajout de résidus en N-terminale à la protéine dont le tag est en C-terminal permet d'augmenter la quantité de holo-OCP (jusqu'à >95% avec 8 résidus). L'extrémité N-terminal a donc un rôle prépondérant dans l'entrée ou la stabilisation du caroténoïde au sein de l'OCP chez *E. coli*. Les OCPs de *Synechocystis* Nter et Cter induisent un quenching de fluorescence des PBs semblable à l'OCP produite chez *Synechocystis*. En revanche, à l'obscurité, dès lors que l'extrémité N-terminal est modifiée, l'OCP se détache plus rapidement du phycobilosome que l'OCP isolée de *Synechocystis*. Les

versions de l'OCP contenant des modifications en Nter et un HisTag en Cter (Syn-NC6aaCtag-ECN-OCP, figure 6 de l'article), le quenching induit est faible.

Toutes les OCPs produites d'après les séquences extraites de *Synechocystis*, *A. variabilis* ou *A. platensis* et contenant l'ECN ou la CAN sont photoactives (figure 3). Cependant, l'OCP de *A. variabilis* n'est convertie à la forme rouge que si elle lie la CAN (Figure 4). L'OCP de *A. platensis* peut induire un large quenching de fluorescence (96%), légèrement supérieur à celui de l'OCP de *Synechocystis* (93%, sup fig. 6). En revanche, l'OCP de *A. variabilis* ne peut induire autant de quenching de fluorescence que l'OCP de *Synechocystis*, que ce soit l'ECN (60%) ou la CAN (20%) qui soit lié (Sup Fig 6).

Conclusion

Le système de production de l'OCP chez *E. coli* permet une obtention rapide d'OCP. Cependant, certaines constructions sont contaminées par la présence de protéine ne liant pas de caroténoïde, l'apoOCP. L'extrémité Nter de l'OCP est essentielle dans l'attachement du caroténoïde à l'apoprotéine, dans la photoactivation et l'attachement de l'OCP aux PBs. Les OCPs de *Synechocystis*, *A. variabilis* et *A. platensis* présentent une affinité différente pour les caroténoïdes et les PBs malgré leur très forte homologie. L'OCP de *A. variabilis* ne peut induire qu'un faible quenching de fluorescence. Nous n'avons pas réussi à expliquer ces différences d'après la séquence primaire de ces protéines, et des expériences complémentaires sont requises pour les expliquer.

Biosynthesis of soluble carotenoid holoproteins in *Escherichia coli*

Published in Scientific Reports in 2015

Céline Bourcier de Carbon, Adrien Thurotte, Adjélé Wilson, François Perreau and Diana Kirilovsky

Abstract

Carotenoids are widely distributed natural pigments that are excellent antioxidants acting in photoprotection. They are typically solubilized in membranes or attached to proteins. In cyanobacteria, the photoactive soluble Orange Carotenoid Protein (OCP) is involved in photoprotective mechanisms as a highly active singlet oxygen and excitation energy quencher. Here we describe a method for producing large amounts of holo-OCP in *E. coli*. The six different genes involved in the synthesis of holo-OCP were introduced into *E. coli* using three different plasmids. The choice of promoters and the order of gene induction were important: the induction of genes involved in carotenoid synthesis must precede the induction of the *ocp* gene in order to obtain holo-OCPs. Active holo-OCPs with primary structures derived from several cyanobacterial strains and containing different carotenoids were isolated. This approach for rapid heterologous synthesis of large quantities of carotenoproteins is a fundamental advance in the production of antioxidants of great interest to the pharmaceutical and cosmetic industries.

Introduction

The human body is constantly exposed to external (ultraviolet radiation, pollution, cigarette smoke, toxic chemicals) and internal (side reactions of respiration, oxidation of nutrients) factors which induce the formation of Reactive Oxygen Species (ROS). Due to the harmful effects of ROS, the pharmaceutical and cosmetic industries have a significant interest in the production of new antioxidant molecules. For many applications, the medium in which the anti-oxidant effect is desired is water-based and requires a hydrophilic antioxidant. Water soluble carotenoid proteins fit these requirements. Carotenoids are widely distributed natural pigments which play important roles in photosynthesis, nutrition and illness prevention. They have a protective role in photosynthetic and non-photosynthetic organisms including humans by serving as protective colorants or by quenching singlet oxygen (1O_2) and free radicals induced by exogenous sensitizers or produced by metabolic processes (reviews^{[142][143][144][145]}). Carotenoids which are relatively hydrophobic molecules, typically occur solubilized in membranes or non-covalently attached to membrane or soluble proteins. In photosynthetic organisms, they are mainly found in the membrane-embedded, chlorophyll-containing-antennae where they have the dual activities of harvesting solar energy and quenching excess energy and 1O_2 (see reviews^{[142][146]}). A number of water soluble carotenoid proteins from photosynthetic organisms have also been characterized^{[147][84][148]}. The cyanobacterial Orange Carotenoid Protein (OCP) is one of the best characterized soluble carotenoid proteins. We have recently demonstrated that the OCP is an excellent antioxidant—better than vitamin C, trolox, tocopherol and isolated carotenoids^[141]. The OCP protects cyanobacteria by quenching the 1O_2 formed in reaction centers and antennae^[141]. It was first described by Holt and Krogmann in 1981^[93] and is present in the majority of cyanobacteria containing phycobilisomes (PBs), the large extra-membrane antenna formed by blue and red phycobiliproteins^[149]. The OCP is a photoactive protein^[97]; it is essential for a photoprotective mechanism that decreases the excitation energy arriving at photochemical reaction centers^[109]. The OCP has an α -helical N-terminal domain (residues 15-165) and an α β C-terminal domain (residues 190-317) (Fig. 1A)^[95]. The carotenoid, 3'-hydroxyechinenone (hECN), spans both domains which are joined by a flexible linker. The presence of a ketocarotenoid is essential for OCP photoactivity^[96]. Light absorption

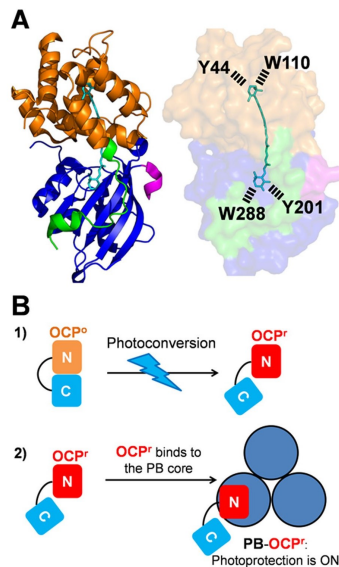


Figure 3.1 – The OCP and photoprotection. (A) Structure of the OCP from *Synechocystis* PCC 6803 (Protein Data Bank ID : 3MG1) The OCP monomer in the OCP_o state. The N-terminal arm (residues 1-22) (green) interacts with the C-terminal domain (residues 196-315) (blue). The C-terminus is colored in rose. The N-terminal domain (residues 22-165) is orange. The hydroxy-echinenone (hECN) spans both domains of the protein. Tyr201 and Trp288 of the C-terminal domain hydrogen bond to the carbonyl group of hECN. Tyr44 and Trp110 of the N-terminal domain interact with the hydroxyl ring of hECN. The OCP was modified adding amino acids in the N-terminus (green) and/or the C-terminus (rose). (B) Model of the OCP-related photoprotective mechanism. 1) Upon light absorption the orange OCP_o is converted into the active open red OCP_r . 2) OCP_r binds to the PB core and induces fluorescence and excitation energy quenching.

by the carotenoid induces conformational changes in the carotenoid and in the protein that are essential for its photoprotective function^[97] (Fig. 1B). In darkness, the OCP is orange (OCP_o); upon illumination, it becomes red. The red form (OCP_r) is the active form of the protein^[97]^[96]. Only OCP_r is able to bind the PBs. Once the OCP_r is bound, the carotenoid interacts with a chromophore of the PB core and quenches the excitation energy^[81]^[150]^[98]. This photoprotective mechanism is activated by blue-green light but not by orange or red light that are not absorbed by the carotenoid. However, the OCP photoprotects cyanobacteria from strong orange-red light; this protection is related to the 1O_2 quenching activity of the OCP^[141].

Due to the outstanding antioxidant properties of carotenoids and their role in human health, substantial effort has been devoted to the engineering of noncarotenogenic bacteria to produce high quantities of these colorants; this also requires the development of methods to isolate the carotenoids from the engineered microorganisms (reviews^[151]^[152]^[153]^[154]). For many applications, a water soluble antioxidant is needed. The soluble OCP which is an excellent 1O_2 quencher, is an ideal candidate. However, to-date there are no reports showing that it is possible to insert a carotenoid molecule in a protein in *E. coli*. To-date, genes encoding carotenoid proteins have been expressed in *E. coli* to isolate the apo-protein (protein without carotenoid) and then the carotenoid is attached to the apo-protein by *in vitro* reconstitution (examples^[152]^[155]^[156]^[157]). The OCP has been isolated from the WT cyanobacterial strains *Synechocystis* PCC 6803 (thereafter *Synechocystis*) and *Arthrospira maxima* or from *Synechocystis* mutants overexpressing WT or mutated OCPs^[97]^[95]^[135]^[113]^[158]. This, however, is a labor-intensive process because of the low concentration of the OCP in cyanobacterial cells. Indeed, even when using OCP-overexpressing strains purification requires 3 weeks to obtain 40 mg protein from 30 L of cyanobacteria cells (A. Wilson and D. Kirilovsky, unpublished data). Here we describe the construction of *E. coli* strains that are able to synthesize large amounts of OCP homologs from different cyanobacterial strains incorporating various carotenoids *in vivo*. This fast holo-OCP production has already enabled us to further understand the function of

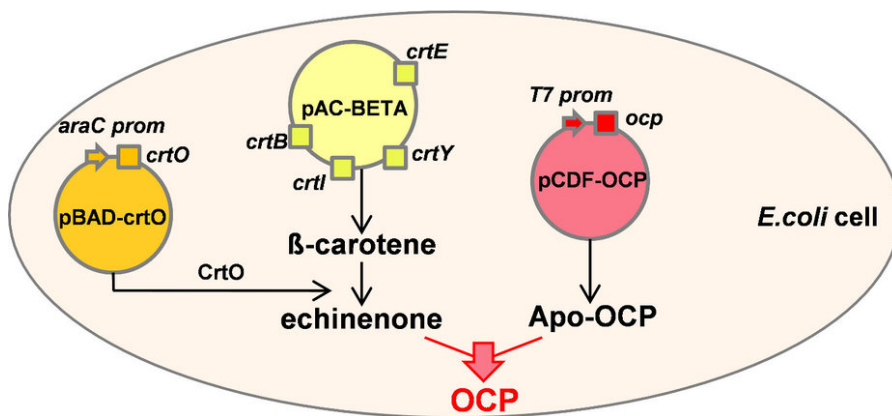


Figure 3.2 – Schematic representation of OCP production in *E. coli*. *E. coli* producing ECN-OCP contain three plasmids : pAC-Beta (yellow) carries the genes involved in β -carotene synthesis (*crtB*, *crtE*, *crtI* and *crtY*) under the control of the *crtE* promoter; pBAD-*crtO* (orange), carries the *crtO* gene under the control of *araC* promoter (arabinose inducible); CrtO enzyme converts β -carotene in echinenone; pCDF-OCP carries the *ocp* gene under the control of an IPTG inducible promoter (red).

different carotenoids in OCPs, for example that canthaxanthin-OCPs are very good energy and 1O_2 quenchers. The work described here is important not only to accelerate the elucidation of the OCP photoprotective mechanism by rapid synthesis of variant OCPs, but promises to enable the isolation and characterization of other carotenoid proteins with potential applications for promoting human health.

Results

Biosynthesis of His-tagged holo-OCPs in *E. coli* cells

The aim of our work was to synthesize holo-OCPs (OCPs attaching one carotenoid molecule) from *Synechocystis*, *Arthrospira* and *Anabaena* strains in *E. coli* cells. For this purpose, the genes coding for enzymes involved in the synthesis of the desired carotenoids (supplementary Fig. 1) and the *ocp* gene must be expressed in the same cell. It is known that in *Synechocystis* and *Arthrospira* OCP binds hECN^{[93][95]}. Previous work showed that it is difficult to obtain large quantities of this carotenoid in *E. coli* cells^{[159][160]}. We decided to express the *ocp* gene in the presence of two other ketocarotenoids : echinenone (ECN) and canthaxanthin (CAN). *Synechocystis* OCP is able to bind ECN and the ECN-OCP is photoactive and induces PB fluorescence quenching^[113]. In contrast, *Arthrospira* OCP weakly binds ECN^[37]. Prior to this study, nothing was known about the *Anabaena* OCP. Although we did not know if CAN-OCP would be active, we hypothesized that the carbonyl groups present in CAN rings could allow photoactivity and stabilization of the carotenoid binding. The *E. coli* cells producing holo-OCPs carried three plasmids. The first plasmid, pAC-BETA (or pACCAR16 Δ crtX)^{[161][162]}, contained the *Erwinia herbicola* (or *Erwinia uredovora*) operon carrying the four genes (*crtB*, *crtE*, *crtI*, *crtY*), which are necessary to synthesize β -carotene. In the second plasmid the *crtO* gene of *Synechocystis* or the *crtW* gene from *Anabaena* PCC 7210 was introduced. While CrtO is a monoketolase synthesizing mostly echinenone from β -carotene^[163], CrtW is a diketolase that catalyses the formation of canthaxanthin^[164] (supplementary Fig. 1). The *ocp* genes were cloned in a third plasmid (pCDFDuet-1). In order to maintain the three plasmids within the same *E. coli* cell, the use of three different and compatible replication origins and three different antibiotic resistances was required (Fig. 2).

The operon containing the *crtB*, *crtE*, *crtI* and *crtY* genes was under the control of the constitutive *crtE* promoter. Thus, the β -carotene was constitutively synthesized in the *E. coli* cells. The *crtO* and *crtW* genes were under the control of the arabinose inducible promoter *araBAD*. The transcription of *ocp* genes was controlled by the IPTG-inducible T7lac promoter. A sequential induction of these genes was essential to isolate high quantities of holo-OCP. The expression of *crtO* or *crtW* genes was induced in *E. coli* containing a relatively high concentration of β -carotene. Sub-

sequently the expression of *ocp* genes was induced in *E. coli* cells containing high concentrations of ECN or CAN in their membranes. The *E. coli* cells carrying the *crtO* gene contained 15-25% of β -carotene, 70-80% ECN and 4-6% CAN. When the *ocp* genes were expressed in the presence of ECN, a mixture of apo- and holo-OCPs was obtained in all cases, but the proportion of holo-OCP varied (Table 1). The presence of photoactive holo-OCPs was already detected *in vivo*. Orange cells containing holo-OCPs became red when they were illuminated with high intensities of white light (Fig. 3A). In contrast, yellow and orange *E. coli* cultures containing only genes involved in β -carotene and ECN (without the *ocp* gene) did not change colour upon illumination (Fig. 3A). Once the cells were broken, a notable difference in the colour of the supernatants (soluble fraction) was observed. The supernatant derived from *E. coli* cells synthesizing only carotenoids, because the *ocp* gene was not induced, was clear (Fig. 3Bb) and the orange colour was concentrated in the membranes (Fig. 3Ba). The slight colour observed in the supernatant is attributable to a leak of the T7 promoter and the presence of a small concentration of OCP. In contrast, in the cells in which the *ocp* gene was induced, the supernatant was distinctly orange (Fig. 3Bc) indicating the presence of high concentrations of holo-OCPs. Moreover, under illumination the supernatant became red (Fig. 3Bd). Nevertheless, the membranes remained orange indicating that the presence of apo-proteins was not related to a lack of ECN in the cells.

Table 1 – Percentage and amount of holo-OCP obtained from 1 L culture of *E. coli*. The Sequences of amino acids added to the N- or C-terminus are given

| Name of OCP | Added Amino acid sequence | Total OCP mg/L | Holo -OCP % total | Holo -OCP mg/L |
|-----------------------------|---------------------------|----------------|-------------------|----------------|
| ECN – Syn. | | | | |
| Syn-Ctag-ECN | -HHHHHH-StopCodon | 4–6 | 25–40 | 1–5 |
| Syn-pDuet-ECN | MGSS-HHHHHH-SQDP- | 18–22 | 35–45 | 5–10 |
| Syn-MIX15aaCtag-ECN | MGSSRLDNPEKTDIEP- | 20–22 | 30–40 | 5–10 |
| Syn-NC15aaCtag-ECN | MGSSNQANQVTLNPQV- | 18–21 | 45–55 | 8–10 |
| Syn-NC10aaCtag-ECN | MGSSNQANQVT- | 9–11 | >95 | 10 |
| Syn-NC8aaCtag-ECN | MGSSNQANQ- | 15–17 | >95 | 15 |
| Syn-NC6aaCtag-ECN | MGSSNQA- | 19–21 | 78–85 | 15–20 |
| Syn-NC3aaCtag-ECN | MGSS- | 8–10 | 70–80 | 5–10 |
| Syn-C8aaCtag-ECN | MGSSKKRRA- | 20–22 | 78–85 | 15–20 |
| Syn-3aaNtag-ECN | MGSS-HHHHHH- | 30–35 | ≥95 | 30–35 |
| ECN-Ana. and Arthro. | | | | |
| Ana-3aaNtag-ECN | MGSS-HHHHHH- | 50–60 | 50–60 | 25–35 |
| Ana-Ctag-ECN | -HHHHHH-StopCodon | 50–60 | 40–45 | 20–25 |
| Arthro-3aaNtag-ECN | MGSS-HHHHHH- | 25–30 | 30–40 | 5–15 |
| Arthro-Ctag-ECN | -HHHHHH-StopCodon | 15–20 | 10–25 | 1–5 |
| CAN | | | | |
| Arthro-3aaNtag-CAN | MGSS-HHHHHH- | 25–30 | 60–65 | 15–20 |
| Arthro-Ctag-CAN | -HHHHHH-StopCodon | 30–35 | 50–60 | 15–20 |
| Syn-3aaNtag-CAN | MGSS-HHHHHH- | 8–10 | 75–85 | 5–10 |
| Ana-3aaNtag-CAN | MGSS-HHHHHH- | 60–70 | 40–45 | 25–30 |
| Ana-Ctag-CAN | -HHHHHH-StopCodon | 60–70 | 50–60 | 30–45 |

The first *Synechocystis* *ocp* overexpressed genes contained an addition of 18 nucleotides coding for six His just before the stop codon (Syn-Ctag) or an extension of 45 nucleotides after the first ATG (Syn-pDuet) (supplementary Fig. 2 and Table 1). This extension which includes the sequence coding for six-His is already inclu-

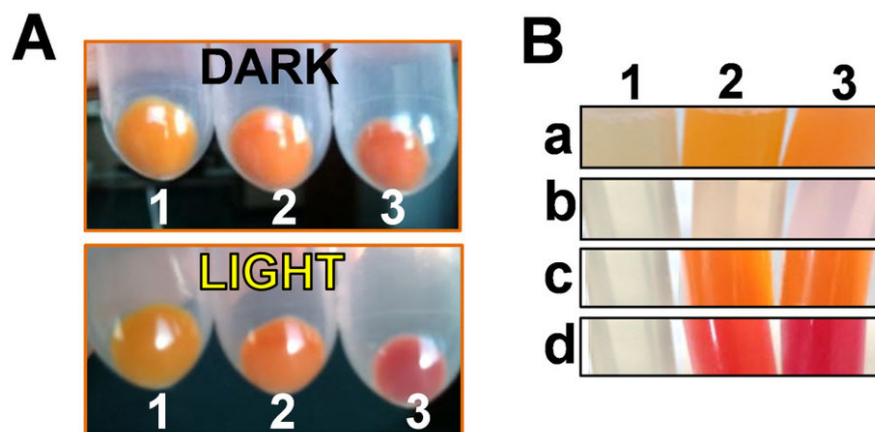


Figure 3.3 – Photoactivity of the OCPs produced in *E. coli*. (A) Photoactivity observed in cells. Cell pellets carrying the three plasmids required for the synthesis of OCP in darkness or after 5 min illumination ($5000\mu\text{moles de photons} * m^{-2} * sec^{-1}$ of white light) : cells pellet without any induction (1), cells after arabinose induction producing ECN (2), cells after induction with both, arabinose and IPTG, producing holo-OCP (3). (B) Location of carotenoids and OCPs in *E. coli* cells : (a) membrane and (b) soluble fractions of *E. coli* cells without plasmids (1) or with the three plasmids and after arabinose induction, synthesizing ECN (2) or CAN (3). (c) and (d) show the soluble fractions of cells producing ECN-OCP (2) and CAN-OCP (3) in darkness (c) and after illumination (d).

ded in the commercial pCDFDuet-1 plasmid. Analysis of the resulting two isolated *Synechocystis* OCPs revealed that the addition of the N-terminal extension of 45 nucleotides increased the total amount of OCP present in the cells (from 4-6 mg/L to 18-22 mg/L) and the yield of holoOCP (from 25-40% (Syn-Ctag-ECN) to 35-45% (Syn-pDuet-ECN)) (Table 1).

Since a modification on the N-terminus seemed to increase the yield of holoOCPs in *E. coli* cells, other *ocp* modifications were tested. In all cases (with the exception of one), nine to 45 nucleotides coding for a series of non-charged or charged amino acids were added just after the first ATG in the *ocp* gene containing a sequence coding for six-His in its 3' end (supplementary Fig. 2 and Table 1). In the construction lacking the C-terminal His-tag, 27 nucleotides including the sequence coding for six-His were added to the 5' end (Syn-3aaNtag) (supplementary Fig. 2 and Table 1). Analysis of the isolated *Synechocystis* OCPs showed that addition of 8 to 10 amino acids largely increased the yield of holo-OCP. More than 95% of isolated *Synechocystis* OCP contained a carotenoid molecule. When only 3 to 6 amino acids were added, the yield of holoprotein also increased but slightly less (Table 1). Finally, addition of 9 amino acids, including 6 His, in the N-terminus, in the absence of C-terminal His-tag (Syn-3aaNtag) allowed the isolation of the largest quantity of holo-OCP containing almost no apo-protein : 30-35 mg holo-OCP (Table 1). All of the isolated *Synechocystis* holo-OCPs contained more than 95% ECN with only traces of CAN (supplementary Fig. 3). These results suggested that a slight destabilisation of the OCP N-terminal arm is necessary to increase and/or to stabilize OCP carotenoid binding. In the *OCP_o*, this arm interacts with the C-terminal domain and seems to stabilize the closed structure of the orange form^{[95][98]} (Fig. 1).

Arthrospira and *Anabaena* *ocp* genes, containing sequences coding for an His-tag in the N- or the C-terminus, were also expressed in *E. coli* cells synthesizing ECN. Although the His-tag in the N-terminus increased the yield of holo *Anabaena* and *Arthrospira* OCPs (to 60 and 40%, respectively), still a large amount of apo-OCP was present (Table 1). Since the membranes remained coloured indicating the presence of ECN, the low concentration of holo-OCPs was not related to insufficient carotenoid production. The holo*Anabaena* and holo*Arthrospira* OCPs contained mostly ECN with traces of CAN (supplementary Fig. 3).

In an attempt to increase the yield of *Arthrospira* and *Anabaena* holo-OCPs, the *ocp* genes were induced in *E. coli* cultures synthesizing CAN. This strain, carrying the *crtW* gene, contained 15-25% of β -carotene, 50-60% CAN, 7-9% ECN and 5-7% of an

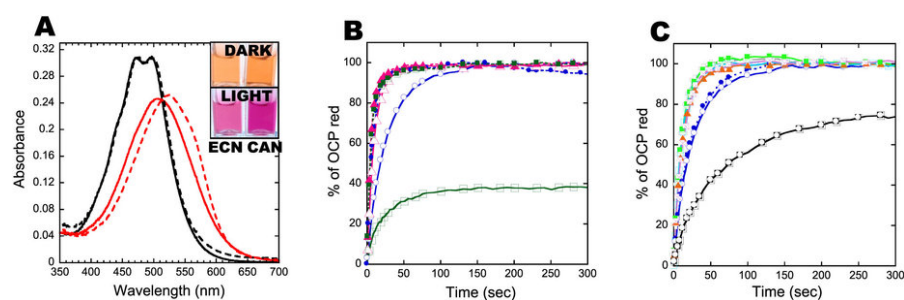


Figure 3.4 – Absorbance spectra and kinetics of photoactivity of recombinant OCPs. (A) Absorbance spectra of *Synechocystis*-3aaNtag-ECN-OCP (solid line) and of *Synechocystis*-3aaNtag-CAN-OCP (dashed line). In darkness (black) or after 5 min illumination (at 18°C) with $5000 \mu\text{moles de photons} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ of white light (red). Inset : The OCPs in darkness (orange) and after 5 min illumination (red). (B) Kinetics of photoactivity (OCP_o to OCP_r) at 18°C of Syn-3aaNtag-ECN-OCP (open circle), Syn-3aaNtag-CAN-OCP (closed circle), Arthro-3aaNtag-ECN-OCP (open triangle), Arthro-3aaNtag-CAN-OCP (closed triangle), Ana-3aaNtag-ECN-OCP (open square) and Ana-3aaNtag-CAN-OCP (closed square). (C) Influence of N-terminal modifications in accumulation of OCP_r . Light-induced OCP_o to OCP_r conversion at 18°C in Tris-HCl pH8.0 (40 mM) of Syn-Ctag-ECN-OCP (closed circle), Syn-3aaNtag-ECN-OCP (open circle), Syn-NC15aaCtag-ECN-OCP (closed triangle), Syn-NC10aaCtag-ECN-OCP (open cross), Syn-NC8aaCtag-ECN-OCP (closed square), Syn-NC6aaCtag-ECN-OCP (open square), Syn-NC3aaCtag-ECN-OCP (square with cross), Syn-C8aaCtag-ECN-OCP (square with open circle). Syn = *Synechocystis*; Ana = *Anabaena*; Arthro = *Arthrospira*.

unknown carotenoid. Indeed, the presence of CAN increased the yield of holoprotein to 60% in the case of *Arthrospira* OCP. In contrast, the presence of CAN decreased the yield of holo-*Synechocystis*-OCP (Syn-3aaNtag-CAN) to 75-85% and of holo *Anabaena*-OCP (Ana-3aaNtag-CAN) to 40-45% (table 1). While the holo-*Anabaena*-CAN-OCP contained mostly CAN with only traces of ECN, holo-*Synechocystis*-CAN-OCP contained 50-70% CAN and holo-*Arthrospira*-CAN-OCP contained only 50-55% CAN (supplementary Fig. 3). Our results indicated that the binding and/or the stability of carotenoids in the protein differs between *Synechocystis* and *Arthrospira* or *Anabaena* OCPs. Most probably only the presence of hECN will allow the isolation of more than 95% of holo *Arthrospira* or *Anabaena* OCPs.

Characteristics of the isolated OCPs

The isolated proteins are orange in darkness and red in strong light (Fig. 4A). Orange ECN-OCPs (OCP_o) absorbance spectra show maxima at 472 and 496 nm and a shoulder at 450 nm, comparable to the native cyanobacteria OCPs (Fig. 4A and supplementary Fig. 4A). The absorbance spectra of CAN- OCP_o s were slightly red shifted compared to ECN- OCP_o s with maxima at 475 and 500 nm (Fig. 4A and supplementary Fig. 4B). The maximum of OCP_r absorbance spectra was at 510 nm for ECN-OCPs and 525 nm for CAN-OCPs.

All three CAN-OCPs and *Synechocystis* and *Arthrospira* ECN-OCPs completely converted to their red form (OCP_r) under illumination (Fig. 4B). The kinetics of OCP_o to OCP_r photoconversion of the three CAN-OCPs and ECN-*Arthrospira*-OCP were similar ($t_{1/2} = 4-7$ sec) and faster than that of the ECN-*Synechocystis* OCP ($t_{1/2} = 18$ sec) (Fig. 4B). The slower photoconversion of *Synechocystis* OCP compared to *Arthrospira* OCP was previously observed when the proteins were overexpressed in *Synechocystis* cells^[37]. *Anabaena* ECN- OCP_o only partially converted to OCP_r , suggesting slight differences in the carotenoid-protein interaction in this protein (Fig. 4B). N- and C-terminal His-tagged *Synechocystis* OCPs presented similar conversion kinetics from OCP_o to OCP_r (Fig. 4C). When both termini of the protein were modified, an acceleration of OCP_r accumulation was observed, suggesting a destabilization of the closed OCP_o (Fig. 4C). Only one exception was observed : the addition of 8 charged amino acids hindered the conversion OCP_o to OCP_r (Fig. 4C).

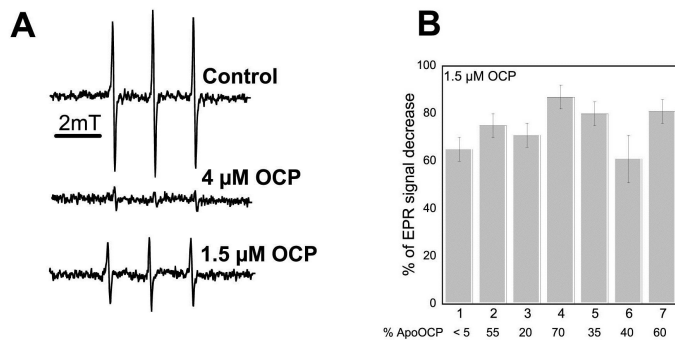


Figure 3.5 – $^1\text{O}_2$ Quenching activity of the recombinant OCPs. $^1\text{O}_2$ was produced by illumination of $10\ \mu\text{M}$ methylene blue for 3 min in the presence of TEMPD-HCl and in the absence (control) or the presence of different OCPs. (A) EPR signal observed in the absence of OCP (control) or in presence of $4\ \mu\text{M}$ or $1.5\ \mu\text{M}$ of *Synechocystis*-3aaNtag-ECN-OCP. (B) Comparison of $^1\text{O}_2$ quenching activity of different OCPs. The percentage (%) of EPR signal decrease induced by Syn-3aaNtag-ECN-OCP (1), Syn-Ctag-ECN-OCP (2), Syn-3aaNtag-CAN-OCP (3), Arthro-3aaNtag-ECN-OCP (4), Arthro-3aaNtag-CAN-OCP (5), Ana-3aaNtag-ECN-OCP (6) and Ana-3aaNtag-CAN-OCP (7). Syn = *Synechocystis*; Ana = *Anabaena*; Arthro = *Arthrospira*. The % of apo-OCP is indicated at the bottom of the figure.

The capacity of N-terminal His-tagged ECN- and CAN-OCPs to quench $^1\text{O}_2$ was studied. Electron paramagnetic resonance (EPR) spin trapping was applied for $^1\text{O}_2$ detection using TEMPD-HCl (2,2,6,6-tetramethyl-4-piperidone). When this nitron reacts with $^1\text{O}_2$, it is converted into the stable nitroxide radical, which is paramagnetic and detectable by EPR spectroscopy. The production of $^1\text{O}_2$ was induced by illumination of the photosensitizer methylene blue. Figure 5A shows the typical EPR signal of the nitroxide radical obtained after 3 min illumination ($1000\ 5000\ \mu\text{moles de photons} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$) of a solution containing methylene blue and TEMPD-HCl in the absence or presence of the OCP. The presence of only $1.5\ \mu\text{M}$ holo-OCP decreased the EPR signal between 65 and 85% and $4\ \mu\text{M}$ OCP quenched nearly the entire EPR signal. These results indicated that all of the *E. coli*-derived OCPs are very good $^1\text{O}_2$ quenchers (Fig. 5B). The slight differences in the efficiency of $^1\text{O}_2$ quenching are likely due to the presence of higher concentrations of apo-protein for the same concentration of holo-OCP (see supplementary Fig. 5). Nevertheless, our results suggested that *Arthrospira* OCP has a slightly better activity as $^1\text{O}_2$ quencher than *Synechocystis* OCP. In contrast, *Anabaena*-ECN-OCP had a slightly lower activity as $^1\text{O}_2$ quencher. The nature of the bound carotenoid seemed not to influence the activity, as previously suggested⁹.

Finally, the ability of the OCPs to quench PB fluorescence was tested. *Arthrospira* and *Synechocystis* OCPs isolated from *E. coli* were able to induce a large PB fluorescence quenching (Fig. 6A and supplementary Fig. 6A). *Arthrospira* OCPs induced the fastest kinetics of fluorescence quenching and *Anabaena* OCPs the slowest, as previously observed with native OCPs (Ref. [37] and supplementary Fig. 6C). The activity of *Synechocystis* ECN-OCP was higher than that of *Synechocystis* CAN-OCP; the opposite was observed with *Anabaena* OCP since ECN-OCP was only partially converted to the red form (Fig. 6A). Addition of 6 to 10 amino acids to the N-terminus of the C-terminal His-tagged OCP partially inhibited the fluorescence quenching, suggesting that OCP binding to PBs is hindered (Fig. 6B and supplementary Fig. 6B). The weaker binding of these modified OCPs was confirmed by the rapid fluorescence recovery observed when PBs-OCP complexes were incubated in darkness (Fig. 6C). In contrast, C-terminal His-tagged OCP remained mostly attached to the PBs (Fig. 6C). Both N- and C-terminal His-tagged *Synechocystis* ECN-OCPs isolated from *E. coli* were able to induce a large fluorescence quenching (Fig. 6A). However, a fast fluorescence recovery was observed only with *Synechocystis* N-terminal His-tagged OCPs (Fig. 6C). Thus, the behaviour of OCPs is affected by the location of the His-tag.

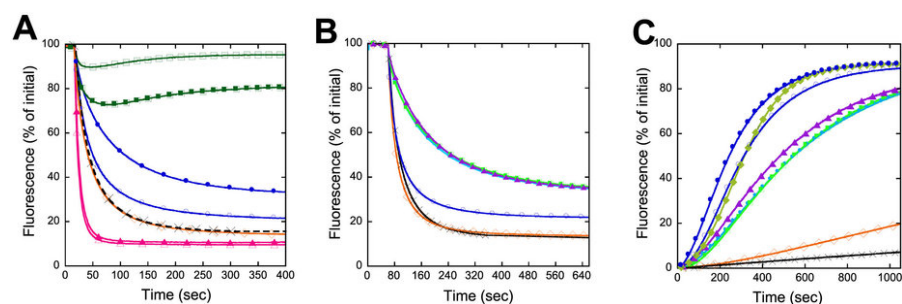


Figure 3.6 – Induction of PB fluorescence quenching by OCP_r and fluorescence recovery WT *Synechocystis* PBs ($0.012 \mu\text{M}$) were incubated in 0.5 M phosphate buffer with pre-converted OCP_r ($0.48 \mu\text{M}$) during 5 min under blue-green light illumination ($900 \mu\text{moles photons m}^{-2} \text{ s}^{-1}$). (A and B) Fluorescence quenching under illumination and (C) fluorescence recovery in darkness. In (A) Native *Synechocystis* OCP (cross), Syn-Ctag-ECN-OCP (open diamond), Syn-3aaNtag-ECN-OCP (open circle), Syn-3aaNtag-CAN-OCP (closed circle), Ana-3aaNtag-ECN-OCP (open square), Ana-3aaNtag-CAN-OCP (closed square), Arthro-3aaNtag-ECN-OCP (open triangle), Arthro-3aaNtag-CAN-OCP (closed triangle). In (B) Native *Synechocystis* OCP (cross), Syn-3aaNtag-ECN-OCP (open circle), Syn-Ctag-ECN-OCP (open diamond), Syn-NC6aaCtag-ECN-OCP (closed circle), Syn-NC8aaCtag-ECN-OCP (closed square), Syn-NC10aaCtag-ECN-OCP (closed triangle). In (C) Native *Synechocystis* OCP (cross), Syn-Ctag-ECN-OCP (open diamond), Syn-NC8aaCtag-ECN-OCP (closed square), Syn-NC10aaCtag-ECN-OCP (closed triangle), Syn-3aaNtag-ECN-OCP (open circle), Syn-3aaNtag-CAN-OCP (closed circle), Syn-pDuet-ECN-OCP (closed diamond).

Discussion

The use of soluble carotenoproteins as antioxidants to promote human health is an area of active research and, consequently, methods to produce them in high yields are important. The aim of our work was to synthesize holo-OCPs (OCPs attaching the carotenoid) in *E. coli* to develop a method for obtaining high quantities of carotenoproteins. Using the method described here, we obtained 200 times more holo-OCP in 20% of the time of previously established purifications involving overexpression in cyanobacterial cells (C. Bourcier de Carbon, A. Wilson and D. Kirilovsky, unpublished data). In only four days more than 30 mg holo-*Synechocystis* OCP can be obtained from 1 L of *E. coli* cells using the construction Syn-3aaNtag-ECN. The key elements of this production method are the choice of promoters and the sequential induction of genes. β -carotene must be present in the membrane before induction of *ctrO* or *crtW* genes leading to the synthesis of ECN or CAN, respectively. More importantly, ECN and CAN have to be synthesized in advance and present in the membrane before induction of the *ocp* gene. The presence of IPTG in the growth medium inhibits cell growth even at low concentrations. In addition, the T7lac promoter cannot be induced at temperatures higher than 30°C . In contrast, arabinose enhances cell growth and the *araBAD* promoter allows induction at 37°C . Thus, the carotenoid genes must be induced first with arabinose at 37°C to obtain a high concentration of carotenoid-containing cells and then, the carotenoprotein gene could be induced by IPTG addition at lower temperatures (20 to 28°C) to slow down protein synthesis, allowing protein folding and carotenoid binding.

The possibility of isolate holo-OCPs from *E. coli* cells constitutes a major advance for the investigation of the molecular mechanism of OCP since it facilitates rapid isolation of mutant proteins with new characteristics. The method has already permitted us to further characterize OCPs revealing different phenotypes related to specificity and strength of carotenoid binding. We demonstrate here that all OCPs are able to bind CAN and that CAN-OCPs are photoactive and able to induce large PB fluorescence quenching, like the native hECN-OCPs. This demonstration was not previously possible when OCPs were isolated by overexpression in *Synechocystis* cells since they contain only traces of CAN.

We also show here that *Arthrospira*, *Anabaena* and *Synechocystis* OCPs are characterized by different phenotypes in terms of specificity and strength of carotenoid

binding. *Synechocystis* OCP binds and stabilizes ECN better than CAN. In contrast, *Arthrospira* and *Anabaena* OCPs preferentially bind CAN over ECN and have a low affinity for both carotenoids. It is difficult to explain these differences based in the comparison of *Arthrospira* and *Synechocystis* OCP_o structures due to the high sequence identity among amino acids forming the carotenoid-binding pocket and the similar carotenoid orientation in the proteins (supplementary Fig 9A). Nothing is known about how the carotenoid is introduced in the apo-OCP. We can hypothesize that the OCP is synthesized by membrane bound ribosomes and that the carotenoid is introduced during the synthesis of the N-terminal domain. It could be possible that the amino acids involved in this initial binding differ from those in the carotenoid-binding pocket of mature OCP_o. Many of the amino acid substitutions among the three OCPs are localized on the outer surface of the N-terminal domain and could have a role in primary binding (supplementary Fig 9B and 9C).

The most unexpected result was the different behavior in PBs binding of *Synechocystis* OCPs with a His-Tag on the N-terminus compared to those with the tag on the C-terminus. In the past, all OCP characterizations and the construction of stable OCP-PBs complexes were made with isolated C-terminal His-tagged OCPs^{[97][81][158]}. These OCPs, once bound to PBs at 0.5 or 0.8 M phosphate, remain almost permanently attached and the PBs remain quenched^{[81][158]}. This characteristic allowed the isolation of quenched OCP-PB complexes^{[81][158]}. Here, we show that the absence of the C-terminal His-tag largely accelerates the dark recovery of PB fluorescence, suggesting a decreased stability of bound OCP_r. In contrast, OCP_r binding kinetics were only slightly affected. In addition, a longer N-terminus in C-terminal His-tagged OCPs hinders the OCP binding and destabilizes the strong OCP_r attachment to PBs. Our results strongly suggest that while the C-terminal His-tag increases the stability of OCP_r-PB complexes, a longer N-terminus destabilizes this attachment.

Production of antioxidant molecules and proteins is a topic of considerable general interest to plant and human biologists since oxidative stress is involved in many processes leading to cell death or tissue damage. Here we show that all three recombinant OCPs show excellent activity as ¹O₂ quencher. Other soluble carotenoproteins also display good antioxidant properties like Asta P6 and could be used in nutraceuticals, cosmetics, etc. The possibility of engineering noncarotenogenic bacteria to produce carotenoproteins, like the OCP, which are present at relative low concentrations in the native organisms, constitutes a major breakthrough in efforts to obtain large quantities of carotenoid molecules as antioxidants.

In addition, the ability to synthesize holo-OCPs in *E. coli* is an important step in the construction of a biofuel (biomass) producing minimal microbe using sunlight as natural source of energy. In order to create these minimal entities new synthetic reaction centers containing the minimal number of components needed for electron transport are being constructed and antenna molecules are being attached to them to expand the spectral range for light absorption (for example^{[165][166][167][168][169]}). Presently, nothing is done to protect these systems. The OCP, a good quencher of excitation energy and singlet oxygen, is an excellent candidate for this function. It can regulate the excitation energy arriving to the reaction centers and quench the singlet oxygen formed by the inevitable secondary, dangerous reactions.

Methods

Amplification and cloning of Crt genes encoding enzymes involved in carotenoid synthesis and of ocp gene

The plasmids pAC-BETA (gift of Prof F. Cunningham) and pACCAR16ΔcrtX (gift of Prof G. Sandmann), which contain a P15A origin of replication and the crtB, crtE, crtI and crtY genes under the control of the promoter of crtE from *Erwinia herbicola* and *Erwinia uredovora* respectively, were used^{32,33}. All results presented in this article were obtained with pAC-BETA. The crtO and crtW genes were cloned into a modified Plasmid pBAD/gIII A (from Invitrogen) which contains a PBR322 origin of replication, an arabinose inducible promoter (araBAD) and an ampicillin resistance marker. The Plasmid pBAD/gIII A was first modified to avoid the export of the recombinant protein into the periplasmic space of the cells. For this purpose, the region encoding the “gene III signal sequence” was deleted. Primers used for the

PCR mutagenesis were pBAD/gIIIAmut (F and R) (supplementary Fig. 7). The modified plasmid pBAD/gIII A was named pBAD. The Plasmid pBAD was digested with BgIII and EcoRI restriction enzymes to clone the crtO gene (slr0088) of *Synechocystis* PCC6803 or with NcoI and EcoRI restriction enzymes to clone the crtW gene (alr3189) of *Anabaena* PCC7120. Primers CrtO (F and R) and CrtW (F and R) were used to amplify crtO and crtW genes respectively (supplementary Fig. 7). The resulting plasmids were named pBAD-CrtO and pBAD-CrtW. The ocp gene was cloned in the plasmid pCDFDuet-1 (from Novagen). The plasmid pCDFDuet-1 contains a CDF origin of replication, T7lac promoter and Streptomycin/Spectinomycin resistance. The sequences of synthetic oligonucleotides (primers) used in the amplification and modification of all the genes are described in supplementary Fig. 7.

N-terminal His-tagged OCP

The pCDFduet-1 plasmid was digested with EcoRI and NotI to clone the different ocp genes (slr1963 from *Synechosystis* PCC6803, NIES39_N00720 from *Arthrospira* Platensis PCC7345 and all3149 from *Anabaena* PCC 7120). The primers OCPSyn-pDuet (F and R) were used to amplify the *Synechocystis* ocp gene (1104 nucleotides) using genomic DNA of *Synechocystis* PCC6803 as template. The primers OCPAna-pDuet (F and R) were used to amplify the *Anabaena* ocp gene (1076 nucleotides) using genomic DNA of *Anabaena* PCC 7120 as template. The primers OCPArthro-pDuet (F and R) were used to amplify the *Arthrospira* ocp gene (1355 nucleotides) using the plasmid pOF7345 as template^[37]. The resulting PCR products were introduced into pCDFDuet-1 to create the pCDF-OCPSyn-pDuet, pCDF-OCPArthro-pDuet and pCDF-OCPAna-pDuet plasmids. In the OCP isolated from *E. coli* strains carrying these plasmids, an extension of 15 amino acids was present in the N-terminal of the OCP protein. This extension contains a His-tag comprising 6 His residues.

C-terminal His-tagged OCP

To obtain a C-terminal His-tagged *Synechocystis* OCP, it was first, necessary to abolish a NcoI site in the *Synechocystis* ocp gene sequence; accordingly the GCC sequence coding for Ala73 was changed to GCG (also coding for an alanine) using the plasmid pSK-OCPsyn-CterHisTagΔFRP^[97]. Then, pCDFDuet-1 was digested with NcoI and NotI to excise the N-terminal extension containing the His-tag initially present in this plasmid. The ocp genes containing a C-terminal His-Tag from *Synechosystis* PCC6803, *Arthrospira* Platensis PCC7345 and *Anabaena* PCC 7120 were cloned in the plasmid. The primers OCPsyn-Ctag (F and R) were used to amplify the ocp gene tagged in C-terminal domain from the plasmid pSK-OCPsyn-CterHisTagΔFRP- A73A^[97]. The primers OCPArthro-Ctag (F and R) were used to amplify the ocp gene from the plasmid p2A7345His^[37]. The primers OCPana-Ctag (F and R) were used to amplify the ocp gene from genomic DNA of *Anabaena* PCC 7120, the C-terminal His-tag was then added by PCR mutagenesis. The resulting PCR products were introduced into pCDFDuet-1 to create the pCDF-OCPsyn-Ctag, pCDF-OCPArthro-Ctag and pCDF-OCPana-Ctag plasmids.

Modifications in His-tagged OCPs

The sequences added after the first ATG of the ocp *Synechocystis* gene are described in supplementary Fig. 2. The modifications (NC15, NC10, NC8, NC6, NC3, Mix15 and C8, table 1) were introduced by directed mutagenesis, using the pCDF-OCPsyn-Ctag plasmid as template and the different oligonucleotides described in supplementary Fig. 7. The modification 3aaNtag (table 1) was created by site-directed mutagenesis using the pCDF-OCPsyn-pDuet plasmid as template and the oligonucleotides described in supplementary Fig. 7. This mutagenesis causes the deletion of part of the OCP N-terminal extension present in the pCDF-OCPsyn-pDuet plasmid. The modification 3aaNtag was also created in the ocp genes of *Arthrospira* and *Anabaena* using the pCDF-OCPArthro-pDuet and pCDF-OCPana-pDuet plasmids as templates and the oligonucleotides described in supplementary Fig. 7.

Transformation of E. coli cells and induction of genes

E. coli BL21-Gold (DE3) cells from Agilent Technologies (F- ompT hsdS(rB - mB-) dcm+ Tetr gal Λ (DE3) endA Hte) were used for OCP production. BL21 cells were transformed simultaneously with three plasmids : pAC-BETA, pBAD-CrtO (or pBAD-CrtW) and pCDF-OCP. The pCDF-OCP plasmid contains WT or modified sequences of ocp genes. The transformed *E. coli* cells were grown in the presence of three antibiotics (ampicillin (50 μ g/ml), chloramphenicol (17 μ g/ml) and streptomycin (25 μ g/ml)) to maintain the three different plasmids in the same *E. coli* cell. For induction of the different genes, transformed *E. coli* were grown in TB medium at 37°C for 3-4 hours until OD₆₀₀ = 0.6. Then arabinose was added (0.02%) and the culture was grown overnight at 37°C. In the morning the cells are diluted with fresh medium and Arabinose 0.02% and they are grown at 37°C till OD₆₀₀ = 1-1.2. Then isopropyl b-D-thiogalactoside (IPTG) (0.2 mM) was added and the cells incubated overnight at 28°C. In the morning, the cultures were harvested and pellets were stored at -80°C until they were used.

OCP isolation and calculation of holo-OCP concentration

E. coli frozen cells were resuspended in the lysis buffer containing 40 mM Tris pH 8, 10% glycerol and 300 mM NaCl and were broken in dim light using a French Press. The membranes were pelleted and the supernatant was loaded onto a nickel column (Ni-Probond resin, Invitrogen). The OCP was eluted with 200 mM Imidazol. For isolation of *Synechocystis*, *Arthrospira* and *Anabaena* OCPs overexpressed in *Synechocystis*, an initial 50 mL *Synechocystis* culture was daily diluted during 2 weeks until reaching 30 L culture at OD₈₀₀ = 0.8. The cells were precipitated and broken using a French Press. The OCP was isolated from the supernatant using two columns as described in 12. Complete isolation took three weeks. Total OCP concentration was measured using the Bradford method. At least five independent Bradford measurements of each isolated OCP were done. The concentration in mg/mL obtained by this method was converted to molar concentration using a MW of 35 kDa for the OCP. Holo-OCP concentration was calculated based in the fact that each holo-OCP binds one carotenoid molecule and thus, the molar concentrations of carotenoid and holo-OCP are identical. Carotenoid concentration was first calculated in mg/mL from the carotenoid absorbance at 496 nm and using $A_{1\%1\text{ cm}} = 2158$ and then converted to molar concentration. The ratio between the molar concentration of holo-OCP and total OCP gives the percentage of holo-protein described in table 1. When this ratio is around 1, we estimated that the preparation contained 100% holo-protein.

Absorbance spectra and photoactivity kinetics of OCP

Absorbance spectra and kinetics of photoactivity (illumination with 5000 μ mol photons m⁻² s⁻¹ of white light) and dark recovery were measured in a Specord S600 (Analytischena) at 18°C.

Measurements of OCP fluorescence quenching activity

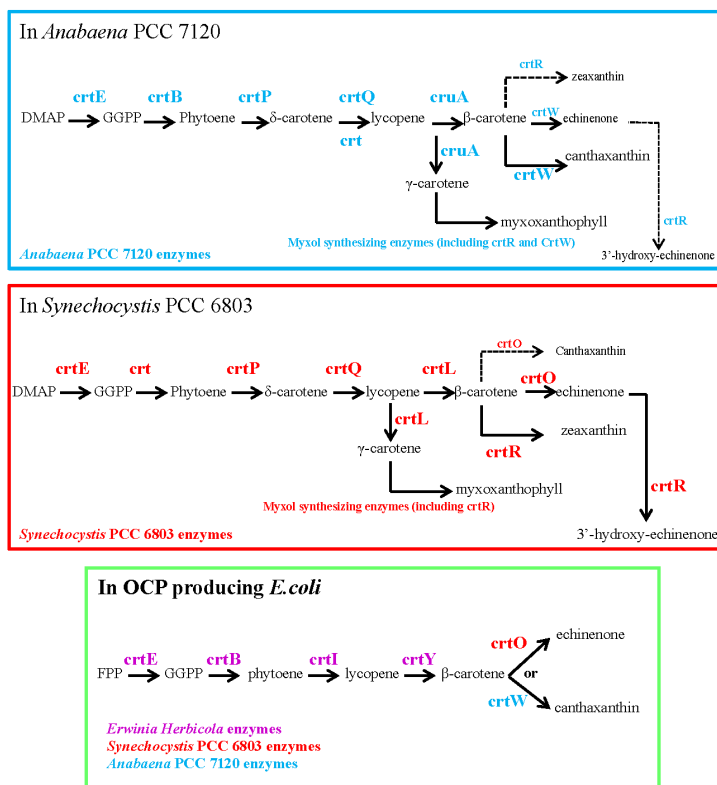
Isolated *Synechocystis* PBs in 0.5 or 0.8 M phosphate were incubated in the presence of different modified OCP_rs under illumination with strong blue-green light. The high concentration of phosphate was needed to maintain the integrity of PBs. Phosphate also influences the strength of OCP binding to PBs; it is stronger at 0.8 M than at 0.5 M phosphate^[81]. The ratio of OCP to PB was 40 in all the experiments as previously described^{[81][98]}. The concentration of the OCP for these experiments was calculated from the carotenoid absorbance spectra since only the OCP attaching a carotenoid is able to be photoactivated and to bind to PBs. The presence of apo-OCP did not hinder OCP_r binding (supplementary Fig. 8). Fluorescence quenching and recovery were monitored with a pulse amplitude modulated fluorometer (101/102/103-PAM; Walz, Effelrich, Germany). The fluorescence quenching was induced by 900 μ mol photons m⁻² s⁻¹ of blue-green light (400-550 nm). All measurements were carried out at 23°C in 0.5 or 0.8 M phosphate buffer. The OCP was pre-converted to the red form by 10 min illumination with 5000 μ mol photons m⁻² s⁻¹ of white light at 4°C.

¹O₂ detection by EPR spin trapping

Electron paramagnetic resonance (EPR) spin trapping was applied for ¹O₂ detection using TEMPD-HCl (2,2,6,6-tetramethyl-4-piperidone) (100 mM). When this nitron reacts with ¹O₂, it is converted into the stable nitroxide radical, which is paramagnetic and detectable by EPR spectroscopy. The production of ¹O₂ was induced by illumination of the photosensitizer methylene blue (10 μM). The measurements were done in buffer 100 mM Tris-HCl pH 8 in the absence or in the presence of different concentrations of purified OCPs. The samples were illuminated for 3 min with white light (1000 μmol photons m⁻² s⁻¹). The EPR settings were as follows : hall center field = 3467.270 G, microwave frequency = 9.74 Ghz, power = 4.450 mV and number of scans = 12.

Measurement of carotenoid content in OCPs

The carotenoid content of *E. coli* cells and the isolated OCPs was analysed by High-Performance Liquid Chromatography (HPLC) and Mass spectrometry as described in^[141].



Sup Fig 1: Schematic carotenoid biosynthetic pathway. Carotenoid biosynthesis in *Synechocystis* and *Anabaena* cyanobacteria strains and in *E. coli* strains producing ECN or CAN-OCPs created in this work.

A- Sequence including the His-tag added in the C-terminus of WT OCPs

Syn-Ctag: *ocp* gene-CACCACCACCACCACCAC-STOPcodon

B- Sequences including the His-Tag added on the N-terminus of WT OCPs

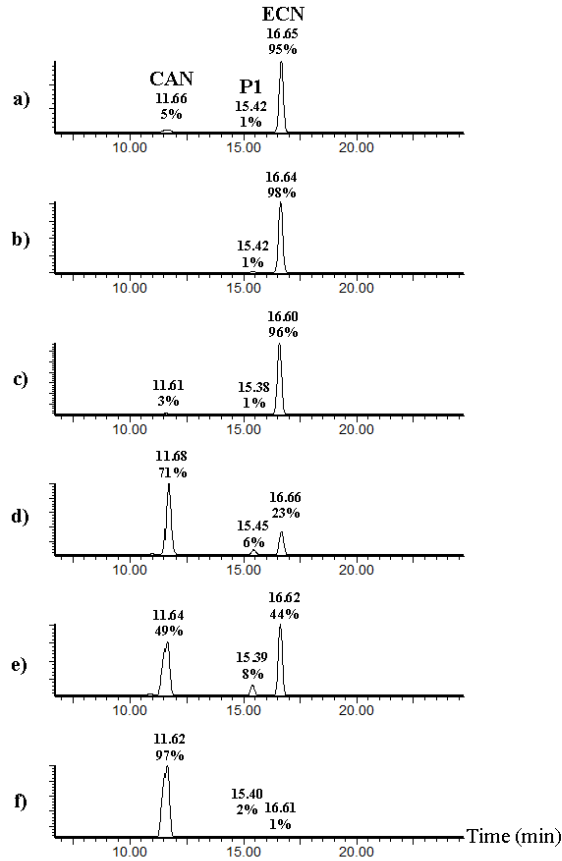
Syn-pDuet: ATGGGCAGCAGCCATCACCATCATCACCACAGCCAGGATCCG-*ocp* gene-STOPcodon

Syn-3aaNtag: ATGGGCAGCAGCCATCACCATCATCACCAC-*ocp* gene-STOPcodon

C- Sequences added on the N-terminus of C-terminal His-tagged *Synechocystis* OCP

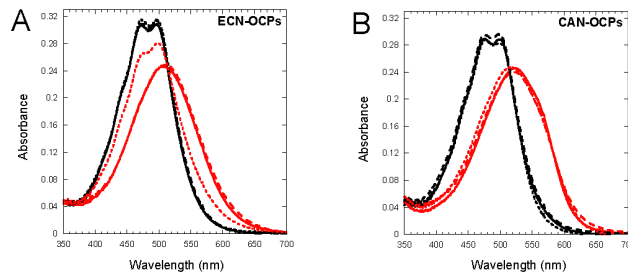
| Name of OCP | Sequence added on the N-terminus |
|-------------------------------|--|
| Syn-NC15aaCtag (nucleotids) | ATGGGCAGCAGCAATCAGGCTAATCAGGTGACTCTAAATCCACAAGTT |
| Syn-NC15aaCtag (amino acids) | MGSSNQANQVTLNPQV |
| Syn-NC10aaCtag (nucleotids) | ATGGGCAGCAGCAATCAGGCTAATCAGGTGACT |
| Syn-NC10aaCtag (amino acids) | MGSSNQANQVT |
| Syn-NC8aaCtag (nucleotids) | ATGGGCAGCAGCAATCAGGCTAATCAG |
| Syn-NC8aaCtag (amino acids) | MGSSNQANQ |
| Syn-NC6aaCtag (nucleotids) | ATGGGCAGCAGCAATCAGGCT |
| Syn-NC6aaCtag (amino acids) | MGSSNQA |
| Syn-NC3aaCtag (nucleotids) | ATGGGCAGCAGC |
| Syn-NC3aaCtag (amino acids) | MGSS |
| Syn-MIX15aaCtag (nucleotids) | ATGGGCAGCAGCCGACTAGACAATCCAGAAAAAACTGACATTGAACCA |
| Syn-MIX15aaCtag (amino acids) | MGSSRLDNPEKTDIEP |
| Syn-C8aaCtag (nucleotids) | ATGGGCAGCAGCAAAAAGAGAAGGGCT |
| Syn-C8aaCtag (amino acids) | MGSSKKRRA |

Sup Fig 2: Nucleotides and amino acids sequences added to the 3' and 5' ends of *ocp* gene (N-terminus and C-terminus of *Synechocystis ocp* gene).



Sup Fig 3: UV Chromatograms (450 nm) of isolated OCPs from *E.coli* culture

a) *Syn*-3aaNtag-ECN-OCP, b) *Arthro*-3aaNtag-ECN-OCP, c) *Ana*-3aaNtag-ECN-OCP, d) *Syn*3aaNtag-CAN-OCP, e) *Arthro*-3aaNtag-CAN-OCP and f) *Ana*-3aaNtag-CAN-OCP. Retention time (min) and relative integration of peak area indicated (% of shown). CAN: canthaxanthin, P1: unknown carotenoid of MW 548, ECN: echinenone.



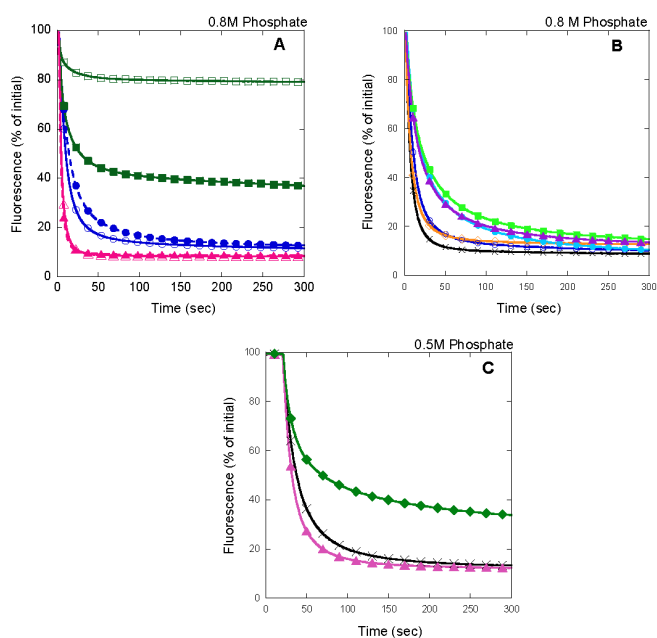
Sup Fig 4: Photoactivity of recombinant OCPs

(A) Absorbance spectra of the dark (black) and light (red) forms of the ECN-OCPs. OCP-*Syn*-3aaNtag-ECN (solid line), OCP-*Arthro*-3aaNtag-ECN (dashed line) and OCP-*Ana*-3aaNtag-ECN (dotted line). To obtain the spectrum of the light form, the OCP was illuminated with $5000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ of white light, at 18°C , for 5 min.

(B) Absorbance spectra of the dark (black) and light (red) forms of the CAN-OCPs. Symbols are the same as in (A).

| OCP | % of EPR signal decrease | % of Apo-OCP |
|------------------------------|--------------------------|--------------|
| Syn-3aaNtag-ECN | 65 | < 5 |
| Syn-3aaNtag-ECN + 20% ApoOCP | 67 | 20 |
| Syn-3aaNtag-ECN + 40% ApoOCP | 71 | 40 |
| Syn-3aaNtag-ECN + 60% ApoOCP | 73 | 60 |
| Syn-Ctag-ECN | 72 | 60 |

Sup Fig 5: Apo-OCP influence on singlet oxygen quenching.



Sup Figure 6: Induction of PB fluorescence quenching by OCP^F and fluorescence recovery WT *Synechocystis* PBs (0.012 μM) were incubated in 0.8 M (A,B) or 0.5 M (C) phosphate buffer with preconverted OCP^F (0.48 μM) during 5 min under blue-green light illumination (900 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). In (A) *Syn*-3aaNtag-ECN (open circle), *Syn*-3aaNtag-CAN (closed circle), *Ana*-3aaNtag-ECN (open square), *Ana*-3aaNtag-CAN (closed square), *Arthro*-3aaNtag-ECN (open triangle), *Arthro*-3aaNtag-CAN (closed triangle). In (B) fluorescence quenching under illumination of native *Synechocystis* OCP (cross), *Syn*-3aaNtag-ECN (open circle), *Syn*-Ctag-ECN (open diamond), *Syn*-NC6aaCtag-ECN (closed circle), *Syn*-NC8aaCtag-ECN (closed square), *Syn*-NC10aaCtag-ECN (closed triangle); in (C) Native *Ana*-OCP (closed diamond), Native *Syn*-OCP (cross) and Native *Arthro*-OCP (closed triangle). The differences in the kinetics of fluorescence quenching are bigger at 0.5 M phosphate than at 0.8M phosphate. Compare Figs 6A and 6 B with sup Figs 6A and 6B respectively.

| Primer names | Primer sequences |
|------------------------------------|---|
| pBADgIII-CrtO (BglII/EcoRI) | Cloning using pBAD/gIII A (Invitrogen) 5'-AACAGAAAGATCTATCACCACCGATGTTGTC-3' 5'-TCTGTTGAATTCGCTTCACTTGCCATACAC-3' |
| pBAD-CrtO | Mutagenesis using pBADgIII-CrtO 5'-CAGGAGGAATTAACCATGATCACCACCGATGTTGTCATTATGGGGCG-3' 5'-CATGGTTAATTCCTCTCTGTTAGCCAAAAAACGGGTATGGAGAAACAG-3' |
| pBAD | Mutagenesis using pBAD/gIII A 5'-CAGGAGGAATTAACCATGACCATGGAGCTCGAGATCTGCAGCTGGTAC-3' 5'-CATGGTTAATTCCTCTCTGTTAGCCAAAAAACGGGTATGGAGAAACAG-3' |
| pBAD-crtW (NcoI/ EcoRI) | pBAD-crtW NcoI/ EcoRI Cloning using pBAD and primers : 5'- GATATAACCATGGTTCAGTGTCAACCATCATC- 3' 5'- GCATCGGAATTCAGTCGTATTCCAGCAG -3' |
| OCPSyn-pDuet (EcoRI/NotI) | 5'-AACAGAGAATTCTCCATTACCATTGACTCT-3' 5'-TCTGTTGCGGCCGCTGTAACATTAACCTTATGA-3' |
| OCPSyn-Ctag | Mutagenesis of NcoI site [GCC(A)73GCG(A)] in OCP 5'-CAAGGAAATTCAGCGATGGGGCCCTC-3' 5'-GAGGGCCCCATCGCTTGAATTCCTTG-3' Amplification of the modified <i>ocp</i> gene: 5'-AACAGACCATGGCATTACCATTGACTCT-3' 5'-TCTGTTGCGGCCGCTGTAACATTAACCTTATGA-3' |
| OCPSyn-3aaNtag | 5'-CATCACCATCATCACCACCCATTACCATTGAC-3' 5'-GTGGTGATGATGGTGATGGCTGCTGCCATGGT-3' |
| OCPArthro-Ctag | 5'-AACAGACCATGGCATTACCATTGACTCG-3' 5'-TCTGTTGCGGCCGCTTATCATTACCTAATTGAGCATT-3' |
| OCPArthro-3aaNtag | 5'-CATCACCATCATCACCACCCATTACCATTGAC-3' 5'-GTGGTGATGATGGTGATGGCTGCTGCCATGGT-3' |
| OCPAna-Ctag | 5'-AACAGACCATGGCAATTACTATCGATTCC-3' 5'-TCTGTTGCGGCCGACTCAATCTTAACTTGTA-3' |
| OCPAna-3aaNtag | 5'-CATCACCATCATCACCACGCAATTACTATCGATTCC-3' 5'-GTGGTGATGATGGTGATGGCTGCTGCCATGGT-3' |

| Primer names | Primer sequences |
|---------------------------|---|
| OCPSyn-NC15aaCtag | Mutagenesis using pCDF-OCPsynCter 5'-AATCAGGCTAATCAGGTGACTCTAAATCCACAAGTCCATTACCATTGACTCTGCC-3' 5'-AACTTGTTGATTAGAGTCACTGATTAGCCTGATTGCTGCTGCCATGGTATATCTCCT-3' |
| OCPSyn-MIX15aaCtag | 5'-CGACTAGACAATCCAGAAAAACTGACATTGAACCACCCATTACCATTGACTCTGCC-3' 5'-TGGTCAATGTCAGTTTTTCTGGATTGTCTAGTCGGCTGCTGCCATGGTATATCTCCT-3' |
| OCPSyn-NC10aaCtag | 5'-AATCAGGCTAATCAGGTGACTCCATTACCATTGACTCTGCCCGCG-3' 5'-AGTCACCTGATTAGCCTGATTGCTGCTGCCATGGTATATCTCCT-3' |
| OCPSyn-NC8aaCtag | 5'-AATCAGGCTAATCAGCCATTACCATTGACTCTGCCCGCGGA-3' 5'-CTGATTAGCCTGATTGCTGCTGCCATGGTATATCTCCT-3' |
| OCPSyn-NC6aaCtag | 5'-CCATTACCATTGACTCTGCCCGCGGAATT-3' 5'-AGAGTCAATGGTGAATGGAGCCTGATTGCTGCTGCCATGGTATATCT-3' |
| OCPSyn-NC3aaCtag | 5'-CCATTACCATTGACTCTGCCCGCGGAATT-3' 5'-AGAGTCAATGGTGAATGGGCTGCTGCCATGGTATATCTCCT-3' |
| OCPSyn-C8aaCtag | 5'-AAAAAGAGAAGGGCTCCATTACCATTGACTCTGCCCGCGGA-3' 5'-AGCCCTTCTTTTTGCTGCTGCCATGGTATATCTCCT-3' |

Sup Fig 7: List of synthetic oligonucleotides used to amplify and to clone *ocp* genes and genes involved in carotenoid synthesis.

Dans le chapitre 3, nous rapportons l'importance de l'extrémité N-terminale de l'OCP dans la stabilisation du caroténoïde et dans l'attachement aux PBs. L'extrémité N-terminale de l'OCP présente une structure singulière. L'OCP est formée de deux domaines globulaires, N- et C-terminaux, reliés entre eux par un linker. Une hélice α portée par un appendice à l'extrémité N-terminale est plaquée contre le domaine C-terminal de l'OCP. L'ensemble correspond aux 20 premiers acides aminés de l'OCP et sera nommé plus bas « bras N-terminal ». Ce bras est conservé chez la quasi-totalité des OCP. Il a été proposé que la photoactivation de l'OCP implique la transisomérisation d'une proline^[112]. La proline n°22, située à la jonction entre bras N-terminal et domaine N-terminal est un bon candidat car son isomérisation changerait la position du bras N-terminal. Nous avons également proposé que l'aspartate n°6 puisse stabiliser le bras via une liaison hydrogène avec l'arginine n°229. L'article rapporte l'identification de la fonction de ce bras dans la régulation de l'activité de l'OCP.

4.0.1 Résumé de l'article Thurotte et al., 2015

Nous avons créé et isolé chez *E. coli* deux OCPs dépourvues de leur extrémité N-terminale et comportant un HisTAG en C-ter ou en N-ter (OCP Δ 2-15). 25% de l'OCP Δ 2-20 est présente sous forme rouge à l'obscurité (figure 2). La photoactivation de l'OCP Δ 2-20 est plus rapide que le WT, et la forme rouge plus stable (figure 3). L'OCP Δ 2-20 induit un quenching de fluorescence des PBs identique ou légèrement plus faible que l'OCP WT. En revanche, une fois attaché, le complexe OCP Δ 2-20-PBs est plus stable qu'avec l'OCP WT. L'ajout de FRP permet de séparer l'OCP Δ 2-20 des PBs beaucoup plus efficacement que le complexe impliquant l'OCP WT.

Le spectre d'absorption (figure 2), les cinétiques de photoconversion (figure 3) ainsi que les cinétiques d'attachement aux PBs et de détachement (figure 4 et 5) de D6L sont très proches du WT. Aussi, l'aspartate n°6 n'est pas impliquée dans la position du bras N-terminal dans la forme orange ou rouge.

La modification P22V crée une protéine dont la photoconversion est légèrement plus rapide que celle de l'OCP WT (figure 3). En revanche, cette OCP présente une faible affinité pour les PBs (figure 4 et 5). L'isomérisation de la Proline n°22 ne semble donc pas requise pour la photoactivation de l'OCP. La faible affinité pour les PBs est probablement due à une position incorrecte du bras N-terminal.

Conclusion

Le bras N-terminal stabilise la forme fermée inactive orange de l'OCP en interagissant avec le domaine C-terminal, et diminue l'action de la FRP qui détache l'OCP des phycobilisomes. Sa présence permet donc d'éviter un déclenchement inopportun du mécanisme de photoprotection par l'OCP, et de le maintenir plus longtemps efficace une fois activée (complexe OCP-PBs formé). La Proline n°22 n'est pas essentielle pour la photoactivation de l'OCP, et ne subit très probablement pas d'isomérisation. L'interaction entre domaine N-terminal de l'OCP et PBs est plus forte en l'absence du bras N-terminal, et un changement de la position de ce dernier (OCP P22V) diminue l'affinité de l'OCP modifiée pour les PBs.

Des résultats complémentaires à cet article sont présentés à la fin du chapitre page 95.

Regulation of Orange Carotenoid Protein Activity in Cyanobacterial Photoprotection

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abstract

Plants, algae, and cyanobacteria have developed mechanisms to decrease the energy arriving at reaction centers to protect themselves from high irradiance. In cyanobacteria, the photoactive Orange Carotenoid Protein (OCP) and the Fluorescence Recovery Protein are essential elements in this mechanism. Absorption of strong blue-green light by the OCP induces carotenoid and protein conformational changes converting the orange (inactive) OCP into a red (active) OCP. Only the red orange carotenoid protein (OCP_r) is able to bind to phycobilisomes, the cyanobacterial antenna, and to quench excess energy. In this work, we have constructed and characterized several OCP mutants and focused on the role of the OCP N-terminal arm in photoactivation and excitation energy dissipation. The N-terminal arm largely stabilizes the closed orange OCP structure by interacting with its C-terminal domain. This avoids photoactivation at low irradiance. In addition, it slows the OCP detachment from phycobilisomes by hindering fluorescence recovery protein interaction with bound OCP_r . This maintains thermal dissipation of excess energy for a longer time. Pro-22, at the beginning of the N-terminal arm, has a key role in the correct positioning of the arm in OCP_r , enabling strong OCP binding to phycobilisomes, but is not essential for photoactivation. Our results also show that the opening of the OCP during photoactivation is caused by the movement of the C-terminal domain with respect to the N-terminal domain and the N-terminal arm.

Introduction

Full sunlight is dangerous for plants, algae, and cyanobacteria. It can cause oxidative damages leading to the destruction of the photosynthetic apparatus and to cell death. A short-term photoprotective mechanism developed by oxygenic photosynthetic organisms is the reduction of excitation energy being funneled into the photochemical reaction centers by dissipating excess energy as heat at the level of the antennae (Niyogi and Truong, 2013). In plants and green algae, this mechanism involves the membrane chlorophyll antennae, the light-harvesting complex (for review, [170-172]), and in cyanobacteria, the extramembrane phycobiliprotein-containing antennae, the phycobilisomes (PBSs; for review, see [83,173]). Despite these differences in composition and structure of their antennae, carotenoids have an essential role in both plants and cyanobacteria. In plants, high irradiance leads to acidification of the lumen that triggers conformational changes in the light-harvesting complexes and in their organization in the membrane, switching the light-harvesting complex into an effective energy-dissipating form. In cyanobacteria, high irradiance photoactivates a soluble carotenoid protein, the Orange Carotenoid Protein (OCP), that acts as the stress sensor and the energy quencher. In both cases, changes in pigment-pigment interactions (carotenoid-chlorophyll, carotenoid-bilin, chlorophyll-chlorophyll) enable thermal dissipation of excitation energy via three different possible mechanisms: excitation energy transfer [102,103], charge transfer [99,104], or excitonic interactions between the pigments [105].

The study of the photoactivation of the OCP and its interaction with the phycobilisome is essential to elucidate the mechanism of energy quenching in cyanobacterial photoprotection. The OCP is a soluble 35-kD protein constituted by an α -helical N-terminal domain (residues 1-165) and an α -helix/ β -sheet C-terminal domain (residues 190-317; [95,113]; Fig. 1A). A flexible linker of 25 amino acids connects both domains. The ketocarotenoid 3'-hydroxyechinenone (3'-hECN), having a carbonyl (keto) group in one of the rings and a hydroxyl group in the other one, spans both

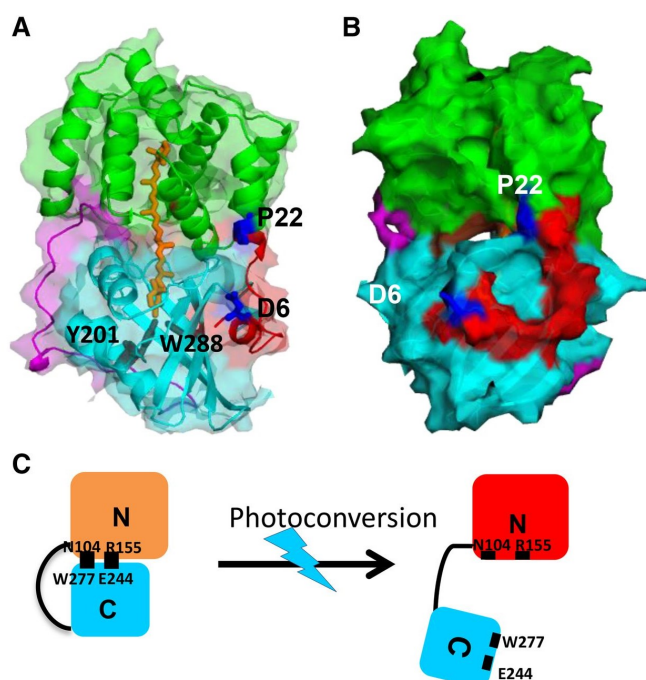


Figure 4.1 – A and B, Structure of the OCP from *Synechocystis* sp. PCC 6803 (Protein Data Bank identifier : 3MG1). The OCP monomer is represented in the orange state. The N-terminal arm (residues 1-22; red) interacts with the C-terminal domain (residues 196-315; sky blue). The Pro-22 and the Asp-6 are marked in blue. The N-terminal domain (residues 22-165) is green in the figure, and the linker between N-terminal and C-terminal domains is colored in violet. C, Model of photoactivation. Upon light absorption, the orange OCP_o is converted into the active red OCP_r . Changes in the carotenoid conformation induce conformational changes in the C-terminal domain, leading to the breakage of the interactions between the N-terminal and C-terminal domains and the opening of the protein.

domains of the protein, with the carbonyl group residing in a hydrophobic pocket of the C-terminal domain. Tyr-201 and Trp-288 interact via hydrogen bonds to the carotenoid keto group. In the dark, the OCP is orange (OCP_o). Absorption of blue-green light by the carotenoid induces conformational changes in the carotenoid and in the protein converting the orange form into the active red form (OCP_r ; [97]; Fig. 1C). The photoconversion reaction has a very low quantum yield, and the rate of OCP_r accumulation largely depends on light intensity [97]. Thus, accumulation of the red form occurs only under high irradiance [97]. Both OCP_o and OCP_r are energetically suitable to quench PBS fluorescence and excitation energy [174,175], but only OCP_r is able to bind to the PBS and dissipate most of the excess energy as heat [81]. In OCP_o , strong interactions exist between the N- and C-terminal globular domains, including salt bridges between residues Trp-277-Asn-104 and Arg-155-Glu-244 [95,113]; Upon photoactivation, these bonds are broken, leading to the solvent exposure of Arg-155, which plays an essential role in OCP binding to PBS [98] (Fig. 1C). The PBSs from *Synechocystis* sp. PCC 6803 (used in this work and hereafter simply referred to as *Synechocystis*) are formed by a core of allophycocyanin (APC) trimers. These trimers are organized in three cylinders from which rods containing phycocyanin hexamers radiate (for review [30,34,107]). OCP_r binds to one APC trimer, and its open structure allows the interaction between the OCP carotenoid and one APC bilin [98]. The first site of energy and fluorescence quenching is an APC trimer emitting at 660 nm [99,100,176,177]. Once OCP_r is attached to PBS, thermal dissipation increases and less energy arrives at both photosystems [109,112,178]. When the light becomes less intense, full antenna capacity is required. The Fluorescence Recovery Protein (FRP) is essential for this process. FRP accelerates the OCP_r to OCP_o dark conversion and facilitates OCP detachment from PBS [81,86,114]. The active FRP is a nonchromophorylated dimer that interacts with the C-terminal domain of the OCP_r [114].

Previously, it has been demonstrated that the N-terminal globular domain of the OCP (green in Fig. 1, A and B) is a constitutively active energy quencher^[150]. Thus, its interaction with the C-terminal globular domain is essential for inhibiting OCP binding to PBS and energy quenching under low irradiance. This process must be tightly regulated. Little is known about this regulation. One possibility is that the N-terminal arm of the protein (red in Fig. 1, A and B), which in *OCP_o* interacts with the C-terminal globular domain, could have a role in this regulation.

According to the OCP structure Asp-6, could form a hydrogen bond with Arg-229, which could stabilize the closed form of *OCP_o*. Pro-22 is located at the bent junction between the N-terminal arm and the N-terminal globular domain. It has been proposed that a cis-trans Pro isomerization could be involved in OCP photoactivation^[112], suggesting that Pro-22 isomerization could help the movement of the N-terminal arm and its detachment from the C-terminal domain during OCP photoactivation. In this work, we studied the effect of deleting the N-terminal arm and the mutations Asp-6-Leu and Pro-22-Val on photoactivity and OCP interaction with PBS and FRP (for the position of the N-terminal arm in the structure of *OCP_o*, see in Fig. 1, A and B).

Results

Construction of N-terminal Arm OCP Mutants in *Escherichia coli*

Modified OCPs lacking the N-terminal arm were created : in the first one, the first 14 amino acids after the Met were deleted, and in the second one, the first 19 amino acids. Also, two single mutants, Pro-22-Val and Asp-6-Leu, were constructed (for positions of mutations, see Fig. 1). We constructed both N-terminal (OCP-HisN) and C-terminal (OCP-HisC) His-tagged mutant OCPs. The modified holo-OCPs (OCP containing carotenoid) were synthesized in *E. coli* cells using a protocol recently developed in our laboratory^[140]. The *E. coli* strain used for OCP production contained three plasmids (Supplemental Fig. S1). The first plasmid carried the operon needed for the synthesis of β -carotene from its precursor (farnesyl-diphosphate) containing the genes *crtE*, *crtY*, *crtI*, and *crtB* from *Erwinia herbicola*. The operon was under the control of the constitutive *crtE* promoter. The second plasmid carried the *Synechocystis crtO* gene coding for a β -carotene ketolase, which is necessary to synthesize echinenone (ECN). This gene was under the control of the arabinose-inducible pBAD promoter. The third plasmid contained the *ocp* gene under the control of the T7 RNA polymerase promoter (induction with isopropyl β -d-thiogalactoside [IPTG]). A sequential induction of the *crtO* and *ocp* genes was essential to obtain holo-OCPs. The *crtO* gene was first induced in *E. coli* containing relatively high concentrations of β -carotene followed by the induction of the *ocp* gene. In these cells, which contained 15% to 25% of β -carotene, 70% to 80% ECN, and 4% to 6% canthaxanthin, OCP incorporated only ECN^[140].

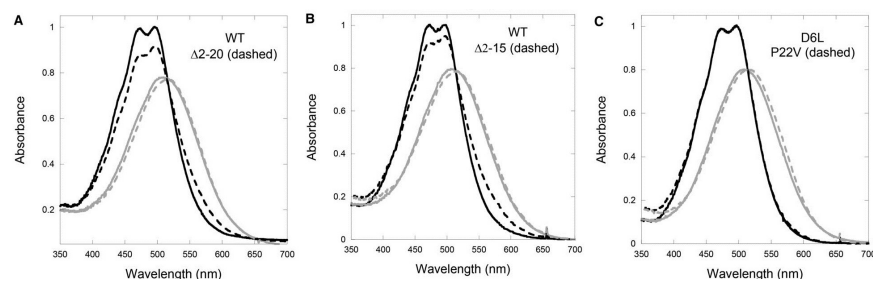


Figure 4.2 – Light and dark absorbance spectra of OCPs. A and B, Absorbance spectra of wild-type (WT; solid line; A and B), Δ 2-20 OCP (dashed line; A), and Δ 2-15 OCP (dashed line; B) in darkness (black) and after 5-min illumination with strong white light ($5,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$; gray). C, Absorbance spectra of Asp-6-Leu (solid line) and Pro-22-Val OCPs (dashed line) in darkness (black) and after 5-min illumination with strong white light ($5,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$; gray).

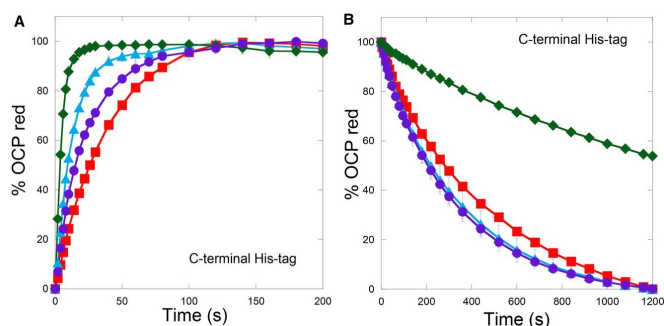


Figure 4.3 – Photoactivation (OCP_0 to OCP_r ; A) and dark OCP_r to OCP_0 (B) kinetics of wild-type and mutated OCPs. A, OCP_0 to OCP_r photoconversion during illumination with strong white light ($5,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 18°C of OCPs-HisC : wild-type (circles), $\Delta 2-20$ (diamonds), Asp-6-Leu (squares), and Pro-22-Val (triangles) OCPs. B, Recovery in darkness using the same symbols. The error bars represent the sd. The results obtained with the N-terminal His-tagged OCPs are shown in Supplemental Figure S4.

OCP Photoactivation

The mutated OCPs were isolated and characterized. Whereas wild-type, Asp-6-Leu, and Pro-22-Val OCP-HisN preparations contained mostly holo-proteins (more than 90%), large quantities of apo-proteins (around 85%; for calculations, see “Materials and Methods”) were present in those of $\Delta 2-20$ OCP-HisN. The lack of the N-terminal arm could destabilize binding of the carotenoid that can escape from the mutated protein. However, we could not disregard that changes in the rate of OCP translation could influence the concentration of holo-proteins. All the OCP-HisC preparations contained more than 40% apo-protein.

All the results obtained with the $\Delta 2-15$ OCP mutant were identical to those observed with the $\Delta 2-20$ OCP mutant related to photoactivity and interaction with PBS and with FRP. For clarity, in the figures, we show only the results obtained with the $\Delta 2-20$ OCP mutant, and the results obtained with the $\Delta 2-15$ OCP mutant are shown in Supplemental Fig. S2.

Figure 2 shows the dark and light spectra of the mutated OCPs compared with the control OCPs. The dark spectra of the single Asp-6-Leu and Pro-22-Val OCP mutants were identical to that of wild-type OCP_0 . The dark spectra of the $\Delta 2-20$ and $\Delta 2-15$ OCPs indicated a partial conversion of the OCP_0 to OCP_r with higher absorbance at 496 nm and lower at 467 nm. We estimate that 25% to 30% of the mutated OCP was in the OCP_r form in the dark (for calculations, see “Materials and Methods.” Due to the presence of OCP_r in darkness, $\Delta 2-20$ and $\Delta 2-15$ OCPs were able to induce PBS fluorescence quenching in the absence of strong blue-green light (Supplemental Fig. S3). After 5-min illumination with strong white light, all of the OCP_0 s were completely converted to OCP_r , indicating that all mutated OCPs were photoactive.

Photoconversion (OCP_0 to OCP_r) and recovery (OCP_r to OCP_0) kinetics were followed by measuring absorbance changes at 550 nm during 5-min illumination with strong white light followed by 20 min in the dark (Figs. 2, A and B, and 3; Supplemental Fig. S4). The rate for photoconversion of the single mutant Asp-6-Leu was slower (HisC, $t_{1/2} = 26$ s and HisN, $t_{1/2} = 18$ s, where $t_{1/2}$ is the time needed to convert one-half of the OCP orange into OCP red) than that of the wild-type OCP ($t_{1/2} = 15$ s). In contrast, those of the $\Delta 2-15$ and $\Delta 2-20$ and Pro-22-Val OCP mutants were faster ($t_{1/2} = 3.8, 4,$ and 9.5 s, respectively). The rate of recovery was similar for the wild-type, Asp-6-Val, and Pro-22-Val mutants but much slower for the $\Delta 2-20$ and $\Delta 2-15$ OCP mutants (Fig. 3; Supplemental Figs. S2 and 4).

OCP Interaction with PBS : In Vitro-Induced PBS Fluorescence Quenching

In vitro reconstitution experiments, developed by Gwizdala et al. (2011), were used to study the interaction between OCPs and PBS to elucidate the role of mutations in this interaction. Isolated *Synechocystis* PBSs were incubated with preilluminated wild-type and mutated OCP_r s in 0.5 M phosphate under strong blue-green light

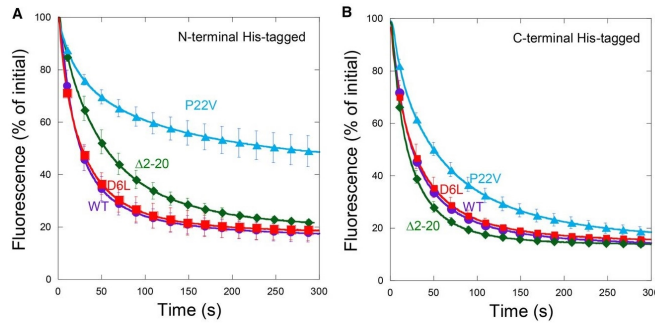


Figure 4.4 – PBS fluorescence quenching induced by wild-type (WT) and mutated N-terminal (A) and C-terminal (B) His-tagged OCP_r s wild-type (circles), $\Delta 2-20$ (diamonds), Asp-6-Leu (squares), and Pro-22-Val (triangles). The OCPs were preilluminated for 10 min with strong white light ($5,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 4°C . Then the OCP_r s were added to the PBSs in 0.5 M phosphate and illuminated with blue-green light ($900 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 23°C . Ratio of OCP to PBS = 40. The error bars represent the sd.

at 23°C . The decrease of PBS fluorescence during the light incubation (5 min) and the following fluorescence dark recovery (15 min) were followed by measurements in a pulse amplitude modulated (PAM) fluorometer (Fig. 4). The experiments were done with the same concentration of holo-OCP. The presence of apo-protein does not affect the kinetics of fluorescence quenching^[140].

The wild-type and $\Delta 2-20$ OCP_r -HisC induced the largest and fastest quenching, suggesting that the absence of the N-terminal arm does not affect OCP_r binding to PBS (Fig. 4). The introduction of the His-tag to the N terminus of the $\Delta 2-20$ OCP appeared to hinder OCP binding since slower and smaller fluorescence quenching was induced by this modified OCP compared with the OCP-HisC (Fig. 4). The Asp-6-Leu and wild-type OCP-HisNs also induced a slightly slower and smaller fluorescence quenching than the OCP-HisCs (Fig. 4). The most affected mutant was the Pro-22-Val-HisN, which induced only 40% of PB fluorescence quenching (Fig. 4).

Effect of Mutations on PBS Fluorescence Recovery and FRP Interaction with Bound OCP_r

To study the effect of the OCP mutations on the FRP interaction with OCP bound to PBS, we compared the dark PBS fluorescence recovery kinetics in the absence and presence of FRP. When the quenched PBS-OCP complexes were transferred to darkness, an increase in fluorescence associated with OCP detachment from PBS was observed (Fig. 5). As has been previously described, the location of the His-tag affects fluorescence recovery^[140]. The OCP-HisN largely detached faster than the OCP-HisC (Fig. 5). Interestingly, the $\Delta 2-20$ OCP-HisN largely recovered slower than the wild-type OCP-HisN (compare Fig. 5, A and B). The fastest fluorescence recovery was observed for Pro-22-Val-OCP-HisN as expected because of its weak binding to PBS (Fig. 5).

The presence of FRP during the dark incubation accelerated the recovery of fluorescence in all cases. However, this effect was much greater when the N-terminal arm was absent. FRP largely increased the rate of dark fluorescence recovery of PB-OCP complexes containing the mutated $\Delta 2-15$ and $\Delta 2-20$ OCPs (Fig. 5; Supplemental Fig. S2).

Effect of the Lack of the N-terminal Arm on the OCP-Related Photoprotection in Vivo

Next, the effect of the absence of the OCP N-terminal arm in the OCP-related photoprotective mechanism in *Synechocystis* cells was investigated. Two *Synechocystis* C-terminal His-tagged OCP mutants were constructed (Supplemental Fig. S5). One lacked the N-terminal arm ($\Delta 2-15$ OCP), and in the second one, only one part of the arm was removed ($\Delta 9-15$ OCP). Unexpectedly, western-blot analysis using the anti-OCP antibody clearly showed that the OCP was absent in the $\Delta 2-15$ OCP mutant

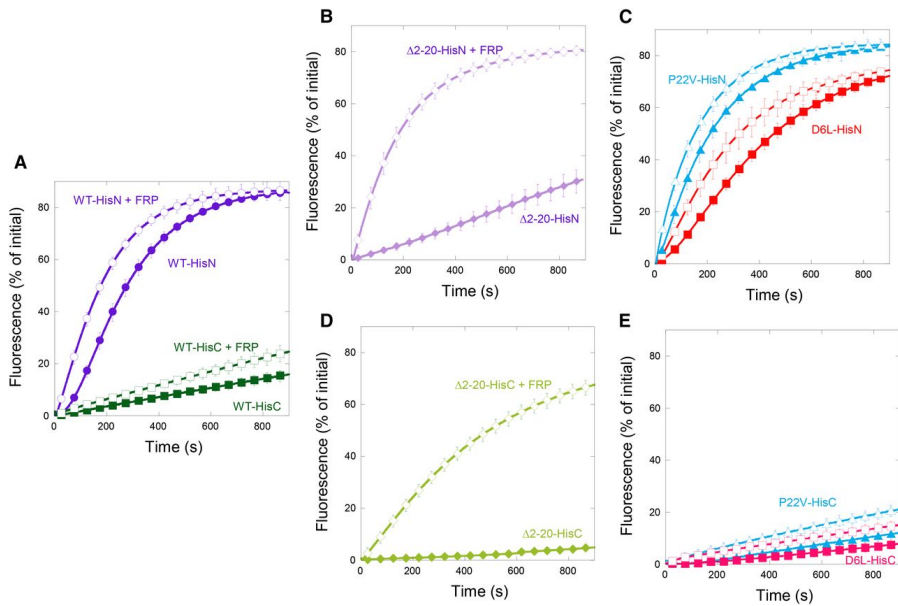


Figure 4.5 – Effect of FRP on dark PBS fluorescence recovery kinetics. Dark PBS fluorescence recovery in quenched OCP-PB complexes in the absence (colored symbols) or presence of FRP (white symbols). A, Wild-type (WT) OCP-HisC (squares) and wild-type OCP-HisN (circles). B, $\Delta 2-20$ OCP-HisN (diamonds). C, Asp-6-Leu OCP-HisN (squares) and Pro-22-Val OCP-HisN (triangles). D, $\Delta 2-20$ OCP-HisC (diamonds). E, Asp-6-Leu OCP-HisC (squares) and Pro-22-Val OCP-HisC (triangles). The error bars represent the sd.

(Supplemental Fig. S5). Reverse transcription-PCR experiments demonstrated that, although the mutated OCP was absent, the corresponding mRNA was present (Supplemental Fig. S5). The presence of a very stable hairpin ($\Delta G = -9.4$) in the mRNA coding for the modified ocp gene lacking the first 42 nucleotides ($\Delta 2-15$ OCP) may have led to an absence of translation due to a hindering of ribosome binding (Supplemental Fig. S6). In the mRNA coding the $\Delta 9-15$ OCP, the hairpin is destabilized (Supplemental Fig. S6) and the $\Delta 9-15$ OCP protein was detected by western-blot analysis (Supplemental Fig. S5). Although the protein was synthesized in the overexpressing $\Delta 9-15$ OCP *Synechocystis* strain, its concentration was substantially lower than in the overexpressing wild-type OCP strain and only slightly higher than in wild-type *Synechocystis* cells (Supplemental Fig. S5). In addition, although wild-type OCP only incorporates ECN and hECN^[96], the $\Delta 9-15$ OCP attached several carotenoids : 13% myxoxanthophyll, 8% zeaxanthin, 2% canthaxantin, 16% ECN, and only 61% hECN. Only the OCPs binding ketocarotenoids are photoactive^[96].

The $\Delta 9-15$ OCP was isolated from *Synechocystis* cells and characterized. The absorbance spectrum of the dark-orange $\Delta 9-15$ OCP, similar to the wild-type OCP, presented two peaks at 476 and 496 nm. However, the peak at 496 nm was higher than that at 476 nm (as in the $\Delta 2-15$ and $\Delta 2-20$ OCPs isolated from *E. coli*), suggesting the presence of OCP_r in the dark (Fig. 6). The $\Delta 9-15$ OCP induced PBS fluorescence quenching in the dark similar to $\Delta 2-20$ OCP (Supplemental Fig. S3). Illumination with strong white light induced a change in the absorbance spectrum, indicating that the modified OCPs binding ECN and hECN were still photoactive (Fig. 6). The rate of $\Delta 9-15$ OCP photoconversion was faster than that of the wild-type OCP, and the light form was very stable (Fig. 6). Despite this, the $\Delta 9-15$ OCP induced much less PBS fluorescence quenching than the wild-type OCP (Fig. 6). This behavior was similar to that of the *E. coli* $\Delta 2-20$ OCP-HisN. Finally, a large effect of the FRP was observed during PBS fluorescence recovery. FRP largely increased the rate of dark fluorescence recovery of the PBS- $\Delta 9-15$ OCP complexes as was observed in the complexes containing $\Delta 2-15$ and $\Delta 2-20$ OCPs (Fig. 6).

In *Synechocystis* cells containing the mutated $\Delta 9-15$ OCP, no fluorescence quenching was induced by strong blue light (Supplemental Fig. S7). This phenotype could be explained by several factors : (1) the low concentration of $\Delta 9-15$ OCP produced by the mutant compared with the wild type, (2) the weak $\Delta 9-15$ OCP binding to PBS,

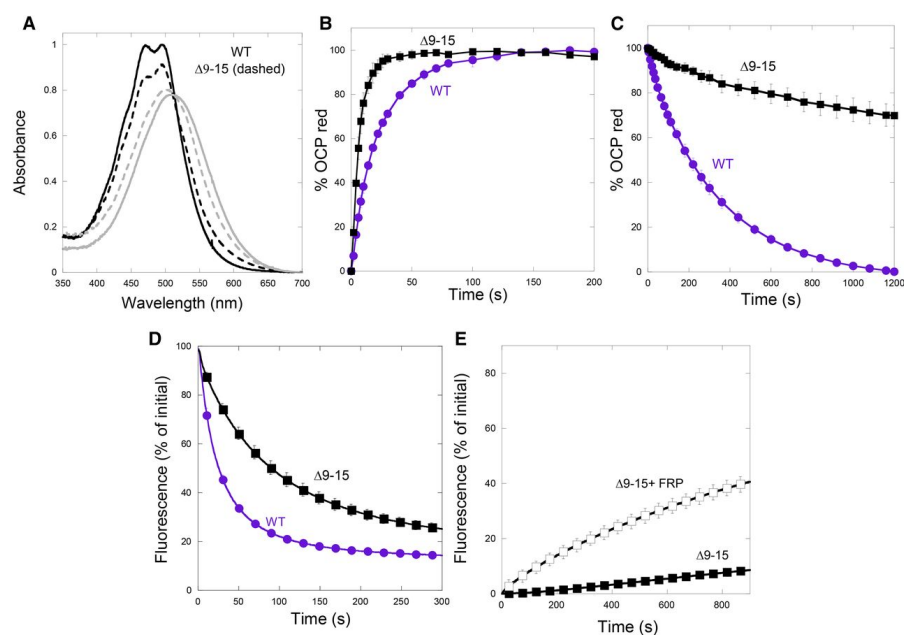


Figure 4.6 – he $\Delta 9-15$ OCP : photoactivation and interaction with PBS and FRP. A, Absorbance spectra of wild-type (WT; solid line) and $\Delta 9-15$ OCP (dashed line) in darkness (black) and after 5-min illumination with strong white light ($5,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$; gray). Photoactivation (B) and dark recovery kinetics (C) of wild-type (circles, violet) and $\Delta 9-15$ OCP (squares, black). OCP_o to OCP_r photoconversion at 18°C during illumination with strong white light ($5,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). The error bars represent the sd. D, PBS fluorescence quenching induced by wild-type (violet circles) and $\Delta 9-15$ (black squares) OCP_r s during illumination with blue-green light ($900 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 23°C . The OCPs were preilluminated for 10 min with strong white light ($5,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 4°C . E, Dark fluorescence recovery in the absence (white symbols) or presence of FRP (black symbols) in quenched PBS- $\Delta 9-15$ OCP complexes. The error bars represent the sd. In some curves, the error bars are smaller than the symbols.

and (3) the greater effect of FRP leading to a fast detachment of OCP from PBS and, as a consequence, fast fluorescence recovery. Since the characteristics of the isolated $\Delta 9-15$ OCP are similar to those of the $\Delta 2-15$ and $\Delta 2-20$ OCPs, it is reasonable to hypothesize that *Synechocystis* mutants containing the latter OCPs will have the same phenotype than that containing the $\Delta 9-15$ OCP.

Discussion

The first evidence for the importance of the N-terminal arm to OCP activity was that, in *Synechocystis* cells with N-terminal His-tagged OCPs, strong blue-green light was able to induce less fluorescence quenching than in those with C-terminal His-tagged OCPs (A. Wilson and D. Kirilovsky, unpublished data). Thus, all OCPs isolated from *Synechocystis* cells have contained a His-tag at their C terminus. In contrast, in *E. coli* cells, the highest yield of holo-OCP was obtained when the His-tag was added to the N terminus. This addition destabilizes the N-terminal arm and facilitates carotenoid incorporation^[140]. However, the *E. coli* OCP-HisN induces less PBS fluorescence quenching than the OCP-HisC. Moreover, when the quenched PBS with the OCP-HisC attached are put in the dark, they remain mostly quenched in the absence of FRP, whereas those with the OCP-HisN recover the initial fluorescence in only several minutes, indicating a weak OCP binding to PBS^[140] (Fig. 5). In the present work, we have further studied the role of the N-terminal arm in OCP activity using different OCP mutants with modifications in the N terminus of the protein.

The Role of the N-terminal Arm in OCP Photoactivation

In the past, we have proposed that OCP photoactivation involves an opening of the protein, which causes the breaking of the interactions between the N- and C-terminal domains at the central interface between the two globular domains^[98] (see Fig. 1B). We observed that photoactivation and opening of the protein occur more readily when any of these interactions is weakened or removed by mutations. For example, when mutations induced cleavage of the Arg-155-Glu-244 hydrogen bond, the closed OCP_0 conformation was destabilized^[98]. Other results from our laboratory and others have suggested that the opening of the protein also involves breaking interactions at the interface between the C-terminal globular domain and the N-terminal arm. Based on a model obtained by computational analysis, we proposed that FRP, which binds only to OCP_r , interacts with a β -sheet region of the C-terminal domain that is solvent exposed in OCP_r but partially obstructed in the closed OCP_0 by the first 15 to 20 amino acids of the N-terminal domain^[114] (N-terminal arm). Thus, OCP photoactivation must involve disruption of the interactions between the C-terminal domain and the N-terminal arm. Indeed, mass spectrometry footprinting experiments have shown that this interaction region is more solvent exposed in OCP_r than in OCP_0 ^[179]. It has been proposed that the detachment of the N-terminal arm from the C-terminal domain could facilitate putative OCP monomerization that occurs, at least *in vitro*, upon photoactivation^[158,179]. In this work, we show that the absence of the N-terminal arm accelerates OCP_r accumulation and stabilizes the OCP_r form in a manner similar to that of the mutations of Arg-155 or Glu-244^[98]. From these results, we conclude that the interaction of the N-terminal arm with the C-terminal domain that stabilizes the closed OCP_0 configuration is broken during photoactivation. The replacement of Asp-6 by a Leu has no evident effect on photoactivation, suggesting that the hydrogen bond between Arg-229 and Asp-6 does not play an important role in the conformational changes of the OCP during photoactivation.

As mentioned in the introduction, Pro-22 is located at the bent junction between the N-terminal arm and the N-terminal globular domain (Fig. 1). As it is known that Pro can isomerize, Pro isomerization could contribute to the movement of the N-terminal arm and its detachment from the C-terminal domain during OCP photoactivation. However, our results indicate that isomerization of Pro is not necessary for OCP photoactivation. When Pro is replaced by a Val, which cannot isomerize, the mutated OCP is still photoactive. Moreover, this change seems to facilitate the detachment of the N-terminal arm from the C-terminal domain during photoactivation rather than hinder it, leading to faster accumulation of the OCP_r in Pro-22-Val-OCP than in wild-type OCP.

The Role of the N-terminal Arm in OCP-PBS Interaction

No significant differences in quenching activity were observed between the wild-type and $\Delta 2-20$ ($\Delta 2-15$) OCP-HisC, indicating that the absence of the N-terminal arm does not destabilize OCP-PBS interaction. In contrast, Pro-22-Val OCP induced less fluorescence quenching, indicating that it was bound more weakly to PBS than the wild-type OCP. This can be explained by free movement of the N-terminal arm and/or a wrong position of the arm in the mutant that could hinder the OCP-PBS interaction. Wild-type OCP-HisN, $\Delta 2-20$ OCP-HisN, and $\Delta 9-15$ OCP-HisC, which contain artificial extensions in the N terminus, also induced slower and smaller fluorescence quenching activity than the wild-type OCP-HisC. Thus, this extension most likely also hinders OCP binding to PBS. Since $\Delta 2-20$ OCP-HisN induces less fluorescence quenching than the wild-type OCP-HisN, we expected faster fluorescence recovery in the presence of the latter. However, the opposite was observed. Both N- and C-terminal His-tagged $\Delta 2-20$ OCPs presented very slow fluorescence recovery. This could be explained by a higher stability of these mutated OCP_r s. In darkness, a large quantity of these OCPs remains red and might reattach to the PBS, creating new PB-OCP-quenched complexes, thereby lowering the increase of fluorescence. Nevertheless, we cannot disregard the possibility that, in the absence of the N-terminal arm, the binding is stronger. It is unlikely that detection of OCP bound to PBS by western blots will help to resolve which of the two hypotheses is correct, since in both cases, a larger concentration of bound OCP would be detected^[98].

Effect of Mutations on FRP Interaction with Free and Bound OCP_r

FRP, by interacting with the C-terminal domain of OCP_r, induces or accelerates the recovery of the PBS fluorescence in OCP-PBS complexes^[86,114]. The absence of the N-terminal arm facilitated the action of the FRP and largely accelerated the detachment of the OCP_r from the PBS as seen by a largely faster recovery of PBS fluorescence. This result suggests that the absence of the N-terminal arm allows FRP to better access the bound OCP. In conclusion, the N-terminal arm in the wild-type OCP partially hinders the FRP-OCP_r interaction and slows down the recovery of fluorescence. On the other hand, when motion of the arm is facilitated, as in the case of the Pro-22-Val mutant, the N-terminal arm hinders FRP binding still more than in the wild-type OCP.

Model of OCP Photoactivation

Based on past and present results, we can speculate about the steps involved in photoactivation (Fig. 7). In darkness, the OCP is in a closed conformation, and the carotenoid spans both domains. The three-dimensional structure of OCP shows hydrogen bonds between the carbonyl of the hECN and Tyr-201 and Trp-288 of the C-terminal domain^[95,113]. We have demonstrated that only ketocarotenoids, hECN, ECN, or cantaxanthin, render the OCP photoactive^[96,140]. OCP is able to bind zeaxanthin, but the zeaxanthin-OCP is not photoactive. Moreover, mutations of Tyr-201 and Trp-288 render the OCP completely photoinactive^[132]. Thus, we proposed that the first step of the photoactivation is the breaking of hydrogen bonds induced by light absorption^[132]. This, most likely, induces a subsequent reorganization of the β -sheet core, involving, for example, changes in the hydrogen bonding between Asp-304 and Arg-289 as proposed by Liu et al.^[179], leading to the movement of the C-terminal domain. During the opening of the protein, the amino acid interactions at the interface between both protein domains and those between the N-terminal arm and the C-terminal domain, which stabilize the closed conformation of OCP_o, are broken^[98,179]. Based on the results obtained with the Pro-22-Val OCP mutant in this work, we propose that the N-terminal arm remains in the same position in OCP_o and OCP_r, and that the isomerization of Pro-22 does not occur during photoactivation. The presence of a Pro at position 22, which confers some rigidity to the protein secondary structure, seems to be essential to correctly fix the position of the arm in OCP_o and in OCP_r. Replacement of Pro-22 by a Val weakens the interaction between the N-terminal arm and the C-terminal domain (faster photoactivation) and hinders OCP_r attachment to PBS (less fluorescence quenching). This behavior can be attributable to a less-constrained positioning of the N-terminal arm in the Pro-22-Val-OCP compared with the wild-type OCP. This can induce a slight opening of the N-terminal arm in Pro-22-Val-OCP_o and a freer movement of the N-terminal arm in OCP_r that largely affect OCP_r binding to PBS. However, we cannot completely disregard the possibility that Pro-22 isomerizes during photoactivation and the N-terminal arm adopts a new rigid position.

Conclusion

We conclude that the N-terminal arm is a key element in both OCP_o and OCP_r. In OCP_o, the relatively strong interaction between the N-terminal arm and the C-terminal domain avoids OCP photoactivation in darkness and low irradiance. For this reason, energy quenching does not occur at low light intensity, which would otherwise decrease photosynthesis rate and, therefore, the energy available for cell metabolism. It can be hypothesized that light energy is mainly needed to successively break hydrogen bonds and Van der Waals interactions that stabilize the closed OCP_o conformation. Since this process requires high energy, the accumulation of OCP_r, and as a consequence, energy quenching, occurs only under high light intensities. In OCP_r, the position of the N-terminal arm controls the rate of recovery, and its presence slows the action of FRP, allowing larger energy quenching under light stress conditions.

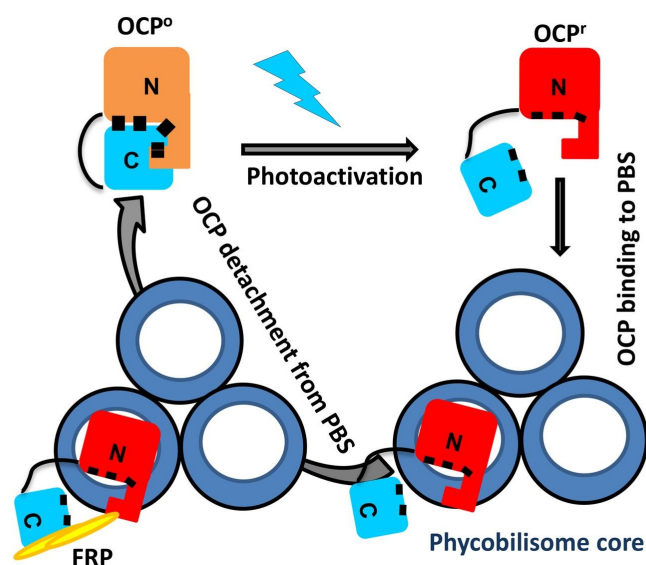


Figure 4.7 – Model of the OCP-related photoprotective mechanism. Strong blue-green light induces conformational changes in the carotenoid, provoking conformational changes in the C-terminal domain of the OCP, leading to the breakage of the interactions between the two domains, including the interactions between the N-terminal arm and the C-terminal domain. This involves the movement of the C-terminal domain, but the N-terminal arm seems to remain in the same position as in OCP_o . In the open OCP_r , Arg-155 can interact with negative charges in the APC trimer of the PBS core and induces energy and fluorescence quenching. FRP induces (or accelerates) the detachment of OCP_r from the PBS. The presence of the N-terminal arm seems to slow down the FRP action, avoiding a rapid OCP detachment.

Materials and methods

Strains and Culture Conditions

The freshwater cyanobacteria *Synechocystis* sp. PCC 6803 wild type and mutants were grown photoautotrophically in a modified BG11 medium^[137], containing double the amount of sodium nitrate. Cells were kept in a rotary shaker (120 rpm) at 30°C, illuminated by fluorescent white lamps giving a total intensity of about 30 to 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ under a CO₂-enriched atmosphere. The cells were maintained in the logarithmic phase of growth and were collected at optical density at 800 nm = 0.6 to 0.8.

Construction of *Synechocystis* Mutant Strains

The nucleotide deletion in the *ocp* gene (*slr1963*) was obtained by site-directed mutagenesis using the QuikChange SL site-directed mutagenesis kit (Stratagene) and the synthetic primers in each case (Supplemental Figure S8). To obtain the mutant $\Delta 2-15$ OCP, a previously described plasmid^[98], containing the *ocp* gene (*slr1963*) under the control of the *psbA2* promoter, and the *frp* gene (*slr1964*) was used as a template with the primers *PsbA2-Rev* and *F-RCP-15*. For the addition of the sequence encoding for the first eight amino acids (taking into account the Met-1), we have used *R-PsbA2-8a* and *F-RCP15-8a* as primers and the plasmid containing the $\Delta 2-15$ construction as a template. The plasmids $\Delta 2-15$ OCP and $\Delta 9-15$ OCP were used to transform the wild-type *Synechocystis* cells. The endogenous *slr1963* gene was then interrupted using the $\Delta\text{OCP-FRP}$ plasmid construct bearing a spectinomycin/streptomycin resistance cassette^[98]. Segregation of the mutants was confirmed by PCR and by DNA sequencing.

DNA and RNA Isolation, Manipulation, and Analysis

Total RNA from *Synechocystis* cells (between 3 and 5 μg chlorophyll·mL⁻¹) was isolated using the method described by Ausubel et al.^[180] and treated with DNase-

RNase free (Ambion). Reverse transcription-PCR was carried out using Omniscript reverse transcriptase (Qiagen). The primer used to construct the complementary DNAs was R-RCP-His. Then, the complementary DNAs were amplified by PCR using the oligonucleotides R-RCP-His and 63-41-F.

Construction of Plasmids for Escherichia coli Production of Wild-Type and Mutated OCPs

The *ocp* (slr1963) gene from *Synechocystis* has been cloned and expressed in *E. coli* cells producing ECN as described in de Carbon et al.^[140]. Construction of the plasmids pCDF-OCPsynCtag and pCDF-OCPsynNtag carrying the slr1963 sequence with a sequence coding for a His-tag in the C terminus or the N terminus, respectively, was described in Bourcier de Carbon et al.^[140]. To obtain the pCDF- Δ 2-20 OCP and pCDF- Δ 2-15 OCP plasmid, the nucleotides coding for the 19 or the 14 first amino acids (after Met-1) of the *ocp* coding sequence were deleted by site-directed mutagenesis using the plasmid pCDF-OCPsyn-Ctag or pCDF-OCPsyn-3aaNtag as template and synthetic primers (Supplemental Fig. S8). The point mutations Pro-22-Val and Asp-6-Leu were introduced by directed mutagenesis, using the pCDF-OCPsyn-Ctag or pCDF-Syn-3aaNtag plasmid as template and mismatching primers (Supplemental Fig. S8).

Transformation of E. coli Cells and Induction of Gene Expression

E. coli BL21-Gold (DE3) cells from Agilent Technologies were used for OCP production. BL21 cells were transformed simultaneously with three plasmids to produce holo-OCPs : pAC-BETA (gift from Francis X. Cunningham, University of Maryland ;^[161]), pBAD-CrtO^[140], and pCDF-OCP plasmids. The transformed *E. coli* cells were grown in the presence of three antibiotics (ampicillin [50 μ g mL⁻¹], chloramphenicol [17 μ g mL⁻¹], and streptomycin [25 μ g mL⁻¹]) to maintain the different plasmids in the *E. coli* cells. For induction of different promoters, transformed *E. coli* cells were grown in Terrific Broth medium at 37°C for 3 to 4 h until OD₆₀₀ = 0.8. Then, 0.02% (w/v) arabinose was added and the culture was grown overnight at 37°C. In the morning, the cells were diluted with fresh medium and 0.02% arabinose, and they were grown at 37°C until OD₆₀₀ = 1 to 1.2. Then, IPTG (0.2 mM) was added, and the cells were incubated overnight at 28°C. In the morning, the cultures were harvested and pellets were stored at -80°C until they were used.

E. coli BL21-Gold (DE3) cells were transformed with only pCDF-OCP to produce apo-OCP. The cells were grown in the presence of streptomycin (50 μ g mL⁻¹). The *ocp* transcription was induced by addition of IPTG (0.2 mM), and the cells were incubated overnight at 28°C.

OCP Isolation and Carotenoid Analysis

His-tagged Δ 9-15 overexpressed in *Synechocystis* was isolated as described in Wilson et al. (2008). For isolation of OCP from *E. coli* cells, frozen *E. coli* cells were re-suspended in the lysis buffer containing 40 mM Tris-HCl (pH 8), 10% glycerol, and 300 mM NaCl and were broken in dim light using a French Press. The membranes were pelleted, and the supernatant was loaded on a nickel column (Ni-ProBond resin, Invitrogen). OCP was eluted with 200 mM imidazole and then dialyzed against Tris-HCl (40 mM).

The carotenoid content of the mutated Δ 9-15 OCP isolated from *Synechocystis* cells was analyzed as described in Punginelli et al.^[96] and Bourcier de Carbon et al.^[140].

Holo-OCP Concentration Calculation

Total OCP was measured using the Bradford method. At least five independent Bradford measurements of each isolated OCP were realized. The concentration in milligrams per milliliter obtained by this method was converted to molar concentration using a molecular mass of 35 kD for the OCP. Holo-OCP concentration was calculated based on the fact that each holo-OCP binds one carotenoid molecule, and thus, the molar concentrations of carotenoid and holo-OCP are identical. Carotenoid concentration was first calculated in milligrams per milliliter from the carotenoid

absorbance at 496 nm and using an absorbance of 2,158 for a solution containing 1% (w/v) of carotenoid measured with a cuvette of 1 cm and then converted to molar concentration. The molar ratio of holo-OCP to total OCP gives the percentage of holo-protein. When this ratio is around 1, we considered that the preparation contains 100% holo-protein.

Absorbance Spectra and Photoactivity Kinetics of OCP

Absorbance spectra and the kinetics of photoactivity (illumination with 5,000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ white light) and dark recovery were measured in a Specord S600 (Analytik Jena) at 9°C or 18°C in the cuvette.

PBS Isolation

Synechocystis PBSs were isolated as described in Gwizdala et al.^[81]. In brief, after reaching optical density = 1 at 800 nm, cyanobacterium cells were pelleted. Cells were broken with glass beads, and PBSs were solubilized by Triton X-100 treatment and then isolated on a Suc gradient.

PAM Fluorometer

Fluorescence yield quenching was monitored using a pulse amplitude fluorometer (101/102/103-PAM, Walz). Measurements were performed in a 1-cm path-length stirred cuvette. In vitro reconstitution was handled as described in Gwizdala et al.^[81] with a PBS concentration of 0.012 μM in 0.5 M potassium phosphate buffer (pH 7.5) at 23°C. Fluorescence quenching was followed under 900 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ blue-green light (halogen white light filtered by a Corion cutoff 550-nm filter; 400-550 nm).

The ratio of OCP to PBS was 40 in all experiments. The concentration of OCP for these experiments was calculated from the carotenoid absorbance spectra since only the OCP attached to a carotenoid is able to be photoactivated and to bind to PBS. OCP was previously converted to the red form by 10-min illumination with 5,000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ white light at 4°C.

Calculation of Percentage of Conversion of $\Delta 2-20$ OCP

To estimate the percentage of the red form present in $\Delta 2-20$ OCP in darkness, we fit the dark spectra with a combination of wild-type OCP_o and OCP_r spectra: we multiplied the OCP wild-type orange absorbance spectrum by a factor a and the OCP red absorbance spectrum by a factor b ($a + b = 1$). The best fit was obtained using the values $a = 0.25$ and $b = 0.75$ for $\Delta 2-20$ C-terminal His-tagged OCP and $a = 0.30$ and $b = 0.70$ for $\Delta 2-20$ N-terminal His-tagged OCP (see Supplemental Fig. S9).

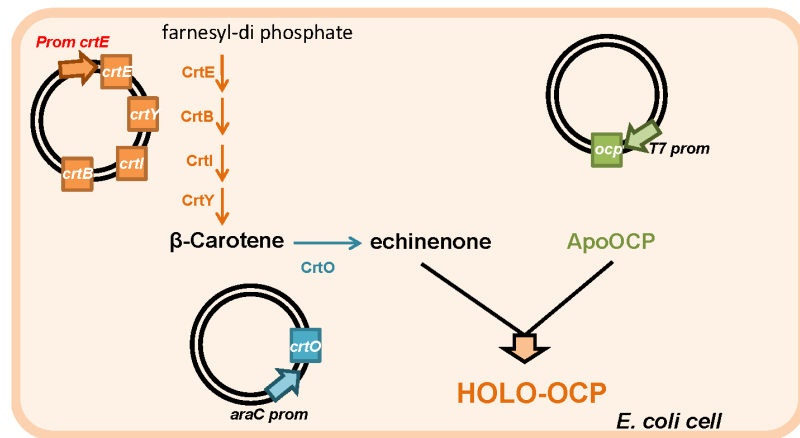
Gel Electrophoresis and Protein Gel Blots

Total cell proteins of the OCP mutants were analyzed by SDS-PAGE on a 12% polyacrylamide/2 M urea gel in a Tris/MES system^[139]. After electrophoresis, proteins were transferred to a membrane, and the OCP was detected using a polyclonal antibody against OCP. Finally, OCP antibody was monitored by an alkaline phosphatase colorimetric reaction.

Acknowledgments

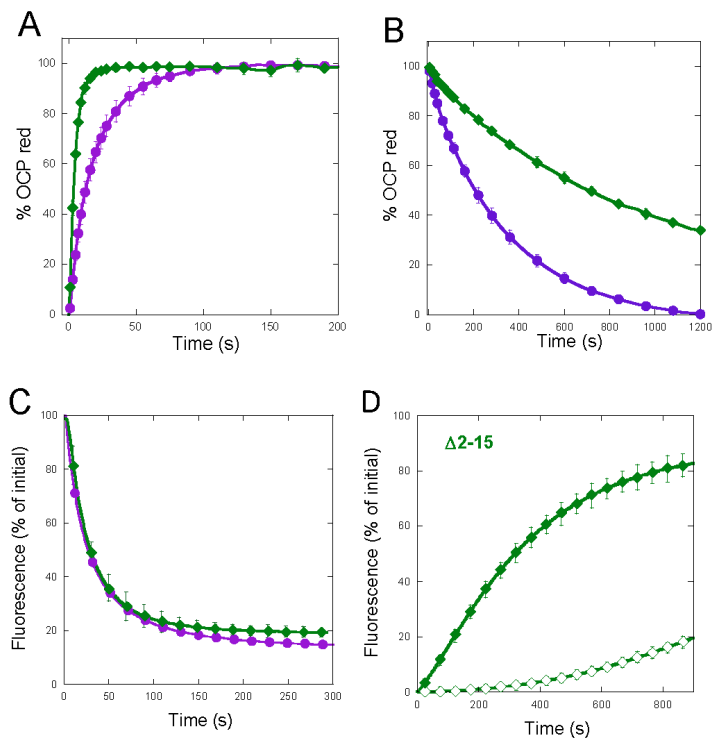
We thank Francis X. Cunningham for the gift of pAC-BETA plasmid, François Perreau for the determination of the carotenoid content in the OCP isolated from *Synechocystis* cells, Herbert van Amerongen for critical reading of the article and stimulating and fruitful discussions, Dr. Sun Un for critical reading of the article and improving the English, Dr. Jose Antonio Escudero for help with mfold utilization, and Sandrine Cot for technical assistance.

Supplemental Data



Supplemental Figure 1 : Scheme of the *E. coli* cells producing holo-OCPs.

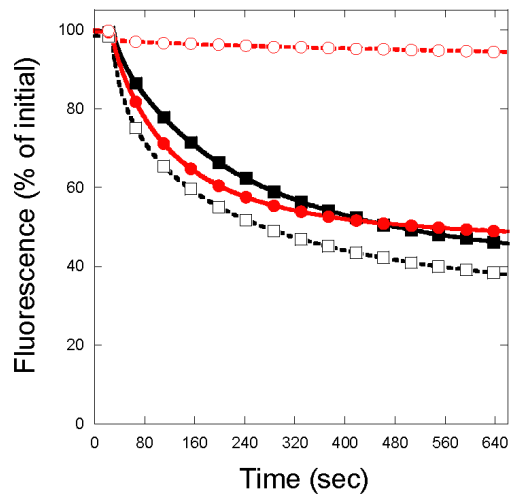
Enzymatic pathway farnesyl-diphosphate to echinenone is composed by enzymes CrtE, CrtB, CrtI, CrtY and CrtO. Coding sequences are cloned in two plasmids, pAC-Beta (CrtE, CrtB, CrtI and CrtY) and pBAD (CrtO). The third plasmid (pCDF) contains OCP coding sequence.



Supplemental Figure 2 :

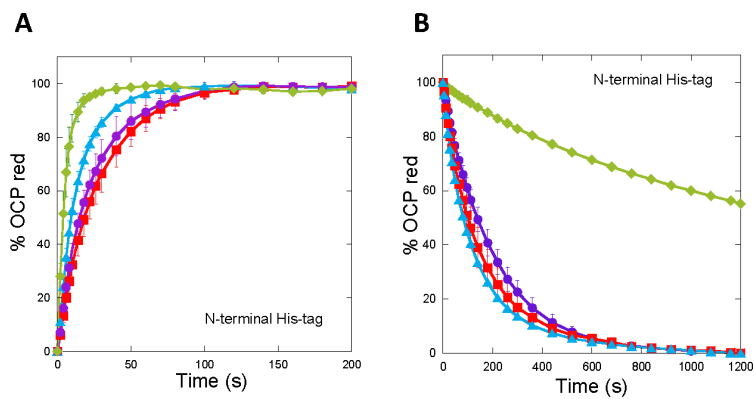
Photoactivation (OCP^o to OCP^r) (A) and dark recovery (OCP^r to OCP^o) (B) kinetics of WT (circles, violet) and $\Delta 2-15$ OCP-HisC (diamonds, green) isolated from *E. coli*. (A) OCP^o to OCP^r photoconversion during illumination with strong white light ($5000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 18°C and (B) recovery in darkness. The error bars represent SD.

C) Phycobilisome Fluorescence Quenching induced by WT (violet circle) and $\Delta 2-15$ (green diamond) OCP^rs during illumination with blue-green light ($900 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 23°C . The OCPs were pre-illuminated for 10 min with strong white light ($5000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 4°C . (D) Dark fluorescence recovery in the absence (open symbols) or in the presence of FRP (closed symbols) in quenched PBS- $\Delta 2-15$ OCP (E) complexes. The error bars represent SD. In some curves, the error bars are smaller than the symbols.

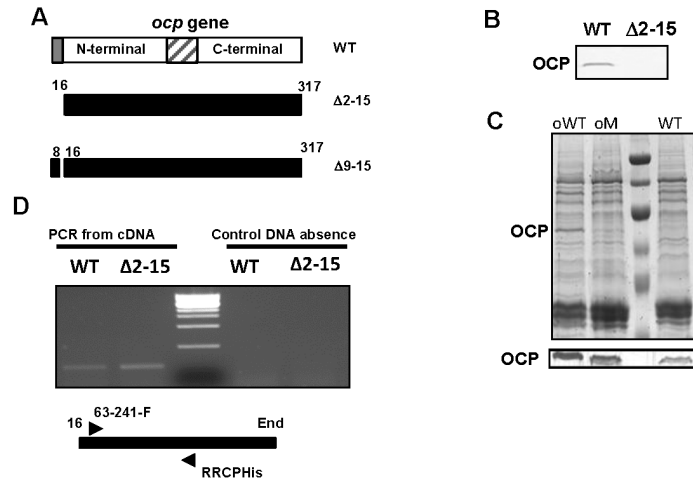


Supplemental Figure 3: Phycobilisome fluorescence quenching induced by $\Delta 2-20$ and $\Delta 9-15$ OCPs in darkness.

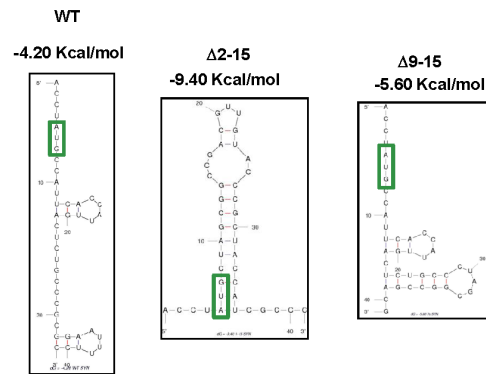
Phycobilisome fluorescence quenching induced by WT (red open circles), N-terminal $\Delta 2-20$ (black close squares), C-terminal $\Delta 2-20$ (black open squares) and C-terminal $\Delta 9-15$ (red closed circles) OCPs followed by a PAM fluorometer in the absence of actinic light at 23°C. A representative experiment is shown in the figure.



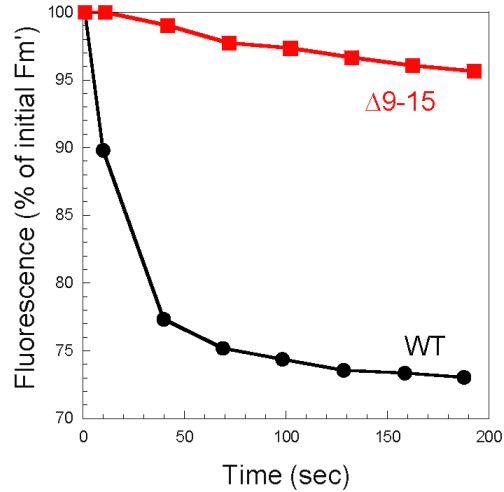
Supplemental Figure 4: Photoactivation (A) and Recovery (B) of WT and mutated OCPs-HisN. (A) OCP^o to OCP^r photoconversion during illumination with strong white light ($5000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 18°C of WT (circles), $\Delta 2-20$ (diamonds), D6L (squares) and P22V (triangles) OCPs. (B) Recovery in darkness with the same symbols. The error bars represent SD.



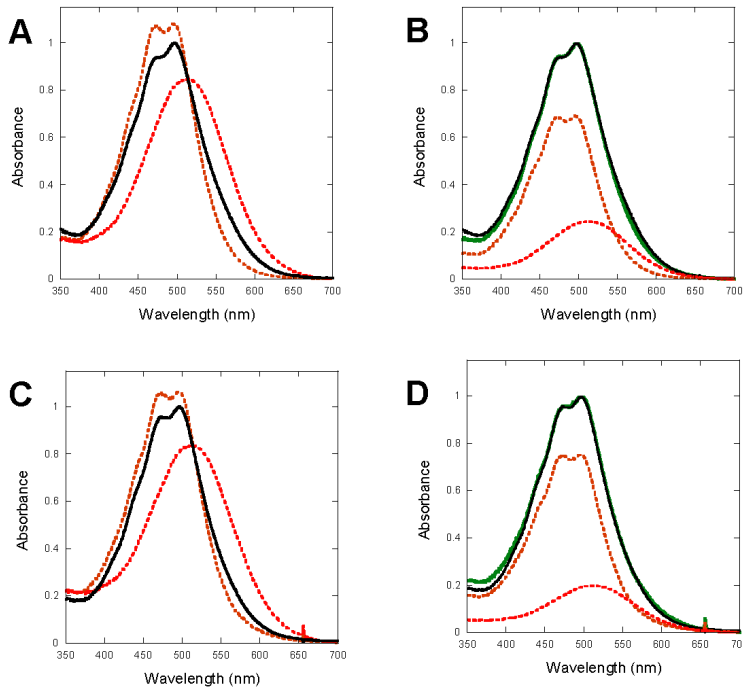
Supplemental Figure 5: Expression of WT and mutant OCPs in *Synechocystis* cells. (A) Diagram of constructed OCPs; (B) Western blot detection of OCP with anti- *Synechocystis* OCP in total proteins extracted from WT *Synechocystis* and from a *Synechocystis* mutant overexpressing the $\Delta 2-15$ OCP mutant; (C) Coomassie Blue gel electrophoresis and western blot analysis with anti-OCP of total proteins extracted from WT *Synechocystis* cells (WT) and from *Synechocystis* strains overexpressing the WT (oWT) and the $\Delta 9-15$ (oM) OCPs; (D) RT-PCR analysis using total RNA isolated from *Synechocystis* cells overexpressing the WT and the $\Delta 2-15$ OCP. The primer RRCPhis was used for the cDNA synthesis, and the same primer with 63-241-F was used for PCR analysis using cDNA as a template in RT-PCR and an equivalent quantity of total RNA as template like control of DNA absence.



Supplemental Figure 6: Predictable hairpins in the mRNAs encoding WT and different mutated OCPs. Taking the sequence corresponding to -4 to $+37$ nucleotide position relative to translation start of WT, $\Delta 2-15$ and $\Delta 9-15$ OCPs, the ΔG was calculated by a program to fold RNA sequence (see Material and Methods). A lower ΔG (in absolute value) indicates that the mRNA secondary structure is easier to break by the translation machinery. Thus, high ΔG (in absolute value) indicates that the protein synthesis can be hindered or stopped.



Supplemental Figure 7: Fluorescence quenching induced in WT (black circles) and in overexpressing $\Delta 9-15$ OCP (red squares) *Synechocystis* cells by strong blue-green light ($750 \mu\text{mol photons m}^{-2} \text{s}^{-1}$).



Supplemental figure 9 : Spectra of N-terminal His-tagged $\Delta 2-20$ OCP (A, B) and C-ter $\Delta 2-20$ C-terminal His-tagged OCP (C,D) and fit of spectra using a combination of OCP° and OCP^r spectra (B, D)

In (A and B) $\Delta 2-20$ N-terminal His-tagged OCP and in (C and D) $\Delta 2-20$ C-terminal His-tagged OCP are represented with a black line. In all spectra, WT OCP° (orange dotted line) and WT OCP^r (red dotted line) forms are shown. In (B and D) the green line represents the fit of the spectra of dark $\Delta 2-20$ His-tagged OCPs.

$$\text{Fit } \Delta 2-20 \text{ N-terminal His-tagged OCP} = 0.70 * \text{OCP}^\circ + 0.30 * \text{OCP}^r$$

$$\text{Fit } \Delta 2-20 \text{ C-terminal His-tagged OCP} = 0.75 * \text{OCP}^\circ + 0.25 * \text{OCP}^r$$

4.0.2 Régulation de la traduction de l'OCP d2-15

Le premier essai de purification de l'OCP dépourvue du bras N-terminal modifiée a été réalisé chez *Synechocystis*. Pour construire cette OCP, les résidus 2 à 15 ont été supprimés ($\Delta 2-15$). La purification de cette OCP chez *Synechocystis* s'est révélée impossible, pourtant, l'ARN messager correspondant est bien présent (Thurotte et al., figure supplémentaire 5). La traduction de la protéine est donc très probablement affectée. En effet, la modélisation des structures secondaires de l'ARN révèle une structure secondaire importante ($\Delta G = -9,10$ kcal/mol) dans la construction créée chez *Synechocystis* (Thurotte et al, Figure S 6).

Afin de déterminer si la présence de cette structure est suffisante pour expliquer l'absence de la protéine, j'ai créé et isolé deux versions de la protéine chez *E.coli* : une contenant la structure secondaire et une autre dont la structure secondaire est cassée diminuant le ΔG à $-2,6$ kcal/mol. La quantité de protéines produite sans la structure secondaire est bien plus importante (7,7 mg/l avec la structure contre 139 mg/l sans).

En revanche, en l'absence de structure secondaire, une fraction seulement des protéines purifiées attache un caroténoïde chez *E. coli*. Une forte concentration en apoOCP peut être expliquée par deux raisons, toutes deux liées à l'augmentation probable de la vitesse de traduction. La première est que la quantité de caroténoïdes présents dans la cellule est trop faible comparé à la quantité d'OCP traduite.

Dans l'article précédent, la quantité maximale d'OCP attachant l'échinénone est de 35mg par litre de culture obtenus avec les constructions Syn-Ntag-3aa-ECN et Ana-3aaNtag-ECN^[140]. Ainsi, il pourrait y avoir une carence en caroténoïde et les molécules d'OCP traduites n'attacheraient pas de caroténoïde. La seconde explication est que l'augmentation de la vitesse de traduction ne permette pas une intégration

La structure secondaire de l'ARN messager est cassée par deux modifications silencieuses CUAGCG → CUGCCG.

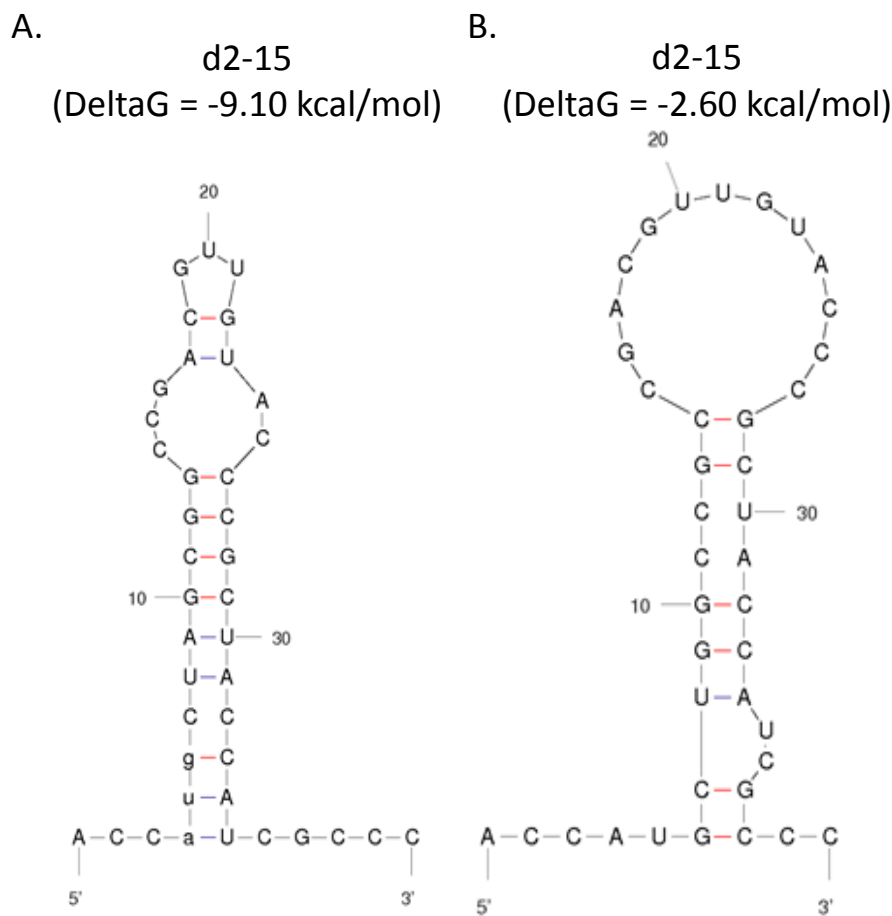


Figure 4.8 – Structure secondaire prédite dans la région -5/+40 pour les ARNm modifiés (A) d2-15 et (B) d2-15 dont la structure secondaire est affaiblie (mfold^[181]).

tion du caroténoïde dans l'OCP. Nous observons en effet que les constructions dont l'extrémité N-terminal est modifiée présentent des taux d'apoOCP très variables de 5 à 70% selon les constructions, malgré une production totale d'OCP plus faible que celle de notre référence, l'OCP Syn-Ntag-3aa-ECN qui est exempt (moins de 5%) d'apoOCP et dont le rendement de production est de 30-35mg/L.

En conclusion, les deux explications restent plausibles, à savoir que la forte concentration en apoOCP dans la construction sans la structure secondaire puisse être due à une carence en caroténoïdes ou une augmentation de la vitesse de traduction.

4.0.3 Influence de l'apo-protein dans les mesures d'activité de l'OCP

Les purifications d'OCP dans l'organisme endogène ont toujours donné des échantillons exempts d'apoOCP. Les apoprotéines peuvent être reconnues et dégradées par une machinerie spécifique ou bien l'entrée du caroténoïde au sein de l'OCP est finement régulée, et l'OCP ne peut être entièrement traduite que si elle intègre un caroténoïde. Cependant certaines OCPs modifiées produites chez *E.coli* peuvent contenir un taux élevé d'apoOCP probablement lié à l'absence de mécanismes de régulation de la traduction de l'OCP (traduction trop rapide pour permettre l'attachement du caroténoïde) et/ou de dégradation d'apoOCP. Il convient donc d'évaluer l'effet de l'apoOCP au cours des expériences menées au laboratoire. Résultats

Afin d'évaluer l'effet de l'ApoOCP sur les cinétiques de conversion, de reconversion à la forme orange en présence et en absence de FRP, ainsi que dans la reconstitution *in vitro* du mécanisme de photoprotection, j'ai décidé d'effectuer ces expériences avec des concentrations différentes d'apoOCP. L'apoOCP est produite de manière analogue à l'OCP décrite dans le chapitre 2^[140], mais en l'absence de caroténoïde (plasmides pAC- β et pBAD absents). La concentration de l'apoOCP (mg/ml) est mesurée par Bradford puis la concentration en microM est calculé en utilisant la masse molaire de l'OCP (MW=34,7). Le mélange est ensuite effectué directement dans la cuvette de mesure. J'ai choisi cinq concentrations en holoOCP différentes pour les expériences de photoconversion (10%, 30%, 60%, 80% et 100% d'holoOCP) et deux pour les expériences de reconstitution *in vitro* (100% et 10% d'holoOCP).

La présence d'apoOCP n'influence pas la cinétique de photoconversion des OCPs qui est identique en présence ou en l'absence d'apoOCP (Figure 4.9.A). Afin de déterminer les conséquences de la présence d'apoOCP dans la cuvette de mesure lors d'une cinétique de reconversion en présence de FRP, j'ai ajouté une même quantité de FRP (rapport holoOCP : FRP de 1 : 1) et observé la cinétique de retour à la forme orange des OCPs en présence d'apoOCP. La présence d'apoOCP diminue l'action de la FRP (Figure 4.9B).

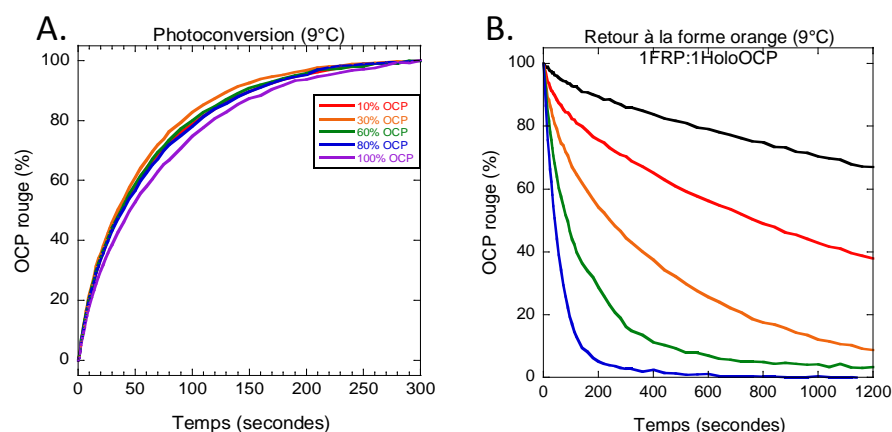


Figure 4.9 – Effet de l'apoOCP lors de la mesure d'une cinétique de retour à la forme orange en présence de FRP (A) Cinétique de photoactivation par une forte lumière blanche ($5000 \mu\text{moles de photons} * \text{m}^{-2} * \text{sec}^{-1}$) et (B) cinétique de retour à la forme orange à l'obscurité en présence de FRP (1FRP :1 holoOCP) d'échantillons contenant 10% (rouge), 30% (orange), 60% (vert), 80% (bleu) ou 100% d'holoOCP.

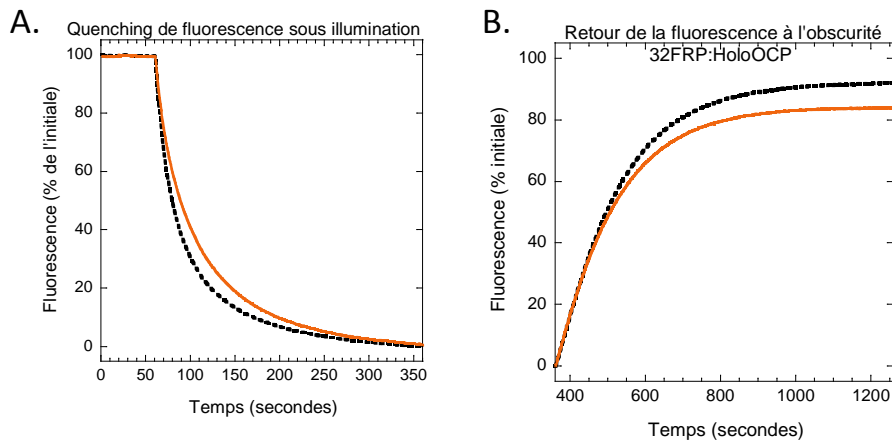


Figure 4.10 – Effet de l’apoOCP lors de la mesure d’une cinétique de retour de la fluorescence des PBs après le quenching de fluorescence induit par l’OCP (A) Quenching de fluorescences des PBs à 23°C induit par l’OCP WT en l’absence (noir) et en présence de 90% d’apoOCP (orange) pré-illuminés 5 minutes par une forte lumière blanche ($5000 \mu\text{moles de photons} * \text{m}^{-2} * \text{sec}^{-1}$) à 4°C. Les PBs sont illuminés avec une forte lumière bleu-verte (30 secondes) puis l’OCP rouge est ajoutée (60 secondes). (B) Après 5 minutes d’illumination, la lumière est éteinte. Ratio 40 holoOCP :1PBs, tampon K-phosphate 0,5M.

J’ai ensuite testé l’effet d’une concentration élevée d’apoOCP (90% d’apoOCP) dans les conditions standard de la reconstitution *in vitro* du mécanisme de photoprotection (32 FRP pour 1 OCP-PBS complex). La cinétique de quenching de fluorescence des PBs est très similaire à celle obtenue avec de l’OCP pure. L’ajout de FRP en large excès induit une récupération de fluorescence semblable, indifférente de la présence de l’apoOC.

Conclusion

La présence d’apoOCP induit un biais dans la mesure de cinétiques de reconversion à la forme orange. Avec des échantillons contaminés en apoOCP, il convient donc d’utiliser une référence autant contaminée en apoOCP, et de travailler en large excès de FRP. En revanche, lors de la reconstitution *in vitro* du mécanisme de photoprotection, l’ajout d’apoOCP n’est pas nécessaire. Ceci est probablement due au large excès de FRP présent lors de l’expérience.

4.0.4 Importance de la phénylalanine n°227 dans la photoconversion de l’OCP

Au cours de la rédaction de l’article sur le rôle du bras N-terminal de l’OCP, le résidu n°227 avait attiré mon attention. Ce dernier est localisé dans le domaine C-terminal, en vis-à-vis de la proline n°22. Résultats

J’ai créé par mutagenèse ponctuelle (primers utilisés en annexe 1) une version modifiée de l’OCP de *Synechocystis* dont la phénylalanine n°227 est modifié en Leucine et isolé la protéine chez *E. coli*. La figure 4.11 présente les résultats obtenus en cinétique de photoconversion et retour à la forme orange à l’obscurité à 18°C et à 9°C en absence et présence de FRP. La photoconversion de F227L à 18°C et à 9°C est plus rapide que l’OCP non modifiée. Le retour à la forme orange est plus lent. Ainsi, la forme rouge de l’OCP F227L est plus stable que celle de l’OCP WT. La figure 4.12 montre que l’activité de la FRP n’est pas impactée sur cette OCP modifiée ponctuellement. Des expériences de reconstitution *in vitro* du mécanisme de photoprotection ont également été réalisées. La diminution de fluorescence des PBs de *Synechocystis*, induite par l’ajout d’OCP préconvertie à la forme rouge, a été suivie dans le temps à l’aide d’un fluorimètre. Afin de maintenir l’OCP sous forme rouge, une forte lumière bleu-verte éclaire la cuvette durant les 5 premières minutes, puis elle est éteinte. Le quenching de fluorescence induit par l’OCP modifiée est plus faible que celui induit par l’OCP WT (Figure 4.12) et ressemble au phénotype de l’OCP dont la proline

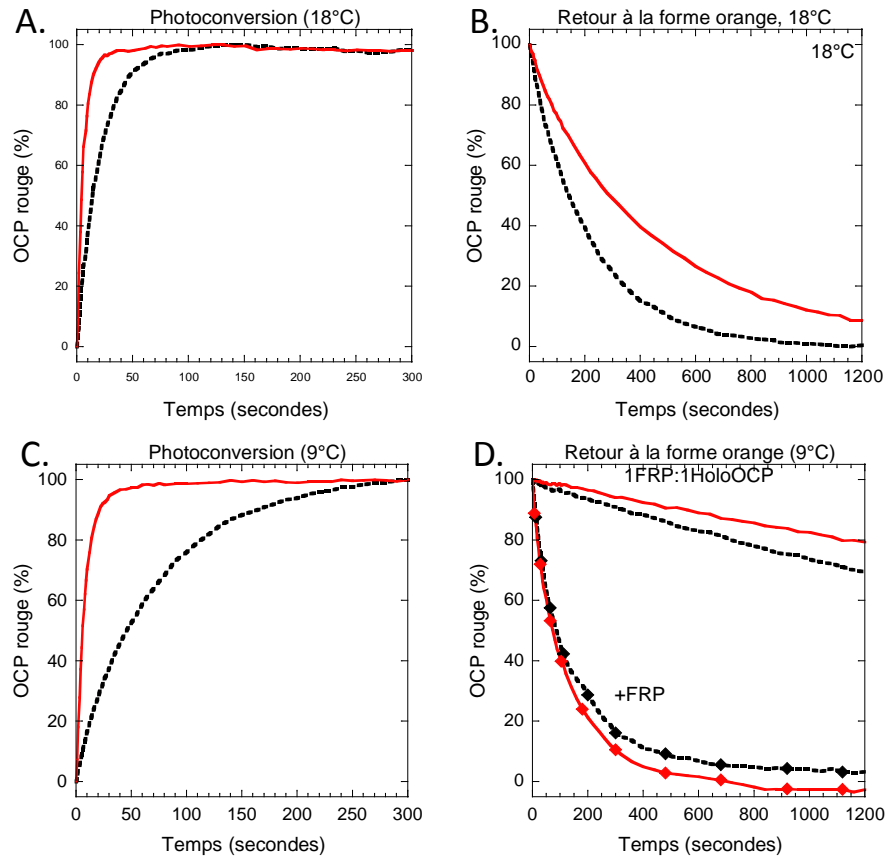


Figure 4.11 – Photoactivation et retour à la forme orange à l’obscurité de l’OCP F227L. Cinétique de photoactivation par une forte lumière blanche ($5000 \mu\text{moles de photons} * m^{-2} * sec^{-1}$) des OCP F227L (rouge) et WT (noir en pointillé) à 18°C (A) ou 9°C (C). (B) Retour à la forme orange à 18°C et (C) retour à la forme orange à 9°C en absence (aucun symbol) ou en présence (losanges) de 1FRP :1HoloOCP. La préparation de F227L contient 20% d’apoOCP, aussi, le contrôle utilisé contient également 20% d’apoOCP.

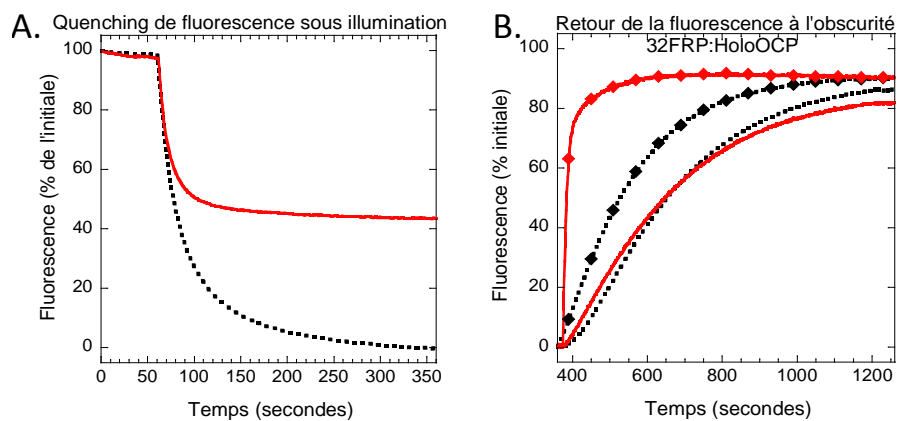


Figure 4.12 – Reconstitution *in vitro* du quenching de fluorescence par l’OCP F227L (A) Quenching de fluorescences à 23°C des PBs induit par les OCP F227L (rouge) et WT (noir en pointillé) pré-illuminés 5 minutes par une forte lumière blanche ($5000 \mu\text{moles de photons} * m^{-2} * sec^{-1}$) à 4°C . Les PBs sont illuminés avec une forte lumière bleu-verte puis l’OCP rouge est ajoutée à 60 secondes d’illumination. Ratio 40 holoOCP :1PBs, tampon K-phosphate 0,5M. (F) Après 5 minutes d’illumination, la lumière est éteinte et la récupération de la fluorescence est suivi au cours du temps.

n°22 est modifiée en valine. En revanche, l'action de la FRP sur les complexes OCP F227L-PBS est beaucoup plus importante qu'avec ceux avec une OCP non modifiée.

Conclusion

La photoactivation de l'OCP F227L est plus rapide que celle de l'OCP rouge suggérant que la F227 pourrait interagir avec des acides aminés du N-terminal bras (par exemple P22) ou du domaine N-terminal (T152 ou N156 qui sont localisés à moins de 4Å de la F227). En absence de la Phénylalanine la protéine pourrait s'ouvrir plus facilement. Cependant, bien que la forme rouge soit plus stable, l'OCP F227L induit moins de quenching de fluorescence des PBS. De plus, la FRP est capable de détacher très efficacement l'OCP des PBS alors que la cinétique de détachement en l'absence de FRP est la même que l'OCP WT. Je n'ai pas pour le moment d'explication à ce résultat, cependant la modification des prolines n°225 ou n°226 aussi induisent moins de quenching de PBS fluorescence suggèrent que cette région est importante pour la photoactivation ou l'interaction avec le PBS.

LE CAROTÉNOÏDE EST TRANSLATÉ DE 12 Å LORS DE LA PHOTOACTIVATION

COLLABORATION AVEC RYAN LEVERENZ (BERKELEY)

Le modèle de la photoactivation de l'OCP présenté à la fin de l'introduction est étayé par des articles récents qui suggèrent fortement que l'interface entre les domaines C-terminal et N-terminal est exposé au solvant dans la forme rouge active de l'OCP^[179] [98]. Selon le modèle de photoactivation établi d'après ces données, la protéine s'ouvre lors de sa photoactivation. Cette ouverture rend la moitié du caroténoïde accessible au solvant. Cependant, le caroténoïde est très hydrophobe et une telle forme ouverte ne pourrait être stabilisé qu'au contact des PBs. Afin d'étayer ce modèle, nous avons cherché à cristalliser la forme rouge active de l'OCP. Cependant, lors du processus de cristallisation, l'OCP est toujours présente en forme orange, même lorsque nous travaillons avec des OCPs modifiées dont la forme rouge est plus stable (R155E par exemple) ou sous forte lumière. Nous avons décidé de changer d'approche en cristallisant uniquement le domaine N-terminal, qui est le domaine actif de l'OCP. La séquence codant pour le domaine N-terminal de l'OCP a été clonée dans un plasmide et exprimé chez *E. coli*. La protéine ainsi synthétisée est la « RCP » (Red Carotenoid Protein). La position du caroténoïde dans la RCP est la même que dans la forme rouge active de l'OCP, et la RCP peut s'attacher aux PBs et induire le quenching de la fluorescence (Leverenz et al., 2014). L'article rapporte la structure cristalline de la RCP, et démontre que le caroténoïde subit un mouvement de 12Å lors de la photoactivation. J'ai contribué à cet article en créant toutes les différentes variantes d'OCP dont les acides aminés impliqués dans la stabilisation du caroténoïde dans la forme active rouge de la protéine.

5.0.1 Résumé de l'article Leverenz et al., 2015

La RCP, correspondant aux résidus 20 à 165 de l'OCP de *Synechocystis* a été clonée et surexprimé chez *E. coli* produisant soit l'ECN (échinénone), soit la CAN (canthaxanthine) (chapitre 2^[140]). La RCP-CAN induit un quenching de fluorescence similaire à celui obtenu avec la RCP créée par protéolyse de l'OCP^[150]. En conséquence, le choix pour la cristallisation s'est porté sur la RCP-CAN. La structure de la RCP-CAN a été résolue à 1,54Å, ainsi que celle de l'OCP-CAN à 1,90Å afin de disposer d'une structure de référence. La superposition des structures de la RCP et de l'OCP révèle la translocation du caroténoïde qui est transféré de 12Å vers le domaine N-terminal. Dans sa nouvelle position, il est intégré complètement dans le domaine N-terminal (figure 1). Les changements intervenant dans l'accessibilité au solvant de résidu ou de clusters de résidus ont été évalués par x-ray hydroxyl radical footprinting mass spectrometry chez l'OCP orange et l'OCP rouge (XF-MS, figure 3 et table S5). Le résidu W41, dont l'interaction avec le caroténoïde augmente lors du passage à la forme rouge, est moins accessible au solvant dans la RCP que dans l'OCP. De plus, le résidu M284 ainsi que le cluster P276, W277, F278 du domaine C-terminal qui entoure le caroténoïde dans l'OCP orange voient leur exposition au solvant augmenter. La translocation de la CAN rend les chaînes latérales de ces résidus accessibles. Par ailleurs, l'augmentation de l'exposition au solvant de l'arginine n°155 est une preuve de plus de l'ouverture de la protéine lors de sa photoactivation (Figure S5). Afin de confirmer la translocation du caroténoïde lors du passage de l'OCP orange à l'OCP rouge, nous avons modifié dans l'OCP des résidus conservés localisés à moins de 4Å de l'extrémité supérieure du caroténoïde : le E34, la P126 et la Y129 (figure 2). Nous avons également modifié la C84 qui est à une distance de 4,1Å du caroténoïde qui est également localisée en haut de la protéine (figure 2C). Les cinétiques de photoconversion et de retour à la forme orange à l'obscurité ont été mesurées pour ces OCPs modifiées. La stabilité de la forme rouge de chacune des OCPs est altérée (figure 2D et 2E). Le résultat le plus drastique est obtenu avec l'OCP P126V-Y129F qui n'est plus photoconvertible.

L'analyse des structures révèle que la structure protéique du domaine N-terminal de l'OCP et de la RCP sont très proches (RMSD=1,6). La principale différence réside dans une boucle reliant deux hélices α (résidus 45 à 65). Le caroténoïde qui était courbé et vrillé dans l'OCP orange est droit et plan dans la RCP (figure S4). Ces modifications de structure et d'environnement lui conférant des propriétés chromiques différentes, expliquant le changement de couleur d'orange à rouge de la protéine après photoactivation. Le déplacement du caroténoïde s'effectue au sein d'un tunnel hydrophobique déjà présent dans la forme orange (Leverenz, Figure S5).

Conclusion

L'article rapporte la résolution de la structure de la RCP, et les résultats démontrent qu'une translocation de 12Å du caroténoïde intervient lors de la photoactivation. Un tel mouvement pour un chromophore n'avait jamais été décrit dans le monde du vivant. De plus, le déplacement du caroténoïde est effectué au sein d'un tunnel déjà présent dans l'OCP orange.

A 12 Å carotenoid translocation in a photoswitch associated with cyanobacterial photoprotection

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Abstract

Pigment-protein and pigment-pigment interactions are of fundamental importance to the light-harvesting and photoprotective functions essential to oxygenic photosynthesis. The orange carotenoid protein (OCP) functions as both a sensor of light and effector of photoprotective energy dissipation in cyanobacteria. We report the atomic-resolution structure of an active form of the OCP consisting of the N-terminal domain and a single noncovalently bound carotenoid pigment. The crystal structure, combined with additional solution-state structural data, reveals that OCP photoactivation is accompanied by a 12 angstrom translocation of the pigment within the protein and a reconfiguration of carotenoid-protein interactions. Our results identify the origin of the photochromic changes in the OCP triggered by light and reveal the structural determinants required for interaction with the light-harvesting antenna during photoprotection.

Article

Photosynthetic organisms balance light harvesting against the toxic effects of oxidative intermediates produced under excess light^[182]. Thermal dissipation of excess absorbed energy manifested as a quenching of antenna fluorescence known as non-photochemical quenching (NPQ)^[85,183] is the predominant photoprotective mechanism. Carotenoid pigments play critical roles in NPQ^[77,85,102,105,146,183–187], including a likely role as a direct quencher of excitation energy in "flexible" NPQ mechanisms^[184] that operate reversibly on short time scales (seconds to minutes) and under dynamic light conditions^[77,102,105,146,186,187]. In cyanobacteria, a relatively simple carotenoid-dependent NPQ mechanism is associated with the light-harvesting antenna protein complex, the phycobilisome (PB). Here, NPQ is triggered by photoactivation of the soluble orange carotenoid protein (OCP), a blue-light photoreceptor that noncovalently binds a single carotenoid^[85]. Activation of the OCP occurs when its dark (orange) state, *OCP_o*, absorbs blue light and forms the quenching active (red) state, *OCP_r*^[97]. *OCP_r* binds to the PB and initiates PB-associated NPQ^[81,97]. Structurally, the OCP is composed of two domains, a mixed α/β C-terminal domain (CTD) and a N-terminal domain (NTD) with an all α -helical fold unique to cyanobacteria^[95] (fig. S1, A and B). A 4-keto carotenoid (fig. S1C) spans both domains^[95,96] and is almost entirely enclosed by protein (4% solvent-exposed; fig. S1B). The isolated, carotenoid-binding NTD, referred to as the red carotenoid protein (RCP), functions as an effector domain; it binds to PBs and quenches PB fluorescence^[150]. The CTD serves as the regulatory (sensory) domain^[150,179] conferring photochemical activity to the OCP and providing the site of interaction with the fluorescence recovery protein (FRP), which catalyzes the *OCP_r*-to-*OCP_o* conversion^[114]. In the absence of the CTD, the RCP is a constitutively active quencher; its activity and spectroscopic properties are essentially identical to those of *OCP_r*^[150]. However, dissociation or absence of the CTD would leave nearly half of the carotenoid accessible to solvent. This raises a fundamental question about how the hydrophobic carotenoid is structurally accommodated in *OCP_r* and RCP prior to interaction with the PB. To probe the molecular details of carotenoid-protein interactions in RCP/*OCP_r*, we produced RCP by expressing a synthetic *rcp* gene [encoding residues 20 to 165 of *Synechocystis* PCC6803 (hereafter *Synechocystis*) OCP] in echinenone (ECN)- or canthaxanthin (CAN)-producing *E. coli* strains. In both strains, the OCP binds a mixture of CAN

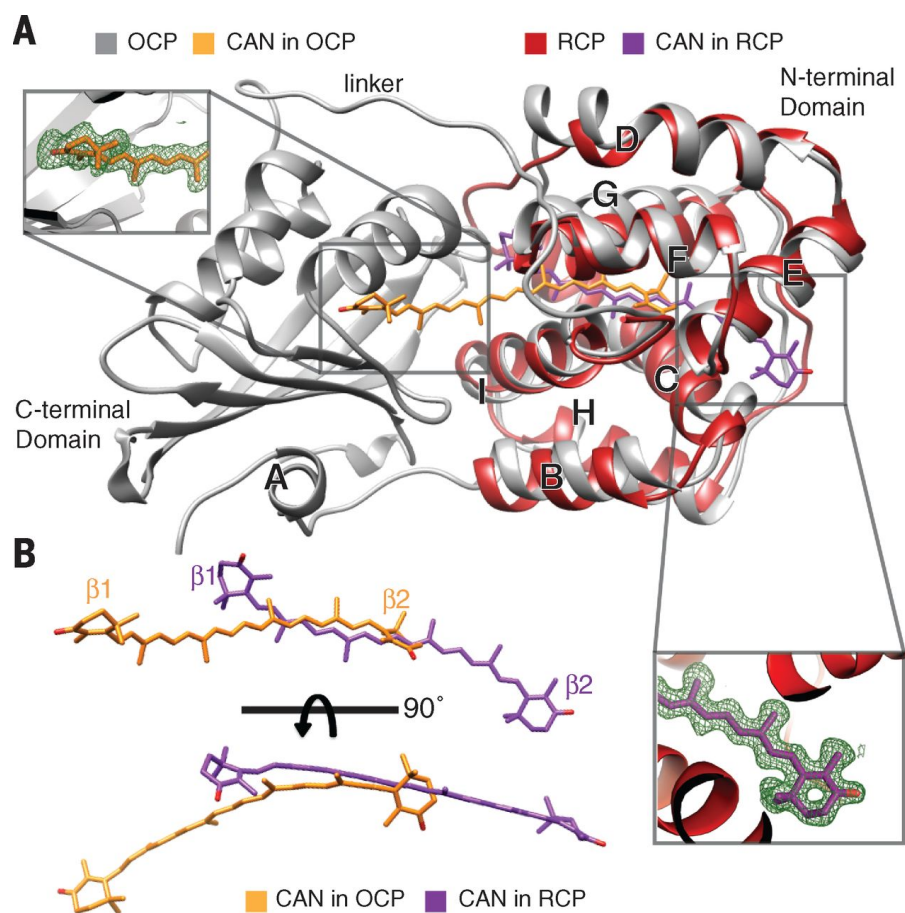


Figure 5.1 – Crystal structures of the orange carotenoid protein (OCP) and red carotenoid protein (RCP) binding canthaxanthin (CAN). (A) Superimposed ribbon structures of OCPCAN (gray) and RCPCAN (red). CAN is shown in orange sticks in OCP, purple sticks in RCP. Inset panels show representative electron density for the carotenoid in each structure (complete carotenoid Fobs - Fcalc maps are shown in fig. S4). (B) CAN structures in OCP and RCP show increased planarity of the polyene chain in RCP and distinctly different β -ring configurations.

and ECN, with a higher relative amount of CAN binding in the CAN-producing strain (table S1). RCPCAN (binding exclusively CAN) was more active than RCPECN (binding exclusively ECN) and induced PB fluorescence quenching comparable to that of RCP obtained by partial proteolysis^[150] of the OCP purified from *Synechocystis* (fig. S2). Accordingly, we structurally characterized RCPCAN and its cognate OCP.

The 1.90Å resolution structure of *Synechocystis* OCP_oCAN (table S2) aligns closely with the structure of *Synechocystis* OCPECN^[113] [root mean square deviation (RMSD) 0.17Å over 304 α -carbon atom pairs]. The carotenoid conformation is also consistent with previously reported OCP_o structures binding ECN^[113] or hydroxyechinenone^[95] (table S3), and there is well-defined electron density for each CAN carbonyl oxygen (Fig. 4.1A and fig. S4A). The OCP-CAN was photoactive and able to induce PB fluorescence quenching (fig. S3). Moreover, the nearly identical UV-visible absorbance spectra for RCPCAN and OCP_rCAN (fig. S2) indicates that the pigment-protein environments are comparable in OCP_rCAN and RCPCAN, as reported for the *Arthrospira* homologs^[150], substantiating their structural and functional homology.

We also determined the RCPCAN structure to 1.54Å resolution (table S2). The protein backbone of the RCP superimposes on the NTD of OCP_o-CAN (Fig. 5.1A), with a RMSD of 1.24Å (104 α -carbon pairs), indicating that large protein conformational changes in the NTD are not involved in PB binding or quenching. However, there is a remarkable difference in the position and conformation of the carotenoid in RCP in comparison to OCP_oCAN. In the active form, the carotenoid is transloca-

ted more than 12Å deeper into the NTD (Fig. 5.1, A and B). Due to the burrowing of the carotenoid into the NTD, it is only sparingly solvent-accessible (8% solvent-exposed) in RCP, specifically in the vicinity of the two terminal β-ionone rings (β1 and β2, Fig. 5.1B, Fig. 5.2A, and fig. S4). Each ring adopts different configurations about the C6-C7 (C6'-C7') single bond in the two structures (Fig. 5.1B and Fig. 5.2A) and the out-of-plane torsions of each ring are decreased relative to those of CAN in *OCP_o* (table S3). The polyene chain is completely encompassed by protein; it assumes a highly planar conformation in RCP, whereas it is bowed and twisted in *OCP_o* (Fig. 5.1B and fig. S4). The increased planarity of the polyene and reduced β-ring torsions observed for CAN in RCP are consistent with previously published electronic absorption and Raman spectroscopy data that indicate extended effective p-conjugation and a planar all-trans configuration for the carotenoid in both quenching-active RCP and *OCP_r* [97,150].

The large displacement of the carotenoid has profound consequences for its interactions with the protein. Specifically, the amino acids comprising the CAN binding pockets in the *OCP_o* and RCP structures (Fig. 5.2A and table S4) occupy two distinct carotenoid-protein configurations (cpcs). In *cpcO* (corresponding to CAN in *OCP_o*), 11 residues of the NTD are in close (<4Å) proximity to the carotenoid. Retrospectively, the hydrophobic tunnel for translocation of the carotenoid further into the NTD is present in *OCP_o* (fig. S1B and fig. S5). In *cpcR* (CAN in RCP) an additional nine residues in this NTD tunnel interact with the carotenoid (Fig. 5.2A). Modest side chain conformational changes accompany translocation (Fig. 5.2B and fig. S5A). A perturbed local electrostatic environment for CAN in *cpcR* versus *cpcO* (fig. S5D), in addition to new H-bonding interactions between solvent and CAN's 4-keto groups in *cpcR*, likely contribute to altered photophysical properties of the carotenoid (i.e., stabilization of an intramolecular charge transfer state) that may be connected to quenching function [106].

The conservation of residues unique to *cpcR* observed in genes encoding for full-length OCPs (figs. S6 and S7) implicate the carotenoid shift as an integral part of OCP function. Several of the conserved residues within 4Å of CAN in *cpcR* were probed by mutagenesis in the OCP. For certain mutations (i.e., Glu34 → Ala), the CAN:ECN binding ratio was observed to change markedly relative to the wild-type OCP (table S1), indicating that these residues influence carotenoid binding specificity in OCP. The OCP single mutants Cys84 → Ala, Tyr129 → Phe, Pro126 → Val, and Glu34 → Ala reduced the stability of the *OCP_r* form, as evidenced by decreased steady-state accumulation of *OCP_r* after illumination and accelerated *OCP_r*-to-*OCP_o* dark-reversion (Fig. 5.2, D and E); these mutants induced less than 40% PB quenching (fig. S8B). The OCP double mutant Pro126 → Val/Tyr129 → Phe remained orange even under prolonged, strong illumination (Fig. 5.2D and fig. S8), which suggests that these exposed residues, relatively distant from the carotenoid in *cpcO*, play a critical role in OCP photochemistry. Collectively, these results implicate the CAN-binding residues in *cpcR* (as observed in the RCP structure) in the stabilization of the carotenoid in the active *OCP_r*. To obtain solution-state structural evidence for carotenoid translocation in the *OCP_o*-to-*OCP_r* photoconversion, we used x-ray hydroxyl radical footprinting mass spectrometry (XF-MS) to identify changes in side-chain solvent accessibility after illumination [188]. X-ray dose response plots show that some of the largest solvent accessibility changes after photoconversion occurred in CAN binding residues (Fig. 5.3A and table S5). The largest solvent accessibility decreases are for peptides containing the NTD residue Trp41 (Fig. 5.3A). The decrease in solvent accessibility for this residue is consistent with an increased interaction with CAN due to CAN translocation. XF-MS analysis of RCP samples exhibited a similarly prominent SA decrease at Trp41 (table S5). Furthermore, CTD residues (Pro276-Trp277-Phe278 and Met284 in *OCP_r*; Fig. 5.3A) that contact the CAN polyene chain in *cpcO* (table S4) had a large increase in solvent accessibility. CAN translocation exposes these side chains to a solvent accessible region in the surface cleft between the CTD and NTD (fig. S1B). Correlated solvent accessibility changes in CAN binding CTD residues (increased solvent accessibility) and NTD residues (decreased solvent accessibility) support carotenoid translocation during OCP activation (Fig. 5.3B). XF-MS data also confirms that CAN translocation accompanies a separation of the CTD and NTD: The factor of 10 solvent accessibility increase in Arg155 (table S5) supports the proposed breakage of the Arg155-Glu244 salt bridge in *OCP_r* [98,179].

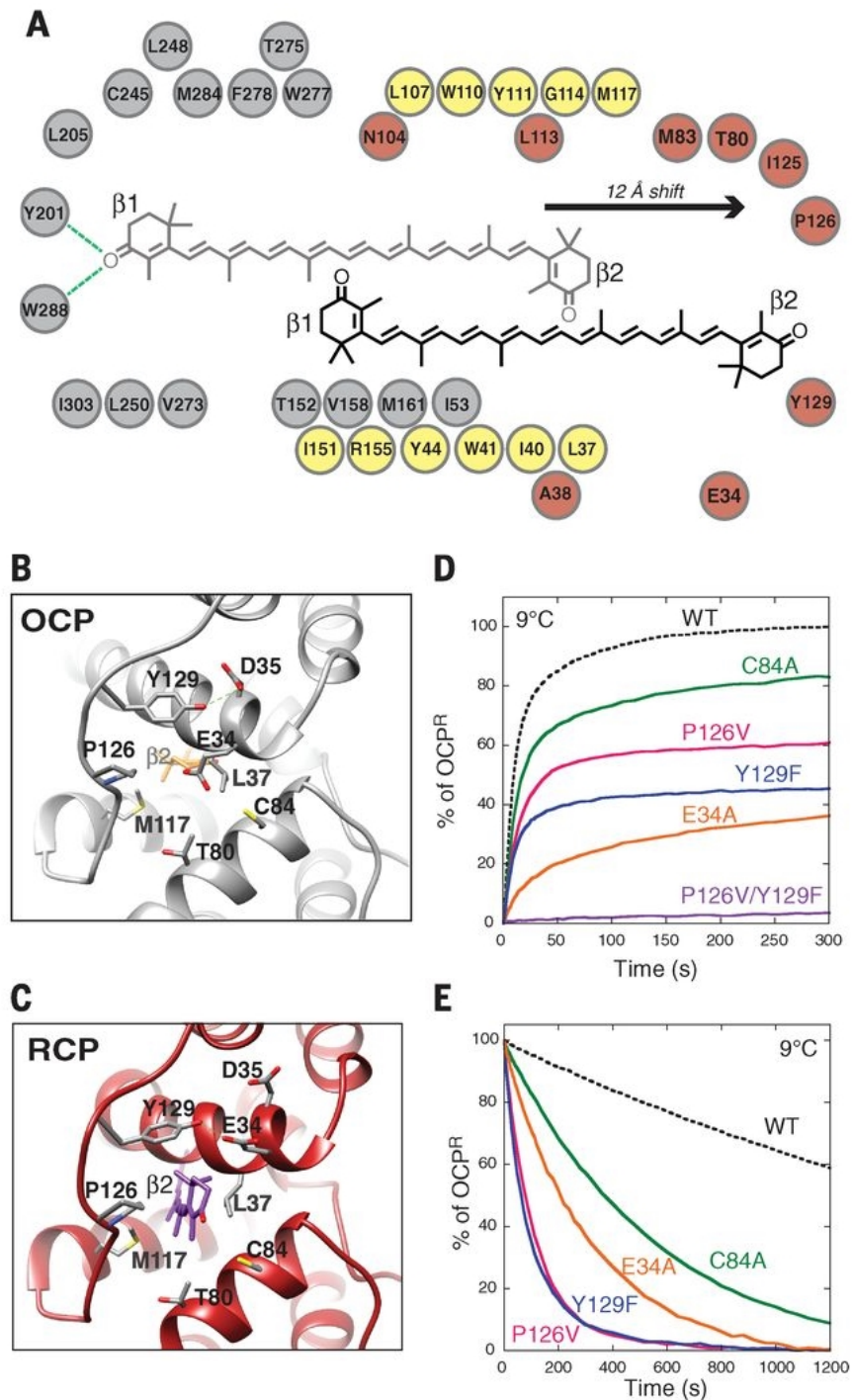


Figure 5.2 – Distinct carotenoid-protein configurations (cpcs) observed in the OCP and RCP structure. (A) Diagram of carotenoid associated residues ($<4 \text{ \AA}$) unique to cpcO (gray circles), unique to cpcR (red circles), or common to both cpcs (yellow circles). Chemical structures of CAN are shown with C6-C7 and C6'-C7' bond configurations, depicted as observed in the crystal structures (fig. S4). H-bonds between the 4-keto oxygen of CAN and residues Tyr201 and Trp288 in cpcO are indicated (green dashes). (B and C) H-bonding residues Tyr129, Glu34, and Asp35 (sticks) in the NTD of OCP (B) and RCP (C). Additional residues interacting with the $\beta 2$ ring of CAN in cpcR are also explicitly shown in both structures. (D) OCP_o-to-OCP_r conversion of OCP mutants at 9°C during 5 min of strong white-light illumination. (E) OCP_r-to-OCP_o dark recovery at 9°C for mutants in (D).

Based on the observation of carotenoid translocation accompanying domain dissociation we propose the following sequence of events in the photoactivation of the

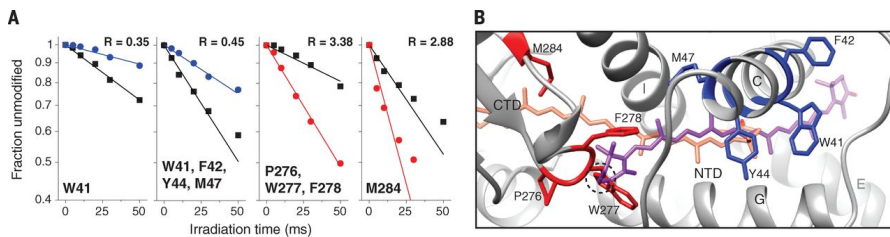


Figure 5.3 – Solvent accessibility changes in OCP_{CAN} as measured by x-ray hydroxyl radical footprinting. (A) Peptide modification as a function of x-ray irradiation dose for Trp41, residue clusters Trp41-Phe42-Tyr44-Met47 and Pro276-Trp277-Phe278, and Met284 for dark-adapted (OCP_o, squares) and illuminated (OCP_r, circles) OCP_{CAN}. Solid lines represent single-exponential fits to the dose-dependent data. The ratio of the modification rates (R) indicates the change in relative SA. (B) Structural view (OCP_{CAN} structure) of CAN binding residues undergoing large (factor of >2) SA changes after illumination. CAN in cpcO (orange sticks) and CAN in cpcR (purple sticks) are both shown. CTD residues Pro276, Trp277, Phe278, and Met284 (red sticks) exhibit a SA increase (R = 3.38, R = 2.88) in OCP_r, whereas residue Trp41 and residue cluster Trp41-Phe42-Tyr44-Met47 (blue) exhibit SA decreases (R = 0.35, R = 0.45). A clash between Trp277 and CAN in cpcR is indicated (black circle).

OCP (Fig. 5.4A). Light absorption triggers structural changes in the carotenoid, perturbing its interaction with the CTD (e.g., perturbing H-bonds with Tyr201/Trp288). Light-induced displacement of the N-terminal α helix from the CTD, proposed to occur based on structural similarities to the Per-Arnt-Sim family of photosensors^{[113][189]}, has recently been demonstrated by chemical footprinting experiments^[179]. Analogous to the photochemical mechanism of PYP^[190], it is possible that partial "ejection" of the carotenoid chromophore, driven by a transient, strained cis-carotenoid geometry may be coupled to CTD structural changes. An accompanying reorganization of side chain-pigment interactions has the net effect of destabilizing carotenoid binding in cpcO; translocation of the carotenoid drives the reconfiguration to cpcR. Chaotrope-induced formation of an activated state of the OCP suggests that the transition to cpcR can take place in the absence of light^[191], implying that translocation may be largely driven by protein-carotenoid binding free energies. In contrast to cpcO, where the carotenoid serves as a structural element bridging the CTD and NTD, carotenoid translocation coupled with dissociation of the α A helix from the CTD^[179] is required for full domain separation in OCP_r.

The separation of the NTD and CTD in OCP_r leads to solvent exposure of both CAN β rings. The regions surrounding the solvent-exposed β rings (β_1 , face 1; β_2 , face 2) include the two largest patches of conserved residues on the surface of RCP (fig. S7). Positive potential, in part due to the critical PB binding residue Arg155^[98], dominates face 1, whereas face 2 is relatively negatively charged (Fig. 5.4B). The distinct differences in surface charge between face 1 and face 2 suggest an electrostatically driven directionality in the NTD-PB interaction. Because the conformation of the NTD is essentially unchanged in the active form of the OCP, NTD-PB binding is likely tied to selective exposure of regions of the NTD occluded in OCP_o (face 1 and Arg155), or to the carotenoid translocation itself.

NTD-PB interaction in the vicinity of the exposed β -ring would also be expected for carotenoid-dependent energy quenching, given the importance of interpigment distances in energy transfer efficiency^[192]. Although the atomic-resolution structure of the fully assembled PB is unknown, in silico docking simulations between RCP and PB subunits implicated in OCP-binding^[37,101,158] show reduced bilin-carotenoid distances as compared to identical simulations with OCP_o (fig. S9A); RCP-PB complexes with face 1 CAN-bilin distances as low as 3.1 Å were identified (fig. S9B). Such close interaction would permit participation of the carotenoid in either direct bilin-carotenoid energy transfer^[106] or charge transfer quenching mechanisms^[99]. The translocation observed concomitant with activation of the protein raises the possibility of additional carotenoid structural changes and/or movement after binding to the PB (Fig. 5.4C) to further reduce carotenoid-bilin distances or change the relative orientations of pigments in the OCP-PB complex. More broadly, the light-driven

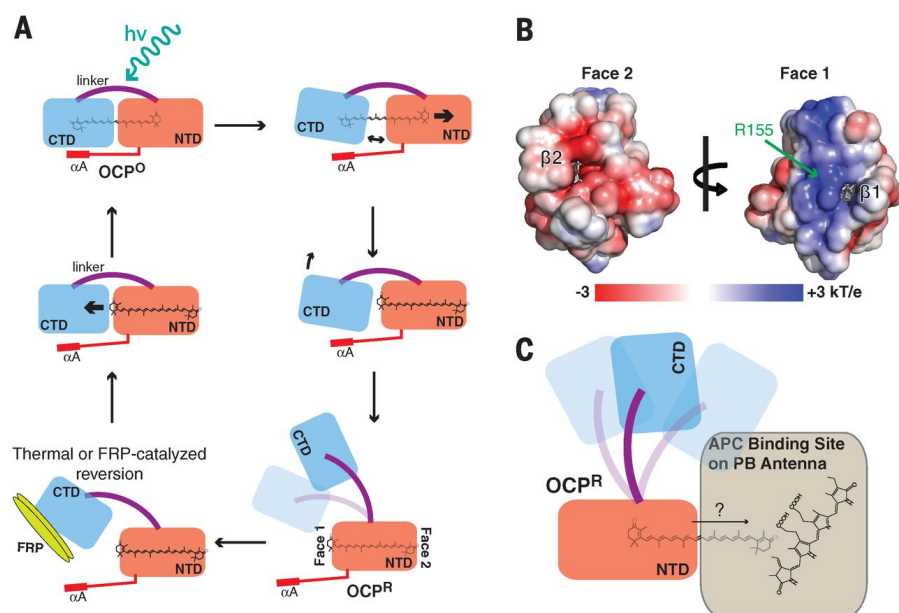


Figure 5.4 – Proposed models for OCP photoactivation and the site of OCP/RCP-phycobilisome interactions. (A) Proposed mechanism for OCP photochemistry, including carotenoid translocation, after light absorption by OCP_o (top left). Structural changes after absorption are localized primarily to the CTD (i.e., dissociation of the αA helix) and are coupled to the translocation (right). Translocation precedes complete NTD-CTD dissociation in OCP_r (bottom right). OCP_r reverts to OCP_o in darkness (thermal reversion) or when catalyzed by an interaction between the fluorescence recovery protein (FRP) and the CTD (18) (bottom left); subsequent protein refolding and carotenoid translocation into the CTD (middle left) restores the OCP_o ground state. (B) Electrostatic surface potential mapped on the RCP molecular surface colored from red to blue (-3 to +3 kT/e). (C) OCP:PB interaction illustrating binding at face 1 and a hypothetical carotenoid translocation after binding.

change in carotenoid-protein interactions observed in the OCP prompts a reexamination of other carotenoid binding protein complexes for the possibility of transient, activation-dependent movement of the noncovalently bound carotenoids in those systems.

Materials and methods

Construction of the RCP plasmid for expression in *E. coli*

To obtain the NTD/RCP of *Synechocystis* OCP, we cloned a C-terminal His-tagged *Synechocystis* OCP in a pCDFDuet-1 plasmid creating the plasmid pCDF-OCPsynCtag as previously described^[140]. To obtain the pCDF-RCP-Syn-1-165Ctag plasmid, the nucleotides encoding the last 169 amino acids (CTD) of the *ocp* gene were deleted by site-directed mutagenesis using the plasmid pCDF-OCPsynCtag as template and synthetic primers (table S6A). The deletion of the nucleotides coding for the first 20 amino acids of the RCP was obtained using the pCDF-RCP-Syn-1-165Ctag as template and the synthetic primers (table S6B) to obtain the plasmid pCDF-RCP-Syn-20-165-Ctag.

Site-directed mutagenesis

The point mutations P126, Y129, P126/Y129, E34 and C84 were introduced by directed mutagenesis, using the pCDF-Syn-3aaNtag plasmid as template and mismatching primers (table S6C). When possible, restriction sites were added by silent mutation (Mfe for P126, Y129 and P126Y129 or XmaI for C84A) to check the presence of the point mutations. Sequencing of the cloned gene confirmed the presence of the mutations and the absence of unwanted mutations.

Holoprotein production in E. coli

E. coli BL21-Gold (DE3) cells from Agilent Technologies were used for OCP/RCP production. BL21 cells were transformed simultaneously with three plasmids : (1) pAC- BETA (described previously (32)), containing the *CrtB-CrtE-CrtI, CrtY* operon need to synthesize-carotene (2) pBAD-CrtO or pBAD-CrtW (*CrtO* and *CrtW* catalyze mainly the transformation of -carotene into echinenone and canthaxanthin, respectively), and (3) pCDF-OCP or pCDF-RCP plasmids, containing *Synechocystis* *ocp* or *rcp* genes, respectively. The construction of pBAD-CrtO, pBAD-CrtW and pCDF-OCP is described elsewhere^[140]. Transformed cells were grown in the presence ampicillin (50 $\mu\text{g/ml}$), chloramphenicol (17 $\mu\text{g/ml}$), and streptomycin (25 $\mu\text{g/ml}$). For induction of the different genes, transformed *E. coli* cells were grown in TB medium at 37°C for 3-4 hours until OD₆₀₀=0.8, at which point arabinose was added (0.02%). Following overnight growth at 37°C, cell cultures were diluted with fresh medium and arabinose (0.02%) and grown at 37°C until OD₆₀₀ = 1-1.2. Isopropyl-D-thiogalactoside (IPTG) (0.2 mM) was added and the cells incubated overnight at 28°C. In the morning, the cultures were harvested and pellets were stored at -80°C until used for protein purification.

Carotenoid content analysis

Carotenoids were extracted as described previously^[141]. Relative quantification of carotenoids was calculated using the results of liquid chromatography-mass spectrometry (LC-MS) analysis as described previously^[96].

Purification of OCP and RCP holoproteins

His-tagged OCP overexpressed in *Synechocystis* were isolated as described previously^[97]. OCP and RCP expressed in *E. coli* were isolated as described elsewhere^[140]. Briefly, cells are resuspended in lysis buffer (40 mM Tris pH 8/10% glycerol/300mM NaCl/1mM EDTA/1mM PMSE, 1mM caproic acid/1mM benzamidic acid/ 50 $\mu\text{g mL}^{-1}$) were broken in dim light using a French press. The membranes were pelleted and the supernatant was loaded on a nickel affinity column (Ni-Probond resin, Invitrogen). OCP was eluted with 200 mM imidazole and then dialyzed against 40 mM Tris-HCl. Purification of OCPCAN and RCPCAN by affinity chromatography yielded a mixture of apo- and holo-proteins. The RCPCAN holoprotein was isolated using a combination of hydrophobic interaction chromatography (HIC) and size exclusion chromatography (SEC). The OCPCAN holoprotein was isolated by SEC alone. HIC was performed using a linearly decreasing (NH₄)₂SO₄ gradient (1.5 M to 0 M) to elute protein bound to a Toyopearl Super-Q 650-S Phenyl resin (Tosoh Biosciences, 1.5 cm D x 15 cm H resin bed dimensions). SEC was performed using isocratic elution (50 mM Tris-HCl, 200 mM NaCl mobile phase) on a HiLoad 16/60 Superdex-75 column (GE Healthcare).

Protein crystallization

The purified N-terminal His-tagged OCP and C-terminal His-tagged RCP holoproteins were exchanged into 10 mM Tris-HCl pH 7.4 and concentrated using centrifugal concentrators (Amicon 0.5, 10 kDa [EMD Millipore]) prior to crystallization. RCPCAN (2 mg/mL) was crystallized at 22°C in sitting drops containing 2 μL of protein plus 1 μL of crystallization solution (100 mM citric acid BIS-TRIS propane pH 5.5, 24% poly-ethylene glycol 3350). OCPCAN (3 mg/mL) was crystallized at 22°C in sitting drops containing 2 μL of protein plus 1 μL of crystallization solution (100 mM sodium acetate pH 4.5, 10% poly-ethylene glycol 20,000, 3% glycerol). Crystals were flash frozen in liquid nitrogen after being transferred to a cryoprotectant solution (crystallization solution plus 15% ethylene glycol for RCPCAN; crystallization solution plus 30% glycerol for OCPCAN).

Diffraction data collection, structure determination and visualization

Diffraction data were collected at the Advanced Light Source at Lawrence Berkeley National Laboratory beamline 5.0.2 (1 Å wavelength, 100K). Diffraction data were integrated with XDS^[193] and scaled with SCALA (CCP4^[194]). The structure of

RCP was solved by molecular replacement with phenix.mr rosetta^[195] with a starting model based on the N-terminal domain of OCP (PDB ID 3MG1). Refinement was performed with phenix.refine^[196] alternating with model building using 2Fo-Fc and Fo-Fc maps visualized in COOT^[197]. 99% of the residues were in the favored region of the Ramachandran plot, and the remaining 1% were in the allowed region for both structures. Statistics for diffraction data collection, structure determination and refinement are summarized in Table S2. Figures of crystal structures were prepared using pymol (www.pymol.org) and UCSF Chimera molecular visualization software^[198].

Structural Analysis

OCP and RCP structures were aligned in UCSF Chimera using the MatchMaker tool and default alignment parameters. The reported distance between the CAN chromophores in the two structures (12 AA) is the distance between the C6 carbons of the two CANs in the alignment (see fig. S1C for carbon numbering). Solvent accessibility of CAN was determined using the areaimol^[199] program in CCP4^[194]. Electrostatics calculations were done using the APBS plugin for PyMOL^[200]. Primary Structure Analysis and Sequence Logo: A full-length OCP sequence logo was generated from a MUSCLE alignment of 168 OCP gene sequences from publicly available genomes in IMG (<http://img.jgi.doe.gov>). The logo was built using the WebLogo application (<http://www.weblogo.berkeley.edu>)^[201].

UV-Visible spectroscopy and measurements of photochemical kinetics

Absorbance spectra, kinetics of photoactivity (illumination with 5000 $\mu\text{moles de photons} * \text{m}^{-2} * \text{sec}^{-1}$ of white light) and dark recovery of the OCP were measured in a spectrophotometer Specord S600 (Analyticjena) at 9°C.

In vitro reconstitution of PB binding

An *in vitro* reconstitution system allows us to monitor OCP to PBs binding by following the fluorescence across time^[81]. Fluorescence yield quenching was monitored using a pulse amplitude modulated fluorometer (101/102/103-PAM, Walz). Measurements were made in a 1-cm pathlength stirred cuvette. OCP was pre-converted to the OCP_r form by 10 min illumination with 5000 $\mu\text{moles de photons} * \text{m}^{-2} * \text{sec}^{-1}$ of white light at 4°C. Then, the OCP_r (0.48 μM) or RCP (0.48 μM) was added to 0.012 μM of PBs in 0.8M and 1.4 M potassium phosphate buffer (pH=7.5) at 23°C under 900 $\mu\text{moles de photons} * \text{m}^{-2} * \text{sec}^{-1}$ blue-green light (white light filtered by a Corion cutoff 550-nm filter; 400-550nm). The ratio of OCP_r (or RCP) to PB was = 40. The concentration of OCP (RCP) was calculated from the carotenoid absorbance spectra since only the OCP holoprotein can be photoactivated and bind to PBs (A1% carotenoid = 2158). RCP isolated by native proteolysis of OCP (OCP from *Synechocystis* ΔCrtR cells) and used in *in-vitro* PB reconstitution experiments was purified as described previously^[150].

X-ray hydroxyl radical footprinting and mass spectrometry

OCPCAN and RCPCAN (expressed in *E. coli*) were exchanged into 20 mM potassium phosphate (pH 7.4), 100 mM NaCl by SEC on a Superdex-75 10/300 GL column prior to XF-MS experiments. Prior to x-ray irradiation of OCP_r , the sample syringe was cooled with an ice pack and illuminated with a blue LED array (470 nm Luxeon Rebel, Philips Lumileds). Protein samples were irradiated in the millisecond and microsecond time range at beamlines 3.2.1 and 5.3.1 at the Advanced Light Source as reported previously^[202]. All samples, including the control (no x-ray irradiation), were subjected to cys-alkylation and salt removal prior to overnight Trypsin and GluC digestion at pH 8 and 37 C. Proteolyzed samples were analyzed in an Agilent 6550 iFunnel Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA) coupled to an Agilent 1290 LC system (Agilent) using Sigma-Aldrich Ascentis Peptides ES-C18 reverse phase column (2.1 mm x 100 mm, 2.7 μm particle size; Sigma- Aldrich, St. Louis, MO). Approximately 10 pmol of samples were loaded onto the column via an Infinity Autosampler (Agilent) with Buffer A (2%

Acetonitrile, 0.1% Formic Acid) flowing at 0.400 mL/min. The peptides were separated and eluted into the mass spectrometer via a gradient with initial condition of 5% buffer B (98% Acetonitrile, 0.1% Formic Acid) increasing to 70% B over 15 minutes. Subsequently, B was increased to 90% over 1 minute and held for 3 minutes at a flow rate of 0.6 ml/min followed by a ramp back down to 5% over 1 minute where it was held for minutes to re-equilibrate the column to the original condition. Peptides were introduced to the mass spectrometer from the LC using a Jet Stream source (Agilent) operating in positive-ion mode (3500 V). The data were acquired with MassHunter B.05.00 operating in Auto MS/MS mode whereby the three most intense ions (charge states 2 - 5) within 300 m/z to 1400 m/z mass range above a threshold of 1000 counts were selected for MS/MS analysis. MS/MS spectra were collected with the quadrupole set to "Narrow" resolution and collision energy to optimize fragmentation. MS/MS spectra were scanned from m/z 100 1700 and were collected until 40000 total counts were collected or for a maximum accumulation time of 333 ms. Parent ions were excluded for 0.1 minutes following MS/MS acquisition. MS/MS data of native and modified peptide fragments were interpreted by Mascot MS/MS Ions Search as well as verified manually. The abundance of native and modified peptides at any irradiation time point area were measured (peak area) from their respective extracted ion chromatogram using Agilent Mass Hunter Ver 2.0. Data analysis and dose response plots : The peak area from the extracted ion chromatograms of a specific peptide fragment with a particular mass-to-charge ratio and associated +16-, +32- or +48- Da sidechain modifications was used to quantify the amount of modification at a given irradiation time^[188]. Increasing irradiation progressively reduces the fraction of unmodified products and provides a site-specific dose response plot (as in Figure 3A). The hydroxyl radical reactivity rate (k), which depends on both intrinsic reactivity and SA, was obtained by fitting the dose response to a single exponential decay (based on a pseudo-first-order reaction scheme using Origin 7.5 (OriginLabs®). The ratio (R) of the measured reactivity of the side chains residues between $OC P_o - OC P_r$ ($R = kOC P_o / kOC P_r$) and $OC P_o - RCP$ ($R = kOC P_o / kOC P_r$) gave information on SA changes independent of the intrinsic reactivity^[202].

RCP-phycoobilisome docking analysis

The RCPCAN and $OC P_o$ were docked to the ApcAB (PDB ID 4FoU^[134]) using half of an ApcAB trimer (1 ApcA and 2 ApcB) using rosetta-dock^[203]. Protein structures were energy minimized using the rosetta-relax application prior to the docking step. Unconstrained docking simulations were performed for 25'000 runs and the top 1000 docking solutions according to the Rosetta score functions were analyzed for carotenoid- bilin distances. When using constrained docking the C4 atom of the RCP CAN was restrained to O5 of ApcA using a harmonic function with $x_0=4 \text{ \AA}$ and a sd of 1 \AA .

Acknowledgments

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Coordinates have been deposited in the RCSB Protein Data Bank under accession codes 4XB4 (RCPCAN) and 4XB5 (OCPCAN).

Fig. S1.

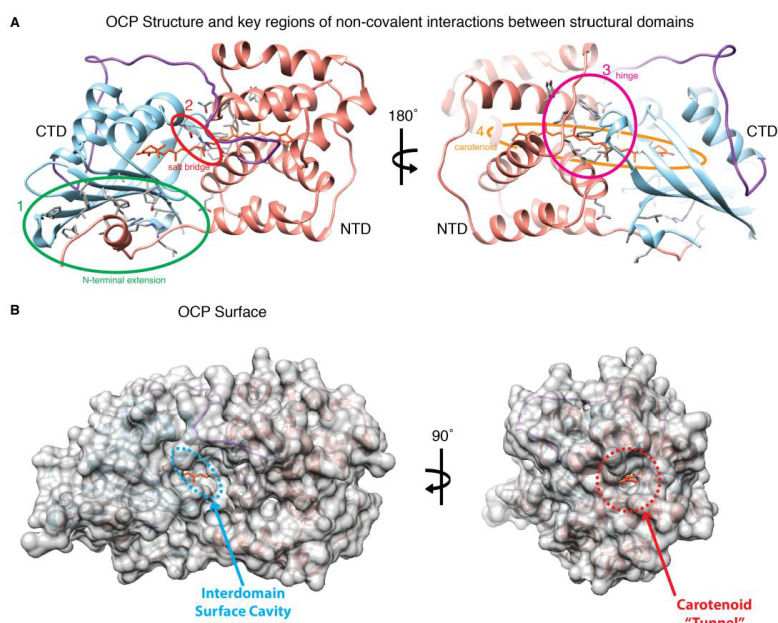
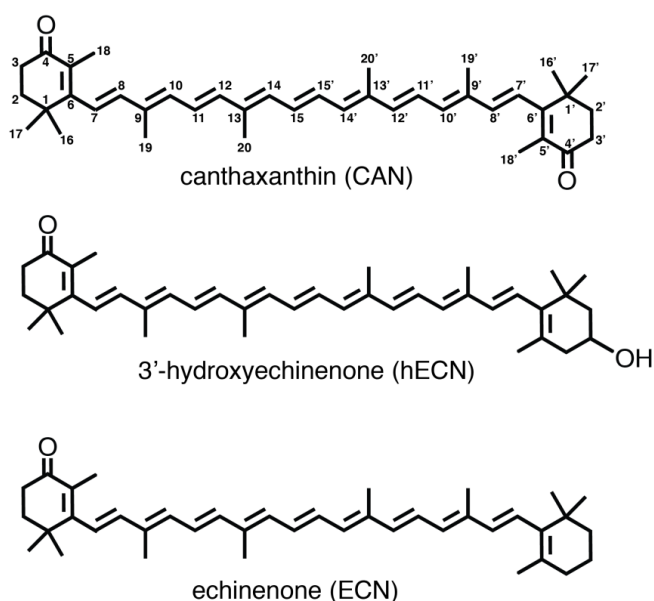


Figure S1. Views of OCP structure illustrating key NTD-CTD interactions and surface cavities. (A) Four primary regions of NTD/CTD interaction are indicated (circled) on the ribbon structures: 1.) the N-terminal extension (including the α A helix), which forms extensive H-bonding and non-bonding contacts to the CTD β -sheet (green circled area), 2.) the R155:E244 salt bridge (red circled area) observed at a large surface cleft between the NTD and CTD that exposes CAN to solvent, 3.) the putative W277 CTD hinge region dominated by hydrophobic contacts with the NTD and CAN (magenta circled area), 4.) the carotenoid chromophore, which is in extensive hydrophobic contact with residues in both domains and H-bonds to residues Y201 and W288 in the CTD. (B) CAN in OCP⁰ is solvent exposed primarily via a large surface cavity (circled in blue) at the NTD/CTD interface. A second solvent accessible region of CAN exists in the NTD near residues Y44 and W41.²⁵ A hydrophobic tunnel (its opening is circled in red) extends from the carotenoid to the surface of the N-terminal domain. Structures were modeled using OCP_{CAN} coordinates from this study (PDB ID 4XB5).

C C40 carotenoids non-covalently binding to photoactive OCPs



(C) Molecular structures of the 4-keto carotenoids (hECN, ECN, and CAN) known to bind to functionally active OCP. Carbon atom numbering is shown for CAN.

Fig. S2

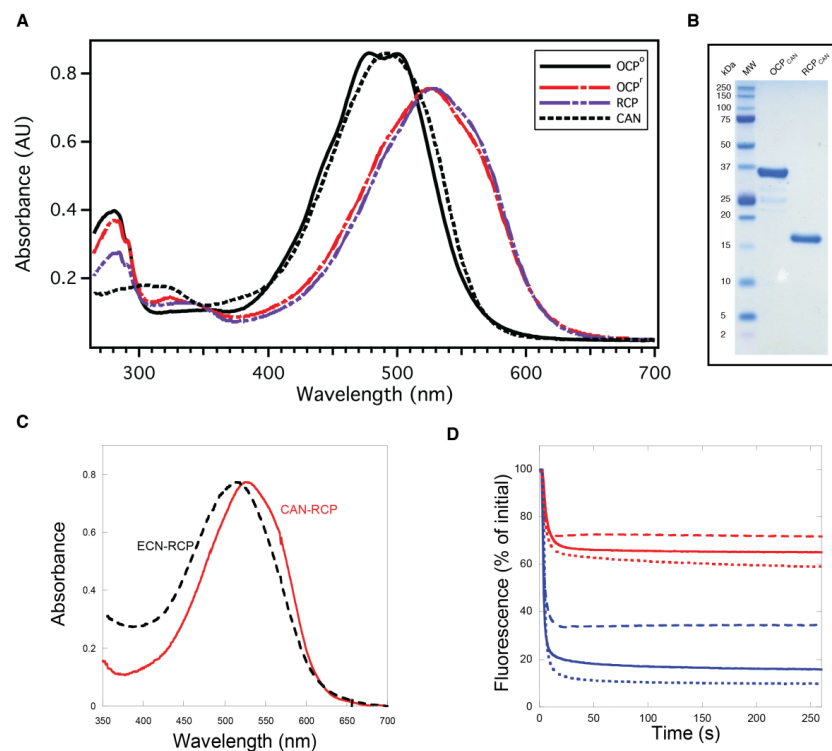


Figure S2. Characterization and comparison of OCP and RCP proteins binding ECN or CAN. (A) UV-visible absorption spectrum of purified OCP^O (black), OCP^R (red), and RCP (purple) holoproteins binding CAN. The absorbance spectrum of CAN in DMSO (black, dotted) is also shown. The CAN and RCP spectra were normalized to the OCP^O and OCP^R spectra, respectively. Slight differences in the OCP^R and RCP spectra are attributed to differences in the ECN/CAN binding ratios in the two samples (Table S1). (B) SDS-PAGE of OCP_{CAN} and RCP_{CAN} samples used for protein crystallography and XF-MS experiments. (C) direct comparison of the UV-Visible spectra of RCP binding ECN (black dashed line) or CAN (red solid line). (D) PB fluorescence quenching at 0.8 M phosphate (red traces) or 1.4 M phosphate (blue traces) induced by RCPs binding either ECN or CAN. RCP-CAN (solid lines), RCP-ECN (dashed lines) and RCP from native proteolysis of OCP (dotted lines). RCP-ECN isolated from *E. coli* cells contained large concentrations of apo-protein (only 1-2% of holoprotein) and reduced solubility (as compared to RCP-CAN) that affected the amplitude of quenching. RCP-CAN also contained about 30-40% of apo-protein that slightly decreased the amplitude of fluorescence quenching relative to the proteolytic RCP.

Fig. S3

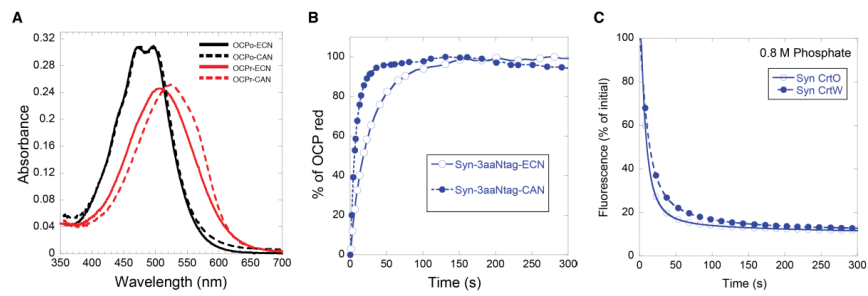


Figure S3. Characterization of *Synechocystis* CAN-OCP and ECN-OCP isolated from *E. coli* cells. (A) Absorbance spectra of dark (black) and light (red) forms of CAN-OCP (dashed) and ECN-OCP. The spectrum of the CAN-OCP^R (max 530 nm) is red-shifted compared to ECN-OCP^R (515 nm) and the photoconversion to OCP^R is faster. (B) Photoactivity (OCP^R accumulation) of CAN-OCP (closed circles) and ECN-OCP (open circles) during illumination with strong white light. (C) PB fluorescence quenching induced by CAN-OCP^R (closed circles) and ECN-OCP^R (open circles) during illumination with strong blue-green light ($900 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) in 0.8 M phosphate at 23°C. The OCP was pre-activated by illumination with strong white light.

Fig. S4

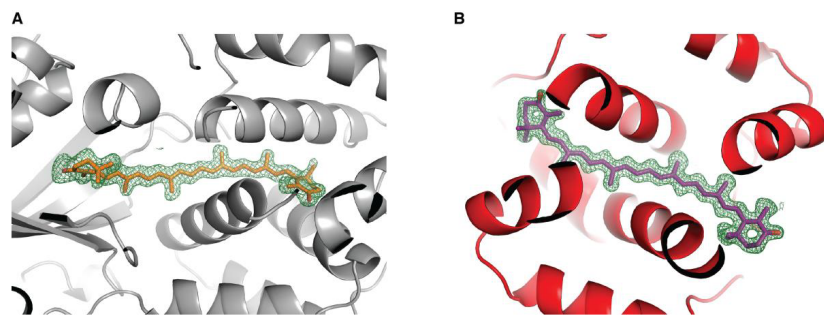


Figure S4. Fo-Fc maps for CAN in OCP and RCP. (A), (B) Fo-Fc maps from simulated annealing refinement runs without canthaxanthin, contoured at +2.0 sigma around the carotenoid (green mesh). The OCP (A, protein in grey ribbon and CAN in orange sticks) and the RCP (B, protein in red ribbon CAN in purple sticks) are shown in the same orientation and color scheme as in Fig. 1a but are slabbed to allow a better view of the carotenoid.

Fig. S5

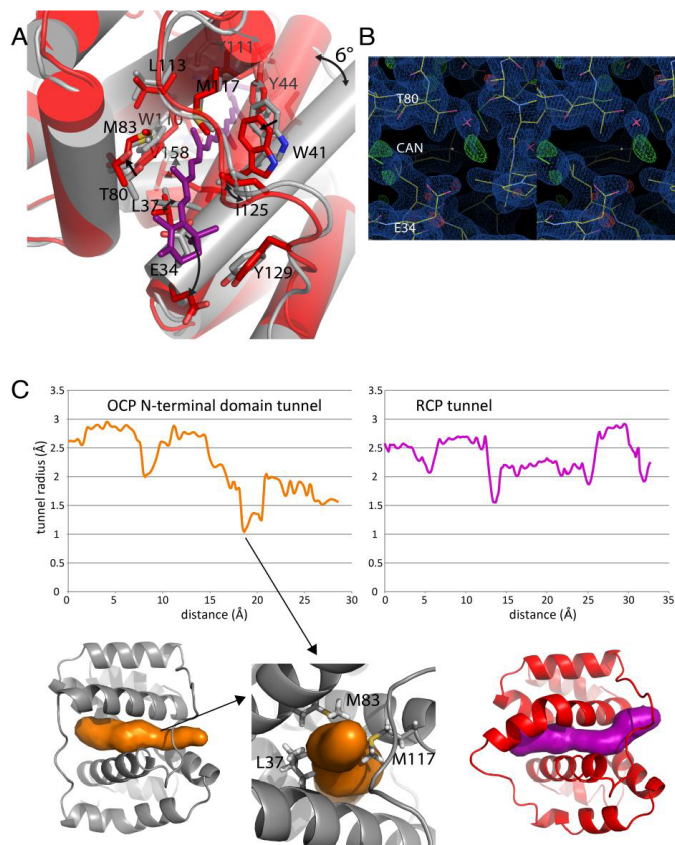


Figure S5. Detailed view of the NTD carotenoid tunnel and analysis of tunnel structure. (A) Alignment of the N-terminal domain of OCP (grey) and RCP (red) with the carotenoid in cpcR configuration (purple) on the site where the carotenoid is translated inwards. Differences between carotenoid interacting sidechains are indicated with single arrows for movement or double arrows indicating a different rotamer. Movements are generally small except for E34 and a 6° rotation of helix C (B) Stereo view of the density of the tunnel where the carotenoid is inserted in cpcR (2foc map in blue, 0.22 e/Å³, foc in green(+)/red(-), 0.26e/ Å³). There is only one ordered water molecule bound on the outside of the tunnel. (C) Comparison of the tunnels for both the N-terminal domain of OCP and RCP. Tunnel diameter plots (top) going in the same direction as the surface visualizations below. The smallest diameter in the OCP form is illustrated in detail below as a constriction formed by L37/M83/M117. Tunnel calculations were done using MOLEonline (47).

Fig. S5

D

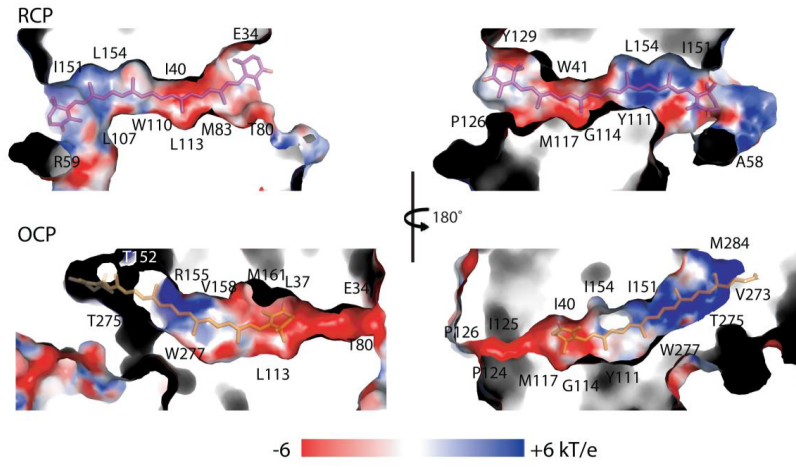


Figure S5. Detailed view of the NTD carotenoid tunnel and analysis of tunnel structure. (D) Electrostatics of the interior surfaces of the N-terminal carotenoid tunnel in RCP and OCP from two slabbed views related by a 180 degree rotation. Positions of residues are indicated to facilitate orientation.

Fig. S6

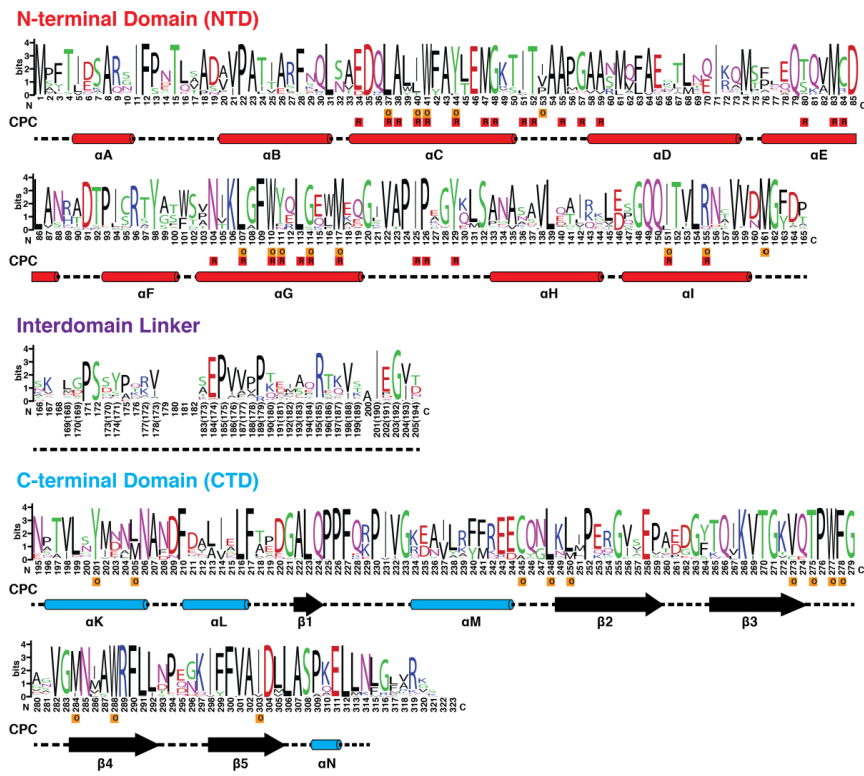


Figure S6. Primary structure sequence logo for full length OCP showing the location of secondary structure and carotenoid binding residues in the OCP and RCP structures. Sequence logos for the NTD, CTD, and interdomain linker are shown separately. Low occupancy insertions (< 1% occurrence) in the NTD and CTD were manually removed to maintain numbering consistency with *Synechocystis* OCP. Low occupancy insertions in the interdomain linker region and C-terminus appear without residue identifiers. Numbers shown in parenthesis in the alignment for the interdomain linker correspond to sequence numbering for *Synechocystis* OCP.

Fig. S7

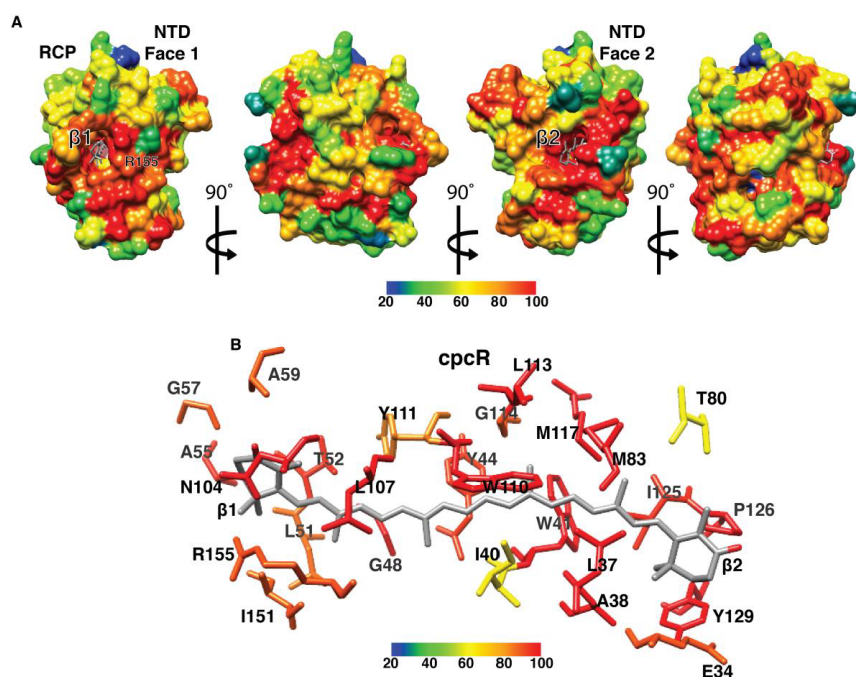


Figure S7. Amino acid sequence conservation mapped onto OCP and RCP structures. (A) Sequence conservation of full-length OCP orthologs mapped on the RCP-can structure. Sequence conservation was determined from an alignment of 168 full-length OCP amino acid sequences (see Methods). Maps are colored by percent conservation as indicated by the inset color bar. (B) Detailed view of the sequence conservation of CAN binding residues in *cpcR* (CAN in grey sticks, residues in sticks colored by percent conservation).

Fig. S8

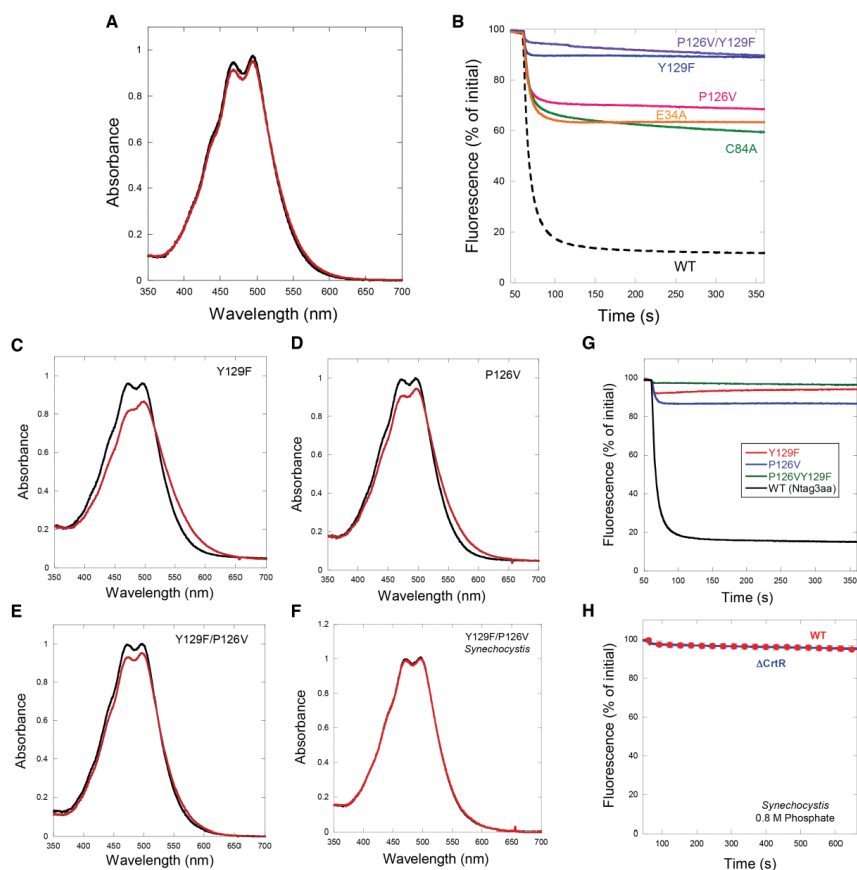


Figure S8. UV-Visible spectra and PB fluorescence quenching of OCPs with mutated cpcR carotenoid binding residues. (A) Absorbance spectra of the dark (black) and light (red) forms of the double mutant P126V/Y129F CAN-OCP isolated from *E. coli* cells. (B) PB fluorescence quenching induced by WT and mutated CAN-OCPs at 0.8 M phosphate during illumination at 900 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of blue-green light and 23°C. The OCP were previously illuminated with strong white light to photoactivate them. (C)-(F) Absorbance spectra of the dark (black) and light (red) forms of single and double mutants of ECN-OCP isolated from *E. coli* or *Synechocystis*. The OCPs were illuminated at 9°C with strong white light during 5 min. (G),(H) PB fluorescence quenching induced by WT and mutated ECN-OCPs isolated from *E. coli* (G) or *Synechocystis* (P126V.Y129F mutant isolated from WT and ΔCrtR background strains) (H) in 0.8 M phosphate, under 900 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ blue-green light and 23°C. The OCPs were pre-illuminated with strong white light before adding to the PBs.

Fig. S9

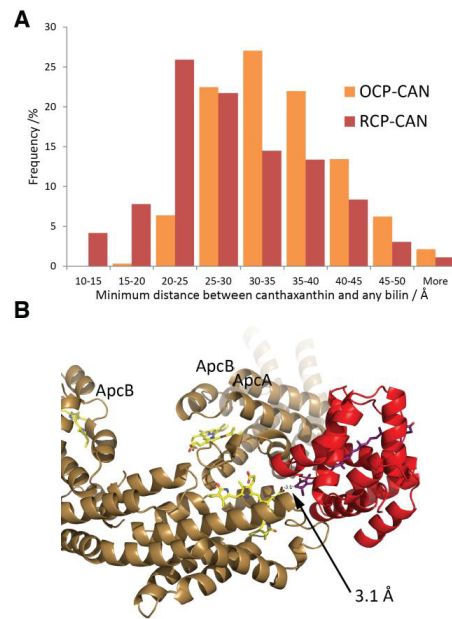


Figure S9. (A) Comparison of the minimal distance between the CAN and any of the bilin chromophores resulting from unconstrained random docking of OCP_{CAN} or RCP_{CAN} to ApcAB. The Y axis indicates how frequent a certain distance range occurs in the 1000 top docking solutions chosen from 25'000 docking runs by highest Rosetta score. (B) Result from a constrained docking run indicating that the minimal distance can be as low as 3.1 Å. The situation *in vivo* is likely very different since the exact binding site and mode is not known.

Table S1. Carotenoid content of OCPs and RCPs used in this study. The carotenoid content of purified OCPs and RCPs determined by LC-MS. For proteins expressed in *E. coli*, β -carotene (β -CAR) producing *E. coli* BL21 cells with either pBAD-CrtW or pBAD-CrtO plasmids were used to produce the carbonyl carotenoids CAN and ECN *in vivo* (see Methods). All the OCPs isolated from *E. coli* cells with the pBAD-CrtO plasmid contained around 95% ECN and 5% CAN. The OCPs produced in cells containing the pBAD-CrtW were observed to bind a mixture of ECN and CAN although the cells contained less than 10% of ECN and more than 50% CAN. Certain mutant OCPs (i.e. E34A) were observed to bind ECN and CAN in significantly different amounts as compared to wildtype (WT) OCP, suggesting an importance of epcR carotenoid-binding residues to the carotenoid binding specificity of OCP. Small amounts of a carotenoid of unknown identity (m/z = 548) were detected for a number of samples, but constituted < 5% of total carotenoid content in all cases. P126V/Y129F OCP mutants expressed in wildtype *Synechocystis* contained moderate amounts of hECN and zeaxanthin (ZEA).

| Sample | Carotenoid content (%) | | | | | |
|--|------------------------|------|---------------------|--------------|------|------|
| | CAN | ECN | Unknown (m/z = 548) | β -CAR | hECN | ZEA |
| WT OCPs from <i>E. coli</i> (CrtO or CrtW) | | | | | | |
| OCP-ECN (CrtO) | 5.0 | 95.0 | 0 | 0 | 0 | 0 |
| OCP-CAN (CrtW) | 43.4 | 52.6 | 4.0 | 0 | 0 | 0 |
| Mutant OCPs from <i>E. coli</i> (CrtW) | | | | | | |
| OCP C84A | 54.6 | 42.1 | 3.3 | 0 | 0 | 0 |
| OCP E34A | 15.9 | 80.4 | 3.7 | 0 | 0 | 0 |
| OCP Y129F | 31.6 | 65.2 | 3.2 | 0 | 0 | 0 |
| OCP P126V | 52.5 | 44.6 | 2.7 | 0.2 | 0 | 0 |
| OCP P126V/Y129F | 45.8 | 49.3 | 4.9 | 0 | 0 | 0 |
| WT RCPs from <i>E. coli</i> (CrtW) | | | | | | |
| RCP-CAN | 100 | 0 | 0 | 0 | 0 | 0 |
| Mutant OCPs from <i>Synechocystis</i> | | | | | | |
| P126V/Y129F (wildtype <i>Synechocystis</i>) | 0 | 72 | 0 | 0 | 9.5 | 18.5 |
| P126V/Y129F (Δ CrtR <i>Synechocystis</i>) | 0.6 | 99.4 | 0 | 0 | 0 | |

Table S2. X-ray crystallography data collection and refinement statistics for OCP_{CAN} and RCP_{CAN} data sets. Statistics for the highest-resolution shell are shown in parentheses.

| | RCP-CAN | OCP-CAN |
|---------------------------------------|---------------------------------|---------------------------------|
| Data collection | | |
| Resolution range (Å) | 39 - 1.54 (1.60 - 1.54) | 37 - 1.90 (1.97 - 1.90) |
| Space group | P 1 2 ₁ 1 | P 3 ₂ 2 1 |
| Unit cell dimensions | 51.3 64.1 53.6 Å 90 115 90 ° | 82.9 82.9 87.2 Å 90 90 120 ° |
| Total reflections | 91995 (8763) | 55545 (5518) |
| Unique reflections | 46069 (4431) | 27757 (2759) |
| Multiplicity | 7.2 (6.9) | 20.7 (20.3) |
| Completeness (%) | 99.6 (96.3) | 99.9 (99.4) |
| Mean I/sigma(I) | 13.8 (2.5) | 23.9 (2.1) |
| Wilson B-factor | 16.3 | 28.9 |
| R-merge | 0.025 (0.32) | 0.017 (0.36) |
| R-meas | 0.035 | 0.023 |
| CC ^{1/2} | 0.999 (0.799) | 1 (0.839) |
| CC* | 1 (0.943) | 1 (0.955) |
| Refinement | | |
| Number of reflections | 46063 | 27746 |
| Number of reflections used for R-free | 1998 | 2008 |
| R-work (%) | 14.74 (25.95) | 18.09 (43.46) |
| R-free (%) | 15.99 (29.45) | 20.76 (43.19) |
| Number of non-hydrogen atoms | 2644 | 2694 |
| macromolecules | 2196 | 2410 |
| ligands | 84 | 48 |
| solvent | 364 | 236 |
| Protein residues | 282 | 315 |
| RMS (bonds, Å) | 0.007 | 0.006 |
| RMS (angles, °) | 1.04 | 0.90 |
| Ramachandran favored (%) | 99 | 99 |
| Ramachandran allowed (%) | 1 | 1 |
| Ramachandran outliers (%) | 0 | 0 |
| Clashscore | 0.67 | 1.64 |
| Average B-factor (Å ²) | 21.5 | 33.7 |
| macromolecules (Å ²) | 19.3 | 33.3 |
| ligands (Å ²) | 21.2 | 24.1 |
| solvent (Å ²) | 35.0 | 39.8 |

Table S3. Comparison of canthaxanthin dihedral angles observed in OCP and RCP structures. Dihedral angles were measured using atomic PDB coordinates for CAN carbon atoms. Dihedral angles for 3⁺-hECN binding (PDB ID: 1M98) and ECN binding (PDB ID: 3MG1) OCPs are also included for comparison.

| Dihedral | RCP _{CAN} (Chain A) | RCP _{CAN} (Chain B) | OCP _{CAN} | Synechocystis OCP (3MG1 - Chain A) | Synechocystis OCP (3MG1 - Chain B) | Arthrospira OCP (1M98 - Chain A) | Arthrospira OCP (1M98 - Chain B) |
|---------------------------|---------------------------------|---------------------------------|--------------------|------------------------------------|------------------------------------|----------------------------------|----------------------------------|
| C5-C6-C7-C8 (β1 ring) | -63.5 | 39.8 | 116.3 | 146.4 | 148.6 | 118.5 | 131.9 |
| C6-C7-C8-C9 | 177.9 | 179.0 | 175.4 | 171.0 | 172.2 | 172.8 | 174.7 |
| C7-C8-C9-C10 | -146.5 | -170.0 | -155.3 | -179.5 | 179.5 | -163.7 | -176.8 |
| C8-C9-C10-C11 | 177.6 | 178.0 | 175.8 | 169.3 | 170.9 | 177.9 | 171.1 |
| C9-C10-C11-C12 | -173.5 | 177.2 | 178.8 | 173.2 | 173.5 | 165.2 | 173.6 |
| C10-C11-C12-C13 | 176.1 | 176.8 | 174.4 | 169.6 | 171.4 | 175.4 | 175.5 |
| C11-C12-C13-C14 | -178.9 | -172.8 | 167.8 | 172.8 | 178.0 | 177.5 | 175.9 |
| C12-C13-C14-C15 | 171.1 | 175.8 | 173.0 | -177.3 | -178.8 | 171.0 | 170.0 |
| C13-C14-C15-C16 | 177.1 | 173.9 | -171.4 | -175.2 | 179.8 | -175.7 | 171.5 |
| C14-C15-C15'-C14' | 178.3 | 176.0 | 172.7 | 168.7 | 167.7 | 170.7 | 171.4 |
| C15-C15'-C14'-C13' | -176.9 | -175.8 | 169.8 | 178.9 | 178.0 | 179.6 | -168.2 |
| C15'-C14'-C13'-C12' | -179.1 | 178.4 | 173.0 | 170.9 | 170.4 | 172.8 | 169.8 |
| C14'-C13'-C12'-C11' | -175.0 | -178.6 | -153.3 | -172.4 | -171.5 | -161.6 | 171.4 |
| C13'-C12'-C11'-C10' | -178.2 | -179.7 | 173.1 | 171.4 | 171.0 | 174.8 | 170.9 |
| C12'-C11'-C10'-C9' | 179.6 | 179.7 | 171.9 | -177.3 | -178.1 | 170.3 | -158.8 |
| C11'-C10'-C9'-C8' | -179.9 | 178.5 | 175.2 | 174.2 | 174.6 | 174.0 | -179.7 |
| C10'-C9'-C8'-C7' | -179.5 | 168.7 | -160.3 | -180.0 | -179.4 | -170.2 | 174.9 |
| C9'-C8'-C7'-C6' | 178.8 | 177.5 | 177.6 | 177.2 | 176.8 | 177.3 | 178.6 |
| C8'-C7'-C6'-C5' (β2 ring) | 163.3 | -146.1 | -72.1 | -52.7 | -52.8 | -66.0 | -51.9 |

Table S4.

(A) Canthaxanthin binding residues observed in the OCP carotenoid-binding pocket. The shortest distance between CAN and a particular residue is listed, along with the specific atoms (in PDB atom numbering) that define the distance of closest approach. Sequence conservation is based on the alignment described in the Methods. Shared residues in cpcO and cpcR are listed in bold.

| cpcO - CAN Binding Residues in OCP | | | | |
|------------------------------------|--------------|----------|--------------|--|
| Residue | Residue Atom | CAN Atom | Distance (Å) | Sequence Conservation |
| Leu37 | CD2 | O02 | 3.34 | L (1.00) |
| Ile40 | CG2 | C16 | 3.41 | I (0.649), L(0.280), T(0.071) |
| Trp41 | N | C06 | 3.66 | W (1.00) |
| Tyr44 | CG | C14 | 3.26 | Y(0.958), F(0.042) |
| Ile53 | CG2 | C40 | 3.96 | V(0.369), I(0.369), P(0.226), R(0.018), A(0.018) |
| Leu107 | CD2 | C36 | 3.78 | L(1.00) |
| Trp110 | CD2 | C22 | 3.61 | W(1.00) |
| Tyr111 | N | C28 | 3.61 | Y(0.851), N(0.071), F(0.071), W(0.006) |
| Gly114 | CA | C13 | 3.56 | G(0.917), S(0.060), A(0.024) |
| Met117 | SD | C13 | 3.81 | M(1.00) |
| Ile151 | O | C41 | 3.64 | I(0.899), L(0.101) |
| Thr152 | OG1 | C39 | 3.51 | T(0.958), S(0.030), Q(0.012) |
| Arg155 | CD | C35 | 3.71 | R(0.911), Q(0.083), H(0.006) |
| Val158 | CG2 | C22 | 3.66 | V(0.994), I(0.006) |
| Met161 | CE | O02 | 3.66 | M(1.00) |
| Tyr201 | OH | O01 | 2.56 | Y(1.00) |
| Leu205 | CD2 | O01 | 3.61 | L(0.565), M(0.435) |
| Cys245 | SG | C12 | 3.54 | C(0.887), A(0.107), G(0.006) |
| Leu248 | CD1 | C07 | 3.95 | L(1.00) |
| Leu250 | CD2 | O01 | 3.66 | L(0.821), M(0.167), I(0.012) |
| Val273 | CG2 | C21 | 3.56 | V(0.905), C(0.095) |
| Thr275 | CB | C33 | 3.79 | T(0.982), S(0.018) |
| Trp277 | CD2 | C40 | 3.73 | W(1.00) |
| Phe278 | CE2 | C42 | 3.91 | F(0.982), A(0.012), V(0.006) |
| Met284 | CE | C31 | 3.57 | M(0.899), I(0.095), L(0.006) |
| Trp288 | NE1 | O01 | 2.87 | W(1.00) |
| Ile303 | CG1 | O01 | 3.66 | I(0.935), V(0.065) |

Table S4.

(B) Canthaxanthin binding residues observed in the RCP carotenoid-binding pocket. Distances and atom numbering are the same as in (S4.A.).

| cpcR - CAN Binding Residues in RCP | | | | |
|------------------------------------|------------------|------------------|--------------------|--|
| Residue | Residue Atom | CAN Atom | Distance (Å) | Sequence Conservation |
| Glu34 | O (A), O (B) | C11 (A), C11 (B) | 3.43 (A), 3.61 (B) | E(0.899), D(0.101) |
| Leu37 | CG (A), CD2 (B) | C29 (A), C23 (B) | 3.43 (A), 3.58 (B) | L(1.00) |
| Ala38 | N (A), N (B) | C11 (A), C11 (B) | 3.97 (A), 3.78 (B) | A(0.994), G(0.006) |
| Ile40 | O (A), O (B) | C42 (A), C42 (B) | 3.41 (A), 3.30 (B) | I(0.649), L(0.280), T(0.071) |
| Trp41 | CB (A), CB (B) | C33 (A), C33 (B) | 3.68 (A), 3.57 (B) | W(1.00) |
| Tyr44 | CB (A), CB (B) | C40 (A), C42 (B) | 3.56 (A), 3.51 (B) | Y(0.958), F(0.042) |
| Met47* | SD(A) | C32(A) | 3.75(A) | M(0.964), T(0.012), L(0.012), I(0.012) |
| Gly48* | CA (B) | C32 (B) | 3.91 (B) | G(0.994), A(0.006) |
| Leu51* | O (B) | C14 (B) | 3.85 (B) | I(0.887), L(0.077), V(0.036) |
| Thr52* | OG1 (B) | C24 (B) | 3.55 (B) | T(0.970), S(0.024), I(0.006) |
| Ala55* | CB (B) | C06 (B) | 2.72 (B) | A(0.988), T(0.006), S(0.006) |
| Gly57* | O (B) | C06 (B) | 3.64 (B) | G(0.911), D(0.030), Q(0.024), S(0.018), A(0.012), E(0.006) |
| Ala59* | CB (B) | O02 (B) | 3.18 (B) | A(0.899), V(0.077), T(0.024) |
| Thr80 | CG2 (A), CG2 (B) | C21 (A), C21 (B) | 3.16 (A), 3.44 (B) | T(0.607), S(0.321), A(0.042), E(0.018), M(0.006), F(0.006) |
| Met83 | CE (A), CE (B) | C27 (A), C27 (B) | 3.46 (A), 3.54 (B) | M(1.00) |
| Cys84* | SG.A (A) | C21 (A) | 3.88 (A) | C(0.810), R(0.113), Y(0.024), F(0.024), T(0.012), V(0.006), I(0.006), H(0.006) |
| Asn104 | OD1 (A), CB (B) | C13 (A), O02 (B) | 3.28 (A), 3.49 (B) | N(1.00) |
| Leu107 | O (A), CA (B) | C30 (A), C32 (B) | 3.88 (A), 3.84 (B) | L(1.00) |
| Trp110 | O (A), O (B) | C41 (A), C41 (B) | 3.43 (A), 3.40 (B) | W(1.00) |
| Tyr111 | N (A), N (B) | C38 (A), C38 (B) | 3.83 (A), 3.85 (B) | Y(0.851), N(0.071), F(0.071), W(0.006) |
| Leu113 | CB (A), CB (B) | C39 (A), C39 (B) | 3.92 (A), 3.94 (B) | L(1.00) |
| Gly114 | N (A), N (B) | C39 (A), C39 (B) | 3.77 (A), 3.68 (B) | G(0.917), S(0.060), A(0.024) |
| Met117 | SD (A), SD (B) | C39 (A), C39 (B) | 3.64 (A), 3.68 (B) | M(1.00) |
| Ile125 | CG2 (A), CG2 (B) | C25 (A), C23 (B) | 3.77 (A), 3.80 (B) | I(0.899), L(0.101) |
| Pro126 | CD (A), CD (B) | C07 (A), C07 (B) | 3.65 (A), 3.90 (B) | P(1.00) |
| Tyr129 | CE1 (A), CE1 (B) | C05 (A), C05 (B) | 3.44 (A), 3.47 (B) | Y(1.00) |
| Ile151 | CG1 (A), CD1 (B) | C28 (A), C14 (B) | 3.95 (A), 3.60 (B) | I(0.899), L(0.101) |
| Arg155 | NH2 (A), NH2 (B) | C14 (A), C13 (B) | 3.86 (A), 3.82 (B) | R(0.911), Q(0.083), H(0.006) |

Table S5. Peptides and specific modification sites detected by XF-MS and the ratio(s) of hydroxyl radical reactivity. Detected peptides, modification sites, and their corresponding hydroxyl radical reactivity ratios (R) are listed for OCP_{CAN}, RCP_{CAN}, and RCP_{ECN} protein samples.

| Seq. No. ^a | Peptide Sequence ^c | Site of modification ^d | Ratio "R" of hydroxyl radical reactivity ^e | | |
|-----------------------|---|---|---|---------------------------------|---------------------------------|
| | | | $\frac{k_{OCP}^R}{k_{OCP}^O}$ | $\frac{k_{RCP-CAN}}{k_{OCP}^O}$ | $\frac{k_{RCP-ECN}}{k_{OCP}^O}$ |
| 2-9 | PFTIDSAR | P1, F2 (+16 Da) ^g | - ^j | - | - |
| | | A8, R9 (+16 Da) | - | - | - |
| 10-27 | GIFPNTLAADVVPATLAR | Residues 13 to 22 (+16 Da) | 1.37 ± 0.15 | - | 1.10 ± 0.24 |
| 28-49 | FSQLNAEDQLALIWFAYL EMGK | W41 (+48 Da) | 0.35 ± 0.05 | 0.55 ± 0.05 | 0.11 ± 0.03 |
| | | W41, F42, Y44, M47 (+16 Da) | 0.45 ± 0.05 | 0.61 ± 0.06 | 0.15 ± 0.03 |
| | | M47 (+16 Da) | 0.79 ± 0.08 | 0.87 ± 0.09 | 0.33 ± 0.04 |
| 50-69 | TLTAAAPGAASMQLAENA LK | P56 (+16 Da) | 0.71 ± 0.14 | 0.73 ± 0.14 | 0.37 ± 0.08 |
| | | M61 (+16 Da) | 1.45 ± 0.19 | 0.89 ± 0.14 | 0.51 ± 0.08 |
| 70-89 | EIQAMGPLQQTQAMCDL ANR | M74P76M83 (+16 Da) | 1.10 ± 0.24 | 0.82 ± 0.20 | 0.31 ± 0.04 |
| 90-96 | ADTPLCR | P94 (+16 Da) | 0.79 ± 0.12 | 0.79 ± 0.12 | 0.34 ± 0.08 |
| 97-106 | TYASWSPNIK | Y98W101P103 (+16 and + 32 Da) | 2.84 ± 0.63 | 2.72 ± 0.61 | 1.00 ± 0.16 |
| 107-112 | LGFWY | F109W110Y111 (+16 and + 32 Da) | 0.98 ± 0.26 | 1.64 ± 0.35 | 0.74 ± 0.13 |
| 113-155 | LGELMEQGFVAPIPAGYQ LSANANAVLATIQGLESG QQITVLR | M117 (+16 Da) | 0.83 ± 0.12 | 1.22 ± 0.15 | 0.59 ± 0.10 |
| | | Residues 121-126 (+16 Da) ^h | 0.73 ± 0.18 | 1.10 ± 0.23 | 0.40 ± 0.05 |
| 147-160 ^b | SGQQTIVLRNAVVD | R155, N156 (+16 Da) | 10.25 ± 3.54 | 17.25 ± 5.76 | 4.74 ± 0.62 |
| 156-167 | NAVVDMGFTAGK | M161 (+16 Da) | 1.27 ± 0.07 | - | 0.25 ± 0.03 |
| | | F163 (+16 Da) | 0.58 ± 0.20 | - | 0.39 ± 0.06 |
| | | K167 (+16 Da) | 0.78 ± 0.16 | - | 0.55 ± 0.12 |
| 172-185 | IAEPVVPQDTASR | P175P178P179R185 (+16 Da) | 0.67 ± 0.15 | -- ^j | -- |
| 192-215 ^b | GVFNATVLYMDNLAN DFDTLIE | Residues 119 to 215 (+16 Da) ^h | 1.11 ± 0.21 | -- | -- |
| 221-235 ^b | GALQPPFQRPIVGKE | Residues 226 to 231 (+16 Da) ^h | 1.47 ± 0.27 | -- | -- |
| 235-239 | ENVLR | R239 (+16 Da) | 0.76 ± 0.05 | -- | -- |
| 243-249 | EECQNLIK | No modification ^f | - | -- | -- |
| 250-254 | LIPER | P252 (+16 Da) | 0.53 ± 0.06 | -- | -- |
| 255-268 | GVTEPAEDGFTQIK | P259, F264, K268 (+16 Da) | 0.91 ± 0.20 | -- | -- |
| 273-289 | VQTPWFVGGNVGMNIAWR | P276, W277, F278 (+32 Da) | 3.38 ± 0.38 | -- | -- |
| | | M284 (+16 Da) | 2.88 ± 0.32 | -- | -- |
| 290-297 | FLLNPEGK | F190 (+16 Da) | 1.67 ± 0.15 | -- | -- |
| | | L291, L292 (+16 Da) | 0.92 ± 0.06 | -- | -- |
| | | P294 (+16 Da) | 0.97 ± 0.10 | -- | -- |
| 298-310 | IFFVAIDLLASPK | I298, F299 (+16 Da) | 1.89 ± 0.33 | -- | -- |
| | | V301, A302, I303 (+16 Da) | 1.86 ± 0.30 | -- | -- |
| | | P309, K310 (+16 Da) | 0.75 ± 0.15 | -- | -- |

^a 94 % sequence coverage was obtained from the bottom up LC-ESI-MS analysis of OCP_O, OCP_R, RCP-CAN, and RCP-ECN using trypsin and ^b Glu c digestions.

^c sequences of digested fragments identified by mass spectrometry analysis described in experimental procedures

^d positions of modified residues identified mass spectrometry analysis described in experimental procedures. Multiple residues are indicated by sequence number

^e rate constants were estimated by employing a non-linear fit of hydroxyl radical modification data to a first order decay as described in experimental procedures. R is a quantitative measure of the change in the solvent accessibility changes. Reported errors were determined from the maximum and minimum values of R calculated from the non-regression fit parameter (k) and its standard error obtained from a single dose response within 95% confidence interval.

^f no modification was detected

^g mass shift due to side chain modification is show within the parenthesis

^h no data available either due to missing sequence in the contrast or no modification

ⁱ comparison not done due to the absence of CTD in RCPs

^j rate constant measured from OCP^O-ECN sample

Table S6. Sequences of primers used in this study. Listed primers include those used for construction of 6x-His tagged RCPs (**A, B**) and those used for site-directed mutagenesis (**C**) as described in the Methods section

| A. Primers used for construction of RCP 1-165 His6-tagged C-terminal plasmid for expression in <i>E. coli</i> | |
|--|---|
| RCP-Syn-1-165-Ctag-F | CATCATCATCATCATCATTAGATAAACCCTTCAGAGTTTGTCT |
| RCP-Syn-1-165-Ctag-R | ATGATGATGATGATGATGATGAGCGGTGAAGCCCATGTCCAC |
| | |
| B. Primer used for construction of RCP 20-165 His6-tagged C-terminal plasmid for expression in <i>E. coli</i> | |
| RCP-Syn-20-165-Ctag-F | TTAATAAGGAGATATACCATGGTACCCGCTACCATCGCCCGT |
| RCP-Syn-20-165-Ctag-R | CATGGTATATCTCCTTATTAAAGTTAAACAAAATTATTCTA |
| | |
| C. Site-directed mutagenesis primers | |
| P126V - F | GCCCCAATTGTCGCTGGTTACCAACTTTCTGCC |
| P126V - R | GGCAGAAAAGTTGGTAACCAGCGACAATTGGGGC |
| P126A - F | CCAATTGCCGCTGGTTACCAACTTTCTGCC |
| P126A - R | GGCAGAAAAGTTGGTAACCAGCGCAATTGG |
| Y129F - F | GCCCCAATTCGCTGGTTTCCAACCTTTCTGCC |
| Y129F - R | GGCAGAAAAGTTGGAAAACCAGCGGAATTGGGGC |
| P126V/Y129F - F | GCCCCAATTGTCGCTGGTTTCCAACCTTTCTGCC |
| P126V/Y129F - R | GGCAGAAAAGTTGGAAAACCAGCGACAATTGGGGC |
| E34A - F | CTCAATGCCGCAGATCAATTGGCTCTGATTTGGTTTGCTTACC |
| E34A - R | AATCAGAGCCAATTGATCTGCGGCATTGAGTTGGCTAAAACG |
| C84A - F | CAAACCCGGCCATGGCTGACTTGCC |
| C84A - R | GCCAAGTCAGCCATGGCCCGGGTTTGG |

COLLABORATION AVEC MARKUS SUTTER (BERKELEY)

Une fois que l'OCP y est attachée, les PBs lui transmettent la quasi-totalité de l'énergie lumineuse collectée. Une fois le stress lumineux terminé, l'OCP doit être retirée des PBs afin que ces derniers retrouvent leur capacité à transmettre l'énergie aux centres réactionnels. La Fluorescence Recovery Protein (FRP) accélère le détachement de l'OCP des PBs et sa reconversion en forme orange. L'article présenté dans ce chapitre rapporte la structure de l'identification du site actif de la FRP. J'ai contribué à ce travail en isolant des FRPs modifiées (W50F, D54E, D54L et R60K) et en réalisant les cinétiques de reconversion de l'OCP_r en OCP_o à l'obscurité en présence des FRP modifiées.

6.0.1 *Résumé de l'article Sutter et al., 2013*

La structure de la FRP a été résolue à 2.5Å. La FRP est présente sous deux formes dans le cristal : dimérique et tétramérique. Le dimère est la forme active. La forme tétramérique est probablement un artefact de cristallisation, mais on ne peut pas exclure qu'elle soit impliquée dans la régulation de l'activité FRP en créant des clusters de FRP inactifs.

Nous avons proposé que la région très conservée 50-61 soit impliquée dans l'activité de la FRP (figure 2). Les acides aminés les plus conservés ont été modifiés, les protéines isolées, et des cinétiques de conversion de l'OCP rouge en OCP orange à 9°C en présence de FRP mesurées (figure 3 de l'article). Les résidus Trp50, Asp54, His53 et plus particulièrement Arg60, qui forment une structure palindromique dans l'homodimère, correspondent au site actif (figure 3 de l'article). Le changement de l'Arg60 en Lys, qui porte également une charge positive, abolit aussi l'activité de la FRP. La présence d'une charge positive est donc très importante, mais également la distance inter-moléculaire entre OCP et FRP.

Par ailleurs, la FRP interagit avec le domaine C-terminal de l'OCP (Western blot en figure 4 de l'article). Ensemble, ces informations ont permis l'élaboration d'un modèle d'interaction possible entre OCP et FRP prenant en compte le site actif de la FRP, et l'affinité de la FRP pour le domaine C-terminal de l'OCP. La meilleure solution de docking entre FRP et domaine C-terminal de l'OCP est présentée en figure 4 de l'article. Cependant, une fois que le domaine N-terminal de l'OCP est ajouté au modèle de docking, une superposition de la FRP et du domaine N-terminal apparaît. D'après notre modèle de travail l'OCP rouge est une forme ouverte dans laquelle les domaines N- et C-terminal sont séparés. Lors du retour à la forme orange, le repositionnement du domaine N-terminal pourrait éjecter la FRP du domaine C-terminal de l'OCP. Ceci expliquerait que la FRP ne peut agir qu'avec l'OCP rouge et non avec l'OCP orange.

Conclusion

L'ensemble de ces données structurelles et fonctionnelles ont permis d'établir un nouveau modèle d'interaction entre OCP et FRP. La conservation de W50, D54, H53 et R60 assure l'attachement et l'activité de la FRP. La distance entre la charge du résidu 60 et la FRP doit être conservée pour assurer l'activité de la FRP.

Crystal structure of the FRP and identification of the active site for modulation of OCP-mediated photoprotection in cyanobacteria

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Abstract

Photosynthetic reaction centers are sensitive to high light conditions, which can cause damage because of the formation of reactive oxygen species. To prevent high-light induced damage, cyanobacteria have developed photoprotective mechanisms. One involves a photoactive carotenoid protein that decreases the transfer of excess energy to the reaction centers. This protein, the orange carotenoid protein (OCP), is present in most cyanobacterial strains; it is activated by high light conditions and able to dissipate excess energy at the site of the light-harvesting antennae, the phycobilisomes. Restoration of normal antenna capacity involves the fluorescence recovery protein (FRP). The FRP acts to dissociate the OCP from the phycobilisomes by accelerating the conversion of the active red OCP to the inactive orange form. We have determined the 3D crystal structure of the FRP at 2.5 Å resolution. Remarkably, the FRP is found in two very different conformational and oligomeric states in the same crystal. Based on amino acid conservation analysis, activity assays of FRP mutants, FRP:OCP docking simulations, and coimmunoprecipitation experiments, we conclude that the dimer is the active form. The second form, a tetramer, may be an inactive form of FRP. In addition, we have identified a surface patch of highly conserved residues and shown that those residues are essential to FRP activity.

Introduction

Light is vital for the survival and growth of photosynthetic organisms. In natural environments, these organisms are exposed to varying light conditions in addition to the day/night cycle. Too much exposure to light causes the formation of reactive oxygen species that damage the sensitive photochemical reaction centers, and thus, a careful regulation of energy flow is critical. Under low light conditions, an efficient energy collection by the antennae complexes is achieved, whereas under high light conditions, the excess energy has to be diverted from photosynthesis^[204].

Plants and cyanobacteria have evolved different ways to deal with excess energy arriving at the reaction centers. Higher plants and green algae contain antenna complexes consisting of transmembrane proteins that sense the acidification of the thylakoid lumen and react by switching from efficient energy collection to heat dissipation^[185,204]. Cyanobacteria, however, contain antenna complexes called phycobilisomes, which are membrane-anchored and consist of phycobilin proteins. Instead of sensing the effect of high light through pH changes, cyanobacterial phycobilisomes are quenched by a protein capable of directly sensing high light conditions, the orange carotenoid protein (OCP). The 35 kDa OCP consists of two distinct domains that encompass a keto-carotenoid (3'-hydroxyechinenone) in an all trans conformation^[84,95,113,205]. Irradiance with high light changes the OCP from an inactive orange (OCP_o) to an active red form (OCP_r) that is capable of binding to the phycobilisomes to prevent excess energy from flowing to the reaction centers. The low quantum yield of the OCP_o to OCP_r photoconversion together with instability of the OCP_r form lead to effective OCP inactivity under low light conditions; therefore, the OCP acts as a switch for photoprotection triggered by a specific light level^[109]. In darkness, isolated OCP_r spontaneously reverts back to the OCP_o form. This reversion is greatly affected by the presence of the fluorescence recovery protein (FRP)^[86].

In vitro, the FRP accelerates the conversion of free *OCP_r* back to the orange form. In vivo, the FRP is essential to recover the full capacity of the antenna, presumably by playing a role in detaching *OCP_r* from the phycobilisomes^[86]. Mutant strains of cyanobacteria lacking this protein are unable to recover the normal antenna capacity under low light conditions. The FRP is a 13 kDa protein that does not bind a chromophore. It is exclusively found in organisms that also contain the OCP, and the two genes are typically in close proximity in the genome. Of the currently available 130 cyanobacterial genomes, 97 genomes contain a gene for the OCP, and 71 genomes also contain a gene for the FRP^[206] (Table S1). The FRP from *Synechocystis* sp. PCC 6803 was first characterized in a form containing an additional 25 aa compared with the FRP from almost all other strains (the other exception is the FRP encoded in the *Microcystis aeruginosa* genome)^[86]. It has recently been shown that this longer form is caused by a misidentified start site, and the active form *in vivo* is a shorter protein that begins with Met26^[87]; we have used this form for this study. Here, we present the 3D structure of the FRP; the protein crystallized in two different conformations and different quaternary states. We show that the active form of the FRP is a homodimer, with a cluster of highly conserved residues on one surface of the dimer. Based on these observations, we made several single amino acid mutations, analyzed the mutant forms for activity, and thereby, identified the active site of the FRP. Based on the structural information, docking, and coimmunoprecipitation studies, we propose a model for the interaction between the FRP and the OCP. These results provide the foundation for additional studies on the molecular mechanism of the regulation of photoprotection in cyanobacteria.

Results

Structure of the FRP Shows That It Adopts Two Distinct Conformations.

We have determined the crystal structure of *Synechocystis* sp. PCC 6803 FRP at 2.5 Å resolution using iodine phasing (Table S2). There are six FRP polypeptide chains in the asymmetric unit of the P4₁2₁2 space group in two distinct conformations. Four (chains A-D) form dimers, with the twofold axis either in the asymmetric unit or generated by crystal symmetry. Remarkably, the other two chains (chains E and F) in the asymmetric unit exist in a very different conformation: α -helices α_1 and α_2 form one long extended helix α_1' , making a dimer that, together with its symmetry-related chains (E' and F'), forms a four-helix bundle (Fig. 6.1 A and B). Sample electron density for these regions can be found in Fig. S1 A and B. Both forms of the FRP are all helical, and they consist of an extended helical stalk of α_1 and α_2/α_1' (residues from 1 to 65) and a compact C-terminal head domain (residues 66-109). The stalk contains all of the contacts for the dimerization interface and likewise, forms the core of the tetramer. Chains B, D, and F show a higher flexibility, which is reflected in their temperature factor (B factor) because of fewer crystal contacts (Fig. S1C). The availability of noncrystallographically related chains enabled us to build into even weak electron density of regions of chain D, which as a result, have a high B factor (Fig. S1C, red arrow).

Although the structure of the head domain remains essentially the same in both the dimer and tetramer, the conformational change needed for their interconversion involves a rotation of helix α_1 by 180° to form one extended helix α_1' , which includes a short loop and most of the residues from α_2 (Fig. 6.1B). The interactions between the N-terminal part of helix α_1/α_1' and the C-terminal head domain are intramolecular in the dimer form, and although conserved in the tetramer assembly, they are instead intermolecular (Fig. 6.1C).

We analyzed the interfaces between the FRP monomers using the protein interfaces, surfaces, and assemblies (PISA) tool at the European Bioinformatics Institute^[207]. Both the dimer and tetramer forms have a large buried surface, consistent with a native state (Tables S3 and S4). Calculation of the energy of association of the interfaces between the different protein chains (Tables S3 and S4) results in a large negative ΔG for all interfaces, indicative of strong affinity. In addition, we measured the P value for each interface; this number quantifies its specificity. Values larger than 0.5 indicate an artifact of crystal packing. Based on the P value, the dimer interface is more specific (P = 0.048 for AC, P = 0.055 for BD), which is consistent with the large number^[208] of highly conserved residues that it contains. The P value

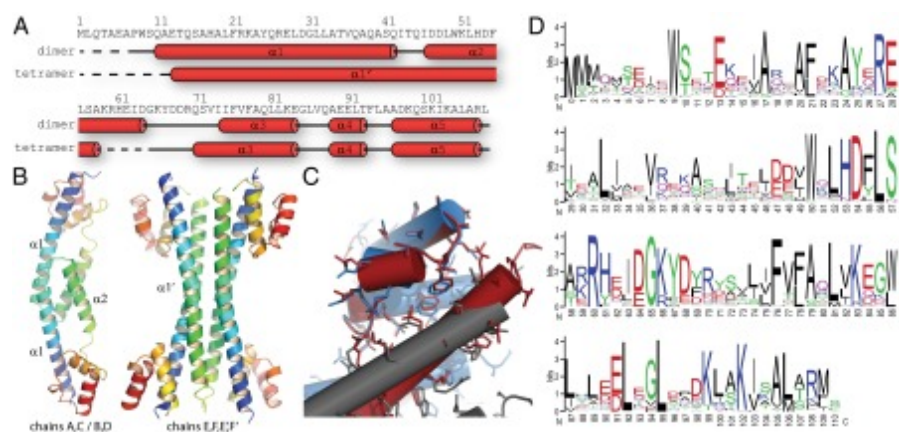


Figure 6.1 – Structural overview of the FRP. (A) Primary and secondary structure of the *Synechocystis* sp. PCC 6803 FRP dimer and tetramer forms. Red tubes indicate α -helices, and the dashed line shows residues disordered in the structure. (B) Cartoon representation of the dimer and tetramer forms of the FRP observed in the crystals. Rainbow coloring from N to C termini from blue to red. (C) Alignment of the head domains of the dimer (red, chain B) and tetramer (blue, chain F; gray, chain E) illustrating the conservation of interactions of the head and stalk domain between the two forms. (D) Sequence conservation logo of the FRP with numbering corresponding to the *Synechocystis* sp. PCC 6803 protein.

close to zero also implicates the dimer as the most stable form; however, the values calculated for the tetramer ($P = 0.338$ for E-F, $P = 0.227$ for E-F') are consistent with a viable alternative native state.

Likewise, analytical size exclusion chromatography of the purified FRP indicates that the dimer form is predominant in solution (Fig. S2A). Additionally, a time course glutaraldehyde cross-linking experiment followed by denaturing SDS/PAGE analysis confirm a preferential monomer-monomer cross-linking (Fig. S2B). When the FRP is stored on ice for more than 1 week before characterization, a size exclusion peak corresponding to a tetramer is occasionally observed (Fig. S2A). However, we cannot exclude that this larger form is a dimer of dimers instead of the four-helix bundle observed in the crystal structure. Efforts to generate the tetramer in solution by varying pH and salt concentration were not successful.

Putative Active Site of the FRP.

In the distribution of conserved amino acids, there is a striking patch of highly conserved residues on one surface of the dimer form of the FRP (Figs. 6.1D and 2A). In contrast, there is very little conservation of the residues on the opposite side. In addition to highly conserved residues on the inside of the patch (L56 and H53), there is a close intermolecular (distance between planes is 3.5 Å) cation- π interaction between W50 and R60, which orients the arginine in the correct position to form an intermolecular salt bridge with D54 (Fig. 6.2B). All of these conserved residues are located around the twofold symmetry axis between residues H53 and S57, forming a symmetrical active site with an extended hydrogen bonding network. In addition to those absolutely conserved residues, there is a histidine residue (H61) that forms a surface bulge. In all of the available primary structures of the FRP, the amino acid at the position corresponding to H61 is an aromatic residue (Phe/Tyr/His) that is also likely to form a similar surface bulge. In the tetramer form, the group of residues that makes up the conserved patch in the dimer is buried in the interface between the two dimers (W50 and H53), involved in interactions with the head domain (D54 and L56), or located in the disordered region between residues 58 and 65 (Figs. 6.1A and 2A). Accordingly, we hypothesized that the region between residues 50 and 61 on one side of the FRP dimer is the active site of the FRP.

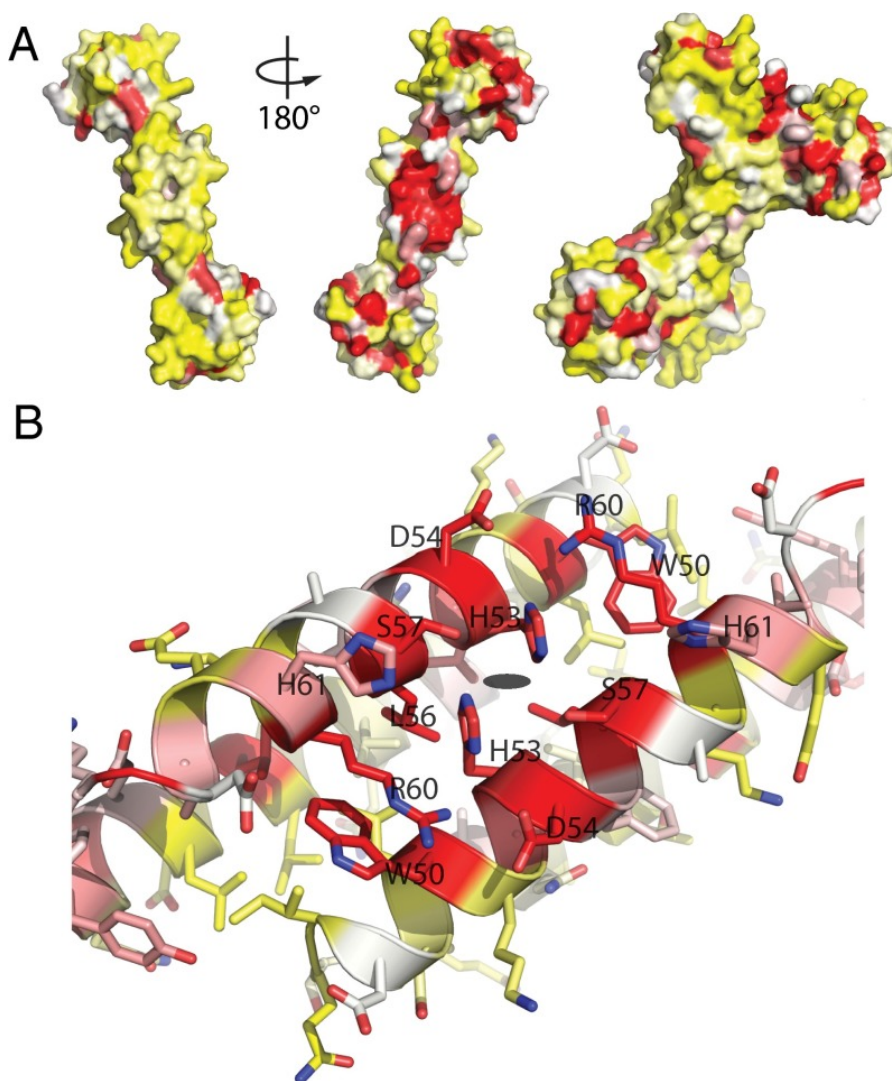


Figure 6.2 – Amino acid conservation mapping onto the structure of the FRP. (A) FRP dimer and tetramer structures shown in surface representation are colored according to conservation (red, high; white, medium; yellow, low). (B) Close-up view of the proposed active site with stick representation of side chains. Side chain oxygen and nitrogen atoms are colored red and blue, respectively. The location of the twofold symmetry axis is indicated with a black ellipse.

Mutations in the Proposed Active Site Alter FRP Activity.

To confirm our putative identification of the FRP active site, we generated several single-point FRP mutants (W9L, W50L, W50F, H53L, D54E, D54L, R60L, and R60K) and tested their activity in an assay measuring the conversion of *OCP_o* to *OCP_r*. The D54L and W50L FRP mutants expressed poorly and tended to precipitate, possibly because of misfolding. These mutants also showed a rather large decrease in activity relative to WT (Fig. 6.3). The H53L mutant was obtained in large quantities as a soluble protein but likewise, was impaired in activity (Fig. 6.3). The W50F and D54E mutants were purified in large quantities, behaved as homogeneous soluble dimers, and showed only a slight reduction in activity. A mutant of a highly conserved tryptophan from the head domain (W9L) showed no reduction in activity.

The most remarkable difference, however, was observed in the R60L and R60K mutants, which behaved similarly to WT during purification and analytical size exclusion chromatography but showed almost no activity, even with the relatively conservative change from arginine to lysine (Fig. 6.3). To exclude the possibility that the R60K mutant was disordered as a consequence of the mutation, we solved its structure. The R60K mutant FRP crystallized in the same space group, and the structure was solved by molecular replacement (Table S2 shows statistics). Although the

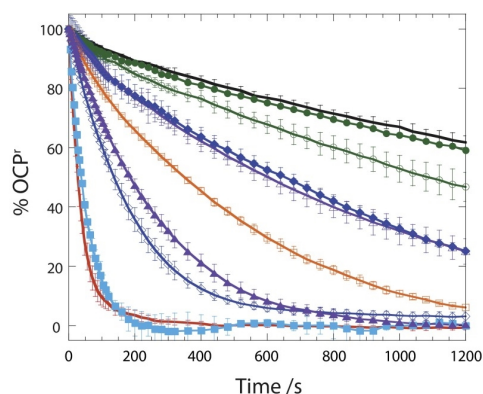


Figure 6.3 – Kinetics of dark reversion of OCP_r to OCP_o at 8 °C in the absence and presence of WT and mutant FRPs with a ratio of OCP to FRP of 2 : 1 : without FRP (black), with WT (red), and with W9L (closed squares; sky blue), H53L (open squares; orange), W50F (closed triangles; violet), W50L (open triangles; violet), R60L (closed circles; green), R60K (open circles; green), D54E (open rhomboids; blue), or D54L (closed rhomboids; blue). Average of three independent experiments. Error bars represent SD.

resolution is comparatively low (3.5 Å), the electron density is clear enough to see that the overall fold of the R60K mutant of the FRP is identical to the fold of the WT (Fig. S1D). Collectively, these data show that R60 is directly involved in the conversion of OCP and that the conserved side of the dimer surface interacts with OCP in the red form.

FRP-OCP Docking Simulations.

We next performed docking simulations between combinations of full-length FRP dimer, tetramer, FRP active site region, and full-length OCP or its C- and N-terminal domains with or without carotenoid. We analyzed the solutions based on Rosetta score^[208] and biological relevance considering the newly identified active site. The most convincing solution was found in a docking simulation between the active site region of the FRP and the C-terminal domain of the OCP lacking the carotenoid (Fig. 6.4). We then expanded the docking refinement to the full-length FRP docking to the C-terminal OCP domain containing the carotenoid based on the initial solution and found a narrow funnel in an rmsd vs. interface energy plot (Fig. S3), indicative of a good solution^[208,209].

The best solution based on score and surface complementarity places the FRP dimer binding to one side of the C-terminal domain (Fig. 6.4 A and B). This region includes a short helix, which is contributed by the N terminus of the protein, including residues T4, I5, and D6. Loops connecting the strands of the C-terminal β -sheet (T218, D220, R229 V232, N236, D262, F264, and F299) are also involved in the interaction. The binding region on the FRP is, as expected, the patch of conserved residues (around 50-62) as well as a contact from the head domain (Q80). This solution resulted from docking with the isolated C-terminal domain only; interestingly, the surface that interacts with the FRP is partially obstructed by the N-terminal domain in the full-length OCP_o form (Fig. 6.4A, shaded circle). The FRP dimer binds to the OCP on a surface groove lined by a cluster of conserved residues (Fig. 6.4B). This finding is notable because although OCP homologs are, in general, very similar, the amino acid conservation is usually highest for the residues interacting with the carotenoid; also, the surface is typically less well-conserved. The electrostatics of the FRP-OCP interface are complementary (Fig. S4, Top Left, shaded circle); there is an overall negative charge around the putative active site of the FRP, which aligns with positive LY charge at the location of the docking site of the OCP (Fig. S4, Bottom Left).

To confirm the predicted interaction between the FRP and the C-terminal domain of the OCP, coimmunoprecipitation experiments were performed. The C-terminal domain of the OCP was purified from a *Synechocystis* PCC 6803 mutant overexpressing a construct encoding residues 170-317 of the OCP (Fig. S5A). For the N-terminal

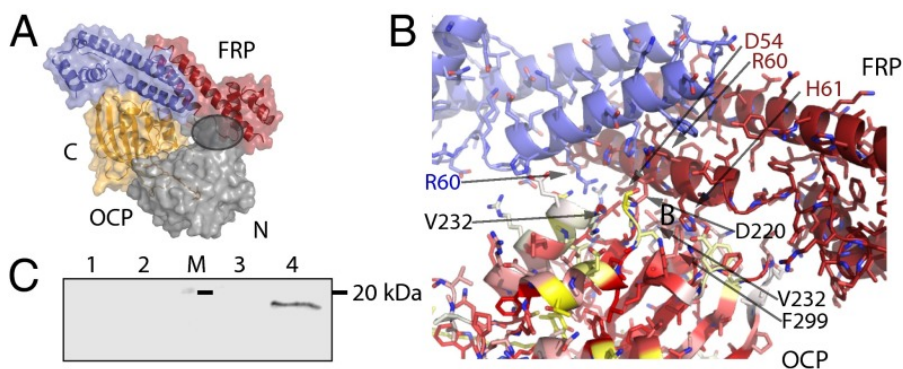


Figure 6.4 – Analysis of the FRP-OCP interaction. (A) Docking solution of the FRP dimer (active site region) to the C-terminal domain of the OCP. Overview of the best solution shown as the surface with the residues involved in the docking as a cartoon and the N-terminal domain in gray, the C-terminal domain in orange. The gray circle indicates the clash between the docked FRP and the N-terminal domain of OCP. (B) Details of the docking solution interaction between OCP and FRP. Amino acids in the OCP involved in the interaction are marked in black, and amino acids of the FRP are marked in blue and red. The surface of the OCP C-terminal domain is colored according to conservation (red, high; white, medium; yellow, low). (C) Anti-OCP immunoblot of the N- (lane 2) and C-terminal domains (lane 4) after coimmunoprecipitation of each domain with the OCP. Control experiments (without FRP) are also shown : N- (lane 1) and C-terminal controls (lane 3). Molecular mass marker (20 kDa; lane M) is denoted with a black line).

domain, we used the 16 kDa red carotenoid protein (RCP), which is an N-terminal proteolytic derivative of the OCP that binds the carotenoid^[205,210] (Fig. S5A). Interaction between the C- and N-terminal domains of the OCP with FRP was tested by coimmunoprecipitation, and OCP was detected using an antibody that recognizes both domains (Fig. S5B). As shown in Fig. 6.4C, only the C-terminal domain of the OCP coprecipitates with the FRP.

Discussion

We have determined the structure of the FRP, which regulates photoprotection in cyanobacteria. The FRP is present in two distinct forms in the crystal : a dimer that we identify as the active state and a tetramer that may be an inactive form. We were not able to purify the tetramer, but the structure shows that the active site that we identified in the dimer is disrupted in the tetramer. The difference between the dimer and tetramer is reminiscent of the conformational change observed in hemagglutinin of the influenza A virus on fusion at low pH, where a loop and an α -helix are drastically rearranged to become a long extended α -helix in the form of a triple-stranded coiled coil^[211]. Despite secondary structure consisting only of α -helices, the tertiary structure of either the active dimer or the tetramer form seems to be unique, because there is no close structural homolog to either in the Protein Data Bank (PDB).

Highly conserved residues forming an intricate network of hydrogen bonds are concentrated on one surface of the FRP dimer (Fig. 6.2A). Mutation of the absolutely conserved amino acids in this region greatly affected the activity of the FRP. Among them, the R60K mutant is almost completely inactive. The dimer form is the predominant form in solution and, based on activity assays, we propose that it is the active form of the FRP.

Based on structural evidence, the tetramer form seems to be a viable alternate conformation of the FRP and could represent an inactive state. The active site residues in the tetramer are either buried or too flexible (e.g., R60) to show ordered electron density. It seems that the crystallization conditions generate the right environment for the tetramer to form. In solution, there is a small fraction of apparent tetramer present, but it is unclear whether this larger form is a dimer of dimers or a tetramer with an extended $\alpha 1'$ helix. Whether the inactive tetramer can be formed

in a cellular environment is an open question. Previous results indicate that the FRP is constitutively active *in vitro* and *in vivo*. *In vitro* the presence of the FRP abolishes/reduces the accumulation of the *OCP_r* and the phycobilisome fluorescence quenching by the OCP^[81]. In *Synechocystis* mutant cells containing a high concentration of the FRP (10 times more than in WT cells), no fluorescence quenching was observed, indicating that, *in vivo*, the FRP is also active under high light conditions^[149]. Nevertheless, it is possible that, *in vivo*, a tetramerization could result in the inactivation of some of the FRP. In addition to the hypothesis that the FRP is inactivated by change in conformation and oligomeric state, there is also the possibility that changes in either pH or redox state coupled to light intensity could perturb the interactions among residues in the active site and thus, reduce its activity. This mechanism would be similar to the regulation of photoprotection in plants and allow a more dynamic regulation in contrast to relying on a difference in expression level of the FRP.

We have identified a patch of residues (W50, D54, H53, and R60), contributed by both chains on the surface of the FRP dimer to be important for the acceleration of the *OCP_r* to *OCP_o* conversion. In contrast, a mutation of a highly conserved residue from the head domain (W9L) did not influence activity at all (Fig. 6.3). The most striking inactivating mutation was changing R60 to lysine. This finding suggests that not only is the positive charge important but also, the intermolecular distance between the FRP and the OCP is critical. Moreover, the observation that mutation of W50 or D54, amino acids interacting with R60, decreases FRP activity also suggested that the exact position of the positive charge of the arginine is fundamental for FRP activity.

Docking simulations suggest that the FRP interacts with the C terminus of the OCP. A recent kinetic study^[112] suggests that the transition from *OCP_o* to *OCP_r* could be limited by *cis-trans* proline isomerization of residues Q224-P225 or P225-P226. Our docking solution places the FRP close to that loop, and therefore, it is possible that it could facilitate that isomerization (Fig. 6.4B). Recently, the OCP has been proposed to interact with the phycobilisome through its N-terminal domain, specifically through the surface surrounding Arg155, which is typically buried in interaction with the C-terminal domain in the *OCP_o*^[98]. We have confirmed the interaction of the FRP with the C-terminal domain of the OCP by coimmunoprecipitation. The FRP specifically interacts with the C-terminal domain, whereas no interaction was observed with the N-terminal domain of the OCP (Fig. 6.4C). It has also been proposed that the OCP might partially open up to expose part of the carotenoid^[113] and that the FRP could act to drive the two domains back together. Alternatively, the binding of the FRP to the C-terminal domain could trigger the conformational change back to the orange form of the OCP (Fig. 6.5). The position of the FRP in the most plausible docking model of FRP-OCP clashes with the N-terminal domain (in the structure of the closed orange form), which is consistent with this hypothesis. The binding of the FRP to *OCP_r* could trigger the conversion, and the following conformational change could cause the dissociation of the FRP.

Additional studies are needed to probe the residues in the OCP involved in the interaction and confirm the site of the interaction of the FRP with the C-terminal domain of the OCP, which is challenging because of the transient nature of the active red form of the OCP. A structure of the complex of the active red form of the OCP and the FRP would provide the information needed to explain how the newly identified active site residues of the FRP can act to convert OCP back to its orange form.

Methods

Site-Directed Mutagenesis.

To obtain a plasmid containing the sequence of the gene encoding the short *Synechocystis* sp. PCC 6803 His-tagged FRP, a deletion of the thrombin sequence and the nucleotides coding for the first 25 aa of FRP was done in the plasmid pCB9 (8) using the synthetic primers petM26Hisfor/petM26hisrev. The construction of the pCB9 plasmid, in which the *frp* gene (*slr1964*) is under control of a T7 promoter, was described in^[86]. The point mutations were added in the short FRP gene by site-directed mutagenesis using the Quickchange XL site-directed mutagenesis kit (Stratagene)

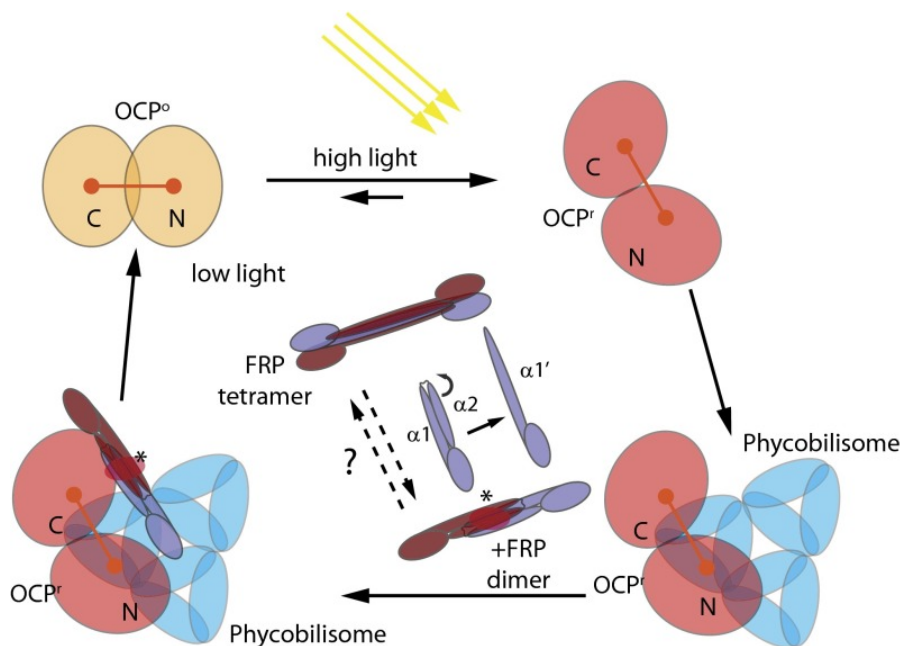


Figure 6.5 – Model of the interplay between the FRP, OCP, and phycobilisome under high and low light conditions. The OCP_o is converted to OCP_r under high light conditions, undergoes a conformational change that exposes part of the carotenoid and R155, and binds to the phycobilisomes to quench excess energy. The FRP can bind to unattached or phycobilisome-attached OCP_r , converting the OCP_r into OCP_o and dissociating it from the phycobilisomes. Under low light conditions, the decrease of OCP_r concentration leads to a situation in which all of the phycobilisomes will be free of OCP and unquenched. The dimer FRP is the active form. The state of the FRP could be regulated by exterior factors that convert it to an inactive tetramer form under high light conditions by rearranging helix $\alpha 2$ to form an extended $\alpha 1'$. The FRP dissociates from the OCP_o after conversion. The red ellipse and asterisk indicate the position of the FRP active site.

and synthetic mutagenic oligonucleotides (all of the oligonucleotides used in this work are described in Table S5). Oligonucleotides used to construct site-directed mutants were synthesized by Eurofins MWG. Incorporated mutations were confirmed by DNA sequencing. DNA containing the desired mutations was transformed into *Escherichia coli* strain BL21 (DE3) for protein expression.

Protein Purification for Crystallization.

E. coli cells containing the frp pcB9-derived plasmid were grown to an OD₆₀₀ of 0.8 at 37 °C, at which point they were cooled to room temperature followed by induction with 0.05 mM isopropylthio- β -d-galactoside and incubation overnight. Harvested cells were resuspended in buffer A (20 mM Tris, pH 8.0, at room temperature, 300 mM NaCl) and lysed using a French Press. Cleared lysate was applied on a 5-mL HisTrap HP column (Amersham) and washed with buffer A containing 20 mM imidazole. His-FRP was eluted with 2 column volumes buffer A containing 300 mM imidazole, concentrated, and then applied on a HiLoad 26/60 Superdex 75 (GE Healthcare) column equilibrated in 20 mM Tris, pH 7.4, at room temperature and 50 mM NaCl for final cleanup. Protein was then concentrated to 10-20 mg/mL for crystallization. Crystals were obtained from sitting drop experiments mixing 3-5 μ L protein solution with 1-5 μ L reservoir solution containing 5-10% (wt/vol) PEG-3350, 100 mM Na-citrate, pH 5.5-5.6, and 2% (vol/vol) tacsimate, pH 5.0 (Hampton Research). Crystals were stabilized by adding an 80% (vol/vol) glycerol solution to the drop for a final concentration of 25% (vol/vol) glycerol, frozen in liquid nitrogen, and measured at beam lines 5.0.1 and 5.0.2 of the Advanced Light Source at Lawrence Berkeley National Laboratory. For iodine soaks, in addition to glycerol, 1 μ L 4 M potassium iodide solution was added to the drop, and crystals were frozen within 5 min.

Structure Determination and Analysis.

Even at 1.0 Å wavelength, the anomalous signal from the iodine was large enough to locate four sites using hkl2map/SHELX C/D/E^[212]. Initial density was obtained using phenix.autosol, and buccaneer successfully built an initial model of 595 residues, which was then manually corrected in Coot combined with refinement runs with phenix.refine^[194,213,214]. The sequence logo was generated at <http://weblogo.berkeley.edu/logo.cgi>^[215] from an alignment of 71 known FRP sequences.

Protein Purification and Activity Assays.

The isolation of the short His-tagged FRP from *E. coli* strain BL21 (DE3) to activity assays is described in^[86]. Briefly, protein expression was induced at an OD₆₀₀ of 0.4 by adding 1 mM isopropylthio-β-d-galactoside and 2% (vol/vol) ethanol. After 12 h of growth at 20 °C, the cells were harvested and lysed by French Press. The supernatant was loaded on a column of Ni-ProBond resin (Invitrogen). The FRP was eluted with 300 mM imidazole and dialyzed during 48 h against 40 mM TrisHCl at pH 8.0. Purity of FRP was checked by SDS/PAGE on 17% (wt/vol) polyacrylamide/2 M urea in a Tris/Mes system. The OCP_r to OCP_o conversion in the absence or presence of FRP was monitored in a Specord S600 (Analytik Jena) spectrophotometer at 8 °C. The OCP was previously photoconverted to the red form by 5 min illumination with 5,000 *mu*mol photons m⁻² s⁻¹ white light at 8 °C.

Construction of the Synechocystis PCC 6803 Strain Overexpressing the His-Tagged C-Terminal Domain of the OCP.

To obtain a strain overexpressing a His6-tagged C-terminal domain of the OCP, a plasmid containing the slr1963 gene under the control of the psbA2 promoter was used. The construction of this plasmid is described in ref.^[97]. The deletion of the nucleotides coding for the first 169 aa (N-terminal domain) of the OCP was obtained by site-directed mutagenesis using the Quickchange XL site-directed mutagenesis kit (Stratagene) and synthetic primers (Table S5). The plasmid obtained was used to transform WT cells. In the strains overexpressing the C-terminal domain, the original WT OCP was suppressed by partial deletion of the slr1963 gene as described in ref.^[98]. The His-tagged C-terminal domain was isolated by Nickel affinity as described in^[97]. The isolated protein did not bind any chromophore.

Coimmunoprecipitation.

Anti-FRP antibodies (Covalab), FRP proteins, and C- or N-terminal OCP domains were incubated at 4 °C overnight. The Sepharose-protein A beads (Sigma) were blocked by incubating them in the presence of 5% BSA overnight. The mixed proteins were then incubated with the blocked Sepharose beads for 2.5 h. The beads were washed with a buffer containing 1% N-β-dodecyl maltoside to remove unbound proteins. The proteins attached to the anti-FRP antibodies were eluted with 2% (wt/vol) SDS buffer and loaded on an SDS/PAGE gel. In the control samples, the FRP protein is absent. Western blot using anti-OCP antibody was performed.

Docking Calculations.

The protein structure of the OCP was modified to include the loop region from amino acids T164 to K170, which is missing in the available structure (PDB ID code 3MG1). Protein structures (OCP : 3MG1 mod; FRP : this structure) were energy-minimized using the rosetta-relax application before the docking step. Then, docking was performed using Rosetta dock^[208]. Unconstrained docking simulations were performed for 100,000 runs, whereas refinement runs were executed for 20,000 runs. The best solutions were chosen based on their Rosetta score and surface complementarity (scripts are in SI Methods) and analyzed for biological significance by hand.

PDB ID Codes.

PDB ID codes are 4JDX for the WT FRP protein and 4JDQ for the R60K mutant.

Acknowledgments

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Supplementary material

Rosetta (Version 3.4) Docking Scripts.
 Executable: docking_protocol
 Full docking flags:
 randomize1
 randomize2
 spin

ex1
 ex2aro
 Refinement docking flags:
 dock_pert 5 8
 ex1
 ex2aro

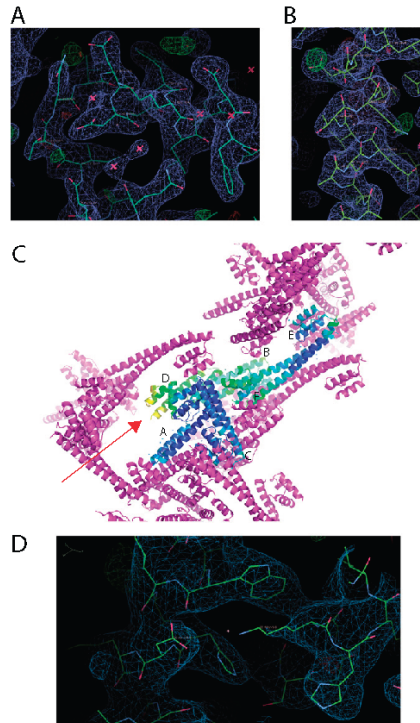


Fig. 51. 2Fo-Fc electron density at 1.5 r.m.s.d. (light blue), Fo-Fc electron density at 3.5 r.m.s.d. (green, positive; red, negative), and the atomic model showing (A) the loop region of chain A (residues 42–47) and (B) the same region in chain E, which is in a helical conformation, (C) B-factor distribution of six fluorescence recovery protein (FRP) chains in the asymmetric unit (rainbow coloring) ranging from 36 to 219 (dark blue to orange) as well as the surrounding symmetry-related chains (magenta). The small crosses are water molecules that were built close to better-ordered parts of the model (shown in blue here). The red arrow indicates a flexible region in chain D with only weak electron density and therefore, high B factors. (D) Electron density of R60K mutant centered on K60, which shows weak side chain density.

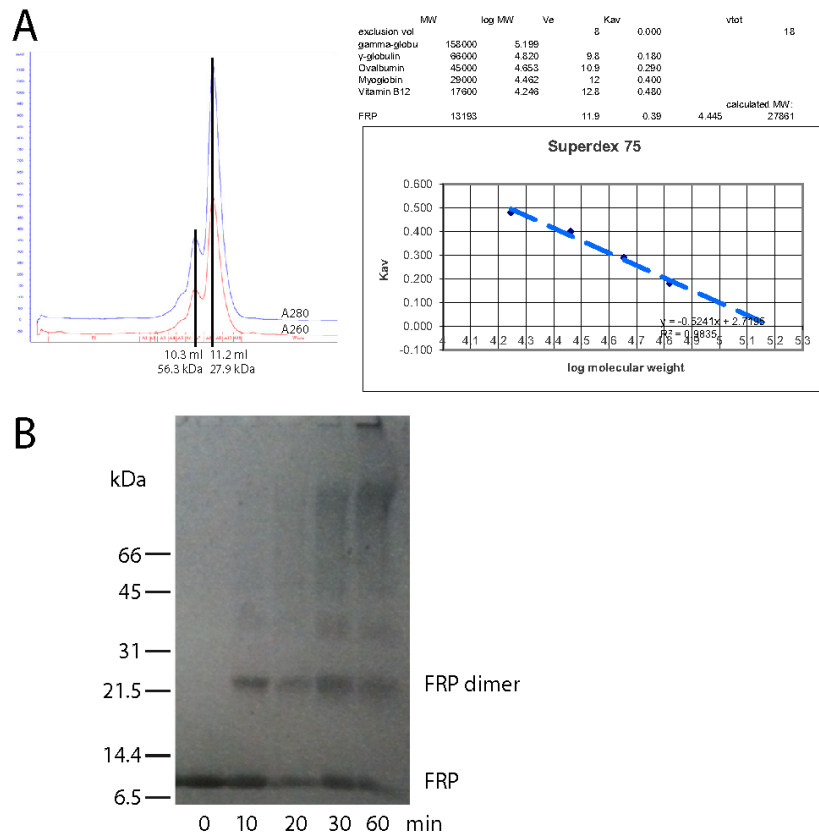


Fig. 52. (A) Size exclusion chromatography of the FRP with calculated molecular mass values and calibration samples. (B) SDS/PAGE analysis of a glutaraldehyde cross-linking experiment using the vapor diffusion method; the positions of the FRP and FRP dimer are indicated on the gel as well as cross-linking times.

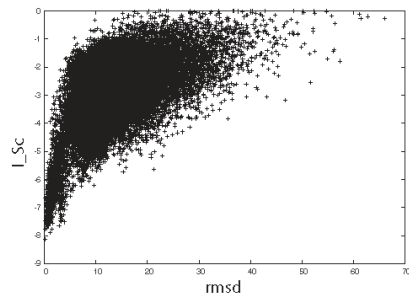


Fig. 53. Surface energy vs. rmsd plot of a docking simulation of the C-terminal domain of orange carotenoid protein (OCP) containing 3'-hydroxyechinenone and full-length FRP dimer. rmsd is from the starting model before refinement.

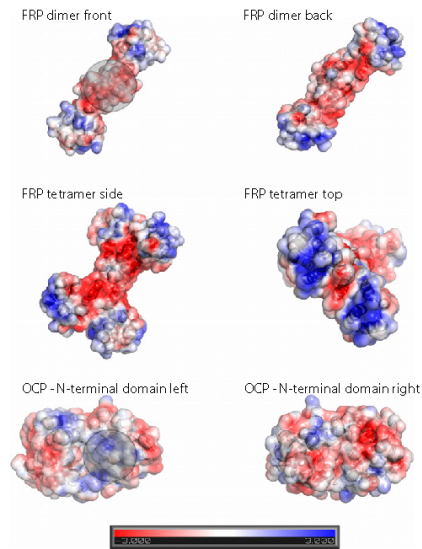


Fig. 54. Electrostatics of full-length FRP dimer and tetramer forms as well as the OCP, with the proposed interaction site marked with a shaded circle. The colors represent potential values, with red at -3 , white at 0 , and blue at 3 kT/e.

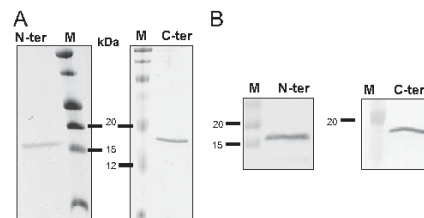


Fig. 55. Purified red carotenoid protein (RCP; N-terminal domain) and C-terminal OCP domain. (A) Coomassie Blue-stained SDS/PAGE of N-terminal OCP domain (RCP; lane N-ter) and C-terminal OCP domain (lane C-ter) and molecular mass marker (lane M). This SDS gel shows the purity of the N- and C-terminal polypeptides used in the coimmunoprecipitation experiment. (B) Western blot analysis. Detection of the RCP and the C-terminal domain using the OCP antibody used for the Western blot of the coimmunoprecipitation experiment. Lane M, molecular mass marker.

Table S1. All known OCP and FRP genes

| gene_oid | Locus tag | Genome |
|------------|------------------------|--|
| FRPs | | |
| 646569345 | Ava_3842 | <i>Anabaena variabilis</i> ATCC 29413 |
| 643171069 | AmxDRAFT_3432 | <i>Arthrospira maxima</i> CS-328 |
| 2507452283 | SPLC1_S542690 | <i>Arthrospira platensis</i> C1 (Draft2 circular genome) |
| 650388689 | NIES39_N00730 | <i>Arthrospira platensis</i> NIES-39 |
| 646132063 | AplaP_010100022156 | <i>Arthrospira platensis</i> Paraca |
| 648385444 | APCC8_010100001194 | <i>Arthrospira</i> sp. PCC 8005 |
| 2504093383 | Cal6303_0404 | <i>Calothrix</i> sp. PCC 6303 |
| 2504097406 | Cal6303_4389 | <i>Calothrix</i> sp. PCC 6303 |
| 2507478830 | Cal7103DRAFT_00057330 | <i>Calothrix</i> sp. PCC 7103 |
| 2505786884 | Chr6712_3123 | <i>Chroococcidiopsis</i> sp. PCC 6712 |
| 2510724890 | CWATWH0003_3605 | <i>Crocospaera watsonii</i> WH 0003 |
| 2503747457 | Cyan10605_3179 | <i>Cyanobacterium aponinum</i> PCC 10605 |
| 2502183528 | CYJSC1_DRAFT_13620 | <i>Cyanobacterium</i> sp JSC-1 |
| 2503365960 | Cyast_0565 | <i>Cyanobacterium stanieri</i> PCC 7202 |
| 2508551229 | Cyagr_0841 | <i>Cyanobium gracile</i> PCC 6307 |
| 647590961 | CPCC7001_233 | <i>Cyanobium</i> sp. PCC 7001 |
| 2507501817 | Cy51472_2676 | <i>Cyanothece</i> sp. BH63E, ATCC 51472 |
| 641676131 | cce_1648 | <i>Cyanothece</i> sp. BH68, ATCC 51142 |
| 640623660 | CY0110_09682 | <i>Cyanothece</i> sp. CCY 0110 |
| 643482686 | PCC7424_4468 | <i>Cyanothece</i> sp. PCC 7424 |
| 648189538 | Cyan7822_4644 | <i>Cyanothece</i> sp. PCC 7822 |
| 647107174 | CRC_02827 | <i>Cylindrospermopsis raciborskii</i> CS-505 |
| 2509552690 | Dacsa_0304 | <i>Dactylococcopsis salina</i> PCC 8305 |
| 2517062860 | PCC9339DRAFT_03718 | <i>Fischerella</i> sp. PCC 9339 |
| 2512976042 | Fis9431DRAFT_0370 | <i>Fischerella</i> sp. PCC 9431 |
| 2516146007 | FIS9605DRAFT_03012 | <i>Fischerella</i> sp. PCC 9605 |
| 2510102424 | Gei7105DRAFT_3679 | <i>Geitlerinema</i> sp. PCC 7105 |
| 2509876956 | Syn6308DRAFT_3663 | <i>Geminocystis herdmanii</i> PCC 6308 |
| 2508646074 | GLO73106DRAFT_00023270 | <i>Gloeocapsa</i> sp. PCC 73106 |
| 2503636725 | PCC7418_1998 | <i>Halotheca</i> sp. PCC 7418 |
| 2509802836 | LepboDRAFT_2178 | <i>Leptolyngbya boryana</i> PCC 6306 |
| 2517693485 | LEP6406DRAFT_2943 | <i>Leptolyngbya</i> sp. PCC 6406 |
| 2509840188 | Lepto7375DRAFT_0201 | <i>Leptolyngbya</i> sp. PCC 7375 |
| 2503887630 | Lepto7376_2016 | <i>Leptolyngbya</i> sp. PCC 7376 |
| 640019822 | L8106_29205 | <i>Lyngbya</i> sp. CCY 8106 |
| 647569609 | MC7420_3635 | <i>Microcoleus chthonoplastes</i> PCC 7420 |
| 641535876 | MAE_18920 | <i>Microcystis aeruginosa</i> NIES-843 |
| 2511040431 | MICAP_02326 | <i>Microcystis aeruginosa</i> PCC 7806 |
| 2509775800 | Lepto7104DRAFT_3021 | <i>Nodosilinea nodulosa</i> PCC 7104 |
| 2503740440 | Nos7107_1803 | <i>Nostoc</i> sp. PCC 7107 |
| 637233535 | all3148 | <i>Nostoc</i> sp. PCC 7120 |
| 2509809328 | Nos7524_1759 | <i>Nostoc</i> sp. PCC 7524 |
| 2509709058 | Pleur7313DRAFT_03046 | <i>Pleurocapsa</i> sp. PCC 7319 |
| 2509710490 | Pleur7313DRAFT_04478 | <i>Pleurocapsa</i> sp. PCC 7319 |
| 638115393 | sync_1805 | <i>Prochlorococcus</i> sp. CC9311 |
| 637771145 | Syncc9902_0971 | <i>Prochlorococcus</i> sp. CC9902 |
| 640543280 | SynWH7803_0927 | <i>Prochlorococcus</i> sp. WH 7803 |
| 637445483 | SYNW1369 | <i>Prochlorococcus</i> sp. WH8102 |
| 2507089991 | Pse6802_4605 | <i>Pseudanabaena</i> sp. PCC 6802 |
| 2504681714 | Pse7367_3252 | <i>Pseudanabaena</i> sp. PCC 7367 |
| 2504584270 | Pse7429DRAFT_1147 | <i>Pseudanabaena</i> sp. PCC 7429 |
| 647108517 | CRD_01082 | <i>Raphidiopsis brookii</i> D9 |
| 2506609769 | Spi6313_2120 | <i>Spirulina major</i> PCC 6313 |
| 2506598110 | Spi9445_1012 | <i>Spirulina subsalsa</i> PCC 9445 |
| 2503798908 | Sta7437_0841 | <i>Stanieria cyanosphaera</i> PCC 7437 |
| 639885019 | BL107_14115 | <i>Synechococcus</i> sp. BL107 |
| 650129106 | SCB01_010100007700 | <i>Synechococcus</i> sp. CB0101 |
| 650132129 | SCB02_010100007628 | <i>Synechococcus</i> sp. CB0205 |
| 2509429363 | Syn6312_1322 | <i>Synechococcus</i> sp. PCC 6312 |
| 641612818 | SYNPCC7002_A2808 | <i>Synechococcus</i> sp. PCC 7002 |
| 647576599 | S7335_1726 | <i>Synechococcus</i> sp. PCC 7335 |
| 647581106 | S7335_121 | <i>Synechococcus</i> sp. PCC 7335 |

Table S1. Cont.

| gene_cid | Locus tag | Genome |
|-----------------------------|------------------------|--|
| 640546947 | SynRCC307_1994 | <i>Synechococcus</i> sp. RCC 307 |
| 638962819 | RS9917_00682 | <i>Synechococcus</i> sp. RS9917 |
| 2507491448 | Syn8016_0173 | <i>Synechococcus</i> sp. WH 8016 |
| 638957797 | WH5701_04000 | <i>Synechococcus</i> sp. WH5701 |
| 639020529 | WH7805_01192 | <i>Synechococcus</i> sp. WH7805 |
| 637011061 | slr1964 | <i>Synechocystis</i> sp. PCC 6803 |
| 651079359 | SYNGTS_1614 | <i>Synechocystis</i> sp. PCC 6803 |
| 2514153953 | SYNPCCN_1613 | <i>Synechocystis</i> sp. PCC 6803, GT-I |
| 2514150736 | SYNPCCP_1613 | <i>Synechocystis</i> sp. PCC 6803, PCC-N |
| OCPs that co-occur with FRP | | |
| 646569346 | Ava_3843 | <i>Anabaena variabilis</i> ATCC 29413 |
| 643171068 | AmaxDRAFT_3431 | <i>Arthrospira maxima</i> CS-328 |
| 2507452282 | SPLC1_5542680 | <i>Arthrospira platensis</i> C1 (Draft2 circular genome) |
| 650388688 | NIES39_N00720 | <i>Arthrospira platensis</i> NIES-39 |
| 646130639 | AplaP_010100014838 | <i>Arthrospira platensis</i> Paraca |
| 648385445 | APCC8_010100001199 | <i>Arthrospira</i> sp. PCC 8005 |
| 2504093384 | Cal6303_0405 | <i>Calothrix</i> sp. PCC 6303 |
| 2504097405 | Cal6303_4388 | <i>Calothrix</i> sp. PCC 6303 |
| 2507478831 | Cal7103DRAFT_00057340 | <i>Calothrix</i> sp. PCC 7103 |
| 2505785423 | Chr6712_1668 | <i>Chroococciopsis</i> sp. PCC 6712 |
| 2505786883 | Chr6712_3122 | <i>Chroococciopsis</i> sp. PCC 6712 |
| 2510724891 | CWATWH0003_3606 | <i>Crocospaera watsonii</i> WH 0003 |
| 2503747458 | Cyan10605_3180 | <i>Cyanobacterium aponinum</i> PCC 10605 |
| 2502181508 | CYJSC1_DRAFT_30550 | <i>Cyanobacterium</i> sp. JSC-1 |
| 2503367326 | Cyast_1904 | <i>Cyanobacterium stanieri</i> PCC 7202 |
| 2508551231 | Cyagr_0843 | <i>Cyanobium gracile</i> PCC 6307 |
| 647590963 | CPCC7001_1352 | <i>Cyanobium</i> sp. PCC 7001 |
| 2507501818 | Cy51472_2677 | <i>Cyanothece</i> sp. BH63E, ATCC 51472 |
| 641676132 | cce_1649 | <i>Cyanothece</i> sp. BH68, ATCC 51142 |
| 640623659 | CY0110_09677 | <i>Cyanothece</i> sp. CCY 0110 |
| 643483577 | PCC7424_5565 | <i>Cyanothece</i> sp. PCC 7424 |
| 643482685 | PCC7424_4467 | <i>Cyanothece</i> sp. PCC 7424 |
| 648189539 | Cyan7822_4645 | <i>Cyanothece</i> sp. PCC 7822 |
| 647107175 | CRC_02828 | <i>Cylindrospermopsis raciborskii</i> CS-505 |
| 2509552689 | Dacsa_0303 | <i>Dactylococopsis salina</i> PCC 8305 |
| 2517065161 | PCC9339DRAFT_06021 | <i>Fischerella</i> sp. PCC 9339 |
| 2517062861 | PCC9339DRAFT_03719 | <i>Fischerella</i> sp. PCC 9339 |
| 2512976041 | Fis9431DRAFT_0369 | <i>Fischerella</i> sp. PCC 9431 |
| 2516145913 | FIS9605DRAFT_02916 | <i>Fischerella</i> sp. PCC 9605 |
| 2516146008 | FIS9605DRAFT_03013 | <i>Fischerella</i> sp. PCC 9605 |
| 2510102423 | Gei7105DRAFT_3678 | <i>Geitlerinema</i> sp. PCC 7105 |
| 2509876957 | Syn6308DRAFT_3664 | <i>Geminocystis herdmanii</i> PCC 6308 |
| 2508646075 | GLO73106DRAFT_00023280 | <i>Gloeocapsa</i> sp. PCC 73106 |
| 2503636727 | PCC7418_2000 | <i>Halotheca</i> sp. PCC 7418 |
| 2509802837 | LepboDRAFT_2179 | <i>Leptolyngbya boryana</i> PCC 6306 |
| 2517693730 | LEP6406DRAFT_3188 | <i>Leptolyngbya</i> sp. PCC 6406 |
| 2509841145 | Lepto7375DRAFT_1162 | <i>Leptolyngbya</i> sp. PCC 7375 |
| 2509840187 | Lepto7375DRAFT_0200 | <i>Leptolyngbya</i> sp. PCC 7375 |
| 2503887628 | Lepto7376_2014 | <i>Leptolyngbya</i> sp. PCC 7376 |
| 640019823 | L8106_29210 | <i>Lyngbya</i> sp. CCY 8106 |
| 647569608 | MC7420_3617 | <i>Microcoleus chthonoplastes</i> PCC 7420 |
| 641535875 | MAE_18910 | <i>Microcystis aeruginosa</i> NIES-843 |
| 2511040432 | MICAP_02327 | <i>Microcystis aeruginosa</i> PCC 7806 |
| 2509778918 | Lepto7104DRAFT_6139 | <i>Nodosilinea nodulosa</i> PCC 7104 |
| 2509775801 | Lepto7104DRAFT_3022 | <i>Nodosilinea nodulosa</i> PCC 7104 |
| 2503742212 | Nos7107_3530 | <i>Nostoc</i> sp. PCC 7107 |
| 2503740439 | Nos7107_1802 | <i>Nostoc</i> sp. PCC 7107 |
| 637233536 | all3149 | <i>Nostoc</i> sp. PCC 7120 |
| 2509809327 | Nos7524_1758 | <i>Nostoc</i> sp. PCC 7524 |
| 2509707823 | Pleur7313DRAFT_01811 | <i>Pleurocapsa</i> sp. PCC 7319 |
| 2509709056 | Pleur7313DRAFT_03044 | <i>Pleurocapsa</i> sp. PCC 7319 |
| 2509710491 | Pleur7313DRAFT_04479 | <i>Pleurocapsa</i> sp. PCC 7319 |

Table S1. Cont.

| gene_oid | Locus tag | Genome |
|-------------------------------|-------------------------|--|
| 638115391 | sync_1803 | <i>Prochlorococcus</i> sp. CC9311 |
| 637771147 | Syncc9902_0973 | <i>Prochlorococcus</i> sp. CC9902 |
| 640543282 | SynWH7803_0929 | <i>Prochlorococcus</i> sp. WH 7803 |
| 637445481 | SYNW1367 | <i>Prochlorococcus</i> sp. WH8102 |
| 2507089992 | Pse6802_4606 | <i>Pseudanabaena</i> sp. PCC 6802 |
| 2504681715 | Pse7367_3253 | <i>Pseudanabaena</i> sp. PCC 7367 |
| 2504584269 | Pse7429DRAFT_1146 | <i>Pseudanabaena</i> sp. PCC 7429 |
| 647108518 | CRD_01083 | <i>Raphidiopsis brookii</i> D9 |
| 2506609770 | Spi6313_2121 | <i>Spirulina major</i> PCC 6313 |
| 2506598109 | Spi9445_1011 | <i>Spirulina subsalsa</i> PCC 9445 |
| 2503798909 | Sta7437_0842 | <i>Stanieria cyanosphaera</i> PCC 7437 |
| 639885017 | BL107_14105 | <i>Synechococcus</i> sp. BL107 |
| 650129104 | SCB01_010100007690 | <i>Synechococcus</i> sp. CB0101 |
| 650132131 | SCB02_010100007638 | <i>Synechococcus</i> sp. CB0205 |
| 2509429361 | Syn6312_1320 | <i>Synechococcus</i> sp. PCC 6312 |
| 641612820 | SYNPCC7002_A2810 | <i>Synechococcus</i> sp. PCC 7002 |
| 647581102 | S7335_655 | <i>Synechococcus</i> sp. PCC 7335 |
| 647576600 | S7335_2791 | <i>Synechococcus</i> sp. PCC 7335 |
| 647581105 | S7335_487 | <i>Synechococcus</i> sp. PCC 7335 |
| 640546945 | SynRCC307_1992 | <i>Synechococcus</i> sp. RCC 307 |
| 638962821 | RS9917_00692 | <i>Synechococcus</i> sp. RS9917 |
| 2507491450 | Syn8016_0175 | <i>Synechococcus</i> sp. WH 8016 |
| 638957799 | WH5701_04010 | <i>Synechococcus</i> sp. WH5701 |
| 639020531 | WH7805_01202 | <i>Synechococcus</i> sp. WH7805 |
| 637011060 | slr1963 | <i>Synechocystis</i> sp. PCC 6803 |
| 651079358 | SYNGTS_1613 | <i>Synechocystis</i> sp. PCC 6803 |
| 2514153952 | SYNPCCN_1612 | <i>Synechocystis</i> sp. PCC 6803, GT-I |
| 2514150735 | SYNPCCP_1612 | <i>Synechocystis</i> sp. PCC 6803, PCC-N |
| 2517700291 | SYN7509DRAFT_4409 | <i>Synechocystis</i> sp. PCC 7509 |
| OCs without FRP in the genome | | |
| 2504135231 | Anacy_5536 | <i>Anabaena cylindrica</i> PCC 7122 |
| 2506493993 | Ana7108_4134 | <i>Anabaena</i> sp. PCC 7108 |
| 2505800904 | Cal7507_2634 | <i>Calothrix</i> sp. PCC 7507 |
| 2510436315 | Cha6605_0316 | <i>Chamaesiphon minutus</i> PCC 6605 |
| 2503615751 | Chro_5460 | <i>Chroococciopsis thermalis</i> PCC 7203 |
| 2504682464 | Cr9333_0045 | <i>Crinalium epipsammum</i> PCC 9333 |
| 2517647922 | A3MYDRAFT_2631 | <i>Cyanobacterium</i> sp. ESFC-1 |
| 2509769046 | CylstDRAFT_3006 | <i>Cylindrospermum stagnale</i> PCC 7417 |
| 2513240909 | DOLIC_03915 | <i>Dolichospermum circinale</i> ACBU02 |
| 2505767326 | FJSC11DRAFT_3982 | <i>Fischerella</i> sp. JSC-11 |
| 637461350 | glr3935 | <i>Gloeobacter violaceus</i> PCC 7421 |
| 637457419 | glr0050 | <i>Gloeobacter violaceus</i> PCC 7421 |
| 2503795025 | Glo7428_2455 | <i>Gloeocapsa</i> sp. PCC 7428 |
| 2517243596 | Mas10914DRAFT_4839 | <i>Mastigocladopsis repens</i> PCC 10914 |
| 2509781001 | Mic7126DRAFT_1752 | <i>Microchaete</i> sp. PCC 7126 |
| 2509433601 | Mic7113_1766 | <i>Microcoleus</i> sp. PCC 7113 |
| 2506348135 | MicvaDRAFT_3718 | <i>Microcoleus vaginatus</i> FGP-2 |
| 640027993 | N9414_13085 | <i>Nodularia spumigena</i> CCY9414 |
| 648050422 | Aazo_2542 | <i>Nostoc azollae</i> 0708 |
| 642604087 | Npun_R5_144 | <i>Nostoc punctiforme</i> PCC 73102 |
| 642599720 | Npun_R0404 | <i>Nostoc punctiforme</i> PCC 73102 |
| 2508875501 | Oscil6407DRAFT_00036220 | <i>Oscillatoria formosa</i> PCC 6407 |
| 2504089918 | Osc7112_3382 | <i>Oscillatoria nigro-viridis</i> PCC 7112 |
| 648859651 | OSCI_3650011 | <i>Oscillatoria</i> sp. PCC 6506 |
| 2509576538 | Ple7327_4656 | <i>Pleurocapsa</i> sp. PCC 7327 |
| 2509576549 | Ple7327_4667 | <i>Pleurocapsa</i> sp. PCC 7327 |
| 2510089267 | Riv7116_4121 | <i>Rivularia</i> sp. PCC 7116 |
| 2510090232 | Riv7116_5086 | <i>Rivularia</i> sp. PCC 7116 |
| 2508688806 | Syn7502_02006 | <i>Synechococcus</i> sp. PCC 7502 |
| 2517697059 | SYN7509DRAFT_1176 | <i>Synechocystis</i> sp. PCC 7509 |
| 2517700424 | SYN7509DRAFT_4542 | <i>Synechocystis</i> sp. PCC 7509 |

Because some of the genomes are still unfinished, there is a slight chance that there may be additional copies of FRP and OCP in some of those organisms.

Table S2. Data collection and refinement statistics

| | Native | R60K mutant | KI soak |
|---|----------------------------------|----------------------------------|----------------------------------|
| Data collection | | | |
| Space group | P4 ₁ 2 ₁ 2 | P4 ₁ 2 ₁ 2 | P4 ₁ 2 ₁ 2 |
| Cell dimensions | | | |
| <i>a</i> , <i>b</i> , <i>c</i> (Å) | 87.33, 87.33, 230.8 | 87.41, 87.41, 229.48 | 87.70, 87.70, 231.17 |
| α , β , γ (°) | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 |
| Resolution (Å) | 39–2.50 (2.57–2.50)* | 39–3.51 (3.70–3.51) | 40–2.92 (2.99–2.92) |
| <i>R</i> _{sym} (%) | 11.3 (75.1) | 26.6 (160.0) | 10.6 (69.4) |
| <i>I</i> / σ | 13.3 (1.51) | 8.6 (1.6) | 19.0 (2.45) |
| Completeness (%) | 99.7 (99.7) | 99.6 (98.0) | 99.7 (97.0) |
| Redundancy | 6.9 (7.2) | 13.6 (13.4) | 7.0 (4.8) |
| Refinement | | | |
| Resolution (Å) | 39–2.50 | 39–3.50 | |
| No. reflections | 31,597 | 20,893 | |
| <i>R</i> _{work} / <i>R</i> _{free} | 21.6/26.4 | 23.3/27.3 | |
| No. atoms | 9,504 | 4,673 | |
| Protein | 9,426 [†] | 4,673 | |
| Water | 78 | | |
| B factors | | | |
| Protein | 77.0 | 75.2 | |
| Water | 55.1 | | |
| rmsd | | | |
| Bond lengths (Å) | 0.002 | 0.004 | |
| Bond angles (°) | 0.469 | 0.695 | |

*Values in parentheses are for highest-resolution shell.

[†]Includes hydrogen atoms.**Table S3. Protein interfaces, surfaces, and assemblies (PISA) analysis of protein complexes in the FRP protein crystal**

| Chain-chain | Stable | Surface area (Å ²) | Buried area (Å ²) | ΔG_{int} (kcal/mol) | ΔG_{diss} (kcal/mol) |
|-------------|--------|--------------------------------|-------------------------------|-----------------------------|------------------------------|
| EFE'F' | Yes | 18,850 | 10,330 | −78.0 | 20.6 |
| BD | Yes | 12,020 | 1,970 | −17.2 | 6.7 |
| AC | Yes | 11,860 | 1,760 | −16.2 | 5.5 |

 ΔG_{diss} , free energy of assembly dissociation; ΔG_{int} , free energy gained on formation of assembly.**Table S4. Protein interfaces, surfaces, and assemblies (PISA) analysis of interfaces between protein chains in the FRP protein crystal**

| Interface analysis | No. of interface atoms/residues | Interface area (Å ²) | ΔiG (kcal/mol) | ΔiG <i>P</i> value |
|--------------------|---------------------------------|----------------------------------|------------------------|----------------------------|
| E–F | 184/43 | 1,697 | −23.8 | 0.338 |
| BD | 99/22 | 983 | −17.2 | 0.048 |
| AC | 87/21 | 880 | −16.2 | 0.055 |
| E–F' | 73/18 | 716 | −12.4 | 0.227 |

 ΔiG *P* value measures the probability of getting a lower than obtained ΔiG if interface atoms are picked randomly from a protein surface such as to amount to the observed interface. ΔiG , solvation free energy gain on formation of the interface.**Table S5. Primers used in this study**

| Primer name | Primer sequence |
|----------------------|---|
| FRP W50L (TGG → CTG) | 5'- GATCGATGATCTTCTGAACTCCATG-3' |
| FRP W50L reverse | 5'- CATGGAGTTTCAGAAAGATCATCGATC-3' |
| FRP W50F (TGG → TTC) | 5'- ATTACGCAGATCGATGATCTTTCAAACCTCCATGATTTTTGAGTGC-3' |
| FRP W50F reverse | 5'- GCACTCAAAAAATCATGGAGTTTGAAGATCATCGATCTGCGTAAT-3' |
| FRP H53L (CAT → CTG) | 5'- CTTTGAAACTCCTGAGTTTTTGGAGTG-3' |
| FRP H53L reverse | 5'- CACTCAAAAAATCCAGGAGTTTCCAAAG-3' |
| FRP D54L (GAT → CTG) | 5'- CTTTGAAACTCCATCTGTTTTTGGAGTG-3' |
| FRP D54L reverse | 5'- CACTCAAAAAACAGATGGAGTTTCCAAAG-3' |
| FRP D54E (GAT → GAA) | 5'- GAAACTCCATGAATTTTTGAGTGCAA-3' |
| FRP D54E reverse | 5'- TTGCACTCAAAAAATCATGGAGTTTC-3' |
| FRP R60L (CGC → CTG) | 5'- GAGTGCAAAAACAGCACGAAATGATGGC-3' |
| FRP R60L reverse | 5'- GCCATCAATTTCTGTGAGTTTTGCACTC-3' |
| FRP R60K (CGC → AAG) | 5'- CCATGATTTTTGAGTGC AAAAAGCACGAAATGATGGCAAGTACG-3' |
| FRP R60K reverse | 5'- CGTACTTCCATCAATTTCTGTGTTTTGCACTCAAAAAATCATGG-3' |
| FRP W9L (TGG → CTG) | 5'- GCCGAAAGCACCTCTGTCCCAAGCTG-3' |
| FRP W9L reverse | 5'- CAGCTTGGGACAGAGGTCTTCGGC-3' |
| For C-ter | 5'- CATTACCAATTGACTGTGCCAAACGCATCGCTGAGCCCGTA-3' |
| Rev C-ter | 5'- GGCAGAGTCAATGGTGAATGGCATATGTTATAATTC-3' |
| Pet M26HIS for | 5'- CATCATCATCATCATCACTTACAAACCCGCAAGCACCTTGGTCC-3' |
| Pet M26HIS rev | 5'- GTGATGATGATGATGATGCATGGTATATCTCCTTC-3' |

Mutated bases are marked with red.

La résolution de la structure de la FRP présentée dans le chapitre 5 a permis de modéliser un site d'interaction putatif entre l'OCP et la FRP. Les simulations ont été réalisées en prenant en compte le site d'actif de la FRP et le domaine C-terminal de l'OCP uniquement^[114]. Si on ajoute le domaine N-terminal au modèle, on observe un chevauchement du domaine N-terminal et de l'homodimère FRP. Cependant, d'après notre modèle de travail l'OCP rouge est sous forme ouverte, et donc il n'y aura pas de chevauchement entre domaine N-terminal et la FRP car la FRP s'attache à l'OCP rouge. Au retour à la forme orange, la FRP se détache de l'OCP.

Ce détachement pourrait impliquer le domaine N-terminal qui éjecterait la FRP du domaine C-terminal lorsque l'OCP se referme. Les résidus T218, D220, R229, V232, N236, D262, F264 et F299 de l'OCP localisés au niveau du feuillet β du domaine C-terminal de l'OCP seraient impliqués dans l'interaction avec la FRP selon le modèle proposé par Markus Sutter^[114] (Figure 7.1). Ces résidus sont protubérants et très conservés au sein des différentes séquences codantes d'OCP chez les cyanobactéries.

En étudiant la structure, il m'a semblé que les résidus E261 et N293 pourraient aussi être impliqués dans l'interaction OCP-FRP (Figure 7.1). Ces résidus ne sont pas apparus dans le modèle prédictif car masqués par le bras N-terminal. Considérant les données présentées dans le chapitre 4 et d'après l'article de Liu et al.^[179], le bras ne chevauche pas le domaine C-terminal quand l'OCP est sous forme rouge, et les résidus Q261, N293 et F299 sont exposés au solvant dans la forme rouge de l'OCP.

Une étude cinétique a récemment proposé que la transition de l'OCP orange vers l'OCP rouge pourrait impliquer la cis-trans isomérisation d'une proline^[112]. Ils proposent que l'isomérisation puisse avoir lieu au niveau de la liaison entre Q224-P225 ou P225-P226. La simulation place la FRP proche de ces résidus. L'activité de la FRP étant inconnue, Ryan Leverenz du laboratoire de Prof Cheryl Kerfeld a proposé que cette dernière puisse faciliter l'isomérisation, accélérant le retour à la forme orange (communication personnelle).

Au début de ma thèse, en 2012, le système de production d'OCP modifiées chez *E.coli* n'avait pas encore été mis au point. Aussi, les premières OCPs modifiées ont été isolées chez *Synechocystis*. Les premières OCPs modifiées ne donnant pas les résultats escomptés, j'ai temporairement arrêté cette problématique. Le protocole de purification d'OCP chez *E.coli* présenté dans le chapitre 2 m'aura permis par la suite de créer un plus grand nombre d'OCP modifiées en un temps plus restreint. Les constructions E261K, N293K ont été réalisées uniquement chez *Synechocystis*. Les constructions D220K, D220L, R229E et R229L ont été aussi réalisées chez *Synechocystis*. Ces OCPs modifiées sont celles qui ont donné les résultats les plus prometteurs. Ainsi, je les ai également isolées chez *E.coli* afin de pouvoir les comparer aux OCPs modifiées qui ont été produites uniquement chez *E.coli*. Dans un second temps, les modifications suivantes ont été construites uniquement chez *E. coli* : N236K, D262K, F299R, ainsi que P225V, P226V et P225VP226V dans le cadre de l'activité potentielle proline-isomérase de la FRP.

Matériel et méthodes

Les mutations ponctuelles ont été réalisées par mutagenèse dirigée en utilisant le kit Quickchange SL site-directed mutagenesis (Stratagene) et des primers synthétiques (Annexe I). La présence de la modification de séquence souhaitée et l'absence de modifications inopportunes sont vérifiées par séquençage. Pour les OCPs modifiées produites chez *Synechocystis*, la mutagenèse a été réalisée en utilisant un plasmide, précédemment décrit^[97], contenant le gène de l'OCP (*SLR1963*) additionné d'un HisTag en C-terminal sous le contrôle du promoteur *psbA2*, comme matrice.

Le plasmide est ensuite utilisé pour transformer la cyanobactérie *Synechocystis* Δ CrtR par recombinaison homologue. La copie endogène de l'OCP est ensuite remplacée par un cassette de résistance spectinomycine/streptomycine comme décrit dans (Wilson et al., 2012). Pour les OCP modifiées produites chez *E.coli*, la mutagenèse a été réalisée en utilisant le plasmide pCDF-OCPsynNtag décrit dans le

Synechocystis Δ CrtR ne produit pas de 3'hECN mais seulement de l'ECN (l'enzyme CrtR permet de transformer l'ECN en 3'hECN). L'OCP liant l'ECN a les mêmes propriétés que celle liant le 3'hECN^[132].

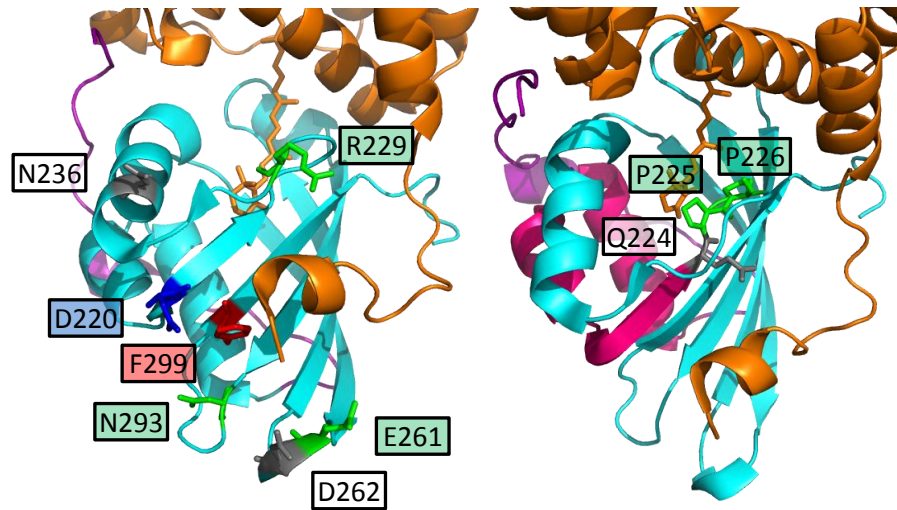


Figure 7.1 – Modifications ponctuelles de l'OCP présentées dans le chapitre 6
 Représentation de la structure tridimensionnelle de l'OCP. Le domaine C-terminal est coloré en cyan, le bras N-terminal en rouge et le caroténoïde en stick oranges. Les résidus (A) potentiellement impliqués dans l'interaction avec la FRP ou (B) pouvant subir une isomérisation sont représentés en sticks. (B) la région comprise entre le linker et la Q224 est représentée en bleu.

chapitre 2. Le plasmide est ensuite utilisé pour transformer des cellules de *E.coli* BL21-Gold (Agilent Technologies) dans lesquelles l'OCP sera produite tel que décrit dans le chapitre 2. Les OCPs ont été purifiées sur colonne His-Tag tel que décrit dans les chapitre 2 (*E.coli*) et 5 (*Synechocystis*), puis caractérisées. Les spectres d'absorbance et les cinétiques de photoconversion (sous une illumination avec 5000 $\mu\text{moles de photons} * m^{-2} * sec^{-1}$ de lumière blanche) et de récupération à l'obscurité ont été mesurées avec un spectrophotomètre SpecordS600 (Analyticjena) à 9°C. Le ratio OCP : FRP est de 2 : 1 dans toutes les expériences présentées dans ce chapitre. La mesure au cours du temps du rendement de fluorescence des PBs est réalisée à l'aide d'un fluorimètre PAM (101/102/103-PAM; Walz). La reconstitution du mécanisme de photoprotection est réalisée tel que décrit dans Gwizdala et al., 2011^[81]. Brièvement, la concentration en PBs utilisée est de 0,012 μM dans un tampon phosphate à 0.5M (pH=7,5) à 23°C. Le quenching de fluorescence est induit par l'OCP (ratio OCP : PBs de 40 : 1) préconvertie sur la glace par 5000 $\mu\text{moles de photons} * m^{-2} * sec^{-1}$. Lors de la cinétique de quenching, une forte lumière bleue verte dirigée vers la cuvette de mesure (900 $\mu\text{moles de photons} * m^{-2} * sec^{-1}$; 400-550nm) maintient l'OCP sous forme rouge. Lors de la cinétique de récupération, la lumière est éteinte et la FRP ajoutée au complexe OCP-PBs (ratio FRP : OCP de 32 : 1)

L'OCP est photoconvertie sur la glace afin de diminuer la vitesse de retour à la forme orange.

Le tampon utilisé contient 0.5 MK contre 0.8 MK habituellement^[81]. Cette faible concentration en phosphate facilite le détachement de l'OCP des PBs et exacerbe l'action de la FRP.

7.0.1 Interaction FRP-OCP Photoactivation et recovery en présence et absence de FRP

La figure 7.2 présente les cinétiques de photoconversion des OCPs modifiées dont l'interaction avec la FRP est peu ou pas diminuée. Les OCPs N236K et D262K se convertissent à la forme rouge aussi vite que l'OCP non modifié (A). Les deux OCPs dont la R229 est modifiée, ainsi que l'OCP E261K se convertissent plus lentement (B et D). En revanche, l'OCP N293K se converti plus vite que l'OCP non modifiée à la forme rouge (C).

A l'obscurité, le retour à la forme orange des OCPs N236K, N293K et E261K est semblable au témoin (D et F). Les cinétiques de reconversion des OCPs D262K, R229L et R229E sont plus rapides (B et D). Vis-à-vis de l'action de la FRP, l'OCP D262K est reconvertie en forme orange aussi vite que l'OCP WT (D). Le retour à la forme orange des OCPs N236K, N293K, E261K et R229L en présence de FRP est à

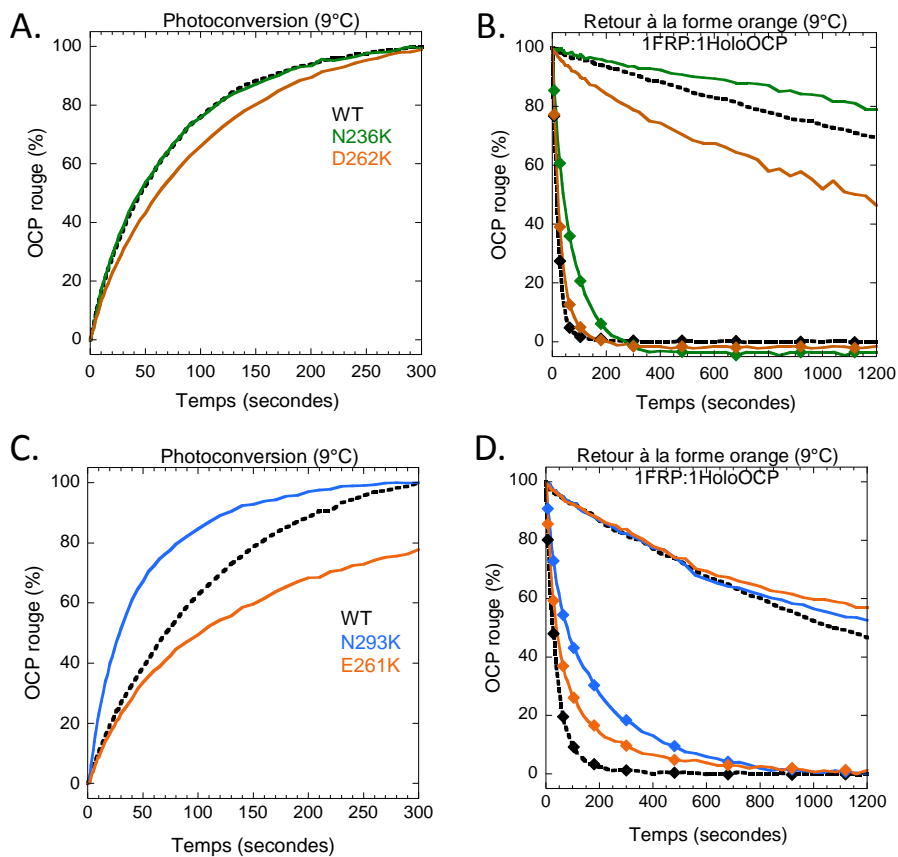


Figure 7.2 – Photoactivation et retour à la forme orange à l’obscurité des OCPs modifiées dont l’action de la FRP est pas ou peu impactée

Pannel de gauche : Cinétique de photoactivation par une forte lumière blanche ($5000 \mu\text{moles de photons} * m^{-2} * sec^{-1}$) des OCP WT en pointillés noirs et (A) N236K en vert et D262K en ocre (isolés chez *E.coli*) ou (C) N293K en bleu et E261K en orange (isolés chez *Synechocystis*) à 9°C

Pannel de droite : Cinétique de retour à la forme orange en en absence (aucun symbole) présence de 1FRP :1OCP (losanges) à 9°C. Mêmes couleurs que dans (A) et (C).

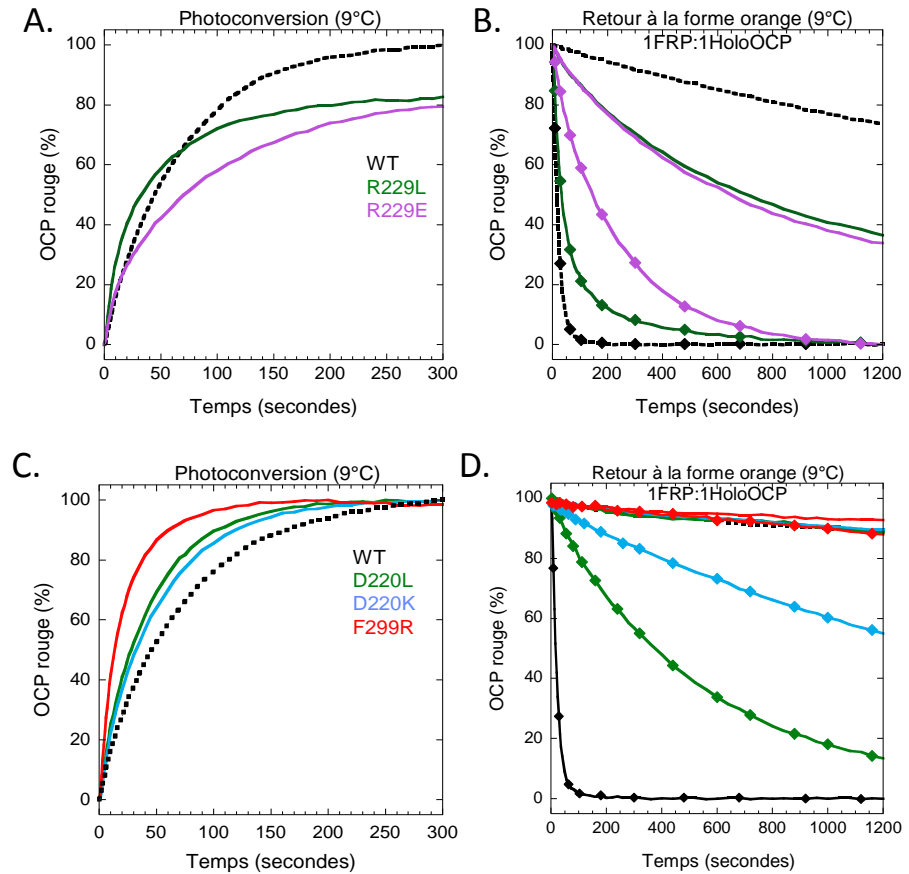


Figure 7.3 – Photoactivation et retour à la forme orange à l’obscurité des OCPs modifiées dont l’action de la FRP est impactée

Pannel de gauche : Cinétique de photoactivation par une forte lumière blanche ($5000 \mu\text{moles de photons} * m^{-2} * sec^{-1}$) des OCP WT en pointillés noirs et (A) R229L en vert et R229E en violet ou (C) D220L en vert, D220K en bleu et F299R en rouge à 9°C

Pannel de droite : Cinétique de retour à la forme orange en absence (aucun symbole) ou présence de 1FRP :1OCP (losanges) à 9°C . Mêmes couleurs que dans (A) et (C). Toutes les OCPs dont les cinétiques sont présentées dans cette figure ont été isolés chez *E.coli*.

peine plus lent qu’avec l’OCP WT (D et F). En revanche, l’action de la FRP sur l’OCP R229E est significativement diminuée.

La figure 7.3 présente les cinétiques de photoconversion des OCPs dont les résidus D220 ou F299 sont modifiés, et avec qui l’activité de la FRP est fortement impactée. La cinétique de photoactivation des OCPs dont l’aspartate n°220 est modifiée est identique au WT. En revanche, quand la phenylalanine 299 est modifiée, la cinétique de photoactivation est plus rapide que le WT. Cette photoactivation plus rapide concorde avec le fait que le retour à la forme orange de F299 est beaucoup plus lent que le WT à l’obscurité (E et F).

A l’obscurité, l’OCP dont l’aspartate 220 est modifiée présente une cinétique de retour à la forme orange similaire au WT.

L’action de la FRP est fortement diminuée lorsque l’aspartate 220 est modifiée et nulle chez le mutant F299R. Dans les deux cas la présence de la FRP accélère très peu la reconversion. En résumé, les résidus D220 et F299 sont les résidus les plus importants pour l’activité de la FRP. R229 est également un bon candidat mais les données demandent à être étayées.

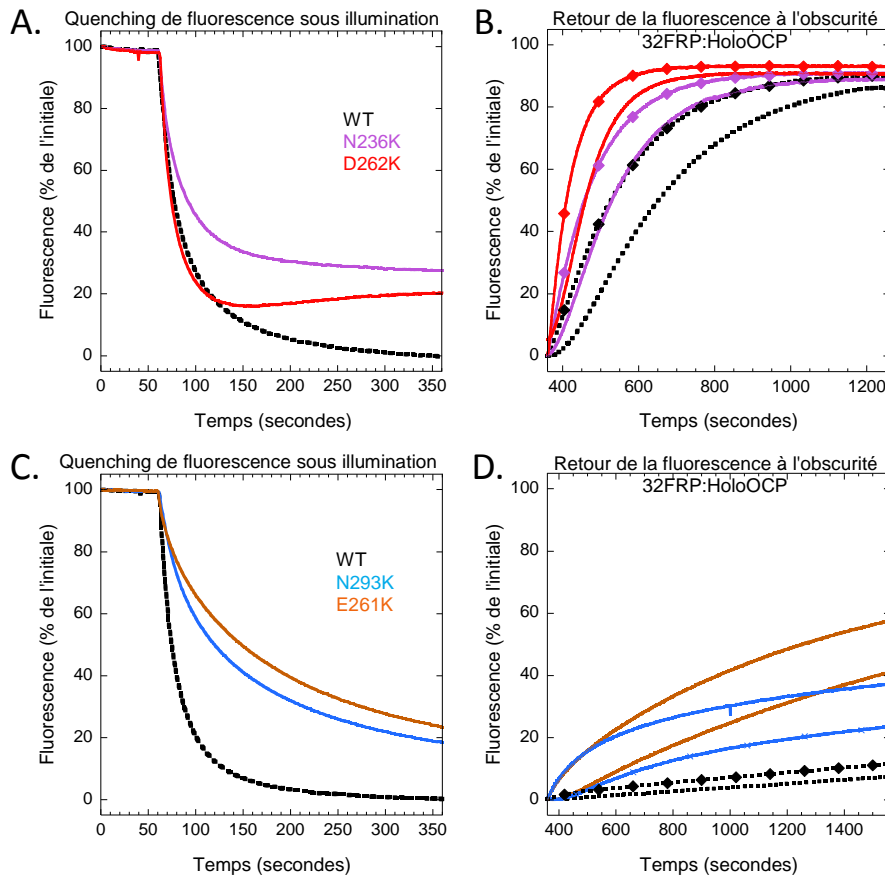


Figure 7.4 – Reconstitution *in vitro* du quenching de fluorescence par les OCP modifiées dont l'action de la FRP est pas ou peu impactée

Pannel de gauche : Quenching de fluorescences à 23°C des PBs induit par les OCP WT en pointillés noirs et (A) N236K en violet et D262K en rouge ou (C) N293K en cyan et E261K en ocre pré-illuminés 5 minutes par une forte lumière blanche ($5000 \mu\text{moles de photons} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$) à 4°C. Les PBs sont illuminés avec une forte lumière bleue-verte puis l'OCP rouge est ajoutée à 60 secondes d'illumination. Ratio 40 holoOCP :1PBs, tampon K-phosphate 0,5M.

Pannel de droite : Après 5 minutes d'illumination, la lumière est éteinte et la récupération de la fluorescence est suivi au cours du temps en absence (aucun symbole) ou présence de 32FRP :1OCP (losanges). Mêmes couleurs que dans (A) et (C).

7.0.2 Interaction OCP-PBS : Diminution de la fluorescence des PBs induite par des OCPs

J'ai ensuite utilisé les OCPs modifiées dans le système de reconstitution *in vitro* afin de tester si l'action de la FRP était bien diminuée. Le quenching de la fluorescence des PBs sous illumination dépend de la capacité de l'OCP de s'attacher aux PBs. Ainsi, une OCP dont la force d'attachement aux PBs est plus faible que le WT se traduit par une diminution moins importante de la fluorescence des PBs que celle induite par l'OCP WT^[81]. Au retour à l'obscurité, l'OCP se détache des PBs et la fluorescence augmente. La vitesse de détachement d'une telle OCP est plus rapide que le contrôle^[81]. Nous avons observé que la plupart des OCPs modifiées (D220, E261, D262, N236 et N293) voient leur capacité d'attachement aux PBs diminuée (Figure 7.4.A). L'OCP dont l'Arginine n°229 est modifiée perd drastiquement son affinité pour les PBs (Figure 7.5.A).

L'OCP F299R est la seule dont la force d'attachement augmente de manière très importante (Figure 7.5.A).

La figure 7.5.B présente les cinétiques de quenching et de récupération de fluorescence des PBs induites par les OCPs dont les acides aminés D220 et F299 sont mo-

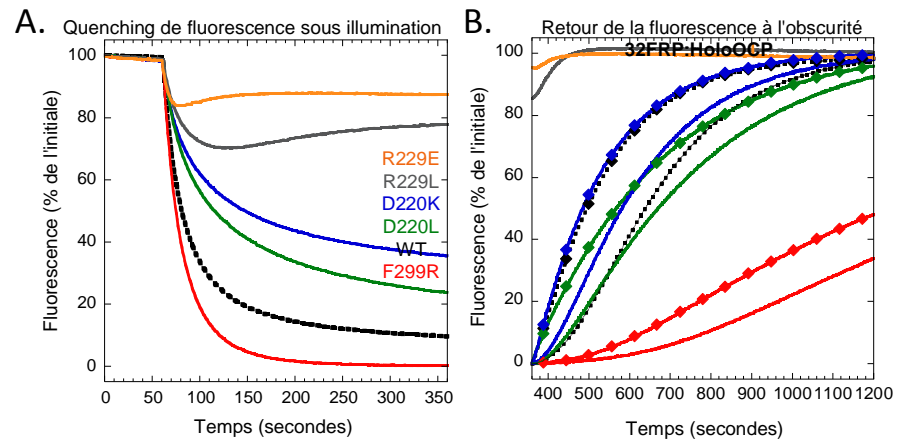


Figure 7.5 – Reconstitution in vitro du quenching de fluorescence par les OCP modifiées dont l'action de la FRP est impactée
 (A) Quenching de fluorescence à 23°C des PBs induit par les OCP WT (pointillés noirs) D220K en bleu, D220L en vert, F299R en rouge, R229L en gris et R229E en orange pré-illuminés 5 minutes par une forte lumière blanche ($5000 \mu\text{moles de photons} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$) à 4°C. Les PBs sont illuminés avec une forte lumière bleu-verte puis l'OCP rouge est ajoutée à 60 secondes d'illumination. Ratio 40 holoOCP :1PBs, tampon K-phosphate 0,5M. (B) Après 5 minutes d'illumination, la lumière est éteinte et la récupération de la fluorescence est suivie au cours du temps en absence (aucun symbole) ou présence de 32FRP :1OCP (losanges). Mêmes couleurs que dans (A) et (C).

difiés, et que nous avons précédemment identifiées comme défailtantes pour l'interaction avec le FRP. Contrairement aux observations réalisées lors des expériences de photoconversion et retour à la forme orange en présence de FRP, l'action de la FRP dans la récupération de la fluorescence semble ne pas être affectée. La FRP réussit à augmenter la vitesse de détachement pour les 3 OCPs modifiées. Étant donné que la température est différente dans les deux expériences (9°C pour la photoconversion contre 23°C pour la reconstitution in vitro), j'ai mesuré la vitesse de retour à la forme orange des deux OCPs les plus affectées (D220K et F299R) en présence et absence de FRP à 23°C (figure 7.5 (C et F)).

Dans le cas de l'D220, l'activité de la FRP est diminuée comme à 9°C mais le retour à la forme orange est presque aussi rapide que dans l'OCP-WT expliquant l'accélération de la récupération de fluorescence. En revanche, la FRP n'accélère pas le retour à la forme orange de l'OCP F299R à 23°C.

7.0.3 Activité Proline Cis-Trans isomerase de la FRP

Afin de tester l'activité cis-trans proline isomérase que pourrait avoir la FRP entre les résidus Q224P225 ou P225P226, j'ai créé trois mutants, P225V, P226V et P225VP226V. Le remplacement de la proline par une valine permet de perdre la capacité d'isomérisation en perdant le moins possible en encombrement stérique. Le résultat des tests de photoconversion est présenté en figure 7.9.

Les mutations des prolines entraînent de lourdes conséquences dans la photoactivation. L'OCP P225V se convertit plus vite à la forme rouge, l'OCP P226V plus lentement et partiellement, et l'OCP P225VP226V est presque sous forme rouge à l'obscurité (80% de forme rouge). Le retour à la forme orange de chacun des mutants est plus rapide que celui de l'OCP WT et la FRP peut accélérer le retour à la forme rouge pour chacun des OCPs modifiées, réfutant l'hypothèse formulée (Figure 7.7).

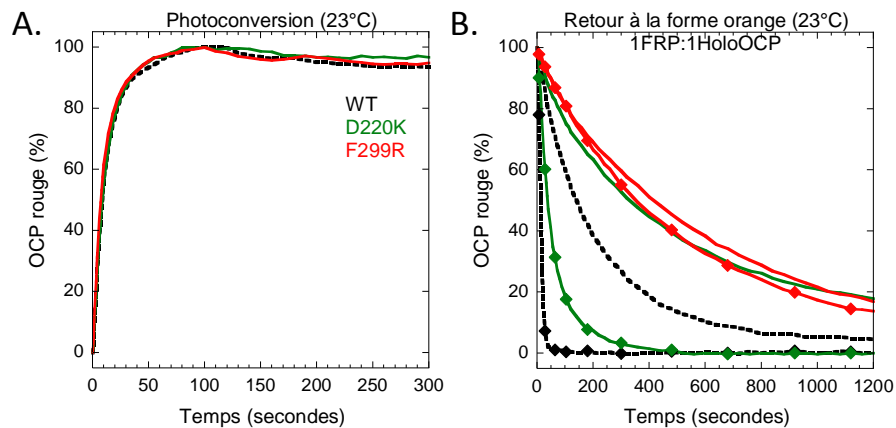


Figure 7.6 – Photoactivation et retour à la forme orange à l’obscurité des OCPs D220K et F299R à 23°C.

(A) Cinétique de photoactivation par une forte lumière blanche ($5\ 000\ \mu\text{moles de photons} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$) des OCP WT en pointillés noirs D220K en vert et F299R en rouge à 23°C. (B) Cinétique de retour à la forme orange en absence (aucun symbole) présence de 1FRP :2OCP (losanges) à 23°C. Mêmes couleurs que dans (A)

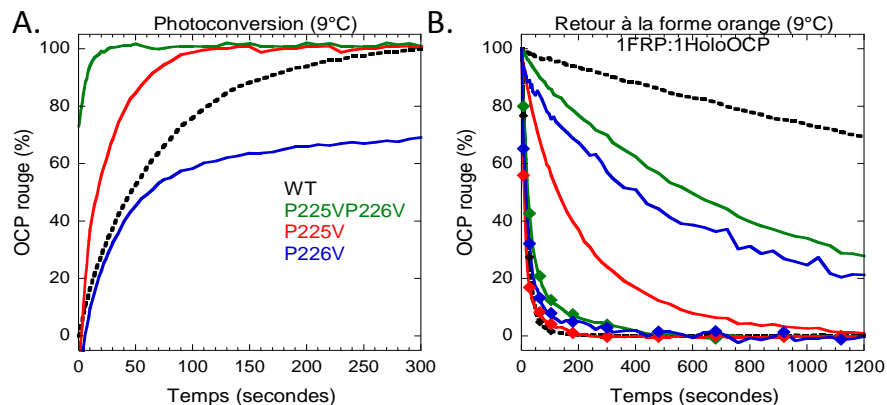


Figure 7.7 – Photoactivation et retour à la forme orange à l’obscurité des OCPs P225V, P226V et P225VP226V.

(A) Cinétique de photoactivation par une forte lumière blanche ($5000\ \mu\text{moles de photons} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$) des OCP WT en pointillés noirs, P225V en rouge, P226V en bleu et P225VP226V en vert à 9°C. 80% de l’OCP P225VP226V est déjà sous forme rouge à l’obscurité. (B) Cinétique de retour à la forme orange en absence (aucun symbole) ou présence de 1FRP :1OCP (losanges) à 9°C. Mêmes couleurs que dans (A).

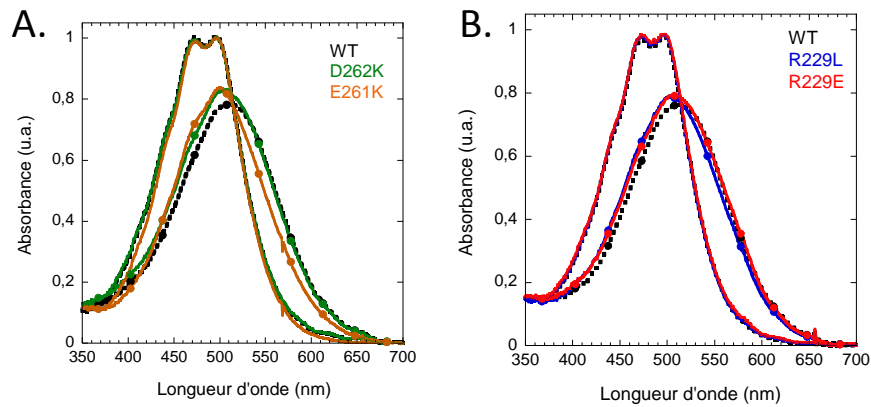


Figure 7.8 – Spectres de la forme orange et rouge des OCPs D262K, E261K, R229E et R229L (aucun symbole) et rouge (ronds) des OCPs WT en pointillés noirs et (A) D262K en vert et E261K en ocre ou (B) R229L en rouge et R229E en bleu à 9°C

7.0.4 Discussion

En résumé, les résidus D220 et F299 sont les résidus importants pour l'activité d'accélération du retour à la forme orange à 9°C. La F299 l'est particulièrement car la FRP n'est pas capable d'agir même à 23°C. En revanche, la FRP est capable de détacher toutes les OCPs des PBs. La FRP disposerait donc de deux activités que le mutant F299 permet de distinguer : reconversion à la forme orange de l'OCP rouge, et détachement de l'OCP des PBs. Le spectre de la forme rouge est différent de celui de la forme rouge de l'OCP WT pour 3 des OCPs modifiées (E261, D262 et R229). Comme attendu d'après les courbes de photoconversion (figure 7.2 page 149), les spectres des OCPs E261, D262 et R229 après 5 min d'illumination sont décalés vers le bleu comparés à celui du WT et indiquent que la conversion à la forme rouge n'est pas complète. Ces trois résidus sont localisés de part et d'autre d'un sillon dans lequel est localisé le bras N-terminal. L'ajout d'une charge positive (R) aux résidus E261 et D262 pourrait fortifier la liaison entre domaine C-terminal et bras N-terminal, et donc stabiliser la forme orange de l'OCP. Cette explication n'est pas satisfaisante pour l'arginine R229 car le remplacement de l'arginine par un glutamate ou une leucine crée une OCP dont la forme orange est stabilisée. C'est donc l'absence de l'arginine qui crée cette caractéristique. La présence à proximité d'une autre charge positive (R9 à 3.7 Å) pourrait créer un effet de répulsion, déstabilisant la position du bras N-terminal et favorisant la forme rouge (Figure 7.9). Le résidu D6 n'est pas impliqué dans la stabilisation du bras N-terminal^[216].

Les résidus 225, 226 et 229 sont localisés en aval du brin β_1 (figure 7.9). Le brin β_1 est en vis-à-vis du brin β_5 , le dernier brin β à l'extrémité C-terminale de la protéine. Les brins β_1 à 5 forment un large feuillet β . Les OCPs dont les résidus P225 et P226 sont modifiés sont très impactées dans leur photoconversion qui est soit plus lente, soit plus rapide que celle de l'OCP WT. Quand les deux résidus sont modifiés, 80% de l'OCP est sous forme rouge à l'obscurité. Les prolines sont connues pour leur contribution à la structure tertiaire des protéines. Ici, elles forment un coude à l'aval du brin β_1 . La surface d'interaction entre brin1 et brin5 est faible comparée à la surface d'interaction entre les autres brins du feuillet β , qui sont certainement très solidaires entre eux. Une séparation du brin β_1 du brin β_5 induite par le changement des deux prolines créerait une brèche permettant l'entrée du solvant, déstabilisant la forme orange. Cependant, nous n'avons aucune donnée expérimentale confortant cette proposition, et aucun indice nous permettant de savoir si le brin β_1 est séparé du brin β_5 lors du processus de photoactivation chez l'OCP WT. En tout état de cause, le fait que les différentes OCPs modifiées soient altérées dans leur affinité pour les PBs suggère qu'au moins une partie du domaine C-terminal interagit avec les PBs.

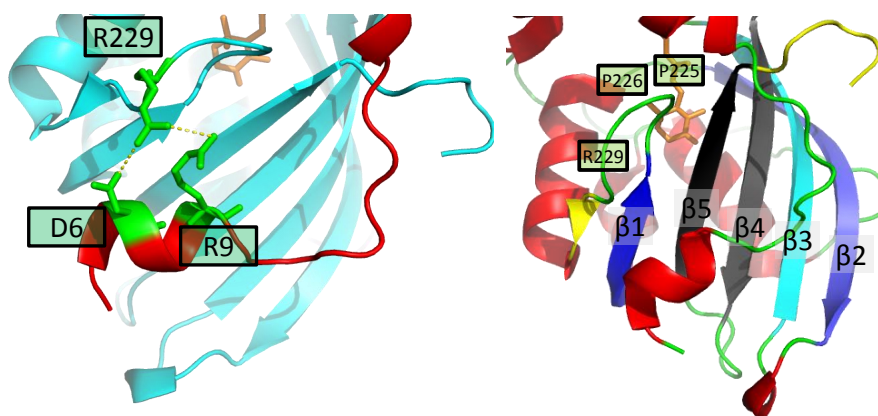


Figure 7.9 – Structure du domaine C-terminal de l'OCP : région de R229 et du feuillet beta.

(A) Représentation de la structure tridimensionnelle de l'OCP. Le domaine C-terminal est coloré en cyan, le N-terminal en orange, la région linker en violet et le caroténoïde en sticks oranges. Les résidus (A) potentiellement impliqués dans l'interaction avec la FRP ou (B) pouvant subir une isomérisation sont représentés en sticks verts. (B) les brins β du feuillet β C-terminal sont colorés β_1 en bleu foncé, β_2 en violet, β_3 en cyan, β_4 en gris et β_5 en noir.

Les données présentées dans ce manuscrit de thèse auront permis une meilleure compréhension de la photoactivation de l'OCP ainsi que de l'interaction entre l'OCP et ses partenaires, la FRP et les phycobilisomes. La figure 8.1 page 158 présente le modèle de la photoactivation de l'OCP et la figure 8.2 page 160 la modèle de photo-protection par l'OCP que les résultats obtenus au cours de ma thèse ont permis de compléter. Le chapitre de discussion est organisé en trois parties. Dans la première, je discute le nouveau modèle de photoactivation de l'OCP. Le second est consacré à l'interaction entre l'OCP et les PBs et le troisième à l'interaction entre OCP et FRP.

8.0.1 Photoactivation : Nouveau modèle

La photoactivation de l'OCP implique deux processus : l'ouverture de la protéine, et le mouvement du caroténoïde. Le caroténoïde, dans son nouvel environnement, donnera une couleur rouge à la protéine au lieu de la couleur orange de la forme inactive (Figure 8.1). Dans l'OCP orange, le caroténoïde chevauche les deux domaines N- et C-terminal. Il forme deux liaisons hydrogène avec les résidus Y201 et W288 en C-terminal et potentiellement Y44 et W110 en N-terminal^[132]. Après photoactivation, le caroténoïde est intégré uniquement au domaine N-terminal et les résidus P126 et Y129 sont indispensables à sa stabilisation dans cette nouvelle conformation^[217]). La translocation de 12 Å du caroténoïde le déplacera dans un nouvel environnement protéique, différent de celui de l'OCP orange.

Le caroténoïde qui était courbé et vrillé dans l'OCP orange est alors beaucoup plus droit et plan. Ces modifications de structure et d'environnement lui conférant des propriétés chromiques différentes, expliquant le changement de couleur d'orange à rouge de la protéine après photoactivation. Le déplacement du caroténoïde s'effectue au cœur de la protéine au sein d'un tunnel déjà présent dans la forme orange (Leverenz, Figure S5). Dans sa nouvelle position, les extrémités du caroténoïde sont plus exposées au solvant que dans l'OCP orange, ce qui pourrait expliquer que l'état d'équilibre est décalé vers la forme orange plutôt que la forme rouge de l'OCP à l'obscurité. Le domaine N-terminal avec le cofacteur caroténoïde est le domaine effecteur capable d'induire le quenching de fluorescence^[150]. L'ouverture de la protéine rend accessible l'interface entre domaine N- et C-terminal et notamment le résidu R155 indispensable à l'activité de l'OCP^[218]^[150]^[217].

Le tunnel au sein duquel le caroténoïde est translaté est déjà présent dans l'OCP orange. Cependant, la forme rouge n'apparaît pas spontanément, mais requiert de l'énergie. Cette énergie est apportée par la capture de la lumière par le caroténoïde. Cette énergie pourra induire la rupture des liaisons hydrogène avec la tyrosine 201 et le tryptophane 288. Hajin Liu^[179] propose que la rupture de la liaison hydrogène entre le caroténoïde et le tryptophane 288 a pour conséquence la réorganisation des β -sheet. Cette réorganisation causerait le détachement du bras N-terminal et permettrait l'entrée du solvant au niveau de l'interface entre les deux domaines de l'OCP, et la rupture des liaisons hydrogène qui maintenaient l'OCP fermée. Le caroténoïde, qui n'est alors plus maintenu dans un environnement quasi-clos et hydrophobe, oscille entre position en forme orange et forme rouge. L'hydrophilicité du solvant aide alors le caroténoïde à se stabiliser en position haute. Pour cela, l'OCP doit rester sous forme ouverte et le domaine C-terminal ne pas rester à l'aplomb du domaine N-terminal. Le cas échéant, le tunnel dans lequel le caroténoïde oscille pourrait se refermer et le caroténoïde pourrait redescendre.

Ce modèle est conforté par les données obtenues avec l'OCP dépourvue du bras N-terminal. D'après le spectre d'absorbance, 25% de l'OCP Δ 2-20 est rouge à l'obscurité^[216]. Cet OCP partiellement rouge est active et peut s'attacher aux PBs à l'obscurité. Le mouvement du caroténoïde au sein de l'OCP peut donc avoir lieu en l'absence de lumière. Sans le bras N-terminal, le solvant peut pénétrer plus facilement au sein de l'OCP, et l'environnement hydrophobe du caroténoïde est alors rompu par la présence de solvant à l'interface entre les deux domaines de l'OCP.

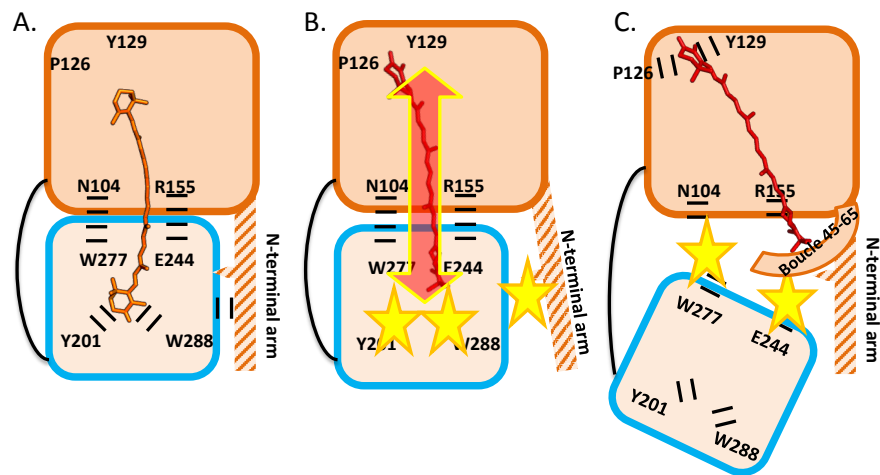


Figure 8.1 – Modèle actualisé de photoactivation de l'OCP

(A) Dans l'OCP orange, le domaine C-terminal (bleu) est maintenu à l'aplomb du domaine N-terminal (orange) au moins par les interactions entre les résidus W277 et N104, R155 et E244, ainsi que celles avec le bras N-terminal (résidus 1 à 21, en hachuré). Les résidus Y201 et W288 stabilisent le caroténoïde (stick orange).

(B) La capture de lumière par le caroténoïde casse les liaisons du carbonyle du caroténoïde avec Y201 et W288. La rupture de ces liaisons induit une légère modification de la position de la chaîne latérale du résidu W288, changeant l'organisation du brin β auquel il appartient et du feuillet beta. Cette cascade aboutit au détachement du bras N-terminal du domaine C-terminal. Le caroténoïde est désormais libre et peut osciller au sein d'un tunnel hydrophobe. L'entrée du solvant sous le bras N-terminal achève d'ouvrir l'OCP et stabilise le caroténoïde dans la position haute, dans laquelle il présente la plus petite surface de contact avec le solvant.

(C) Dans la forme rouge, l'OCP est stabilisée au moins par les résidus P126 et Y129. La boucle entre les résidus 45 à 65 est alors décalée par rapport à sa position dans l'OCP orange et se place sous le caroténoïde, diminuant la surface d'interaction entre ce dernier et le solvant.

Une fraction plus grande d'OCP aura donc son caroténoïde en position haute. Ainsi, le mouvement du caroténoïde pouvant avoir lieu en l'absence de lumière, je propose que le solvant soit le moteur de ce mouvement.

Dans l'OCP non modifiée, le bras N-terminal empêche le solvant d'entrer en contact avec le caroténoïde. La forme orange de cette OCP est très stable en l'absence de lumière et seul une concentration forte en certains sels (1.5 M sodium thiocyanate) est capable de convertir l'OCP orange en OCP rouge^[191], probablement en s'immiscant sous le bras N-terminal et en cassant les liaisons hydrogènes qui maintiennent l'OCP fermée et le caroténoïde protégé du solvant.

Dans l'OCP rouge photoactivée, le domaine C-terminal est séparé du domaine par le solvant. Le degré de liberté du domaine C-terminal n'est pas connu. Cependant, dans l'OCP dépourvue du bras N-terminal, la forme rouge de l'OCP est très stable. Aussi, le bras N-terminal pourrait agir comme un guide pour que le domaine C-terminal ait une probabilité plus importante de retrouver sa position initiale autorisant le retour du caroténoïde dans la position intermédiaire de l'OCP orange. Cet équilibre est régi par la température. A faible température, le mouvement des molécules est plus lent, et le retour du domaine C-terminal à sa position initiale prend plus de temps.

En conclusion, l'énergie lumineuse capturée par le caroténoïde induit des changements qui diminuent l'interaction entre bras N-terminal et domaine C-terminal. L'entrée consécutive du solvant stabilise le caroténoïde dans la position haute. Le caroténoïde ne pourra redescendre et l'OCP être désactivée que quand le domaine C-terminal se retrouvera à nouveau à l'aplomb du domaine N-terminal. La lumière sera alors à nouveau requise pour démarrer un nouveau cycle.

Trans-isomérisation possible d'une proline lors de la photoactivation

Dans un article publié en 2011, il a été proposé que l'un des mécanismes sous-jacent à la photoactivation soit la trans-isomérisation d'une proline^[112]. Trois prolines sont de bons candidats pour cela. Les prolines 22, 224 et 225. La proline 22 est située à la jonction entre le bras N-terminal et le domaine globulaire N-terminal. Les données obtenues avec la protéine dont la proline 22 est modifiée en valine montrent qu'une isomérisation de cette proline n'est pas indispensable pour la photoactivation.^[216] Les prolines 224 et 225 sont situées à la jonction entre un feuillet β et une hélice α au début du domaine C-terminal. Leur isomérisation pourrait induire une modification de l'architecture du domaine C-terminal responsable de la montée du caroténoïde. Les caractéristiques des OCP modifiées dans les prolines 225 et 226 sont difficiles à interpréter car ces mutations induisent probablement des changements structuraux de grande ampleur dans le domaine C-terminal conduisant à la stabilisation du caroténoïde dans sa forme rouge. Cependant, les protéines modifiées peuvent quand même se photo-converter (chapitre 7). Ainsi, je n'ai pas identifié de proline dont l'isomérisation soit indispensable à l'activité de l'OCP.

Importance de la taille du linker dans la photoactivation

Dans les articles présentés dans les chapitre 2^[37] et 3^[140], nous avons rapporté que toutes les OCPs ne présentent pas la même cinétique de photoactivation. La photoconversion de l'OCP de *A. platensis* est plus rapide que celle de *Synechocystis* qui est elle-même plus rapide que celle d'*A. variabilis*. Nous avons supposé en 2013 que la différence dans la cinétique de photoactivation et dans la spécificité entre OCP de *A. platensis* et de *Synechocystis* pouvait être due à la différence de longueur du linker qui possède deux résidus de plus chez *A. platensis*^[37]. Cependant, l'OCP de *Synechocystis* dont on a ajouté les deux résidus manquants ne présente pas les mêmes propriétés que l'OCP de *A. platensis*. La longueur du linker n'est donc pas suffisante pour expliquer les différences entre OCP de *Synechocystis* et *A. platensis*.

Différences entre les OCPs de Synechocystis et A. variabilis

Par ailleurs, l'étude de la séquence protéique révèle que tous les résidus à moins de 4Å du caroténoïde dans la forme orange et la forme rouge sont strictement identiques. La plupart des différences entre les deux OCPs sont localisées à la surface du domaine N-terminal.

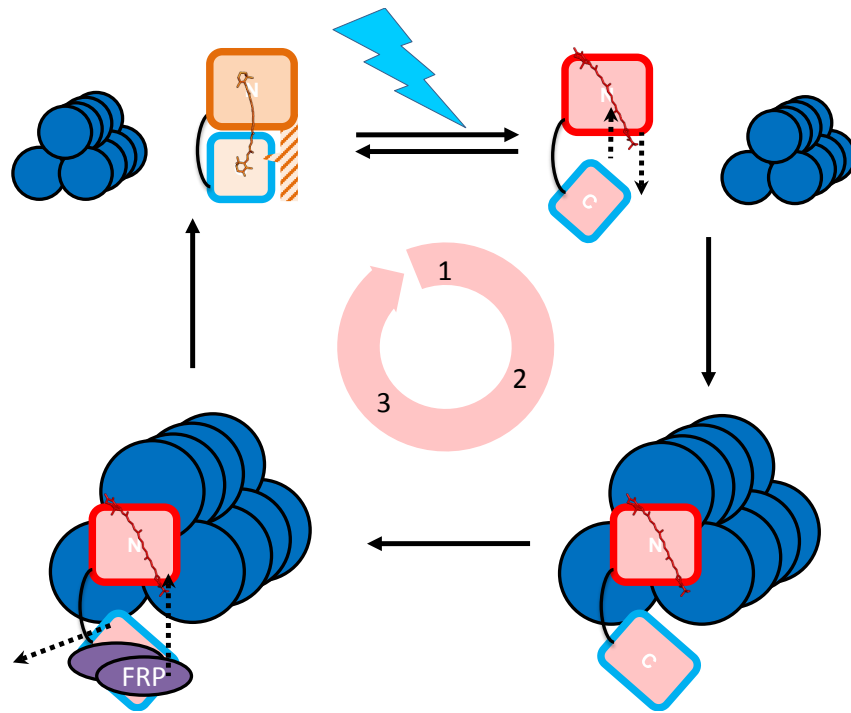


Figure 8.2 – Modèle actualisé d’attachement aux PBs

- (1) L’OCP est photoactivée par la lumière bleu-verte. La photoactivation implique une ouverture de la protéine, et un mouvement de 12angrstrom du caroténoïde au sein de la protéine.
- (2) L’OCP rouge s’attache aux PBs. La région effectrice du quenching de fluorescence est le domaine N-terminal. Le domaine C-terminal semble être également impliqué dans l’interaction avec les PBs.
- (3) La FRP interagit avec le domaine C-terminal de l’OCP, extrait cette dernière des PBs et catalyse sa reconversion en forme orange.

En revanche, le constat est différent lorsque l’OCP de *Synechocystis* est comparée à celle de *A. variabilis*. Plusieurs résidus qui sont situés à moins de 4Å du caroténoïde dans la forme rouge de l’OCP sont différents chez *Synechocystis* et *A. variabilis* (respectivement Ile₄₀ ≠ T₄₀, Y₄₁ ≠ F₄₁, I₅₃ ≠ V₅₃, ainsi que Y₁₁₁ ≠ N₁₁₁). Les résidus 40 et 41 sont substitués par des acides aminés à l’encombrement stérique proche (l’isoleucine₄₀ par une thréonine, et la tyrosine₄₁ par une phénylalanine). On note qu’un groupement alcool est présent dans les deux OCPs, mais qu’il est porté par la T₄₀ chez *Synechocystis* et par la Y₄₁ chez *A. variabilis*.

A la position 53, l’isoleucine est substituée par une valine. Ces deux résidus ont des propriétés physicochimiques et un encombrement stérique similaire. Cependant, ils restent intéressants car ils sont impliqués dans une boucle importante vis-à-vis de la photoactivation dont je vais parler au paragraphe suivant.

Enfin, la substitution de la Y₁₁₁ par une asparagine apporte un groupement NH₂. Il est à noter que le résidu R₁₁₂, non mentionné jusqu’alors car éloigné de plus de 4Å du caroténoïde, n’est pas présent chez *A. variabilis* et qu’il est remplacé par un glutamate. Les deux groupements NH₂ sont donc conservés mais répartis sur deux résidus différents chez *A. variabilis*.

Comparaison des structures tridimensionnelle de l’OCP et de la RCP

La résolution de la structure de la RCP révèle que le domaine N-terminal de l’OCP orange et la RCP ont une structure tridimensionnelle très proche (RMSD=1,6). La différence majeure entre les deux structures est une région comprise entre les résidus 45 à 65 (RMSD=2,46).

Aucune structure tridimensionnelle n’est associée à cette région qui correspond à une boucle reliant deux hélices α du domaine N-terminal. L’un des résidus au facteur β le plus élevé est une proline, la proline 55 (Figure 8.3 page 162. Cette

proline fait partie d'un motif singulier (AAPGAA) composé d'acides aminés dont l'implication sur la structure tertiaire est importante. L'alanine et la glycine sont de très petits acides aminés, et sont, comme la proline, connu pour être impliqués dans des boucles de la structure tertiaire des protéines. Dans la forme rouge, cette boucle se décale pour assurer un environnement plus hydrophobe au caroténoïde (Figure 8.1).

L'un des acides aminés différents entre *Synechocystis* et *A. variabilis* est impliqué dans cette boucle (respectivement I53 et V53). Cependant, cette substitution implique deux acides aminés aux propriétés physicochimiques, et n'est donc probablement pas responsable des différences observées dans la cinétique de photoconversion.

8.0.2 Interaction OCP-PBs

Les phycobilisomes sont de très gros complexes protéiques. Le développement des techniques modernes de single particle electron microscopy permet d'obtenir des cartes de densité électronique de plus en plus détaillées^{[31][32]}. Cependant, aucune équipe n'a encore réussi à cristalliser le complexe PBs-OCP. Par ailleurs, il a été démontré au laboratoire que les émetteurs terminaux, ApcD, ApcE et ApcF ne sont pas essentielles pour l'action de l'OCP^[101] et que l'OCP peut s'attacher à un phycobilisome dont le cœur ne comporte que deux cylindres^[37]. Ainsi, l'OCP se fixe probablement au niveau des trimères d'allophycocyanine qui composent les cylindres basaux du cœur des PBs.

Afin de déterminer le site de fixation exact de l'OCP, plusieurs équipes travaillent en utilisant des expériences de crosslinking associées à la spectrométrie de masse. Deux modèles ont été proposés plaçant l'OCP soit au niveau des chromophores à l'extérieur du phycobilisome^[219] soit à l'intérieur du phycobilisome^[158]. Ces modèles ont été construits avec la structure de l'OCP orange telle que connue jusqu'alors. La résolution de la structure de la RCP remet ces modèles en question.

Implication possible du feuillet β dans l'interaction entre le domaine C-terminal de l'OCP et les PBs

Dans la discussion du chapitre 7 j'ai proposé que les caractéristiques peu attendues des OCPs dont les résidus compris dans la région entre 225 et 229 (P225, P226, F227 et R229) soient dues à la dissociation des brins β_1 et β_5 . Cette dissociation rompra le feuillet β du domaine C-terminal, et pourra expliquer les différences d'envergure observées vis-à-vis de l'attachement aux PBs. En effet, la structure du domaine C-terminal ne serait plus alors un domaine globulaire. La structure résultante est difficile à modéliser. Elle contiendrait probablement le feuillet β et un assemblage d'hélices α du côté du brin β_2 . Cette structure dont l'intérieur du feuillet β , qui est, dans la forme orange, l'environnement du caroténoïde, est très hydrophobe.

Ainsi, on pourrait imaginer que l'intérieur du feuillet β crée de nombreuses liaisons hydrogène avec la surface d'un cylindre d'APC. La modification de résidus potentiellement impliqués dans l'interaction avec la FRP, sur la face externe du feuillet β , pourrait avoir des répercussions sur l'autre face ou sur l'organisation du feuillet β . Ceci expliquant les différences de cinétiques de photoactivation (mouvement du caroténoïde facilité ou diminué) ou les différences d'attachement aux PBs (liaisons entre face interne du feuillet β et PBs plus faible ou plus forte).

La position du bras N-terminal dans l'OCP P22V diminue l'affinité pour les PBs

Nous avons rapporté dans l'article^[216] que la modification de la proline 22 en valine diminue l'affinité de l'OCP pour les PBs, malgré une photoconversion totale à la forme rouge. Cette baisse d'affinité pourrait être due au mauvais positionnement ou à une plus grande flexibilité du bras N-terminal.

Les données disponibles aujourd'hui ne permettent pas d'établir un modèle fiable d'interaction entre l'OCP et les PBs

Malgré les données précédemment discutées concernant la position du caroténoïde et l'importance de la région autour du feuillet β du domaine C-terminal, les données sont aujourd'hui insuffisantes pour créer un modèle fiable d'interaction

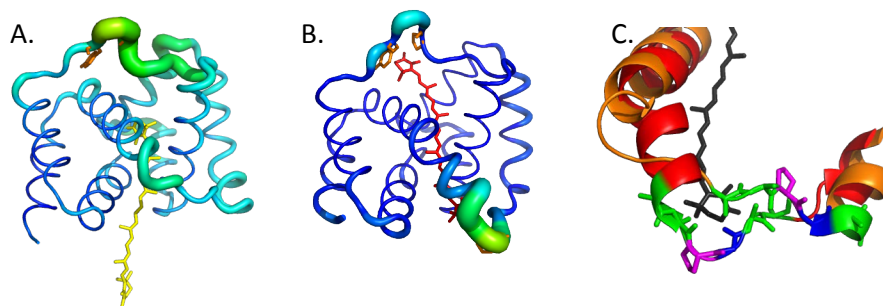


Figure 8.3 – Structure du domaine N-terminal de l'OCP : facteur β et boucle 45-65. Représentation du facteur β obtenus lors de la cristallisation de (A) l'OCP-CAN de *Synechocystis* dont seul le domaine N-terminal est représenté (4XB4) et (B) le RCP-CAN (domaine N-terminal de l'OCP de *Synechocystis*). La couleur bleu foncé traduit un facteur β faible et la couleur jaune un facteur β élevé. (C) représentation de la région 45-65 dans l'OCP-CAN orange (orange) et dans la RCP-CAN (rouge). Les résidus AAPGAA sont respectivement colorés en vert, vert, violet, bleu, vert et vert.

entre OCP et PBs. D'une part, la position relative des domaines N- et C-terminal reste à démontrer. Enfin, si la position du caroténoïde après photoconversion a été démontrée, le caroténoïde ne la conserve pas forcément une fois l'OCP complexée avec les PBs. En effet, les APC pourraient accueillir la moitié du caroténoïde à la manière du domaine C-terminal comme proposé dans^[150] (chapitre 5). Ainsi, le caroténoïde serait positionné idéalement proche des bilines afin de récupérer le plus efficacement possible leur énergie d'excitation.

8.0.3 Interaction OCP-FRP

Il a été montré avant le début de mon doctorat que la FRP accélère la conversion de l'OCP rouge en OCP orange et aide à la séparation de l'OCP du PB^{[86][81]}. Cependant, les expériences conduites jusqu'à aujourd'hui ne permettent pas de savoir si la FRP possède une activité de séparation du complexe OCP-PBs ou bien si la FRP reconvertit l'OCP rouge en OCP orange, ce qui pourrait conduire au détachement de l'OCP des PBs.

Domaine d'interaction entre OCP et FRP

En étudiant les possibles interactions entre la FRP et l'OCP, j'ai observé que deux résidus sont importants pour l'activité de reconversion de l'OCP rouge en OCP orange de la FRP : l'D220 ou la F299. En revanche, ces modifications sont totalement transparentes lorsque l'activité de la FRP est mesurée en suivant la vitesse de détachement de l'OCP des PBs. Ces résultats soutiennent l'hypothèse que la FRP a bien deux activités, et que les modifications effectuées ralentissent (D220) ou annihilent (F299) la capacité de la FRP d'accélérer le retour à la forme orange de l'OCP rouge en solution (pas attaché au PBS).

A contrario, la FRP est capable de détacher très facilement l'OCP dépourvue du bras N-terminal, OCP Δ 2-20. Cette OCP semble présenter une affinité pour les PBs supérieure à l'OCP WT (récupération de la fluorescence plus lente). Le bras semble donc gêner la fixation de l'OCP WT. En revanche, la FRP a également plus de facilité à détacher l'OCP des PBs car le bras N-terminal est absent et ne peut pas déranger l'extraction du domaine N-terminal de l'OCP du PBs. Le bras contribue ainsi à l'équilibre entre PBs dont l'énergie est dissipée sous forme de chaleur et PBs qui ne l'est pas, en évitant que l'OCP soit détachée trop aisément par la FRP.

Modèle d'interaction entre OCP, PBs et FRP

Ensemble, les éléments discutés précédemment permettent d'imaginer un modèle d'interaction entre l'OCP, les PBs et la FRP (Figure 8.4). Il a été montré que 1 à 2 OCPs peuvent s'attacher par PBs^[81]. Cependant, les quatre disques qui constituent

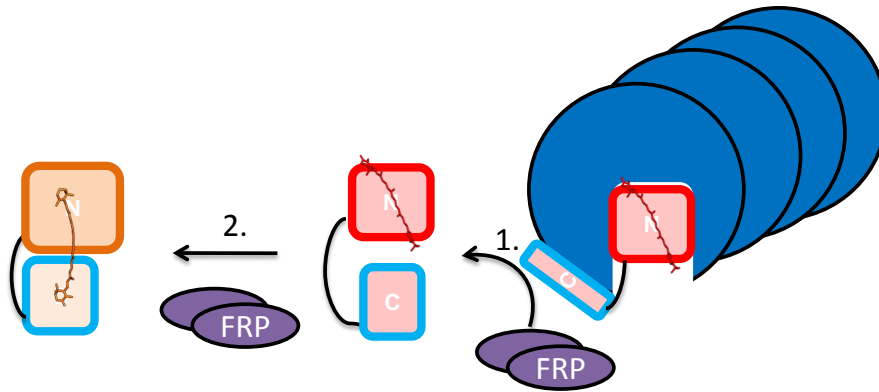


Figure 8.4 – Modèle d'action de la FRP L'OCP rouge (rouge et bleu) est attaché entre deux trimères de l'un des deux cylindres basaux du PBs (un seul cylindre est représenté). Le feuillet β du domaine C-terminal interagit avec l'un des cylindres d'APC. La FRP (dimère représenté en violet). La FRP interagit avec le domaine C-terminal de l'OCP (1) extrait cette dernière des PBs et catalyse sa reconversion en forme orange (2).

chaque cylindre du cœur ne diffèrent que par la position des émetteurs terminaux, qui ne sont pas essentiels à l'induction du quenching de fluorescence^[101]. Compte tenu du fait que l'OCP peut s'attacher à des PBs qui ne contiennent que les deux cylindres basaux^[37], l'hypothèse la plus simple est qu'une seule molécule d'OCP s'attache à un cylindre basal. L'OCP peut potentiellement s'attacher à l'intérieur^[158] ou à l'extérieur des disques d'APC^[219].

Les données présentées dans l'article de Zhang et al.^[158], sont plus convaincantes car elles reposent sur des données expérimentales. Si l'OCP se glisse effectivement entre deux trimères d'APC, alors 3 sites de fixation sont possibles. Les trois sites de fixation potentiels sont différents à cause de la présence des émetteurs terminaux au sein des cylindres basaux.

Cependant, nous savons qu'aucun des émetteurs terminaux n'est indispensable pour l'attachement de l'OCP aux PBs^[101] et que le quenching de fluorescence intervient au niveau d'un trimère APC660 (Tian et al., 2011). Aussi, je pense que l'OCP se fixe entre les deux disques d'APC émettant à 660nm ($(\alpha\beta)_3L_c8$ et $(\alpha\beta)_3$).

Le domaine N-terminal de l'OCP doit pénétrer suffisamment profondément pour pouvoir expliquer que la mauvaise position du bras N-terminal puisse gêner la fixation^[216], de même que les longues prolongations en N-terminal^[140]. Le domaine C-terminal, reste vraisemblablement à l'extérieur du cylindre d'APC pour assurer un point d'ancrage à la FRP.

Le site d'interaction entre domaine C-terminal et PBs pourrait correspondre à l'intérieur du feuillet β qui est normalement en contact avec le caroténoïde. L'autre face du feuillet β restant accessible à la FRP, dont l'activité pourrait être de permettre au domaine C-terminal de retrouver sa forme initiale. Le domaine Linker, qui s'était trouvé allongé lorsque le feuillet β du domaine C-terminal s'est ouvert, se raccourcirait détachant le domaine N-terminal de l'OCP (activité n°1 de la FRP). Ensuite, la FRP pourrait guider la réconciliation des domaines N- et C-terminal de l'OCP (activité n°2 de la FRP), permettant au caroténoïde de redescendre.

Les travaux présentés dans ce manuscrit ont permis de compléter le modèle de photoprotection par l'OCP et de soulever de nouvelles questions. Le rôle du bras N-terminal a été élucidé et l'implication de certaines régions du domaine C-terminal dans l'interaction avec les PBs et la FRP démontrés. Cependant, les sites d'interaction entre OCP et PBs ou FRP restent à être confirmés par le recoupement de données expérimentales obtenues avec plusieurs techniques.

9.0.1 Photoactivation

Un article récent ouvre des perspectives quant à la détermination des mouvements de la structure tertiaire sous-jacents à la photoactivation de l'OCP. Cet article rapporte la résolution dans le temps du mouvement des hélices α et feuillets β photoinduits de la Channelrhodopsin-2^[220]. Brièvement, la structure tridimensionnelle d'une protéine est résolue d'après un cristal composé d'un assemblage ordonné de cette protéine. Avant d'éclairer le cristal avec un laser afin de récupérer les données de diffractions qui permettent de résoudre la structure, ce dernier est plongé dans l'azote liquide pour être congelé (voir matériel et méthode supplémentaire du chapitre 5^[217]). Dans l'article de Müller et al.^[220], les auteurs soumettent le cristal à une source de lumière laser pour induire des changements conformationnels de la Channelrhodopsin-2 puis plongent le support des cristaux dans l'azote liquide en moins de 10ms à l'aide d'un dispositif spécial. La transposition de cette technique, utilisée dans cet article pour caractériser un canal membranaire, pourrait révéler les changements sous-jacents à la photoactivation comme la position du bras N-terminal.

La détermination des mouvements des domaines N- et C-terminal, ainsi que probablement ceux du bras N-terminal peuvent également être mis en évidence par SAXS (small-angle x-ray scattering,^{[221][222]}). La résolution obtenue sera plus faible que celle obtenue avec un cristal, mais probablement suffisante pour déterminer les mouvements de grande amplitude intervenant durant la photoactivation de l'OCP.

Par ailleurs, l'utilisation de la technique SAXS avec les différentes protéines modifiées et notamment P225V-P226V pourrait probablement nous aider à déterminer si leur structure est altérée par les modifications réalisées.

9.0.2 Domaines impliqués dans l'affinité avec les PBs.

Dans l'article Jallet et al., 2014^[37] nous avons montré en comparant deux OCPs très proches (*Synechocystis* et *A. platensis*) que la spécificité de l'OCP était intrinsèque à sa séquence. Dans l'article^[140] nous avons isolé chez *E. coli*, trois OCPs, celle de *Synechocystis*, de *A. platensis* et de *A. variabilis*. Les 3 OCPs présentent une affinité très différente vis-à-vis des PBs de *Synechocystis*. Par ailleurs, certains mutants du domaine C-terminal présentés dans le chapitre 7 présentent une faible affinité pour les PBs. Afin de déterminer les résidus ou domaines importants dans l'affinité de l'OCP pour les PBs, il convient de s'intéresser au domaine N- et C-terminal.

Je propose de créer des protéines fusion comportant une partie de l'OCP de *Synechocystis*, et une partie de l'OCP de *A. platensis* (meilleure affinité) ou d'*A. variabilis* (affinité plus faible). Le domaine C-terminal de ces 3 OCPs est très semblable. Ainsi, il est raisonnable de créer deux protéines fusion comportant le domaine N-terminal de *Synechocystis* et le domaine C-terminal de *A. platensis*. En revanche, le domaine N-terminal est moins conservé, et il y a donc plus de chances que l'explication des différences d'affinité réside dans des différences au niveau N-terminal. Aussi, j'assemblerais une moitié du domaine N-terminal appartenant à *Synechocystis* (1-79) avec l'autre autre moitié du domaine N-terminal (79-169) appartenant à *A. platensis*. Le choix de couper le domaine N-terminal en deux au niveau du résidu 79 s'explique par le fait que la région 63-79 comporte de nombreux résidus non conservés.

En revanche, pour l'OCP d'*A. variabilis*, nous disposons de quatre résidus proches du caroténoïdes (L40, Y41, I53 et Y111) dont un impliqué dans une structure de stabilisation potentielle de la position du caroténoïde en forme rouge (I53). Je propose d'effectuer quatre mutants ponctuels afin de déterminer si l'une de ces modifications est responsable de la faible photoconversion de l'OCP d'*A. variabilis*. Ainsi, par la création de 3 OCPs chimères et 4 mutants ponctuels, il pourra être possible de déterminer quels domaines sont responsables de l'augmentation de l'affinité de l'OCP pour les PBs chez *A. platensis*, et de sa diminution chez *A. variabilis*.

9.0.3 Complexe OCP-FRP

Le complexe OCP-FRP étant très versatile, sa cristallisation est délicate. Selon le modèle actuel, le détachement de la FRP de l'OCP est lié au retour à la forme orange de l'OCP. Si le retour à la forme orange est impossible, alors la FRP reste probablement attachée à l'OCP. Dans le chapitre 6, je présente les résultats d'une co-immunoprécipitation entre domaine C-terminal et FRP, confortant l'hypothèse précédemment mentionnée. Si l'interaction entre le domaine C-terminal et la FRP est stable, alors le complexe peut probablement être cristallisé en l'état. Une autre approche pourrait être d'utiliser les expériences de footprinting et de la spectrométrie de masse pour trouver les résidus qui interagissent ensemble et ainsi valider le site d'interaction entre OCP et FRP.

9.0.4 La carte de densité électronique du complexe OCP-PBs pourrait nous aider à déterminer le lieu d'attachement de l'OCP aux PBs

L'article de Chang publié en 2015^[32] rapporte l'organisation structurale d'un PBs intact déterminé par single-particle electron microscopy. Cette technique permet aux auteurs d'obtenir une carte de densité électronique très précise qu'ils peuvent ensuite associer aux structures publiées des composants isolés des PBs, et ainsi reconstituer l'assemblage précis des bras et du cœur des PBs, et le positionner sur le PSII.

La résolution obtenue pour le complexe PBs-PSII (présenté en figure 1.11 page 29) 2,23Å. Cette résolution serait suffisante pour positionner l'OCP au sein du complexe. Ainsi, réaliser la carte de densité électronique un complexe entre phycobilisome d'*A. variabilis* et OCP d'*A. platensis* pourrait permettre d'identifier le site d'attachement de l'OCP au sein des PBs.

9.0.5 Souches naturellement dépourvues d'OCP

La possibilité de quencher la fluorescence des PBs de *S. elongatus*, une souche dépourvue d'OCP, avec l'OCP de *Synechocystis* à 1.4M phosphate et avec l'OCP de *A. platensis* à 0.8 et 1.4M phosphate a été une surprise pour nous. Cependant, les données présentées dans l'article ont été obtenues uniquement *in vitro*. Les étayer par des données *in vivo* nous permettrait de confirmer que les deux OCPs sont capables de s'attacher aux PBs de *S. elongatus*, et que l'OCP de *A. platensis* est plus efficace que celle de *Synechocystis*. *S. elongatus* est une bonne souche candidate pour une telle approche car les outils de biologie moléculaire permettant de la transformer sont décrits dans la littérature. La question de savoir pourquoi certaines souches de cyanobactéries, comme *S. elongatus*, ont perdu le gène codant pour l'OCP est une question ouverte. Pour l'instant, personne n'a trouvé de corrélation entre milieu de vie, et photoprotection par l'OCP. Les publications récentes de nouveaux génomes offrent une mine de données pour les analyses bio-informatiques et l'écologie des populations. Il serait particulièrement intéressant de déterminer, pour les souches dont les conditions de vie sont connues, si elles possèdent une séquence codante homologue à celle du gène *slr1963* (OCP). Et si c'est le cas, vérifier que le mécanisme de photoprotection est bien présent *in vivo* en cultivant les souches au laboratoire et en évaluant au fluorimètre PAM l'efficacité du mécanisme de photoprotection par l'OCP.

9.0.6 Régulation des gènes

Au début de ma thèse, j'ai souhaité travailler sur la régulation des gènes de l'OCP et de la FRP par des approches de biologie moléculaire. Les expériences n'ont pas donné de résultats pour des problèmes techniques, cependant, le travail de bibliographie réalisé en amont me permet de proposer des perspectives de travail sur la régulation des gènes.

Le transcrit OCP est 4 fois moins abondant après ajout de DBMIB et deux fois moins important après ajout de DCMU. Le transcrit OCP étant moins abondant si les éléments en aval du cytochrome *B₆f* sont oxydés^[223], alors on peut supposer que l'état redox du pool de plastoquinone régule la synthèse d'OCP.

Cependant, les acteurs de cette régulation restent inconnus. Une piste serait que cette régulation soit effectuée par un système à deux composants incluant une protéine à histidine kinase (Hik) et un régulateur de réponse (Rre).

Régulation possible du transcrit OCP par des protéines Hik

Les protéines Hik possèdent un domaine senseur et en réponse à un changement environnemental transfèrent un groupe phosphate depuis l'ATP vers une histidine. Le groupement phosphate est ensuite transféré au régulateur de réponse^[224]. Un article publié en 2004 propose un lien possible entre la régulation de l'OCP et les protéines Hik₃₄ et son régulateur de réponse Rre1^[225].

Les auteurs observent une diminution par 4 de la quantité de transcrits OCP après exposition durant 20 minutes à un stress osmotique (0,5M sorbitol) chez le mutant Hik₃₄ et chez le mutant du régulateur en aval de Hik₃₄, Rre1. Ce résultat est confirmé par Suzuki qui observe une diminution du transcrit de l'OCP chez le surexprimeur de Hik₃₄^[226].

Le transcrit Hik₃₄ étant lui-même régulé par l'état redox du pool de plastoquinone^[227], on peut poser l'hypothèse que la transcription de l'OCP pourrait être contrôlée par l'état redox du pool de plastoquinone via Hik₃₄ et Rre1. Cependant, ces données demandent à être confirmées par des expériences de biologie moléculaire centrées sur l'OCP et à être complétées par des données sur la FRP.

ARN antisens de l'OCP

Par ailleurs un ARN antisens exprimé dans des conditions de culture normales et sous stress pourrait être impliqué dans la régulation des gènes *SLR1963* et *SLR1964*. L'unité de transcription de l'ARN antisens déterminée par Mitchke et al.^[26] correspond à la région 150-210 de l'OCP, soit la région linker reliant domaine N- et C-terminal. Chez certaines cyanobactéries, il existe un ou plusieurs gènes codant pour une protéine à forte homologie avec le domaine N-terminal de l'OCP nommée RCP. Il n'existe pas de séquence codant pour la RCP chez *Synechocystis*. L'ARN antisens présent en aval de la séquence codant le domaine N-terminal de l'OCP chez *Synechocystis* pourrait agir comme terminateur de transcription, créant ainsi des protéines RCP en utilisant la séquence codante de l'OCP. Le rôle de la RCP chez *Synechocystis* reste toutefois à établir.

CONCLUSION

Les couleurs fascinent l'Humanité depuis la nuit des temps. En témoignent les peintures vieilles de 32 000 ans découvertes dans la grotte Chauvet, en Ardèche. Travailler avec tant de couleurs a été pour moi un grand plaisir. En effet, une culture de cynaobactérie est d'un vert profond, les PBs qui en sont extraits sont bleus nuit et leur fluorescence est rouge. Les travaux présentés dans ce manuscrit expliquent en partie comment l'OCP stoppe la quasi-totalité de la fluorescence des PBs. Nous avons également expliqué le changement de couleur unique de l'OCP, qui implique le mouvement de 12Å de son cofacteur caroténoïde. Le dernier partenaire connu de l'OCP, la FRP est transparente. Je ne suis pour autant pas resté sur ma faim, considérant les mystères qui entourent toujours son activité.

Je suis convaincu que le mécanisme de photoprotection par l'OCP continuera d'intéresser de nombreux jeunes chercheurs spécialistes de biologie moléculaire, génomique, cristallographie, biochimie et biophysique, qui seront attirés par cette panoplie de couleurs et qui pourront réfuter, ou peut-être confirmer, les explications proposées dans ce manuscrit.

Annexes

Table 2 – Primers utilisés dans ce travail

| Mutagenèse ponctuelle : Interaction OCP-FRP | | |
|---|---|--|
| D220L | F | GAA TTG TTC ACC TCC CTC GGC GCC CTC C |
| D220L | R | GGA GGG CGC CGA GGG AGG TGA ACA ATT C |
| D220K | F | GTT CAC CTC CAA GGG CGC CCT CCA ACC G |
| D220K | R | CGG TTG GAG GGC GCC CTT GGA GGT GAA C |
| R229L | F | CC TTC CAA CTG CCA ATT GTC GGT AAA G |
| R229L | R | CTT TAC CGA CAA TTG GCA GTT GGA AGG |
| R229E | F | CCC TTC CAA GAG CCA ATT GTC GGT AAA |
| R229E | R | TTT ACC GAC AAT TGG CTC TTG GAA GGG |
| E261K | F | GTT ACT GAA CCC GCT AAA GAT GGT TT |
| E261K | R | AAA CCA TCT TTA GCG GGT TCA GTA AC |
| N293K | F | GCC CTC GGG TTT GAG GAG AAA GCG CCA |
| N293K | R | TGG CGC TTT CTC CTC AAA CCC GAG GGC |
| F299R | F | GAG GGC AAA ATT AGG TTT GTG GCG ATC GAC CTC |
| F299R | R | GAT CGC CAC AAA CCT AAT TTT GCC CTC GGG GTT |
| N236K | R | GTC GGT AAA GAA AAA GTG CTC CGC TTT TTC CGG |
| N236K | R | AAA GCG GAG CAC TTT TTC TTT ACC GAC AAT GGG |
| D262K | F | GAA CCC GCT GAA AAG GGT TTC ACC CAA ATT AAA |
| D262K | R | TTG GGT GAA ACC CTT TTC AGC GGG TTC AGT AAC |
| Mutagenèse ponctuelle : Isomerisation des prolines 225 et 226 | | |
| P225V | F | GCC CTC CAA GTG CCC TTC CAA CGG |
| P225V | R | CCG TTG GAA GGG CAC TTG GAG GGC |
| P226V | F | CGG CGC CCT CCA ACC GGT CTT CCA ACG G |
| P226V | R | CCG TTG GAA GAC CGG TTG GAG GGC GCC G |
| P225VP226V | R | CGG CGC CCT CCA AGT GGT CTT CCA ACG G |
| P225VP226V | R | CCG TTG GAA GAC CAC TTG GAG GGC GCC G |
| Mutagenèse ponctuelle : Phe227 | | |
| F227L-F | F | CTC CAA CCG CCC TTG CAA CGG CCC ATT GTC GGT |
| F227L-R | R | AAT GGG CCG TTG CAA GGG CGG TTG GAG GGC ACC |
| Déletion des 14 premiers acides aminés | | |
| OCP-d2-15Ct | | TTA ATA AGG AGA TAT ACC ATG CTA GCG GCC GAC GTT GTA CCC |
| Déletion des 14 premiers acides aminés et suppression de la structure secondaire | | |
| OCP-d2-15NeutCt | | TTA ATA AGG AGA TAT ACC ATG CTG GCC GCC GAC GTT GTA CCC |
| FRCP15 | | CAT AAG GAA TTA TAA CCA TAT GCT AGC GGC CGA CGT TGT ACC CGC TAC |

BIBLIOGRAPHIE

1. Birger Rasmussen, Ian R. Fletcher, Jochen J. Brocks, and Matt R. Kilburn. Reassessing the first appearance of eukaryotes and cyanobacteria. *Nature*, 455(7216) :1101–1104, October 2008.
2. A. Bekker, H. D. Holland, P.-L. Wang, D. Rumble, H. J. Stein, J. L. Hannah, L. L. Coetzee, and N. J. Beukes. Dating the rise of atmospheric oxygen. *Nature*, 427(6970) :117–120, January 2004.
3. James Barber. Photosynthetic generation of oxygen. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 363(1504) :2665–2674, August 2008.
4. Geoffrey I. McFadden. Origin and Evolution of Plastids and Photosynthesis in Eukaryotes. *Cold Spring Harbor Perspectives in Biology*, 6(4) :a016105, April 2014.
5. Steven G. Ball, Christophe Colleoni, Derifa Kadouche, Mathieu Ducatez, Maria-Cecilia Arias, and Catherine Tirtiaux. Toward an understanding of the function of Chlamydiales in plastid endosymbiosis. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1847(6–7) :495–504, 2015.
6. Daryl Domman, Matthias Horn, T. Martin Embley, and Tom A. Williams. Plastid establishment did not require a chlamydial partner. *Nature Communications*, 6, March 2015.
7. Takashi Yamaoka, Kazuhiko Satoh, and Sakae Katoh. Photosynthetic activities of a thermophilic blue-green alga. *Plant and Cell Physiology*, 19(6) :943–954, September 1978.
8. A. Taton, S. Grubisic, E. Brambilla, R. De Wit, and A. Wilmotte. Cyanobacterial diversity in natural and artificial microbial mats of Lake Fryxell (McMurdo dry valleys, Antarctica) : A morphological and molecular approach. *Applied and Environmental Microbiology*, 69(9) :5157–5169, September 2003. WOS :000185437000014.
9. Heath E. O'Brien, Jolanta Miadlikowska, and François Lutzoni. Assessing population structure and host specialization in lichenized cyanobacteria. *The New Phytologist*, 198(2) :557–566, April 2013.
10. Ana L. Pereira and Vitor Vasconcelos. Classification and phylogeny of the cyanobiont *Anabaena azollae* Strasburger : an answered question? *International Journal of Systematic and Evolutionary Microbiology*, 64(Pt 6) :1830–1840, June 2014.
11. R. K. Pachauri, M. R. Allen, V. R. Barros, J. Broome, W. Cramer, R. Christ, J. A. Church, L. Clarke, Q. Dahe, P. Dasgupta, N. K. Dubash, O. Edenhofer, I. Elgizouli, C. B. Field, P. Forster, P. Friedlingstein, J. Fuglestedt, L. Gomez-Echeverri, S. Hallegatte, G. Hegerl, M. Howden, K. Jiang, B. Jimenez Cisneroz, V. Kattsov, H. Lee, K. J. Mach, J. Marotzke, M. D. Mastrandrea, L. Meyer, J. Minx, Y. Mulugetta, K. O'Brien, M. Oppenheimer, J. J. Pereira, R. Pichs-Madruga, G.-K. Plattner, Hans-Otto Pörtner, S. B. Power, B. Preston, N. H. Ravindranath, A. Reisinger, K. Riahi, M. Rusticucci, R. Scholes, K. Seyboth, Y. Sokona, R. Stavins, T. F. Stocker, P. Tschakert, D. van Vuuren, and J.-P. van Ypserle. *Climate Change 2014 : Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. IPCC, Geneva, Switzerland, 2014.
12. Catherine Quiblier, Wood Susanna, Echenique-Subiabre Isidora, Heath Mark, Villeneuve Aurelie, and Humbert Jean-Francois. A review of current knowledge on toxic benthic freshwater cyanobacteria–ecology, toxin production and risk management. *Water research*, 47(15) :5464–79, July 2013. MEDLINE :23891539.

13. Caterina Batello, Marzio Marzot, Adamou Harouna Touré, Peter Ervin Kenmore, and Food and Agriculture Organization of the United Nations. *The Future is an Ancient Lake : Traditional Knowledge, Biodiversity and Genetic Resources for Food and Agriculture in Lake Chad Basin Ecosystems*. Food & Agriculture Org., 2004.
14. Jacques Simpire, Fatoumata Kabore, Frederic Zongo, Deleli Dansou, Augustin Bere, Salvatore Pignatelli, Daniela M. Biondi, Giuseppe Ruberto, and Salvatore Musumeci. Nutrition rehabilitation of undernourished children utilizing Spiruline and Misola. *Nutrition Journal*, 5(1) :3, January 2006.
15. CEA. Mémento sur l'énergie, June 2015.
16. Marcia Ortega-Ramos, Thichakorn Jittawuttipoka, Panatda Saenkham, Aurelia Czarnecka-Kwasiborski, Hervé Bottin, Corinne Cassier-Chauvat, and Franck Chauvat. Engineering *Synechocystis* PCC6803 for Hydrogen Production : Influence on the Tolerance to Oxidative and Sugar Stresses. *PLoS ONE*, 9(2) :e89372, 2014.
17. Zhengxu Gao, Hui Zhao, Zhimin Li, Xiaoming Tan, and Xuefeng Lu. Photosynthetic production of ethanol from carbon dioxide in genetically engineered cyanobacteria. *Energy & Environmental Science*, 5(12) :9857–9865, November 2012.
18. Patrícia C. M. Da Rós, Caroline S. P. Silva, Maria E. Silva-Stenico, Marli F. Fiore, and Heizir F. De Castro. Assessment of Chemical and Physico-Chemical Properties of Cyanobacterial Lipids for Biodiesel Production. *Marine Drugs*, 11(7) :2365–2381, July 2013.
19. S. Andreas Angermayr, Aleix Gorchs Rovira, and Klaas J. Hellingwerf. Metabolic engineering of cyanobacteria for the synthesis of commodity products. *Trends in Biotechnology*, 33(6) :352–361, 2015.
20. Aravinthan DT Samuel, Jennifer D Petersen, and Thomas S Reese. Envelope structure of *Synechococcus* sp. WH8113, a nonflagellated swimming cyanobacterium. *BMC Microbiology*, 1 :4, April 2001.
21. Michelle Liberton, R. Howard Berg, John Heuser, Robin Roth, and Himadri B. Pakrasi. Ultrastructure of the membrane systems in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. *Protoplasma*, 227(2-4) :129–138, May 2006.
22. Gordon C. Cannon, Sabine Heinhorst, and Cheryl A. Kerfeld. Carboxysomal carbonic anhydrases : Structure and role in microbial CO₂ fixation. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, 1804(2) :382–392, 2010.
23. Benjamin D. Rae, Benedict M. Long, Murray R. Badger, and G. Dean Price. Functions, Compositions, and Evolution of the Two Types of Carboxysomes : Polyhedral Microcompartments That Facilitate CO₂ Fixation in Cyanobacteria and Some Proteobacteria. *Microbiology and Molecular Biology Reviews*, 77(3) :357–379, September 2013.
24. Takakazu Kaneko, Shusei Sato, Hirokazu Kotani, Ayako Tanaka, Erika Asamizu, Yasukazu Nakamura, Nobuyuki Miyajima, Makoto Hirose, Masahiro Sugiura, Shigemi Sasamoto, Takaharu Kimura, Tsutomu Hosouchi, Ai Matsuno, Akiko Muraki, Naomi Nakazaki, Kaoru Naruo, Satomi Okumura, Sayaka Shimpo, Chie Takeuchi, Tsuyuko Wada, Akiko Watanabe, Manabu Yamada, Miho Yasuda, and Satoshi Tabata. Sequence Analysis of the Genome of the Unicellular Cyanobacterium *Synechocystis* sp. Strain PCC6803. II. Sequence Determination of the Entire Genome and Assignment of Potential Protein-coding Regions. *DNA Research*, 3(3) :109–136, January 1996.
25. Miguel A. Hernandez-Prieto and Matthias E. Futschik. CyanoEXpress : A web database for exploration and visualisation of the integrated transcriptome of cyanobacterium *Synechocystis* sp. PCC6803. *Bioinformatics*, 8(13) :634–638, 2012.

26. Jan Mitschke, Jens Georg, Ingeborg Scholz, Cynthia M. Sharma, Dennis Dienst, Jens Bantscheff, Björn Voß, Claudia Steglich, Annegret Wilde, Jörg Vogel, and Wolfgang R. Hess. An experimentally anchored map of transcriptional start sites in the model cyanobacterium *Synechocystis* sp. PCC6803. *Proceedings of the National Academy of Sciences*, 108(5) :2124–2129, February 2011.
27. Ulf Dühring, Ilka M. Axmann, Wolfgang R. Hess, and Annegret Wilde. An internal antisense RNA regulates expression of the photosynthesis gene *isiA*. *Proceedings of the National Academy of Sciences*, 103(18) :7054–7058, May 2006.
28. Yasufumi Umena, Keisuke Kawakami, Jian-Ren Shen, and Nobuo Kamiya. Crystal structure of oxygen-evolving photosystem II at a resolution of 1.9 angstrom. *Nature*, 473(7345) :55–U65, May 2011. WOS :000290218300029.
29. P. Jordan, P. Fromme, H. T. Witt, O. Klukas, W. Saenger, and N. Krauss. Three-dimensional structure of cyanobacterial photosystem I at 2.5 angstrom resolution. *Nature*, 411(6840) :909–917, June 2001. WOS :000169386200034.
30. Noam Adir. Elucidation of the molecular structures of components of the phycobilisome : reconstructing a giant. *Photosynthesis Research*, 85(1) :15–32, July 2005.
31. Ana A Arteni, Ghada Ajlani, and Egbert J Boekema. Structural organisation of phycobilisomes from *Synechocystis* sp. strain PCC6803 and their interaction with the membrane. *Biochimica et biophysica acta*, 1787(4) :272–279, April 2009.
32. Leifu Chang, Xianwei Liu, Yanbing Li, Cui-Cui Liu, Fan Yang, Jindong Zhao, and Sen-Fang Sui. Structural organization of an intact phycobilisome and its association with photosystem II. *Cell Research*, 25(6) :726–737, 2015.
33. A N Glazer. Phycobilisomes : Structure and Dynamics. *Annual Review of Microbiology*, 36(1) :173–198, 1982.
34. Robert MacColl. Cyanobacterial Phycobilisomes. *Journal of Structural Biology*, 124(2–3) :311–334, 1998.
35. Mai Watanabe and Masahiko Ikeuchi. Phycobilisome : architecture of a light-harvesting supercomplex. *Photosynthesis Research*, 116(2-3) :265–276, October 2013.
36. Dr Sidler, J. Bergan, M. Bhupathy, J. Sager, and Rp Volante. The Synthesis of Ltd4 Antagonist Mk-0476. *Abstracts of Papers of the American Chemical Society*, 210 :48–ORGN, August 1995. WOS :A1995RP25900396.
37. Denis Jallet, Adrien Thurotte, Ryan L Leverenz, François Perreau, Cheryl A Kerfeld, and Diana Kirilovsky. Specificity of the cyanobacterial Orange Carotenoid Protein : Influences of OCP and phycobilisome structures. *Plant physiology*, December 2013.
38. Walter A. Sidler. Phycobilisome and Phycobiliprotein Structures. In Donald A. Bryant, editor, *The Molecular Biology of Cyanobacteria*, number 1 in *Advances in Photosynthesis*, pages 139–216. Springer Netherlands, 1994.
39. A. Ducret, W. Sidler, G. Frank, and H. Zuber. The Complete Amino-Acid-Sequence of R-Phycocyanin-I Alpha-Subunit and Beta-Subunit from the Red Alga *Porphyridium-Cruentum* - Structural and Phylogenetic-Relationships of the Phycocyanins Within the Phycobiliprotein Families. *European Journal of Biochemistry*, 221(1) :563–580, April 1994. WOS :A1994NF04200060.
40. Alison E. Murray and Joseph J. Grzymalski. Diversity and genomics of Antarctic marine micro-organisms. *Philosophical Transactions of the Royal Society B-Biological Sciences*, 362(1488) :2259–2271, December 2007. WOS :000250644100006.
41. Alexander N. Glazer. Adaptive Variations in Phycobilisome Structure. In E. Edward Bittar and J. Barber, editor, *Advances in Molecular and Cell Biology*, volume 10 of *Molecular Processes of Photosynthesis*, pages 119–149. Elsevier, 1994.

42. P. J. O'Toole, C. Wolfe, S. Ladha, and R. J. Cherry. Rapid diffusion of spectrin bound to a lipid surface. *Biochimica Et Biophysica Acta*, 1419(1) :64–70, June 1999.
43. Marina G. Rakhimberdieva, Vladimir A. Boichenko, Navassard V. Karapetyan, and Igor N. Stadnichuk. Interaction of Phycobilisomes with Photosystem II Dimers and Photosystem I Monomers and Trimers in the Cyanobacterium *Spirulina platensis*. *Biochemistry*, 40(51) :15780–15788, 2001.
44. Haijun Liu, Hao Zhang, Dariusz M. Niedzwiedzki, Mindy Prado, Guannan He, Michael L. Gross, and Robert E. Blankenship. Phycobilisomes supply excitations to both photosystems in a megacomplex in cyanobacteria. *Science (New York, N.Y.)*, 342(6162) :1104–1107, November 2013.
45. Radek Kaňa, Eva Kotabová, Martin Lukeš, Stěpán Papáček, Ctirad Matonoň, Lu-Ning Liu, Ondřej Prášil, and Conrad W. Mullineaux. Phycobilisome Mobility and Its Role in the Regulation of Light Harvesting in Red Algae. *Plant Physiology*, 165(4) :1618–1631, June 2014.
46. Conrad W Mullineaux and Mary Sarcina. Probing the dynamics of photosynthetic membranes with fluorescence recovery after photobleaching. *Trends in plant science*, 7(6) :237–240, June 2002.
47. Jacco Kromkamp and Anthony E. Walsby. A computer model of buoyancy and vertical migration in cyanobacteria. *Journal of Plankton Research*, 12(1) :161–183, January 1990.
48. Amel Latifi, Marion Ruiz, and Cheng-Cai Zhang. Oxidative stress in cyanobacteria. *FEMS Microbiology Reviews*, 33(2) :258–278, March 2009.
49. Anja Krieger-Liszkay, Christian Fufezan, and Achim Trebst. Singlet oxygen production in photosystem II and related protection mechanism. *Photosynthesis Research*, 98(1-3) :551–564, September 2008.
50. Ateeq Ur Rehman, Krisztián Cser, László Sass, and Imre Vass. Characterization of singlet oxygen production and its involvement in photodamage of Photosystem II in the cyanobacterium *Synechocystis* PCC 6803 by histidine-mediated chemical trapping. *Biochimica Et Biophysica Acta*, 1827(6) :689–698, June 2013.
51. A. William Rutherford, Artur Osyczka, and Fabrice Rappaport. Back-reactions, short-circuits, leaks and other energy wasteful reactions in biological electron transfer : redox tuning to survive life in O(2). *FEBS letters*, 586(5) :603–616, March 2012.
52. Anja Krieger-Liszkay. Singlet oxygen production in photosynthesis. *Journal of Experimental Botany*, 56(411) :337–346, January 2005.
53. Sara Rinalducci, Jens Z. Pedersen, and Lello Zolla. Generation of reactive oxygen species upon strong visible light irradiation of isolated phycobilisomes from *Synechocystis* PCC 6803. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1777(5) :417–424, 2008.
54. Karl-Josef Dietz. Redox Regulation of Transcription Factors in Plant Stress Acclimation and Development. *Antioxidants & Redox Signaling*, 21(9) :1356–1372, September 2014. WOS :000341360100006.
55. G. Storz and J. A. Imlay. Oxidative stress. *Current Opinion in Microbiology*, 2(2) :188–194, April 1999. WOS :000079526800013.
56. Yu Kanesaki, Hiroshi Yamamoto, Kalyanee Paithoonrangsarid, Maria Shumskaya, Iwane Suzuki, Hidenori Hayashi, and Norio Murata. Histidine kinases play important roles in the perception and signal transduction of hydrogen peroxide in the cyanobacterium, *Synechocystis* sp. PCC 6803. *The Plant Journal*, 49(2) :313–324, January 2007.
57. Nils Schuergers and Annegret Wilde. Appendages of the Cyanobacterial Cell. *Life*, 5(1) :700–715, March 2015.

58. Anthony E. Walsby, Ferdinand Schanz, and Martin Schmid. The Burgundy-blood phenomenon : a model of buoyancy change explains autumnal waterblooms by *Planktothrix rubescens* in Lake Zürich. *The New Phytologist*, 169(1) :109–122, 2006.
59. Sarah Joshua and Conrad W. Mullineaux. Phycobilisome Diffusion Is Required for Light-State Transitions in Cyanobacteria. *Plant Physiology*, 135(4) :2112–2119, August 2004.
60. Michael D. McConnell, Randy Koop, Sergej Vasil'ev, and Doug Bruce. Regulation of the Distribution of Chlorophyll and Phycobilin-Absorbed Excitation Energy in Cyanobacteria. A Structure-Based Model for the Light State Transition. *Plant Physiology*, 130(3) :1201–1212, November 2002.
61. Eleonora Sendersky, Noga Kozer, Mali Levi, Yuval Garini, Yaron Shav-Tal, and Rakefet Schwarz. The proteolysis adaptor, NblA, initiates protein pigment degradation by interacting with the cyanobacterial light-harvesting complexes. *The Plant Journal*, 79(1) :118–126, 2014.
62. I. Luque, Jago de Alda, C. Richaud, G. Zabulon, J. C. Thomas, and J. Houmard. The NblAI protein from the filamentous cyanobacterium *Tolypothrix PCC 7601* : regulation of its expression and interactions with phycobilisome components. *Molecular Microbiology*, 50(3) :1043–1054, November 2003. WOS :000185988400026.
63. R. Bienert, K. Baier, R. Volkmer, W. Lockau, and U. Heinemann. Crystal structure of NblA from *Anabaena* sp PCC 7120, a small protein playing a key role in phycobilisome degradation. *Journal of Biological Chemistry*, 281(8) :5216–5223, February 2006. WOS :000235426200076.
64. Anne Karradt, Johanna Sobanski, Jens Mattow, Wolfgang Lockau, and Kerstin Baier. NblA, a Key Protein of Phycobilisome Degradation, Interacts with ClpC, a HSP100 Chaperone Partner of a Cyanobacterial Clp Protease. *Journal of Biological Chemistry*, 283(47) :32394–32403, November 2008.
65. Robert Lehmann, Rainer Machné, Jens Georg, Manuela Benary, Ilka Axmann, and Ralf Steuer. How cyanobacteria pose new problems to old methods : challenges in microarray time series analysis. *BMC bioinformatics*, 14 :133, 2013.
66. Laetitia Houot, Martin Floutier, Benoit Marteyn, Magali Michaut, Antoine Picciocchi, Pierre Legrain, Jean-Christophe Aude, Corinne Cassier-Chauvat, and Franck Chauvat. Cadmium triggers an integrated reprogramming of the metabolism of *Synechocystis PCC6803*, under the control of the Slr1738 regulator. *BMC genomics*, 8 :350, 2007.
67. Claudia Hackenberg, Jan Huege, Annerose Engelhardt, Floyd Wittink, Michael Laue, Hans C. P. Matthijs, Joachim Kopka, Hermann Bauwe, and Martin Hagemann. Low-carbon acclimation in carboxysome-less and photorespiratory mutants of the cyanobacterium *Synechocystis* sp. strain PCC 6803. *Microbiology (Reading, England)*, 158(Pt 2) :398–413, February 2012.
68. Yagut Allahverdiyeva, Janne Isojärvi, Pengpeng Zhang, and Eva-Mari Aro. Cyanobacterial Oxygenic Photosynthesis is Protected by Flavodiiron Proteins. *Life*, 5(1) :716–743, March 2015.
69. Pengpeng Zhang, Marion Eisenhut, Anna-Maria Brandt, Dalton Carmel, Henna M. Silén, Imre Vass, Yagut Allahverdiyeva, Tiina A. Salminen, and Eva-Mari Aro. Operon *flv4-flv2* provides cyanobacterial photosystem II with flexibility of electron transfer. *The Plant Cell*, 24(5) :1952–1971, May 2012.
70. Nataliya Yeremenko, Robert Jeanjean, Peerada Prommeenate, Vladimir Krasikov, Peter J. Nixon, Wim F. J. Vermaas, Michel Havaux, and Hans C. P. Matthijs. Open Reading Frame *ssr2016* is Required for Antimycin A-sensitive Photosystem I-driven Cyclic Electron Flow in the Cyanobacterium *Synechocystis* sp. PCC 6803. *Plant and Cell Physiology*, 46(8) :1433–1436, August 2005.

71. Gábor Bernát, Jens Appel, Teruo Ogawa, and Matthias Rögner. Distinct roles of multiple NDH-1 complexes in the cyanobacterial electron transport network as revealed by kinetic analysis of P700+ reduction in various Ndh-deficient mutants of *Synechocystis* sp. strain PCC6803. *Journal of Bacteriology*, 193(1) :292–295, January 2011.
72. Hongqiao Zhang and Henry Jay Forman. Glutathione synthesis and its role in redox signaling. *Seminars in Cell & Developmental Biology*, 23(7) :722–728, September 2012.
73. Ildikó Domonkos, Mihály Kis, Zoltán Gombos, and Bettina Ughy. Carotenoids, versatile components of oxygenic photosynthesis. *Progress in Lipid Research*, 52(4) :539–561, October 2013.
74. W. C. Neely, J. M. Martin, and S. A. Barker. Products and Relative Reaction Rates of the Oxidation of Tocopherols with Singlet Molecular Oxygen. *Photochemistry and Photobiology*, 48(4) :423–428, October 1988.
75. Christiane Funk and Wim Vermaas. A Cyanobacterial Gene Family Coding for Single-Helix Proteins Resembling Part of the Light-Harvesting Proteins from Higher Plants. *Biochemistry*, 38(29) :9397–9404, 1999.
76. Qingfang He, Nadia Dolganov, Olle Björkman, and Arthur R. Grossman. The High Light-inducible Polypeptides in *Synechocystis* PCC6803 EXPRESSION AND FUNCTION IN HIGH LIGHT. *Journal of Biological Chemistry*, 276(1) :306–314, January 2001.
77. Hristina Staleva, Josef Komenda, Mahendra K. Shukla, Václav Šlouf, Radek Kaňa, Tomáš Polívka, and Roman Sobotka. Mechanism of photoprotection in the cyanobacterial ancestor of plant antenna proteins. *Nature Chemical Biology*, 11(4) :287–291, 2015.
78. Danny Yao, Thomas Kieselbach, Josef Komenda, Kamoltip Promnares, Miguel A. Hernández Prieto, Martin Tichy, Wim Vermaas, and Christiane Funk. Localization of the small CAB-like proteins in photosystem II. *The Journal of Biological Chemistry*, 282(1) :267–276, January 2007.
79. Alexander N. Melkozernov, Thomas S. Bibby, Su Lin, James Barber, and Robert E. Blankenship. Time-resolved absorption and emission show that the CP43' antenna ring of iron-stressed *synechocystis* sp. PCC6803 is efficiently coupled to the photosystem I reaction center core. *Biochemistry*, 42(13) :3893–3903, April 2003.
80. Nataliya Yeremenko, Roman Kouřil, Janne A. Ihalainen, Sandrine D'Haene, Niels van Oosterwijk, Elena G. Andrizhiyevskaya, Wilko Keegstra, Henk L. Dekker, Martin Hagemann, Egbert J. Boekema, Hans C. P. Matthijs, and Jan P. Dekker. Supramolecular Organization and Dual Function of the IsiA Chlorophyll-Binding Protein in Cyanobacteria. *Biochemistry*, 43(32) :10308–10313, 2004.
81. Michal Gwizdala, Adjélé Wilson, and Diana Kirilovsky. In vitro reconstitution of the cyanobacterial photoprotective mechanism mediated by the Orange Carotenoid Protein in *Synechocystis* PCC 6803. *The Plant cell*, 23(7) :2631–2643, July 2011.
82. Diana Kirilovsky and Cheryl A Kerfeld. The Orange Carotenoid Protein : a blue-green light photoactive protein. *Photochemical & photobiological sciences : Official journal of the European Photochemistry Association and the European Society for Photobiology*, 12(7) :1135–1143, July 2013.
83. Diana Kirilovsky and Cheryl A Kerfeld. The orange carotenoid protein in photoprotection of photosystem II in cyanobacteria. *Biochimica et biophysica acta*, 1817(1) :158–166, January 2012.
84. Cheryl A Kerfeld. Water-soluble carotenoid proteins of cyanobacteria. *Archives of biochemistry and biophysics*, 430(1) :2–9, October 2004.

85. Diana Kirilovsky. Photoprotection in cyanobacteria : the orange carotenoid protein (OCP)-related non-photochemical-quenching mechanism. *Photosynthesis research*, 93(1-3) :7–16, September 2007.
86. Clémence Boulay, Adjélé Wilson, Sandrine D’Haene, and Diana Kirilovsky. Identification of a protein required for recovery of full antenna capacity in OCP-related photoprotective mechanism in cyanobacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 107(25) :11620–11625, June 2010.
87. Michal Gwizdala, Adjélé Wilson, Amin Omairi-Nasser, and Diana Kirilovsky. Characterization of the Synechocystis PCC 6803 Fluorescence Recovery Protein involved in photoprotection. *Biochimica et biophysica acta*, 1827(3) :348–354, March 2013.
88. Y. Hihara, A. Kamei, M. Kanehisa, A. Kaplan, and M. Ikeuchi. DNA microarray analysis of cyanobacterial gene expression during acclimation to high light. *The Plant Cell*, 13(4) :793–806, April 2001.
89. Maria A Shoumskaya, Kalyanee Paithoonrangsarid, Yu Kanesaki, Dmitry A Los, Vladislav V Zinchenko, Morakot Tanticharoen, Iwane Suzuki, and Norio Murata. Identical Hik-Rre systems are involved in perception and transduction of salt signals and hyperosmotic signals but regulate the expression of individual genes to different extents in synechocystis. *The Journal of biological chemistry*, 280(22) :21531–21538, June 2005.
90. Lixuan Huang, Michael P. McCluskey, Hao Ni, and Robert A. LaRossa. Global Gene Expression Profiles of the Cyanobacterium Synechocystis sp. Strain PCC 6803 in Response to Irradiation with UV-B and White Light. *Journal of Bacteriology*, 184(24) :6845–6858, December 2002.
91. Hong-Liang Wang, Bradley L. Postier, and Robert L. Burnap. Alterations in Global Patterns of Gene Expression in Synechocystis sp. PCC 6803 in Response to Inorganic Carbon Limitation and the Inactivation of ndhR, a LysR Family Regulator. *Journal of Biological Chemistry*, 279(7) :5739–5751, February 2004.
92. Miguel A. Hernández-Prieto, Verena Schön, Jens Georg, Luísa Barreira, João Varela, Wolfgang R. Hess, and Matthias E. Futschik. Iron deprivation in Synechocystis : inference of pathways, non-coding RNAs, and regulatory elements from comprehensive expression profiling. *G3 (Bethesda, Md.)*, 2(12) :1475–1495, December 2012.
93. Thomas Kay Holt and David W. Krogmann. A carotenoid-protein from cyanobacteria. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 637(3) :408–414, October 1981.
94. Y P Wu and D W Krogmann. The orange carotenoid protein of Synechocystis PCC 6803. *Biochimica et biophysica acta*, 1322(1) :1–7, November 1997.
95. Cheryl A Kerfeld, Michael R Sawaya, Vishnu Brahmamdam, Duilio Cascio, Kwok Ki Ho, Colleen C Trevithick-Sutton, David W Krogmann, and Todd O Yeates. The crystal structure of a cyanobacterial water-soluble carotenoid binding protein. *Structure (London, England : 1993)*, 11(1) :55–65, January 2003.
96. Claire Punginelli, Adjélé Wilson, Jean-Marc Routaboul, and Diana Kirilovsky. Influence of zeaxanthin and echinenone binding on the activity of the orange carotenoid protein. *Biochimica et biophysica acta*, 1787(4) :280–288, April 2009.
97. Adjélé Wilson, Claire Punginelli, Andrew Gall, Cosimo Bonetti, Maxime Alexandre, Jean-Marc Routaboul, Cheryl A Kerfeld, Rienk van Grondelle, Bruno Robert, John T M Kennis, and Diana Kirilovsky. A photoactive carotenoid protein acting as light intensity sensor. *Proceedings of the National Academy of Sciences of the United States of America*, 105(33) :12075–12080, August 2008.

98. Adjélé Wilson, Michal Gwizdala, Alberto Mezzetti, Maxime Alexandre, Cheryl A Kerfeld, and Diana Kirilovsky. The essential role of the N-terminal domain of the orange carotenoid protein in cyanobacterial photoprotection : importance of a positive charge for phycobilisome binding. *The Plant cell*, 24(5) :1972–1983, May 2012.
99. Lijin Tian, Ivo H. M. van Stokkum, Rob B. M. Koehorst, Aniek Jongerius, Diana Kirilovsky, and Herbert van Amerongen. Site, rate, and mechanism of photoprotective quenching in cyanobacteria. *Journal of the American Chemical Society*, 133(45) :18304–18311, November 2011.
100. Lijin Tian, Michal Gwizdala, Ivo H. M. van Stokkum, Rob B. M. Koehorst, Diana Kirilovsky, and Herbert van Amerongen. Picosecond kinetics of light harvesting and photoprotective quenching in wild-type and mutant phycobilisomes isolated from the cyanobacterium *Synechocystis* PCC 6803. *Biophysical Journal*, 102(7) :1692–1700, April 2012.
101. Denis Jallet, Michal Gwizdala, and Diana Kirilovsky. ApcD, ApcF and ApcE are not required for the Orange Carotenoid Protein related phycobilisome fluorescence quenching in the cyanobacterium *Synechocystis* PCC 6803. *Biochimica et biophysica acta*, 1817(8) :1418–1427, August 2012.
102. Alexander V. Ruban, Rudi Berera, Cristian Iliaia, Ivo H. M. van Stokkum, John T. M. Kennis, Andrew A. Pascal, Herbert van Amerongen, Bruno Robert, Peter Horton, and Rienk van Grondelle. Identification of a mechanism of photoprotective energy dissipation in higher plants. *Nature*, 450(7169) :575–U22, November 2007. WOS :000251158500053.
103. Rudi Berera, Michal Gwizdala, Ivo H. M. van Stokkum, Diana Kirilovsky, and Rienk van Grondelle. Excited states of the inactive and active forms of the orange carotenoid protein. *The Journal of Physical Chemistry. B*, 117(31) :9121–9128, August 2013.
104. N. E. Holt, D. Zigmantas, L. Valkunas, X. P. Li, K. K. Niyogi, and G. R. Fleming. Carotenoid cation formation and the regulation of photosynthetic light harvesting. *Science*, 307(5708) :433–436, January 2005. WOS :000226492300050.
105. Stefan Bode, Claudia C. Quentmeier, Pen-Nan Liao, Nour Hafi, Tiago Barros, Laura Wilk, Florian Bittner, and Peter J. Walla. On the regulation of photosynthesis by excitonic interactions between carotenoids and chlorophylls. *Proceedings of the National Academy of Sciences of the United States of America*, 106(30) :12311–12316, July 2009.
106. Rudi Berera, Ivo H M van Stokkum, Michal Gwizdala, Adjélé Wilson, Diana Kirilovsky, and Rienk van Grondelle. The photophysics of the orange carotenoid protein, a light-powered molecular switch. *The journal of physical chemistry. B*, 116(8) :2568–2574, March 2012.
107. A. R. Grossman, M. R. Schaefer, G. G. Chiang, and J. L. Collier. The phycobilisome, a light-harvesting complex responsive to environmental conditions. *Microbiological Reviews*, 57(3) :725–749, September 1993.
108. Nicole Tandeau de Marsac. Phycobiliproteins and phycobilisomes : the early observations. *Photosynthesis Research*, 76(1-3) :193–205, 2003.
109. Adjélé Wilson, Ghada Ajlani, Jean-Marc Verbavatz, Imre Vass, Cheryl A Kerfeld, and Diana Kirilovsky. A soluble carotenoid protein involved in phycobilisome-related energy dissipation in cyanobacteria. *The Plant cell*, 18(4) :992–1007, April 2006.
110. Marina G. Rakhimberdieva, Igor N. Stadnichuk, Irina V. Elanskaya, and Navassard V. Karapetyan. Carotenoid-induced quenching of the phycobilisome fluorescence in photosystem II-deficient mutant of *Synechocystis* sp. *FEBS letters*, 574(1-3) :85–88, September 2004.

111. Tomás Polívka, Cheryl A Kerfeld, Torbjörn Pascher, and Villy Sundström. Spectroscopic properties of the carotenoid 3'-hydroxyechinenone in the orange carotenoid protein from the cyanobacterium *Arthrospira maxima*. *Biochemistry*, 44(10) :3994–4003, March 2005.
112. Maxim Y. Gorbunov, Fedor I. Kuzminov, Victor V. Fadeev, John Dongun Kim, and Paul G. Falkowski. A kinetic model of non-photochemical quenching in cyanobacteria. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1807(12) :1591–1599, December 2011.
113. Adjele Wilson, James N Kinney, Petrus H Zwart, Claire Punginelli, Sandrine D'Haene, François Perreau, Michael G Klein, Diana Kirilovsky, and Cheryl A Kerfeld. Structural determinants underlying photoprotection in the photoactive orange carotenoid protein of cyanobacteria. *The Journal of biological chemistry*, 285(24) :18364–18375, June 2010.
114. Markus Sutter, Adjélé Wilson, Ryan L Leverenz, Rocio Lopez-Igual, Adrien Thurotte, Annette E Salmeen, Diana Kirilovsky, and Cheryl A Kerfeld. Crystal structure of the FRP and identification of the active site for modulation of OCP-mediated photoprotection in cyanobacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 110(24) :10022–10027, June 2013.
115. Clémence Boulay, Leyla Abasova, Christophe Six, Imre Vass, and Diana Kirilovsky. Occurrence and function of the orange carotenoid protein in photoprotective mechanisms in various cyanobacteria. *Biochimica Et Biophysica Acta*, 1777(10) :1344–1354, October 2008.
116. An Glazer. Phycobilisome - a Macromolecular Complex Optimized for Light Energy-Transfer. *Biochimica Et Biophysica Acta*, 768(1) :29–51, 1984. WOS :A1984SQ18500002.
117. Bryant DA. Cyanobacterial phycobilisomes : progress towards a complete structural and functional analysis via molecular genetics. *The Molecular Biology of Plastids and Mitochondria*. Academic Press, New York - L Bogorad, IK Vasil, eds, pages 257–300, 1991.
118. JD Zhao, JH Zhou, and DA Bryant. Energy transfer processes in phycobilisomes as deduced from mutational analyses. *Photosynth Res*, 34(83), 1992.
119. G. Shen, S. Boussiba, and W. F. Vermaas. *Synechocystis* sp PCC 6803 strains lacking photosystem I and phycobilisome function. *The Plant Cell*, 5(12) :1853–1863, December 1993.
120. Ghada Ajlani, Claudie Vernotte, Lisa DiMagno, and Robert Haselkorn. Phycobilisome core mutants of *Synechocystis* PCC 6803. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1231(2) :189–196, September 1995.
121. Ghada Ajlani and Claudie Vernotte. Construction and characterization of a phycobiliprotein-less mutant of *Synechocystis* sp. PCC 6803. *Plant Molecular Biology*, 37(3) :577–580, 1998.
122. V. Capuano, A. S. Braux, N. Tandeau de Marsac, and J. Houmard. The "anchor polypeptide" of cyanobacterial phycobilisomes. Molecular characterization of the *Synechococcus* sp. PCC 6301 *apce* gene. *Journal of Biological Chemistry*, 266(11) :7239–7247, April 1991.
123. V. Capuano, J. C. Thomas, N. Tandeau de Marsac, and J. Houmard. An in vivo approach to define the role of the LCM, the key polypeptide of cyanobacterial phycobilisomes. *Journal of Biological Chemistry*, 268(11) :8277–8283, April 1993.
124. M. Glauser, W. A. Sidler, K. W. Graham, D. A. Bryant, G. Frank, E. Wehrli, and H. Zuber. Three C-phycoerythrin-associated linker polypeptides in the phycobilisome of green-light-grown *Calothrix* sp. PCC 7601 (cyanobacteria). *FEBS letters*, 297(1-2) :19–23, February 1992.

125. A. Ducret, S. A. Müller, K. N. Goldie, A. Hefti, W. A. Sidler, H. Zuber, and A. Engel. Reconstitution, characterisation and mass analysis of the pentacylindrical allophycocyanin core complex from the cyanobacterium *Anabaena* sp. PCC 7120. *Journal of Molecular Biology*, 278(2) :369–388, May 1998.
126. Judith A. Kipe-Nolt, S. Edward Stevens, and Donald A. Bryant. Growth and Chromatic Adaptation of *Nostoc* sp. Strain MAC and the Pigment Mutant R-MAC. *Plant Physiology*, 70(5) :1549–1553, November 1982.
127. D. J. Lundell, R. C. Williams, and A. N. Glazer. Molecular architecture of a light-harvesting antenna. In vitro assembly of the rod substructures of *Synechococcus* 6301 phycobilisomes. *Journal of Biological Chemistry*, 256(7) :3580–3592, April 1981.
128. A. Ducret, W. Sidler, E. Wehrli, G. Frank, and H. Zuber. Isolation, characterization and electron microscopy analysis of a hemidiscoidal phycobilisome type from the cyanobacterium *Anabaena* sp PCC 7120. *European Journal of Biochemistry*, 236(3) :1010–1024, March 1996. WOS :A1996UX16200034.
129. Pinthipya Nomsawai, Nicole Tandeau de Marsac, Jean Claude Thomas, Morakot Tanticharoen, and Supapon Cheevadhanarak. Light Regulation of Phycobilisome Structure and Gene Expression in *Spirulina platensis* C1 (*Arthrospira* sp. PCC 9438). *Plant and Cell Physiology*, 40(12) :1194–1202, January 1999.
130. Irina Piven, Ghada Ajlani, and Anna Sokolenko. Phycobilisome Linker Proteins Are Phosphorylated in *Synechocystis* sp. PCC 6803. *Journal of Biological Chemistry*, 280(22) :21667–21672, June 2005.
131. T. Sudhakar Babu, Ashok Kumar, and Ajit K. Varma. Effect of Light Quality on Phycobilisome Components of the Cyanobacterium *Spirulina platensis*. *Plant Physiology*, 95(2) :492–497, February 1991.
132. Adjélé Wilson, Claire Punginelli, Mohea Couturier, François Perreau, and Diana Kirilovsky. Essential role of two tyrosines and two tryptophans on the photoprotection activity of the Orange Carotenoid Protein. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1807(3) :293–301, March 2011.
133. Delphine Lagarde, Laurent Beuf, and Wim Vermaas. Increased Production of Zeaxanthin and Other Pigments by Application of Genetic Engineering Techniques to *Synechocystis* sp. Strain PCC 6803. *Applied and Environmental Microbiology*, 66(1) :64–72, January 2000.
134. Ailie Marx and Noam Adir. Allophycocyanin and phycocyanin crystal structures reveal facets of phycobilisome assembly. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1827(3) :311–318, March 2013.
135. Igor N. Stadnichuk, Mikhail F. Yanyushin, Evgeni G. Maksimov, Evgeni P. Lukashev, Sergei K. Zharmukhamedov, Irina V. Elanskaya, and Vladimir Z. Paschenko. Site of non-photochemical quenching of the phycobilisome by orange carotenoid protein in the cyanobacterium *Synechocystis* sp. PCC 6803. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1817(8) :1436–1445, August 2012.
136. F. I. Kuzminov, N. V. Karapetyan, M. G. Rakhimberdieva, I. V. Elanskaya, M. Y. Gorbunov, and V. V. Fadeev. Investigation of OCP-triggered dissipation of excitation energy in PSI/PSII-less *Synechocystis* sp. PCC 6803 mutant using non-linear laser fluorimetry. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1817(7) :1012–1021, 2012.
137. M. Herdman, S. Delaney, and Ng Carr. New Medium for Isolation and Growth of Auxotrophic Mutants of Blue-Green-Alga *Anacystis-Nidulans*. *Journal of General Microbiology*, 79(DEC) :233–237, 1973. WOS :A1973R793100007.
138. G. Yamanaka, A. N. Glazer, and R. C. Williams. Cyanobacterial phycobilisomes. Characterization of the phycobilisomes of *Synechococcus* sp. 6301. *Journal of Biological Chemistry*, 253(22) :8303–8310, November 1978.

139. Y. Kashino, H. Koike, and K. Satoh. An improved sodium dodecyl sulfate-polyacrylamide gel electrophoresis system for the analysis of membrane protein complexes. *Electrophoresis*, 22(6) :1004–1007, April 2001.
140. Celine Bourcier de Carbon, Adrien Thurotte, Adjele Wilson, Francois Perreau, and Diana Kirilovsky. Biosynthesis of soluble carotenoid holoproteins in *Escherichia coli*. *Scientific Reports*, 5 :9085, March 2015. WOS :000351149500016.
141. Arezki Sedoud, Rocío López-Igual, Ateeq Ur Rehman, Adjélé Wilson, François Perreau, Clémence Boulay, Imre Vass, Anja Krieger-Liszkay, and Diana Kirilovsky. The Cyanobacterial Photoactive Orange Carotenoid Protein Is an Excellent Singlet Oxygen Quencher. *The Plant cell*, April 2014.
142. G. A. Armstrong. Genetics of eubacterial carotenoid biosynthesis : a colorful tale. *Annual Review of Microbiology*, 51 :629–659, 1997.
143. G. Britton. Structure and properties of carotenoids in relation to function. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 9(15) :1551–1558, December 1995.
144. R. Edge, D. J. McGarvey, and T. G. Truscott. The carotenoids as anti-oxidants — a review. *Journal of Photochemistry and Photobiology B : Biology*, 41(3) :189–200, 1997.
145. Wilhelm Stahl and Helmut Sies. Antioxidant activity of carotenoids. *Molecular Aspects of Medicine*, 24(6) :345–351, 2003.
146. H. A. Frank and R. J. Cogdell. Carotenoids in photosynthesis. *Photochemistry and Photobiology*, 63(3) :257–264, March 1996.
147. Shinji Kawasaki, Keisuke Mizuguchi, Masaru Sato, Tetsuya Kono, and Hirofumi Shimizu. A novel astaxanthin-binding photooxidative stress-inducible aqueous carotenoprotein from a eukaryotic microalga isolated from asphalt in midsummer. *Plant & Cell Physiology*, 54(7) :1027–1040, July 2013.
148. Jodi Pilbrow, Daniel Garama, and Alan Carne. Carotenoid-binding proteins ; accessories to carotenoid function. *Acta Biochimica Polonica*, 59(1) :163–165, 2012.
149. Cheryl A. Kerfeld and Diana Kirilovsky. Structural, Mechanistic and Genomic Insights into OCP-Mediated Photoprotection. In *Advances in Botanical Research*, pages 1–26. Elsevier BV, 2013.
150. Ryan L Leverenz, Denis Jallet, Ming-De Li, Richard A Mathies, Diana Kirilovsky, and Cheryl A Kerfeld. Structural and Functional Modularity of the Orange Carotenoid Protein : Distinct Roles for the N- and C-Terminal Domains in Cyanobacterial Photoprotection. *The Plant cell*, January 2014.
151. Prakash Bhosale and Paul S. Bernstein. Microbial xanthophylls. *Applied Microbiology and Biotechnology*, 68(4) :445–455, September 2005.
152. Prakash Bhosale, Alexander J. Larson, Jeanne M. Frederick, Katie Southwick, Craig D. Thulin, and Paul S. Bernstein. Identification and characterization of a Pi isoform of glutathione S-transferase (GSTP1) as a zeaxanthin-binding protein in the macula of the human eye. *The Journal of Biological Chemistry*, 279(47) :49447–49454, November 2004.
153. Amitabha Das, Sang-Hwal Yoon, Sook-Hee Lee, Jae-Yean Kim, Deok-Kun Oh, and Seon-Won Kim. An update on microbial carotenoid production : application of recent metabolic engineering tools. *Applied Microbiology and Biotechnology*, 77(3) :505–512, December 2007.
154. Victor M. Ye and Sujata K. Bhatia. Pathway engineering strategies for production of beneficial carotenoids in microbial hosts. *Biotechnology Letters*, 34(8) :1405–1414, April 2012.
155. Prakash Bhosale and Paul S. Bernstein. Synergistic effects of zeaxanthin and its binding protein in the prevention of lipid membrane oxidation. *Biochimica Et Biophysica Acta*, 1740(2) :116–121, May 2005.

156. E. Giuffra, D. Cugini, R. Croce, and R. Bassi. Reconstitution and pigment-binding properties of recombinant CP29. *European journal of biochemistry / FEBS*, 238(1) :112–120, May 1996.
157. H. Paulsen and W. Rüdiger. Reconstitution of Chlorophyll A,B-Containing Complexes from LHCP Overproduced in Bacteria. In M. Baltscheffsky, editor, *Current Research in Photosynthesis*, pages 2633–2636. Springer Netherlands, 1990. DOI : 10.1007/978-94-009-0511-5_595.
158. Hao Zhang, Haijun Liu, Dariusz M. Niedzwiedzki, Mindy Prado, Jing Jiang, Michael L. Gross, and Robert E. Blankenship. Molecular Mechanism of Photoactivation and Structural Location of the Cyanobacterial Orange Carotenoid Protein. *Biochemistry*, 53(1) :13–19, January 2014.
159. Karin Lemuth, Kristin Steuer, and Christoph Albermann. Engineering of a plasmid-free Escherichia coli strain for improved in vivo biosynthesis of astaxanthin. *Microbial Cell Factories*, 10 :29, 2011.
160. T. Makino. Characterization of cyanobacterial carotenoid ketolase CrtW and hydroxylase CrtR by complementation analysis in Escherichia coli. *Plant & Cell Physiology*, 49 :1867–1878, 2008.
161. F. X. Cunningham. Functional analysis of the beta and epsilon lycopene cyclase enzymes of Arabidopsis reveals a mechanism for control of cyclic carotenoid formation. *Plant Cell*, 8 :1613–1626, 1996.
162. N. Misawa. Structure and Functional-Analysis of a Marine Bacterial Carotenoid Biosynthesis Gene-Cluster and Astaxanthin Biosynthetic-Pathway Proposed at the Gene Level. *Journal Of Bacteriology*, 177 :6575–6584, 1995.
163. B. Fernandez-Gonzalez, G. Sandmann, and A. Vioque. A new type of asymmetrically acting beta-carotene ketolase is required for the synthesis of echinenone in the cyanobacterium Synechocystis sp. PCC 6803. *J Biol Chem*, 272 :9728–9733, 1997.
164. P. D. Fraser, Y. Miura, and N. Misawa. In vitro characterization of astaxanthin biosynthetic enzymes. *J Biol Chem*, 272 :6128–6135, 1997.
165. J. L. Anderson. Constructing a man-made c-type cytochrome maquette : electron transfer, oxygen transport and conversion to a photoactive light harvesting maquette. *Chemical Science*, 5 :507–514, 2014.
166. N. J. Claassens, M. Volpers, V. A. dos Santos, J. van der Oost, and W. M. de Vos. Potential of proton-pumping rhodopsins : engineering photosystems into microorganisms. *Trends In Biotechnology*, 31 :633–642, 2013.
167. T. A. Farid. Elementary tetrahelical protein design for diverse oxidoreductase functions. *Nature Chemical Biology*, 9 :826–833, 2013.
168. K. Hingorani. Photo-oxidation of tyrosine in a bio-engineered bacterioferritin reaction centre-a protein model for artificial photosynthesis. *Biochim Biophys Acta*, 1837 :1821–1834, 2014.
169. E. T. Johnson and C. Schmidt-Dannert. Light-energy conversion in engineered microorganisms. *Trends In Biotechnology*, 26 :682–689, 2008.
170. P. Horton, A. V. Ruban, and R. G. Walters. Regulation of light harvesting in green plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, 47 :655–684, 1996. WOS :A1996UT11900025.
171. Peter Horton and Alexander Ruban. Molecular design of the photosystem II light-harvesting antenna : photosynthesis and photoprotection. *Journal of Experimental Botany*, 56(411) :365–373, January 2005.
172. Peter Jahns and Alfred R. Holzwarth. The role of the xanthophyll cycle and of lutein in photoprotection of photosystem II. *Biochimica Et Biophysica Acta-Bioenergetics*, 1817(1) :182–193, January 2012. WOS :000298823000014.

173. Diana Kirilovsky. Modulating energy arriving at photochemical reaction centers : orange carotenoid protein-related photoprotection and state transitions. *Photosynthesis Research*, August 2014.
174. Tomáš Polívka, Pavel Chábera, and Cheryl A Kerfeld. Carotenoid-protein interaction alters the S(1) energy of hydroxyechinenone in the Orange Carotenoid Protein. *Biochimica et biophysica acta*, 1827(3) :248–254, March 2013.
175. Dariusz M. Niedzwiedzki, Haijun Liu, and Robert E. Blankenship. Excited State Properties of 3'-Hydroxyechinenone in Solvents and in the Orange Carotenoid Protein from *Synechocystis* sp. PCC 6803. *The Journal of Physical Chemistry. B*, 118(23) :6141–6149, June 2014.
176. Lijin Tian, Ivo H. M. van Stokkum, Rob B. M. Koehorst, and Herbert van Amerongen. Light harvesting and blue-green light induced non-photochemical quenching in two different C-phycoerythrin mutants of *Synechocystis* PCC 6803. *The Journal of Physical Chemistry. B*, 117(38) :11000–11006, September 2013.
177. Hiroko Takahashi, Sophie Clowez, Francis-André Wollman, Olivier Vallon, and Fabrice Rappaport. Cyclic electron flow is redox-controlled but independent of state transition. *Nature Communications*, 4 :1954, 2013.
178. Marina G. Rakhimberdieva, Irina V. Elanskaya, Wim F. J. Vermaas, and Navasard V. Karapetyan. Carotenoid-triggered energy dissipation in phycobilisomes of *Synechocystis* sp. PCC 6803 diverts excitation away from reaction centers of both photosystems. *Biochimica Et Biophysica Acta*, 1797(2) :241–249, February 2010.
179. Haijun Liu, Hao Zhang, Jeremy D. King, Nathan R. Wolf, Mindy Prado, Michael L. Gross, and Robert E. Blankenship. Mass spectrometry footprinting reveals the structural rearrangements of cyanobacterial orange carotenoid protein upon light activation. *Biochimica Et Biophysica Acta*, 1837(12) :1955–1963, December 2014.
180. Frederick M Ausubel. *Current protocols in molecular biology*. J. Wiley, [New York], 2006.
181. Michael Zuker. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research*, 31(13) :3406–3415, July 2003.
182. Roberta Croce and Herbert van Amerongen. Natural strategies for photosynthetic light harvesting. *Nature Chemical Biology*, 10(7) :492–501, 2014.
183. P. Horton, A. V. Ruban, and R. G. Walters. Regulation of Light Harvesting in Green Plants (Indication by Nonphotochemical Quenching of Chlorophyll Fluorescence). *Plant Physiology*, 106(2) :415–420, October 1994.
184. Krishna K. Niyogi and Thuy B. Truong. Evolution of flexible non-photochemical quenching mechanisms that regulate light harvesting in oxygenic photosynthesis. *Current Opinion in Plant Biology*, 16(3) :307–314, June 2013.
185. Andrew A. Pascal, Zhenfeng Liu, Koen Broess, Bart van Oort, Herbert van Amerongen, Chao Wang, Peter Horton, Bruno Robert, Wenrui Chang, and Alexander Ruban. Molecular basis of photoprotection and control of photosynthetic light-harvesting. *Nature*, 436(7047) :134–137, July 2005.
186. Tae Kyu Ahn, Thomas J. Avenson, Matteo Ballottari, Yuan-Chung Cheng, Krishna K. Niyogi, Roberto Bassi, and Graham R. Fleming. Architecture of a Charge-Transfer State Regulating Light Harvesting in a Plant Antenna Protein. *Science*, 320(5877) :794–797, May 2008.
187. Peter Jomo Walla, Christoph-Peter Holleboom, and Graham R. Fleming. Electronic Carotenoid-Chlorophyll Interactions Regulating Photosynthetic Light Harvesting of Higher Plants and Green Algae. In Barbara Demmig-Adams, Gyoza Garab, William Adams III, and Govindjee, editors, *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, number 40 in *Advances in Photosynthesis and Respiration*, pages 229–243. Springer Netherlands, 2014. DOI : 10.1007/978-94-017-9032-1_9.

188. Sayan Gupta, Rhijuta D'Mello, and Mark R. Chance. Structure and dynamics of protein waters revealed by radiolysis and mass spectrometry. *Proceedings of the National Academy of Sciences*, 109(37) :14882–14887, September 2012.
189. C. A. Kerfeld, Diana Kirilovsky, and M. Alexandre. *Carotenoids : physical, chemical, and biological functions and properties*. CRC Press, Boca Raton, 2010.
190. Ulrich K. Genick, Gloria E. O. Borgstahl, Kingman Ng, Zhong Ren, Claude Pradervand, Patrick M. Burke, Vukica Šrajer, Tsu-Yi Teng, Wilfried Schildkamp, Duncan E. McRee, Keith Moffat, and Elizabeth D. Getzoff. Structure of a Protein Photocycle Intermediate by Millisecond Time-Resolved Crystallography. *Science*, 275(5305) :1471–1475, March 1997.
191. Jeremy D. King, Haijun Liu, Guannan He, Gregory S. Orf, and Robert E. Blankenship. Chemical activation of the cyanobacterial orange carotenoid protein. *FEBS letters*, 588(24) :4561–4565, December 2014.
192. Gregory D. Scholes and Kenneth P. Ghiggino. Electronic Interactions and Interchromophore Excitation Transfer. *The Journal of Physical Chemistry*, 98(17) :4580–4590, 1994.
193. Wolfgang Kabsch. XDS. *Acta Crystallographica. Section D, Biological Crystallography*, 66(Pt 2) :125–132, February 2010.
194. Martyn D. Winn, Charles C. Ballard, Kevin D. Cowtan, Eleanor J. Dodson, Paul Emsley, Phil R. Evans, Ronan M. Keegan, Eugene B. Krissinel, Andrew G. W. Leslie, Airlie McCoy, Stuart J. McNicholas, Garib N. Murshudov, Navraj S. Pannu, Elizabeth A. Potterton, Harold R. Powell, Randy J. Read, Alexei Vagin, and Keith S. Wilson. Overview of the CCP4 suite and current developments. *Acta Crystallographica. Section D, Biological Crystallography*, 67(Pt 4) :235–242, April 2011.
195. Frank DiMaio, Nathaniel Echols, Jeffrey J. Headd, Thomas C. Terwilliger, Paul D. Adams, and David Baker. Improved low-resolution crystallographic refinement with Phenix and Rosetta. *Nature Methods*, 10(11) :1102–1104, November 2013.
196. Paul D. Adams, Pavel V. Afonine, Gábor Bunkóczi, Vincent B. Chen, Nathaniel Echols, Jeffrey J. Headd, Li-Wei Hung, Swati Jain, Gary J. Kapral, Ralf W. Grosse Kunstleve, Airlie J. McCoy, Nigel W. Moriarty, Robert D. Oeffner, Randy J. Read, David C. Richardson, Jane S. Richardson, Thomas C. Terwilliger, and Peter H. Zwart. The Phenix software for automated determination of macromolecular structures. *Methods (San Diego, Calif.)*, 55(1) :94–106, September 2011.
197. P. Emsley, B. Lohkamp, W. G. Scott, and K. Cowtan. Features and development of Coot. *Acta Crystallographica. Section D, Biological Crystallography*, 66(Pt 4) :486–501, April 2010.
198. Eric F. Pettersen, Thomas D. Goddard, Conrad C. Huang, Gregory S. Couch, Daniel M. Greenblatt, Elaine C. Meng, and Thomas E. Ferrin. UCSF Chimera—a visualization system for exploratory research and analysis. *Journal of Computational Chemistry*, 25(13) :1605–1612, October 2004.
199. B. Lee and F. M. Richards. The interpretation of protein structures : estimation of static accessibility. *Journal of Molecular Biology*, 55(3) :379–400, February 1971.
200. Nathan A. Baker, David Sept, Simpson Joseph, Michael J. Holst, and J. Andrew McCammon. Electrostatics of nanosystems : Application to microtubules and the ribosome. *Proceedings of the National Academy of Sciences*, 98(18) :10037–10041, August 2001.
201. Gavin E. Crooks, Gary Hon, John-Marc Chandonia, and Steven E. Brenner. WebLogo : A Sequence Logo Generator. *Genome Research*, 14(6) :1188–1190, June 2004.

202. Sayan Gupta, Jin Chai, Jie Cheng, Rhijuta D'Mello, Mark R. Chance, and Dax Fu. Visualizing the kinetic power stroke that drives proton-coupled zinc(II) transport. *Nature*, 512(7512) :101–104, August 2014.
203. Sidhartha Chaudhury and Jeffrey J. Gray. Conformer selection and induced fit in flexible backbone protein-protein docking using computational and NMR ensembles. *Journal of Molecular Biology*, 381(4) :1068–1087, September 2008. WOS :000258971300021.
204. Krishna K Niyogi. Safety valves for photosynthesis. *Current Opinion in Plant Biology*, 3(6) :455–460, December 2000.
205. Cheryl A Kerfeld. Structure and function of the water-soluble carotenoid-binding proteins of cyanobacteria. *Photosynthesis research*, 81(3) :215–225, 2004.
206. C. A. Kerfeld and D. Kirilovsky. *Structure, Function and Evolution of OCP-based Photoprotection in Cyanobacteria*. *Genomics of Cyanobacteria*, eds., Franck Chauvat and Corrine Cassier-Chauvat., 2013.
207. Evgeny Krissinel and Kim Henrick. Inference of macromolecular assemblies from crystalline state. *Journal of Molecular Biology*, 372(3) :774–797, September 2007. WOS :000249494800018.
208. Chu Wang, Philip Bradley, and David Baker. Protein-protein docking with backbone flexibility. *Journal of Molecular Biology*, 373(2) :503–519, October 2007.
209. Sidhartha Chaudhury, Monica Berrondo, Brian D. Weitzner, Pravin Muthu, Hannah Bergman, and Jeffrey J. Gray. Benchmarking and analysis of protein docking performance in Rosetta v3.2. *PLoS One*, 6(8) :e22477, 2011.
210. Pavel Chábera, Milan Durchan, Patrick M. Shih, Cheryl A. Kerfeld, and Tomáš Polívka. Excited-state properties of the 16kda red carotenoid protein from *Arthrospira maxima*. *Biochimica Et Biophysica Acta*, 1807(1) :30–35, January 2011.
211. D. C. Wiley and J. J. Skehel. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annual Review of Biochemistry*, 56 :365–394, 1987.
212. George M. Sheldrick. A short history of SHELX. *Acta Crystallographica. Section A, Foundations of Crystallography*, 64(Pt 1) :112–122, January 2008.
213. Pavel V. Afonine, Ralf W. Grosse-Kunstleve, Nathaniel Echols, Jeffrey J. Headd, Nigel W. Moriarty, Marat Mustyakimov, Thomas C. Terwilliger, Alexandre Urzhumtsev, Peter H. Zwart, and Paul D. Adams. Towards automated crystallographic structure refinement with phenix.refine. *Acta Crystallographica. Section D, Biological Crystallography*, 68(Pt 4) :352–367, April 2012.
214. Paul Emsley and Kevin Cowtan. Coot : model-building tools for molecular graphics. *Acta Crystallographica. Section D, Biological Crystallography*, 60(Pt 12 Pt 1) :2126–2132, December 2004.
215. Thomas D. Schneider and R. Michael Stephens. Sequence logos : a new way to display consensus sequences. *Nucleic Acids Research*, 18(20) :6097–6100, October 1990.
216. Adrien Thurotte, Rocio Lopez Igual, Adjélé Wilson, Léa Comolet, Céline Bourcier de Carbon, Fugui Xiao, and Diana Kirilovsky. Regulation of Orange Carotenoid Protein activity in cyanobacterial photoprotection. *Plant Physiology*, page pp.00843.2015, July 2015.
217. Ryan L. Leverenz, Markus Sutter, Adjélé Wilson, Sayan Gupta, Adrien Thurotte, Céline Bourcier de Carbon, Christopher J. Petzold, Corie Ralston, François Perreau, Diana Kirilovsky, and Cheryl A. Kerfeld. A 12 Å carotenoid translocation in a photoswitch associated with cyanobacterial photoprotection. *Science (New York, N.Y.)*, 348(6242) :1463–1466, June 2015.

218. Eugene G. Maksimov, Evgeny A. Shirshin, Nikolai N. Sluchanko, Dmitry V. Zlenko, Evgenia Y. Parshina, Georgy V. Tsoraev, Konstantin E. Klementiev, Gleb S. Budylin, Franz-Josef Schmitt, Thomas Friedrich, Victor V. Fadeev, Vladimir Z. Paschenko, and Andrew B. Rubin. The Signaling State of Orange Carotenoid Protein. *Biophysical Journal*, 109(3) :595–607, 2015.
219. Igor N. Stadnichuk, Mikhail F. Yanyushin, Gábor Bernát, Dmitry V. Zlenko, Pavel M. Krasilnikov, Evgeny P. Lukashov, Evgeny G. Maksimov, and Vladimir Z. Paschenko. Fluorescence quenching of the phycobilisome terminal emitter LCM from the cyanobacterium *Synechocystis* sp. PCC 6803 detected in vivo and in vitro. *Journal of Photochemistry and Photobiology. B, Biology*, 125 :137–145, August 2013.
220. Maria Müller, Christian Bamann, Ernst Bamberg, and Werner Kühlbrandt. Light-induced helix movements in channelrhodopsin-2. *Journal of Molecular Biology*, 427(2) :341–349, January 2015.
221. Matthew J. Cuneo, Anita Changela, Aleksandr E. Miklos, Lorena S. Beese, Joanna K. Krueger, and Homme W. Hellinga. Structural analysis of a periplasmic binding protein in the tripartite ATP-independent transporter family reveals a tetrameric assembly that may have a role in ligand transport. *The Journal of Biological Chemistry*, 283(47) :32812–32820, November 2008.
222. Alexey G. Kikhney and Dmitri I. Svergun. A practical guide to small angle X-ray scattering (SAXS) of flexible and intrinsically disordered proteins. *FEBS letters*, August 2015.
223. Masayuki Muramatsu and Yukako Hihara. Acclimation to high-light conditions in cyanobacteria : from gene expression to physiological responses. *Journal of plant research*, 125(1) :11–39, January 2012.
224. Kristin K. Koretke, Andrei N. Lupas, Patrick V. Warren, Martin Rosenberg, and James R. Brown. Evolution of Two-Component Signal Transduction. *Molecular Biology and Evolution*, 17(12) :1956–1970, December 2000.
225. Kalyanee Paithoonrangsarid, Maria A Shoumskaya, Yu Kanesaki, Syusei Satoh, Satoshi Tabata, Dmitry A Los, Vladislav V Zinchenko, Hidenori Hayashi, Morakot Tanticharoen, Iwane Suzuki, and Norio Murata. Five histidine kinases perceive osmotic stress and regulate distinct sets of genes in *Synechocystis*. *The Journal of biological chemistry*, 279(51) :53078–53086, December 2004.
226. Iwane Suzuki, Yu Kanesaki, Hidenori Hayashi, John J. Hall, William J. Simon, Antoni R. Slabas, and Norio Murata. The Histidine Kinase Hik34 Is Involved in Thermotolerance by Regulating the Expression of Heat Shock Genes in *Synechocystis*. *Plant Physiology*, 138(3) :1409–1421, July 2005.
227. Yukako Hihara, Kintake Sonoike, Minoru Kanehisa, and Masahiko Ikeuchi. DNA Microarray Analysis of Redox-Responsive Genes in the Genome of the Cyanobacterium *Synechocystis* sp. Strain PCC 6803. *Journal of Bacteriology*, 185(5) :1719–1725, March 2003.

Study of Functional domains involved in the interaction between the photoprotective cyanobacterial Orange Carotenoid Protein and its partners

Keywords : Cyanobacteria, Phycobilisome, OCP

Cyanobacteria, as procaryote photosynthetic organism, harvest light for living. But harvesting too much light can be harmful. To protect themselves against this stress, cyanobacteria have developed several photoprotective mechanisms. This manuscript reports my PhD thesis about one of these mechanisms by combined approaches of molecular biology, biochemistry and biophysics.

Cyanobacterial light harvesting antenna are extra-membranous complexes called phycobilisome. They funnel harvested energy to the photosynthetic reaction centers. Under high light, high energy input induces the formation of reactive oxygen species (ROS), which are harmful in excess. One of the existing photoprotective mechanisms helps to avoid ROS formation by decreasing the energy arriving at the reaction centers. The main actor of this mechanism is the photoactive Orange Carotenoid Protein (OCP) that binds to the phycobilisome, and induces an increase of the part of energy dissipated as heat. The OCP is a protein composed of two globular domains (called N- and C-terminal) and binds a carotenoid cofactor. High intensity of blue-green light triggers conformational changes in the inactive orange OCP, which turns red and is now able to bind the PBs. Under low light conditions, this mechanism is turned off by another protein, the Fluorescence Recovery Protein (FRP).

The first article (chapter 2) presented in this manuscript reports the study of the specificity of OCPs isolated from two strains for different classes of phycobilisomes whose core architecture differs. The second one (chapter 3) presents the protocol of overexpression and purification of OCP in *E.coli* setup at the laboratory. Furthermore, it reports the characterization of OCP cloned from *Synechocystis*, *A. variabilis* and *A. platensis*. The third (chapter 4) one presents the tridimensional structure of the N-terminal domain of the OCP. In this chapter, we demonstrate that the carotenoid undergoes a 12Å movement upon photoactivation. The fourth (chapter 5) reports that the N-terminal arm of the OCP helps to maintain the inactive orange OCP closed in darkness or low light, avoiding activation and consequent unwanted PBs fluorescence quenching. The fifth (chapter 6) presents the resolution of the structure and the identification of the active site of the FRP. These data allow to compute a predictionnal model of interaction between OCP and FRP. I assess the validity of the model by isolating several modified OCPs. Results shown in chapter 7 report that the aspartate 220 and the phenylalanine 299 are required for effective FRP action.



Étude des domaines fonctionnels impliqués dans l'interaction entre la protéine cyanobactérienne photoprotective Orange Carotenoid Protein et ses partenaires

Keywords : Cyanobacterie, Phycobilisome, OCP

Les cyanobactéries sont des organismes procaryotes photosynthétiques qui utilisent l'énergie lumineuse pour vivre. Cependant, un excès de cette énergie essentielle est délétère. Afin de se protéger, elles ont acquis plusieurs mécanismes de photoprotection. La thématique de ma thèse est l'étude de l'un d'eux par des approches combinées de biologie moléculaire, biochimie et biophysique.

Les antennes collectrices de lumière des cyanobactéries sont des complexes extra-membranaires solubles appelés les phycobilisomes. Ils permettent de capturer la lumière et canaliser l'énergie vers les centres réactionnels des photosystèmes. Sous forte lumière, l'afflux d'énergie y parvenant augmente la génération d'espèces réactives de l'oxygène délétères pour la cellule. L'Orange Carotenoid Protein (OCP) est impliquée dans un mécanisme de photoprotection qui diminue l'énergie arrivant au niveau des centres réactionnels en augmentant la part d'énergie dissipée sous forme de chaleur. L'OCP est une caroténo-protéine composée de deux domaines globulaires N- et C-terminal qui lie un caroténoïde. Cette protéine photoactivable est à la fois le senseur et l'acteur du mécanisme de photoprotection. Le mécanisme est désactivé par une seconde protéine, la FRP (Fluorescence Recovery Protein).

Le premier article (chapitre 2) présenté dans ce travail de thèse rapporte l'étude de la spécificité des OCPs isolées chez deux souches différentes pour différentes classes de phycobilisomes aux architectures du coeur différentes. Le second article (chapitre 3) présente la méthode mise au point au laboratoire de production de l'OCP chez *E.coli*, ainsi que la caractérisation d'OCPs clonées depuis le génome de *Synechocystis*, *A. variabilis* et *A. platensis* et surexprimés chez *E. coli*. Le troisième (chapitre 4) présente la structure tridimensionnelle du domaine N-terminal, qui est le domaine effecteur de l'OCP. Dans cet article, nous démontrons que le cofacteur caroténoïde se déplace de 12 Å au sein de l'OCP lors de la photoactivation. Le quatrième (chapitre 5) rapporte que le bras N-terminal de l'OCP est une structure singulière qui maintient la protéine fermée à l'obscurité, évitant que l'OCP ne s'active sous faible lumière ou à l'obscurité. Le cinquième (chapitre 6) présente la résolution de la structure et l'identification du site actif de la FRP, qui nous ont permis de prédire *in silico* le site d'attachement putatif de la FRP sur le domaine C-terminal de l'OCP. Dans le chapitre 7, je rapporte que deux résidus (l'aspartate 220 et la phénylalanine 299) sont requis pour que l'activité de la FRP soit maximale, confirmant le site d'interaction prédit.

