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Anja Korn

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Numéro d'ordre 9782

**Respective roles of the ferredoxin:NADP-oxidoreductase  
isoforms in the cyanobacterium**

*Synechocystis* sp. PCC 6803

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# Contents

<b>Résumé</b>	<b>xi</b>
<b>Abbreviations</b>	<b>xvii</b>
<b>Acknowledgements</b>	<b>xix</b>
<b>1 Introduction</b>	<b>1</b>
1.1 Photosynthesis and bioenergetics . . . . .	1
1.1.1 Linear electron transfer and membrane complexes . . . . .	2
1.1.2 Respiration . . . . .	8
1.1.3 Alternative electron sinks and cyclic electron transfer . . . . .	8
1.1.4 Cyanobacteria . . . . .	11
<i>Synechocystis</i> sp. strain PCC6803 . . . . .	12
1.2 Light-harvesting antenna . . . . .	13
1.2.1 Phycobilisome . . . . .	13
Phycobilisome structure . . . . .	13
Phycobilisome function . . . . .	16
1.2.2 Phycobilisome rod mutants . . . . .	19
1.3 Photosystem I and its electron acceptors . . . . .	19
1.3.1 Photosystem I . . . . .	19
1.3.2 Ferredoxin . . . . .	20
1.3.3 Ferredoxin:NADP oxidoreductase . . . . .	23
Structure of ferredoxin:NADP oxidoreductase . . . . .	23



Mechanism of ferredoxin:NADP oxidoreductase . . . . .	28
Isoforms of ferredoxin:NADP oxidoreductase . . . . .	33
Ferredoxin:NADP oxidoreductase mutants . . . . .	35
Growth characteristics . . . . .	37
1.4 Objective . . . . .	38
<b>2 In vitro studies</b>	<b>39</b>
2.1 Results and discussion . . . . .	39
2.1.1 Purification of FNR <sub>L</sub> -PC . . . . .	39
2.1.2 FNR quantification in FNR <sub>L</sub> -PC . . . . .	43
2.1.3 Reconstitution of PBS-FNR <sub>L</sub> . . . . .	44
2.1.4 NADPH oxidase activity . . . . .	46
Ferricyanide reductase activity . . . . .	46
Ferredoxin-mediated cytochrome <i>c</i> reductase activity . . . . .	47
NADP <sup>+</sup> inhibition of cyt <i>c</i> reduction . . . . .	50
2.1.5 NADP <sup>+</sup> reductase activity . . . . .	53
Single reduction by reduced Fd . . . . .	54
Catalytic turnover of FNR isoforms during NADP <sup>+</sup> reduction . . . . .	59
2.1.6 Catalytic properties of cyanobacterial FNR <sub>S</sub> . . . . .	61
NADPH oxidase activities . . . . .	61
NADP <sup>+</sup> reductase activities . . . . .	62
2.1.7 Catalytic properties of plant FNR <sub>S</sub> . . . . .	63
NADPH oxidase activities . . . . .	63
NADP <sup>+</sup> reductase activity . . . . .	64
2.2 Conclusion . . . . .	65
Catalytic properties of FNR isoforms . . . . .	66

<b>3</b>	<b><i>In vivo</i> studies</b>	<b>69</b>
3.1	Results and discussion	69
3.1.1	NADP <sup>+</sup> /NADPH ratio	70
3.1.2	P700 oxidation kinetics using white light	71
3.1.3	P700 oxidation kinetics using far-red light	74
	P700 oxidation kinetics in the presence of inhibitors	77
	Induction of cyclic ET under low CO <sub>2</sub>	79
3.1.4	PQ reduction in the dark	82
3.2	Conclusion	83
<b>4</b>	<b>Conclusions and Perspectives</b>	<b>87</b>
4.1	Conclusions	87
4.2	Perspectives	89
<b>5</b>	<b>Materials and Methods</b>	<b>91</b>
5.1	Bacterial growth conditions	91
5.2	Biochemical techniques	92
5.2.1	Chlorophyll quantification	92
5.2.2	Purification of photosystem I, ferredoxin and short FNR isoform	92
5.2.3	Purification of FNR <sub>L</sub> -PC	93
	Phycobilisome isolation	93
	IMAC purification of FNR <sub>L</sub> -PC	94
	Gel filtration of FNR <sub>L</sub> -PC	94
5.2.4	Quantification of apoprotein and active protein	95
	FNR apoprotein quantification	95
	FNR holoenzyme quantification	95
5.3	<i>In vitro</i> studies	96
5.3.1	NADPH oxidase activities	96
5.3.2	NADP <sup>+</sup> reductase activities	97

Single reduction of FNR . . . . .	97
Multiple catalytic turnover . . . . .	98
5.3.3 Fittings and calculations . . . . .	98
Single reduction of FNR . . . . .	98
Multiple catalytic turnover . . . . .	99
5.4 <i>In vivo</i> studies . . . . .	100
5.4.1 NADP <sup>+</sup> /NADPH quantification . . . . .	100
5.4.2 Absorption spectra +/- CO <sub>2</sub> . . . . .	101
5.4.3 P700 oxidation and reduction kinetics . . . . .	102
5.4.4 Monitoring of the transient increase in chlorophyll fluorescence . . . . .	104
<b>Bibliography</b>	<b>105</b>

# List of Figures

1.1	The structure of thylakoid membrane-protein complexes. . . . .	3
1.2	The structure of photosystem II. . . . .	3
1.3	The cofactors of photosystem II. . . . .	4
1.4	The structure of cytochrome- <i>b<sub>6</sub>f</i> complex and its cofactors. . . . .	5
1.5	The structure of photosystem I and its cofactors. . . . .	6
1.6	A composite model for the structure of the chloroplast F-ATPase. . . . .	7
1.7	Scheme of the thylakoid membrane. . . . .	8
1.8	Scheme of the thylakoid membrane 2. . . . .	10
1.9	A <i>Synechocystis</i> 6803 cell. . . . .	12
1.10	Types of phycobilisomes. . . . .	14
1.11	Phycocyanobilin. . . . .	15
1.12	Phycocyanin hexamer. . . . .	16
1.13	Phycobilisome representation. . . . .	17
1.14	Energy transfer in PBS. . . . .	18
1.15	Phycobilisome mutant. . . . .	19
1.16	Cofactors of photosystem I. . . . .	20
1.17	Kinetics of charge separation in photosystem I. . . . .	21
1.18	Stromal subunits of photosystem I. . . . .	21
1.19	Ferredoxin:NADP oxidoreductases. . . . .	24
1.20	Ferredoxin:NADP oxidoreductase structure. . . . .	25
1.21	Ferredoxin : ferredoxin:NADP oxidoreductase complex. . . . .	27

1.22	Ferredoxin:NADP oxidoreductase mechanism. . . . .	29
1.23	Ferredoxin:NADP oxidoreductase mechanisms proposed. . . . .	32
1.24	Representation of FNR <sub>S</sub> and FNR <sub>L</sub> primary structures. . . . .	35
1.25	<i>Synechocystis</i> WT, FS1 and MI6 strains. . . . .	36
1.26	<i>Synechocystis</i> WT, FS1 and MI6 strains immunoblot. . . . .	36
1.27	WT, FS1 and MI6 strains immunoblot under stress conditions. . . . .	37
2.1	Phycobilisome isolation and IMAC purification of the FNR <sub>L</sub> -PC complex. . . . .	40
2.2	Gel filtration chromatogram. . . . .	41
2.3	Purification of the FNR <sub>L</sub> -PC complex. . . . .	42
2.4	FAD release from the FNR <sub>L</sub> -PC complex. . . . .	44
2.5	Reconstitution of PBS-FNR <sub>L</sub> . . . . .	45
2.6	Scheme of ferricyanide reduction. . . . .	47
2.7	Ferricyanide reductase activities of FNR <sub>S</sub> and FNR <sub>L</sub> -PC. . . . .	48
2.8	Scheme of Fd-mediated cyt <i>c</i> reduction. . . . .	49
2.9	Fd-mediated cyt <i>c</i> reductase activities of FNR <sub>S</sub> and FNR <sub>L</sub> -PC. . . . .	50
2.10	Inhibition of cyt <i>c</i> reductase activities of FNR <sub>L</sub> -PC. . . . .	52
2.11	Lineweaver-Burk plot of the inhibition of FNR <sub>L</sub> -PC cyt <i>c</i> reductase activities. . . . .	52
2.12	Schemes of single FNR reduction. . . . .	54
2.13	Flash titration of FNR <sub>S</sub> and FNR <sub>L</sub> -PC in the presence of NADP <sup>+</sup> under single reduction conditions. . . . .	57
2.14	Flash titration of FNR <sub>S</sub> and FNR <sub>L</sub> -PC in the absence of NADP <sup>+</sup> under single reduction conditions. . . . .	58
2.15	Flash titration of FNR <sub>S</sub> and FNR <sub>L</sub> -PC in the presence of NADP <sup>+</sup> under catalytic turnover conditions. . . . .	60
3.1	Scheme of the situation <i>in vivo</i> . . . . .	70
3.2	P700 oxidation and reduction kinetics of WT strain under high CO <sub>2</sub> . . . . .	72
3.3	Blue-green fluorescence of WT and M55. . . . .	73
3.4	P700 oxidation and reduction kinetics in high CO <sub>2</sub> of WT, FS1 and MI6 mutant strains. . . . .	75

3.5	Scheme of P700 oxidation in WT and MI6. . . . .	76
3.6	Scheme of P700 oxidation and reduction in FS1. . . . .	76
3.7	P700 oxidation and reduction in the presence of inhibitors. . . . .	78
3.8	P700 oxidation and reduction kinetics in low CO <sub>2</sub> of WT, FS1- and MI6 mutant strains. . . . .	80
3.9	P700 oxidation kinetics under high and low CO <sub>2</sub> for WT. . . . .	80
3.10	P700 oxidation kinetics under high and low CO <sub>2</sub> of MI6 and FS1 mutant strains. . . . .	81
3.11	PQ reduction for WT and FS1. . . . .	82
3.12	Scheme of FNR <sub>S</sub> and FNR <sub>L</sub> association. . . . .	84
4.1	Crystal structures of FNR <sub>S</sub> and the PC hexamer. . . . .	89
5.1	Differential absorption spectrum for FNR single reduction by Fd. . . . .	98
5.2	Calibration curve of NADP <sup>+</sup> quantification. . . . .	101
5.3	Normalized absorbance spectra of WT. . . . .	102
5.4	Normalized absorbance spectra of MI6 and FS1. . . . .	102
5.5	Scheme of the pulse amplitude modulation (PAM) chlorophyll fluorometer . . . . .	103



# List of Tables

2.1	Quantification of FNR <sub>L</sub> and FAD in FNR <sub>L</sub> -PC. . . . .	43
2.2	Extinction coefficients for cofactors. . . . .	43
2.3	Calculated FNR <sub>L</sub> stoichiometries for CBH- and CBFS PBS. . . . .	46
2.4	Ferricyanide reductase activity of FNR <sub>L</sub> -PC and FNR <sub>S</sub> . . . . .	48
2.5	Cyt <i>c</i> reductase activity of FNR <sub>L</sub> -PC and FNR <sub>S</sub> . . . . .	50
2.6	Inhibition of cyt <i>c</i> reductase activity for FNR <sub>L</sub> -PC. . . . .	51
2.7	Single reduction of FNR <sub>L</sub> -PC and FNR <sub>S</sub> by Fd <sub>red</sub> in the presence of NADP <sup>+</sup> . . . . .	59
2.8	Single reduction of FNR <sub>L</sub> -PC and FNR <sub>S</sub> by Fd <sub>red</sub> in the absence of NADP <sup>+</sup> . . . . .	59
2.9	Multiple turnover of FNR <sub>L</sub> -PC and FNR <sub>S</sub> . . . . .	61
2.10	NADPH oxidation of the <i>Anabaena</i> FNR <sub>S</sub> . . . . .	62
2.11	Multiple turnover of FNR <sub>S</sub> . . . . .	63
3.1	Averaged NADP <sup>+</sup> /NADPH molar ratios for WT, FS1 and MI6. . . . .	71
3.2	P700 oxidation kinetics under high and low CO <sub>2</sub> for WT, MI6 and FS1. . . . .	82





# Résumé

Ce travail de thèse concerne la photosynthèse oxygénique, le processus utilisé par les cyanobactéries, les algues et les plantes pour convertir la lumière solaire en énergie chimique et stocker cette énergie. Lors des étapes initiales dépendant de la lumière, ce processus rejette de l'oxygène et forme de l'ATP et du NADPH, qui sont produits lors d'un flux linéaire d'électrons. Ces deux molécules énergétiques sont utilisées pour réduire le CO<sub>2</sub> et l'assimiler sous forme de sucres. Des modes alternatifs de transfert d'électrons, cyclique et pseudo-cyclique, conduisent seulement à la formation d'ATP. Deux flux d'électrons cycliques majeurs ont été proposés: un flux qui dépend de la ferrédoxine et un flux qui dépend du NADPH. Le premier et le deuxième flux sont respectivement les flux cycliques majeurs dans les plantes et les cyanobactéries. D'une part, le transfert cyclique implique chez les cyanobactéries le recyclage des excès de NADPH vers le pool des plastoquinones (PQ) dans la membrane thylakoïdale. D'autre part, le transfert pseudo-cyclique implique chez les cyanobactéries la formation de NADPH, dont les électrons ne sont pas recyclés vers le pool de PQ mais "perdus" pour réduire l'oxygène en eau.

Dans les membranes photosynthétiques des cyanobactéries et également à un degré moindre des chloroplastes se déroulent des transferts d'électrons respiratoires, producteurs d'ATP à partir du NAD(P)H lui-même issu de la dégradation des sucres. La cyanobactérie étudiée ici - *Synechocystis* sp. PCC6803 (*Synechocystis*) - est un organisme non seulement photoautotrophe mais aussi hétérotrophe facultatif.

Le phycobilisome (PBS) est le complexe majeur collecteur de lumière chez les cyanobactéries. Il transfère l'énergie capturée essentiellement vers le photosystème (PS) II. De plus, il constitue jusqu'à 30% des protéines dans la cyanobactérie et peut être dégradé en conditions de carence en nutriments. Le PS I (PSI) est responsable de la formation photosynthétique de NADPH, les électrons étant transportés des accepteurs du PSI vers la ferrédoxine (Fd) et finalement vers le NADP<sup>+</sup> *via* la ferrédoxine:NADP oxydoréductase (FNR).

Chez les plantes, différentes isoformes de Fd et de FNR sont présentes dans différents tissus et codées par différents gènes. Ainsi dans les chloroplastes de feuilles, des Fds réduisent la FNR photosynthétique pour former le NADPH. Par contre, dans les plastes des racines, la FNR

hétérotrophe réduit une autre Fd en oxydant le NADPH et la Fd ainsi réduite est impliquée entre autres dans l'assimilation de l'azote. Chez *Synechocystis*, la Fd codée par le gène *fed1* est indispensable en conditions de croissance photoautotrophe aussi bien qu'hétérotrophe cependant que trois autres gènes codant des Fd photosynthétiques sont faiblement exprimés. Le produit du gène *fed1* est donc probablement impliqué dans la réduction du NADP<sup>+</sup> et l'oxydation du NADPH.

A partir d'un seul gène, deux isoformes de FNR de taille différente ont été trouvées chez *Synechocystis*. Grâce à un domaine de type linker, la plus grande, FNR<sub>L</sub>, est liée au PBS, contrairement à la plus petite, FNR<sub>S</sub>, qui est dépourvue de ce domaine. Ces observations posent deux questions qui ont motivé ce travail de thèse: quelle est la fonction de l'attachement de FNR<sub>L</sub> au PBS et quelles sont les rôles respectifs des deux isoformes de FNR? Des mutants exprimant uniquement une des isoformes ont été récemment obtenus. De l'étude de la croissance des mutants en différentes conditions a été formulée l'hypothèse de ce travail que FNR<sub>L</sub> est impliquée dans la réduction du NADP<sup>+</sup> (transfert d'électrons linéaire) et FNR<sub>S</sub> dans l'oxydation du NADPH (transfert d'électrons respiratoire/cyclique).

Ce travail de thèse a fait l'objet de deux approches différentes. D'une part, les activités catalytiques des deux isoformes ont été étudiées *in vitro*. D'autre part, les mutants de FNR ont été comparés au type sauvage (WT) dans différentes études *in vivo*.

FNR<sub>L</sub> étant toujours liée au PBS *in vivo* et étant protéolysée lorsqu'elle n'est pas liée, nous avons choisi de purifier un sous-complexe du PBS comprenant FNR<sub>L</sub> et un hexamère de phycocyanine (PC) du PBS. Ce complexe, appelé FNR<sub>L</sub>-PC, est stable tout en présentant des propriétés d'absorption compatibles avec des études de spectroscopie d'absorption. FNR<sub>L</sub>-PC a été purifié à partir d'une souche mutée de *Synechocystis* possédant d'une part un seul hexamère de PC par bâtonnet (au lieu de trois) et d'autre part une étiquette histidine insérée dans le domaine charnière de la FNR<sub>L</sub> (situé entre le domaine linker et les domaines catalytiques). Puisqu'une à deux FNR<sub>L</sub> sont attachées par PBS aussi bien dans le mutant que dans le WT, nous avons multiplié par trois le rapport FNR<sub>L</sub>/PC dans le matériel de départ. De plus, la présence de l'étiquette histidine a facilité la purification du complexe par l'utilisation d'une étape de chromatographie d'affinité au nickel, les impuretés restantes étant ensuite éliminées par filtration sur tamis moléculaire.

Grâce à cette approche, nous avons atteint notre premier objectif qui était de purifier FNR<sub>L</sub>-PC à l'homogénéité et d'augmenter son rendement de purification. Le complexe FNR<sub>L</sub>-PC contient FNR<sub>L</sub>, un hexamère de PC et un linker bâtonnet-coeur appelé L<sub>RC</sub>, pour une masse moléculaire d'environ 330 kDa. Il possède un groupe prosthétique FAD par FNR<sub>L</sub> et est stable à 4°C, ce qui confirme que la liaison à l'hexamère de PC protège FNR<sub>L</sub> de la protéolyse. Nous avons également initié des études de reconstitution du PBS et de FNR<sub>L</sub>, ce qui permet d'envisager la production de grandes quantités de complexe en vue d'une analyse structurale.

Nous avons ensuite effectué une étude enzymologique détaillée des activités NADP<sup>+</sup>-réductase et NADPH-oxydase de FNR<sub>L</sub>-PC que nous avons comparées à celles de FNR<sub>S</sub>. Nous avons caractérisé l'activité d'oxydation du NADPH par des tests classiques d'enzymologie et commencé l'étude de l'inhibition par le produit de la réaction. L'activité NADP<sup>+</sup>-réductase a été mesurée par spectroscopie d'absorption différentielle résolue en temps en présence de PSI.

Bien que dans l'ensemble assez proches, les mesures d'activité présentent quelques différences majeures entre FNR<sub>L</sub>-PC et FNR<sub>S</sub>. La différence la plus importante concerne l'affinité réduite de la Fd oxydée (Fd<sub>ox</sub>) pour FNR<sub>L</sub>-PC *vs.* FNR<sub>S</sub> lors de l'oxydation du NADPH. L'effet observé peut s'expliquer par l'encombrement stérique de l'hexamère de PC dans le complexe FNR<sub>L</sub>-PC. Comme il est généralement admis que la dissociation de Fd<sub>ox</sub> est cinétiquement limitante lors de la réduction du NADP<sup>+</sup>, cet effet pourrait favoriser la réduction du NADP<sup>+</sup> par le complexe. Ceci est en accord avec les caractéristiques de croissance des mutants de FNR, le mutant exprimant uniquement FNR<sub>L</sub> poussant mieux en photoautotrophie (réduction du NADP<sup>+</sup>). Cet effet d'encombrement s'est aussi manifesté pour les cinétiques de réduction de la FNR par Fd<sub>red</sub>.

L'augmentation d'affinité pour le NADPH de FNR<sub>L</sub>-PC *vs.* FNR<sub>S</sub> a été également observée. Cette augmentation pourrait ne pas avoir d'effet négatif sur l'oxydation *in vivo* du NADPH par FNR<sub>S</sub>, car l'induction de FNR<sub>S</sub> chez le WT en conditions de stress ou d'hétérotrophie est corrélée à une augmentation, au moins transitoire, de la concentration de NADPH. Une meilleure affinité pour le substrat NADP<sup>+</sup>/NADPH pourrait par contre renforcer la réduction du NADP<sup>+</sup> par FNR<sub>L</sub>-PC. Nous avons de plus mis en évidence que dans les conditions de force ionique élevée que nous utilisons pour garder intact FNR<sub>L</sub>-PC, la première réduction de la FNR par Fd<sub>red</sub> est limitante pour la réduction du NADP<sup>+</sup>, ce qui n'est pas le cas à faible force ionique.

Les différences que nous avons observées sont en désaccord avec les différences observées chez les isoformes de FNR de plantes (feuilles *vs.* racines). Les isoformes de *Synechocystis* correspondent peut-être mieux aux différentes isoformes trouvées dans les feuilles d'un même organisme. D'autres facteurs que les propriétés catalytiques, tels que la disponibilité des substrats (Fd<sub>ox</sub>/Fd<sub>red</sub> et NADP<sup>+</sup>/NADPH) et la localisation de la FNR, sont probablement essentiels pour expliquer les rôles physiologiques respectifs des deux isoformes de la FNR de *Synechocystis*. Ainsi FNR<sub>L</sub> est liée au PBS tandis que FNR<sub>S</sub> est soit soluble, soit liée à la membrane, soit liée à des complexes membranaires (cytochrome *b<sub>6</sub>f*, NADPH déshydrogénase NDH-1).

C'est pourquoi nous avons commencé des études *in vivo* sur le WT ainsi que sur des mutants appelés MI6 et FS1 qui expriment une seule isoforme, respectivement FNR<sub>L</sub> et FNR<sub>S</sub>. Nous avons effectué trois types de mesure. D'abord nous avons caractérisé l'état rédox du pool de NADP par la mesure du rapport NADP<sup>+</sup>/NADPH. Ensuite nous avons mesuré la réduction transitoire du pool des PQ de la membrane, à l'obscurité juste après une période d'éclairement, par des mesures de fluorescence chlorophyllienne. Enfin nous avons identifié le(s) mode(s) prédominant(s) de

transfert d'électrons (linéaire *vs.* respiratoire/cyclique) par les cinétiques de photooxydation du donneur primaire P700 du PSI lors d'une excitation sélective du PSI. L'interprétation de ces dernières données requiert l'utilisation d'inhibiteurs qui bloquent sélectivement les transferts d'électrons du PSII (DCMU) ou du cytochrome *b<sub>6</sub>f* (DBMIB) ou qui inhibent les réactions de recombinaison du PSI (méthyl viologène).

Les trois souches (WT, MI6 et FS1) ont d'abord été étudiées en conditions de croissance photoautotrophes en présence d'un excès de CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>, conditions dans lesquelles seule FNR<sub>L</sub> est exprimée dans le WT. Nous avons déterminé que le pool de NADP est plus oxydé dans FS1 que dans le WT et MI6, ce qui suggère que FNR<sub>S</sub> est soit peu apte à réduire le NADP<sup>+</sup>, soit recycle efficacement les électrons du NADPH vers le pool de PQ. Les mesures de réduction transitoire du pool de PQ à l'obscurité sont en plein accord avec la deuxième possibilité, avec un signal de réduction plus élevé pour FS1 que pour WT/MI6. Les cinétiques de photooxydation du P700 sont également cohérentes avec les mesures précédentes: la photooxydation est rapide chez WT/MI6 et beaucoup plus lente chez FS1. Ces résultats s'expliquent par un transfert d'électrons linéaire (et peut-être pseudo-cyclique) dominant chez WT/MI6 et un transfert d'électrons respiratoire/cyclique beaucoup plus efficace chez FS1.

Nous avons ensuite répété les mesures de photooxydation du P700 sur les trois souches cultivées à faible CO<sub>2</sub> (CO<sub>2</sub> atmosphérique, pas de bicarbonate) car ces conditions sont connues pour induire le transfert d'électrons cyclique/respiratoire. MI6 et FS1 montrent pas ou peu de différences phénotypiques avec les observations précédentes tandis que le comportement du WT se rapproche de celui du FS1: le P700 est beaucoup plus lentement photooxydé. Nous proposons que cela est dû à l'accumulation de FNR<sub>S</sub> qui se produit généralement chez le WT en situation de stress (manque d'azote, excès de lumière). La mesure de MI6 (comportement identique à fort ou faible CO<sub>2</sub>) renforce cette interprétation car ce mutant est incapable d'exprimer FNR<sub>S</sub>. L'accumulation de FNR<sub>S</sub> dans le WT favorise ainsi un transfert d'électrons respiratoire/cyclique, en accord avec l'idée que ces modes de transfert d'électrons sont induits à faible CO<sub>2</sub>, lorsque le cycle de Calvin est ralenti.

L'implication de FNR<sub>L</sub> dans le transfert d'électron linéaire/pseudo-cyclique et de FNR<sub>S</sub> dans le transfert d'électron respiratoire/cyclique est donc confirmée par nos études *in vivo*. Pour remplir son rôle, FNR<sub>S</sub> pourrait se lier à d'autres complexes membranaires comme le cytochrome *b<sub>6</sub>f* ou les complexes NDH-1. Nous favorisons la dernière hypothèse car le mode dominant de transfert cyclique ainsi que la respiration chez les cyanobactéries implique les complexes NDH-1 et qu'un des complexes NDH-1 est fortement induit à faible CO<sub>2</sub>.

Les résultats *in vitro* ainsi que les mesures de l'état rédox du pool de NADP sont décrits dans un article publié en 2009 dans "The Journal of Biological Chemistry" joint à la fin du manuscrit.

Ce travail ouvre de nombreuses perspectives pour des études *in vitro* et *in vivo*. Les études

d'inhibition de l'oxydation du NADPH par le produit de la réaction (NADP<sup>+</sup>) permettront de comparer les affinités relatives de FNR<sub>S</sub> pour le NADP<sup>+</sup> et le NADPH. La production massive d'une FNR<sub>L</sub> contenant une étiquette histidine favorisera l'obtention par reconstitution de grandes quantités de complexes FNR<sub>L</sub>-PC pour des études structurales ou spectroscopiques. Les études *in vivo* suivantes doivent être entreprises pour préciser le rôle de FNR<sub>S</sub> et ses partenaires dans la réduction du pool de PQ chez les cyanobactéries hétérotrophes facultatives: accumulation de FNR<sub>S</sub> dans le WT à faible CO<sub>2</sub>, étude de l'état rédox du pool de PQ à l'obscurité et de sa réduction transitoire à fort et faible CO<sub>2</sub>, mesures en temps réel du NADPH par fluorescence. L'étude transcriptomique des mutants de FNR permettra d'identifier les régulations induites par l'accumulation d'une seule isoforme. Une étude génétique est en cours afin d'étudier le rôle de la région 5' non-codante de l'ARN messenger du gène *petH* dans l'accumulation de FNR<sub>S</sub>.



# Abbreviations

$A_0^-$	Chlorophyll of PSI, reduced in the primary electron transfer
AL	Actinic light
AP	Allophycocyanin PB subunit of an phycobiliprotein, PB indicates the protein
ATP	Adenosine 5'-triphosphate
$\alpha^{PC}$ and $\beta^{PC}$	Subunits of phycocyanin
$b_{6f}$	Cytochrome $b_{6f}$ complex
chl	Chlorophyll
CP43	Core antenna complex with apparent mass of 43 kDa
CP47	Core antenna complex with apparent mass of 47 kDa
Cys	Cysteine
cyt <i>c</i>	Cytochrome <i>c</i>
Da	Dalton
DBMIB	2,5-Dibromo-3-methyl-6-isopropylbenzoquinone
DCMU	3-(3',4'-Dichlorophenyl)-1,1-dimethylurea
DCPIP	2,6-Dichlorophenolindophenol
EDTA	Ethylene diaminetetraacetic acid
$E_m$	Midpoint redox potential
ET	Electron transfer
( $F_A, F_B$ )	(4Fe-4S) Clusters, the terminal acceptors of PSI
Fd	Ferredoxin
(4Fe-4S) and (2Fe-2S)	Iron sulfur clusters
FNR	Ferredoxin-NADP(H)-oxidoreductase
FNR <sub>L</sub>	Large <i>Synechocystis</i> FNR isoform, <i>Synechocystis</i> indicates the cyanobacterium
FNR <sub>S</sub>	Small <i>Synechocystis</i> FNR isoform, <i>Synechocystis</i> indicates the cyanobacterium
FNR <sub>sq</sub>	Singly reduced FNR/semiquinone form
FR	Far red
GDH	Glucose dehydrogenase
HC	high CO <sub>2</sub>



IMAC	Immobilized metal affinity chromatography
LC	low CO <sub>2</sub>
L <sub>C</sub>	Core-linker polypeptide
L <sub>CM</sub>	Core-membrane-linker polypeptide
L <sub>RC</sub>	Rod-core-linker polypeptide
L <sub>R</sub> (L <sub>R</sub> <sup>M</sup> )	Rod-linker polypeptide, M indicates its molecular mass
MES	2-Morpholinoethanesulfonic acid
MV	Methylviologen
NADP <sup>+</sup> (NADPH)	Nicotinamide adenine dinucleotide phosphate (reduced form)
NDH	NADPH dehydrogenase
OD	Optical density
P680	Primary electron donor of photosystem II
P700	Primary electron donor of photosystem I
PAM	Pulse amplitude modulation
PBP	Phycobiliprotein
PBS	Phycobilisome
Pc	Plastocyanin
PC	Phycocyanin, an αβ protomer
PCB	Phycocyanobilin
PCC	Pasteur Culture Collection
PCR	Polymerase chain reaction
pdb	protein data bank
PQ	Plastoquinone
ps	picosecond, 1 ps=10 <sup>-12</sup> s
PSI	Photosystem I
PSII	Photosystem II
Q <sub>A</sub>	Primary electron acceptor quinone of photosystem II
Q <sub>B</sub>	Secondary electron acceptor quinone of photosystem II
SDS	Sodium dodecyl sulphate
SDS-PAGE	Polyacrylamide gel electrophoresis in the presence of SDS
<i>Synechocystis</i>	<i>Synechocystis</i> sp. PCC6803
TCA	Trichloroacetic acid
Tricine	N-[2-Hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine
Tris	Tris hydroxymethyl methylamine
UV-Vis	Ultraviolet-visible
Y <sub>Z</sub>	Tyrosine residue of photosystem II
WT	Wild type

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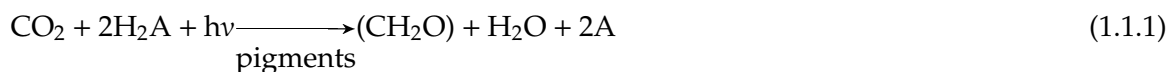
# Chapter 1

## Introduction

The field of photosynthesis research is very broad and comprises research at various levels - from eco-systems to isolated proteins [Messinger et al., 2009]. We will introduce in the following sections photosynthesis and bioenergetics and present various pathways of electron transfer. The antenna complexes and the photosystem I acceptor side are described in detail.

### 1.1 Photosynthesis and bioenergetics

Photosynthesis is the process that transforms light energy into electrochemical energy following the basic stoichiometry shown in Reaction 1.1.1 [Kiang et al., 2007]:



When  $\text{H}_2\text{A}$  is  $\text{H}_2\text{O}$ , this reaction is called oxygen-evolving photosynthesis. This reaction is divided in the photochemical reactions (formerly known as light reactions) and a series of enzymatic reactions involved in the Calvin-Benson-Bassham cycle (Calvin cycle) for  $\text{CO}_2$  assimilation (formerly known as dark reactions) [Dubbs and Tabita, 2004]. The different stages of energy storage will be detailed later. In the following, we will describe the photosynthetic organisms.

Autotrophs derive all their cellular carbon from  $\text{CO}_2$ , whereas heterotrophs derive cellular carbon from organic carbon compounds [Blankenship, 2002]. In this way, heterotrophs depend on autotrophs to provide them with the organic carbon compounds. A further distinction can be made concerning the energy source. Phototrophs derive their energy from sunlight, whereas chemotrophs derive energy from different chemical compounds. Organisms that derive their

energy from the sunlight and derive all their cellular carbon from CO<sub>2</sub> are called photoautotrophs. Most of the photosynthetic organisms grow photoautotrophically. Species capable of performing photosynthesis are among prokaryotes and eukaryotes.

Five distinct major groups of prokaryotes are found that are capable of photosynthesis. Four of them are anoxygenic. H<sub>2</sub>A does not correspond to water in this case. Thus, oxygen is not produced as a byproduct. The anoxygenic phototrophic bacteria include purple bacteria, green sulfur bacteria, green nonsulfur bacteria and heliobacteria. The only oxygen-evolving group of bacteria are called cyanobacteria (formerly known as blue-green algae).

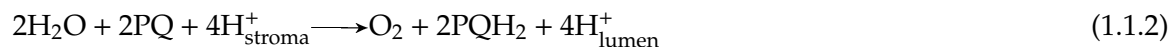
Eukaryotic photosynthetic organisms such as plants and algae contain a subcellular organelle (plastid) called chloroplast. Chloroplasts originated by endosymbiosis from cyanobacteria. Initially a cyanobacterial-like cell was a symbiont with a protoeukaryotic cell and became a semi-autonomous part of the host cell. This explains the similar mechanism of photosynthesis of cyanobacteria compared to that of photosynthetic eukaryotes.

In the chloroplast as well as in cyanobacteria an extensive internal membrane system called the thylakoid contains chlorophyll and the electron transport chain that carries out initial light energy capture and storage.

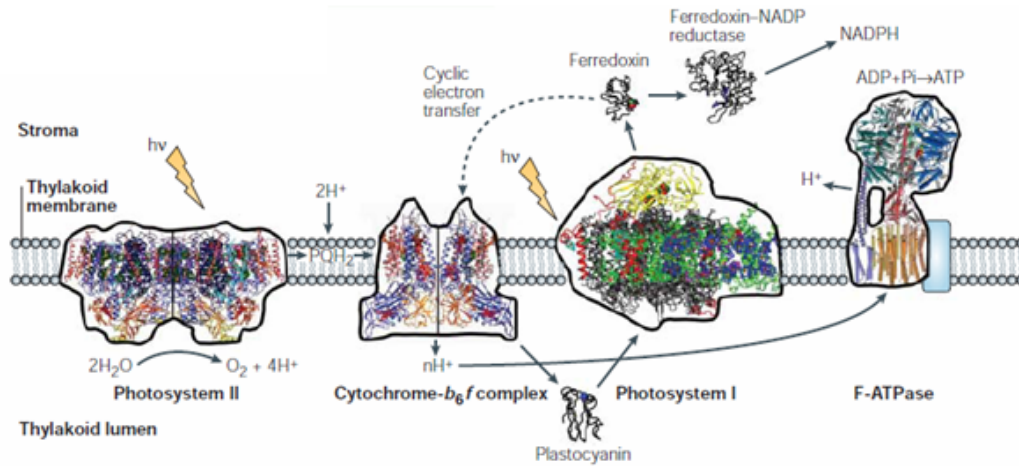
### 1.1.1 Linear electron transfer and membrane complexes

In oxygenic photosynthetic organisms, the major mode of electron transfer (ET) is the linear ET (noncyclic). It involves water oxidation to molecular oxygen and the reduction of NADP<sup>+</sup> into NADPH. This is achieved by two sequential photoreactions involving two photosystems (Figure 1.1). We will further introduce the four integral membrane protein complexes involved in photosynthetic electron transfer and ATP build-up that are photosystem II (PSII), cytochrome *b<sub>6</sub>f* (cyt *b<sub>6</sub>f*), photosystem I (PSI) and ATP-synthase (ATPase).

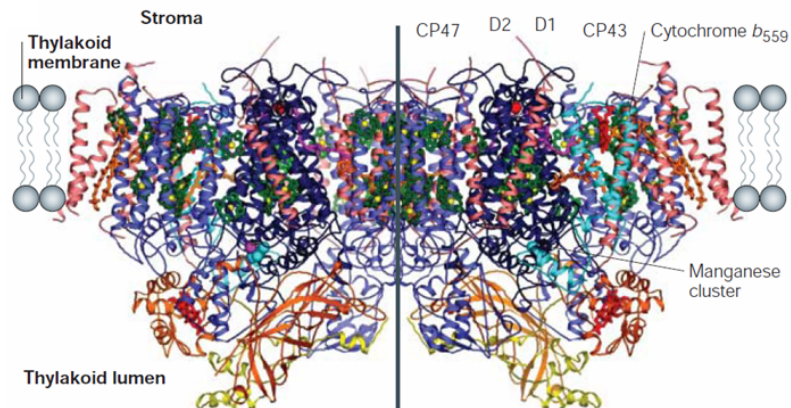
PSI and PSII contain numerous pigments that harvest light and funnel the excitation to the primary electron donors, which can reduce an electron acceptor and accept electrons from specific electron donors. The cyt *b<sub>6</sub>f* complex mediates electron transport between PSII and PSI and converts redox energy into a high-energy intermediate (protonmotive force; pmf) for ATP formation. The reaction catalyzed by PSII is shown in Equation 1.1.2:



The reaction involves the reduction of plastoquinone (PQ) into plastoquinol (PQH<sub>2</sub>) and the oxidation of water into molecular oxygen. The PSII reaction center is composed of two similar

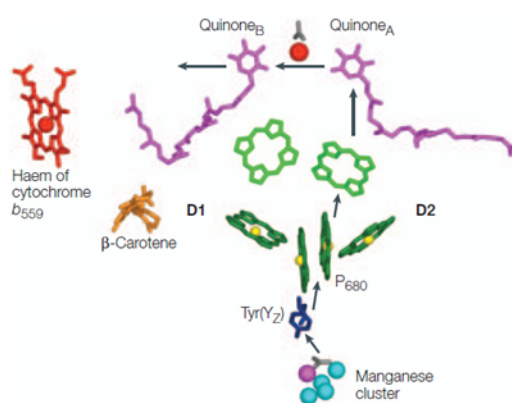


**Figure 1.1** The structures of the four large membrane-protein complexes in thylakoid membranes that drive oxygenic photosynthesis taken from [Nelson and Ben-Shem, 2004].



**Figure 1.2** The structure of photosystem II from the cyanobacterium *Thermosynechococcus elongatus* [Ferreira et al., 2004].

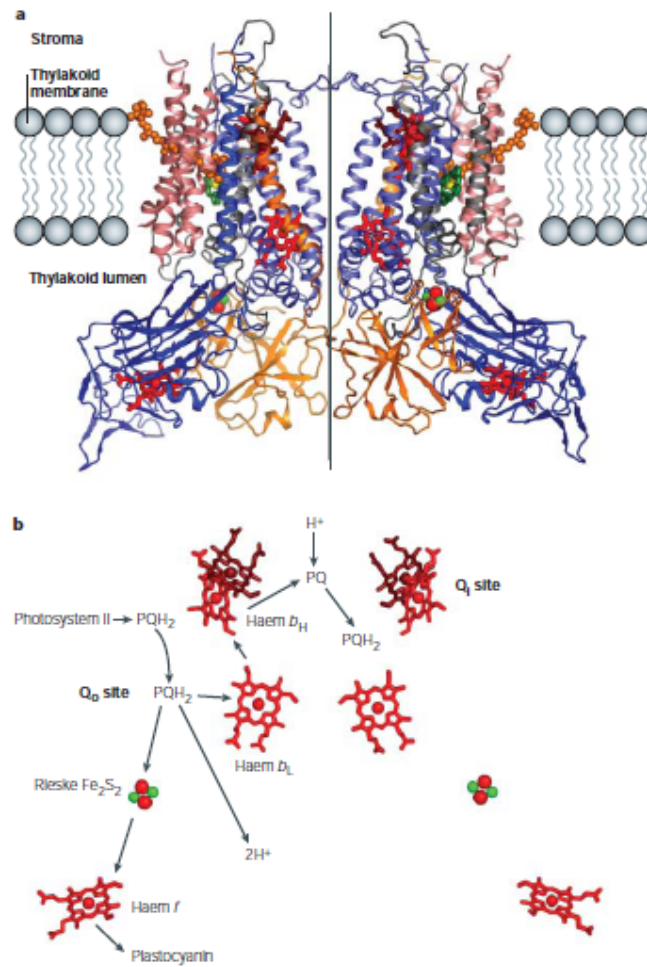
proteins (D1 and D2). These proteins coordinate both the manganese cluster of PSII and all of the electron-transfer components involved in the main pathway of charge separation and stabilization (Figure 1.2). Following photoexcitation, a charge separation involving  $P680^+$  and a reduced pheophytin occurs rapidly. To avoid recombination of the charges, a series of fast secondary electron transfer reactions is carried out. The main electron carriers in PSII include a bound PQ,  $Q_A$ , and a dissociable PQ,  $Q_B$ , after the pheophytin (Figure 1.3). The oxygen-evolving complex (OEC) *via* Tyr<sub>Z</sub> reduces the  $P680^+$  back to  $P680$  and accumulates in this way the oxidizing equivalents necessary to oxidize water. The reduced  $Q_B$  then transfers electrons to the cyt  $b_6f$ . The widely used inhibitor 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU) displaces  $Q_B$  from its PSII binding site.



**Figure 1.3** The cofactors of photosystem II published in [Ferreira et al., 2004].

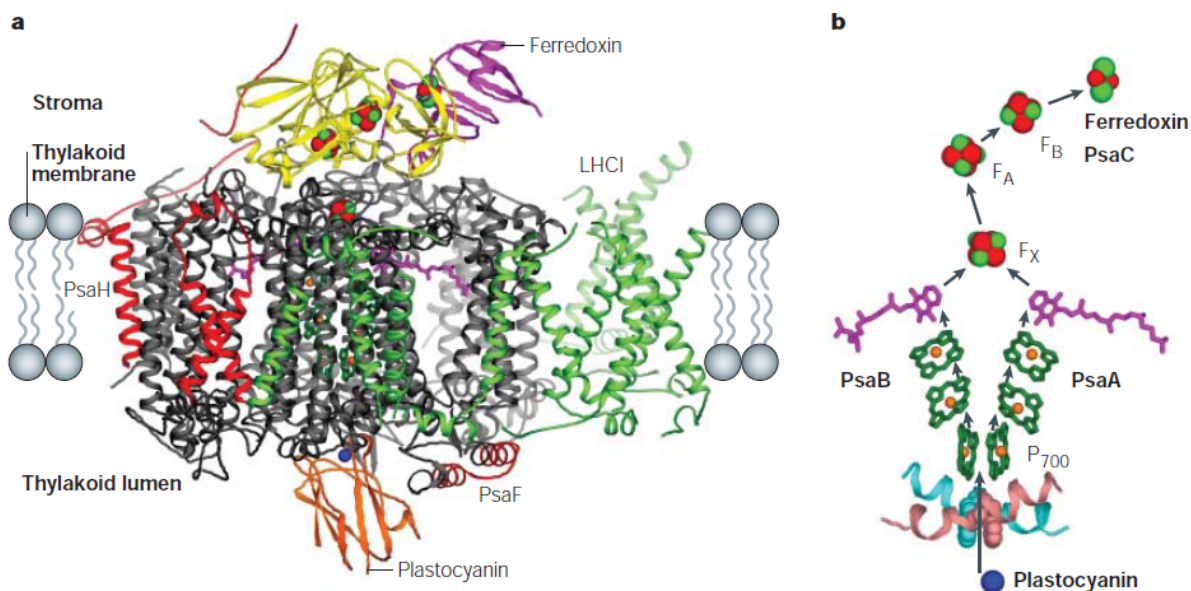
The cyt  $b_6f$  complex (Figure 1.4a) is also called "plastoquinol-plastocyanin oxidoreductase". The complex operates following the "Q-cycle" [Mitchell, 1976, Trumpower, 1990] and is similar in structure and function to the cytochrome  $bc_1$  complex from the respiratory ET chain. It exhibits two PQ binding sites, the  $Q_o$  site close to haem  $b_L$  binds quinol (lumenal side) and the  $Q_i$  site close to haem  $b_H$  binds quinone (stromal side). The  $PQH_2$  loses a first electron, which is transferred to the Rieske iron-sulfur protein, the cyt  $f$  and finally to plastocyanin. The second electron from  $PQH_2$  is transferred *via* two cyt  $b$  to the  $Q_i$  site where another PQ is reduced (Figure 1.4b). The quinone analogue 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB) acts as an inhibitor of cyt  $b_6f$  as it is thought to bind at the  $Q_o$  site. The presence of a further haem group that is covalently bound to cytochrome  $b_6$  is puzzling [Kurusu et al., 2003, Stroebel et al., 2003]. Overall, two protons will be translocated across the thylakoid membrane for one electron that is passed to PSI *via* plastocyanin.

Plastocyanin (Pc), a single copper containing protein, transfers its electron to PSI (Figure 1.5a). Controlled by the Cu availability in the growth media, cytochrome  $c_6$  can replace Pc in its function in many algae and cyanobacteria. The bulk of the reaction center of PSI is built of two,



**Figure 1.4** The structure of cytochrome- $b_6f$  complex (a) and its cofactors (b) from the alga *Chlamydomonas reinhardtii* [Stroebel et al., 2003].



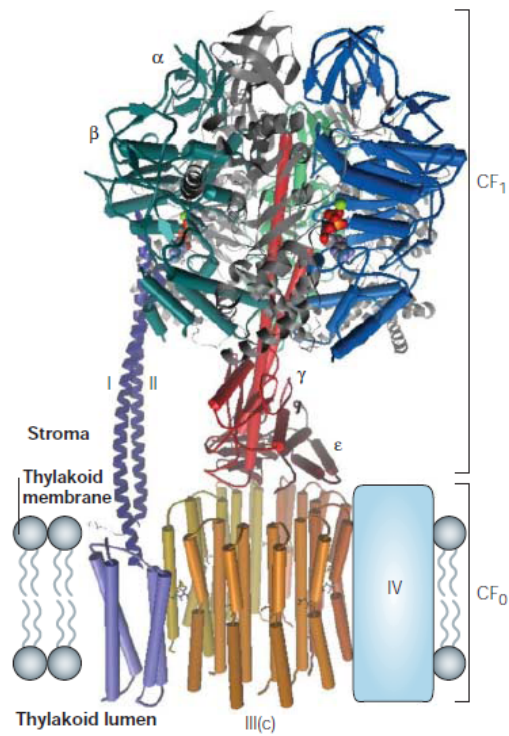


**Figure 1.5** The model of a super-complex containing photosystem I, plastocyanin and ferredoxin (a) and their cofactors (b) from a higher plant (*Pisum sativum* var. *alaska*) [Ben-Shem et al., 2003].

homologous, large subunits (PsaA and PsaB) that harbor most of the PSI pigments and all of the cofactors up to F<sub>X</sub>. Photoexcitation gives rapidly (ps time range) P700<sup>+</sup> and A<sub>1</sub><sup>-</sup>. An electron from Pc (or cyt *c*<sub>6</sub>) regenerates the special pair P700. The electron on the phylloquinone A<sub>1</sub> is passed through the F<sub>X</sub> and F<sub>A</sub>/F<sub>B</sub> iron sulfur centers (Figure 1.5b). The electrons at the acceptor side of PSI are transferred to the soluble ferredoxin (Fd) and finally lead to NADP<sup>+</sup> reduction. This reaction is catalyzed by the enzyme ferredoxin-NADP<sup>+</sup> oxidoreductase (FNR) which is the subject of this thesis and will be described in section 1.3.3. N,N-dimethyl-4,4'-bipyridinium dichloride (methylviologen; MV) is an efficient electron acceptor, from (F<sub>A</sub>,F<sub>B</sub>) and Fd. Reduced MV reacts very rapidly with O<sub>2</sub> (contrary to (F<sub>A</sub>,F<sub>B</sub>) and Fd which react much more slowly).

A total of six protons are translocated, for 2 PSII charge separations and cyt *b*<sub>6</sub>f ET, to the lumen that lead to an electrochemical-potential gradient (proton motive force; pmf) across the thylakoid membrane. The pmf will be used in the fourth complex, the ATPase, in order to synthesize ATP. During this process, protons flow from the lumen to the stroma through the integral membrane part of the ATPase CF<sub>0</sub> and ATP is generated at the level of the soluble part CF<sub>1</sub> (Figure 1.6).

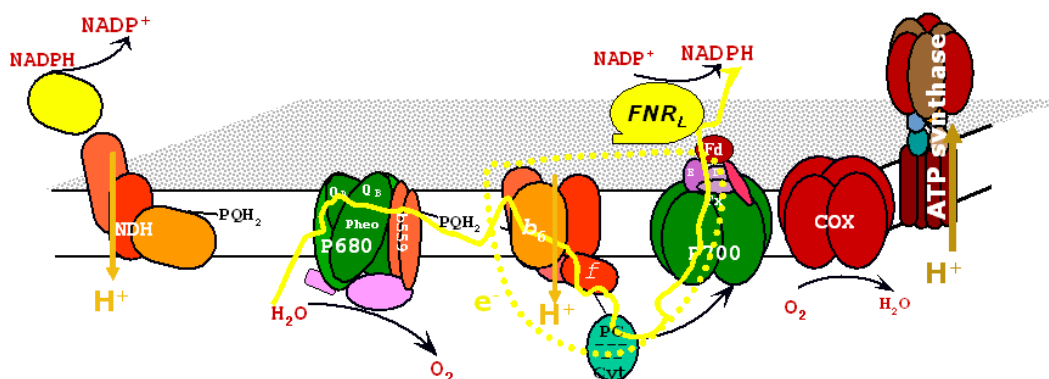
Most of the details shown so far concern the photosynthetic electron transfer in higher plants and algae [Nelson and Ben-Shem, 2004]. All the complexes cited above are identical in cyanobacteria. Photosynthetic and respiratory electron transfers are carried out in the same compartment in cyanobacteria. This phenomenon is also present in chloroplasts and is called chlororespiration [Bennoun, 1982, Rumeau et al., 2007].



**Figure 1.6** A composite model for the structure of the chloroplast F-ATPase. This model was created by W. Frasch using available structural data for mitochondrial F-ATPase subcomplexes [Abrahams et al., 1994, Stock et al., 1999, Gibbons et al., 2000].

### 1.1.2 Respiration

Both photosynthesis and respiration use membrane protein complexes located in the thylakoid membrane (Figure 1.7). Several components such as PQ, the cyt  $b_6f$  complex and soluble electron transporters are common to both bioenergetic processes.



**Figure 1.7** Scheme of the thylakoid membrane. The different electron translocating complexes are shown. Respiratory complexes involve the COX: cytochrome oxidase complex, NDH: NAD(P)H dehydrogenase (NDH-1) complex.

Succinate dehydrogenase (SDH) is the only enzyme of the tricarboxylic acid pathway which is found attached to the membrane. This complex functions in the respiratory ET as complex II. Evidence was found for the implication of SDH in the PQ pool reduction in *Synechocystis* [Cooley et al., 2000].

In the thylakoids of both cyanobacteria and plastids, distinct NAD(P)H dehydrogenases are found that oxidize NAD(P)H (NDH-1) and are equivalent to complex I involved in respiration. In cyanobacteria, the NDH-1 is extensively studied and is involved in a variety of functions like respiration, cyclic electron flow (introduced later) around PSI and CO<sub>2</sub> uptake [Battchikova and Aro, 2007].

In addition to that, three respiratory terminal oxidases (RTOs, complex IV) exist in *Synechocystis*: cytochrome c oxidase (CCox), quinol oxidase (Cyd), and alternative RTO (ARTO) [Pils and Schmetterer, 2001, Hart et al., 2005]. ET through the respiratory complexes lead to a proton gradient, hence to ATP formation, at the expense of reductants (NADPH and succinate).

### 1.1.3 Alternative electron sinks and cyclic electron transfer

Light-induced linear electron transfer between the two photosystems generate ATP and reducing equivalents in the form of NADPH. ATP and NADPH are used in a variety of metabolic processes. Under photoautotrophic growth conditions, CO<sub>2</sub> assimilation in the Calvin cycle constitutes the major electron sink for NADPH. However, stress conditions may lead to electron redirection

toward alternative electron sinks. Alternative electron sinks involve essentially the Mehler-reaction, cyclic electron transfer or respiration.

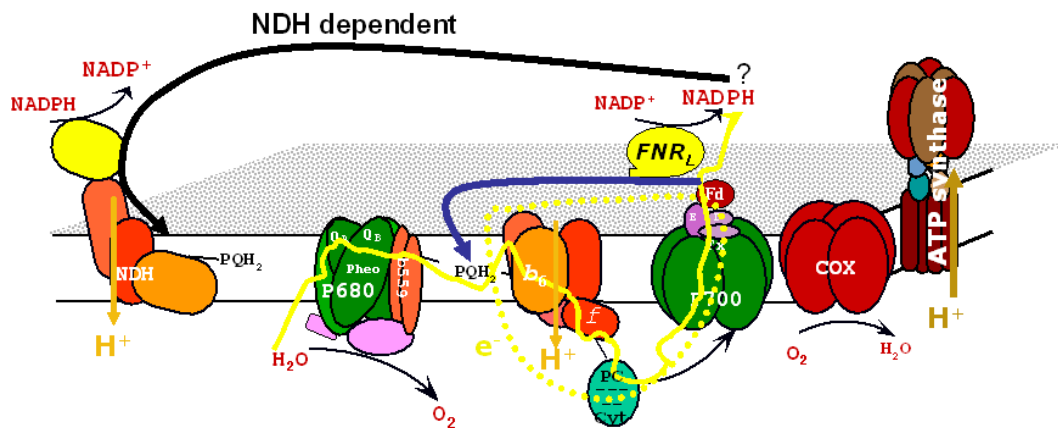
A substantial part of electrons can be transferred from PSI to molecular oxygen, which results in photoreduction of O<sub>2</sub> *via* superoxide anion to H<sub>2</sub>O<sub>2</sub> in chloroplasts, *i.e.* the Mehler-reaction [Mehler, 1951, Asada, 1999]. The produced reactive oxygen species (ROS) are quickly detoxified by the combined action of superoxide dismutase and peroxidases. Thereby, the photoreduction of O<sub>2</sub> acts as an electron sink (pseudocyclic ET) under certain conditions, where up to 30% of the electrons from the light reactions can be directed to oxygen [Hackenberg et al., 2009]. For *Synechocystis* sp. PCC6803 (*Synechocystis*), it was shown that O<sub>2</sub> is reduced directly to water in one reaction mediated by A-type flavoproteins [Helman et al., 2003]. The genome of *Synechocystis* encodes four putative A-type flavoproteins, but only two of them, Flv1 and Flv3, are apparently involved in light-dependent O<sub>2</sub> reduction activity [Helman et al., 2003]. Recently, a role in the photoprotection of PSII has been shown for the two other *Synechocystis* flavoproteins, Flv2 and Flv4 [Zhang et al., 2009]. For pseudocyclic ET, which involves the Mehler-reaction or photorespiration, redox poisoning was proposed as a plausible function [Allen, 2003].

Under CO<sub>2</sub>-limiting conditions, the Calvin cycle activity is strongly reduced and photorespiration, a Rubisco oxygenase reaction, increases. The so-called photorespiratory 2PG-metabolism helps to avoid depletion of Calvin-cycle intermediates. Due to the efficient inorganic carbon concentrating mechanism [Badger et al., 2006], it was assumed that cyanobacteria do not possess a photorespiratory 2PG-metabolism. In contrast to this earlier view, it was recently demonstrated that an active photorespiratory 2PG-metabolism involving three different pathways exists in *Synechocystis* [Eisenhut et al., 2008]. The complete loss of all three pathways leads to a high-CO<sub>2</sub>-requiring-phenotype and highlights the essential function of photorespiratory 2PG-metabolism for cyanobacteria despite the carbon concentrating mechanism [Eisenhut et al., 2008]. The contribution of the Mehler-reaction may be controlled by inorganic carbon [Badger et al., 2000]. Transcription of one of the A-type flavoproteins, Flv3, essential for photoreduction of O<sub>2</sub> in cyanobacteria, is increased under high-light and low-CO<sub>2</sub> conditions [Eisenhut et al., 2007]. This may indicate an increase in pseudocyclic ET under low CO<sub>2</sub>.

We will focus in the following especially on cyclic electron flow. This is an alternative electron flow that generates exclusively a proton gradient to build up ATP without accumulation of NADPH. It is assumed to involve PSI and the *cyt b<sub>6</sub>f* (Figure 1.8). In addition to that, several partners are proposed to catalyze donation of electrons from the acceptor side of PSI (Fd, FNR, NADPH) back into the PQ pool.

The assimilation of CO<sub>2</sub> in the Calvin cycle requires ATP and NADPH in a 3:2 ratio [Allen, 2002]. The number of protons that are translocated through the membrane per ATP is function-specific [Stock et al., 1999, Seelert et al., 2000]. On one side, it was recently calculated in spinach

chloroplasts that the 3:2 ratio cannot be completely satisfied by linear electron transfer [Seelert et al., 2000]. On the other side, a new reconstitution method and a chemiosmotic model system were described to determine the  $H^+$ /ATP ratio of the ATP synthase from spinach chloroplasts and resulted in a value of exactly 3 ATP /2 NADPH [Turina et al., 2003]. Thus, the importance of cyclic electron transfer to participate in building up an additional proton gradient without accumulating NADPH is still not shown under non-stressed photoautotrophic growth. We should however keep in mind that NADPH and ATP are both used for a variety of metabolic processes (e.g. nitrogen, carbon and sulfur assimilation, transport).



**Figure 1.8** Scheme of the thylakoid membrane. The different electron and proton translocating complexes are shown. The two major pathways for cycling of electrons are indicated with a blue and a black arrow. They involve the respiratory NDH-1 complex (NDH dependent; from NADPH) and a non-identified Ferredoxin:Quinone reductase (FQR; from Ferredoxin).

Photophosphorylation requires a redox poise - a balance in its input and output of electrons. Hence, photosynthetic systems try to maintain a poised plastoquinone pool. Over-reduction of the plastoquinone pool is expected when the Calvin cycle is unable to use NADPH, and one reason for this is insufficient ATP [Allen, 2003]. It was suggested that linear ET alone would not be sufficient to generate the ATP required for  $CO_2$  fixation and an obligatory role for cyclic ET was proposed [Golding et al., 2004]. Another role that has been postulated for cyclic ET in plants is to generate a trans-thylakoid pH gradient ( $\Delta pH$ ) [Heber and Walker, 1992]. The debate about the role of cyclic ET is fuelled by the difficulty of measuring it directly. Two approaches have been commonly used -  $P700^+$  steady-state measurements and  $P700^+$  relaxation following far-red illumination.

What is for sure is the fact that cyclic electron transfer is triggered under excess of NADPH - for example under high light or low  $CO_2$  [Miyake et al., 2005]. High light leads to a fast accumulation of electrons on the acceptor side of PSI and the utilization of these reducing equivalents can be limited through the Calvin cycle. Under low  $CO_2$  conditions, the Calvin cycle is limited by



substrate availability and NADPH accumulates. In addition to that, pseudocyclic ET involving the Mehler-reaction is triggered under low CO<sub>2</sub> [Hackenberg et al., 2009].

What is already known about the pathways of cyclic electron flow? According to Joliot [Joliot and Joliot, 2005], it is generally believed that the pathway starting from ferredoxin, the ferredoxin:quinone reductase (FQR) dependent pathway, constitutes the major pathway for cycling of electrons in plants (shown by the blue arrow in Figure 1.8). This pathway was originally found by inhibiting cyclic electron transfer using the cyt *bc*<sub>1</sub> specific inhibitor antimycin A and no biochemical evidence for the FQR pathway was obtained so far. However, Pgr5 and PgrL1 ([DalCorso et al., 2008] and references therein) were found to participate in this cyclic electron transfer. In plants, there are two partially redundant pathways taken by electrons in PSI cyclic ET [Shikanai, 2007, Munekage et al., 2002, 2004, 2008].

In cyanobacteria, the FQR pathway is generally considered to be a minor pathway for cycling of electrons whereas the NDH dependent pathway (Figure 1.8 black arrow) is believed to constitute the major pathway.

In *Synechocystis*, a mutant called M55 was constructed, defective in *ndhB* which is the single gene coding for the subunit NdhB of the NDH-1 complex. Due to this mutant the various functions of NDH-1 complexes were discovered. M55 is characterized by impaired cyclic ET [Mi et al., 1992b, 1994, 1995], impaired respiration and presents an impaired CO<sub>2</sub> uptake [Ogawa, 1991]. Due to multiple copies of genes *ndhD* and *ndhF*, distinct NDH-1 complexes with distinct functions were identified [Ohkawa et al., 2000].

NDH-1 is expressed only in low levels under high CO<sub>2</sub> photoautotrophic growth. Two distinct NDH-1 complexes are implicated in the carbon concentrating mechanism (CCM) and the expression of one of these complexes is induced under low CO<sub>2</sub> [Battchikova and Aro, 2007].

In addition to the major pathways, several other pathways have been proposed. In chloroplasts, association of FNR to PSI and/or cyt *b*<sub>6</sub>*f* has sometimes been taken as a structural evidence for different pathways of cyclic ET [DalCorso et al., 2008]. Supercomplex formation - e.g. PSI/cyt *b*<sub>6</sub>*f*/Fd - has also been proposed to support cyclic ET [Joliot and Joliot, 2005] but there is no clear biochemical evidence yet for such supercomplexes in cyanobacteria (see [Peng et al., 2008] for chloroplasts). During our study we wanted to address the issue of FNR involvement in these different cycling routes.

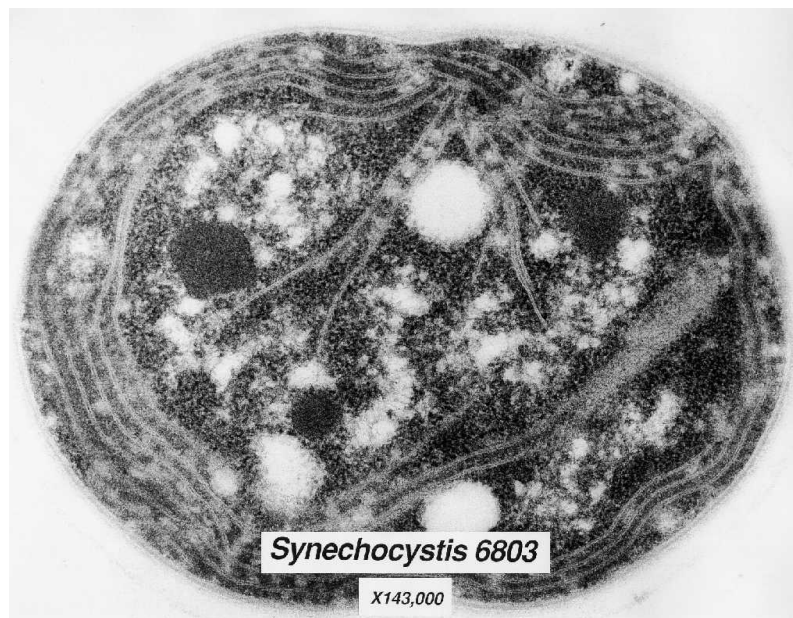
#### 1.1.4 Cyanobacteria

Cyanobacteria constitute a large and diverse group of photosynthetic prokaryotes. They inhabit almost any illuminated environment (freshwater, marine or terrestrial). All cyanobacteria are photoautotrophs and some species can grow as well photoheterotrophically.

All oxygen-evolving photosynthetic bacteria are cyanobacteria. Many species of cyanobacteria can fix nitrogen although the enzyme responsible for  $N_2$  fixation (nitrogenase) is sensitive to  $O_2$ . All cyanobacteria contain chlorophylls and carotenoids as photosynthetic pigments. They generally use chlorophyll *a* and lack chlorophyll *b*.

### *Synechocystis* sp. strain PCC6803

The unicellular, non-nitrogen-fixing cyanobacterium *Synechocystis* sp. strain PCC6803, hereafter referred to as *Synechocystis*, was the first photosynthetic organism and the second bacterium to have its genome fully sequenced [Kaneko et al., 1996]. The strain was originally isolated from a freshwater lake in California (Figure 1.9) [Zhang, 2006].



**Figure 1.9** Electron micrograph of a thin section through a *Synechocystis* 6803 cell. Taken from [www.nsf.gov/news/mmg/media/images/](http://www.nsf.gov/news/mmg/media/images/).

In addition to the cell envelope, these organisms have an internal system of thylakoid membranes (closely spaced membranes in Figure 1.9) where the electron transfer reactions of photosynthesis and respiration occur. The three-dimensional organization of the cytoplasm has been investigated using standard transmission electron microscopy and electron tomography. On one hand, it was shown that the thylakoid membranes are physically discontinuous from the plasma membrane [Liberton et al., 2006]. On the other hand, close connections between thylakoids and cytoplasmic membrane systems were observed [van de Meene et al., 2006]. Therefore, this debate is not closed yet.

We will now introduce the differences between cyanobacteria and chloroplasts that are important for the following study. We will start by a detailed description of the light-harvesting

complex - the phycobilisome - in cyanobacteria followed by an introduction on structure and function of FNR isoforms and the acceptor side of PSI in general.

## 1.2 Light-harvesting antenna

Prior to charge separation, a photon is absorbed by a pigment-protein (antenna) complex. The resulting excitation energy is transferred using radiationless steps to the chlorophylls involved in primary charge separation. These chlorophylls are generally associated to several hundred pigment molecules serving as light harvesting. This is necessary so that photosynthesis is less light-limited [Blankenship, 2002, Glazer, 1989].

Light-harvesting complexes can be divided into integral and external membrane complexes. Furthermore, they can be classified into seven families. These families include the core antenna complexes (e.g. CP47 and CP43 for PSII), the proteobacterial antenna complexes (e.g. LH1, LH2 and LH3 of purple bacteria), the eucaryotic LHC superfamily (e.g. LHC1 associated to PSI in algae and plants), the peridinin-Chl a protein (in Dinoflagellate algae), the chlorosome (in green sulfur bacteria and green filamentous bacteria) and the phycobilisome (in cyanobacteria and red algae) [Ughy, 2005]. The major difference between cyanobacteria/red-algae and green algae/brown algae/plants is the presence of a giant antenna complex, the phycobilisome (PBS; [Adir, 2005]).

### 1.2.1 Phycobilisome

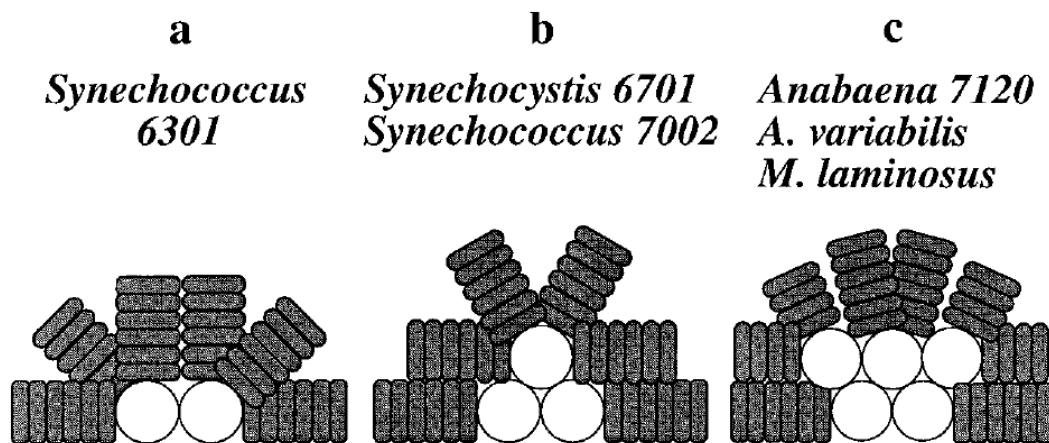
The phycobilisome is an external membrane complex that is attached to the stromal side of the photosynthetic membrane and constitutes an accessory antenna. It allows species bearing these antennas to harvest light in the spectral gap (500-660 nm) between the major chlorophyll absorbing bands and thus to utilize the entire visible range of sunlight [Glazer, 1989, Adir, 2005].

In the following, we will further detail some advances in (ultra)structure determination and function of these large macromolecular protein complexes (7 to 15 Megadaltons).

#### Phycobilisome structure

Different types of PBS exist. We will focus on the hemidiscoidal PBS because they are present in *Synechocystis*. They are composed of a core subdomain and peripheral rods. The hemidiscoidal PBS are divided in three types (shown in Figure 1.10). They include bicylindrical, tricylindrical and pentacylindrical PBS (Figure 1.10; [MacColl, 1998]). In *Synechocystis*, the common tricylindrical hemidiscoidal PBS is present (Figure 1.10b). In addition to these classical PBS, minor PBS were identified in *Synechocystis* that do not contain core subunits [Kondo et al., 2005, 2007, 2009].





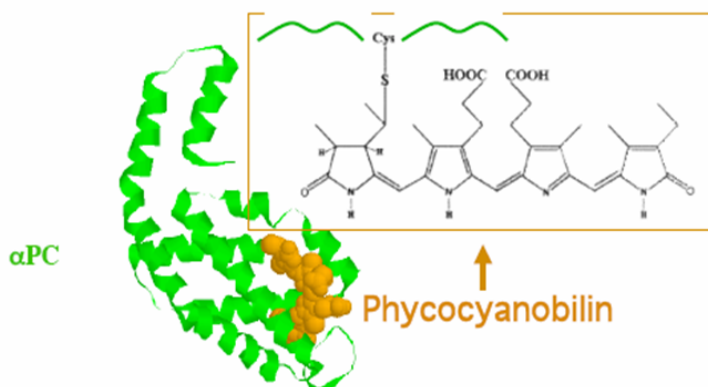
**Figure 1.10** Schematic representation of the three types of hemidiscoidal PBS. a, bicylindrical; b, tricylindrical; c, pentacylindrical. Taken from [Ducret et al., 1998].

**Phycobiliproteins** The PBS is composed of phycobiliproteins (PBP) and mostly colorless linker polypeptides. Four major subgroups of PBP are found. They include the allophycocyanin of the core (AP,  $\lambda_A^{\max}=652$  nm), phycocyanin that is always present close (proximal) to the core in the rods (PC,  $\lambda_A^{\max}=620$  nm), phycoerythrin (PE,  $\lambda_A^{\max}=560$  nm) and phycoerythrocyanin (PEC,  $\lambda_A^{\max}=575$  nm) far away (distal) to the core in the rods when present. The PBPs are physically arranged to favor an energy gradient from PE (or PEC) through PC to AP and finally to the reaction center ( $\lambda=670$ -680 nm).

The smallest PBP unit are  $\alpha$  and  $\beta$  ( $\approx 17$  and 18 kDa) subunits that form a heterodimer - the "( $\alpha\beta$ ) monomer (protomer)". Each subunit contains one or more covalently attached bilins. Bilins are linear tetrapyrrole prosthetic groups. The covalent attachment of the bilins in the amino acid sequence is conserved,  $\alpha 84$  in the  $\alpha$  monomer,  $\beta 84$  and  $\beta 155$  in the  $\beta$  monomer. An  $\alpha$  subunit of PC with the phycocyanobilin (PCB) covalently attached to a cysteinyl residue (ring A) is shown in Figure 1.11. In *Synechocystis*, only AP and PC are present in the core and rod subdomains, respectively. They are both composed of subunits that contain exclusively PCB.

**Linker polypeptides** In addition to the PBP, a variety of linker polypeptides are present. Except for the  $L_{CM}$ , these do not contain any chromophore. Different linkers are specifically responsible for each level of PBP assembly and function to stabilize the PBS and optimize its absorption and energy transfer characteristics. There exist linkers that anchor the core to the photosynthetic membrane ( $L_{CM}$ ), linkers that connect the rods to the core ( $L_{RC}$ ) and linkers that are only present in the core and the rods:  $L_C$  and  $L_R$ , respectively. *CpcC2*, *cpcC1* and *cpcD* encode the rod linkers  $L_R^{30}$ ,  $L_R^{33}$  and  $L_R^{10}$  (Figure 1.13). Two independent genes (*cpcG1* and *cpcG2*) encode the rod-core linker ( $L_{RC}$ ) [Ughy and Ajlani, 2004].

The core-membrane linker  $L_{CM}$  is a high molecular weight polypeptide. This major linker is



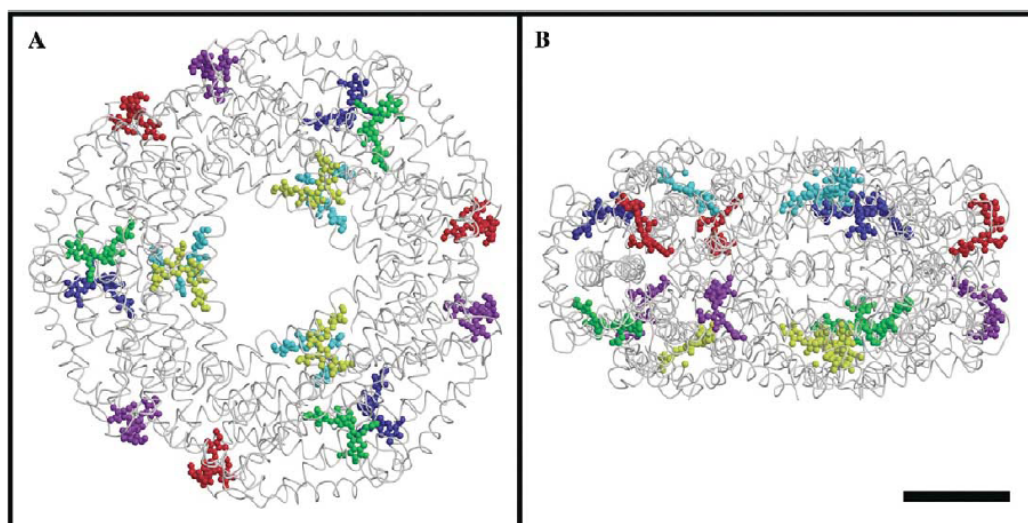
**Figure 1.11**  $\alpha$  subunit of phycocyanin (PC) with its chromophore phycocyanobilin (PCB).

responsible for the assembly of AP discs into cylinders and core formation.  $L_{CM}$  also plays a key role in anchoring the PBS to the photosynthetic membrane and in tuning the properties of the bound pigment cofactors such that absorbed light is funnelled towards the photosystems. Two copies of this multifunctional polypeptide (mass 75-125 kDa) are present per PBS core [Capuano et al., 1991, Arteni et al., 2009].

**Phycobilisome Assembly** PBS substructures are built up from stacked PBP discs made either of trimers (AP) or of hexamers (PC) of PBP subunits. X-ray crystallography was used to determine the structure of PBP discs. The crystal structure of allophycocyanin showed two loosely stacked trimers [Liu et al., 1999]. C-phycocyanin is a hexameric disc of 110Å diameter and 60Å thickness (Figure 1.12 and [Nield et al., 2003]). In many structures of PC, the two  $(\alpha\beta)_3$  trimers that form a hexameric disk  $(\alpha\beta)_6$  were positioned face to face. The hexamer is easily disassembled in  $(\alpha\beta)$  monomer in diluted solutions, indicating that just a limited number of salt-bridges and/or hydrogen bonds are involved in monomer stability. Except in one case [Reuter et al., 1999], the available structures do not contain any linker.

Each of the cylinders in the tri-cylindrical core is composed of four trimeric AP discs. These discs have slightly different compositions. They involve simple AP trimers,  $L_C$ -containing AP trimers, trimers containing an alternative AP-B  $\alpha$  subunit and trimers that possess a red-shifted  $\beta$  isoform and an  $\alpha$  subunit provided by the  $L_{CM}$ . As indicated above,  $L_{CM}$  together with the  $L_C$  assemble the AP discs into cylinders and into a core substructure.

The rod-core linkage position is always occupied by a PC hexamer that is attached due to



**Figure 1.12** *S. elongatus* C-PC hexameric cluster. The 3 chromophores of each  $\alpha\beta$  heterodimer are depicted in the same color. Three  $\alpha\beta$  heterodimers form a disk around the three-fold axis. Bar represents 25 Å. A, projection parallel to the three-fold axis; B, projection normal to the three-fold axis. Taken from [Nield et al., 2003].

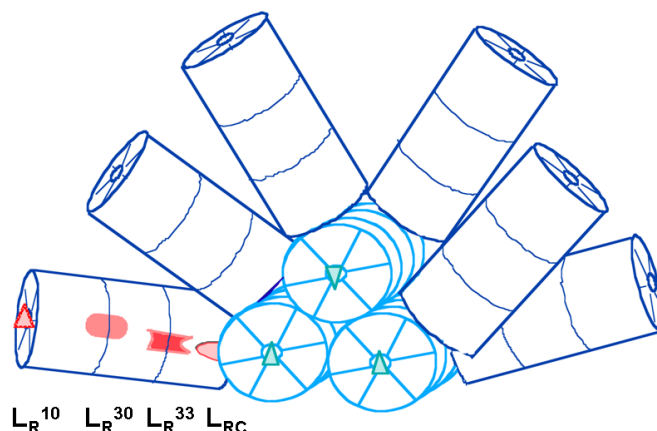
$L_{RC}$ . For each disc at a particular rod location there exists a specific linker. Six rods radiate in a hemidiscoidal array from the core. In *Synechocystis*, rods are composed of three stacked PC hexamers. The position of the rod linkers  $L_R^{10}$ ,  $L_R^{30}$ ,  $L_R^{33}$  and  $L_{RC}$  in the PC hexamers is indicated in Figure 1.13.

Although many isolated components of different PBSs were crystallized, the structure of the entire PBS and its association to PSII is only studied by electron microscopy [Arteni et al., 2009].

### Phycobilisome function

Attached primarily to reaction centers of PSII, the PBS can functionally link more than 600 energy-absorbing pigments to a single PSII dimer in addition to the PSII integral antenna subunits CP43 and CP47. On one side, direct measurements of fluorescence recovery after photobleaching [Mullineaux, 2004] indicated that the 10 Megadalton PBS is quite mobile *in vivo*, much more than the photosystems. On the other side, ultrastructures of the *Synechocystis* photosynthetic membranes indicated that the width of the stromal space between two membranes matches the PBS height (see Figure 1.9). Therefore, it seems difficult to imagine highly mobile PBS between the closely spaced thylakoids.

In addition to light absorption, the PBS can function as a source of nutrients under starvation conditions. There exists a mechanism of ordered PBS disassembly that requires the presence of a number of gene products. Keeping in mind that the PBS can account for up to 30 % of the total protein mass in a cyanobacterial cell, it constitutes a significant reservoir.



**Figure 1.13** Representation of a hemi-discoidal PBS, as seen from the side. Kindly provided by Dr. Ajlani.

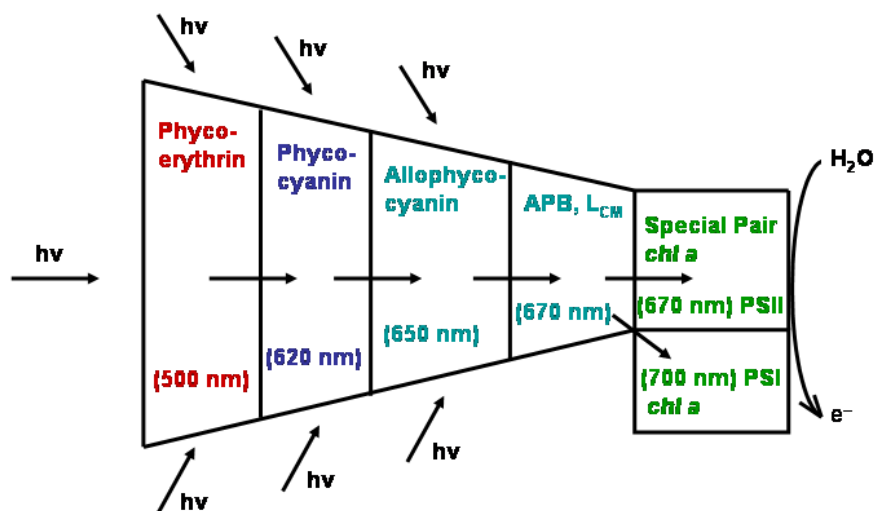
**Energy transfer within the PBS** The absorbed light energy harvested at the periphery of the PBS is transferred to the PSII reaction center complex by radiationless excitation energy transfer with an efficiency of  $> 95\%$ . This implies that the energy-transfer mechanism must proceed rapidly in order to avoid energy losses by competing radiative or non-radiative decay processes. Light energy is absorbed mainly by the peripheral rods, where the shortest wavelength absorbing PBP (PE or PEC) are located. The excitation energy is then transferred by a radiationless resonance energy transfer to C-PC and then to AP. Energy is finally transmitted to PSII and partially to PSI reaction centers through the terminal emitters of the PBS (Figure 1.14) [Sidler, 1994].

The spectroscopic properties of the bilins are modified by the protein in two ways which are critical for their role in light absorption and energy transfer:

1. the bilins in all PBP are held rigidly in extended conformations,
2. the excited state lifetime of the pigment in the protein is long (*vs.* the isolated pigment).

The extended conformations allows the strong absorption in the visible part of the light spectrum. The long excited-state lifetime avoids the loss of excitation energy by radiationless de-excitation pathways. The absorption and transfer of light energy is performed by chromophores that are called donors. Acceptors can both absorb excitation energy and fluoresce. Thus, the steady state fluorescence emission originates almost exclusively from the acceptors.

In C-PC, the bilins at  $\alpha 84$  and  $\beta 155$  are donors and the bilin at  $\beta 84$  is the acceptor. PCB  $\beta 84$  extends into the center of the trimeric disc, whereas those at  $\alpha 84$  and  $\beta 155$  lie toward the



**Figure 1.14** Energy flow in PBS of cyanobacteria and red algae. Radiationless excitation energy transfer from short-wavelength (PE) to long-wavelength-absorbing pigment-protein complexes (AP). Energy is finally transferred to and distributed between PSII and PSI. Adapted from [Sidler, 1994].

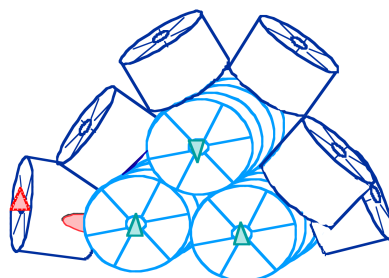
periphery. In the face-to-face arrangement of double discs in the rods, the consecutive discs are arranged to favor rapid energy transfer. Picosecond energy transfer measurements showed that the excitation energy absorbed by any bilin at the periphery is rapidly localized on the centrally located acceptor bilins (yellow and light blue chromophores in Figure 1.12A) [Glazer, 1989].

Directional energy transfer is promoted through the PBS. Between the PC discs, interaction with different linker polypeptides confer distinctive spectroscopic properties to the acceptor bilins. The absorption and emission spectra of the  $(\alpha^{PC}\beta^{PC})_6L_{RC}$  complex are shifted towards the red relative to those of  $(\alpha^{PC}\beta^{PC})_6L_R^{33}$ . In consequence, the favored direction of transfer is from the distal disc to the PC disc proximal to the core.

In summary, the energy absorbed by any of the bilins in the PBS localizes rapidly ( $< 8$  ps) on the four terminal acceptor bilins (APB and  $L_{CM}$ ) in the core. The emission of these bilins overlaps precisely the absorption spectrum of the reaction center of PSII. The light-guide function of the PBS is completed when energy is transferred radiationless from the terminal acceptors in the PBS to the reaction center [Glazer, 1989].

### 1.2.2 Phycobilisome rod mutants

PBS mutants were constructed in three rod-linker-coding genes located in the *cpc* operon of *Synechocystis* [Ughy and Ajlani, 2004]. *CpcC1* and *cpcC2* encode  $L_R^{33}$  and  $L_R^{30}$ , respectively.  $L_R^{33}$  and  $L_R^{30}$  are linker polypeptides that attach the middle and the distal PC hexamer of the rods (Figure 1.13). During *in vitro* studies, we used a mutant called CB in which *cpcC1* and *cpcC2* were deleted. The PBS contained only one PC hexamer per rod (Figure 1.15) [Ughy and Ajlani, 2004].



**Figure 1.15** Representation of the PBS in the CB mutant. WT PBS contains 3 hexamers of PC per rod, whereas CB contains only 1 hexamer of PC per rod. Kindly provided by Dr. Ajlani.

## 1.3 Photosystem I and its electron acceptors

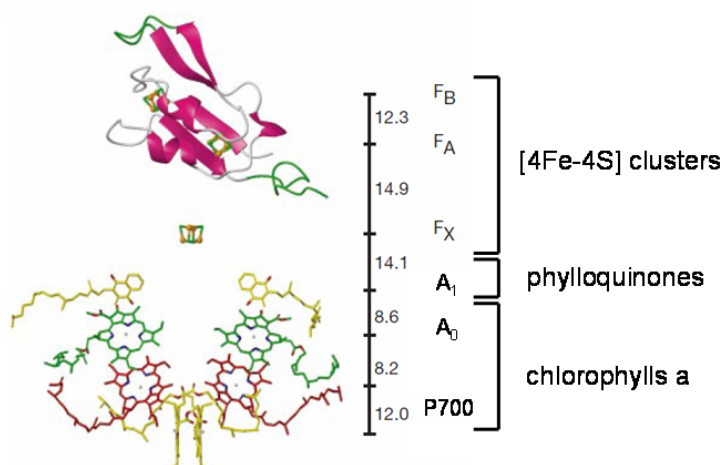
We were interested in the acceptor side of PSI. Electrons following the linear electron transfer are designated to reduce  $NADP^+$ , building up the reducing power NADPH. We will now further introduce PSI, Fd and FNR.

### 1.3.1 Photosystem I

The three-dimensional structure of cyanobacterial PSI was solved by Jordan et al. in [2001]. It provided atomic details of the 12 subunits and 127 cofactors comprising 96 chlorophylls, two phylloquinones, three [4Fe-4S] clusters, carotenoids and lipids. The cofactors involved in ET are located within the membrane subunits PsaA/PsaB and the stromal subunit PsaC (Figure 1.16). It can be seen that the chlorophyll pairs are arranged in two branches labelled A and B. There has been some controversy about whether the two branches work [Brettel and Leibl, 2001] and this issue is now settled in favor of the theory that the two branches work. The first of the three [4Fe-4S] cluster  $F_X$  is located in the middle between PsaA and PsaB. The following two [4Fe-4S]  $F_A$  and  $F_B$  are provided by the stromal subunit PsaC. We will now further introduce the ET kinetics inside PSI.

Electron transfer in PSI was reviewed [2001] by Brettel and Leibl. Standard free energy levels and kinetics of charge separation are shown in Figure 1.17. Charge recombination between  $P700^+$





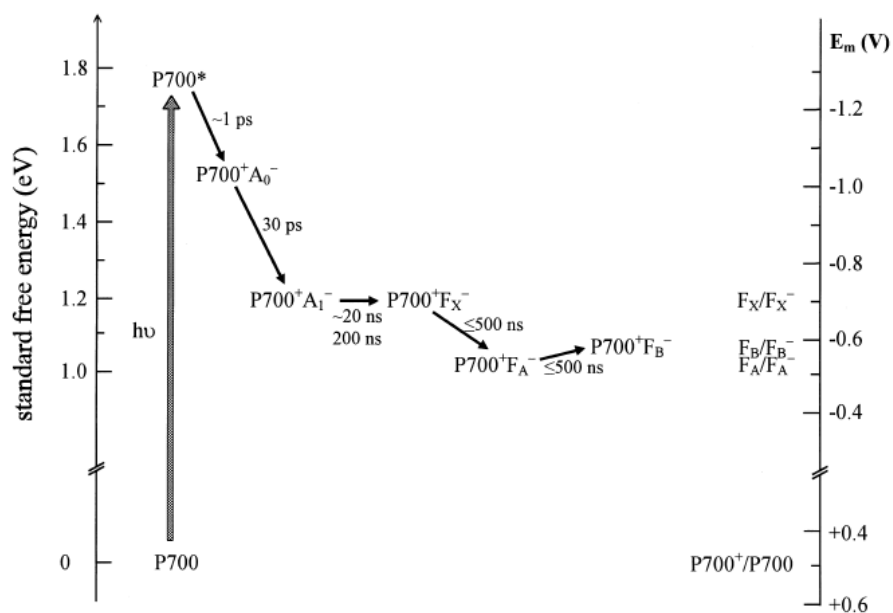
**Figure 1.16** Cofactors of the electron transfer chain (ETC) and of PsaC. View parallel to the membrane plane. The pairs of chlorophylls of the ETC are arranged in two branches A and B. The chlorophylls a (P700 and A<sub>0</sub>), the phylloquinones (A<sub>1</sub>) and the [4Fe-4S] clusters (F<sub>X</sub>, F<sub>A</sub> and F<sub>B</sub> according) are labelled to their spectroscopic terms. The center-to-center distances between the cofactors (black lines) are given in Å. Adapted from [Jordan et al., 2001].

and the reduced form of any one of the electron acceptors can be observed when forward electron transfer to the subsequent acceptor is blocked. In addition to that, reduction midpoint potentials are indicated on the right in Figure 1.17. The reduction midpoint potentials are very similar for F<sub>A</sub> and F<sub>B</sub> and it is generally thought that F<sub>A</sub> and F<sub>B</sub> undergo fast (< 1 μs) redox equilibrium [Setif, 2001]. They are both higher than the midpoint electron potential for F<sub>X</sub>/F<sub>X</sub><sup>-</sup>. We will be furthermore interested in the stromal subunits of PSI as they provide the docking site for Fd binding.

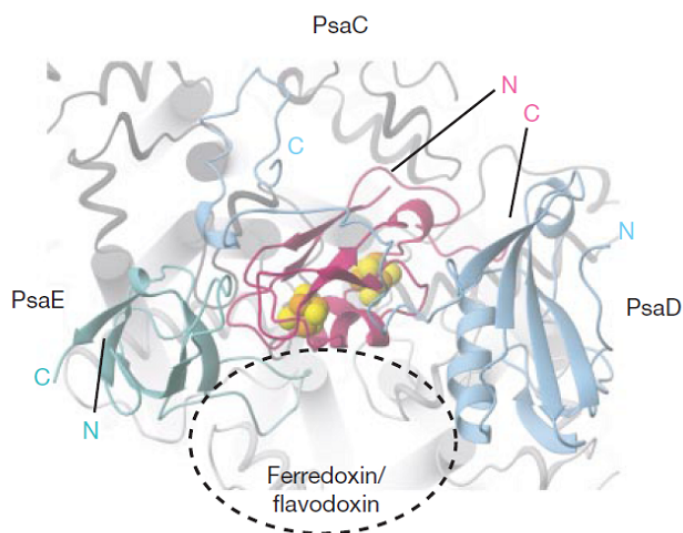
The three stromal subunits of PSI are shown in Figure 1.18. In addition to providing the two terminal electron acceptors of PSI, they provide the docking side for Fd that is shown as a dashed ellipse in Figure 1.18. This complex formation is important for efficient electron transfer. We will now further introduce Fd structure and function in plants and cyanobacteria.

### 1.3.2 Ferredoxin

Ferredoxin (Fd) is a soluble, low molecular weight protein (*ca.* 11 kDa) that mediates transfer of one electron from a donor to an acceptor. The redox active center is a [2Fe-2S] cluster with a highly negative redox potential (-350 to -450 mV), making reduced Fd a powerful reductant. The [2Fe-2S] cluster is ligated by four highly conserved Cys residues [Bottin and Lagoutte, 1992,



**Figure 1.17** Approximate standard free energy levels and kinetics of charge separation in PSI. The standard free energy of the dark state (P700) was arbitrarily set to zero. Reduction midpoint potentials (versus NHE) obtained by redox titrations of intact PSI are indicated on the right-hand scale. Taken from Brettel and Leibl [2001].



**Figure 1.18** View along the membrane normal from the stromal side showing subunits PsaC, PsaD and PsaE. They cover some of the loop regions and helices of PsaA and PsaB (light grey). Dashed ellipse: putative docking site of ferredoxin, covering loops of PsaA. Taken from [Jordan et al., 2001].



[Hanke et al., 2004b](#)].

Fd is best known for its photosynthetic role by accepting electrons from PSI and donating them to the enzyme FNR for photoreduction of NADP<sup>+</sup>. Donation of electrons by Fd has been demonstrated to many other enzymes essential for cellular processes including nitrogen assimilation (*e.g.* nitrite reductase), sulfur assimilation (sulfite reductase) and redox regulation (Fd:thioredoxin reductase) [[Knaff, 1996](#)]. In addition to PSI, Fd may be reduced by NADPH oxidation by FNR. Flavodoxin can substitute Fd under conditions of iron starvation in most cyanobacteria and some algae and may be efficient in reducing most or all of the soluble electron acceptors.

Fds are present as multiple isoforms in many plants and algae. We will further introduce the isoforms existing in plants and cyanobacteria.

**Plant isoforms** Higher plants contain distinct leaf and root Fd isoforms with conserved differences, reflecting the different electron donors to Fd in photosynthetic and non-photosynthetic tissues. Functional differences have been demonstrated between leaf and root Fds, with respect to redox potential and activity in assays of NADP<sup>+</sup> photoreduction and NADPH oxidation. These differences are highly conserved among species: Leaf Fds have a redox potential around 50 mV more negative than root Fds; during NADP<sup>+</sup> photoreduction, leaf FNR has an affinity around 10-fold higher for leaf Fds than for root Fds, and during NADPH oxidation root FNR has an affinity around five times higher for root Fds than for leaf Fds.

As the redox potential of leaf Fd is around -420 mV, a 50 mV difference may not appear dramatic, but the flux through photosynthesis is so vast that small changes in efficiency are likely to have a profound physiological impact. It was stated that the concentration of Fd in the chloroplast is of the same order as the concentration of Fd-dependent enzymes, which could therefore be in competition, giving great physiological significance to even small differences in affinity and activity [[Gou et al., 2006](#)].

**Cyanobacteria isoforms** Analogously to plants, cyanobacteria also possess several molecular forms of [2Fe-2S] Fd encoded by distinct genes. The most abundant protein form has been termed Fd1 (Fed1). The *fed1* gene (*ssl0020* in *Synechocystis*) was found to be strongly expressed as a light-induced transcript. The other *fed*-like genes appeared to be silent or moderately expressed. *fed1* was found to be critical to *Synechocystis* viability in spite of *fed*-like genes *slr0150*, *sll1382* or flavodoxin induction, even after the addition of glucose that compensates for the loss of photosynthesis [[Poncelet et al., 1998](#)]. We used during our studies only the major Fd isoform assuming that it is involved in all the metabolic pathways under our conditions. This is in

contrast to the plant isoforms, where tissue specificity ensures the function of distinct isoforms in distinct metabolic pathways.

**Kinetics of ferredoxin reduction** After PSI photoexcitation, several fast kinetic components (submicrosecond and microsecond) for Fd reduction have been identified *in vitro*. The rates of the fast kinetic components did not depend on the concentration of the partners. In addition to that, a slow kinetic component was also identified characterized by a rate that depends linearly on the Fd concentration. The fast and slow kinetic components are thus called first-order and second-order phases, respectively. The first-order phases are thought to correspond to ET processes which occur within PSI/Fd complexes. The second-order phase corresponds to a diffusion-limited ET which is observable in the fraction of PSI which does not bind Fd before flash excitation [Setif, 2001].

At least two or three first-order components were necessary to describe the first-order kinetics of Fd reduction. The spectra of the three phases obtained in *Synechocystis* were shown to be consistent with Fd reduction from  $(F_A, F_B)^-$ . At least 80% of Fd is reduced within the PSI/Fd complex at pH 8 in *Synechocystis*. The two slower first-order processes might result from some rate limitation either in ET from  $F_B$  to Fd or during intramolecular PSI ET. The distal cluster  $F_B$  is photoreduced in the submicrosecond time range in PSI. Heterogeneity of ET kinetics is an intrinsic property of Fd reduction, and was ascribed to different conformations of the PSI/Fd complex.

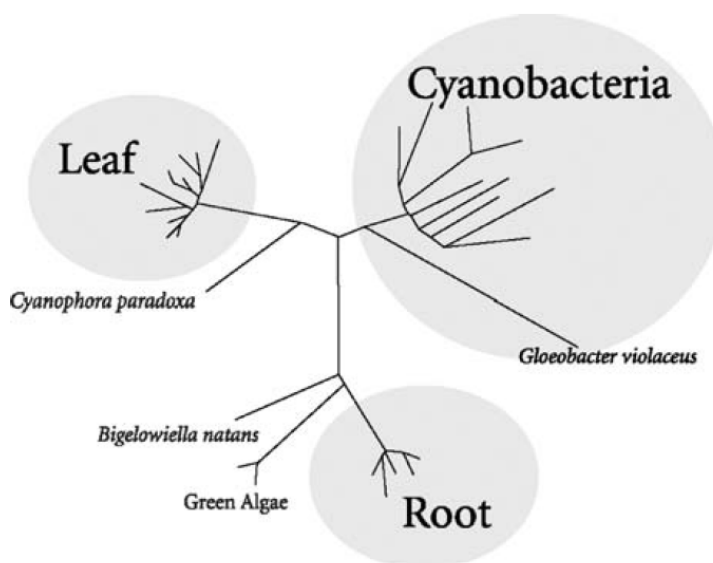
Fd reduction should compete efficiently with the recombination reaction between  $P700^+$  and  $(F_A, F_B)^-$  and this would imply a  $t_{1/2}$  of several orders of magnitude faster than the recombination (30-100 ms). Furthermore, a high efficiency of Fd may be required for avoiding reduction of oxygen from  $(F_A, F_B)^-$  which is potentially harmful for PSI. It has been speculated that, *in vivo*, complex formation is useful, though not critical, for promoting efficient reduction of the soluble acceptors and avoiding reduction of oxygen by  $(F_A, F_B)^-$  [Karplus and Faber, 2004].

Complex formation was found to occur as well between Fd and the enzyme FNR in plants and cyanobacteria [Hanke et al., 2004b]. As stated above, FNR receives two electrons from two Fds to finally reduce  $NADP^+$  to NADPH during linear electron flow. We will now introduce the structure and function of FNR that catalyzes the last step of the building up of the NADPH.

### 1.3.3 Ferredoxin:NADP oxidoreductase

#### Structure of ferredoxin:NADP oxidoreductase

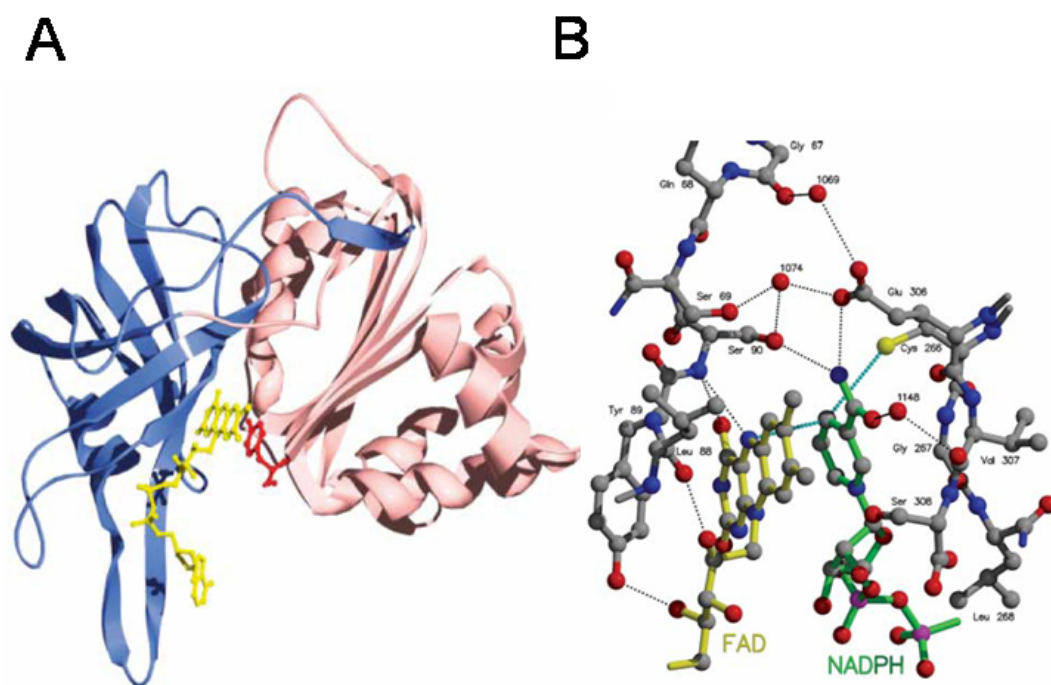
From their primary sequences, the different ferredoxin:NADP oxidoreductases (FNRs) can be grouped in three major branches. The plant-leaf chloroplast FNRs are on one branch, the widely



**Figure 1.19** Relationships among various FNR amino-acid sequences. In the unrooted dendrogram shown, each branch represents 1 of 30 known plastid FNR sequences. The lengths of the branches are proportional to the level of sequence difference. Taken from [Karplus and Faber, 2004].

diverse cyanobacterial FNRs are on a second branch, and the root plastid enzymes together with the enzymes from green algae chloroplasts are on the third branch (Figure 1.19). All of the plastid type FNRs share sequence identities of over 40%. Crystallographic structures have been determined for six different FNRs: four leaf-type enzymes, one root-type enzyme and one cyanobacterial enzyme (*Anabaena variabilis*). These enzymes all have equivalent structures including two classical structural domains (Figure 1.20A). The amino-terminal residues (*ca.* 150) form the FAD-binding domain (blue in Figure 1.20A) and the carboxy-terminal residues (*ca.* 150) form the NADP<sup>+</sup> binding domain (pink in Figure 1.20A). The plant chloroplast enzymes are structurally similar and the root and cyanobacterial enzymes are structurally variable. One major difference in the corn root enzyme structure compared to leaf is that the amino terminus packs in a completely different position. Based on sequence comparisons, this amino-terminal packing appears to be conserved among root-type enzymes. With regard to amino-acid residue conservation, among the *ca.* 40 known FNR sequences, about 25% of the residues in the protein are conserved. It was stated that this high level of conservation over such long evolutionary distances implies a fairly stringent level of selection [Karplus and Faber, 2004].

**FAD binding** The FAD binding site in FNRs is quite highly conserved for the FMN half of the prosthetic group that contains the isoalloxazine (upper part in Figure 1.20A). In contrast, the adenosine portion of FAD (lower part in Figure 1.20A) shows significant variation in its position of binding. The isoalloxazine moiety is the best defined portion of the FAD and the adenosine



**Figure 1.20** Ferredoxin:NADP oxidoreductase structure. A: The C $\alpha$  polypeptide backbone of plant-type ferredoxin:NADP oxidoreductase. FNR is a two-domain flavoprotein. The computer graphic is based on X-ray diffraction data for the spinach enzyme, with the FAD binding domain shown in blue, the NADP(H) binding domain in pink, and the FAD prosthetic group in yellow. Taken from [Carrillo and Ceccarelli, 2003]. B: Geometry of the productive NADPH-FAD Michaelis charge transfer complex. A view is shown including all atoms surrounding the locus of hydride transfer, that is the nicotinamide C4- and the FAD N5-atoms. The model is that of the pea FNR Y308S-NADPH complex (pdb entry 1QFZ chain A). Taken from [Karplus and Faber, 2004].

portion is highly mobile.

**NADP(H) binding** The originally published structures showed that the C-terminal Tyr residue (shown in red in Figure 1.20A) was blocking nicotinamide access to the flavin. A technique of crystal soaking with NADP<sup>+</sup> had resulted only in clear density for the 2'-phospho-5'-AMP (PAMP) part of the dinucleotide. The 2'-phosphate itself has been proposed to be the primary recognition feature, with the adenine and 5'-phosphate being of secondary importance. Because of how NADP binds to FNR, we will introduce the NADP as two halves, the PAMP half and the nicotinamide-mononucleotide (NMN) half (in Figure 1.20B the NMN part is shown). From crystallographic studies, it was observed that only in a small fraction of the enzyme (15% pea FNR, [Piubelli et al., 2000]) and an even smaller fraction of cyanobacterial enzymes [Hermoso et al., 2002] the C-terminal Tyr swings out of the way so that the NMN half of NADP binds properly. A more complete understanding of nicotinamide binding was finally obtained using a mutant of pea FNR with the C-terminal Tyr converted to Ser [Deng et al., 1999, Piubelli et al., 2000]. A surprise compared to other NAD(P) dependent flavoenzymes was that the nicotinamide was not co-planar with the flavin, but made a 30° angle with it [Karplus and Faber, 2004].

Roles of active site residues were proposed by Deng et al. [1999] and involved the boat-like conformation of the nicotinamide ring to facilitate hydride transfer. In addition to that, the C-terminal Tyr does not play an active role in hydride transfer, but is primarily a placeholder for the nicotinamide that modulates the binding thermodynamics of NADP and protects the flavin from reaction with oxygen. It was also speculated that reduction of the flavin or Fd binding might promote the movement of Tyr, even if dynamics of Tyr movements may be sufficient to support catalysis [Karplus and Faber, 2004].

**Ferredoxin binding** Protein-protein interaction is an important determinant for electron transfer between Fd and FNR. The X-ray crystal structures of complexes formed between Fd and FNR from the cyanobacterium *Anabaena* 7120 [Morales et al., 2000], maize leaf [Kurisu et al., 2001] and maize root have been solved (see Figure 1.21 for the cyanobacterial Fd-FNR complex). In the complex structure of Fd and FNR, Fd binds to a concave region on the FAD-binding domain of FNR, bringing the [2Fe-2S] cluster into close proximity to that of FAD. Different orientations of Fd relative to FNR have been found in cyanobacteria, plant-leaf and plant-root complexes. The relative buried surface areas differ as well, the root complex having a decreased buried surface area [Hanke et al., 2004b].

The complex in general is largely electrostatic in nature. The pattern of interaction between Fd and FNR is composed of a core of hydrophobic interactions surrounding the prosthetic groups, stabilized by a series of interactions between charged side chains and through hydrogen bonds.

Hydrophobic effects originating from dehydration of water molecules in the protein-protein interface may also give a significant contribution. In *Anabaena*, a total of ten hydrogen-bonding and ionic-bridge interactions stabilize the complex [Morales et al., 2000]. The side chains involved in intermolecular charge interactions concern mainly acidic Fd residues and basic FNR residues [Hurley et al., 2002].



**Figure 1.21** Ribbon drawing of *Anabaena* Fd : FNR complex (PDB code: 1EWY). One intermolecular salt bridge is shown as ball-and-stick model: 1, FNRLys75-FdGlu94. Taken from [Hanke et al., 2004b].

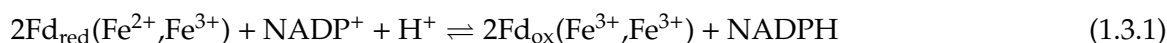
Redox potentials may change when binding occurs [Batie and Kamin, 1984b]. A negative redox shift in the potential of the [2Fe-2S] cluster of Fd was observed that would be advantageous to ET in the photosynthetic direction [Hanke et al., 2004b]. The more weakly binding complexes seem to yield more rapid ET and catalytic turnover. Thus, it could be that effective *in vivo* ET involves very short-lived (nearly collisional) complexes, rather than a tight, highly specific complex [Karplus and Faber, 2004].

**FNR superfamily** Given the early origin of photosynthesis, FNRs would be expected to be ancient proteins. Consistent with this, there exists a large and diverse family of oxidoreductases which have as a catalytic core the two-domain FNR-like module [Karplus et al., 1991]. They are diverse enough that some use FAD and others use FMN whereas some use NADP and others use NAD. Structurally known members of the family are sulfite reductase, NO synthase, NADPH: cytochrome P450 reductase, *etc.*



### Mechanism of ferredoxin:NADP oxidoreductase

Early studies [Shin and Arnon, 1965] showed that the physiological role of the chloroplast oxidoreductase was to catalyze the final step of photosynthetic electron transfer, namely, the electron transfer from the iron-sulfur protein Fd, reduced by PSI, to NADP<sup>+</sup> (Equation 1.3.1).

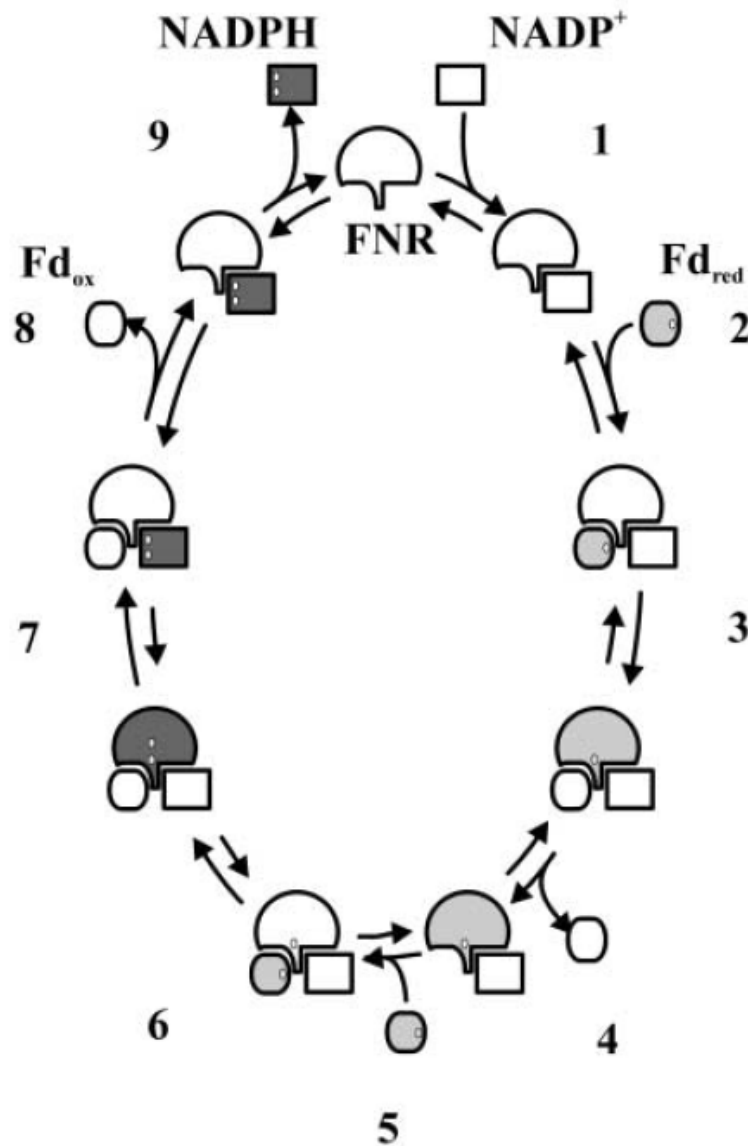


Equation 1.3.1 shows the ability of FNR to exchange electrons between obligatory one- and two-electron carriers, which is a direct consequence of its prosthetic group. FAD and other flavins can exist in three different redox states: oxidized, one-electron reduced (semiquinone radical) and fully reduced (hydroquinone), containing 18, 19 and 20 electrons in a  $\pi$  orbital system, respectively. The isoalloxazine ring also provides lone electron pairs for protonation, which results in tautomers. FNR functions are not confined to photosynthesis. The backreaction of Equation 1.3.1 is actually more often found in nature in heterotrophic tissues such as plant roots, heterotrophic bacteria, animal and yeast mitochondria, *etc.*. Following the backreaction, NADPH is oxidized and reduced Fd is available for numerous metabolic pathways (detailed in paragraph on Fd).

As indicated above, FNR forms complexes with NADP(H). The enzyme actually displays a strong preference for NADP(H) and is a poor NAD(H) oxidoreductase. FNR was stated to be characterized by its plasticity as a catalyst [Ceccarelli et al., 2004] and is ubiquitous among living organisms. Steady-state and rapid kinetic measurements have resulted in a comprehensive model describing the various reactions of Equation 1.3.1 [Batie and Kamin, 1984a].

The overall reaction was interpreted as an ordered two-substrate process, with NADP<sup>+</sup> binding first (Reaction 1 in Figure 1.22). Under these assumptions, the kinetics were consistent with the formation of ternary complexes as intermediates of the catalytic mechanism (Reaction 2 and 5 in Figure 1.22). We will first introduce the individual steps of the NADP<sup>+</sup> reductase activity of FNR and discuss the NADPH oxidation later in detail.

**NADP(H) binding (Reactions 1 and 9 in Figure 1.22)** NADP<sup>+</sup> is probably the leading substrate during FNR turnover even if no chemistry is expected to occur between the oxidized nicotinamide and the oxidized flavin. For example, NADP<sup>+</sup> greatly accelerates the full reduction of FNR by reduced Fd [Batie and Kamin, 1984a]. Formation of the binary NADP:FNR complex was studied using differential spectroscopy. As indicated above, the C-terminal Tyr must be displaced to allow stacking of the nicotinamide ring onto the re-face of the isoalloxazine moiety. This



**Figure 1.22** The electron transfer mechanism of ferredoxin-NADP(H) reductase. The various steps of the catalytic pathway were initially proposed by Batie and Kamin [1984a] on the basis of kinetic and binding experiments on the spinach FNR. Oxidized forms are white, one-electron reduced forms are light grey and two-electron reduced forms are dark grey. An error was found in the present scheme, between steps 5 and 6 the FNR should be in light grey as already one-electron reduced. Taken from [Carrillo and Ceccarelli, 2003].



thermodynamically unfavored process results in a decrease of the binding affinity for NADP(H) [Carrillo and Ceccarelli, 2003].

Binding of NADP(H) to FNR might thus be interpreted as a two-step binding of the nucleotide to a bipartite site. First, a strong interaction between the PAMP part and FNR is carried out and is followed by isomerization that favors NMN part stacking onto the isoalloxazine moiety to facilitate hydride transfer. The second step of NADP(H) binding is energetically costly and weakens the entire interaction to a remarkable extent [Carrillo and Ceccarelli, 2003]. Reaction 9 in Figure 1.22 also represents the initial event of the reverse reaction, the reduction of Fd due to NADPH oxidation.

**ET from Fd<sub>red</sub> to FNR (Reactions 2-4 in Figure 1.22)** First electron reduction of FNR<sub>ox</sub> by Fd<sub>red</sub> to the radical semiquinone is too fast to be measured by rapid mixing techniques. The molecular association of FNR with its electron partners is steered by electrostatic interactions. This could explain the interchangeable accommodation of Fd and flavodoxin to FNR, that share very low sequence similarity. It was determined that the surface electrostatic potentials of Fd and flavodoxin overlap completely [Ullmann et al., 2000].

As shown in Figure 1.21, binary complexes of oxidized FNR and Fd have been resolved by X-ray crystallography for *Anabaena* and maize couples [Kurusu et al., 2001, Morales et al., 2000]. As indicated above, the FAD and [2Fe-2S] redox centers were sufficiently close for direct electron transfer and the relevance of complementary patches of basic and acidic residues in FNR and Fd was confirmed. It is believed that first a nonproductive complex is built up due to polar interactions. Then, several fine adjustments stabilized by hydrogen bonds, salt bridges, van der Waals interactions and hydrophobic packing forces originate from the dehydration of the protein-protein interface [Carrillo and Ceccarelli, 2003].

Differences between maize and cyanobacterial complexes were found that indicate different protein-protein interactions. Hurley et al. [2002] proposed that crucial parameters for Fd and flavodoxin binding might be proximity of the prosthetic groups in a nonpolar environment to facilitate direct electron transfer.

**Building up of FNR<sub>red</sub> (Reactions 5-7 in Figure 1.22)** First electron reduction of FNR by Fd<sub>red</sub> on one hand was too fast to be observed [Batie and Kamin, 1984a]. The second electron reduction on the other hand was too slow to be compatible with steady-state catalysis in the absence of NADP<sup>+</sup>. This process actually involves various steps: dissociation of Fd<sub>ox</sub> (Reaction 4 in Figure 1.22), binding of Fd<sub>red</sub> (Reaction 5) and flavin reduction (Reaction 6). The reaction is inhibited by Fd<sub>ox</sub> and stimulated by NADP<sup>+</sup>, indicating that Reaction 4 is the rate-limiting step. In addition

to that,  $\text{NADP}^+$  seems to facilitate  $\text{Fd}_{\text{ox}}$  release which allows the entire reaction to proceed at a rapid pace through Reactions 4 and 8 [Batie and Kamin, 1984a].

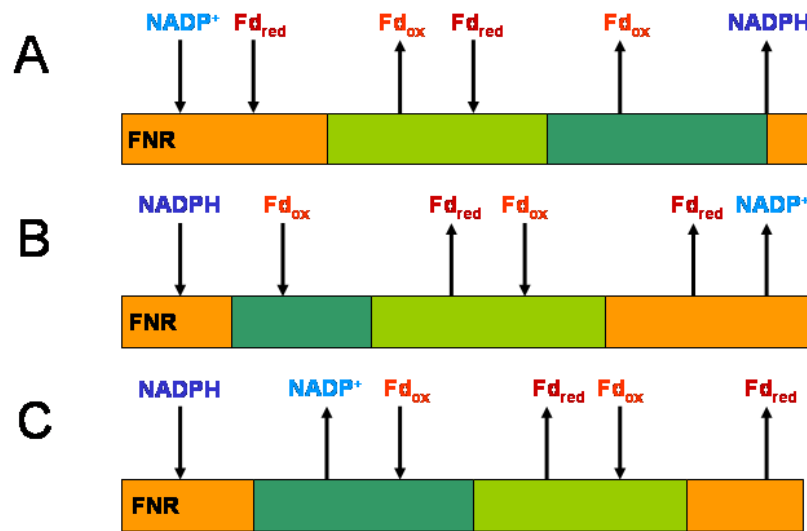
Furthermore, a ternary complex including  $\text{Fd}_{\text{ox}}$ ,  $\text{FNR}_{\text{ox}}$  and  $\text{NADPH}$  is readily formed. A strong case of negative cooperativity for binding was observed, that decreases  $\text{Fd}$  binding in the presence of  $\text{NADP(H)}$  and *vice versa*. The reciprocal negative cooperativity is translated into positive cooperativity at the kinetic level [Batie and Kamin, 1984a, 1986]. These observations are compatible with the proposed mechanistic cycle shown in Figure 1.22.

**$\text{NADP}^+$  reduction and product release (Reactions 8 and 9 in Figure 1.22)** The sequence of Reactions 8 and 9 proposed in Figure 1.22 is one example. They involve  $\text{NADP}^+$  reduction prior to  $\text{Fd}_{\text{ox}}$  dissociation and as the final step  $\text{NADPH}$  dissociation from the  $\text{FNR}_{\text{ox}}$ . Alternative pathways such as first  $\text{Fd}_{\text{ox}}$  dissociation before  $\text{NADP}^+$  reduction might be envisaged. Again, it was Batie and Kamin [1984a] that provided evidence that  $\text{NADP}^+$  reduction is faster than dissociation of  $\text{Fd}$  from  $\text{FNR}$ .

**$\text{NADPH}$  oxidation - the backward reaction** Ferredoxin reduction is the most widely distributed function of  $\text{FNR}$ -type proteins.  $\text{NADPH}$  binding to oxidized  $\text{FNR}$  leads to rapid hydride exchange between the nucleotide and the oxidoreductase, resulting in a succession of charge-transfer complexes involving flavin and nicotinamide. The appearance of these species can be followed by long-wavelength absorbance signals.

In Figure 1.23 two different mechanisms for  $\text{FNR}$  catalysis are shown. The reaction mechanism in A corresponds to the mechanism discussed above during  $\text{NADP}^+$  reduction. The reaction mechanism in B shows the same ordered mechanism for the  $\text{NADPH}$  oxidation (backward reaction of  $\text{FNR}$ ). It was proposed that the  $\text{NADPH}$  oxidation involves the same mechanism as the  $\text{NADP}^+$  reduction as all the reactions involved are in principle reversible. The reaction mechanism in C corresponds to a so-called ping-pong mechanism (two-step mechanism). No ternary complexes are involved in this reaction mechanism. First,  $\text{NADPH}$  passes two electrons on  $\text{FNR}$ , that are stored and  $\text{NADP}^+$  dissociates from  $\text{FNR}$  before the first  $\text{Fd}_{\text{red}}$  is fixed. Two  $\text{Fds}$  receive two electrons and thereby reoxidize  $\text{FNR}$  (see Figure 1.23C).

To distinguish between these two mechanisms during  $\text{NADPH}$  oxidation, the velocity of the reactions are systematically plotted versus one of the two substrate concentrations in a double reciprocal plot. This plot is called a Lineweaver-Burk plot and consists in a graphical method to distinguish between mechanisms. Spinach  $\text{Fd}$  reduction was measured *in vitro* and resulted in parallel lines by increasing the inhibitor concentration,  $\text{NADP}^+$ . This indicates the mechanism in Figure 1.23C to operate, without formation of a ternary complex [Forti and Sturani, 1968].



**Figure 1.23** The electron transfer mechanisms of ferredoxin-NADP(H) reductase. **A:** NADP<sup>+</sup> reduction following the mechanism proposed by Batie and Kamin [1984a]. **B:** NADPH oxidation following the same reaction mechanism as NADP<sup>+</sup> reduction in reverse order. **C:** NADPH oxidation following a two-step mechanism. NADP<sup>+</sup> bright blue, NADPH dark blue, Fd<sub>ox</sub> bright red, Fd<sub>red</sub> dark red, FNR<sub>ox</sub> orange, FNR<sub>sq</sub> bright green, FNR<sub>red</sub> dark green. Adapted from [Carrillo and Ceccarelli, 2003].

This was surprising, as distinct kinetic constraints must operate to justify different reaction mechanisms.

This could indeed be the case concerning  $Fd_{ox}$  release. This step may be rate-limiting during  $NADP^+$  reduction. Ternary-complex formation prevents rate limitation *via*  $Fd_{ox}$  release during this reaction. It was proposed that this requirement could be relieved in the reverse reaction if  $Fd_{red}$  dissociates from  $FNR_{sq}$  and  $FNR_{ox}$  at rates compatible with steady-state catalysis [Carrillo and Ceccarelli, 2003].

Product inhibition studies could also be revealing to obtain informations concerning the kinetic mechanism of NADPH oxidation. Following an ordered pathway as determined for the  $NADP^+$  reduction, a competitive inhibition by  $NADP^+$  is expected during NADPH oxidation (product inhibition). If the mechanism on the other hand follows a two-step kinetic mechanism,  $NADP^+$  inhibition is characterized by mixed-type inhibition (see Chapter 2).

### Isoforms of ferredoxin:NADP oxidoreductase

We will first introduce the isoforms of FNR in plants and afterwards introduce similarities and differences to cyanobacterial FNRs.

**Plant isoforms** These isoforms are tissue-specific, *i.e.* leaf isoforms (photosynthetic; pFNR) are primarily required for the photoreduction of  $NADP^+$ , and root isoforms (heterotrophic; hFNR) generate reduced Fd following NADPH oxidation. Root FNR was observed in one early report on radish roots [Morigasaki et al., 1990]. In plant roots, genetically distinct, soluble, root-type FNRs were found and characterized *e.g.* from tomato [Green et al., 1991] and corn [Onda et al., 2000, Aliverti et al., 2001] roots. Similar to Fd isoforms, root FNRs were found to be shifted in the redox potential, being more adapted to provide physiological  $Fd_{red}$  in nonphotosynthetic tissues [Aliverti et al., 2001]. Best affinities were obtained between root Fd and root FNR and between leaf Fd and leaf FNR isoforms [Onda et al., 2000].

Interestingly, the redox potentials of these oxidoreductases as those of their corresponding Fds have been tuned by evolution to favor the physiological direction of electron transport. However, the four proteins can be readily exchanged *in vitro* when assayed in a variety of reactions, indicating that the major parameter driving  $NADP^+$  or Fd reduction *in vivo* would be the substrate availability [Carrillo and Ceccarelli, 2003].

In addition to leaf-type and root-type FNR, different isoforms have been found in maize [Okutani et al., 2005] and wheat leaves [Gummadova et al., 2007]. They vary in localization and might be implicated in meeting changing metabolic capacity and reductant demands [Gummadova et al., 2007]. Concerning the localization, it is interesting to note that one isoenzyme

was exclusively soluble in maize, a second isoenzyme was found only attached to the thylakoid membrane and a third one had a dual location [Okutani et al., 2005].

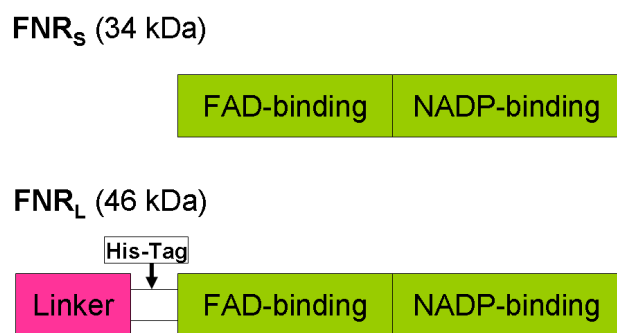
**Cyanobacterial isoforms** The *petH* gene encoding FNR in the cyanobacterium *Synechococcus* sp. PCC 7002 was cloned and sequenced [Schluchter and Bryant, 1992]. An additional N-terminal domain was identified that was 78% similar to the phycocyanin associated linker protein CpcD. The resulting molecular mass was around 45 kDa for this FNR versus 35 kDa for the plant plastid FNR. Thus, the N-terminal extension of FNR serves to localize the protein on the rods of the antenna complex in cyanobacteria, the PBS [Schluchter and Bryant, 1992].

Although the binding to PBS is now established, a controversy still exists concerning the exact localization of FNR on the PBS. On one side, it was proposed that FNR attaches to the distal PC hexamer due to the highest similarity of its N-terminus to the distal rod linker  $L_R^{10}$  [Schluchter and Bryant, 1992, Gómez-Lojero et al., 2003]. On the other side, a localization close to the core on the proximal PC hexamer [Arteni et al., 2009] was proposed. It was also proposed that the N-terminal extension may help localizing the FNR directly to the thylakoid membrane, providing an anchor domain and the question was raised what may be the function of the attachment of FNR to the antenna complex [van Thor et al., 2000].

It was recently established, that a second FNR isoform is generated by an in-frame initiation of translation [Thomas et al., 2006] in *Synechocystis*. These two isoforms are encoded by a unique *petH* gene and differ in size - 34 kDa and 46 kDa - for the small and the large isoforms, respectively. The latter is the isoform identified by [Schluchter and Bryant, 1992]. The smaller isoform was previously identified as a proteolytic degradation product. However, the authors in [Thomas et al., 2006] identified the small isoform as a product of an internal ribosome entry site (IRES) within the *petH* open reading frame (ORF). We denote the small isoform as FNR<sub>S</sub> and the large isoform as FNR<sub>L</sub>.

As for plant-like FNR, FNR<sub>S</sub> is composed of the two catalytic domains (green): the NADP and the FAD binding domains that are typical (Figure 1.24). In Figure 1.24, the N-terminal extension of FNR<sub>L</sub> is shown in white and pink for the hinge- and the linker-like domain. The latter is responsible for the attachment of FNR<sub>L</sub> to PBS.

The two isoforms are translated from distinct methionines (Met), Met1 and Met113 for FNR<sub>L</sub> and FNR<sub>S</sub>, respectively. Thomas et al. [2006] showed that the linker-like domain of FNR<sub>L</sub> undergoes proteolysis in the absence of PC, just as PBS linkers are known to be highly sensitive to proteolysis when not attached to PBPs. They also showed that obligate photoautotrophic cyanobacteria expressed only FNR<sub>L</sub> and FNR<sub>S</sub> accumulated in *Synechocystis* and most probably in other facultatively heterotrophic cyanobacteria under conditions where heterotrophic metabolism was needed.



**Figure 1.24** Representation of FNR<sub>S</sub> and FNR<sub>L</sub> primary structures highlighting their functional domains.

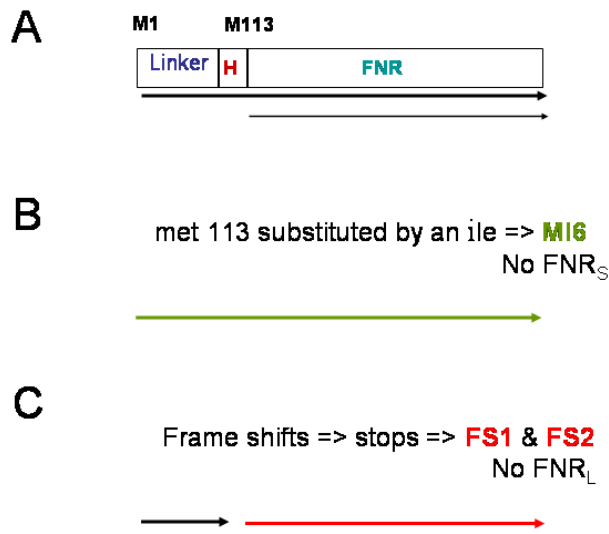
Therefore, Thomas et al. [2006] concluded that FNR<sub>L</sub> probably sustains photoautotrophic growth (NADP<sup>+</sup> reduction) and FNR<sub>S</sub> might be more adapted to provide electrons for heterotrophic growth (NADPH oxidation). Mutants were constructed in *Synechocystis* which translate either the small or the large FNR isoform only. These mutations are introduced in the next section.

#### Ferredoxin:NADP oxidoreductase mutants

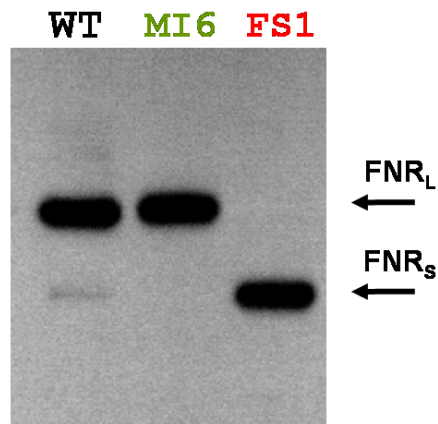
In Figure 1.25A the representation of the FNR<sub>L</sub> polypeptide with the linker, hinge (H), and enzymatic (FNR) domains is shown for the WT. As a consequence of the presence of the two methionines Met-1 and Met-113, in principle both isoforms can be expressed in the WT under different growth conditions. Under standard photoautotrophic conditions, the major isoform is FNR<sub>L</sub> as indicated by the wider arrow in Figure 1.25A.

Missense and frame-shift mutations were introduced in *petH* to elucidate the function of the two isoforms in facultative heterotrophic cyanobacteria. The missense mutation in MI6 changed the Met113 into an isoleucine (see Figure 1.25B). Frame-shift mutations, created by a single base deletion or insertion, caused premature translation stops upstream and downstream of Met-113 in FS1 and FS2, respectively (see Figure 1.25C) [Thomas et al., 2006].

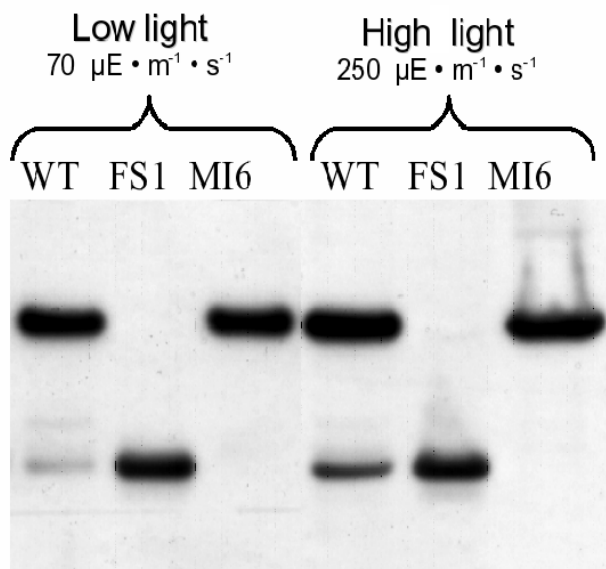
As shown in Figure 1.26, cell extracts from WT contained FNR<sub>L</sub> as major isoform with only trace amounts of FNR<sub>S</sub>. MI6 contained only FNR<sub>L</sub> (Figure 1.26), consistent with FNR<sub>S</sub> originating from an internal ribosome entry site (IRES) at Met-113. In cell extracts from FS1, FNR<sub>S</sub> is present at the WT FNR<sub>L</sub> level, whereas FNR<sub>L</sub> is absent (Figure 1.26). It was shown in [Thomas et al., 2006], that FNR<sub>S</sub> does not bind to the PBS due to the missing linker-like domain.



**Figure 1.25** *Synechocystis* WT, FS1 (FNR<sub>S</sub>) mutant and MI6 (FNR<sub>L</sub>) mutant strains.



**Figure 1.26** Immunoblot of total protein extracts for *Synechocystis* WT, FS1 and MI6 strains. Kindly provided by Dr. Ajlani.



**Figure 1.27** Immunoblot of total protein extracts for *Synechocystis* WT, FS1 and MI6 strains under low and high light from [Thomas et al., 2006].

Different growth conditions have been tested for the newly constructed mutants. First, under photoautotrophic growth, the light intensity was varied and WT, FS1 and MI6 strains were tested for their FNR isoforms by performing immunoblots on the total protein extracts. In Figure 1.27 are shown immunoblots for WT, FS1 and MI6 strains under low and high light. The WT exhibits accumulation of FNR<sub>S</sub> under high light compared to low light [Thomas et al., 2006].

This may be explained by the stress conditions of high light. Normally, high light results in increased turnover of the photosynthetic machinery, thus building up increased levels of NADPH. The induction of FNR<sub>S</sub> may help evacuate the excess of NADPH by the reverse reaction catalyzed by FNR. In this way, the excess NADPH is oxidized into NADP<sup>+</sup> and the electrons are fed back in the PQ pool by respiratory or cyclic electron flow. One interesting photoautotrophic condition may be limiting CO<sub>2</sub>. If the main substrate for the Calvin-cycle is limiting, the electrons may accumulate at the acceptor side of PSI, probably in the form of NADPH. We will summarize the results of different growth characteristics tested by Dr. Ajlani for the WT, FS1 and MI6 strains.

### Growth characteristics

Different growth conditions have been tested by Dr. Ajlani for the WT, MI6 and FS1 strains. WT can grow under photoautotrophic (light and CO<sub>2</sub>), chemoheterotrophic (dark and glucose) as well as bleaching (under N-starvation) conditions.

Under photoautotrophic conditions, mutant FS1 is characterized by a slower growth. The



MI6 mutant grows just as the WT. This can be correlated to the FNR isoforms expressed under these conditions. Both the WT and MI6 exhibit FNR<sub>L</sub> as the major isoform, whereas FS1 contains only FNR<sub>S</sub>. Apparently, FNR<sub>S</sub> cannot sustain photoautotrophic growth to the same extent as FNR<sub>L</sub>.

Under chemoheterotrophic conditions, FS1 exhibits an increased growth compared to WT. MI6 failed to grow. Apparently, FNR<sub>S</sub> in the FS1 mutant is better adapted to sustain heterotrophic growth which includes the oxidation of NADPH for reducing Fd just as in nonphotosynthetic tissues in plants. MI6 seems to have become an obligatory photoautotroph due to the presence of only FNR<sub>L</sub>. However the WT strain can adapt to heterotrophic growth but not to the same extent as the FS1 mutant.

In the study of [Thomas et al., 2006], extensive studies under N-starvation have been carried out. These growth conditions are characterized by the trimming of PBS using the rods as nitrogen source. Analogously to the chemoheterotrophic growth but to a lesser extent, FS1 exhibits fast PBS degradation compared to WT whereas MI6 exhibited a limited possibility to adapt to N-starvation. Again, FNR<sub>S</sub> in FS1 allows a better adaptation to N-starvation conditions probably due to the fact that FNR<sub>S</sub> participates to the heterotrophic metabolism induced in the early stages of N-starvation. WT can still grow under these conditions, trimming the PBS and inducing FNR<sub>S</sub> expression. There may be transformation of FNR<sub>L</sub> into FNR<sub>S</sub> due to proteolysis when the linker-like domain is not protected due to PC association. MI6 cannot induce FNR<sub>S</sub> and thus will only after extended bleaching get proteolysed into FNR<sub>S</sub>.

After this introduction, we can now state the objective of the PhD and briefly outline different approaches.

## 1.4 Objective

The objective of this PhD is to determine the function of the attachment of FNR to PBS in facultative heterotrophic cyanobacteria and the respective roles of the two FNR isoforms. First, we compared the two FNR isoforms for their intrinsic, catalytic activities (Chapter 2). To approach *in vivo* conditions, we purified an FNR<sub>L</sub>-PBS subunit complex and compared it to FNR<sub>S</sub>. In addition to that, preliminary reconstitution studies of PBS-FNR<sub>L</sub> binding were carried out using biochemical techniques. Second, we compared FNR mutants to WT following *in vivo* studies (Chapter 3).

# Chapter 2

## *In vitro* studies

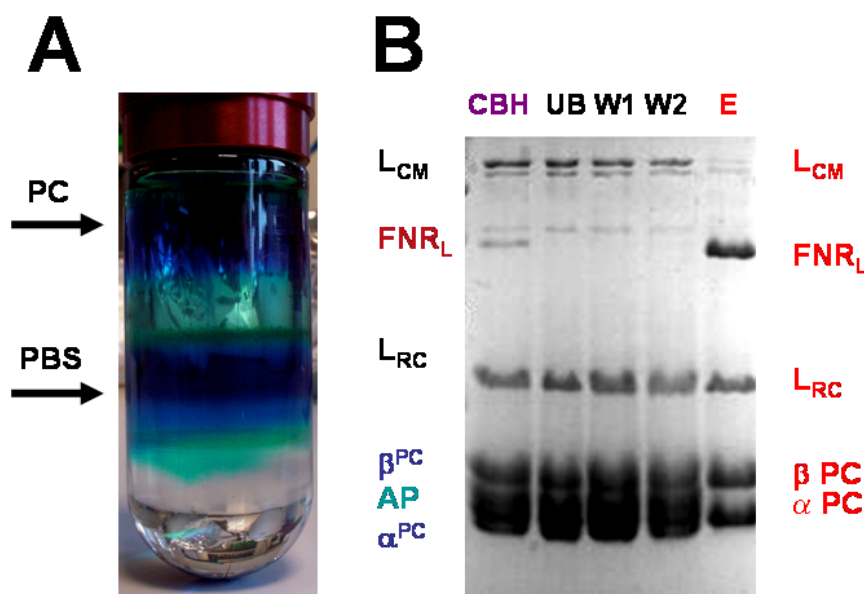
### 2.1 Results and discussion

We were interested in comparing the *in vitro* activities of the two FNR isoforms as different roles were proposed for the FNR isoforms of *Synechocystis* [Thomas et al., 2006]. The small isoform FNR<sub>S</sub> on one hand was shown to accumulate in the WT under heterotrophic or nitrogen starvation conditions. On the other hand, the large isoform FNR<sub>L</sub> is the major isoform under photoautotrophic conditions. It was proposed that FNR<sub>L</sub> could be implicated in the linear photosynthetic reactions (NADP<sup>+</sup> reduction), whereas FNR<sub>S</sub> would be implicated in cyclic photosynthetic or respiratory reactions (NADPH oxidation, see Chapter 1). This was our working hypothesis.

#### 2.1.1 Purification of FNR<sub>L</sub>-PC

Previous studies [Schluchter and Bryant, 1992, Nakajima et al., 2002, Thomas et al., 2006] have shown that when FNR<sub>L</sub> is not bound to PC, its N-terminal part is easily proteolysed just as the PBS linkers. Therefore intact FNR<sub>L</sub> is not detected *in vivo* in mutants lacking PC [Thomas et al., 2006]. One to two PC hexamers per PBS contained the native FNR<sub>L</sub> attached to it [van Thor et al., 1999b, Gómez-Lojero et al., 2003] and we decided to purify a native complex composed of FNR<sub>L</sub> and a PC hexamer in order to preserve the native conformation of FNR<sub>L</sub>. In the WT, the PBS contains six rods composed of three PC hexamers each. For the purification, we used the CB mutant, which contains only one PC hexamer per rod [Ughy and Ajlani, 2004]. Additionally, six histidines were inserted between the catalytic domains (FAD- and NADP-binding domain) and the linker-like domain, in the hinge domain (Figure 1.24 in Chapter 1). In this way, neither the enzymatic activity nor the binding to PC was hindered by the His-tag insertion. This mutant is named CBH. CBH grows normally and its PBS composition is not altered.

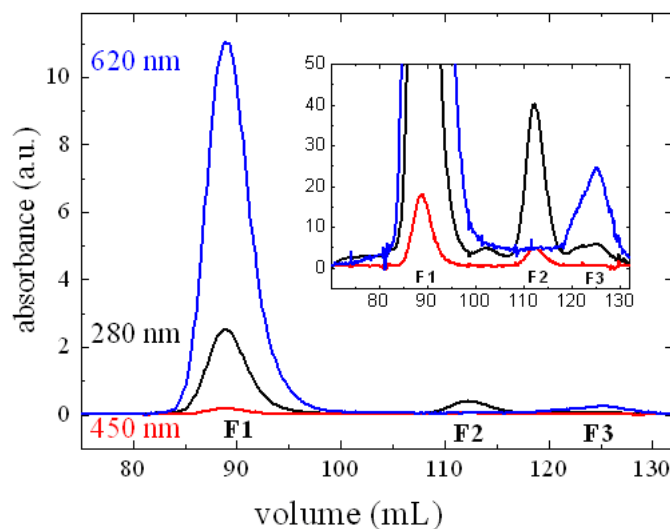
A first step consisted in purifying sufficient amounts of homogeneous  $\text{FNR}_L$ -PC to measure the catalytic activities. The procedure, described in Chapter 5, started with PBS isolation on a sucrose step gradient (Figure 2.1A). The lower blue band corresponds to the PBS complex and an additional centrifugation was carried out to concentrate the PBS complex. The intact PBS was then selectively dissociated into AP and PC (PBS fraction, lane CBH in Figure 2.1B). The mixture was incubated with a Ni-resin where  $\text{FNR}_L$  and associated polypeptides were bound due to the His-Tag on  $\text{FNR}_L$  (unbound fraction, lane UB in Figure 2.1B). After extensive washing (lanes W1-W2), the sample was eluted *via* competitive binding of imidazole to the Ni-resin (lane E). The eluted fraction was highly enriched in  $\text{FNR}_L$  compared to entire PBS as shown in Figure 2.1B. Additionally, a linker called  $L_{RC}$  and the  $\alpha$  and  $\beta$  PC were found. Contamination by a linker called  $L_{CM}$  was also detected in the eluted fraction.



**Figure 2.1** Phycobilisome isolation and IMAC purification of the  $\text{FNR}_L$ -PC complex. A: density gradient showing PC and PBS fractions. B: SDS-PAGE analysis of the different fractions. Taken from [Bordot, 2005]. CBH: entire PBS from the mutant CBH, UB: unbound fraction, W1 and W2: washing fractions, and E: elution fraction. Polypeptides in CBH are indicated on the left. Polypeptides in E are indicated on the right.

To determine the size of the complex and ensure its homogeneity, gel filtration was performed on a preparative column and three fractions were recovered (Figure 2.2). The first and major fraction F1 was recovered at 89 mL. The shape of the peak indicates a homogeneous complex. F1 had an estimated molecular mass of around 330 kDa. This is in agreement with a PC hexamer (230 kDa) with  $\text{FNR}_L$  (46 kDa) and  $L_{RC}$  (28 kDa) attached to it. Furthermore, as shown in the

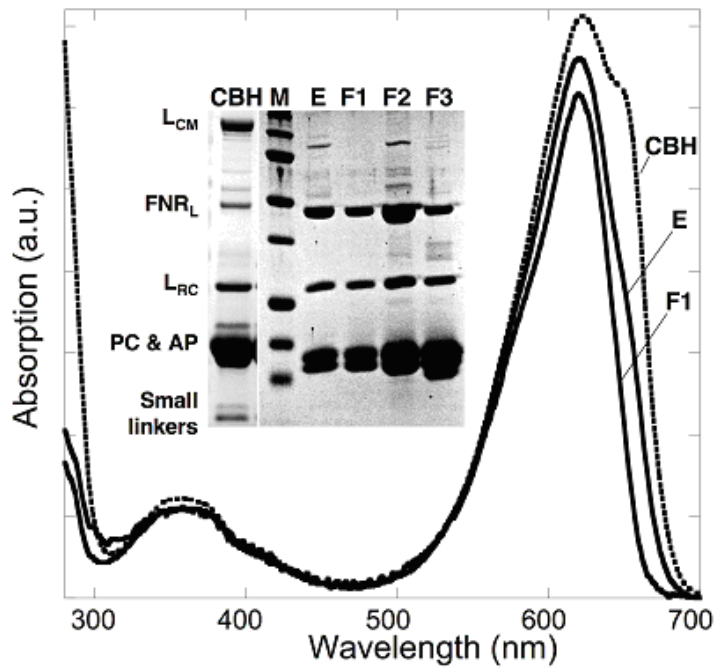
inset of Figure 2.2, all three characteristic wavelengths for peptide-, FAD- and PC-absorption were present in contrast to minor fractions, F2 and F3, that were recovered at 112 and 125 mL. The inset of Figure 2.2 shows on one hand that F2 contains only a background absorption of PC and on the other hand that F3 contains no FAD-characteristic absorption. In addition, these fractions were found at a lower molecular mass. Therefore, we assigned F2 and F3 to dissociation products of the FNR<sub>L</sub>-PC complex.



**Figure 2.2** Gel filtration chromatogram. The chromatogram was followed at 620, 450 and 280 nm which corresponds to characteristic PC-, FAD-cofactor and peptide absorption. The inset shows a zoom in low absorbance region (ma.u.) of the recovered fractions F1, F2 and F3.

The different fractions were monitored *via* UV-Vis absorption spectroscopy and SDS-PAGE (Figure 2.3). The specific absorption of the CBH PBS at 650 nm corresponding to AP strongly decreased during the different purification steps. This absorption decreased significantly after Ni-affinity chromatography (*E vs.* CBH in Figure 2.3). After gel filtration, the absorption at 650 nm was completely abolished (F1 fraction in Figure 2.3). This indicates that we purified an FNR<sub>L</sub> complex that contains PC only.

In summary, we purified a homogeneous PBS subcomplex that contains a PC hexamer and FNR<sub>L</sub> as well as L<sub>RC</sub> attached to it. Fraction F1 described above was used to carry out activity measurements.



**Figure 2.3** Overview of the purification of the FNR<sub>L</sub>-PC complex. Absorption spectroscopy for the CBH PBS, E (Ni-affinity: elution fraction) and F1 (gel filtration: major fraction) are shown. In the inset, SDS-PAGE electrophoretic fractions are shown. CBH: entire PBS from this mutant, M: molecular mass marker, E: elution fraction during Ni-affinity chromatography, F1, F2 and F3: fractions obtained during gel filtration chromatography. Polypeptides are indicated on the left.

### 2.1.2 FNR quantification in FNR<sub>L</sub>-PC

Before performing *in vitro* activity measurements, the FNR<sub>L</sub> in the complex has to be quantified. First, the apoenzyme quantification was carried out (Table 2.1). The density of the different bands on SDS-PAGE was determined. A stoichiometry for the complex of about 1:1:1 for FNR<sub>L</sub> : ( $\alpha^{\text{PC}} \beta^{\text{PC}})_6$  : L<sub>RC</sub> was found.

sample	FNR <sub>L</sub> concentration ( $\mu\text{M}$ )	FAD concentration ( $\mu\text{M}$ )	FAD/FNR <sub>L</sub>
1	$0.43 \pm 0.02$	$0.39 \pm 0.02$	$0.91 \pm 0.09$
2	$0.55 \pm 0.04$	$0.49 \pm 0.05$	$0.89 \pm 0.16$
3	$1.22 \pm 0.03$	$1.19 \pm 0.04$	$0.98 \pm 0.06$

**Table 2.1** Quantification of FNR<sub>L</sub> and FAD in FNR<sub>L</sub>-PC. Each sample represents an average of three measurements.

The next step was the quantification of the holoenzyme (FAD concentration, see Table 2.1). Only the FAD-containing enzyme can perform catalysis. This is why the quantification of the holoenzyme is essential before starting *in vitro* activity measurements. For FNR<sub>S</sub>, several methods were developed. First, a holoenzyme quantification can be determined *via* the FAD absorption at 450 nm. This method cannot be performed with the FNR<sub>L</sub>-PC complex, as the characteristic FAD absorption is hidden behind the enormous absorption of the cyanobilin attached to PC (Table 2.2).

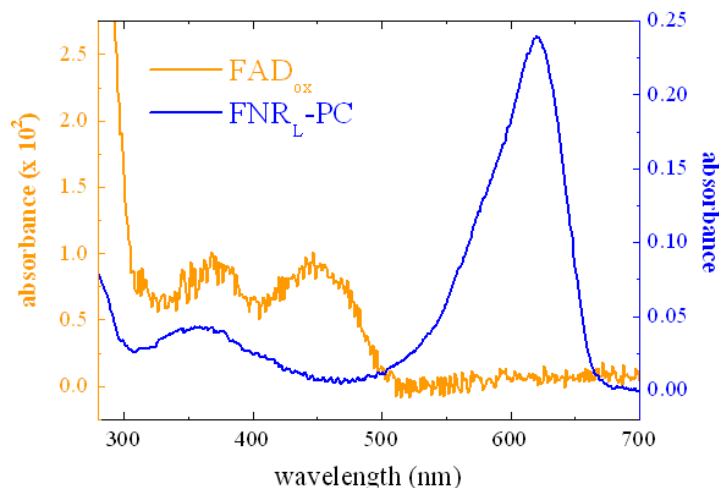
wavelength (nm)	$\epsilon$ ( $\text{M}^{-1} \text{cm}^{-1}$ )
620 (PBS cofactors)	14,400,000
620 (PC cofactors)	2,370,000
450 (free FAD)	11,300

**Table 2.2** Extinction coefficients for cofactors.

For the same reason, the photochemical reduction with deazaflavin/EDTA was difficult to perform [Massey et al., 1978]. We tried to obtain difference spectra under anaerobic conditions. Instead, the PC absorption was altered under the action of blue light. This made FAD quantifications in the complex difficult. Holoenzyme quantification by extraction *via* SDS was hindered for the same reason in FNR<sub>L</sub>-PC. Finally, the TCA extraction method was combined together with standard apoprotein quantification (Micro-BCA) and absorption due to PC chromophore.

In summary, the TCA extraction method was the only holoenzyme quantification working for the purified FNR<sub>L</sub>-PC. This is due to the covalently-linked bilins in PC and the non-covalently linked FAD in FNR<sub>L</sub>. TCA treatment extracts selectively FAD and proteins containing bilins are precipitated. Three independent samples were treated with TCA and the non-covalently bound

FAD was extracted. The holoenzyme quantification is shown in Table 2.1. The procedure is detailed in Chapter 5. A ratio between 0.92-1.00 for [FAD]/[FNR<sub>L</sub>-PC] was obtained (Table 2.1). The FAD release from FNR<sub>L</sub>-PC is shown in Figure 2.4.



**Figure 2.4** FAD release from the FNR<sub>L</sub>-PC complex. The blue trace (right y-scale) shows the characteristic FNR<sub>L</sub>-PC absorption, at a concentration of 0.1 μM. The PC-cofactor determines essentially the FNR<sub>L</sub>-PC absorption with an absorption maximum at 620 nm and an extinction coefficient shown in Table 2.2. The orange trace (left y-scale) shows the FAD absorption, after extraction by TCA from FNR<sub>L</sub>-PC, at a concentration of 0.81 μM ( $\epsilon$  for FAD shown in Table 2.2). The characteristic FAD absorption in solution exhibits absorption maxima at 450 and 378 nm.

### 2.1.3 Reconstitution of PBS-FNR<sub>L</sub>

We performed preliminary reconstitution measurements of PBS with FNR<sub>L</sub>. These biochemical studies could provide us with higher amounts of entire PBS or subcomplexes, although not native.

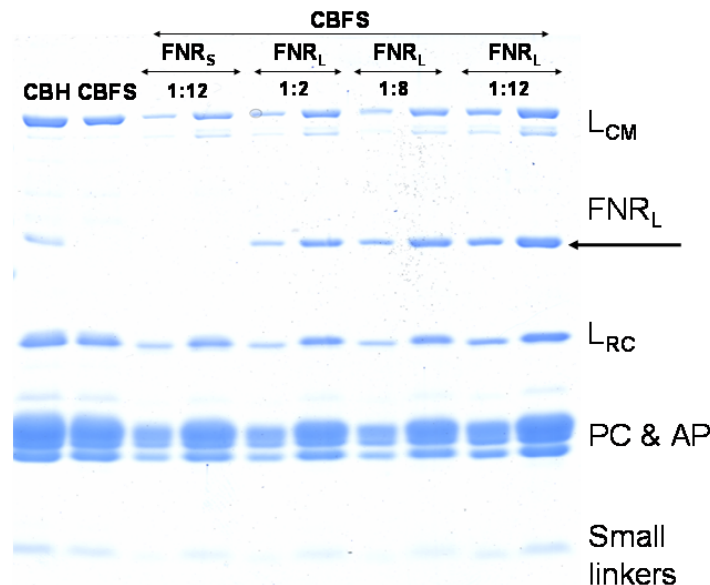
To do so, we isolated and purified the PBS of two mutant strains. We used two PBS and FNR mutant strains called CBH and CBFS. CBFS contains the same mutation as CBH on the PBS and, in addition to that, a FNR mutation equivalent to the one in FS1, resulting in the absence of FNR<sub>L</sub>. In parallel, we purified, from *E. coli*, a recombinant FNR<sub>L</sub> and incubated FNR<sub>L</sub> with PBS in different ratios of PBS:FNR<sub>L</sub> (1:0, 1:2, 1:8, 1:12). The next step consisted in performing a second density step-gradient in order to purify the FNR<sub>L</sub> bound to the PBS as in [Gómez-Lojero et al., 2003].

The authors in [Gómez-Lojero et al., 2003] studied sedimentation profiles of sucrose gradients combined with diaphorase activity profiles (FNR activity) in *Synechococcus* sp. PCC 7002. They



showed that binding of exogenously added  $\text{FNR}_L$  to PBS was possible and that a maximum of six binding sites are available for  $\text{FNR}_L$  in the WT and the mutant similar to CB PBS. In the WT PBS, one to two  $\text{FNR}_L$  per PBS were found [Gómez-Lojero et al., 2003, van Thor et al., 1999b]. We observed 1.2  $\text{FNR}_L$  per CBH PBS (lane CBH in Figure 2.5 and Table 2.3) which was in agreement with [Gómez-Lojero et al., 2003] (1.0-1.6  $\text{FNR}_L$ /PBS in the WT).

CBFS did not contain  $\text{FNR}$ , as the unique  $\text{FNR}_S$  isoform present in this mutant is missing the linker-like domain responsible for the attachment to PBS (lane CBFS in Figure 2.5). The latter mutant was used for reconstitution experiments with  $\text{FNR}_L$ . A control was carried out by incubating the PBS of CBFS with a 12-fold excess of  $\text{FNR}_S$  per PBS. No bound  $\text{FNR}_S$  was detected, as observed (lanes CBFS  $\text{FNR}_S$  1:12). We observed a significant increase of  $\text{FNR}_L$  amounts from the CBFS PBS fraction of the density gradient (lanes CBFS PBS: $\text{FNR}_L$  1:2, 1:8 and 1:12 in Figure 2.5). The density of the  $\text{FNR}_L$  bands were analyzed and the data were normalized assuming that there are six copies of  $L_{RC}$  linker per PBS. The samples CBFS PBS: $\text{FNR}_L$  1:2, 1:8 and 1:12 resulted in 4.5, 6.4 and 5.9  $\text{FNR}_L$  per PBS (Table 2.3) and indicated saturation of the binding sites. Therefore, CBFS PBS seems to incorporate around 6  $\text{FNR}_L$  per PBS which is in agreement with 6 binding sites available for  $\text{FNR}_L$  in *Synechococcus* PCC7002 [Gómez-Lojero et al., 2003].



**Figure 2.5** Reconstitution of PBS- $\text{FNR}_L$ . CBH: PBS from a mutant that contains 1 PC hexamer per rod and  $\text{FNR}_L$  in natural amounts; CBFS: PBS from a mutant that in addition does not contain  $\text{FNR}_L$ ; PBS: $\text{FNR}_S$  1:12; PBS: $\text{FNR}_L$  1:2, PBS: $\text{FNR}_L$  1:8 and PBS: $\text{FNR}_L$  1:12. All the reconstituted PBS: $\text{FNR}$  samples are loaded twice in 2 different amounts.

These measurements are very promising to finally answer the question about the possible localization of  $\text{FNR}_L$  in the PBS [van Thor et al., 1999b, 2000, Gómez-Lojero et al., 2003, Arteni et al., 2009]. In the future, these reconstitution studies can be facilitated by the availability of a



Lane	FNR <sub>L</sub> per PBS
CBH	1.19
CBFS:FNR <sub>L</sub> 1:0	0
CBFS:FNR <sub>L</sub> 1:2	4.5
CBFS:FNR <sub>L</sub> 1:8	6.4
CBFS:FNR <sub>L</sub> 1:12	5.9

**Table 2.3** Calculated FNR<sub>L</sub> stoichiometries for CBH- and CBFS PBS. The number of FNR<sub>L</sub> was obtained by densitometric analysis of SDS-PAGE.

His-Tag containing FNR<sub>L</sub> that can be overexpressed in *E.coli*. This would result in a facilitated and up-scaled purification of FNR<sub>L</sub> which is needed in high amounts for such studies.

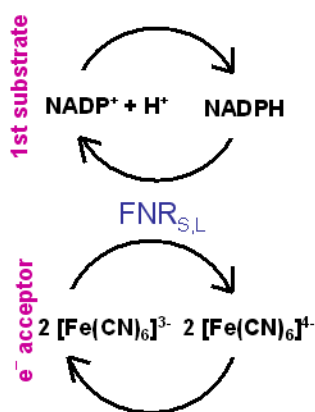
#### 2.1.4 NADPH oxidase activity

In order to test our working hypothesis, we wanted to compare the catalytic constants of FNR<sub>S</sub> and FNR<sub>L</sub>-PC. For this, we were carrying out two different assays. The first assay is called ferricyanide reductase activity. The second assay is called Fd-mediated cytochrome *c* reductase activity.

##### Ferricyanide reductase activity

We performed the ferricyanide reductase activity assay (also called diaphorase activity) to measure the affinity to the substrate NADPH, to determine the catalytic turnover and the catalytic efficiency during NADPH oxidation. This was done in the presence of an artificial electron acceptor, potassium ferricyanide (K<sub>3</sub>[Fe(CN)<sub>6</sub>]). Ferricyanide in the oxidized state exhibits a characteristic absorption with a maximum around 420 nm ( $\epsilon_{420\text{nm}} = 1020 \text{ M}^{-1}\text{cm}^{-1}$ ). When this external electron acceptor receives one electron, its absorption is abolished. Thus, the reaction was followed spectrophotometrically.

The reaction starts with the addition of the substrate NADPH. After a hydride transfer, the reduced FAD in the enzyme FNR reduces ferricyanide in two one-electron transfer reactions (Figure 2.6). The initial velocity of the catalyzed reaction is recorded, analyzed and plotted against the initial substrate concentration of NADPH (Figure 2.7). The only rate-limiting step is hydride transfer from NADPH to FNR<sub>S/L</sub> as an excess of ferricyanide was used. By varying the initial substrate concentration of NADPH, we obtained informations about the affinity to NADPH and the catalytic turnover. We obtained the catalytic efficiency as well which is calculated from these two values. The obtained Michaelis-Menten catalytic constants are listed in Table 2.4. The Michaelis-Menten constant  $K_m(\text{NADPH})$  is shown for the two FNR isoforms. A 30% decrease



**Figure 2.6** Scheme of ferricyanide reduction.

was observed for FNR<sub>L</sub>-PC compared to FNR<sub>S</sub>. The Michaelis-Menten constant is related to the affinity. Thus, an increase in affinity for NADPH of about 30% was observed in the FNR<sub>L</sub>-PC complex compared to FNR<sub>S</sub>.

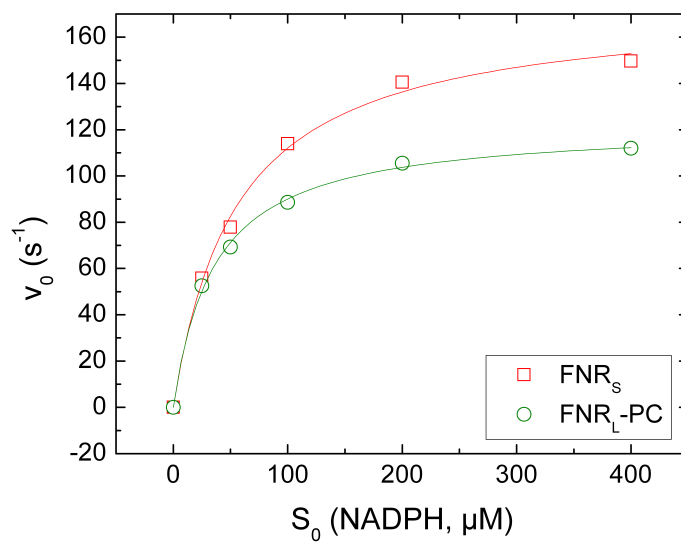
We want to recall that, following our working hypothesis, FNR<sub>S</sub> is catalyzing the NADPH oxidation under heterotrophic growth. Heterotrophic growth is accompanied at least transiently by increased NADPH concentrations where catabolism contributes to NADPH built-up. Thus, the physiological conditions may not require a high affinity of NADPH to FNR<sub>S</sub>.

In contrast, FNR<sub>L</sub> is catalyzing the NADP<sup>+</sup> reduction under photoautotrophic growth. These conditions may require a better NADP<sup>+</sup> affinity. If we assume that the NADP<sup>+</sup> affinity is similar to NADPH affinity, the increased affinity of FNR<sub>L</sub>-PC for NADPH may be correlated to a faster turnover of NADP<sup>+</sup> reduction. We have seen in Chapter 1 that Fd<sub>ox</sub> dissociation can be rate-limiting for NADP<sup>+</sup> reduction. This limitation is decreased by *prior* NADP<sup>+</sup> binding and by the mechanism of positive kinetic cooperativity. Increased NADP<sup>+</sup> affinity for FNR<sub>L</sub>-PC may thus favor the physiologically catalyzed reaction, *i.e.* NADP<sup>+</sup> reduction.

Furthermore, the catalytic turnover  $k_{\text{cat}}$  is shown for the two FNR isoforms. A 30% decrease was observed for FNR<sub>L</sub>-PC compared to FNR<sub>S</sub>. Apparently, the increase in affinity to NADPH limits turnover. This is in agreement with the physiologically catalyzed reactions for the two FNR isoforms (see above). The ratio of the catalytic turnover over the Michaelis-Menten constant  $k_{\text{cat}}/K_{\text{m}}$  reflects the catalytic efficiency of the two FNR isoforms. Similar values were observed for FNR<sub>L</sub>-PC and FNR<sub>S</sub>.

### Ferredoxin-mediated cytochrome *c* reductase activity

We performed ferredoxin-mediated cytochrome *c* reductase activity tests to measure the affinity to the second substrate Fd, to determine the catalytic turnover and the catalytic efficiency in



**Figure 2.7** Ferricyanide reductase activities of  $\text{FNR}_S$  and  $\text{FNR}_L\text{-PC}$ .

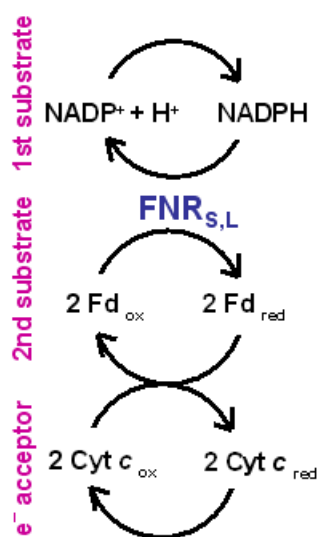
Ferricyanide reductase activity (unit)		$\text{FNR}_L\text{-PC}$	$\text{FNR}_S$
$K_m(\text{NADPH})$	( $\mu\text{M}$ )	$40 \pm 3$	$55 \pm 5$
$k_{\text{cat}}$	( $\text{s}^{-1}$ )	$124 \pm 3$	$174 \pm 5$
$k_{\text{cat}}/K_m$	( $\mu\text{M}^{-1} \text{s}^{-1}$ )	$3.1 \pm 0.3$	$3.2 \pm 0.4$

**Table 2.4** Ferricyanide reductase activity of  $\text{FNR}_L\text{-PC}$  and  $\text{FNR}_S$ .

an alternative and more physiological NADPH oxidase activity using both natural substrates of FNR (Fd and NADPH).

This was achieved in the presence of Fd as an intermediate and cytochrome *c* as the final artificial electron acceptor. When cyt *c* receives an electron, a characteristic absorption maximum builds up ( $\Delta\epsilon_{550\text{nm}} = 21.1 \text{ mM}^{-1} \text{ cm}^{-1}$ ; Sigma Aldrich C2506). Thus, the absorption change permitted us to follow the reaction spectrophotometrically [Shin and Pietro, 1971, Zanetti et al., 1980].

The reaction is started by the addition of the substrate NADPH. After hydride transfer, the reduced FAD in the FNR reduces the oxidized ferredoxin and finally the cytochrome *c* in two one-electron transfer reactions (Figure 2.8).

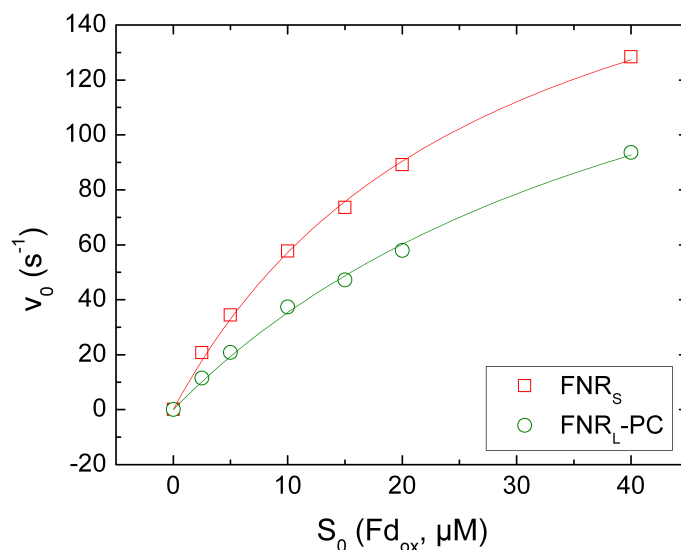


**Figure 2.8** Scheme of Fd-mediated cyt *c* reduction.

The initial velocity of the catalyzed reaction is plotted against the initial  $\text{Fd}_{\text{ox}}$  concentration (Figure 2.9). The only rate-limiting step is the electron transfer from  $\text{FNR}_{\text{S/L}}$  to Fd as both NADPH and cyt *c* were used in excess. By varying the initial Fd concentration, we obtained information about the affinity to  $\text{Fd}_{\text{ox}}$ , the catalytic turnover and the catalytic efficiency. The Michaelis-Menten catalytic constants are listed in Table 2.5. The Michaelis-Menten constant  $K_{\text{m}}(\text{Fd})$  is shown for the two FNR isoforms. A 70% increase was observed for  $\text{FNR}_{\text{L-PC}}$  compared to  $\text{FNR}_{\text{S}}$ . Thus, a decrease of about 70% of the affinity to NADPH was observed for  $\text{FNR}_{\text{L-PC}}$  compared to  $\text{FNR}_{\text{S}}$ . Such an important decrease in the affinity for  $\text{FNR}_{\text{L-PC}}$  may be explained by the presence of the additional PC hexamer. Due to its considerable size (230 kDa), it may cause steric hindrance and thus complex formation of  $\text{Fd}_{\text{ox}}\text{-FNR}_{\text{red}}$  or  $\text{Fd}_{\text{ox}}\text{-FNR}_{\text{sq}}$  prior to electron transfer may be limited.

Furthermore, the catalytic turnover  $k_{\text{cat}}$  was determined for the two FNR isoforms. No significant decrease was observed for  $\text{FNR}_{\text{L-PC}}$  compared to  $\text{FNR}_{\text{S}}$ . Therefore, a change in

$K_m(\text{Fd}_{\text{ox}})$  is not correlated to a change in turnover, in contrast to the case of NADPH. A 44% decrease was observed for the catalytic efficiency  $k_{\text{cat}}/K_m$  of the  $\text{FNR}_L\text{-PC}$  complex compared to  $\text{FNR}_S$ . This would mean that  $\text{FNR}_S$  is slightly better in performing NADPH oxidation than  $\text{FNR}_L\text{-PC}$ . This difference in the catalytic efficiency is in agreement with our working hypothesis.



**Figure 2.9** Fd-mediated cyt *c* reductase activities of  $\text{FNR}_S$  and  $\text{FNR}_L\text{-PC}$ .

Cyt <i>c</i> reductase activity (unit)	$\text{FNR}_L\text{-PC}$	$\text{FNR}_S$
$K_m(\text{Fd})$ ( $\mu\text{M}$ )	$47 \pm 6$	$28 \pm 2$
$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$202 \pm 17$	$215 \pm 9$
$k_{\text{cat}}/K_m$ ( $\mu\text{M}^{-1} \text{s}^{-1}$ )	$4.3 \pm 0.9$	$7.7 \pm 0.8$

**Table 2.5** Cyt *c* reductase activity of  $\text{FNR}_L\text{-PC}$  and  $\text{FNR}_S$ .

### NADP<sup>+</sup> inhibition of cyt *c* reduction

In order to get further informations about the mechanism of the catalysis, we measured the apparent inhibition constant (see Chapter 1). To measure the inhibition of NADPH oxidation, we repeated the Fd-mediated cyt *c* reductase reactions for  $\text{FNR}_L\text{-PC}$  in the presence of  $50 \mu\text{M}$  NADP<sup>+</sup>. The results for the  $\text{FNR}_L\text{-PC}$  are shown in Figure 2.10 together with Michaelis-Menten fits and the obtained catalytic constants are shown in Table 2.6.

On one side, the apparent Michaelis-Menten constant  $K_{\text{mapp}}$  is almost doubled in the presence of NADP<sup>+</sup>. Thus, only about half the  $\text{Fd}_{\text{ox}}$  affinity is observed. On the other side, the

catalytic turnover remains constant. This decreased the catalytic efficiency to almost half of the catalytic efficiency in the absence of NADP<sup>+</sup>. The apparent inhibition constant  $K_I$  for NADP<sup>+</sup> was determined (Equation 2.1.1).

$$K_I = [I] \cdot \frac{K_m}{K_{mapp} - K_m} \quad (2.1.1)$$

We did not repeat this experiment at other NADP<sup>+</sup> concentrations as these assays are very Fd-consuming. We cannot exclude that the apparent inhibition constant might vary as a function of the concentration of NADP<sup>+</sup> used in the assay.

It has been shown that *prior* NADP<sup>+</sup> binding to FNR inhibits NADPH oxidase activity [Carrillo and Ceccarelli, 2003]. As introduced in the catalytic cycle (see Chapter 1), the binding of NADP<sup>+</sup> is actually the first step of NADP<sup>+</sup> reduction and may be as well considered as the last step of the reverse reaction (NADPH oxidation). Therefore NADP<sup>+</sup> inhibition could be ascribed to product inhibition.

Cyt <i>c</i> reductase activity NADP <sup>+</sup>	(unit) ( $\mu$ M)	FNR <sub>L</sub> -PC 0	FNR <sub>L</sub> -PC 50
$K_m$ (Fd)	( $\mu$ M)	$47 \pm 6$	$85 \pm 32$
$k_{cat}$	(s <sup>-1</sup> )	$202 \pm 17$	$207 \pm 47$
$k_{cat}/K_m$	( $\mu$ M <sup>-1</sup> s <sup>-1</sup> )	$4.3 \pm 0.9$	2.4
$K_I$	( $\mu$ M)		46.6

**Table 2.6** Inhibition of cyt *c* reductase activity for FNR<sub>L</sub>-PC.

Furthermore, we drew a Lineweaver-Burk plot of the inhibition data (Figure 2.11) which in this case meant plotting the  $1/v_0$  vs.  $1/Fd_{ox}^0$ . Details of the catalyzed reaction can be obtained from such plots if different concentrations of the inhibitor result in parallel lines or intersecting lines. Parallel lines indicate a simple two-reaction pathway (also called ping-pong mechanism) if the two substrates are involved in an alternative manner (Figure 1.23). Intersecting lines indicate the involvement of a ternary complex. In our case the ternary complex would contain FNR, Fd and NADP(H). As the figure 2.11 presents intersecting lines (although for only one concentration of NADP<sup>+</sup>), the involvement of a ternary complex is indicated. This is in contrast with results obtained by Forti and Sturani on spinach FNR [Forti and Sturani, 1968]. Following the same assay, they found parallel lines using different concentrations of NADP<sup>+</sup> and thus proposed a different mechanism during NADPH oxidation with no involvement of ternary complexes.

Involvement of ternary complexes have been found for FNR<sub>S</sub>-like plant FNR during NADP<sup>+</sup> reduction [Batie and Kamin, 1984a, Carrillo and Ceccarelli, 2003]. Here, inhibition data were

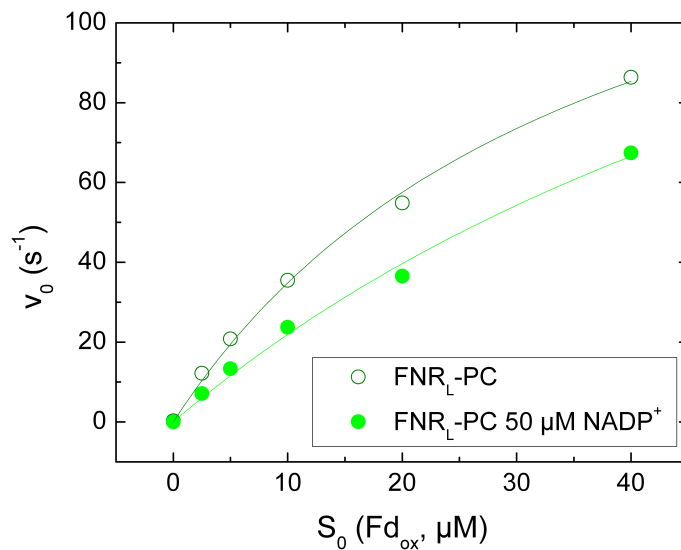


Figure 2.10 Inhibition of cyt *c* reductase activities of FNR<sub>L</sub>-PC.

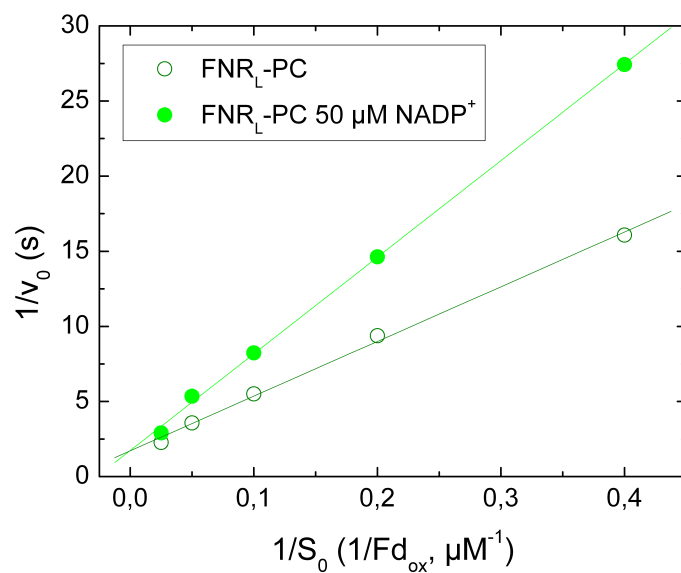


Figure 2.11 Lineweaver-Burk plot of the inhibition of FNR<sub>L</sub>-PC cyt *c* reductase activities.

obtained for the first time for a native FNR<sub>L</sub> attached to a PBS subunit. The result indicates the involvement of a ternary complex during NADPH oxidation. This would mean that the reaction mechanism should be the same for NADP<sup>+</sup> reduction and NADPH oxidation. Assuming that reaction mechanisms are similar, the Fd<sub>ox</sub> affinity may be smaller during NADP<sup>+</sup> reduction for FNR<sub>L</sub>-PC *vs.* FNR<sub>S</sub>. As this reaction is limited by Fd dissociation, this would result in faster NADP<sup>+</sup> reduction and by contrast, decreased Fd<sub>ox</sub> affinity correlates with slower NADPH oxidation.

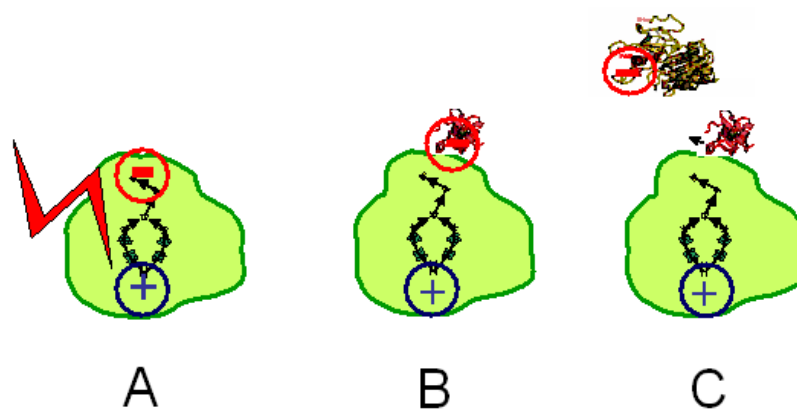
### 2.1.5 NADP<sup>+</sup> reductase activity

NADP<sup>+</sup> reduction is the major reaction catalyzed by FNR during photoautotrophic growth. Following this FNR activity, the reducing power is built up in the form of NADPH. In order to test our working hypothesis, we wanted to compare FNR<sub>S</sub> and FNR<sub>L</sub>-PC catalytic constants. First, catalytic constants can be determined for the one-electron reduction of FNR. Second, multiple turnovers can be determined experimentally. Using the catalytic constants from single reduction, turnover can be calculated for the case that the first one-electron reduction is limiting the overall reaction. Experimental turnover can then be compared to the calculated turnover.

Compared to the NADPH oxidase activity, the assays for NADP<sup>+</sup> reductase activity involved an additional partner: PSI. The kinetics of FNR reduction and Fd reoxidation were measured in the ternary mixture PSI/Fd/FNR by laser flash absorption spectroscopy [Cassan *et al.*, 2005]. First, a laser flash at 700 nm excites the reaction center of PSI. This results in oxidation of P700 and in charge separation (scheme A in Figure 2.12). We will further regard especially the acceptor side of PSI. At the end of the charge separation, the final acceptor in PSI, (F<sub>A</sub>F<sub>B</sub>), is reduced (PSI<sub>red</sub> in Equation 2.1.2). Afterwards, a cascade of single electron transfers is occurring. The first electron transfer occurs from the reduced acceptor of PSI, (F<sub>A</sub>F<sub>B</sub>)<sup>-</sup>, to oxidized Fd (first reaction in Equation 2.1.2 and scheme B in Figure 2.12). This results in reduced Fd and oxidizes the terminal acceptor. Electrons from reduced Fd are then transferred to FNR in single electron transfers (reaction 2 in Equation 2.1.2 and scheme C in Figure 2.12). We were performing NADP<sup>+</sup> reductase activity under two different conditions. The first condition permits only the first one-electron reduction of FNR<sub>S/L</sub> by Fd<sub>red</sub>. The second condition is adapted for multiple turnover of FNR<sub>S/L</sub>.







**Figure 2.12** Reaction and scheme of single FNR reduction.

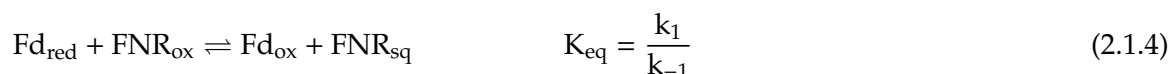
### Single reduction by reduced Fd

We performed measurements in the presence and the absence of the second substrate  $\text{NADP}^+$ . After the actinic laser flash (at  $t=0$ ) has triggered a charge separation in PSI, an electron transfer cascade is occurring towards FNR, *via* Fd. Concentrations of PSI (and the concentrations of photoreduced Fd) are chosen inferior to FNR to transfer only one electron per FNR and single electron transfer to FNR can be followed. The first one-electron reduction of oxidized FNR results in the build-up of a radical species called semiquinone. This radical species exhibits a differential absorption spectra with a maximum around 520 nm (Figure 5.1 in Chapter 5). We can thus follow the build-up of the semiquinone radical by following the absorption changes at 520 nm.

The kinetics in Figure 2.13 show the events due to reduction of the PSI electron acceptors, Fd and  $\text{FNR}_{\text{S/L}}$ . The contribution of  $\text{P700}^+$  has been eliminated as explained in Chapter 5. In the absence of  $\text{FNR}_{\text{S/L}}$  (black traces in Figure 2.13A and B), only a single kinetic component is observed. The reduction of the final electron acceptor of PSI, ( $\text{F}_\text{A}\text{F}_\text{B}$ ), is not time-resolved on this timescale. It corresponds to the decrease in absorption that is seen just after the flash. The kinetic component that can be seen in the black trace is due to reduction of Fd. This results in a further decrease of absorption at 520 nm. Fd reduction can be further divided in a fast (submicrosecond and microsecond; small fraction) and a slower (millisecond) component (see Chapter 1). The fast component accounts for less than 10% of the full Fd reduction signal. This component corresponds to a fast electron transfer in the preformed PSI-Fd complex [Setif and Bottin, 1994]. The slower component accounts for a second-order diffusion-limited reduction of Fd.

**Single reduction of  $\text{FNR}_{\text{S}}$  in the presence of  $\text{NADP}^+$ .** In the presence of  $\text{NADP}^+$ , we observed the two isoforms acting according to the proposed catalytic cycle [Batie and Kamin, 1984a]. It

has been shown in this article, that the  $\text{NADP}^+$  binds first to the FNR before binding Fd (ordered binding). The red, green, blue and purple traces in Figure 2.13A correspond to increasing concentrations of  $\text{FNR}_S$  as indicated on the right. Here, the same initial fast decay is observed but, in addition to that, an absorption increase is observed at 520 nm. This component is ascribed to the reduction of  $\text{FNR}_S$  by  $\text{Fd}_{\text{red}}$  with rate constants  $k_1$  and  $k_{-1}$ . The rates and the final amplitudes of this absorption change increase with the  $\text{FNR}_S$  concentration. We used the following kinetic model to simulate the observed kinetics:



In Equation 2.1.3,  $\text{PSI}_{\text{red}}$  stands for PSI with the reduced terminal acceptor ( $F_A, F_B$ ). Reduction of this terminal acceptor of PSI is not considered on this time scale. Following Equation 2.1.3, one electron is passed to Fd. In Equation 2.1.4, the FNR is reduced once by  $\text{Fd}_{\text{red}}$ . This results in the build-up of the radical species, the semiquinone FNR. In order to solve the kinetic model analytically, it has to be further simplified. This has been done by considering all the reactions as pseudo first-order. The solution equations are shown in detail in Chapter 5. By applying these solution equations, we could perform a global fit analysis. We obtained the following values for the rate constants:  $k_r = 50.0 \mu\text{M}^{-1} \text{s}^{-1}$ ,  $k_1 = 15.2 \mu\text{M}^{-1} \text{s}^{-1}$ ,  $k_{-1} = 5.4 \mu\text{M}^{-1} \text{s}^{-1}$  for  $\text{FNR}_S$ . Furthermore, we calculated the equilibrium constant  $K_{\text{eq}}$  from Equation 2.1.4 ( $K_{\text{eq}} = k_1/k_{-1}$ ) and obtained  $K_{\text{eq}} = 2.84$ . From the equilibrium constant, the midpoint redox potential  $E_m$  can be calculated in the following way:

$$\Delta E_m = E_m(\text{FNR}_{\text{ox}}/\text{FNR}_{\text{sq}}) - E_m(\text{Fd}_{\text{ox}}/\text{Fd}_{\text{red}}) = (RT/F) \ln(K_{\text{eq}})$$

We wanted to obtain the midpoint redox potential for the first one-electron reduction of FNR,  $E_m(\text{FNR}_{\text{ox}}/\text{FNR}_{\text{sq}})$ . We can assume that  $E_m(\text{Fd}_{\text{ox}}/\text{Fd}_{\text{red}}) = -412 \text{ mV}$  for *Synechocystis* Fd as obtained from [Bottin and Lagoutte, 1992]. With this, we calculated the midpoint redox potential for the first one-electron reduction of FNR. We obtained  $E_m(\text{FNR}_{\text{ox}}/\text{FNR}_{\text{sq}}) = -385 \text{ mV}$  for  $\text{FNR}_S$ . In previous studies,  $\text{FNR}_{\text{sq}}$  was only hardly observed during redox titrations [Corrado et al., 1996] because the semiquinone radical is thermodynamically unstable. The values of the rate constants, equilibrium constant and the midpoint redox potential are summarized in Table 2.7.

**Single reduction of FNR<sub>L</sub>-PC in the presence of NADP<sup>+</sup>.** The same experiment as for FNR<sub>S</sub> was performed with FNR<sub>L</sub>-PC. Due to limited amounts of this complex, the kinetics were obtained at lower concentrations of the complex. The kinetics are shown in Figure 2.13B in the absence (black trace) and the presence of FNR<sub>L</sub>-PC (red, green, blue and purple traces).

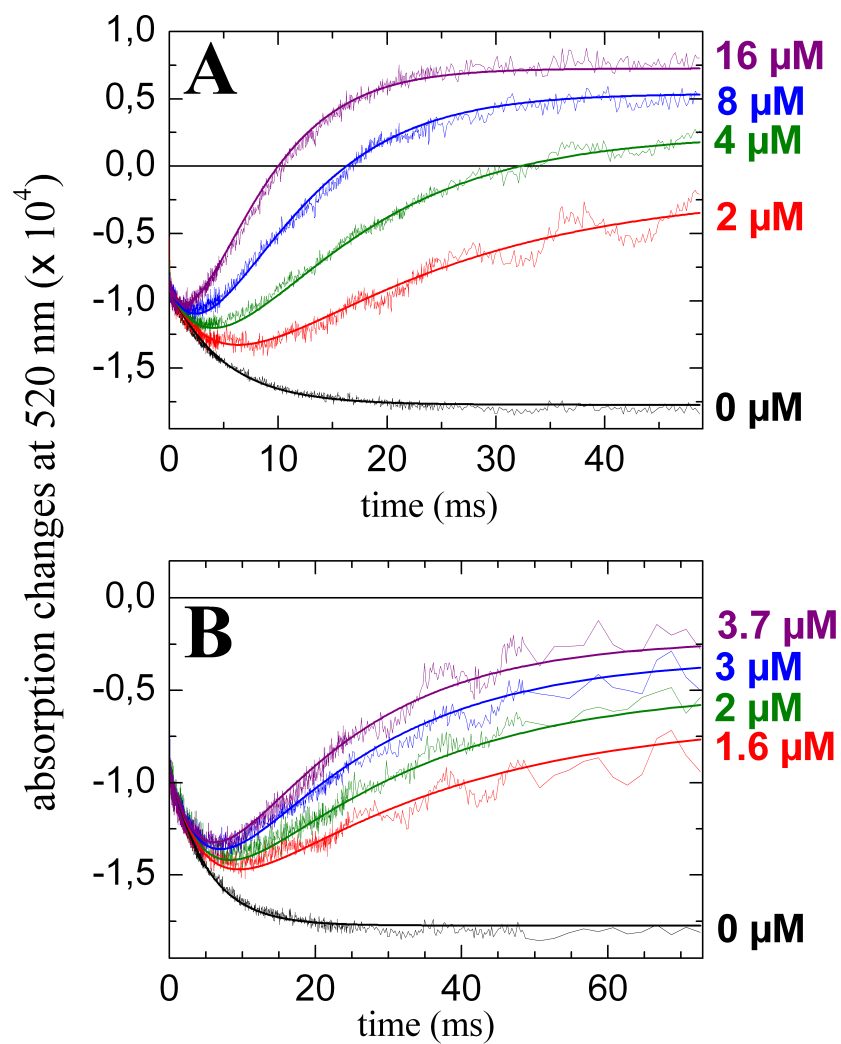
The experimental data were fitted as for the FNR<sub>S</sub> and the obtained rate constants, equilibrium constant and midpoint redox potential are shown in Table 2.7. On one side, a 25-30% decrease of  $k_1$  and  $k_{-1}$  is obtained in FNR<sub>L</sub>-PC *versus* FNR<sub>S</sub>. This is in contradiction to our working hypothesis, that the FNR<sub>L</sub>-PC might be more adapted to perform NADP<sup>+</sup> reductase activity. The FNR receives the electron from Fd<sub>red</sub>. As discussed above in 2.1.4, the presence of the additional PC hexamer might result in steric hindrance when Fd is involved. This might explain the decrease in rate constants for our complex compared to FNR<sub>S</sub>.

On the other side, the equilibrium constant and the midpoint redox potential for the first one-electron reduction of FNR were quite similar. This indicates that in the presence of NADP<sup>+</sup>, the electrostatic environment of FAD is not modified in our complex compared to FNR<sub>S</sub>. Deduced from first one-electron FNR reduction, both isoforms seem to be able to perform NADP<sup>+</sup> reduction.

**Single reduction of FNR<sub>S/L</sub> in the absence of NADP<sup>+</sup>** We repeated the same measurements in the absence of NADP<sup>+</sup>. This is in contrast to the proposed catalytic cycle [Batie and Kamin, 1984a]. Normally, the NADP<sup>+</sup> is bound to FNR *prior* to Fd binding and electron transfer. This condition might mimic a physiological stressed state, when NADPH is in excess and hence NADP<sup>+</sup> is limiting.

The kinetics are shown in Figure 2.14 in the absence (black trace) and the presence of FNR<sub>S</sub> (red, green, blue and purple traces) and FNR<sub>L</sub>-PC (red, green and blue traces). It would be interesting to compare single FNR reduction +/- NADP<sup>+</sup>. This is indeed possible for the  $k_1$  rate constant. However, the rate constant  $k_{-1}$  and thus the derived values  $K_{eq}$  and  $E_m$  are obtained with a large uncertainty (data not shown). This might result from the measurement itself, favoring first one-electron reduction and thus unfavoring the back-reaction on a short timescale. This results in overlapping uncertainties for  $k_{-1}$ ,  $K_{eq}$  and  $E_m$ . We will now discuss the differences that are significant in our measurements.

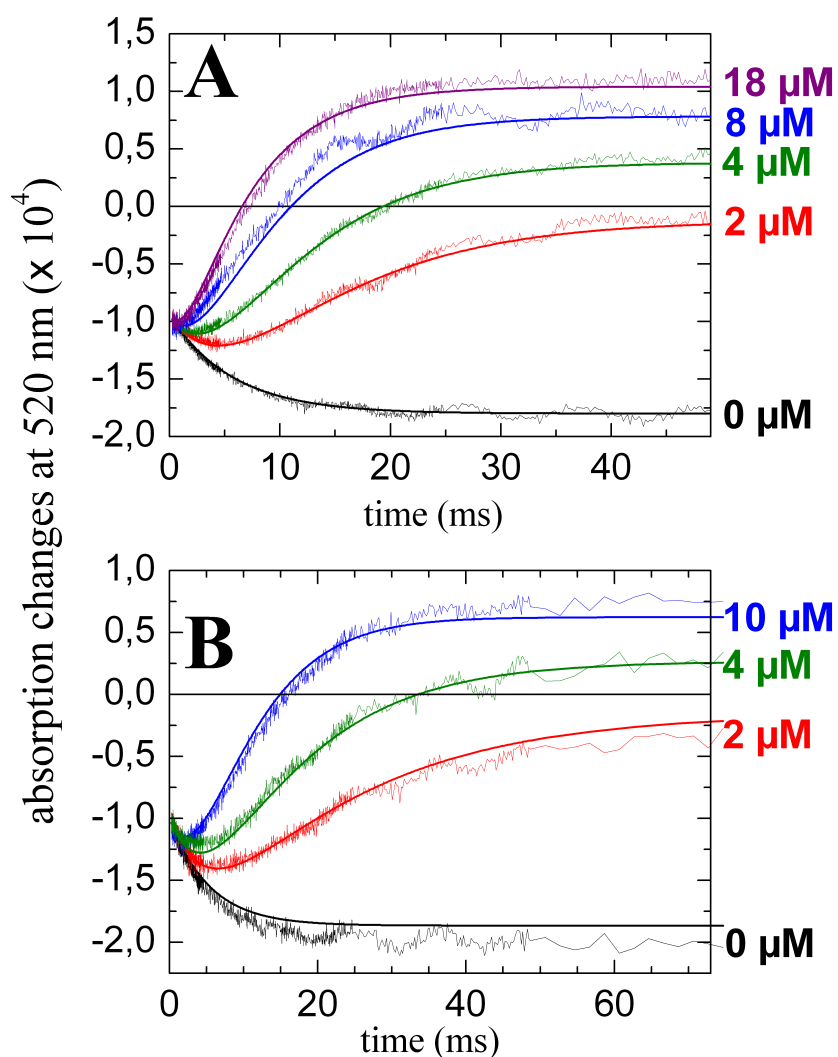
The rates, equilibrium constants and midpoint electron potentials are summarized in Table 2.8. Overall, the rate constants for both FNR isoforms are larger without than with NADP<sup>+</sup>. This agrees with a previous study [Cassan et al., 2005]. This effect was attributed in this article to a repulsive electrostatic effect between the phosphate moiety of NADP<sup>+</sup> and the negatively charged Fd. The rates  $k_1(+NADP^+)/k_1(\text{no } NADP^+)$  are 0.77 and 0.61 for FNR<sub>L</sub>-PC and FNR<sub>S</sub>,



**Figure 2.13** Flash titration of  $\text{FNR}_S$  (A) and  $\text{FNR}_L\text{-PC}$  (B) in the presence of  $\text{NADP}^+$  under single reduction conditions. Thin line, data; bold line, fit.

respectively. Compared to FNR<sub>S</sub>, FNR<sub>L</sub>-PC presents a decrease for  $k_1$  and  $k_{-1}$ . Thus, FNR<sub>L</sub>-PC exhibits steric hindrance due to PC in the complex in the absence and presence of NADP<sup>+</sup>.

As a summary, NADP<sup>+</sup> reduction measurements does not seem to favor our working hypothesis. Especially single FNR reduction may not be able to explain different growth characteristics of the FNR mutants. It has to be noted that several steps are involved during catalysis following first one-electron FNR reduction (see Chapter 1). Multiple turnover during NADP<sup>+</sup> reduction may help comparing the steps involved during catalysis after the first one-electron reduction of FNR.



**Figure 2.14** Flash titration of FNR<sub>S</sub> (A) and FNR<sub>L</sub>-PC (B) in the absence of NADP<sup>+</sup> under single reduction conditions. Thin line, data; bold line, fit.

	Single reduction of FNR by Fd <sub>red</sub>	(unit)	FNR <sub>L</sub> -PC	FNR <sub>S</sub>
+ NADP <sup>+</sup>	Second-order forward rate $k_1$	( $\mu\text{M}^{-1} \text{s}^{-1}$ )	10.8	15.2
	Second-order reverse rate $k_{-1}$	( $\mu\text{M}^{-1} \text{s}^{-1}$ )	4.0	5.4
	$K_{\text{eq}} = k_1/k_{-1}$	-	2.73	2.84
	$E_m(\text{FNR}_{\text{ox}}/\text{FNR}_{\text{sq}})$	(mV)	-386	-385

**Table 2.7** Single reduction of FNR<sub>L</sub>-PC and FNR<sub>S</sub> by Fd<sub>red</sub> in the presence of NADP<sup>+</sup>.

	Single reduction of FNR by Fd <sub>red</sub>	(unit)	FNR <sub>L</sub> -PC	FNR <sub>S</sub>
- NADP <sup>+</sup>	Second-order forward rate $k_1$	( $\mu\text{M}^{-1} \text{s}^{-1}$ )	14.1	24.9
	Second-order reverse rate $k_{-1}$	( $\mu\text{M}^{-1} \text{s}^{-1}$ )	4.6	10.6
	$K_{\text{eq}} = k_1/k_{-1}$	-	3.06	2.35
	$E_m(\text{FNR}_{\text{ox}}/\text{FNR}_{\text{sq}})$	(mV)	-384	-390

**Table 2.8** Single reduction of FNR<sub>L</sub>-PC and FNR<sub>S</sub> by Fd<sub>red</sub> in the absence of NADP<sup>+</sup>.

### Catalytic turnover of FNR isoforms during NADP<sup>+</sup> reduction

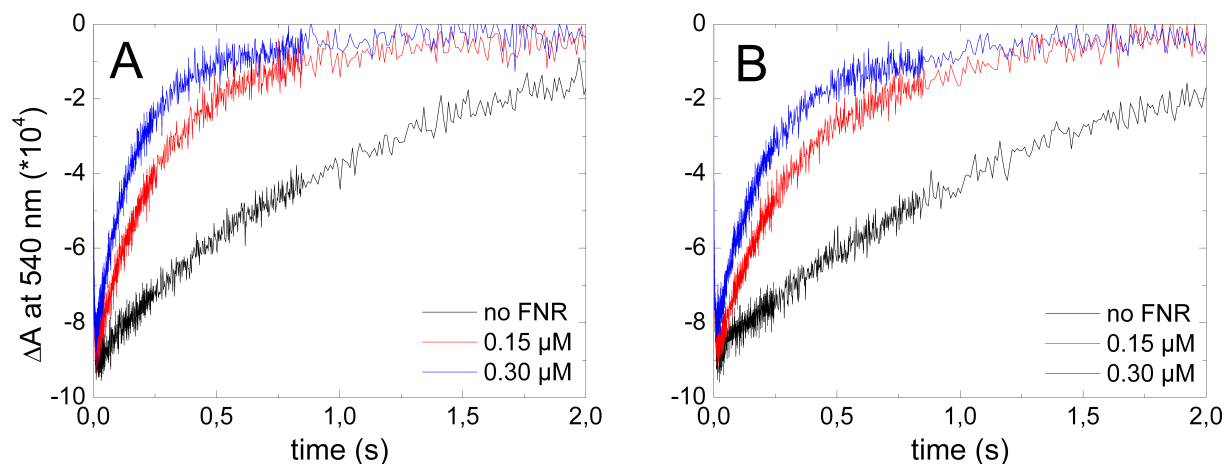
We performed a second type of measurements on NADP<sup>+</sup> reduction: the catalytic turnover of the two FNR isoforms. We measured the kinetics obtained for a ternary mixture PSI/Fd/FNR in the presence of NADP<sup>+</sup>. As the product of this reaction is NADPH, one might on one hand follow the build-up of NADPH. This was however not easily possible in our set-up. On the other hand, in our laboratory the redox changes of the second substrate Fd can be easily followed [Cassan et al., 2005]. Electrons flow from PSI to Fd and, under the action of FNR, NADPH build-up is followed. Alternative measurements using continuous light illumination during photoreduction were performed with FNR in excess so that FNR catalysis is not rate-limiting. On the other side our set-up is specifically adapted to determine rate-limitation due to FNR catalysis (see below).

Conditions for multiple catalytic turnover were characterized by PSI in large excess over FNR (3.75  $\mu\text{M}$  vs 0.15/0.3  $\mu\text{M}$ ). In addition to that, Fd is added in excess over PSI (Fd<sub>ox</sub> concentration 8  $\mu\text{M}$ ). The reaction starts with photoexcitation of PSI (3.75  $\mu\text{M}$ ). The same concentration of Fd<sub>ox</sub> will get rapidly photoreduced from PSI<sub>red</sub>. The large excess of reduced Fd will afterwards doubly reduce FNR. Following the catalytic cycle proposed by Batie and Kamin in [1984a], FNR reduction by Fd<sub>red</sub> is involved at steps 3 and 6 (see Chapter 1). We can thus follow spectrophotometrically the reoxidation of Fd<sub>red</sub>. Furthermore, we subtracted the decay rate in the absence of FNR<sub>S/L</sub> ( $k_{\text{noFNR}}$ ) from the reoxidation in the presence of FNR ( $k_{\text{FNR}}$ ). Reoxidation of Fd<sub>red</sub> in the absence of FNR is mainly due to slow reduction of oxygen. We would like to point out that no maximum turnover rate ( $k_{\text{cat}}$ ) is measured *via* these measurements.

**Multiple turnover of FNR<sub>S</sub>.** We obtained values of 2.2 and 4.1 s<sup>-1</sup> for (k<sub>FNR</sub> - k<sub>noFNR</sub>) with 0.15 and 0.3 μM FNR<sub>S</sub> (Figure 2.15A). We calculated the monoexponential decay rate as indicated in Chapter 5. This results in 55 and 51 reoxidized Fd<sub>red</sub> per second and per FNR<sub>S</sub>. The average value of 53 is indicated in Table 2.9.

**Multiple turnover of FNR<sub>L</sub>-PC.** The same type of measurements were performed using FNR<sub>L</sub>-PC. We obtained similar rates of 2.14 and 3.69 s<sup>-1</sup> for (k<sub>FNR</sub> - k<sub>noFNR</sub>) with 0.15 and 0.3 μM FNR<sub>L</sub>-PC (Figure 2.15B). This results in the averaged value of 50 reoxidized Fd<sub>red</sub> per second and FNR<sub>L</sub>-PC (Table 2.9).

Overall, the multiple turnover rates for the two isoforms are similar. From these results, both isoforms are equally capable of catalyzing the NADP<sup>+</sup> reductase activity of FNR under multiple turnover conditions. This was in contrast to our working hypothesis.



**Figure 2.15** Flash titration of FNR<sub>S</sub> (A) and FNR<sub>L</sub>-PC (B) in the presence of NADP<sup>+</sup> under catalytic turnover conditions.

**Calculated turnover rate.** In the previous paragraph, we have presented experimental catalytic turnover rates for the two FNR isoforms. We have seen that these turnovers are relatively slow. Due to special conditions during the measurements (discussed below), the first one-electron reductions were observed to be slow, too. We wanted to check if the two observations might be related, that is if first one-electron reduction might limit the overall reaction.

For this, we calculated a catalytic turnover rate by using the experimental rate  $k_1$  of single FNR reduction and by supposing that this rate limits overall turnover. This calculation was performed according to Equation 5.3.1 in Chapter 5. We obtained 40.5 and 57 reoxidized Fd per second and FNR<sub>S/L</sub> for FNR<sub>S</sub> and FNR<sub>L</sub>-PC, respectively (Table 2.9). The smaller value obtained

for FNR<sub>L</sub>-PC results from a smaller rate  $k_1$ . These rates are in agreement with the experimental turnovers (Table 2.9).

As explained in Chapter 1, rate limiting steps during NADP<sup>+</sup> reduction were proposed to be Fd<sub>ox</sub> dissociation or NADPH release [Aliverti et al., 2001, Batie and Kamin, 1984a, Carrillo and Ceccarelli, 2003] and, contrary to our results, no indications were found for a rate limitation by ET from Fd<sub>red</sub> to FNR. We attribute our results to the high ionic strength which leads to a decrease in  $k_1$  strong enough that it becomes the limiting rate.

Multiple turnover: Initial rate of reoxidation of 3.75 $\mu$ M Fd <sub>red</sub> (reoxidized Fd <sub>red</sub> per second and per FNR)	FNR <sub>L</sub> -PC	FNR <sub>S</sub>
Observed rate	50	53
Calculated rate (with limiting $k_1$ )	40.5	57

**Table 2.9** Multiple turnover of FNR<sub>L</sub>-PC and FNR<sub>S</sub>.

### 2.1.6 Catalytic properties of cyanobacterial FNR<sub>S</sub>

Our first objective was the *in vitro* characterization of FNR<sub>S</sub> in comparison to FNR<sub>L</sub>-PC. These *in vitro* activities were performed under high ionic strength (high salt) conditions that ensure the stability of the purified FNR<sub>L</sub>-PC. The interprotein ET reactions depend on the salt concentration (see Chapter 1). This makes comparison with previously obtained rate constants and catalytic parameters not straightforward. However, comparison may be useful to understand which steps or reactions are mostly affected by ionic strength. Thus, we will compare our results with results obtained on cyanobacterial FNR<sub>S</sub> under low salt [Medina et al., 1998, Cassan et al., 2005].

#### NADPH oxidase activities

Extensive NADPH oxidation measurements were carried out on *Anabaena* sp. FNR<sub>S</sub> [Medina et al., 1998]. We will compare catalytic parameters concerning ferricyanide reduction and Fd-mediated cytochrome *c* reduction.

**Ferricyanide reduction.** On one hand, catalytic turnovers  $k_{cat}$  were found to be quite similar to high-salt results. On the other hand, affinities for NADPH were found to be two-fold decreased in our case (Table 2.10). This indicates that high salt is not detrimental for this oxidase activity. This also means that the affinity to NADPH is decreased in high salt.



**Fd-mediated cytochrome *c* reduction.** On one hand, catalytic turnovers  $k_{\text{cat}}$  were found to be similar (Table 2.10). On the other hand, affinities to Fd were found to be 3-fold decreased in our case (Table 2.10). Electrostatic interactions are known to be weakened in the presence of high salt. This might explain the strong decrease in affinity to Fd in our conditions. The important decrease in affinity to Fd might also explain the decreased catalytic efficiencies.

Ferricyanide reductase activity	(unit)	<i>Anabaena</i> FNR <sub>S</sub>
$K_m(\text{NADPH})$	( $\mu\text{M}$ )	$23.0 \pm 1.2$
$k_{\text{cat}}$	( $\text{s}^{-1}$ )	$225 \pm 3$
$k_{\text{cat}}/K_m$	( $\mu\text{M}^{-1} \text{s}^{-1}$ )	$9.82 \pm 0.15$
Cytochrome <i>c</i> reductase activity		
$K_m(\text{NADPH})$	( $\mu\text{M}$ )	$11.0 \pm 2.0$
$k_{\text{cat}}$	( $\text{s}^{-1}$ )	$200 \pm 10$
$k_{\text{cat}}/K_m$	( $\mu\text{M}^{-1} \text{s}^{-1}$ )	$18.2 \pm 1.0$

**Table 2.10** NADPH oxidation of the *Anabaena* FNR<sub>S</sub>.

### NADP<sup>+</sup> reductase activities

Extensive NADP<sup>+</sup> reductase activities were carried out previously in our laboratory on *Synechocystis* FNR<sub>S</sub> [Cassan et al., 2005] in low salt. We will compare catalytic parameters concerning single FNR reduction and multiple turnover of FNR.

**Single FNR reduction.** Single FNR reduction by Fd<sub>red</sub> was strongly inhibited in high salt. The second-order rate constant  $k_1$  was 27-fold decreased in our case compared to low salt ( $15.2$  vs.  $417 \mu\text{M}^{-1}\text{s}^{-1}$  see Table 2.11). As it has been found in NADPH oxidase activities, electrostatic interactions are known to be weakened in the presence of high salt and this results in this strong decrease in the rate of single FNR reduction.

**Multiple turnover of FNR.** The data concerning multiple turnover of FNR in low salt were recalculated using  $\epsilon_{461\text{nm}}(\text{FNR}_S) = 9000 \text{ M}^{-1}\text{cm}^{-1}$ . This results in 330 reoxidized Fd<sub>red</sub> per second and FNR. The rate of Fd<sub>red</sub> reoxidation was 6 times smaller in our case ( $53$  vs.  $330$ ; Table 2.11). This indicates that high salt has a strong effect on overall turnover. However, the effect is smaller than on single FNR reduction. The calculated turnover (calculated from the single FNR reduction) is 27 times smaller in our case ( $57$  vs.  $1564$ ; Table 2.11).

Under low salt, catalytic turnover is increased. As can be seen in Table 2.11, single FNR reduction works at an even more increased rate ( $k_1 = 417 \mu\text{M}^{-1}\text{s}^{-1}$ ) which results in a 4-5 times increased calculated turnover rate ( $1564$  vs.  $330$ ). This indicates that in low salt, first FNR

reduction is not limiting. We have seen before that first electron reduction was limiting for both FNR isoforms under high salt conditions whereas under low salt, another first-order process is limiting. This may be  $Fd_{ox}$  dissociation, hydride transfer or NADPH release.

Single reduction of FNR by $Fd_{red}$ (unit)	high salt	low salt
Second-order forward rate $k_1$ ( $\mu M^{-1} s^{-1}$ )	15.2	417
Multiple turnover: Initial rate of reoxidation of $3.75 \mu M Fd_{red}$		
(reoxidized $Fd_{red}$ per second and per FNR)		
Observed rate	53	330
Calculated rate (with limiting $k_1$ )	57	1564

**Table 2.11** Multiple turnover of FNR<sub>S</sub> under high and low salt.

### 2.1.7 Catalytic properties of plant FNR<sub>S</sub>

Previous work has been performed on different FNR isoforms in plants under low salt. These FNR isoforms have a size corresponding to FNR<sub>S</sub>. They are encoded by different genes and thus are composed of slightly different catalytic domains. They are called leaf (photosynthetic; p) FNR and root (heterotrophic; h) FNR according to their respective localization. In plants there are as well different Fd present in leaves and roots. Here we compare our results with results obtained on different plant FNR isoforms under low salt.

#### NADPH oxidase activities

NADPH oxidase activities have been extensively studied in maize FNR [Onda et al., 2000] and spinach and corn FNR isoforms [Aliverti et al., 2004]. The different Fd isoforms have been compared in *Arabidopsis thaliana* [Hanke et al., 2004a, Gou et al., 2006]. A review summarizes the knowledge on Fd:FNR electron transfer complexes [Hanke et al., 2004b]. We will compare catalytic parameters concerning ferricyanide reduction and Fd-mediated cytochrome *c* reduction.

**Ferricyanide reduction.** The leaf and root FNR isoforms from plants differ mainly in four catalytic or thermodynamic parameters. The first one implies ferricyanide reduction. The Michaelis-Menten constant  $K_m(\text{NADPH})$  has been found 3-10 fold higher for leaf FNR compared to root FNR. This indicated a decreased affinity of NADPH to the leaf FNR. According to our working hypothesis, we will tentatively make the parallel between FNR<sub>L</sub>-PC from cyanobacteria and leaf FNR from plants on the one hand, and between FNR<sub>S</sub> from cyanobacteria and root FNR from plants on the other hand. We observed a 30% difference for the  $K_m(\text{NADPH})$  between the

FNR<sub>L</sub>-PC and FNR<sub>S</sub>. However, the difference was observed in the direction opposite to what was expected. This indicated in our case an increased affinity of NADPH to FNR<sub>L</sub>-PC.

**Fd-mediated cytochrome *c* reduction.** Two other differences between the plant isoforms have been found. They imply the Fd-mediated cytochrome *c* reductase activity. First, the Michaelis-Menten constant  $K_m$ (leaf Fd) has been found 5-10 fold smaller for leaf FNR compared to root FNR. This indicated an increased affinity of leaf Fd to the leaf FNR. We have found a 70% difference for the  $K_m$ (Fd) between the FNR<sub>L</sub>-PC and FNR<sub>S</sub>. Again, the difference was observed in the opposite direction. This indicated in our case a decreased affinity of Fd to FNR<sub>L</sub>-PC. Second, the plants isoforms differ in the catalytic turnover  $k_{cat}$ . The  $k_{cat}$  of cyt *c* reduction is 3-4 fold smaller for leaf FNR compared to root FNR. In our case, we did not observe a significant difference for the catalytic turnover of cyt *c* reduction.

### NADP<sup>+</sup> reductase activity

Another major difference observed between the plant isoforms concerns NADP<sup>+</sup> reductase activity. The midpoint potential  $E_m$ (FNR<sub>ox</sub>/FNR<sub>red</sub>) for the two-electron reduction has been found 20 mV higher for the corn root FNR than for spinach leaf FNR [Aliverti et al., 2001]. However, we cannot compare the midpoint potential for two plant FNR isoforms from the same organism as it is not yet available. We have obtained the midpoint potential for the first-electron reduction only. No significant difference was found for the two cyanobacterial isoforms concerning the  $E_m$ (FNR<sub>ox</sub>/FNR<sub>sq</sub>).

The differences in cyanobacterial FNR isoforms are in contrast with the differences observed in plants. In addition to the presence of leaf or photosynthetic FNR (pFNR) and root or heterotrophic FNR (hFNR) isoforms, multiple pFNR isoforms have been found e.g. in *Arabidopsis* (2 pFNR) [Hanke et al., 2005], *Oryza sativa* (2 pFNR) [Ohyanagi et al., 2006] and *Zea mays* (maize; 3 pFNR) [Okutani et al., 2005]. Another study was carried out in wheat [Gummadova et al., 2007]. This study has identified the presence of multiple forms of FNR in wheat leaves with varied expression and N-terminal processing. Two *Arabidopsis* mutant lines have been studied in detail, each lacking one of the pFNR isoforms [Lintala et al., 2009]. The deficiency of FNR affected electron transfer properties of the mutant plants, especially cyclic electron transfer around PSI. Moreover, a distinct difference in the function of FNR1 and FNR2 became evident upon low-temperature acclimation of *Arabidopsis*.

It is essential to consider different Fd isoforms that may interact with different FNR isoforms. In plants, the midpoint potential of root Fd was found to be much higher compared to leaf Fd (50-100 mV) [Hanke et al., 2004a, Gou et al., 2006, Aliverti et al., 1995, Akashi et al., 1999]. This

might be an important factor favoring NADPH oxidation in roots compared to NADP<sup>+</sup> reduction in leaves. In *Synechocystis*, 4 genes have been identified for *fed*-like genes [Poncelet et al., 1998]. It is usually considered that only one major isoform encoded by *fed1* is responsible for bioenergetic electron flows under photoautotrophic conditions. The Fd encoded by *fed1* exhibits a similar redox potential to leaf type Fd.

The differences between heterotrophic and photosynthetic plant FNR isoforms do not correspond to the differences present in cyanobacterial FNR isoforms (see above). In addition to that, there is no evidence that the two FNR isoforms present in cyanobacteria interact with different Fd partners. The different leaf isoforms should interact exclusively with photoautotrophic Fd (tissue specificity). Differences in membrane attachment were found for leaf FNR isoforms, too [Hanke et al., 2005, Okutani et al., 2005]. Maybe the differences between the cyanobacterial FNR isoforms resemble more the differences observed for different leaf FNR isoforms that are summarized above.

## 2.2 Conclusion

In the first part of my PhD, we were interested in determining the catalytic properties of the FNR isoforms of *Synechocystis*. Functional specificity of FNR was supported by a recent study [Thomas et al., 2006]. In this article, it was proposed that FNR<sub>S</sub> is involved in oxidative metabolism (NADPH oxidation) and that FNR<sub>L</sub> sustains NADPH production under reductive metabolism (NADP<sup>+</sup> reduction). This was our working hypothesis. Thus, we compared *in vitro* the two isoforms: FNR<sub>S</sub> and FNR<sub>L</sub>. We wanted to approach as close as possible the *in vivo* situation. FNR<sub>L</sub> is always bound to PBS *in vivo* and is degraded when it is not attached to PBS. Thus, we purified a complex called FNR<sub>L</sub>-PC that is stable and compatible with absorption spectroscopy studies. A first objective was to scale up the purification of FNR<sub>L</sub>-PC and to purify the complex to homogeneity. We used a mutant that was composed only of one disk of PC hexamer per rod (CB, [Ughy and Ajlani, 2004]). On average, one to two FNR<sub>L</sub> is found attached per PBS [van Thor et al., 1999b, Gómez-Lojero et al., 2003]. By using the CB mutant, we increased the FNR<sub>L</sub> amount per phycocyanin (total of 6 hexamers per PBS instead of 18 in the WT) in the starting material by a factor of three. The purification was further facilitated due to a His-Tag inserted in the hinge domain. This mutant was constructed by Dr. Ajlani and called CBH. Thus, we could use IMAC (Immobilized Metal Affinity Chromatography) for the purification. Further impurities were eliminated during preparative gel filtration. Our results indicated a pure and homogeneous complex composed of a PC hexamer, FNR<sub>L</sub> and a rod-core linker L<sub>RC</sub> with a stoichiometry of the apoproteins around 1:1:1 for FNR<sub>L</sub>:L<sub>RC</sub>:( $\alpha^{PC}, \beta^{PC}$ )<sub>6</sub> (complex size 330 kDa). Our complex was stable at 4°C. It can thus be concluded that the purification of FNR<sub>L</sub> in our

complex favors protection from proteolytic degradation, as previously observed [Schluchter and Bryant, 1992, Nakajima et al., 2002]. We also carried out preliminary studies on reconstitution of PBS with FNR<sub>L</sub>. These reconstitution studies open enormous possibilities in terms of up-scaled purification and structure determination of the FNR<sub>L</sub>-PC complex.

Several articles discussed the exact localization of FNR<sub>L</sub> in the PBS and different proposals of FNR<sub>L</sub> binding to core-proximal [van Thor et al., 1999b, Arteni et al., 2009], core-distal [Gómez-Lojero et al., 2003] PC hexamers as well as attachment to the PBS core and thylakoid membrane [van Thor et al., 1999a, 2000] were proposed. Little was known about the function of FNR<sub>L</sub> attachment to the PBS. This study deals with the roles of the FNR isoforms and contributes to determine the function of the attachment of FNR<sub>L</sub> to the PBS.

We have identified the exact composition of the FNR<sub>L</sub>-PC complex and quantified the holoenzyme in this complex. We wanted to elucidate the function of the selective attachment of the FNR<sub>L</sub> isoform to the PBS. For this, we performed a detailed functional characterization and observed differences in NADP<sup>+</sup> reduction and NADPH oxidation between FNR<sub>S</sub> and FNR<sub>L</sub>-PC.

### Catalytic properties of FNR isoforms

We have found several catalytic parameters that are similar for the two *Synechocystis* isoforms. Most of the differences we have observed can be explained by steric hindrance brought by the additional PC hexamer in the FNR<sub>L</sub>-PC complex. However, we have also observed some differences that cannot be easily explained by steric hindrance.

The catalytic parameters that were similar for FNR<sub>S</sub> and FNR<sub>L</sub>-PC are as follows: During NADPH oxidation, the presence of the PC did not prevent the NADPH/ferricyanide oxidoreduction. The interaction with the second substrate Fd resulted in similar maximal velocities during Fd-mediated NADPH oxidation. During first-electron FNR reduction, the electrostatic environment of the FAD cofactor was not changed. In addition to that, the two isoforms seem to be able to perform NADP<sup>+</sup> reduction to the same extent under multiple turnover.

However, some significant differences were observed in the NADP<sup>+</sup> reductase and NADPH oxidase activities between FNR<sub>S</sub> and FNR<sub>L</sub>-PC. We will summarize now the catalytic parameters that were different for FNR<sub>S</sub> and FNR<sub>L</sub>-PC and could be explained by steric hindrance brought by the additional PC in the FNR<sub>L</sub>-PC complex. We found a decreased affinity of Fd for FNR<sub>L</sub>-PC compared to FNR<sub>S</sub> during Fd-mediated NADPH oxidation. Furthermore, we obtained indications that the FNR<sub>L</sub>-PC complex was less able to perform Fd-mediated NADPH oxidation. This was in agreement with our working hypothesis. The same effect of steric hindrance was observed during NADP<sup>+</sup> reduction during single electron reduction of FNR. The rate  $k_1$  for FNR single

reduction by  $\text{Fd}_{\text{red}}$  was smaller for  $\text{FNR}_{\text{L}}\text{-PC}$  compared to  $\text{FNR}_{\text{S}}$ . These effects might be alternatively explained by electrostatic repulsion. However, electrostatic interactions are expected to be of limited importance at high salt. Overall, the rate  $k_1$  for FNR single reduction is in contrast to our working hypothesis. This would mean that  $\text{FNR}_{\text{L}}$  is less adapted to perform  $\text{NADP}^+$  reduction.

Finally, we will summarize the catalytic parameters that are different for  $\text{FNR}_{\text{S}}$  and  $\text{FNR}_{\text{L}}\text{-PC}$  and cannot be explained by steric hindrance. The  $K_{\text{m}}(\text{NADPH})$  is decreased and the catalytic turnover is decreased to the same extent for  $\text{FNR}_{\text{L}}\text{-PC}$  compared to  $\text{FNR}_{\text{S}}$  during  $\text{NADPH}$  oxidation. The size of the substrate  $\text{NADPH}$  excludes the effect of steric hindrance brought by  $\text{PC}$ . We propose that increased  $\text{NADP}^+/\text{NADPH}$  affinity for  $\text{FNR}_{\text{L}}\text{-PC}$  might favor the physiological reaction. Following the working hypothesis,  $\text{FNR}_{\text{L}}$  is catalyzing  $\text{NADP}^+$  reduction under photoautotrophic growth. We have seen in Chapter 1 that  $\text{Fd}_{\text{ox}}$  dissociation can be rate-limiting for  $\text{NADP}^+$  reduction. This limitation is decreased by *prior*  $\text{NADP}^+$  binding and by the mechanism of positive kinetic cooperativity. Following the working hypothesis, the isoform  $\text{FNR}_{\text{S}}$  is catalyzing the  $\text{NADPH}$  oxidation under heterotrophic growth, starvation or high-light conditions. These conditions are probably accompanied by an increased  $\text{NADPH}$  concentration. Therefore, a higher affinity of  $\text{NADPH}$  to  $\text{FNR}_{\text{S}}$  may not be necessary under physiological conditions.

To summarize, the results obtained for the  $\text{Fd}$ -mediated  $\text{NADPH}$  oxidation are in agreement with the working hypothesis. In addition, the results for the ferricyanide-mediated  $\text{NADPH}$  oxidation indicate adaptation to physiological conditions. Furthermore, both isoforms are capable of catalyzing the  $\text{NADP}^+$  reduction under multiple turnover conditions to the same extent. Finally, by comparing calculated and experimental turnover for  $\text{NADP}^+$  reduction, we found evidence, that under our conditions, the first electron reduction is rate-limiting.

The extent of the observed differences contrast with large differences between leaf and root FNR isoforms in plants. The situation of the cyanobacterial isoforms might resemble more the case of different leaf FNR isoforms present in plants. We propose that the main photosynthetic  $\text{Fd}$  [Poncelet et al., 1998] is involved *in vivo* in electron transfer with both isoforms. Thus, the observed *in vitro* differences of the two *Synechocystis* FNR isoforms might not fully explain the *in vivo* properties of the mutants expressing only one of the isoforms. We have seen in Chapter 1 that FNR mutants that express only  $\text{FNR}_{\text{L}}$  or  $\text{FNR}_{\text{S}}$  show large differences in growth under photoautotrophic and heterotrophic growth. These differences were explained by the hypothesis of function specificity of FNR isoforms [Thomas et al., 2006]. This study proposed that  $\text{FNR}_{\text{L}}$  could be implicated in the photosynthetic reaction ( $\text{NADP}^+$  reduction), whereas the  $\text{FNR}_{\text{S}}$  could be implicated in the reverse reaction ( $\text{NADPH}$  oxidation).

Instead of their intrinsic catalytic properties, it would then be necessary to invoke their localization and their association to other complexes. It can be *e.g.* speculated that  $\text{FNR}_{\text{S}}$  is

involved in cyclic/respiratory electron flow because it is free to bind to other membrane complexes such as NADPH dehydrogenase (NDH-1) or cytochrome *b<sub>6</sub>f*. By contrast, PBS-bound FNR<sub>L</sub> would not be able to play such a role but would be dedicated only to NADP<sup>+</sup> photoreduction. For both types of activities, substrate availability (Fd<sub>red</sub>/Fd<sub>ox</sub> and NADP<sup>+</sup>/NADPH) might also be key *in vivo* characteristics for the activity of the two isoforms. This situation would be reminiscent of what has been described recently for the different plant leaf isoforms, where catalytic activities appear to depend upon their variable attachment to the thylakoid membrane [Palatnik et al., 1997, Hanke et al., 2008].



## Chapter 3

# *In vivo* studies

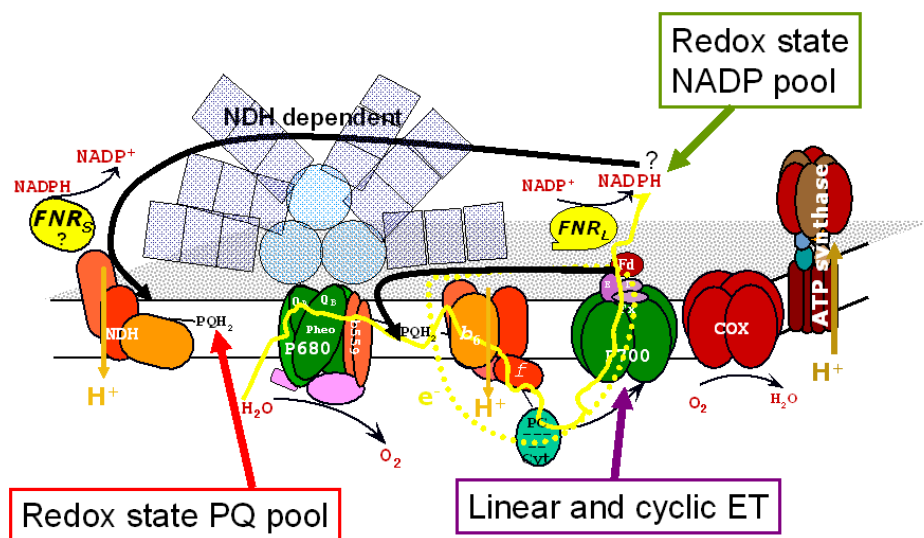
### 3.1 Results and discussion

We studied the implication of the FNR isoforms in linear-, cyclic-, pseudocyclic and respiratory ET that are present in thylakoid membranes. In addition to that, recombination reactions involving  $P700^+$  can occur under conditions where electrons cannot be evacuated on the acceptor side of PSI.

As discussed in Chapter 2, substrate (Fd, NADP(H)) availability and enzyme localization might well be important for the FNR isoforms to perform different roles *in vivo*. On one hand, the localization of FNR<sub>L</sub> is always close to the thylakoid membrane because of the attachment to the PBS. FNR<sub>S</sub> on the other hand may be mobile or attached to other complexes. Growth characteristics of the mutants expressing only one FNR isoform (see Chapter 1) indicates specific roles for each isoform: FNR<sub>L</sub> could be implicated in NADP<sup>+</sup> reduction, whereas FNR<sub>S</sub> could be implicated in NADPH oxidation (see Chapter 1) [Thomas et al., 2006]. However, from a detailed functional characterization *in vitro*, we observed small differences in the NADP<sup>+</sup> reductase and NADPH oxidase activities of FNR<sub>S</sub> and FNR<sub>L</sub>-PC [Korn et al., 2009]. Thus, *in vivo* studies of the FNR mutants compared to the WT should help to determine their respective roles *in vivo*.

We studied mutants containing either FNR<sub>L</sub> (MI6) or FNR<sub>S</sub> (FS1). We determined the phenotypes for these mutants by comparing P700 oxidation kinetics (purple in Figure 3.1) where various ET processes in the FNR mutants and WT are analyzed. We also determined the redox state of the NADP pool (green in Figure 3.1) where we obtained informations about the output of PSI ET. Furthermore, NADPH can be reoxidized *via* the NDH-dependent cyclic ET (black arrow in Figure 3.1). Finally, we measured in collaboration with D. Kirilovsky the PQ pool reduction in the dark. The PQ pool (red arrow in Figure 3.1) is reduced by different cyclic electron transfer pathways besides PSII. Reduced PQ represents the input to PSI electron transfer *via* the *cyt b<sub>6</sub>f* complex.





**Figure 3.1** Scheme of the situation *in vivo*. Possible measurements are indicated. Adapted from G. Ajlani.

The FNR mutants and WT have been analyzed under different growth conditions. First, they were analyzed under standard photoautotrophic growth conditions. *Synechocystis* is usually grown under high CO<sub>2</sub> conditions (air enriched in CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> in the medium, detailed in Chapter 5) in the presence of light, oxygen and minimal medium. Several reports suggest a significant contribution of cyclic and pseudocyclic ET in the normal operation of photosynthesis in plants and cyanobacteria [Badger et al., 2000, Rumeau et al., 2007, Mi et al., 1992a,b]. The contribution of cyclic and pseudocyclic ET to overall ET was furthermore shown to be enhanced in conditions such as low CO<sub>2</sub> and high light in plants and cyanobacteria [Harbinson and Foyer, 1991, Miyake et al., 2005, Battchikova and Aro, 2007, Hackenberg et al., 2009, Eisenhut et al., 2007]. We compared the FNR mutants and WT under high- (HC) and low-CO<sub>2</sub> (LC) conditions.

### 3.1.1 NADP<sup>+</sup>/NADPH ratio

The NADP pool redox state was the first phenotype that we characterized for the FS1 and MI6 mutants, that contain only FNR<sub>S</sub> and FNR<sub>L</sub>, respectively. NADPH build-up represents the output of PSI ET. A method has been previously used to determine NADPH fluorescence in *Synechocystis* [Mi et al., 2000]. We used the quantification of the cellular NADP<sup>+</sup> and NADPH to determine the NADP pool redox state because no blue fluorescence device was available.

We adapted a commercial kit to carry out separate extraction of NADP<sup>+</sup> and NADPH. Once extracted, the concentration of NADP<sup>+</sup> and NADPH were determined *via* colorimetric cycling and the NADP<sup>+</sup>/NADPH ratio was calculated (see Chapter 5). We compared NADP<sup>+</sup>/NADPH ratios in FS1 and MI6 mutants to WT.

The NADP<sup>+</sup>/NADPH ratio in the WT was found to be 2.6 (see Table 3.1). This is in agreement with previously published values of 3 [Mi et al., 2000]. FS1 showed an increased NADP<sup>+</sup>/NADPH ratio (4.3) compared to WT while MI6 showed a slight decreased NADP<sup>+</sup>/NADPH ratio (2.0) compared to WT (Table 3.1).

These results are in agreement with our working hypothesis that is based on previously published results [Thomas et al., 2006]. On one side, FS1 showed a decreased growth under photoautotrophic conditions. The increased NADP<sup>+</sup>/NADPH ratio indicates an insufficient build-up of NADPH during photoautotrophic growth. On the other side, MI6 showed a similar growth compared to WT.

Interestingly, previous work on mutant M55 in *Synechocystis* showed a lower redox state of the NADP pool. Values from 80%- [Mi et al., 2000] to up to 100%-reduced NADP pool [Cooley and Vermaas, 2001] were previously observed which corresponds to NADP<sup>+</sup>/NADPH ratios of 0.25 and < 0.01. This can be correlated to the absence of the NDH-1 complexes in the M55 mutant. The authors in [Mi et al., 2000] concluded that the NDH-1 is one major site of NADPH reoxidation by respiratory or cyclic ETs. For FS1, we obtained differences in the redox state of the NADP pool that are opposite to that of M55 compared to WT. This indicates that FS1 is characterized by an increased level of NADPH reoxidation *via* respiratory or cyclic ETs. The increased NADP<sup>+</sup>/NADPH ratio in FS1 may also be the result of an increased pseudocyclic ET involving the Mehler-reaction. This pathway involves Flv1 and Flv3 that are NAD(P)H dependent flavoproteins responsible for the light-dependent O<sub>2</sub> reduction (Chapter 1).

NADP <sup>+</sup> /NADPH	
WT	2.6 ± 0.2
FS1	4.3 ± 0.8
MI6	2.0 ± 0.4

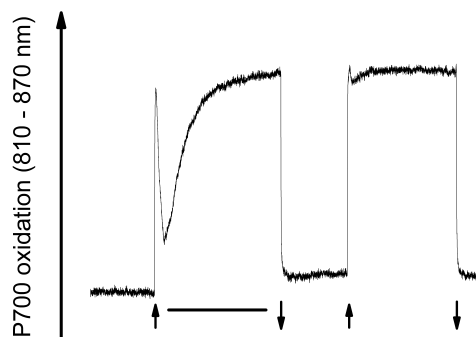
**Table 3.1** Averaged NADP<sup>+</sup>/NADPH molar ratios for WT, FS1 and MI6.

These measurements strengthen our working hypothesis assigning a specific role for each of the isoforms. In MI6 on one hand, FNR<sub>L</sub> ensures a redox state of the NADP pool similar to that of the WT. We have seen in Chapter 1 that under photoautotrophic conditions, FNR<sub>L</sub> is the major isoform in the WT. In FS1 on the other hand, FNR<sub>S</sub> is unable of building up a similar NADP<sup>+</sup>/NADPH ratio.

### 3.1.2 P700 oxidation kinetics using white light

Different actinic light qualities such as white light [Golding et al., 2004] and far-red (FR) light [Mi et al., 1992b] are applied to drive photosynthesis and to follow P700 oxidation and reduction kinetics. Also light quantities change from moderate continuous light to intense laser flashes.

Here, we present results obtained with continuous light illumination. First, we performed preliminary measurements on WT under white actinic light (white AL; Figure 3.2), which excites both PSI and PSII and drives linear ET. Figure 3.2 shows the kinetics of P700<sup>+</sup> formation and decay. After switching on the light, the P700 oxidation kinetics is characterized by a first fast rise which is followed by a dip phase and then a second slow rise results eventually in a steady-state. In Figure 3.2 the reduction kinetics of P700<sup>+</sup> is also shown. When the light is switched off, the oxidized P700 is reduced back to P700 rather quickly and the initial level of absorption is recovered. A second light-induced P700 oxidation is shown which exhibits a much less-pronounced dip phase.



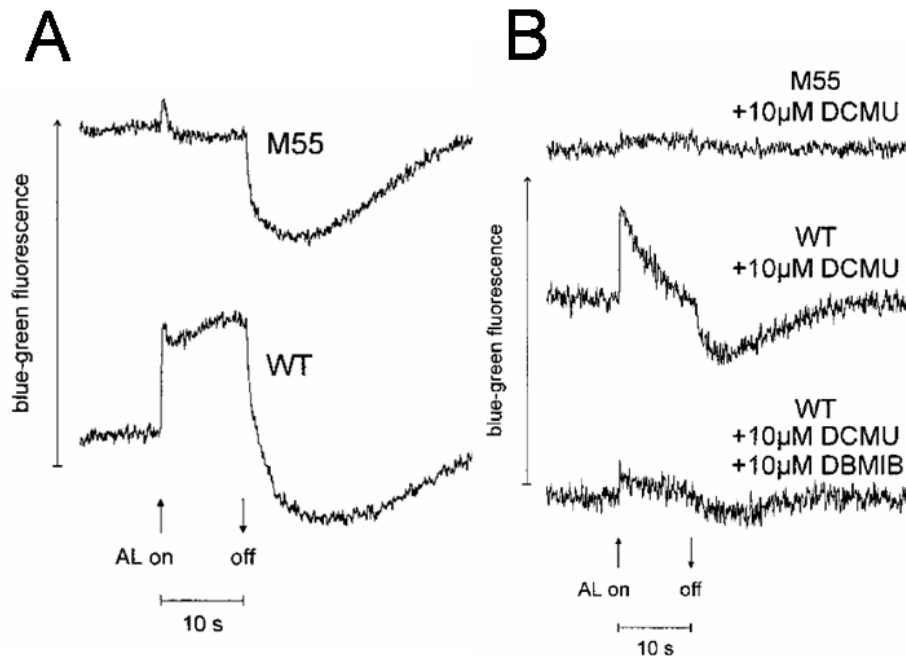
**Figure 3.2** P700 oxidation and P700<sup>+</sup> reduction kinetics of WT strain under high CO<sub>2</sub>. Characteristic curves for white light illumination are shown. Arrows under the trace indicate the light switch-on and switch-off. Horizontal bar represents 10 s.

Our observed complex kinetics of WT are in agreement with previously obtained traces of P700 oxidation [Trubitsin et al., 2005] and with blue-green fluorescence kinetics [Mi et al., 2000]. The latter authors identified the changes in blue-green fluorescence as changes in the redox state of the NADP pool. Dark-light-dark induction transients of blue-green fluorescence for *Synechocystis* WT and the *ndhB*-defective mutant M55 are shown in Figure 3.3A. This represents changes in the output of PSI ET and constitutes a complementary view to previous studies on the NADP pool redox state (see above).

WT exhibited the same two different fast and slow rising phases separated by the dip phase. The studies of [Mi et al., 2000] were carried out after repetitive dark-light-dark induction transients. This would explain why the amplitude of the dip resembles more the amplitude seen in the second P700 oxidation kinetics in Figure 3.2. The authors in [Mi et al., 2000] proposed the following explanations for the different phases upon onset of AL:

- First rapid rise: light driven accumulation of NADPH;
- Dip phase: oxidation of NADPH *via* Calvin cycle activity;

- Secondary rise phase: accumulation of NADPH as its oxidation in the Calvin cycle becomes limited, possibly going along with a limitation in ATP-supply;
- Stationary phase: matched rates of light-driven NADP<sup>+</sup> reduction and NADPH oxidation via the Calvin cycle.



**Figure 3.3** Dark-light-dark induction transients of blue-green fluorescence of *Synechocystis* WT and its *ndhB*-defective mutant M55. A: blue-green fluorescence of WT and M55. B: blue-green fluorescence of WT and M55 in the presence of inhibitors DCMU and DBMIB. Taken from [Mi et al., 2000].

P700 oxidation kinetics under white light observed in our study could also be explained in a similar way. The different phases would then correspond to P700 oxidation events as followed:

- First rapid rise: oxidation of P700 resulting in accumulation of NADPH;
- Dip phase: reduction of P700<sup>+</sup> results in decrease in light-driven NADP<sup>+</sup> reduction;
- Secondary rise: further oxidation of P700 resulting in accumulation of NADPH; Calvin cycle is limited by ATP;
- Stationary phase: matched rates of light-driven PSI oxidation and PSI reduction *via* luminal (Pc/cyt *c*<sub>6</sub>) and membrane (PQ) donors.

In Figure 3.3B dark-light-dark induction transients are shown in the presence of DCMU and DBMIB for WT and M55 mutant. These inhibitors of PSII and cyt *b*<sub>6</sub>*f*, respectively, are widely

used as control measurements. The authors proposed that, in the WT, the transient reduction of NADPH in the presence of DCMU showed high capacity of cyclic electron flow around PSI *via* the NDH-1 which ensured reduction of the intersystem electron chain even when electron donation by PSII was abolished [Mi et al., 1992a,b]. The M55 mutant did not present a transient reduction of NADPH due to deletion of all NDH-1. The transient NADPH build-up may also be explained by the presence of prereduced donors of PSI. This reduced donor pool may be less important in the M55 mutant.

Another approach for observing P700 oxidation kinetics consists in exciting preferentially PSI. This can be achieved either by adding the PSII inhibitor DCMU or by changing the light quality to FR (> 695 nm). These approaches are regularly performed for measuring P700 oxidation and reduction kinetics in oxygen-evolving organisms [Mi et al., 1992b, Breyton et al., 2006, Ivanov et al., 2007, Lintala et al., 2007, DalCorso et al., 2008, Lintala et al., 2009]. We have chosen the FR light continuous illumination to characterize the WT and the FNR mutants.

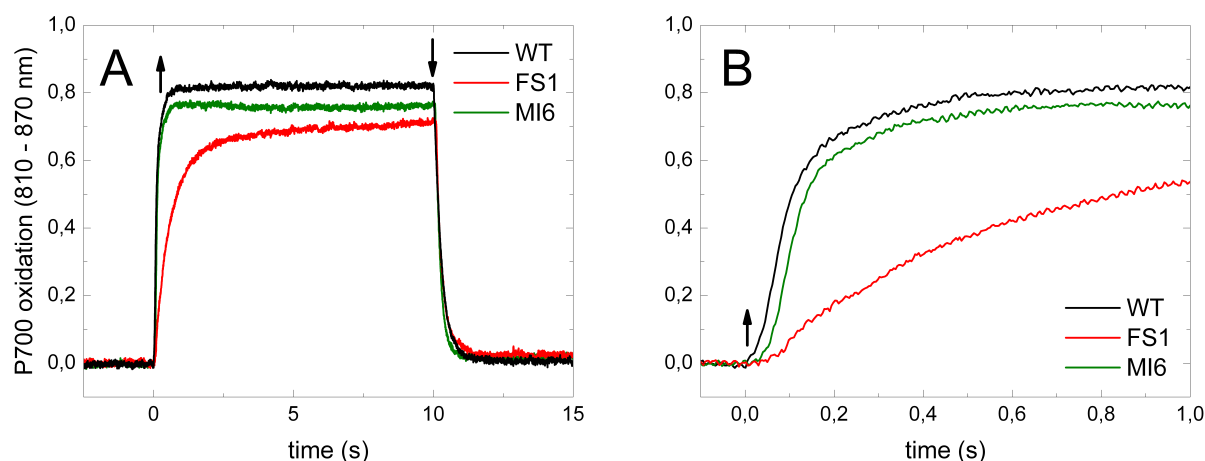
### 3.1.3 P700 oxidation kinetics using far-red light

We have seen a clear increase in the NADP<sup>+</sup>/NADPH ratio for the FS1 mutant. Here, we determined if the oxidized NADP pool in FS1 is due to an increase of an alternative electron transfer. We performed P700 oxidation and reduction kinetics on cells grown under standard photoautotrophic conditions. Under these conditions, the WT expresses FNR<sub>L</sub> as the major isoform. The WT behavior should then be close to MI6, which can only express the FNR<sub>L</sub> whereas FS1 may present differences in the kinetics as it contains only FNR<sub>S</sub>.

Characteristic curves for FNR-mutant- and WT strains under HC are shown in Figure 3.4. Cells were dark adapted for 5' and FR light was applied for 10 s. A very fast P700 oxidation rate with a half time of less than 0.1 s was observed for WT (Figure 3.4, black trace). In comparison with white-light illumination, no dip phase followed by a secondary slow rise were observed. This gives indications that the participation of PSII in ET is negligible under these conditions. This is explained by the use of FR light as actinic light source and the elevated PSI/PSII ratio in *Synechocystis* (PSI/PSII = 3) in comparison with *e.g.* plants. The oxidized P700 under steady-state conditions was around 80% in WT under HC, the WT exhibited the maximum amount of oxidized P700 under steady-state conditions compared to both FNR mutants. The WT under standard photoautotrophic growth is expected to perform linear ET as the major ET (see above). However, up to 20% of oxidized P700 that are missing for the WT under steady state may represent the contribution of cyclic and/or respiratory ET. We will in the following consider the WT as a reference for mainly linear ET compared to FNR mutants.

MI6, that contains only FNR<sub>L</sub>, exhibits a similar P700 oxidation rate with a half-time of

around 0.1 s (see Figure 3.4A and B). The oxidized P700 under steady-state conditions was slightly decreased compared to wild type, around 75%. As MI6 is close to WT, we can conclude that this mutant probably performs linear electron transfer as the major electron transfer. Note that both strains contain FNR<sub>L</sub> as the major or only FNR isoform under these conditions. Linear electron transfer is cartooned in Figure 3.5. We observed a quick rise in the signal corresponding to oxidized P700 for WT and MI6. This is represented with a positive charge on P700 in Figure 3.5. The electrons flow from P700 to NADP<sup>+</sup> *via* Fd and FNR<sub>L</sub>. We will consider this flow of electrons as the output of PSI ET. Electrons are further evacuated *via* NADPH to the major electron sink: the Calvin cycle. We attribute this behavior to linear ET (Figure 3.5) but pseudocyclic ET may be present. WT and MI6 may perform alternative ETs as respiratory or cyclic ET that account for the difference with 100% oxidized P700 in the presence of DBMIB.



**Figure 3.4** P700 oxidation and P700<sup>+</sup> reduction kinetics in high CO<sub>2</sub> of WT (black), FS1 (red) and MI6 (green) mutant strains. A: overall P700 oxidation and reduction kinetics. B: zoom on P700 oxidation kinetics. The three strains were shifted for their onset of illumination with WT being the first, MI6 the second and FS1 the last strain. The arrows indicate the FR light that was switched on and off. Switch-on and -off of the light could not be determined precisely (see Chapter 5).

FS1, that contains only FNR<sub>S</sub>, showed very different P700 oxidation kinetics (Figure 3.4A and B). First, a much smaller P700 oxidation rate was observed with a half time of around 0.6 s -6 times increased compared to WT. Second, a decrease in the maximal P700<sup>+</sup> signal on the same time scale was observed. After 10 s of FR light, no real steady state was reached in this mutant under HC (around 65% at a pseudo-steady state). P700 oxidation and reduction in FS1 are depicted in Figure 3.6. In addition to the output of electrons by linear ET, P700<sup>+</sup> may be rapidly reduced or the output of PSI may be perturbed, leading to slowed P700 oxidation kinetics.

The P700 oxidation kinetics of WT, MI6 and FS1 are in agreement with the NADP pool redox states found for the three strains. These measurements revealed a striking difference in the P700

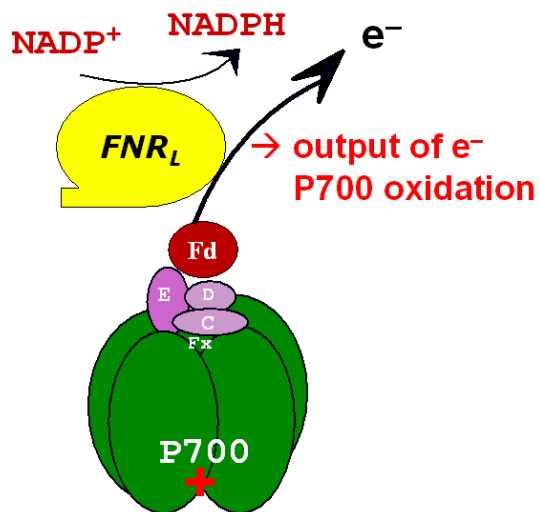


Figure 3.5 Representation of P700 oxidation by linear ET in WT and MI6.

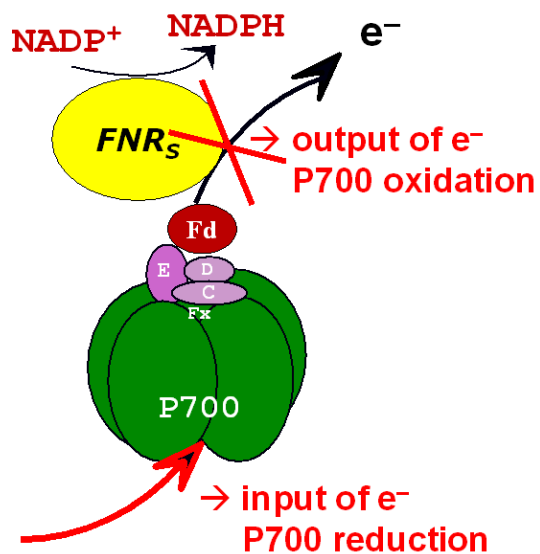


Figure 3.6 Representation of P700 oxidation and reduction in FS1.



oxidation kinetics of FS1 that has to be further studied by P700 oxidation measurements in the presence of inhibitors and complementary *in vivo* measurements as PQ pool redox state and immunoblots to determine the metabolic pathways involved.

### P700 oxidation kinetics in the presence of inhibitors

The difference in the phenotype of FS1 compared to WT and MI6 was further investigated during control measurements. This consisted in adding inhibitors of electron flow. Measurements with sequential addition of DCMU, MV and finally DBMIB are shown in Figure 3.7 for WT. DCMU, MV and DBMIB are known to inhibit PSII activity (PQ reduction), recombination reactions involving P700<sup>+</sup> and cyt *b<sub>6</sub>f* activity (PQ oxidation), respectively. In the presence of DCMU, the WT was characterized by a small rate increase of P700 oxidation and similar P700<sup>+</sup> reduction kinetics. We concluded that the contribution of PSII photochemistry was very small under HC (FR light, high PSI/PSII ratio in *Synechocystis*).

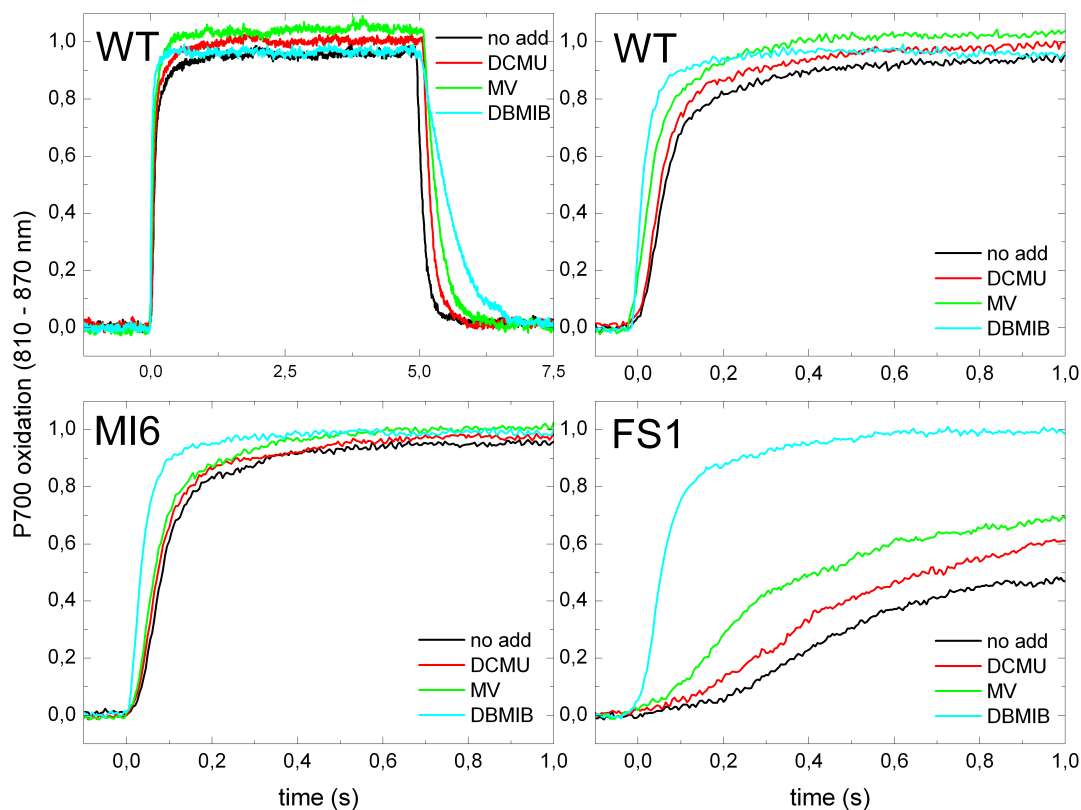
In the presence of DCMU and MV, the WT was characterized by a small rate increase in the P700 oxidation kinetics and a significant rate decrease in the P700<sup>+</sup> reduction kinetics (Figure 3.7). On one hand, we concluded that the contribution of recombination reactions involving P700<sup>+</sup> was rather small. On the other hand, the contribution of pseudocyclic and cyclic ET with this control measurement was not possible (see Chapter 5). We focused on the P700 oxidation kinetics as this step was especially affected in FS1.

In the presence of DCMU, MV and DBMIB, once the available reduced donors are exhausted, a very fast P700 oxidation is observed. In addition, all oxidizable P700 should be observable in the presence of DBMIB. Surprisingly, the WT was characterized by a somewhat lower steady-state of the P700<sup>+</sup> signal. We have no plausible explanation for this decrease in steady-state of P700<sup>+</sup>. There may be some interference of the three different inhibitors. However, the P700 oxidation kinetics was further accelerated and the P700<sup>+</sup> reduction kinetics was slowed down (Figure 3.7).

The P700 oxidation kinetics for MI6 are shown in Figure 3.7. Overall, the kinetics in the presence of DCMU, MV and DBMIB were similar for MI6 *vs.* WT. Only small increases in the rate of P700 oxidation were observed in the presence of DCMU and MV. This indicated a small contribution of PSII photochemistry and recombination reactions involving P700<sup>+</sup> in this mutant. In the presence of DBMIB, a further significant though small increase in the rate of P700 oxidation was observed. We have obtained evidence that WT and MI6 exhibit mainly linear ET as the contribution of recombination reactions involving P700<sup>+</sup> was rather small and the kinetics in the presence of MV and DBMIB did not show much difference. The difference between MV and DBMIB traces indicated that alternative ET such as cyclic and respiratory ET were poorly inhibited in the presence of MV (see Chapter 5).



The P700 oxidation kinetics for FS1 are shown in Figure 3.7. The sequential addition of inhibitors (DCMU/MV/DBMIB) resulted in a sequential increase in the rate of P700 oxidation. Thus a small contribution of PSII photochemistry is apparent in the FS1 P700 oxidation. Addition of MV resulted in a significant increase of P700 oxidation kinetics (Figure 3.7). This increase can be attributed to inhibition of recombination reactions and/or to partial inhibition of cyclic/respiratory ET. The addition of DBMIB resulted in a strong increase in the rate of P700 oxidation (Figure 3.7). The *cyt b<sub>6</sub>f* complex is involved in respiratory and cyclic ETs. The addition of DBMIB eliminates all these ETs and thus very fast P700 oxidation kinetics were obtained. The difference between MV and DBMIB indicated a major contribution of cyclic and/or respiratory ET in FS1.



**Figure 3.7** P700 oxidation and P700<sup>+</sup> reduction in the presence of inhibitors. WT left: overall P700 oxidation and reduction kinetics shown. WT right: zoom on P700 oxidation kinetics. MI6 and FS1: zoom on P700 oxidation kinetics. Traces in black, red, green and cyan correspond to no addition and sequential additions in the order of DCMU, MV and DBMIB, respectively.

### Induction of cyclic ET under low CO<sub>2</sub>

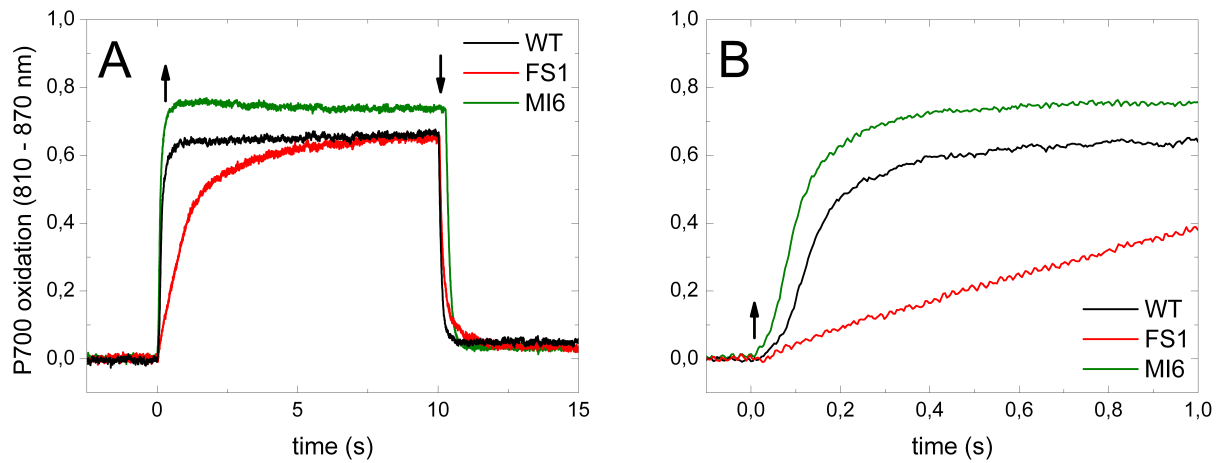
We have obtained strong indications that FS1 may present increased respiratory or cyclic electron flows. To further test this hypothesis, we repeated some of the measurements under LC. Several reports presented evidence that LC induces cyclic and pseudocyclic ET [Harbinson and Foyer, 1991, Miyake et al., 2005, Hackenberg et al., 2009]. We may be able to confirm the involvement of FNR<sub>S</sub> in the reoxidation of NADPH under LC.

LC decreases growth dramatically. We first checked the impact of LC on the pigments composition and confirmed the expected phenotype of decrease in PBP content in the three strains (Chapter 5). We will present detailed comparison of WT and mutant strains under LC concerning the P700 oxidation kinetics. If cyclic/respiratory ET was observed under HC for the FS1 mutant, we may be able to observe the same effect on WT under conditions of induced cyclic and pseudocyclic ETs (LC).

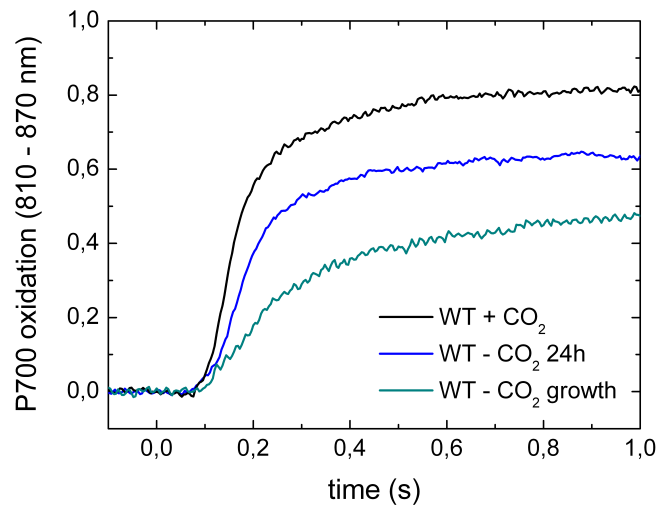
We repeated the P700 oxidation and reduction kinetics under LC. First, we will present the overall P700 oxidation and reduction kinetics and then focus on the P700 oxidation kinetics for WT, FS1 and MI6. In Figure 3.8, characteristic curves for the WT and mutant strains are shown after growth under LC for 24 hours. WT exhibited a rate decrease in P700 oxidation kinetics. Furthermore, the steady-state of oxidized P700 was also significantly smaller. MI6 on the contrary did not exhibit significant changes. Under LC, MI6 exhibited the largest steady-state amount of oxidized P700. FS1 under LC exhibited enhancement of the phenotype present in HC, the half time for the P700 oxidation rate almost doubled and kinetics had clearly two phases. The steady-state oxidized P700 was slightly smaller and similar to that of the WT under the same conditions. The oxidation kinetics for WT, MI6 and FS1 are now compared separately under HC and LC.

WT was adapted to LC for 24 hours or for entire growth (8-9 days). Figure 3.9 shows WT kinetics under HC and LC on a 1 s time scale. As a function of duration in CO<sub>2</sub> limitation, the rate of P700 oxidation decreased. The half time for P700 oxidation increased from 0.09 s to 0.12 s and finally 0.29 s for HC, LC for 24h and entire growth, respectively (Table 3.2). The steady state of oxidizable P700 decreased in the same way. The steady state decreased from initially 80% to 65% and finally to 45% (Table 3.2).

Under LC the WT P700 oxidation resembles that of FS1. Linear ET was hindered due to CO<sub>2</sub> limitation. Alternative electron sinks may operate to evacuate the electrons accumulated on the PSI acceptor side. If cyclic/respiratory ET was observed under HC in FS1, the WT may be able to induce the same alternative ET under LC. We hypothesize that the induction of that behavior in the WT correlates with FNR<sub>S</sub> accumulation in the WT. This would be in agreement with an increased FNR<sub>S</sub> expression under high light [Thomas et al., 2006].



**Figure 3.8** P700 oxidation and reduction kinetics in low  $\text{CO}_2$  of WT (black), FS1 (red) and MI6 (green) mutant strains.

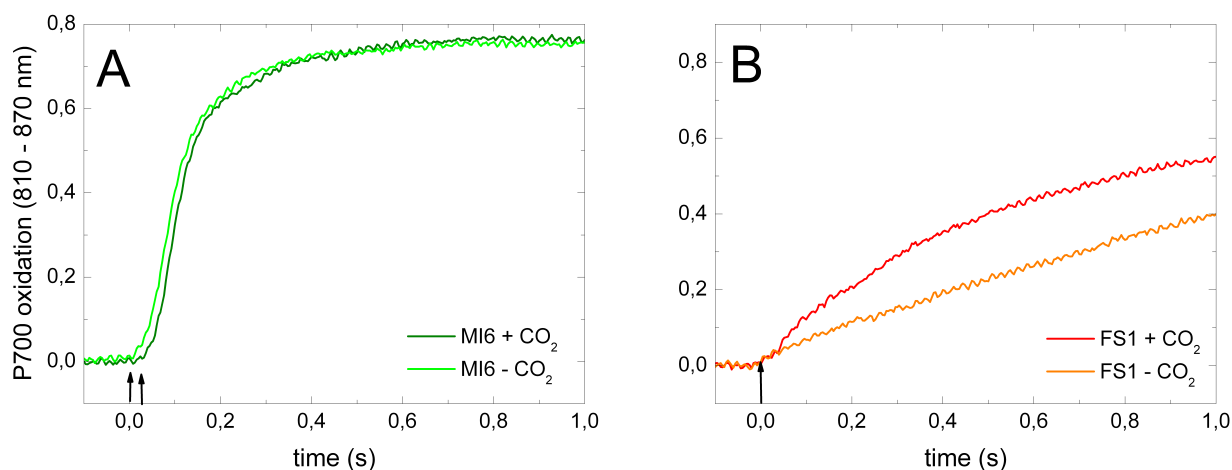


**Figure 3.9** P700 oxidation kinetics under high and low  $\text{CO}_2$  for WT. WT kinetics in high  $\text{CO}_2$  (black), low  $\text{CO}_2$  for 24h (blue) and in low  $\text{CO}_2$  for 8 days (dark cyan) are shown.

For the two mutants, we compared the P700 oxidation kinetics on a shorter time scale (1 s) after 24 hours in LC to those in HC. Contrary to the WT, MI6 did not exhibit changes in the P700 oxidation rate under LC (Figure 3.10A). The half time for the P700 oxidation rate did not change significantly and was 0.12 s and 0.104 s for HC and LC, respectively (Table 3.2). The steady state of oxidized P700 was as well unmodified (75% under both conditions; Table 3.2). Overall, the P700 oxidation kinetics of MI6 did not change under LC for 24 hours.

It is interesting to note that the mutant unable to produce FNR<sub>S</sub> (MI6) was unable to induce the LC behavior as the WT. MI6 was still characterized by a major linear ET under LC. An alternative electron sink may be pseudocyclic ET in the MI6 mutant. Under HC, only a small contribution of pseudocyclic ET must occur in MI6 and WT. Flv3, that is responsible for the Mehler-reaction in *Synechocystis*, is induced under LC [Eisenhut et al., 2007]. The pseudocyclic ET involving the Mehler-reaction can thus result in fast P700 oxidation kinetics under CO<sub>2</sub> limitation eventually involving FNR<sub>L</sub>.

For FS1, oxidation kinetics are shown on a 1 s time scale in Figure 3.10B. Similar phenotypes were observed for FS1 under HC and LC but the phenotype was more pronounced in LC. The half time for the P700 oxidation rate increased about two-fold from 0.56 s to 1.1 s after 24 hours under LC (Table 3.2). The oxidized P700 after 10 s of FR light under quasi-steady state was decreased but to a lower extent than in the WT. In HC, the oxidized P700 under quasi-steady state was about 70%, and decreased to about 65% in LC for 24 hours (Table 3.2).



**Figure 3.10** P700 oxidation kinetics under high and low CO<sub>2</sub> of MI6 (A) and FS1 (B) mutant strains. A: MI6 in high CO<sub>2</sub> (olive) and in low CO<sub>2</sub> for 24h (green). B: FS1 in high CO<sub>2</sub> (red) and in low CO<sub>2</sub> for 24h (orange).

We identified a clear phenotype for FS1 under HC which is accentuated under LC. FS1 is characterized by a decreased rate of P700 oxidation and a decrease of the quasi-steady state of oxidized P700. Under LC, the WT, contrary to MI6, exhibited a behavior similar to that of FS1.

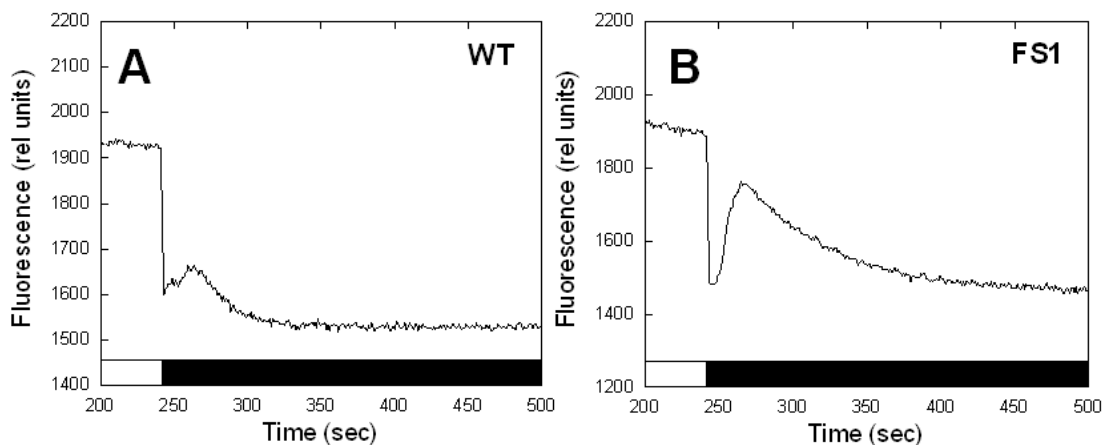
oxidation	WT		MI6		FS1	
	$t_{1/2}$ (s)	P700 <sup>+</sup> (%)	$t_{1/2}$ (s)	P700 <sup>+</sup> (%)	$t_{1/2}$ (s)	P700 <sup>+</sup> (%)
+CO <sub>2</sub>	0.09 ± 0.02	80	0.12 ± 0.01	75	0.56 ± 0.08	70
-CO <sub>2</sub> 24h	0.12 ± 0.02	65	0.104 ± 0.001	75	1.1 ± 0.23	65
-CO <sub>2</sub> growth	0.29 ± 0.14	45	-	-	-	-

**Table 3.2** P700 oxidation kinetics and steady state of P700<sup>+</sup> under high and low CO<sub>2</sub> for WT, MI6 and FS1.

These results could correlate with the accumulation of the two FNR isoforms in the three different strains. MI6 does not contain FNR<sub>S</sub>. WT is expected to induce significant amounts of FNR<sub>S</sub> under stress conditions as was observed under N-starvation and high-light conditions. Further immunoblotting experiments are necessary to confirm this hypothesis. FS1 does not contain FNR<sub>L</sub> and FNR<sub>S</sub> will be either mobile or attached to other complexes (cyt *b<sub>6</sub>f* or NDH-1). Attachment to these membrane complexes may promote respiratory/cyclic ET *via* NADPH oxidation with NADPH being accumulated by linear ET or catabolism.

### 3.1.4 PQ reduction in the dark

We compared ET from the stromal donors to PQ pool in the WT and FNR mutants. The electron flow was monitored by the transient increase in chlorophyll fluorescence (apparent  $F_0$ ) which occurs in darkness after a period of AL illumination [Schreiber *et al.*, 1986]. This transient  $F_0$  rise is attributed to reduction of the PQ pool by the stromal reductants accumulated during illumination period. The WT and MI6 showed similar transient  $F_0$  increase with a maximum between 20-30 s after AL was switched off but FS1 showed significantly larger transient  $F_0$  increases (Figure 3.11).



**Figure 3.11** Transient PQ reduction for WT (A) and FS1 (B). Kindly provided by Dr. Kirilovsky.

This transient  $F_0$  increase was previously observed in cyanobacteria and plants [Shikanai et al., 1998, Deng et al., 2003, Ma and Mi, 2005, Lintala et al., 2009]. It was also shown that this transient increase was significantly reduced in M55 and in the *ndhB*-deficient *Arabidopsis* mutant [Shikanai et al., 1998]. The authors concluded that the NDH-mediated cyclic ET was missing but that Fd-mediated cyclic ET may contribute to remaining  $F_0$  increase. Thus, the increased PQ pool reduction in the dark in FS1 may well be due to an increased NDH-mediated cyclic ET.

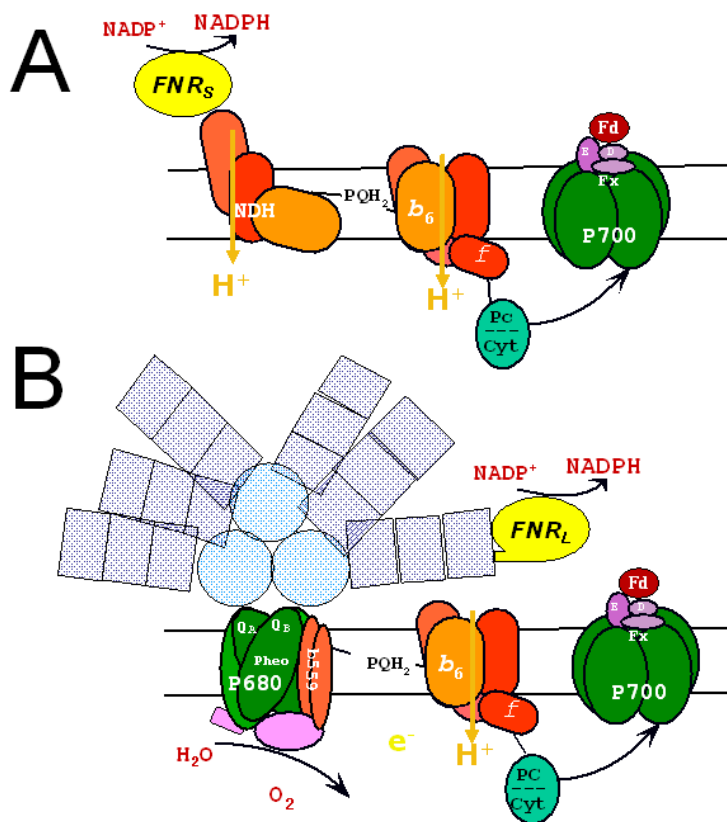
### 3.2 Conclusion

Different ET pathways are operating in WT, MI6 and FS1 and we will present a model for the association of  $FNR_L$  and  $FNR_S$  in view of our *in vivo* results. We obtained evidence that cyclic or respiratory ET is induced when  $FNR_S$  accumulates. We propose that  $FNR_S$  transfers electrons to NDH-1 acting as the dehydrogenase module (Figure 3.12A). Binding of FNR to NDH-1 was proposed previously [Vara and Gómez-Lojero, 1986, Guedeny et al., 1996, Matsuo et al., 1998] and the NDH-1 dependent pathway is expected to be the major cyclic ET in cyanobacteria [Mi et al., 1992a,b]. We propose that this cyclic/respiratory ET is NDH-1 dependent and involves  $FNR_S$ .

The increase of such an alternative electron flow will result in a reduced PQ pool, a slow-down of the rate of P700 oxidation and an oxidized NADP pool. The NADP pool redox state gave indications that the mutant lacking  $FNR_L$  (FS1) presents an increased reoxidation of NADPH. Slower P700 oxidation excluded a large contribution of pseudocyclic ET and our control measurements in the presence of inhibitors showed that recombination reactions involving  $P700^+$  are also not a major contributor in the FS1 P700 oxidation phenotype. Studies on PQ pool reduction in the dark resulted in an increased PQ pool reduction in FS1 compared to WT and MI6. These observations strongly indicate an increase of NADPH oxidation and subsequent injection of electrons to the PQ pool *via* NDH-1.

$FNR_L$  is known to be attached to the PBS and is probably tuned to perform  $NADP^+$  reduction involved in linear ET (Figure 3.12B). WT under HC and MI6 that contain  $FNR_L$  as the major and the only isoform, respectively, performed essentially linear ET and maybe some pseudocyclic ET.

We analyzed the FNR mutants and the WT for their phenotypes under LC conditions that are known to induce cyclic ET [Harbinson and Foyer, 1991, Miyake et al., 2005]. FS1 showed under LC an enhancement of the phenotype present under HC thus indicating an induction of the respiratory or cyclic ET. The WT under LC behaved like FS1 under HC and LC conditions and we propose that the WT P700 oxidation correlates with  $FNR_S$  accumulation under LC which would be in agreement with previous results under other stress conditions (high light and N-starvation) [Thomas et al., 2006].



**Figure 3.12** Representation of the possible association of FNR<sub>S</sub> with NDH-1 (A) and the known association of FNR<sub>L</sub> with PBS (B).

LC conditions also lead to Flv3 accumulation [Eisenhut et al., 2007] which promotes pseudo-cyclic ET and we found evidence that, under LC conditions, MI6 presents pseudocyclic ET. The absence of the LC behavior in MI6 further confirmed the involvement of FNR<sub>S</sub> in this alternative ET present in FS1 and WT under LC.

To summarize, we obtained promising results from the *in vivo* studies indicating respiratory or cyclic ET that is dependent on NDH-1 and involves FNR<sub>S</sub>. Further studies are necessary to establish the mechanism of the backflow of electrons into the PQ pool and the exact partners involved in addition to FNR<sub>S</sub>. First, we will perform immunoblotting on cells grown under LC in order to confirm the accumulation of FNR<sub>S</sub> and of NDH-1 complexes in the WT. Second, fluorescence induction measurements can confirm informations previously obtained about the PQ pool redox state. These measurements are needed to compare the WT and the FNR mutant strains. This will result in a more complete *in vivo* view and should help us understand the mechanism of respiratory/cyclic ET.





## Chapter 4

# Conclusions and Perspectives

### 4.1 Conclusions

We are interested in determining the respective roles of the two FNR isoforms in *Synechocystis*. FNR<sub>L</sub> is attached to the PBS [Schluchter and Bryant, 1992] due to the linker-like domain that FNR<sub>S</sub> is lacking [Thomas et al., 2006]. We wanted to determine the function of FNR<sub>L</sub> attachment to the PBS. Previous studies strongly indicated that FNR<sub>L</sub> sustains photoautotrophic growth (NADP<sup>+</sup> reduction) and FNR<sub>S</sub> is expressed under stress conditions and during heterotrophic growth (NADPH oxidation; working hypothesis) [Thomas et al., 2006].

We performed *in vitro* and *in vivo* studies to determine differences due to intrinsic activities and differences due to *e.g.* enzyme localization. As FNR catalysis involves intermolecular ETs involving various steps that are concentration-dependent, it is not straightforward to compare the results obtained during *in vitro* and *in vivo* studies.

*In vitro* studies resulted in three major differences between FNR<sub>S</sub> and the purified FNR<sub>L</sub>-phycobilisome subcomplex (FNR<sub>L</sub>-PC) beside overall similar enzymologic properties. The strongest effect was observed for the affinity of Fd<sub>ox</sub> to FNR<sub>L</sub>-PC *vs.* FNR<sub>S</sub> during NADPH oxidation, with a decreased affinity for FNR<sub>L</sub>-PC. This effect was attributed to steric hindrance in FNR<sub>L</sub>-PC. As the dissociation of Fd<sub>ox</sub> can be rate-limiting for NADP<sup>+</sup> reduction [Batie and Kamin, 1984a, Carrillo and Ceccarelli, 2003], this difference may result in an increased NADP<sup>+</sup> reduction for FNR<sub>L</sub>-PC. Fd affinities are in agreement with the growth characteristics of the FNR mutants.

Steric hindrance was also observed during single FNR reduction involved in NADP<sup>+</sup> reduction for FNR<sub>L</sub>-PC which was in contradiction with our working hypothesis. The third major difference was a larger NADPH affinity for FNR<sub>L</sub>-PC. An increased NADPH affinity may result

in an enhanced NADP<sup>+</sup> reduction by FNR<sub>L</sub> whereas FNR<sub>S</sub> may still perform NADPH oxidation under heterotrophic or stress conditions, when NADPH concentration is higher.

The differences we obtained for cyanobacterial FNR isoforms do not parallel the differences obtained for plant root and leaf isoforms. In addition to different gene products that are present in different tissues, several plant FNR isoforms were found that are present in the same tissues [Okutani et al., 2005, Gummadova et al., 2007, Lintala et al., 2009]. These plant leaf isoforms may parallel the situation in cyanobacteria as they are present in the same tissues and may catalyze the different FNR reactions.

In parallel to the *in vitro* studies, we performed *in vivo* studies of the WT and FNR mutants expressing only one isoform. It is clear that FNR localization is different for FNR<sub>S</sub> and FNR<sub>L</sub>. FNR<sub>L</sub> is known to be attached to the PBS but FNR<sub>S</sub> may be soluble, membrane-attached [Palatnik et al., 1997], attached to cyt *b<sub>6</sub>f* [Zhang et al., 2001] or NDH-1 [Vara and Gómez-Lojero, 1986, Guedeney et al., 1996, Matsuo et al., 1998]. In addition to that, substrate availability (Fd<sub>ox</sub>/Fd<sub>red</sub> and NADP<sup>+</sup>/NADPH) is probably important for the catalyzed reaction. *In vivo* studies are characterized by the presence of various metabolic pathways that interact with each other (cyt *b<sub>6</sub>f*, PQ pool, NADP pool etc.).

Under photoautotrophic growth, the NADP pool was found to be more oxidized in the mutant lacking FNR<sub>L</sub> (FS1) than in the mutant lacking FNR<sub>S</sub> (MI6) and the WT. We envisaged increased NADPH oxidation by alternative electron flows in FS1 (cyclic, pseudocyclic or respiratory ET), we excluded pseudocyclic ET and recombination reactions involving P700<sup>+</sup> and found further evidence for an increase of respiratory/cyclic ET by measuring P700 oxidation and PQ pool reduction in the dark.

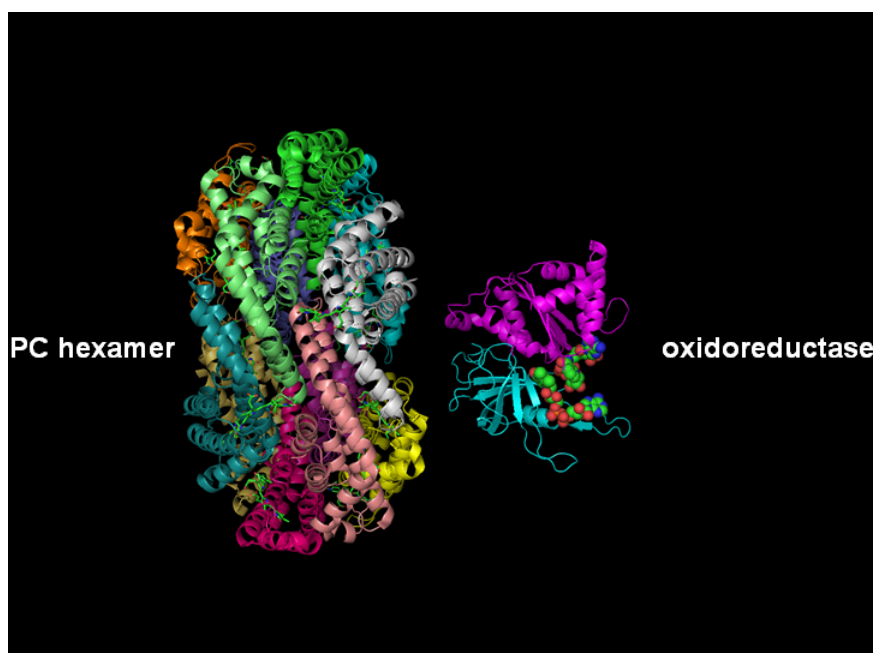
If the FS1 phenotype is FNR<sub>S</sub>-dependent, the WT should under conditions of FNR<sub>S</sub> induction present the same phenotype. WT is known to induce FNR<sub>S</sub> under stress conditions as N-starvation and high light [Thomas et al., 2006]. High light and LC are conditions known to induce pseudocyclic and cyclic ET [Harbinson and Foyer, 1991, Miyake et al., 2005, Hackenberg et al., 2009]. Thus we analyzed the P700 oxidation kinetics for WT, MI6 and FS1 under LC.

WT presented under LC a similar phenotype as FS1 under HC and LC. FS1 presents thus an LC-phenotype. This indicates that the FS1 phenotype is due to the presence of FNR<sub>S</sub>. We proposed the involvement of FNR<sub>S</sub> in the NDH-1-dependent NADPH oxidation that was previously accumulated from reductive (photosynthesis) or oxidative (carbohydrate) metabolism. MI6 mutant under LC may present an increase of pseudocyclic ET.

We clarified the issue of intrinsic activities *in vitro* of the two isoforms and obtained promising results from the *in vivo* studies indicating respiratory or cyclic ET that is dependent on NDH-1 and involves FNR<sub>S</sub>. Further studies are necessary in order to determine the mechanism of the backflow of electrons into the PQ pool and the exact partners involved.

## 4.2 Perspectives

Both *in vitro* and *in vivo* studies have perspectives. First of all, product inhibition of NADPH oxidation and the corresponding  $K_I$  may be measured for FNR<sub>S</sub>. The affinities for NADP<sup>+</sup> and NADPH are thought to be similar, but inhibition can provide more detailed informations on this issue. Another idea for the *in vitro* studies is based on reconstitution studies. A scaled-up purification of FNR<sub>L</sub> could be obtained using a His-tagged FNR<sub>L</sub> overexpressed in *E.coli* for further biochemical and biophysical studies, *e.g.* crystallography. Crystallography may give answers to the issue of conformation changes upon complex formation. This may validate differences observed between the two isoforms. In Figure 4.1, the crystal structures for the separately obtained subunit of phycobilisome - PC hexamer - and the oxidoreductase - FNR<sub>S</sub> - are shown.



**Figure 4.1** Crystal structures of FNR<sub>S</sub> and the PC hexamer are shown side by side for size comparison and for giving a possible suggestion for the orientation of the FNR-PC complex. This Figure was created by P. Sétif using available structural data for PC hexamer and *Anabaena* FNR in the pdb.

Further *in vivo* studies are needed to determine the respective roles of the FNR isoforms present in facultative heterotrophic cyanobacteria. To determine the mechanism of the NADPH-dependent cyclic ET and the exact partners involved, several complementary studies might be useful. First, we need to confirm the accumulation of FNR<sub>S</sub> in the WT under LC *via* immunoblotting. Second, we must confirm the informations previously obtained about the PQ pool redox state in the WT and mutants under HC and LC *via* chlorophyll fluorescence induction measurements. Third, the NADP pool redox state under low CO<sub>2</sub> conditions is expected to exhibit a

more oxidized NADP pool in the WT and FS1 that can be determined using the commercial kit. In addition, a module is now commercially available for the PAM spectrometer for real-time measurements of NADPH fluorescence [Schreiber and Klughammer, 2009]. This would give insights in the NADP<sup>+</sup> reduction kinetics *in vivo*.

Finally, control measurements need to be carried out under LC *via* P700 absorption. In addition, a saturating pulse method was developed to distinguish donor and acceptor side limitation in PSI [Klughammer and Schreiber, 2008]. Thus, we may obtain further informations to explain the phenotype of FS1.

In addition to the presented *in vitro* and *in vivo* perspectives, other techniques may be carried out to study the role of the FNR isoforms. Approaches such as transcriptomic studies with DNA microarrays may be used to determine differences between the mutants and WT. A genetic approach is currently underway to determine the role of the 5' region of the mRNA of the gene *petH* for the accumulation of FNR<sub>S</sub>.

These studies would result in a more complete *in vivo* view and clarify the important issue of the NADPH-dependent cyclic ET into the PQ pool that seems to be enhanced in FS1.

# Chapter 5

## Materials and Methods

### 5.1 Bacterial growth conditions

The cyanobacterium *Synechocystis* sp. PCC6803 was grown photoautotrophically in an orbital incubator at 34°C in a CO<sub>2</sub>-enriched atmosphere (5 L min<sup>-1</sup>) and under continuous light (60 μE m<sup>-2</sup>s<sup>-1</sup>). Allen's medium [Allen, 1968] was modified as follows: 30 μM ferric citrate, 3 μM disodium EDTA, 30 mM sodium nitrate, 250 μM potassium phosphate, 250 μM sodium carbonate, 10 mM sodium bicarbonate and microelements as in Allen's medium [Ughy and Ajlani, 2004]. All chemicals were purchased from Sigma-Aldrich.

For NADP<sup>+</sup>/NADPH quantification, 50 mL were harvested during midexponential phase at OD<sub>580nm</sub>=1.3. The growth conditions were photoautotrophic in the presence of high CO<sub>2</sub> (HC) or low CO<sub>2</sub> (LC) (HC: bicarbonate 10 mM in the medium and 5 L min<sup>-1</sup> in the incubator) which resulted in doubling times of 8 or around 24 hours, respectively.

Complete growth under HC, LC for 24 hours and entire growth under LC was carried out. For 24 hours under LC, cells were grown under HC until OD<sub>580nm</sub>(1 cm)=1.7. The cells were harvested and transferred in LC conditions about 24 hours before P700 oxidation measurements. After 8 to 9 days of exponential growth, the 50 mL cell cultures reached the OD needed to perform P700 oxidation measurements. For all three conditions, 25-50 mL were harvested during mid-exponential phase. They were transferred to fresh medium prior to *in vivo* P700 oxidation measurements. The chlorophyll concentration was adjusted to 10 μg chlorophyll \* mL<sup>-1</sup>.

## 5.2 Biochemical techniques

### 5.2.1 Chlorophyll quantification

No difference in the chlorophyll *a* content per cell was found between the mutants and the WT strain. This is why the calibration to a similar chlorophyll content permits a correct comparison between mutant and WT strains.

10 to 50  $\mu\text{L}$  culture was harvested in 1.5 mL eppendorff tubes and resuspended in 1 mL 100% methanol. Once in methanol, excess light should be avoided in order to avoid chlorophyll to pheophytin conversion. Vortexing for a few seconds extracted 100% of the chlorophylls. One minute of centrifugation at 21,400 g was performed to remove insoluble materials. The supernatant was transferred into cuvettes and the absorbance was measured at 666 nm. The absorbance was divided by 76 (extinction coefficient chlorophyll in 100% methanol in  $\text{mL mg}^{-1} \text{cm}^{-1}$ ) to obtain the chlorophyll concentration ( $\text{mg/mL}$ ).

### 5.2.2 Purification of photosystem I, ferredoxin and short FNR isoform

Photosystem I (PSI) was purified according to [Rogner et al., 1990]. Thylakoid membranes were obtained from French press broken cells after extensive washing with ice-cold 20 mM Tricine, 1 mM EDTA, pH 7.8. PSI was obtained after solubilization with 1% (w/v)  $\beta$ -DM and purified on a sucrose density gradient. The upper green band consisting of highly-enriched monomeric PSI particles was dialyzed against 20 mM Tricine/NaOH, pH 7.8, and 0.03%  $\beta$ -DM and concentrated by ultracentrifugation. The last step of the purification procedure was anion-exchange chromatography on a Mono Q column. One modification to [Rogner et al., 1990] was the substitution of ammonium sulfate for magnesium sulfate as eluting salt.

*Synechocystis* recombinant ferredoxin (Fd) was overexpressed in *Escherichia coli* according to [Barth et al., 2000]. Ferredoxin was supplied thanks to Dr. B. Lagoutte and Ms. V. Mary. A Fd-rich soluble fraction from *Thermosynechococcus elongatus* was a gift from Dr. A. Boussac. Fd from this soluble fraction was purified to homogeneity using the same procedure as for *Synechocystis* Fd.

Recombinant Ferredoxin:NADP oxidoreductase (FNR) was overexpressed in *Escherichia coli* according to [Cassan et al., 2005]. A construct with deletion was designed in a pQE 60 expression vector for overproducing a 34-kDa FNR (corresponding to FNR<sub>S</sub>). Overexpressed FNR<sub>S</sub> was precipitated between 50 and 70% ammonium sulfate saturation. The recovered pellet was solubilized in 20 mM Tricine, pH 7.8, first roughly purified on DE52 (Whatman), and the FNR fraction was further purified by anion exchange chromatography on a Hitrap Q-Sepharose (Amersham Biosciences). The last purification step was performed by hydrophobic chromatography on a

phenyl-Sepharose matrix (HiLoad phenyl-Sepharose 16/10 from Amersham Biosciences). The reverse salt gradient was from 1.6 to 0 M ammonium sulfate in 80 mM Tricine, pH 7.8. FNR fractions were extensively dialyzed against 10 mM Hepes buffer, pH 7.0.

### 5.2.3 Purification of FNR<sub>L</sub>-PC

#### Phycobilisome isolation

Phycobilisome (PBS) complexes were isolated [Elmorjani et al., 1986, Ajlani et al., 1995] from CBH under conditions that are known to preserve inter-subunit interactions [Gantt and Lipschultz, 1972], *i.e.* high KP (potassium phosphate pH 8) concentrations. All steps were carried out at room temperature except breaking of cells. An antiprotease cocktail (Complete, Roche) was used during the isolation of FNR<sub>L</sub>-PC. The procedure was as follows.

18 L cultures of CBH were harvested at late exponential phase and washed twice in 0.5 M KP buffer and resulted in more than 20 mg cells and about 5-6 g of cells were used as starting material for each purification. The cells were resuspended in 15 mL 0.8 M KP, then vortexed 5-6 times for 40 s in an ice-cooled bead beater (Biospec) with half the volume of glass beads (0.1 mm diameter). Triton X-100 was added to the broken cells to a final concentration of 2% (v/v). After incubation for 30 min in the dark, unbroken cells and debris were removed by centrifugation at 25,000 g for 30 min at 18°C. If necessary, a second cycle of Triton X-100 treatment was performed to reduce chlorophyll contamination. 4-8 mL of supernatant were loaded onto sucrose step-gradients prepared as follows in 35 mL ultracentrifuge tubes: 6 mL of 1.0 M, 9 mL of 0.75 M, 9 mL of 0.5 M, and 3 mL of 0.25 M sucrose in 0.9 M KP buffer. The gradients were spun for 12-16 h in a Beckman SW-27 rotor at 26,000 rpm at 20°C. The PBS complex was collected in the 0.75 M zone.

For reconstitution assays the purified PBS complex was mixed with a molar excess of purified FNR<sub>L</sub> (c(PBS)=0.08 μM; 0.95 μM FNR<sub>S</sub>; 0, 0.16, 0.32, 0.64 and 0.95 μM FNR<sub>L</sub>) and incubated for 1-2 hours. After this, a second run of sucrose step-gradients was performed overnight and the fractions were analyzed for their contents in PBS complex and FNR enzyme *via* SDS-PAGE. Densitometric analysis of SDS-PAGE were calibrated to L<sub>RC</sub>, assuming that each PBS contains 6 L<sub>RC</sub>.

An additional step to resuspend the PBS in a different buffer and to concentrate the sample was performed. Harvested PBSs from the 0.75 M zone of sucrose were diluted four times with 0.9 M KP buffer and ultracentrifuged at 44,000 rpm for 6-12 h at 18°C. The PBS complex, that sedimented at the bottom of the tubes, was resuspended in 150 mM KP.



### IMAC purification of FNR<sub>L</sub>-PC

Thanks to previous work a His-tag was inserted between the enzymatic domain and the linker-like domain of FNR<sub>L</sub> (between Gly 98 and Ser 99). Thus, the FNR<sub>L</sub>-PC complex could be purified simply following an IMAC (Immobilized Metal Affinity Chromatography) on a Ni-resin column in low phosphate conditions in order to separate PC from the PBS complex. All the following steps were performed at 4°C on ice.

The PBS complex was resuspended in 150 mM KP buffer in order to dissociate the complex in its subunits: allophycocyanin from the core and phycocyanin from the rods. This dissociation was performed overnight at 4°C in the presence of a protease inhibitor without EDTA (Roche). After preparation of the Ni-resin (ProBond, Invitrogen, France) by washing with milliQ water and 250 mM KP, the Ni-resin was added to the sample. After 1h incubation (for binding), 2 steps of washing in batch with 250 mM KP were performed. Then, the mixture of sample and Ni-resin was transferred to a column and the resin was allowed to settle. Up to 20 times the bed volume of the resin was used to wash. The elution was performed with 150 mM KP buffer containing 150 mM imidazole.

The eluted fractions were concentrated using Vivaspin concentrators (100 kDa cutoff). A first estimation of the concentration was performed by UV-Vis spectroscopy. An absorption coefficient  $\epsilon_{620nm}$  of 2,370,000 M<sup>-1</sup>cm<sup>-1</sup> was used for the phycocyanin hexamer [Glazer, 1989]. SDS-PAGE was performed according to [Ughy and Ajlani, 2004] to control the purification. 12% of acrylamide was used in a Tris/Tricine gel. The following fractions were loaded at 0.3 OD<sub>620nm</sub> · mL except the elution fraction which was loaded at about 0.03 OD<sub>620nm</sub> · mL: entire PBS complex (CBH), not-bound (NB), wash 1 (W1), wash 2 (W2), elution (E). The samples were concentrated by TCA precipitation (10% w/v) prior to loading. Proteins were visualized using Coomassie Blue stain (see Figure 2.1B in Chapter 2).

### Gel filtration of FNR<sub>L</sub>-PC

A final step consisted in checking the size of the PC complex with FNR<sub>L</sub> bound to it and eliminating smaller complexes on a Superdex 200 preparative grade (26/85, bed volume 165 mL, void volume 64 mL) gel filtration column (GE Healthcare) with optimal separation of molecular mass between 10 and 600 kDa.

The column was washed with 3 bed volumes of ethanol and distilled water at a flow rate of 0.2 and 0.1 mL/min respectively. After this, the column was equilibrated with about 5 bed volumes of 250 mM KP buffer at a flow rate of 0.5 mL/min. The sample was injected at a maximal volume of 300 µL and was run at a flow rate of 0.5 mL/min. The peak was eluted with the same buffer after 2 hours.

The molecular mass of the purified FNR<sub>L</sub>-PC complex was determined using calibration of the column by Vitamin B<sub>12</sub> (1.35 kDa), myoglobin (17.0 kDa), ovalbumin (44.0 kDa),  $\gamma$  globulin (158 kDa) and thyroglobulin (670 kDa) as standards. Gel filtration was followed at three wavelengths to check total protein, FAD and PC absorptions at 280 nm, 460 nm and 620 nm, respectively and was further analyzed by SDS-PAGE and UV-Vis. The major fraction was pooled, concentrated with Vivaspin (100 kDa cutoff) and used as the enzyme preparation. All the other fractions were concentrated using Centricon (30 kDa cutoff) and analyzed on SDS-PAGE. Polypeptide quantifications were achieved by measuring the Coomassie blue density of the different bands using an Image scanner II (GE Healthcare). As a further control, different molar amounts of FNR<sub>L</sub>-PC were loaded on an SDS-PAGE and the optical density of the FNR<sub>L</sub> polypeptide was quantified with known concentrations of recombinant FNR<sub>L</sub> that were loaded in neighboring lanes.

#### 5.2.4 Quantification of apoprotein and active protein

##### FNR apoprotein quantification

Two samples of recombinant FNR<sub>S</sub> and recombinant FNR<sub>L</sub> were calibrated on the basis of the absorption maximum of the FAD ( $\epsilon_{461\text{nm}}=9,000 \text{ M}^{-1}\text{cm}^{-1}$ ; see below) and analyzed for their protein content using the micro-BCA protein assay (Pierce Biotechnology). The protein amounts were found to be smaller than expected (92% and 91% of the calculated values for FNR<sub>S</sub> and FNR<sub>L</sub>, respectively), which must be ascribed to some underestimation by the micro-BCA assay. From these measurements, we conclude that there is practically no FAD-free protein in our FNR samples. 461/280 nm absorbance ratios of 0.128 and 0.122 were measured for FNR<sub>S</sub> and FNR<sub>L</sub>, respectively.

##### FNR holoenzyme quantification

Biochemical extraction is one approach to release the FAD from the holoenzyme using SDS for denaturing the enzyme [Aliverti et al., 1999]. First, the concentration of the FNR<sub>S/L</sub> sample was determined with an extinction coefficient of  $10,500 \text{ M}^{-1}\text{cm}^{-1}$  at 461 nm [Forti, 1966, Foust et al., 1969]. Then, a minimal volume of concentrated SDS was added directly in the spectrometer cuvette (final concentration 0.2%). The molar extinction coefficient and the maximal wavelength of the cofactor FAD was altered during denaturation. Released FAD after denaturation of the protein is characterized by an extinction coefficient of  $11,300 \text{ M}^{-1}\text{cm}^{-1}$  at 450 nm. A direct comparison between native and denatured protein can be established in this way. Six different

measurements on six different FNR<sub>S</sub> preparations have resulted in a corrected extinction coefficient in the native enzyme. Instead of 10,500 M<sup>-1</sup>cm<sup>-1</sup> at 461 nm from [Forti, 1966][Foust et al., 1969], a lower value of 9,000 ± 100 M<sup>-1</sup>cm<sup>-1</sup> at 461 nm has been obtained.

The FAD can also be extracted by TCA precipitation of the proteins. First, the enzyme concentration was determined using the same extinction coefficient as for the SDS denaturation. Then, TCA was added to a final concentration of 5% (w/v) and the pellet was washed at the same concentration of TCA. The collected supernatants were extracted three times with diethylether to eliminate TCA. The sample was further neutralized by adding 0.1 M Na phosphate pH 7 [Engel and Massey, 1971]. Released FAD in the buffered solution was characterized by an extinction coefficient of 11,300 M<sup>-1</sup>cm<sup>-1</sup> at 450 nm. The obtained molar extinction coefficient of 9,070 M<sup>-1</sup>cm<sup>-1</sup> at 461 nm was similar to that obtained by SDS denaturation. Therefore, SDS denaturation and TCA extraction give identical results.

### 5.3 *In vitro* studies

#### 5.3.1 NADPH oxidase activities

NADPH and horse-heart cytochrome *c* (cyt *c*) were purchased from Sigma-Aldrich. The enzymatic reactions were monitored with an Uvikon-XL spectrophotometer. The initial velocities were fitted with Origin 7.5 (OriginLab Corp., Northampton, MA) to obtain Michaelis-Menten curves.

Ferricyanide reduction was measured at room temperature (RT) in duplicate with NADPH and potassium ferricyanide (K<sub>3</sub>[Fe(CN)<sub>6</sub>]) as the electron donor and acceptor molecules, respectively [Zanetti et al., 1980]. A range of different FNR<sub>S/L</sub> concentrations (0.025-0.1 μM) was mixed with an excess of potassium ferricyanide (0.7 M) and 5 mM MgCl<sub>2</sub> in 150 mM KP. The reactions were initiated by the addition of a range of different NADPH concentrations (25-400 μM). The absorption decrease at 420 nm (reduction of ferricyanide) was recorded to determine the steady-state kinetic parameters. Reduction of two ferricyanide corresponds to oxidation of one NADPH. This factor of two was taken into account for determining the rate of NADPH oxidation. This rate of oxidized μM NADPH/(s × μM FNR; [s<sup>-1</sup>]) was plotted against the initial substrate concentration of NADPH (μM).

The Fd-mediated cyt *c* reduction of FNR<sub>L</sub>-PC was measured at 25°C in triplicate with Fd and cyt *c* acting as intermediate and terminal electron acceptors [Shin and Pietro, 1971, Zanetti et al., 1980]. Fd from *Thermosynechococcus elongatus* was used for these experiments as it was available in large quantities. A few control measurements were performed with *Synechocystis* Fd giving results identical to those obtained with *Th. elongatus* Fd. The reaction was started by the addition

of an excess of NADPH (400  $\mu\text{M}$  final concentration). Kinetic parameters for the Fd-dependent cyt *c* reductase activity were determined. This was achieved by varying the concentrations of Fd (2.5-40  $\mu\text{M}$ ) in the reaction mixtures and monitoring the resulting absorption increase at 550 nm, corresponding to the reduction of cyt *c* used in excess (40  $\mu\text{M}$  final concentration). The same type of measurements were performed in the presence of the inhibitor  $\text{NADP}^+$  in triplicates. A concentration of 50  $\mu\text{M}$   $\text{NADP}^+$  was used and the Lineweaver-Burk plots of  $1/v_0$  vs.  $1/\text{Fd}_{\text{ox}}^0$  were analyzed for the  $\text{FNR}_{\text{L}}$ -PC isoform.

### 5.3.2 $\text{NADP}^+$ reductase activities

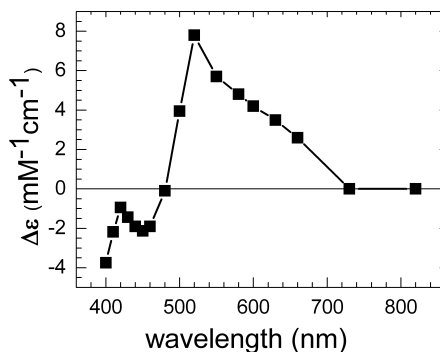
Flash-absorption measurements with a time resolution of 10  $\mu\text{s}$  were performed as described previously [Cassan et al., 2005] at 22°C. Laser excitation (700 nm) was provided by a dye laser (Continuum, Excel Technology, Villebon sur Yvette, France) pumped by a frequency-doubled Nd-Yag laser and was saturating for P700 photochemistry. Conditions were chosen to eliminate any actinic effect of measuring light.

All the spectroscopic measurements were performed under aerobic conditions in 150 mM KP containing 30 mM NaCl and 0.03% (w/v)  $\beta$ -dodecyl maltoside (Biomol, Hamburg, Germany). Sodium ascorbate (2 mM) and 2,6-dichlorophenolindophenol (5-25  $\mu\text{M}$ ) were used to reduce the oxidized P700 between two consecutive flashes. The PSI concentration was estimated using the absorption coefficient  $\varepsilon_{800\text{nm}}=7,740 \text{ M}^{-1}\text{cm}^{-1}$  for  $\text{P700}^+$  [Cassan et al., 2005]. For all flash experiments, the kinetics are shown after subtraction of the  $\text{P700}^+$  contribution. This was done by measuring, in the absence of Fd, the differential absorption coefficients of  $\text{P700}^+$  at 520/540 nm and at 800 nm, using methyl viologen (MV) as an electron acceptor that results in fast reoxidation of the terminal PSI acceptor  $(\text{F}_A, \text{F}_B)^-$ . Using this procedure, the differential absorption coefficient of  $\text{P700}^+$  at 520/540 nm was found to be 50%/34% that of  $\text{P700}^+$  at 800 nm ( $\Delta\varepsilon_{520\text{nm}}=3.9 \text{ mM}^{-1}\text{cm}^{-1} \approx 7.74 \text{ mM}^{-1}\text{cm}^{-1} \times 0.50$ ;  $\Delta\varepsilon_{540\text{nm}}=2.6 \text{ mM}^{-1}\text{cm}^{-1} \approx 7.74 \text{ mM}^{-1}\text{cm}^{-1} \times 0.34$ ). The kinetics probed at 800 nm were subtracted, after multiplication by the normalization factor of 0.50/0.34. In this way, all absorption changes are associated with the reduction of the electron acceptors, *i.e.* those due to  $(\text{F}_A, \text{F}_B)$ , Fd and FNR.

### Single reduction of FNR

Single reduction of FNR by reduced Fd was triggered by flash excitation of PSI. These experiments were performed in the presence/absence of  $\text{NADP}^+$  with FNR in excess over PSI. Under this condition, a single reduction event is favored where the neutral protonated semiquinone is produced. These measurements were performed at 520 nm, which corresponds to an absorption minimum of the  $\text{PSI}/\text{FNR}_{\text{L}}$ -mixture (Fd absorbance is small compared to those of PSI and PC).

Moreover, a large signal is expected at 520 nm for formation of the FNR semireduced form  $\text{FNR}_{\text{sq}}$  as shown in the calculated differential absorption spectrum for FNR single reduction by Fd (Figure 5.1) [Cassan et al., 2005].



**Figure 5.1** Calculated differential absorption spectrum for FNR single reduction by Fd.

### Multiple catalytic turnover

In order to promote multiple catalytic turnover, the PSI concentration was more than 10-fold greater than the enzyme under investigation ( $\text{FNR}_{\text{S/L}}$ ). Under these conditions FNR receives two electrons from Fd and NADPH is formed via hydride transfer. This multiple turnover reaction was monitored by the reoxidation of  $\text{Fd}_{\text{red}}$  at 540 nm. This wavelength was chosen because of the minimal PSI absorption, which allows actinic effects of the measuring light to be minimized in these measurements made on a long time scale [Cassan et al., 2005].

### 5.3.3 Fittings and calculations

#### Single reduction of FNR

The kinetic model used to interpret the experiments of single FNR reduction is shown in Chapter 2. It involves two reactions, the first one describes Fd reduction by PSI and the second one corresponds to the redox equilibrium of the first reduction of FNR by  $\text{Fd}_{\text{red}}$ . Such a model does not take into account complex formation and dissociation, because of the large ionic strength of the medium, which impedes formation of complexes [Hurley et al., 2002, Setif et al., 2002] and hence only considers second-order processes. We assume also that the PSI charge separation leading to the formation of  $(\text{F}_A, \text{F}_B)^-$  is much faster than the subsequent steps since it occurs in the submicrosecond range [Brettel and Leibl, 2001]. The kinetic analysis is further simplified as the

experiments were performed under conditions where one partner is in large excess over the other one for each of the reactions:  $Fd_{ox} > PSI_{red}$ ,  $FNR_{ox} > Fd_{red}$ ,  $Fd_{ox} > FNR_{sq}$ . This allows the system of time-differential equations corresponding to the model to be solved analytically, using the three following first-order rates:  $k_{rFd}=k_r \cdot [Fd]$  with  $[Fd]$  being the total Fd concentration ( $[Fd_{red}] < [Fd_{ox}]$ ),  $k_{red}=k_1 \cdot [FNR]$  with  $[FNR]$  being the total FNR concentration ( $[FNR_{sq}] < [FNR_{ox}]$ ),  $k_{ox}=k_{-1} \cdot [Fd]$ . The solution is then the following:

$$[PSI_{red}](t)=[PSI] \cdot e^{-k_{rFd}t}$$

$$[Fd_{red}](t)=[PSI] \cdot \left[ \frac{k_{rFd}-k_{ox}}{k_{ox}+k_{red}-k_{rFd}} \cdot e^{-k_{rFd}t} - \frac{k_{red}k_{rFd}}{(k_{ox}+k_{red}-k_{rFd})(k_{ox}+k_{red})} \cdot e^{-(k_{ox}+k_{red})t} + \frac{k_{ox}}{k_{ox}+k_{red}} \right]$$

$$[FNR_{sq}](t)=[PSI] \cdot \left[ \frac{-k_{red}}{k_{ox}+k_{red}-k_{rFd}} \cdot e^{-k_{rFd}t} + \frac{k_{red}k_{rFd}}{(k_{ox}+k_{red}-k_{rFd})(k_{ox}+k_{red})} \cdot e^{-(k_{ox}+k_{red})t} + \frac{k_{red}}{k_{ox}+k_{red}} \right]$$

with  $[PSI]$  being the total PSI concentration. The Excel solver (V. 2003, Microsoft, USA) was used to fit the experimental results with the above equations.

### Multiple catalytic turnover

When measuring multiple turnover event from reoxidation of  $Fd_{red}$ , the decay kinetics were able to be fitted with a single exponential component [Cassan et al., 2005]:

$$[Fd_{red}(t)] = [Fd_{red}]_{t=0} \cdot e^{-k_{FNR}t}$$

$$\left( \frac{d[Fd_{red}(t)]}{dt} \right) = -k_{FNR} [Fd_{red}(t=0)] \cdot e^{-k_{FNR}t}$$

$$\text{Initial rate: } -\left( \frac{d[Fd_{red}]_{t=0}}{dt} \right) = +k_{FNR} [Fd_{red}]_{t=0}$$

$$\text{Without FNR: } -\left( \frac{d[Fd_{red}]_{t=0}}{dt} \right) = +k_{noFNR} [Fd_{red}]_{t=0}$$

The initial decay rate  $k_{noFNR}$  in the absence of FNR was subtracted from the exponential rates  $k_{FNR}$  in its presence. The initial turnover rate was then calculated from the equation:

$$-\frac{\left( \frac{d[Fd_{red}]}{dt} \right)_{t=0}}{[FNR]} = (k_{FNR} - k_{noFNR}) \cdot \frac{[PSI]}{[FNR]} \text{ as } [PSI] = [Fd_{red}]_{t=0}$$

This rate can also be calculated from rate  $k_1$  of Equation 2.1.4 (Chapter 2) when this reaction is rate-limiting. From Equation 2.1.4, the decay rate of  $Fd_{red}$  is:

$$\frac{dFd_{red}}{dt} = -k_1 [Fd_{red}] [FNR_{ox}] + k_{-1} [Fd_{ox}] [FNR_{sq}]$$

which gives for  $t=0$ :

$(d[Fd_{red}]/dt)_{t=0} = -k_1 [Fd_{red}]_{t=0} [FNR]$  with  $[FNR]$  being the total concentration of FNR. With  $[PSI] = [Fd_{red}]_{t=0}$ , one gets:

$$-\frac{\left( \frac{d[Fd_{red}]}{dt} \right)_{t=0}}{[FNR]} = k_1 [PSI] \tag{5.3.1}$$

## 5.4 *In vivo* studies

### 5.4.1 NADP<sup>+</sup>/NADPH quantification

The Enzychrom™ NADP<sup>+</sup>/NADPH assay kit (Gentaur, France) was used to quantify the NADP<sup>+</sup>/NADPH ratio. NADP<sup>+</sup> is transformed into NADPH by the enzyme glucose dehydrogenase (GDH) in the presence of an excess of glucose (electron donor; Reaction 5.4.1). Two samples are used for separate extraction of NADP<sup>+</sup> or NADPH. NADPH will reduce 5-methylphenazinium methosulfate (PMS, electron transferring system; Reaction 5.4.2) and then 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2/H/-tetrazolium bromide (tetrazolium salt MTT) as final electron acceptor (absorbing at 565 nm; Reaction 5.4.3). The oxidation of NADPH into NADP<sup>+</sup> is the only rate-limiting step (rls; Reaction 5.4.2).



The different steps for the calibration curve, the reconstitution of the working reagent and the sample preparation were performed as indicated by the supplier. We first tried to optimize the assay for cuvette based measurements. Then we calibrated the assay using NADP<sup>+</sup>. Finally, we measured the NADP<sup>+</sup> and NADPH. From these values we could calculate the respective NADP<sup>+</sup>/NADPH ratio for WT and the mutants. Ratios were calculated for five independent measurements, averaged and standard deviations were calculated.

The calibration curve was done using a NADP<sup>+</sup> standard from 0-10 μM in duplicates. The difference in optical density at 565 nm  $t=30'$  -  $t=0'$  was linear up to 10 μM ( $\Delta\text{OD}_{565\text{nm}}=0.2-1.6$ ) and up to 6 μM, the best linearity was obtained. This corresponded to  $\Delta\text{OD}=1.1$  (Figure 5.2). Values for [NADP<sup>+</sup>] above 10 μM ( $\Delta\text{OD}_{565\text{nm}} > 1.73$ ) led to saturation and could not be fitted.

In this way, the mutants and the WT were quantified for their NADP pool redox state. These measurements were performed with cells in their mid-exponential growth phase.



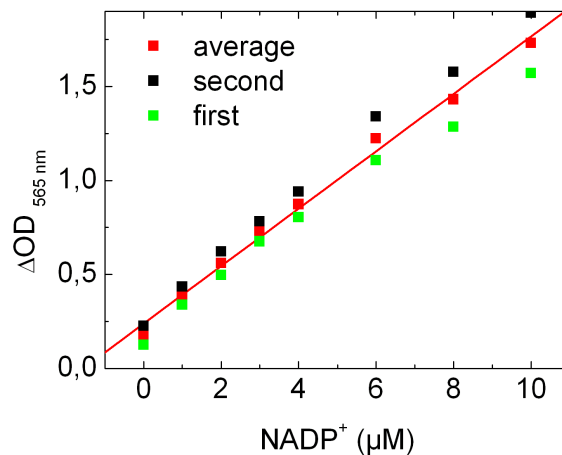


Figure 5.2 Calibration curve of NADP<sup>+</sup> quantification.

#### 5.4.2 Absorption spectra +/- CO<sub>2</sub>

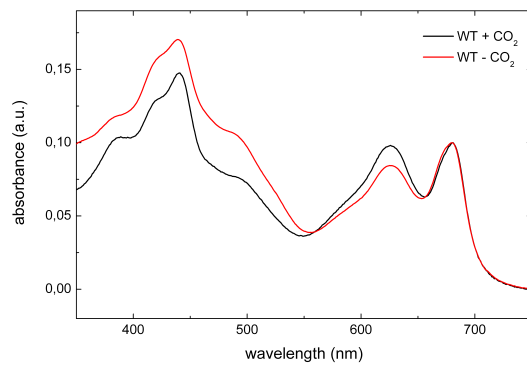
The phycobiliprotein-antenna (PBP-antenna) -, chlorophyll- and carotenoid-content in the cells can be determined by their characteristic absorbance maxima. Whole cell absorption spectra were recorded for two different growth conditions. In order to limit scattering, several precautions were taken. First, cells were harvested and suspended carefully in a solution of 2 M sucrose in 0.9 M KP. Second, the spectra were recorded on a Aminco<sup>TM</sup> DW 2 spectrometer (OLIS INC., Bogart, Georgia, USA). Here, the detector is very close to the sample in order to minimize light scattering. We measured absorption spectra of HC- and LC grown cells for the WT, MI6 and FS1 strains. Absorption spectra give us informations on the impact of the CO<sub>2</sub> limitation on the cell pigment composition in the WT and the mutants.

Spectra were normalized as follows. First, the absorbance of 750 nm was set to zero. Second, the spectra of the mutant and WT strains were normalized at 680 nm to the same chlorophyll *a* absorption maximum. In this way, we determined the difference in absorbance at 620 nm, characteristic for PBP in the PBS antenna.

**Absorption spectra under high and low CO<sub>2</sub>** For WT, the absorption spectra under HC and LC are shown in Figure 5.3. The absorbance at 620 nm was found to be 86% under LC compared to HC. This means that LC conditions result in a decrease in PBP content.

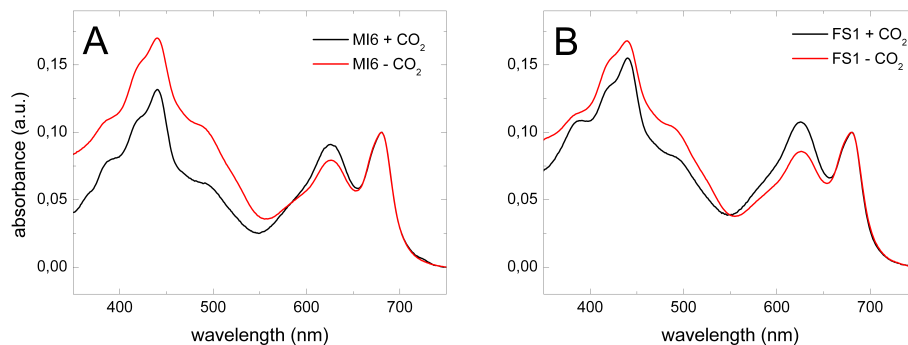
For MI6 and FS1, the absorption spectra under HC and LC are shown in Figure 5.4A and B. Compared to HC, the absorbance at 620 nm under LC was found to be 87% and 80% for MI6 and FS1, respectively. These values are very close to WT. Thus, all the three strains exhibit a similar





**Figure 5.3** Normalized absorbance spectra of WT in high (black) and low (red)  $\text{CO}_2$ .

decrease in PBP content under LC. No significant difference is thus expected concerning PBP degradation and the strains seem to be equally stressed. They can be further compared in their P700 oxidation phenotypes under LC.



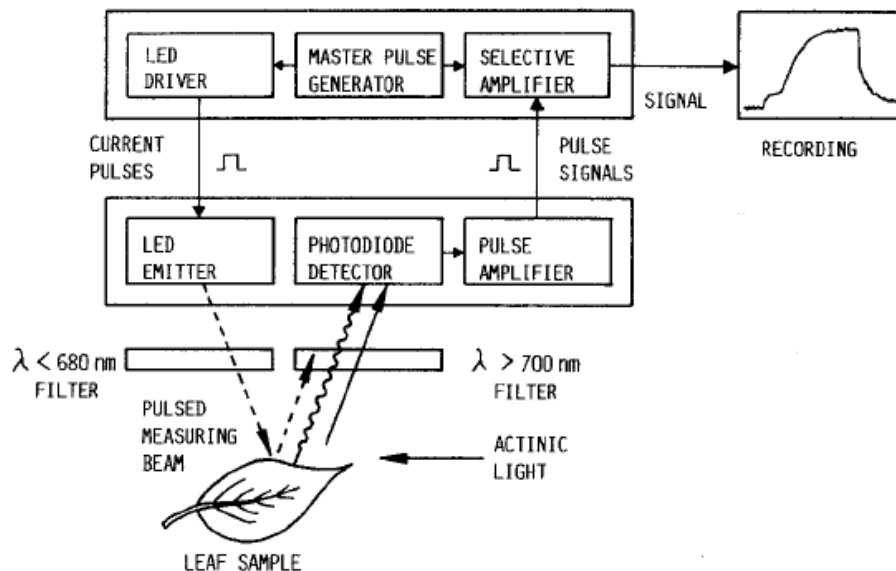
**Figure 5.4** Normalized absorbance spectra of MI6 (A) and FS1 (B) in high (black) and low (red)  $\text{CO}_2$ .

### 5.4.3 P700 oxidation and reduction kinetics

FS1, MI6 and WT were characterized by measuring *in vivo* P700 oxidation and P700<sup>+</sup> reduction. The P700<sup>+</sup> absorption changes around 810 nm were measured with a pulse amplitude modulated fluorometer [Schreiber, 1986, Schreiber et al., 1986].

The pulse amplitude modulated system (PAM 101, 102, 103 chlorophyll fluorometer, H. Walz, Effeltrich, Germany) is equipped with fiber-optics connecting a suspension cuvette with a LED emitter, a photodiode detector and a source for continuous actinic light [Schreiber, 1986, Schreiber et al., 1986]. A modulate measuring beam (1  $\mu\text{s}$  intense pulses from LED applied repetitively at 1.6/100 kHz) was used in addition to the non-modulated actinic light. The amplifier system selected the modulated signal, so that actinic illumination can be varied within wide ranges without corresponding artefactual signal changes. The obtained signal reflects relative

fluorescence yield (fluorescence intensity/light intensity). Figure 5.5 taken from [Schreiber, 1986] shows a schematic diagram of the measuring system.



**Figure 5.5** Schematic diagram of the pulse amplitude modulation (PAM) chlorophyll fluorometer

The only unit that needs to be exchanged when changing from fluorescence to absorbance measurements is the emitter-detector unit of the fluorometer [Schreiber et al., 1988]. The redox changes of P700 were measured *via* the broad band increase in absorbance caused by the P700<sup>+</sup> cation-radical at 810 nm.

In detail, the changes concern:

- 650 nm LED for fluorescence excitation is substituted by a 830 nm LED (Type HE 8811, Hitachi)
- Short-pass filter in front of the LED is exchanged with a high-pass filter (RG 780, Schott). This filter eliminates the short wavelength tail emission of the 830 nm LED
- The RG 9 filter in front of the photodiode is exchanged with a RG 780 filter. With this filter, the photodetector is protected against all visible and photosynthetically active light.

**Sample preparation and analysis** Measurements were performed with dark-adapted (5') cells. The cell suspensions were stirred and thermostated at 32°C. Actinic light illumination used was white light (2800  $\mu\text{mol s}^{-1} \text{m}^{-2}$ ) and FR light (100  $\mu\text{mol s}^{-1} \text{m}^{-2}$ ; about 25 photons absorbed per PSI and per s). P700 oxidation kinetics were carried out under HC and LC. Control measurements

were carried out by sequential addition of inhibitors DCMU (20  $\mu\text{M}$ ), MV (2 mM) and DBMIB (20  $\mu\text{M}$ ).

P700<sup>+</sup> exhibits an absorption around 800 nm. The contribution of plastocyanin (Pc) is expected to be the same between 810 and 870 nm. By subtracting the 870 nm signal from the 810 nm signal, Pc contribution at 810 nm should be efficiently subtracted by the set-up. Curves were analyzed and normalized to the maximum amount of oxidizable P700 in the presence of DBMIB. The set-up used for our measurements did not permit to define exactly the starting point. Thus, short lags before rising signals occurred could not be identified between mutant and WT strains. We will assume that addition of MV in our experiments fully inhibits the recombination processes involving P700<sup>+</sup>. Moreover, our experiments with MV addition on FS1 show that, in that case, MV does not or only partially inhibits cyclic ET.

#### 5.4.4 Monitoring of the transient increase in chlorophyll fluorescence

Cyclic ET around PSI was monitored by the transient increase of dark-level chlorophyll fluorescence after actinic light (AL) [Shikanai et al., 1998]. The PAM spectrometer introduced above was used in the chlorophyll fluorescence mode (repetitive pulses at 1.6 kHz). The saturation pulse method of chlorophyll fluorescence was used for these studies. Cell suspensions were calibrated to 3  $\mu\text{g}$  chlorophyll  $\text{mL}^{-1}$ .

After 5' dark adaptation, strong white AL was applied for around 2' and NADPH was accumulated [Schreiber et al., 1986]. After switching off AL, a transient PQ pool reduction in the dark was observed in the mutants and WT.

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# Ferredoxin:NADP<sup>+</sup> Oxidoreductase Association with Phycocyanin Modulates Its Properties\*<sup>§</sup>

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In photosynthetic organisms, ferredoxin:NADP<sup>+</sup> oxidoreductase (FNR) is known to provide NADPH for CO<sub>2</sub> assimilation, but it also utilizes NADPH to provide reduced ferredoxin. The cyanobacterium *Synechocystis* sp. strain PCC6803 produces two FNR isoforms, a small one (FNR<sub>S</sub>) similar to the one found in plant plastids and a large one (FNR<sub>L</sub>) that is associated with the phycobilisome, a light-harvesting complex. Here we show that a mutant lacking FNR<sub>L</sub> exhibits a higher NADP<sup>+</sup>/NADPH ratio. We also purified to homogeneity a phycobilisome subcomplex comprising FNR<sub>L</sub>, named FNR<sub>L</sub>-PC. The enzymatic activities of FNR<sub>L</sub>-PC were compared with those of FNR<sub>S</sub>. During NADPH oxidation, FNR<sub>L</sub>-PC exhibits a 30% decrease in the Michaelis constant  $K_{m(\text{NADPH})}$ , and a 70% increase in  $K_{m(\text{ferredoxin})}$ , which is in agreement with its predicted lower activity of ferredoxin reduction. During NADP<sup>+</sup> reduction, the FNR<sub>L</sub>-PC shows a 29/43% decrease in the rate of single electron transfer from reduced ferredoxin in the presence/absence of NADP<sup>+</sup>. The increase in  $K_{m(\text{ferredoxin})}$  and the rate decrease of single reduction are attributed to steric hindrance by the phycocyanin moiety of FNR<sub>L</sub>-PC. Both isoforms are capable of catalyzing the NADP<sup>+</sup> reduction under multiple turnover conditions. Furthermore, we obtained evidence that, under high ionic strength conditions, electron transfer from reduced ferredoxin is rate limiting during this process. The differences that we observe might not fully explain the *in vivo* properties of the *Synechocystis* mutants expressing only one of the isoforms. Therefore, we advocate that FNR localization and/or substrates availability are essential *in vivo*.

In cyanobacteria and plastids, ferredoxin:NADP<sup>+</sup> oxidoreductase (FNR)<sup>3</sup> catalyzes the exchange of electrons

between the one-electron carrier ferredoxin (Fd) and the two-electron carrier NADP<sup>+</sup> (1–5):  $2 \text{Fd}_{\text{red}} + \text{NADP}^+ + \text{H}^+ \rightleftharpoons 2 \text{Fd}_{\text{ox}} + \text{NADPH}$ . FNR contains the noncovalently bound FAD cofactor. The NADP<sup>+</sup>-reductase catalytic cycle involves the reduction of FAD to the neutral semiquinone FADH<sup>•</sup> (FNR<sub>sq</sub>) followed by its further reduction to the fully reduced FADH<sup>-</sup> (FNR<sub>red</sub>), with reduced Fd (Fd<sub>red</sub>) binding at a single site (4, 6). Hydride transfer from FADH<sup>-</sup> to NADP<sup>+</sup> completes the catalytic cycle (7) and NADPH is then released. Ternary complexes between the three partners FNR, NADP<sup>+</sup> and Fd have been shown to be involved in NADP<sup>+</sup>-reductase activity (1, 8). This is in line with the fact that fast turnover requires NADP<sup>+</sup> binding before Fd<sub>red</sub> binding, FAD reduction, and Fd<sub>ox</sub> release (1). Such ternary complexes may not be required during the NADPH-oxidase catalytic cycle (7, 9), although this has yet to be established. In the final step of linear photosynthetic electron flow, FNR is involved in NADPH production, which in turn is used in the Calvin cycle. In plant plastids several FNR isoforms are encoded by different genes (10–12). The expressed enzymes are processed to give molecular masses of ~35 kDa. The different isoforms are differentially expressed in roots and leaves (13). The root enzyme is involved in NADPH consumption, reducing Fd for nitrogen fixation, while the leaf enzyme is involved in NADPH formation (14–17).

The biochemical and structural properties of cyanobacterial and plastid FNR are highly similar except that in most phycobilisome (PBS)-containing cyanobacteria, FNR contains an N-terminal domain whose sequence is similar to PBS-linker polypeptides (18). This extension is responsible for FNR<sub>L</sub> attachment to the PBS (18). The conventional PBS is composed of two substructures, the core and the rods. In *Synechocystis* sp. strain PCC6803 (hereafter named *Synechocystis*), the core is composed of allophycocyanin (AP) and each rod contains three phycocyanin (PC) discs. Different linkers are specifically responsible for each level of phycobiliprotein assembly and function to stabilize the PBS and optimize its absorption and energy transfer characteristics (19). FNR<sub>L</sub> has been shown to bind to the PBS rods but its precise binding site is still controversial (20–22). Smaller FNR isoforms have been purified from several cyanobacteria and this was attributed to proteolytic degradation of the N-terminal domain (18, 23). However, it has been recently demonstrated that in *Synechocystis* the small isoform (FNR<sub>S</sub>, ~34 kDa) results from an internal translation initiation and not from proteolysis of the large isoform (24). The same authors proposed that FNR<sub>L</sub> functions as an NADP<sup>+</sup> reductase whereas FNR<sub>S</sub> is a better NADPH oxidase. More precisely, FNR<sub>L</sub> was shown to support photoautotrophic growth in *Synechocystis* whereas it is the only isoform found in obligate

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<sup>3</sup> The abbreviations used are: FNR, ferredoxin:NADP<sup>+</sup> oxidoreductase; AP, allophycocyanin;  $\alpha^{\text{PC}}$  and  $\beta^{\text{PC}}$ , the two subunits of phycocyanin; cyt *c*, cytochrome *c*;  $E_m$ , midpoint redox potential; Fd, ferredoxin; red, reduced; ox, oxidized; FNR<sub>sq</sub>, singly reduced FNR/semiquinone form; FNR<sub>S</sub>, small *Synechocystis* FNR isoform; FNR<sub>L</sub>, large *Synechocystis* FNR isoform; PBS, phycobilisome; PC, phycocyanin, an  $\alpha\beta$  protomer; PSI, photosystem I; P700, primary electron donor of photosystem I; *Synechocystis*, *Synechocystis* sp. PCC6803; L<sub>CM</sub>, core-membrane linker; L<sub>RC</sub>, rod-core linker.



## FNR<sub>L</sub>-Phycocyanin Complex

phototrophic cyanobacteria. Conversely, FNR<sub>S</sub> accumulates when photosynthesis is slowed down, *i.e.* under heterotrophic or starvation conditions (24). These observations support the idea that the two isoforms differ in their NADP<sup>+</sup>-reductase/NADPH-oxidase activities. This can be regarded as analogous to the leaf and root isoforms of plants.

Both *Synechocystis* isoforms being encoded by the same gene, they share identical catalytic domains. The N-terminal extension of FNR<sub>L</sub> or its association to PBS could somehow modify its catalytic properties. As FNR<sub>L</sub> is bound *in vivo* to the core-containing PBS and undergoes proteolysis when not bound to it (25), it is crucial to compare the enzymatic properties of FNR<sub>S</sub> to those of PBS-bound FNR<sub>L</sub>. However, in practical terms, the large extinction coefficient of the PBS makes such experiments virtually impossible since they are based on absorption measurements. This was circumvented by the purification of a PBS subcomplex, termed FNR<sub>L</sub>-PC that contained FNR<sub>L</sub>, a PC hexamer and a PBS rod-core linker (L<sub>RC</sub>). The FNR<sub>L</sub>-PC complex possesses a lower extinction coefficient than that of the whole PBS and thus permits absorption measurements to be undertaken.

In this work, we established that the NADP<sup>+</sup>/NADPH ratio is higher in a mutant containing only FNR<sub>S</sub>. An FNR<sub>L</sub>-PC complex was purified to homogeneity and shown to be stable for several days in 150 mM phosphate buffer. Finally, the catalytic activities and kinetic constants of the two FNR isoforms are compared with each other and to their plant homologues.

### EXPERIMENTAL PROCEDURES

**Materials**—*Synechocystis* strains were grown at 34 °C in a CO<sub>2</sub> enriched atmosphere under 60 μE m<sup>-2</sup> s<sup>-1</sup> illumination in a modified Allen's medium (26). Photosystem I (PSI) was purified from *Synechocystis* wild type (27), whereas Fd, FNR<sub>S</sub>, and FNR<sub>L</sub> were overexpressed in *Escherichia coli* and purified as previously described (5, 28). NADPH and horse-heart cytochrome *c* (cyt *c*) were purchased from Sigma-Aldrich. ProBond Ni-resin was obtained from Invitrogen. An antiprotease mixture (Complete, Roche Applied Sciences) was used during the isolation of FNR<sub>L</sub>-PC.

**NADP<sup>+</sup>/NADPH Quantification**—Absolute and relative amounts of pyridine nucleotides were obtained using an Enzy-Chrom™ NADP<sup>+</sup>/NADPH assay kit (Gentaur, France) for the wild type and the two mutants where only one FNR isoform is expressed, *i.e.* FNR<sub>S</sub> and FNR<sub>L</sub> in the FS1 and MI6 mutants, respectively (24). These measurements were performed with cells in their exponential growth phase under photoautotrophic conditions.

**Construction and Purification of His-tagged FNR<sub>L</sub> in *Synechocystis***—Because the N- and C-terminal domains of the enzyme are buried in the PC hexamer and the NADP binding site of the FNR, respectively, a His tag was inserted into the exposed hinge domain preceding the catalytic FNR<sub>S</sub> domain (Fig. 1). PCR mutagenesis was performed on the *petH* gene of *Synechocystis* to introduce 6 histidines (between Gly-98 and Ser-99). The mutagenic primers were HIF (5'-CCATCAT-CACCATCACTCAGGAGCGGTGGC-3') and HIR (5'-GATGGTGATGATGGTGACCCTTCCTCGG-3'). The overall method was similar to that used in Ref. 24. The modified gene

was introduced in CB, a *Synechocystis* mutant that contains only one PC hexamer per rod instead of three as expressed in the wild type (26). The resulting strain was named CBH.

Phycobilisomes were purified from CBH under conditions that are known (29–31) to preserve PBS-subunit interactions, *i.e.* 0.8 M phosphate (KP: potassium phosphate buffer, pH 8.0). Membranes and chlorophylls were eliminated by Triton X-100 extraction. The PBS complex was then allowed to dissociate overnight at 4 °C by lowering the phosphate concentration to 150 mM KP. The sample was then added to a Ni-resin equilibrated in 250 mM KP and allowed to bind for 1 h. After two washes in the same buffer, the resin was poured into a column. After extensive washing with 150 mM KP, FNR<sub>L</sub> was eluted in the presence of 150 mM imidazole. The eluted fractions were concentrated using Vivaspinn concentrators (100 kDa cut-off). For each fraction, the PC hexamer concentration was determined by absorption spectroscopy ( $\epsilon_{620\text{ nm}} = 2.37 \mu\text{M}^{-1} \text{ cm}^{-1}$ , Ref. 32) prior to gel filtration chromatography (250 mM KP, 26/85 Superdex 200, GE Healthcare). Elution profiles were obtained by monitoring the absorbance at 280, 460, and 620 nm, which are indicative of the relative amounts of protein, FAD, and PC, respectively (supplemental Fig. S1). The polypeptide composition of each fraction was analyzed by SDS-PAGE. Polypeptide quantifications were achieved by measuring the Coomassie Blue density of the different bands using an Image scanner II (GE Healthcare). Different amounts of FNR<sub>L</sub>-PC were loaded and the staining of the FNR<sub>L</sub> polypeptide was compared with known amounts of recombinant FNR<sub>L</sub> that were loaded in neighboring lanes.

**Measurements of FAD Content in the FNR<sub>L</sub>-PC Complex**—The polypeptides of FNR<sub>L</sub>-PC from three different batches were precipitated by the addition of trichloroacetic acid (5% w/v). Under these conditions, the released FAD cofactor is recovered in the supernatant (33). FAD concentrations were calculated from the absorption maxima at 450 nm ( $\epsilon_{450\text{ nm}} = 11,300 \text{ M}^{-1} \text{ cm}^{-1}$ , Ref. 34). This is illustrated in supplemental Fig. S2. In parallel, the pelleted polypeptides were solubilized for SDS-PAGE, and FNR<sub>L</sub> quantified after electrophoretic separation. These two approaches allowed us to compare the FNR<sub>L</sub> and FAD contents.

**Determination of the Absorption Coefficients of FNR<sub>S</sub> and FNR<sub>L</sub>**—As detailed in supplemental data, the FAD cofactor from recombinant FNR<sub>S</sub> and FNR<sub>L</sub> was released and quantified in the presence of 0.02% SDS (w/v). This allowed us to reevaluate the absorption coefficients of both FNR isoforms. They were determined to be 9,000 M<sup>-1</sup> cm<sup>-1</sup> at 461 nm instead of 10,800 M<sup>-1</sup> cm<sup>-1</sup> that was previously reported for plant FNR (35).

**Oxidase Activities**—Enzymatic reactions were monitored with a Uvikon-XL spectrophotometer. The initial velocities were fitted with Origin 7.5 (OriginLab Corp., Northampton, MA) to obtain Henri-Michaelis-Menten curves. Ferricyanide reductase activity was measured at room temperature in duplicate with NADPH and potassium ferricyanide as the electron donor and acceptor molecules, respectively (36). A series of FNR<sub>S</sub>/FNR<sub>L</sub>-PC concentrations (0.025–0.1 μM) was mixed with 0.7 mM potassium ferricyanide and 5 mM MgCl<sub>2</sub> in 150 mM KP. The reactions were initiated by the addition of a range of

different NADPH concentrations (25–400 μM). The absorption decrease at 420 nm (reduction of ferricyanide) was recorded to determine the steady-state kinetic parameters.

The Fd-mediated cyt *c* reductase activity of FNR<sub>S</sub>/FNR<sub>L</sub>-PC was measured at 25 °C in triplicate with Fd and cyt *c* acting as intermediate and terminal electron acceptors (35, 36). The reaction was started by the addition of NADPH (400 μM final concentration). Steady-state kinetic parameters for the Fd-dependent cyt *c* reductase activity were determined by varying the concentrations of Fd (2.5–40 μM) in the reaction mixtures and monitoring the resulting absorption increases at 550 nm (reduction of cyt *c*). Fd from *Thermosynechococcus elongatus* was used for these experiments as it was available in large quantities. A few control measurements were performed with *Synechocystis* Fd giving results identical to those obtained with *T. elongatus* Fd.

**Flash Absorption Experiments for the Measurements of Reductase Activities**—Flash absorption measurements with a time resolution of 10 μs were performed as described previously (5) at 22 °C. Laser excitation (700 nm) was provided by a dye laser (Continuum, Excel Technology, Villebon sur Yvette, France) pumped by a frequency-doubled Nd-Yag laser and was saturating for PSI photochemistry. Conditions were chosen to eliminate any actinic effect of the measuring light.

All spectroscopic measurements were performed under aerobic conditions in 150 mM KP containing 30 mM NaCl and 0.03% (w/v) β-dodecyl maltoside (Biomol, Hamburg, Germany). Sodium ascorbate (2 mM) and 2,6-dichlorophenolindophenol (5–25 μM) were used to reduce the oxidized P700 between two consecutive flashes. The PSI concentration was estimated using the absorption coefficient ε<sub>800 nm</sub> = 7.74 mM<sup>-1</sup> cm<sup>-1</sup> for P700<sup>+</sup> (5). For all flash experiments, the kinetics is shown after subtraction of the P700<sup>+</sup> contribution. This was achieved by measuring, in the absence of Fd, the differential absorption coefficients of P700<sup>+</sup> at 520/540 nm and at 800 nm, using methyl viologen as an electron acceptor that results in fast reoxidation of the terminal PSI acceptor (F<sub>A</sub>, F<sub>B</sub>)<sup>-</sup>. Using this procedure, the differential absorption coefficient of P700<sup>+</sup> at 520/540 nm was found to be 50%/34% that of P700<sup>+</sup> at 800 nm (Δε<sub>520 nm</sub> = 3.9 mM<sup>-1</sup> cm<sup>-1</sup> ≈ 7.74 mM<sup>-1</sup> cm<sup>-1</sup> × 0.50; Δε<sub>540 nm</sub> = 2.6 mM<sup>-1</sup> cm<sup>-1</sup> ≈ 7.74 mM<sup>-1</sup> cm<sup>-1</sup> × 0.34). The kinetics probed at 800 nm was subtracted, after multiplication by the normalization factor of 0.50/0.34. In this way, all absorption changes are associated with the reduction of the electron acceptors, *i.e.* those due to (F<sub>A</sub>, F<sub>B</sub>)<sup>-</sup>, Fd, and FNR.

Single reduction of FNR by reduced Fd was triggered by flash excitation of PSI. These experiments were performed in the presence/absence of NADP<sup>+</sup> with FNR in excess over PSI. Under this condition, a single reduction event is favored where the neutral protonated semiquinone is produced. These measurements were performed at 520 nm, which corresponds to an absorption minimum of the PSI/FNR<sub>L</sub>-PC mixture (Fd absorbance is small compared with those of PSI and PC). Moreover, a large signal is expected at 520 nm for formation of the FNR semireduced form FNR<sub>sq</sub>.

To promote multiple catalytic turnover, the PSI concentration was >10-fold greater than that of the investigated enzyme (either FNR<sub>S</sub> or FNR<sub>L</sub>-PC). Under these conditions, FNR

receives two electrons from Fd and NADPH is formed via hydride transfer. This multiple turnover reaction was monitored by the reoxidation of Fd<sub>red</sub> at 540 nm. This wavelength was chosen because of the minimal PSI absorption, which allows actinic effects of the measuring light to be minimized in these measurements made on a long time scale (5).

**Fittings and Calculations**—The kinetic model used to interpret the single FNR reduction experiments is shown under “Results.” It involves two reactions, the first one describes Fd reduction by PSI and the second one corresponds to the redox equilibrium of the first FNR reduction by Fd<sub>red</sub>. Such a model does not take into account complex formation and dissociation, because of the large ionic strength of the medium, which impedes formation of complexes (2, 37) and hence only considers second-order processes. We assume also that the PSI charge separation leading to the formation of (F<sub>A</sub>, F<sub>B</sub>)<sup>-</sup> is much faster than the subsequent steps since it occurs in the submicrosecond range (38). The kinetic analysis is further simplified as the experiments were performed under conditions where one partner is in large excess over the other one for each of the reactions: [Fd<sub>ox</sub>] ≫ [PSI<sub>red</sub>], [FNR<sub>ox</sub>] ≫ [Fd<sub>red</sub>], [Fd<sub>ox</sub>] ≫ [FNR<sub>sq</sub>]. This allows the system of time-differential equations corresponding to the model to be solved analytically, using the three following first-order rate Equations 1–3,

$$k_{rFd} = k_r \times [Fd] \quad (\text{Eq. 1})$$

with [Fd] as the total Fd concentration ([Fd<sub>red</sub>] ≪ [Fd<sub>ox</sub>]),

$$k_{red} = k_1 \times [FNR] \quad (\text{Eq. 2})$$

with [FNR] as the total FNR concentration ([FNR<sub>sq</sub>] ≪ [FNR<sub>ox</sub>]).

$$k_{ox} = k_{-1} \times [Fd] \quad (\text{Eq. 3})$$

The solution is then shown in Equations 4–6,

$$[PSI_{red}](t) = [PSI] \times e^{-k_{rFd}t} \quad (\text{Eq. 4})$$

$$[Fd_{red}](t) = [PSI] \times \left[ \frac{k_{rFd} - k_{ox}}{k_{ox} + k_{red} - k_{rFd}} \times e^{-k_{rFd}t} - \frac{k_{red} k_{rFd}}{(k_{ox} + k_{red} - k_{rFd})(k_{ox} + k_{red})} \times e^{-(k_{ox} + k_{red})t} + \frac{k_{ox}}{k_{ox} + k_{red}} \right] \quad (\text{Eq. 5})$$

$$[FNR_{sq}](t) = [PSI] \times \left[ \frac{-k_{red}}{k_{ox} + k_{red} - k_{rFd}} \times e^{-k_{rFd}t} + \frac{k_{red} k_{rFd}}{(k_{ox} + k_{red} - k_{rFd})(k_{ox} + k_{red})} \times e^{-(k_{ox} + k_{red})t} + \frac{k_{red}}{k_{ox} + k_{red}} \right] \quad (\text{Eq. 6})$$

with [PSI] as the total PSI concentration. The Excel solver (V. 2003, Microsoft) was used to fit the experimental results with the above equations.

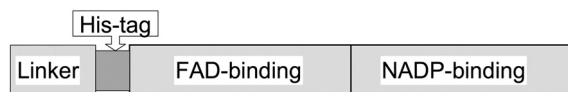
When measuring multiple turnover event from reoxidation of Fd<sub>red</sub>, the decay kinetics were able to be fitted with a single exponential component (5). The initial decay rate  $k_{noFNR}$  in the absence

## FNR<sub>L</sub>-Phycocyanin Complex

FNR<sub>S</sub> (34 kDa)



FNR<sub>L</sub> (46 kDa)



**FIGURE 1. Representation of the FNR primary structures highlighting their functional domains.** FNR<sub>S</sub> is restricted to the catalytic part that is divided into the FAD binding domain and the NADP binding domain. FNR<sub>L</sub> contains an N-terminal extension that comprises the PBS-linker domain and a hinge domain, whose length and primary sequence are variable depending on the cyanobacterium. Because the N- and C-terminal domains of the enzyme are buried in the PBS and the NADP binding site, respectively, the His tag was inserted into the hinge domain.

of FNR was subtracted from the exponential rate  $k_{\text{FNR}}$  in its presence. The initial turnover rate was then calculated from Equation 7,

$$-(d[\text{Fd}_{\text{red}}]/dt)_{t=0}/[\text{FNR}] = (k_{\text{FNR}} - k_{\text{noFNR}}) \times [\text{PSI}]/[\text{FNR}] \quad (\text{Eq. 7})$$

as  $[\text{PSI}] = [\text{Fd}_{\text{red}}]_{t=0}$ .

This rate can also be calculated from rate  $k_1$  of Reaction 2 (see “Results”) when this reaction is rate limiting. From Reaction 2, the decay rate of  $\text{Fd}_{\text{red}}$  is shown in Equation 8,

$$d[\text{Fd}_{\text{red}}]/dt = -k_1[\text{Fd}_{\text{red}}][\text{FNR}_{\text{ox}}] + k_{-1}[\text{Fd}_{\text{ox}}][\text{FNR}_{\text{sq}}] \quad (\text{Eq. 8})$$

which gives, for  $t = 0$ , Equation 9,

$$(d[\text{Fd}_{\text{red}}]/dt)_{t=0} = -k_1[\text{Fd}_{\text{red}}]_{t=0}[\text{FNR}] \quad (\text{Eq. 9})$$

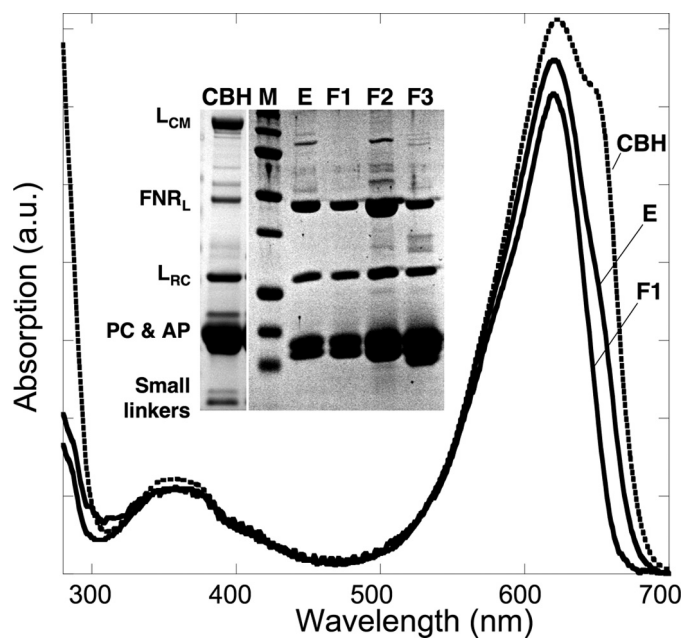
with  $[\text{FNR}]$  as the total concentration of FNR. With  $[\text{PSI}] = [\text{Fd}_{\text{red}}]_{t=0}$  one gets Equation 10.

$$-(d[\text{Fd}_{\text{red}}]/dt)_{t=0}/[\text{FNR}] = k_1[\text{PSI}] \quad (\text{Eq. 10})$$

## RESULTS

**Quantification of NADP<sup>+</sup> and NADPH in Cell Extracts**—The cellular contents of NADP<sup>+</sup> and NADPH were measured in three *Synechocystis* strains grown under photoautotrophic conditions, the wild type and two mutants containing only one of the FNR isoforms, *i.e.* FNR<sub>L</sub> and FNR<sub>S</sub> in MI6 and FS1, respectively (24). The NADP<sup>+</sup>/NADPH ratios were  $2.6 \pm 0.2$ ,  $2.0 \pm 0.4$ , and  $4.3 \pm 0.8$  for the wild type, MI6, and FS1 strains, respectively. Whereas the wild type and MI6 strains exhibit similar NADP<sup>+</sup>/NADPH ratios, FS1 contained a significantly more oxidized NADP pool. As the NADP<sup>+</sup>/NADPH ratio is expected to depend on the PSI/PSII content, we measured the PSI/PSII ratios in the thylakoids by EPR (39). The ratios were found to be similar in the three strains in the 2.5–2.9 range (data not shown).

**Purification of an FNR<sub>L</sub>-PC Complex**—To study FNR<sub>L</sub> under conditions as close as possible to its native conformation, we purified an FNR<sub>L</sub>-PBS subcomplex from CBH, a *Synechocystis* mutant that contained a His tag in FNR<sub>L</sub> (Fig. 1). The tag had no effect on either the cell growth characteristics or its PBS composition (data not shown).



**FIGURE 2. Purification of the FNR<sub>L</sub>-PC complex.** Absorption spectra of sample fractions during the purification steps, CBH: PBS preparation (*dotted line*) where AP ( $\lambda_{\text{max}}$ , 650 nm) and PC ( $\lambda_{\text{max}}$ , 620 nm) peaks are visible, E, the sample after Ni-affinity chromatography contains PC and a shoulder at 650 nm due to traces of AP; F1, the major fraction from gel filtration chromatography exhibits a PC spectrum with a shoulder at 580 nm indicating the presence of L<sub>RC</sub>. The polypeptide composition of the samples was analyzed by SDS-PAGE. *Inset*, CBH; M, molecular markers, E1, after Ni-affinity chromatography; F1, first and major fraction; F2 and F3, minor fractions from gel filtration chromatography. The identities of the polypeptides are indicated on the left.

Fig. 2 illustrates the purification protocol as followed both by UV/visible absorption spectroscopy and by SDS-PAGE. The specific absorption of the PBS starting material at 650 nm (AP contribution) strongly decreases during the different purification steps (PBS gradient, Ni-affinity chromatography, and gel filtration). Denaturing electrophoresis clearly shows an enrichment of FNR<sub>L</sub> after the Ni-column (*lane E*) but the complex contained minor impurities that are ascribed to L<sub>CM</sub> (the core membrane linker of the phycobilisome) and AP subunits as evidenced by the shoulder at 650 nm in the corresponding spectrum. The impurities were then eliminated from FNR<sub>L</sub>-PC by gel filtration. Indeed only Fraction F1 corresponds to pure FNR<sub>L</sub>-PC as it contained only FNR<sub>L</sub>,  $\alpha^{\text{PC}}$ ,  $\beta^{\text{PC}}$ , and L<sub>RC</sub>, as observed by SDS-PAGE (Fig. 2, *lane F1*). The molecular mass of FNR<sub>L</sub>-PC was determined to be 330 kDa. F2 and F3 are minor fractions of lower molecular weight ([supplemental Fig. S1](#)).

The polypeptide composition of the purified FNR<sub>L</sub>-PC was evaluated by densitometry of the Coomassie Blue-stained SDS-PAGE bands and indicates that the protein partners, FNR<sub>L</sub>: L<sub>RC</sub>: ( $\alpha^{\text{PC}}$ ,  $\beta^{\text{PC}}$ )<sub>6</sub>, are in a 1:1:1 stoichiometry. The estimated mass of the complex (330 kDa) closely matches its theoretical mass (303 kDa), which takes into account one phycocyanin hexamer (229 kDa) binding one FNR<sub>L</sub> (46 kDa) and one L<sub>RC</sub> (28 kDa). A direct measurement of the FAD content at 461 nm was impossible due to the large PC absorption. Therefore, an extraction procedure was applied (see “Experimental Procedures” and [supplemental Fig. S2](#)), leading to an occupancy value from 92 to 100% for the FAD cofactor in FNR<sub>L</sub>-PC (Table 1).



TABLE 1

Quantification of FNR<sub>L</sub> and FAD in FNR<sub>L</sub>-PC

Three different FNR<sub>L</sub>-PC samples have been trichloroacetic acid precipitated, and analyzed for their FNR<sub>L</sub> and FAD contents. For each sample, the result is an average of three measurements. Taking together the results of the three samples, one gets a [FAD]/[FNR<sub>L</sub>] ratio of between 0.92 and 1.00.

Sample	FNR <sub>L</sub> concentration $\mu\text{M}$	FAD concentration $\mu\text{M}$	[FAD]/[FNR <sub>L</sub> ]
A	$0.43 \pm 0.02$	$0.39 \pm 0.02$	$0.91 \pm 0.09$
B	$0.55 \pm 0.04$	$0.49 \pm 0.05$	$0.89 \pm 0.16$
C	$1.22 \pm 0.03$	$1.19 \pm 0.04$	$0.98 \pm 0.06$

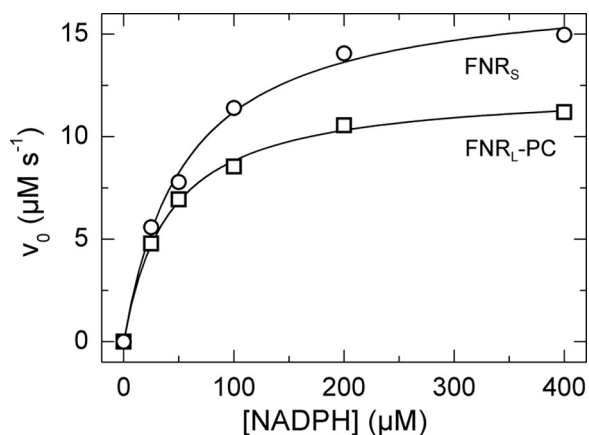


FIGURE 3. Ferricyanide reductase activities of FNR<sub>S</sub> and FNR<sub>L</sub>-PC. K<sub>3</sub>[Fe(CN)<sub>6</sub>] was premixed with 0.025–0.1 μM FNR<sub>S/L</sub> in 150 mM KP pH 8 and 5 mM MgCl<sub>2</sub> at room temperature. The reaction was started by addition of 25–400 μM NADPH. For the FNR<sub>L</sub>-PC preparation, at least two measurements have been carried out at each given point. The data were normalized to an FNR<sub>S/L</sub> concentration of 0.1 μM and were fitted with the Henri-Michaelis-Menten equation: For FNR<sub>S</sub> (open circles) and FNR<sub>L</sub>-PC (open squares),  $K_{m(\text{NADPH})}$  values were  $55 \pm 5 \mu\text{M}$  and  $40 \pm 3 \mu\text{M}$ , respectively. The turnover number  $k_{\text{cat}}$  for FNR<sub>S</sub> and FNR<sub>L</sub>-PC were  $174 \pm 5 \text{ s}^{-1}$  and  $124 \pm 3 \text{ s}^{-1}$ , respectively.

It has been recently reported that two types of PBS could be found in *Synechocystis*, the conventional one that contains L<sub>RC1</sub> encoded by *cpcG1* and another one that lacks the core substructure and contains the *cpcG2* encoded L<sub>RC2</sub> (40, 41). We analyzed the L<sub>RC</sub> polypeptide contained in the FNR<sub>L</sub>-PC complex by MALDI-TOF mass spectrometry, and this polypeptide was identified as L<sub>RC1</sub>.

**Ferricyanide Reductase Activity**—NADPH oxidase activity (also called diaphorase activity) was used to measure the FNR turnover and its affinity for NADPH, in the presence of the artificial electron acceptor potassium ferricyanide. The diaphorase reaction starts with binding of NADPH to FNR, which is followed by the formation of a charge-transfer complex and then hydride transfer from NADPH to FAD (42). Electrons are then transferred to ferricyanide in a non-rate-limiting one-electron reaction. Initial enzyme velocities were plotted as a function of NADPH concentration and fitted according to the Henri-Michaelis-Menten equation (Fig. 3). Both  $K_{m(\text{NADPH})}$  and  $k_{\text{cat}}$  were found to be 30% smaller in FNR<sub>L</sub>-PC than in FNR<sub>S</sub>, which results in similar catalytic efficiencies (Table 2).

**Ferredoxin-mediated Cytochrome c Reductase Activity**—This NADPH oxidase activity was used to measure the affinity for Fd and the turnover of FNR in the presence of its natural electron acceptor Fd. After hydride transfer from NADPH to FNR, electrons flow to Fd, which is then reoxidized by cyt c. To obtain specific information about the Fd reduction step, the initial

TABLE 2

Catalytic properties of the FNR<sub>L</sub>-PC and FNR<sub>S</sub> isoforms

FNR catalytic properties (unit)	FNR <sub>L</sub> -PC	FNR <sub>S</sub>
<b>Ferricyanide reductase activity</b>		
$K_{m(\text{NADPH})}$ (μM)	$40 \pm 3$	$55 \pm 5$
$k_{\text{cat}}$ (s <sup>-1</sup> )	$124 \pm 3$	$174 \pm 5$
$k_{\text{cat}}/K_{m}$ (μM <sup>-1</sup> s <sup>-1</sup> )	$3.1 \pm 0.3$	$3.2 \pm 0.4$
<b>Cytochrome c reductase activity</b>		
$K_{m(\text{Fd})}$ (μM)	$47 \pm 6$	$28 \pm 2$
$k_{\text{cat}}$ (s <sup>-1</sup> )	$144 \pm 12$	$154 \pm 6$
$k_{\text{cat}}/K_{m}$ (μM <sup>-1</sup> s <sup>-1</sup> )	$3.1 \pm 0.7$	$5.5 \pm 0.6$
<b>Single reduction of FNR by Fd<sub>red</sub> no NADP<sup>+</sup></b>		
Second-order forward rate $k_1$ (μM <sup>-1</sup> s <sup>-1</sup> )	14.1	24.9
Second-order reverse rate $k_{-1}$ (μM <sup>-1</sup> s <sup>-1</sup> )	4.6	10.6
$K_{\text{eq}} = k_1/k_{-1}$	3.06	2.35
$E_{m(\text{FNRox}/\text{FNRsq})}^a$ (mV)	-384	-390
<b>1 mM NADP<sup>+</sup></b>		
Second-order forward rate $k_1$ (μM <sup>-1</sup> s <sup>-1</sup> )	10.8	15.2
Second-order reverse rate $k_{-1}$ (μM <sup>-1</sup> s <sup>-1</sup> )	4.0	5.4
$K_{\text{eq}} = k_1/k_{-1}$	2.73	2.84
$E_{m(\text{FNRox}/\text{FNRsq})}^a$ (mV)	-386	-385
<b>Multiple turnover: Initial rate of reoxidation of 3.75 μM Fd<sub>red</sub> (reoxidized Fd<sub>red</sub> per second and per FNR)</b>		
Observed rate	50	$53/(330)^b$
Calculated rate (with limiting $k_1$ )	40.5	$57/(1564)^b$

<sup>a</sup> Vs NHE. Calculated assuming  $E_{m(\text{Fdox}/\text{Fdred})} = -412 \text{ mV}$  (44).

<sup>b</sup> Numbers in italics were obtained at low ionic strength and recalculated from Ref. 5 by using an absorption coefficient of  $9,000 \text{ M}^{-1}\text{cm}^{-1}$  for FNR<sub>S</sub> at 461 nm instead of  $10,800 \text{ M}^{-1}\text{cm}^{-1}$  (giving e.g.  $k_1 = 417 \mu\text{M}^{-1}\text{s}^{-1}$ ).

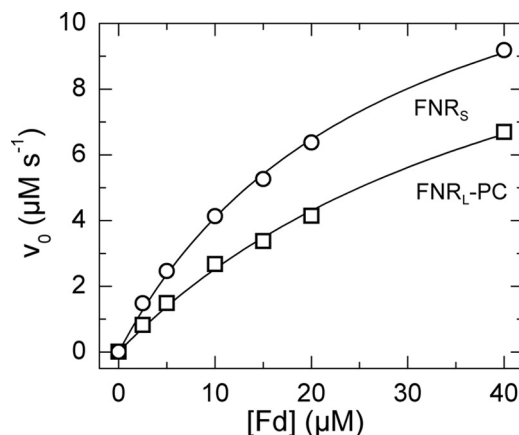
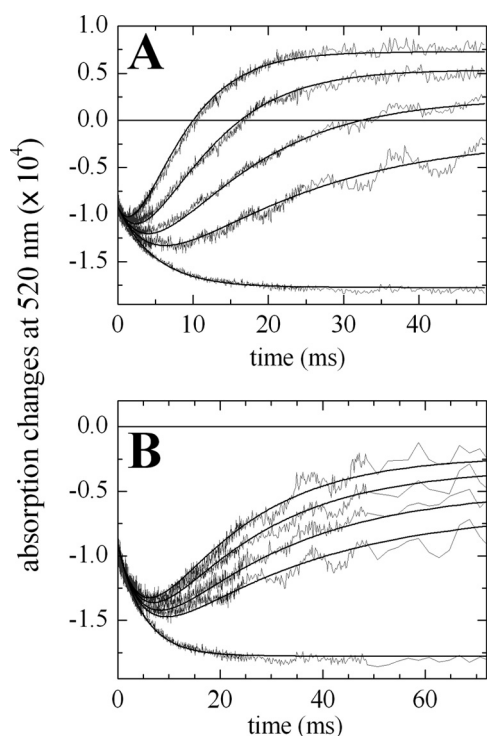


FIGURE 4. Fd-mediated cyt c reductase activities of FNR<sub>S</sub> and FNR<sub>L</sub>-PC. FNR<sub>S/L</sub> was mixed with 2.5–40 μM Fd from *T. elongatus* and 40 μM cyt c in 150 mM KP buffer pH 8 at 25 °C. The reaction was started by injection of 400 μM NADPH. The data were normalized to an FNR<sub>S/L</sub> concentration of 0.1 μM. For FNR<sub>S</sub> (open circles) and FNR<sub>L</sub>-PC (open squares), the  $K_{m(\text{Fd})}$  values were  $28 \pm 2 \mu\text{M}$  and  $47 \pm 6 \mu\text{M}$ , respectively. The turnover number  $k_{\text{cat}}$  for FNR<sub>S</sub> and FNR<sub>L</sub>-PC were  $154 \pm 6 \text{ s}^{-1}$  and  $144 \pm 12 \text{ s}^{-1}$ , respectively.

enzyme velocities were obtained by varying the amount of Fd under saturating concentrations of NADPH and cyt c. The initial rates of cyt c reduction were plotted as a function of Fd concentrations (Fig. 4) and fitted after the Henri-Michaelis-Menten equation. Table 2 highlights the similarities and differences between the two FNR isoforms: similar  $k_{\text{cat}}$  values in both isoforms,  $K_{m(\text{Fd})}$  70% larger and catalytic efficiency 44% smaller in FNR<sub>L</sub>-PC than in FNR<sub>S</sub> were found.

**Single Electron Transfer from Reduced Ferredoxin**—The kinetics of FNR reduction in the ternary mixture PSI/Fd/FNR were measured by flash absorption spectroscopy (5). After the actinic flash has triggered a charge separation in PSI, an electron transfer cascade is occurring toward FNR, via Fd. The

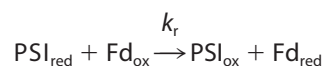
## FNR<sub>L</sub>-Phycocyanin Complex



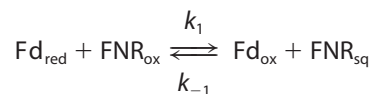
**FIGURE 5. Flash titration of FNR<sub>S</sub> and FNR<sub>L</sub>-PC in the presence of 1 mM NADP<sup>+</sup> under single reduction conditions.** The absorption changes at 520 nm are attributed to electron acceptors as the P700<sup>+</sup> formation and decay have been subtracted. The solid line at zero level corresponds to the baseline before the flash. Concentrations of PSI and Fd were 0.475 μM and 3.96 μM. *A*, concentrations of FNR<sub>S</sub> were 0, 2.0, 4.0, 8.0, and 16.0 μM for curves from bottom to top. The best fit resulted in  $k_r = 50 \mu\text{M}^{-1} \text{s}^{-1}$ ,  $k_1 = 15.2 \mu\text{M}^{-1} \text{s}^{-1}$ , and  $k_{-1} = 5.4 \mu\text{M}^{-1} \text{s}^{-1}$ . *B*, concentrations of FNR<sub>L</sub>-PC were 0, 1.6, 2.1, 3.0, and 3.7 μM for curves from bottom to top. The best fit resulted in  $k_r = 50 \mu\text{M}^{-1} \text{s}^{-1}$ ,  $k_1 = 10.8 \mu\text{M}^{-1} \text{s}^{-1}$ , and  $k_{-1} = 4.0 \mu\text{M}^{-1} \text{s}^{-1}$ .

measurements were performed in the absence/presence of NADP<sup>+</sup> and, in order to favor single FNR reduction, FNR was in large excess over PSI (and therefore over photoreduced Fd). The kinetics in the presence of 1 mM NADP<sup>+</sup> are shown in Fig. 5A for different FNR<sub>S</sub> concentrations, together with a control experiment without FNR<sub>S</sub>. The signals are only due to reduction of the PSI electron acceptors, Fd and FNR as the P700<sup>+</sup> contribution has been eliminated (see “Experimental Procedures”). In the control experiment (bottom trace), two different kinetic components are present. The fastest component is not time resolved and is attributed to the formation of the reduced PSI terminal acceptor (F<sub>A</sub>, F<sub>B</sub>)<sup>-</sup>, as confirmed by a sample without Fd (data not shown). A very small and fast submicrosecond to microsecond signal due to Fd reduction (<10% of the full Fd reduction signal) was observed, corresponding to a small amount of PSI:Fd complex present before flash excitation (data not shown; see also Ref. 43). This is in line with the large dissociation constant  $K_d$  that is expected for the PSI:Fd complex under our conditions (150 mM potassium phosphate, pH 8). The slowest millisecond absorption decrease is due to Fd reduction by a second-order diffusion-limited process. In the presence of FNR<sub>S</sub>, the same initial fast decay is observed but a signal increase, which is ascribed to the reduction of FNR<sub>S</sub> by Fd<sub>red</sub>, dominates the subsequent absorption changes. The rates and the final amplitudes of this signal increase when FNR<sub>S</sub> con-

centration increases. The kinetic model we used to simulate the observed kinetics is shown in Reactions 1 and 2.



REACTION 1



REACTION 2

In Reaction 1, PSI<sub>red</sub> stands for PSI with the terminal acceptor (F<sub>A</sub>, F<sub>B</sub>) reduced. Reduction of (F<sub>A</sub>, F<sub>B</sub>) occurs in the submicrosecond time range and thus does not need to be considered. This kinetic model can be analytically solved in a simplified version when all reactions are considered as pseudo first-order (see “Experimental Procedures” for the solution equations). This allowed us to perform a global fit analysis resulting in the following values:  $k_r = 50.0 \mu\text{M}^{-1} \text{s}^{-1}$ ,  $k_1 = 15.2 \mu\text{M}^{-1} \text{s}^{-1}$ ,  $k_{-1} = 5.4 \mu\text{M}^{-1} \text{s}^{-1}$ . The redox equilibrium constant  $K_{\text{eq}} = 2.84$  can be calculated for Reaction 2 ( $k_1/k_{-1}$ ). This constant is related to the difference in midpoint redox potentials ( $E_m$ ) of the reaction partners in Equation 11.

$$\Delta E_m = E_{m(\text{FNR}_{\text{ox}}/\text{FNR}_{\text{sq}})} - E_{m(\text{Fd}_{\text{ox}}/\text{Fd}_{\text{red}})} = (RT/F) \ln(K_{\text{eq}}) \quad (\text{Eq. 11})$$

Assuming  $E_m = -412$  mV for *Synechocystis* Fd (44), one gets  $-385$  mV for  $E_{m(\text{FNR}_{\text{ox}}/\text{FNR}_{\text{sq}})}$ , which is very close to the value of  $-382$  mV previously determined under conditions of moderate ionic strength (30 mM NaCl, 5 mM MgCl<sub>2</sub>; the value of  $-378$  mV in Ref. 5 has been recalculated using  $\epsilon_{461 \text{ nm}} = 9,000 \text{ M}^{-1} \text{cm}^{-1}$  for FNR). It should be noted that measuring  $E_{m(\text{FNR}_{\text{ox}}/\text{FNR}_{\text{sq}})}$  by standard methods is fairly difficult because the thermodynamically unstable semiquinone (45) is only marginally observed during a redox titration.

The same experiment was performed with FNR<sub>L</sub>-PC and the corresponding kinetics are shown in Fig. 5B. Fitting these data gave similar values of  $K_{\text{eq}}$  and hence of  $E_{m(\text{FNR}_{\text{ox}}/\text{FNR}_{\text{sq}})}$ , whereas the second-order rate constants were 25–30% smaller than with FNR<sub>S</sub> (Table 2). The smaller rate constants measured with FNR<sub>L</sub>-PC are in accordance with its larger  $K_m(\text{Fd})$  observed in the ferredoxin-mediated cyt *c* reduction assay. The above experiments were repeated in the absence of NADP<sup>+</sup>, to compare the kinetics in the presence or absence of a ternary complex Fd/FNR/NADP<sup>+</sup>. These results are summarized in Table 2: For both isoforms the  $E_m$  is very similar to those measured in the presence of NADP<sup>+</sup>. This indicates that the electrostatic environment of FAD is not modified by NADP<sup>+</sup>. In contrast, the  $k_1$  and  $k_{-1}$  rates are larger in the absence of NADP<sup>+</sup>, in agreement with a previous study, where this effect was attributed to a repulsive electrostatic effect between the phosphate moiety of NADP<sup>+</sup> and the negatively charged Fd (5).

**Catalytic Turnover of the Two FNR Isoforms during NADP<sup>+</sup> Reduction**—We also measured FNR-reduction kinetics obtained for a ternary mixture PSI/Fd/FNR in the presence of

NADP<sup>+</sup> under multiple catalytic turnover conditions (5). These conditions were met by using PSI in large excess over FNR (3.75 μM versus 0.15/0.3 μM). Fd (8 μM) is also added in excess over PSI so that Fd<sub>red</sub> at a PSI equivalent concentration, is rapidly formed after PSI photoexcitation. Fd<sub>red</sub> is then slowly monoexponentially reoxidized by FNR (rate  $k_{\text{FNR}}$ ). Taking into account the decay without FNR (rate  $k_{\text{no FNR}}$ ), we obtained values of 2.2 and 4.1 s<sup>-1</sup> for ( $k_{\text{FNR}} - k_{\text{no FNR}}$ ) with 0.15 and 0.30 μM FNR<sub>S</sub>, respectively. This corresponds to 55 and 51 reoxidized Fd<sub>red</sub> per second and per FNR<sub>S</sub>, respectively and the average value of 53 is indicated in Table 2. Using the same enzyme concentrations, the ( $k_{\text{FNR}} - k_{\text{no FNR}}$ ) rates are quite similar to those of FNR<sub>L</sub>-PC (2.14 and 3.69 s<sup>-1</sup>). This corresponds to an averaged value of 50 reoxidized Fd<sub>red</sub> per second and per FNR<sub>L</sub>-PC (Table 2). Overall, the multiple turnover rates are similar for the two isoforms.

As the second-order rate constants  $k_1$  measured for the first FNR reduction are rather small under our conditions (see "Discussion"), it is worth checking if this process could be rate limiting during the catalysis (see Equation 10 under "Experimental Procedures"). The similarity between the calculated and measured turnover rates (Table 2) indicates that this is indeed the case. Thus we have identified under our conditions a limiting step which has not been identified previously. As a control, we also considered FNR<sub>S</sub> under low ionic strength conditions (5) in order to compare the measured and calculated turnovers (bracketed values in Table 2). The 5-fold excess in calculated versus measured turnover shows that in this case of faster turnover, FNR<sub>S</sub> reduction by Fd<sub>red</sub> is not rate limiting.

## DISCUSSION

Based on the observation that in *Synechocystis* FNR<sub>S</sub> accumulates only under heterotrophic or starvation conditions whereas FNR<sub>L</sub> is the major isoform detected under photoautotrophic conditions (24), it was proposed that each isoform plays a specific role. In this work, we have shown that under photoautotrophic conditions the NADP<sup>+</sup>/NADPH ratio is higher in a mutant containing only FNR<sub>S</sub>. Furthermore this observation cannot be attributed to a different PSI/PSII ratio as the ratio was shown to be unchanged in FS1 compared with that of the wild type. This reinforces the hypothesis that the FNR isoforms have different roles. FS1 seems unable to accumulate the NADPH amounts produced in the strains (wild type and MI6) where FNR<sub>L</sub> is the main isoform. This also explains the fact that photoautotrophic growth is impaired in FS1, while MI6 growth is similar to that of the wild type (24). We decided to check whether the *in vivo* differences could be explained by the *in vitro* properties of the two FNR isoforms. In other words, is there any selectivity of the two isolated FNR isoforms for NADP<sup>+</sup> reductase versus NADPH oxidase activities? Such a selectivity has been observed in the case of root and leaf FNR isoforms in plants (3, 12, 14–17, 46).

**Purification of an L<sub>RC</sub>-containing FNR<sub>L</sub>:PC Complex**—The best compromise between approaching the *in vivo* situation and feasibility (stability, compatibility with absorption-spectroscopy studies) was to obtain a PBS subcomplex containing FNR<sub>L</sub> and a PC hexamer ( $\alpha^{\text{PC}}, \beta^{\text{PC}}$ )<sub>6</sub>. The purification was facilitated by a His tag in the hinge domain of FNR<sub>L</sub>. We obtained a

pure and homogeneous complex, as judged by gel filtration, SDS-PAGE analysis, and FAD content. The stoichiometry of FNR<sub>L</sub>:L<sub>RC</sub>:( $\alpha^{\text{PC}}, \beta^{\text{PC}}$ )<sub>6</sub> in the 300-kDa complex was found to be 1:1:1. Furthermore it was verified that the L<sub>RC</sub> polypeptide present in FNR<sub>L</sub>-PC was encoded by *cpcG1*, which was expected since conventional PBS were used for its purification. It was recently proposed, using single particle analysis of CBH PBS, that FNR<sub>L</sub> is located at the interface between the rod and the core (22). From our purification data, it can be further concluded that FNR<sub>L</sub> is bound at only one of the PC hexamers, with no major involvement of the other hexamers. The complex was stable, for at least 2 weeks at 4 °C, with no proteolysis of FNR<sub>L</sub>. This is probably due to protection of the FNR<sub>L</sub> linker-domain by the PC hexamer.

**Effect of High Ionic Strength on the Catalytic Properties of FNR<sub>S</sub>**—With the aim of comparing FNR<sub>S</sub> and FNR<sub>L</sub>-PC, we performed a broad set of measurements on NADPH-oxidase and NADP<sup>+</sup>-reductase activities of the two isoforms as summarized in Table 2. These measurements were performed under high ionic strength conditions (150 mM potassium phosphate) because such conditions are necessary to stabilize the FNR<sub>L</sub>-PC complex. We compared our data to those previously obtained for cyanobacterial FNR<sub>S</sub> at lower ionic strength. The NADPH-oxidase catalytic parameters (measured via ferricyanide reduction) are quite similar to those previously reported for FNR<sub>S</sub> from *Anabaena* sp. ( $k_{\text{cat}}$  20% smaller,  $K_{m(\text{NADPH})}$  about 2-fold greater in our case; Ref. 47). This implies that this ionic strength is not detrimental for diaphorase activity. Regarding the ferredoxin-mediated NADPH-oxidase activity, we found a 3-fold increase in  $K_{m(\text{Fd})}$  and only a 25% decrease in  $k_{\text{cat}}$  between our measurements and those previously measured in the same report with *Anabaena* FNR<sub>S</sub> (47). The  $K_{m(\text{Fd})}$  increase can be attributed to the screening of electrostatic interactions occurring at high ionic strength between FNR and Fd (2). A similar screening effect explains our data concerning FNR<sub>S</sub> reduction by Fd<sub>red</sub> when compared with a previous study, conducted under lower ionic strength (5): the second-order rate constant  $k_1$  of single FNR<sub>S</sub> reduction by Fd<sub>red</sub> is 28-fold smaller and during multiple turnover, the rate of Fd<sub>red</sub> reoxidation is 6 times smaller in the present study. We also obtained evidence that at high ionic strength,  $k_1$  is rate limiting during multiple turnover, which is not the case at lower ionic strength. Under these last conditions, the enzyme turnover is much faster and is limited by one of the first-order processes (Fd<sub>ox</sub> dissociation, hydride transfer, or NADPH release).

**Comparison of the Catalytic Properties of FNR<sub>S</sub> and FNR<sub>L</sub>-PC: an Analogous System to Leaf and Root FNR Isoforms?**—The following catalytic parameters are quite similar for the two *Synechocystis* isoforms: the catalytic efficiency ( $k_{\text{cat}}/K_m$ ) of NADPH/ferricyanide oxidoreduction, the  $k_{\text{cat}}$  of the Fd-mediated cyt *c* reduction, the  $E_m$  (FNR<sub>ox</sub>/FNR<sub>sq</sub>) in the presence/absence of NADP<sup>+</sup> and the initial reoxidation rate of Fd<sub>red</sub> by FNR during multiple catalytic turnover. Differences between the two isoforms were observed: 30% smaller  $K_{m(\text{NADPH})}$  and  $k_{\text{cat}}$  of FNR<sub>L</sub>-PC versus FNR<sub>S</sub> during NADPH/ferricyanide oxidoreduction, a 70% larger  $K_{m(\text{Fd})}$  and a 44% smaller catalytic efficiency of FNR<sub>L</sub>-PC for the Fd-mediated cyt *c* reductase activity, and a 29/43% (NADP<sup>+</sup> present/absent) decrease in  $k_1$ ,



## FNR<sub>L</sub>-Phycocyanin Complex

the rate of single FNR reduction by Fd<sub>red</sub>, for FNR<sub>L</sub>-PC (Table 2). The slight decrease in  $K_{m(\text{NADPH})}$  and  $k_{\text{cat}}$  during ferricyanide reduction indicates that the presence of the PC hexamer slightly modifies the association of NADPH to FNR and/or the following steps leading to FAD reduction. The increase in  $K_{m(\text{Fd})}$  and the decrease in  $k_1$  are likely due to a steric hindrance by, or a conformational effect due to, the PC hexamer moiety of FNR<sub>L</sub>-PC. We favor these explanations over electrostatic repulsion brought by PC, because electrostatic interactions are expected to be of limited importance at high ionic strength. Moreover, the electrostatic environment of FAD appears to be unmodified in FNR<sub>L</sub>-PC as judged by the similar  $E_{m(\text{FNRox}/\text{FNRsq})}$  measured for the two isoforms. Overall, the results obtained for the Fd-mediated cyt *c* reductase activity are in agreement with the different predicted roles for the FNR isoforms. Both isoforms are capable of catalyzing NADP<sup>+</sup> reduction under multiple turnover conditions. Furthermore, we obtained indications that in our conditions electron transfer from Fd<sub>red</sub> is rate limiting.

The leaf and root FNR isoforms from plants differ mainly in four catalytic or thermodynamic parameters (supplemental Table S1): 1)  $K_{m(\text{NADPH})}$ , measured via ferricyanide reduction, is 3–10-fold higher, depending on the authors, for leaf FNR compared with root FNR (14, 16). We observed a 30% decrease in FNR<sub>L</sub>-PC versus FNR<sub>S</sub>. If we tentatively make the parallel between FNR<sub>L</sub>-PC and leaf FNR on the one hand, and between FNR<sub>S</sub> and root FNR on the other hand, the situation seems reversed; 2)  $K_{m(\text{leaf Fd})}$ , measured by Fd-mediated cyt *c* reduction, is 5–10-fold smaller for leaf FNR than for root FNR (3, 14, 17, 46). The 70% difference that we observe is also in the unexpected direction; 3)  $k_{\text{cat}}$  of the Fd-mediated cyt *c* reduction is 3–4-fold smaller for leaf FNR than for root FNR (3, 14, 17, 46). We observed no significant difference in this parameter; 4) The  $E_{m(\text{FNRox}/\text{FNRred})}$  couple, 2-electron reduction) of corn root FNR is 20 mV higher than the midpoint potential of spinach leaf FNR (15). Unfortunately, no comparison is available for two FNR isoforms from the same plant, to our knowledge. We observed no significant difference for  $E_{m(\text{FNRox}/\text{FNRsq})}$  between the two *Synechocystis* isoforms.

Contrary to the case of *Synechocystis*, there are some differences in the catalytic domains of the plant FNR isoforms. Moreover, the existence of Fd isoforms is essential when comparing the processes of NADP<sup>+</sup> reduction and NADPH oxidation in leaves and roots. The  $E_m$  of root Fd was found to be much higher than that of leaf Fd (50–100 mV difference; Refs 17, 46, 48, 49). This probably favors NADPH oxidation in roots versus NADP<sup>+</sup> reduction in leaves. Many different Fd encoding genes have been identified in *Synechocystis* (50). In the present work we studied the major Fd encoded by *fed1*. This Fd shares with the leaf Fd a similar redox potential but both root and leaf Fds appear to be phylogenetically equally distant to the *Synechocystis* Fd (17). At our present state of knowledge, there is no equivalent of the root Fd in cyanobacteria, in terms of redox potential, and the major photosynthetic Fd is generally thought to be involved in all bioenergetically significant electron flows. This was also a basic assumption in our approach. However, the involvement of other Fd isoforms under heterotrophic conditions cannot be excluded.

## CONCLUSION

From recent data (24), specific roles were proposed for the two *Synechocystis* FNR isoforms, which seem to parallel the enzymatic selectivity of plant FNR root and leaf isoforms. Such specificity is also supported by the change in the NADP<sup>+</sup>/NADPH ratios that we measured in *Synechocystis* cells containing only one of the isoforms. However, from a detailed functional characterization, we observed small differences in the NADP<sup>+</sup> reductase and NADPH oxidase activities of FNR<sub>S</sub> and FNR<sub>L</sub>-PC complex. This contrasts with the much larger *in vitro* differences observed between leaf and root FNR isoforms from plants.

If the main photosynthetic Fd (50) is involved *in vivo* in electron transfer with both isoforms (see above), the differences that we observe might not fully explain the *in vivo* properties of the *Synechocystis* mutants expressing only one of the isoforms. Besides the intrinsic catalytic properties of those isoforms, it would be necessary to invoke their localization or association to other complexes. For example, it can be speculated that FNR<sub>S</sub> is involved in cyclic/respiratory electron flow because it is free to bind to other membrane complexes such as NADPH dehydrogenase or cytochrome *b<sub>6</sub>f*. Conversely, PBS-bound FNR<sub>L</sub> cannot play such a role and is therefore dedicated to NADP<sup>+</sup> photoreduction. For both types of activities, substrates availability (Fd<sub>red</sub>/Fd<sub>ox</sub> and NADP<sup>+</sup>/NADPH) might also be key *in vivo* characteristics for the activity of the two isoforms. This situation would be reminiscent of what has been described recently for the different leaf isoforms, where catalytic activities appear to depend upon their variable attachment to the thylakoid membrane (51, 52).

In this context, it would be worth studying the involvement of CpcG2-PBS, which lacks the PBS core, in binding FNR<sub>L</sub>, as it has been hypothesized to be directly bound to PSI (53). However the small amount of CpcG2-PBS versus CpcG1-PBS in the wild type (40) and the effect of *cpcG2* disruption on the PSI/PSII ratio (41) are obstacles, which have to be surmounted for such studies. Further *in vivo* measurements are needed to better understand the reason for which FNR binds the PBS in the majority of PBS-containing cyanobacteria.

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## Abstract

In photosynthetic organisms, ferredoxin:NADP oxidoreductase (FNR) provides NADPH for CO<sub>2</sub> assimilation, but it also utilizes NADPH to provide reduced ferredoxin (Fd). The cyanobacterium *Synechocystis* sp. strain PCC6803 contains two FNR isoforms, a small (FNR<sub>S</sub>, 34 kDa) and a large one (FNR<sub>L</sub>, 46 kDa) that is associated with the phycobilisome (PBS), a light-harvesting complex. We purified a PBS subcomplex comprising FNR<sub>L</sub> (FNR<sub>L</sub>-PC) and compared the enzymatic properties of FNR<sub>L</sub>-PC to FNR<sub>S</sub>. FNR<sub>L</sub>-PC exhibits an increased NADPH affinity, and a decreased Fd affinity in agreement with its predicted lower activity of Fd reduction. FNR<sub>L</sub>-PC shows also a decrease in the rate of single electron transfer (ET) from Fd<sub>red</sub>. Most of the obtained differences are attributed to steric hindrance by the phycocyanin moiety of FNR<sub>L</sub>-PC. Both isoforms seem to catalyze the NADP<sup>+</sup> reduction under multiple turnover conditions to the same extent and we obtained evidence that, under our high ionic strength conditions, ET from Fd<sub>red</sub> is rate limiting. During *in vivo* studies, we presented evidence supporting an increase of NADPH oxidation by respiratory or cyclic ET in a mutant lacking FNR<sub>L</sub> and a similar behavior was observed for the wild type under low CO<sub>2</sub>. The measurements clearly showed that FNR<sub>S</sub> is implicated in this alternative ET and we propose that FNR<sub>S</sub> attaches to NDH-1 acting as the dehydrogenase module. FNR localization and/or substrate availability seem to be essential so that FNR isoforms perform their respective roles *in vivo*. Future studies should result in a more complete *in vivo* view and clarify the important issue of the NADPH-dependent ET into the PQ pool that seems to be enhanced by FNR<sub>S</sub>.

## Résumé

Dans les organismes photosynthétiques, la ferrédoxine:NADP oxydoréductase (FNR) fournit le NADPH nécessaire à l'assimilation du CO<sub>2</sub>, mais elle réduit aussi la ferrédoxine (Fd) à partir du NADPH. La cyanobactérie *Synechocystis* sp. PCC6803 contient deux isoformes de FNR: une forme courte (FNR<sub>S</sub>, 34 kDa) et une forme longue (FNR<sub>L</sub>, 46 kDa) qui est liée au phycobilisome (PBS), un complexe collecteur de lumière. Nous avons purifié un sous-complexe du PBS qui contient la FNR<sub>L</sub> (FNR<sub>L</sub>-PC) et comparé les propriétés enzymatiques de FNR<sub>L</sub>-PC à FNR<sub>S</sub>. Par rapport à FNR<sub>S</sub>, FNR<sub>L</sub>-PC présente des affinités plus faible/forte pour le NADPH/la Fd, conformément aux prédictions des activités relatives des deux isoformes. La plupart des différences observées sont attribuées à l'encombrement stérique amené par la phycocyanine dans FNR<sub>L</sub>-PC. En conditions de turnover multiple, les deux isoformes catalysent de la même manière la réduction de NADP<sup>+</sup> et de plus le transfert d'électrons (TE) depuis Fd<sub>red</sub> est limitant à force ionique élevée. Lors d'études *in vivo*, nous avons observé une augmentation de l'oxydation du NADPH par TE respiratoire ou cyclique chez un mutant ne contenant que la FNR<sub>S</sub> et chez le type sauvage à faible CO<sub>2</sub>. Les mesures ont montré clairement que FNR<sub>S</sub> est impliqué dans ce TE alternative et nous proposons que FNR<sub>S</sub> constitue le module déshydrogénase de NDH-1. La localisation de la FNR et/ou la présence de substrats semblent être essentiels dans les rôles respectifs des isoformes de FNR *in vivo*. Des études futures devraient nous donner une vue plus complète des processus de TE *in vivo* et clarifier le rôle du TE dépendant du NADPH et favorisé par FNR<sub>S</sub> dans la réduction du pool de PQ.