Ferrodoxin: NADP+ reductase and photosynthetic energy transduction in Cyanobacteria

van Thor, J.J.

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CHAPTER 8

General conclusions
Synechocystis PCC 6803 is a cyanobacterium that is well suited as a model organism for studying photosynthetic energy transduction. The composition of the reaction centers and the light harvesting complexes are known, and the entire genome sequence is available. The ability to introduce foreign DNA, via natural transformation, greatly facilitates the study on energy transduction mechanisms. However, once a mutation is introduced, complete segregation of the mutant genotype has to occur in order to obtain a stable mutant. The ‘physiological back-pressure’ will determine the rate at which a certain mutation segregates. For example, deletion of the petH gene proved not to segregate (see chapter 2). Apparently, loss of FNR results in a non-viable, or slow-growing, strain. Others have been able to obtain a petH deletion mutant, in a photosystem I-deficient background of Synechocystis PCC 6803. This double mutant, however, could not be cultured once complete segregation had occurred (W. Vermaas; personal communication). Perhaps FNR has a vital function in scavenging radicals, as the FNR protein of Escherichia coli does. Other explanations for the phenotypic behaviour of this double mutant cannot easily be found. The replacement of the wildtype petH gene with a version that lacks the 5' sequence encoding the CpcD homologous N-terminal domain, is a mutation that efficiently segregates. The phenotype of the resulting mutant has proved to be instructive in understanding the possible physiological functions of the third domain of Synechocystis PCC 6803 FNR.

The experiments presented in this work have led to the conclusion that the N-terminal domain of the cyanobacterial FNR has an effect on one particular electron transport reaction: the reduction of the intersystem chain during photosystem I dependent cyclic electron transport (see chapter 3). Since FNR catalyses several physiologically significant reactions, our goal was to define under which conditions what type of reaction occurs.

We found that under conditions of standard photoautotrophic growth the expression level of petH mRNA (that we determined to be correlated to the protein levels) is relatively constant in the wildtype strain. The mRNA abundance is regulated by the availability of light (chapters 2 and 6), and is even under control of the circadian clock (chapter 6). The ratio of FNR per photosystem I particles is close to 1 under standard photoautotrophic growth conditions, and a large fraction of these molecules are present in the phycobilisomes (chapter 3).

Under conditions of salt stress the petH mRNA was overexpressed, resulting in elevated levels of FNR (chapters 2 and 3). This condition was correlated with an induction of the rate of photosystem I dependent cyclic electron transport. The extrusion of ions from the cell requires ATP which is synthesised by increasing the rate of PSI-dependent cyclic electron transport. The phycobilisomes isolated from salt-stressed cells do not contain significantly higher concentrations of FNR; the average number of FNR molecules per complex remains approximately 2. Therefore, the ‘additional’ (accumulated) FNR molecules represent a ‘new’ pool that differs from the pool that is present under standard photoautotrophic growth conditions. It was found that the N-terminal ‘CpcD-homologous’ domain of FNR specifically directs binding to the thylakoid membrane as well as to the phycobilisomes: N-terminally truncated FNR no longer associated with the membranes, and Green Fluorescent Protein associated only
with the membranes when fused to the ‘CpcD-homologous’ N-terminal domain of FNR (chapter 3). Therefore under conditions of overexpression of FNR, the ‘additional’ molecules are free to associate with the thylakoid membrane. The actual binding site is still not known: membranes from \textit{ndhB} and \textit{psaE} mutant strains still bind FNR, excluding the NAD(P)H dehydrogenase complex and the stromal photosystem I subunit PsaE. In addition, GFP molecules that were fused to the N-terminal domain of FNR did not seem to compete for binding to the thylakoid membranes with wildtype FNR already present in these membranes. Therefore the number of binding sites is probably higher than the number of photosystem I reaction centers (chapter 3).

An attempt was made to reconstitute fluorescently labelled recombinant wildtype FNR into thylakoid membranes, in order to determine the actual number of binding sites. The fluorescence polarisation was used in order to determine if a complex was formed. However, no interaction could be detected with this method. Each FNR molecule contained on average 10 fluoresceine groups, a labelling ‘density’ that is essential to detect the fluorescence polarisation at a nanomolar concentration of the conjugated protein. Probably, the chemical conjugation of this number of amine-groups of the protein with fluorescent label also destroyed its ability to bind to the thylakoid membranes. In principle it should be possible to determine the number of binding sites the thylakoid membrane has for (FNR-) N-terminal domains by titrating the recombinant fusion protein between GFP and the N-terminal domain of FNR. This fusion protein could then be conveniently detected by Western blot analysis, as shown in chapter 3.

The presence of FNR in the thylakoid membrane was correlated with an increase of the rate of PSI dependent cyclic electron transport. After removal of the N-terminal domain from the protein, no increase of the activity of PSI dependent cyclic electron transport was observed when overexpression of the truncated gene was induced (chapter 3). Furthermore, an \textit{in vitro} assay of NADPH dependent cytochrome \textit{c} reduction demonstrated the ability of wildtype FNR to donate electrons to the intersystem chain in isolated thylakoid membranes of wildtype \textit{Synechocystis PCC 6803}. The turnover number of FNR in these assays is about 0.6 e$^{-}$s$^{-1}$, showing that even though the equilibrium constant is clearly in favour of plastoquinone reduction (see also Figure 1), the ‘forward’ electron transport rate constant is very slow, indicative of a poorly catalysed reaction (chapter 3). The free energy difference between FNR and the plastoquinone pool is higher than -400 mV (see Figure 1). The slow forward rate constant may partly be explained as the result of this reaction being in the ‘Marcus inverted region’ (the Gibbs free energy is larger than the reorganisation energy in the ‘Marcus inverted region’, resulting in a decrease of the forward electron transport rate constant; see Moser \textit{et al.}, 1992. Nature 355: 796-802). However, the reorganisation energy for the reaction was not determined. More likely, an unfavourable distance between the flavin group of FNR and the plastoquinone molecules (or other intermediates) result in a slow rate of electron transport.

These kinetics of cytochrome \textit{c} reduction were determined \textit{in vitro}, and are not necessarily representative for the \textit{in vivo} turnover numbers. In an NAD(P)H
FIGURE 1 ELECTRON TRANSPORT CHAIN OF PHOTOSYSTEM I DEPENDENT CYCLIC PATHWAYS

Scheme presenting the midpoint potentials of the redox-active groups and intermediates that are involved in photosystem I dependent cyclic electron transport. It is assumed that reduction of the intersystem chain by either FNR or ferredoxin involves the direct or indirect reduction of plastoquinone.

dehydrogenase deficient background, overexpression of FNR is correlated with an increase in the 'pseudo' first-order rate constant for P700+ reduction with approximately 0.5 s⁻¹ (chapter 3). Assuming a ratio of 1 for (thylakoid-associated) FNR per photosystem I particle, the resulting turnover number for FNR is certainly in the same order of magnitude as the turnover number that was determined in vitro. Possibly the redox-poise of the components that are involved are comparable between these in vivo- and in vitro measurements, since the determination of the P700+ reduction kinetics is performed directly after a period of continuous excitation of photosystem I, which results in the oxidation of the luminal electron carriers and the intersystem chain.

The psaE mutant showed an elevated expression-level of FNR in comparison with the wildtype. Presumably, the higher concentration of FNR in these cells compensates for a decrease of the linear electron transport rate that is the result of the absence of affinity of FNR for photosystem I (chapter 4). The expression level, however, was comparable to the level of FNR in wildtype cells under conditions of salt stress. Therefore the thylakoid-associated pool of FNR is probably competent in linear- as well as in cyclic electron transport. The NADP⁺:NADPH ratio is expected to influence the fluxes of linear and cyclic electron transport that this pool will catalyse. The maximal turnover number during linear electron transport is about 700 e⁻/s⁻¹ (chapters 1 and 4), whereas the turnover number associated with the reduction of the intersystem chain is in the order of 0.6 e⁻/s⁻¹ (chapter 3). Consequently, cyclic electron transport via FNR will only occur the NADP⁺:NADPH ratio is low. It is, however, not known if the determined turnover number of FNR during cyclic activity reflects the maximal rate. The increase in the oxygen production rate of the SM8 mutant, relative to the wildtype, suggests
that FNR may catalyse significant rates of plastoquinone reduction. Kinetic modelling may be possible of the influence of the redox poise of the plastoquinone pool on the levels of QA and QA\textsuperscript{−}, and the resulting influence on the turnover rate of the photosystem II reaction center. A significant over-oxidation of the plastoquinone pool (in the dark) would be expected to occur in a mutant deficient in the NAD(P)H dehydrogenase complex. Intuitively, this would be expected to result in significantly lower QA\textsuperscript{−} levels under conditions of non-saturated photosynthetic electron transport. Measurements of photochemical quenching of fluorescence from PSII indeed suggest this to be the case. Since the mutant SM8 shows a maximal oxygen production rate that is intermediate between the level of the wildtype and the mutant M55, it may indicate that the rate of plastoquinone reduction by FNR under conditions of (white) light-saturated photosynthesis is slower than the rate of reduction via the NAD(P)H dehydrogenase complex. This view would support the findings of the Asada group, who also claim that the NAD(P)H dehydrogenase complex catalyses the highest flux of plastoquinone reduction during PSI dependent cyclic electron transport (chapter 3).

A ternary complex consisting of PSI, ferredoxin and FNR was shown to exist \textit{in vitro} during linear photosynthetic electron transport (chapter 4). Formation of this transient complex was found to require the stromal subunit PsaE of photosystem I, suggesting that a direct interaction between FNR and PsaE occurs. The main effect of the \textit{psaE} mutation on the NADP\textsuperscript{+} photoreduction kinetics of PSI, is an increased apparent \(K_m\) for FNR (chapter 4). The \textit{psaE} mutant apparently compensates for the loss of the ternary complex by increasing the expression of FNR. This would suggest that \textit{in vivo} the ternary complex affects the photoreduction kinetics as well. The N-terminal domain of FNR does not appear to be required for the formation of this complex. However, it is not known if FNR associated to the phycobilisomes as well as FNR bound to the thylakoids can engage in the formation of such a ternary complex. In the wildtype strain, under standard photoautotrophic growth conditions, a significant fraction of FNR is present in the phycobilisome complexes (chapter 3). This fact, by itself, would invite the speculation that phycobilisome-associated FNR indeed interacts with photosystem I in a transient fashion.

In summary, the experimental observations suggest that FNR bound to the thylakoid membranes can participate both in linear- and in cyclic electron transport, whereas FNR bound to the phycobilisomes is expected to function primarily during linear electron transport. The phycobilisome-bound pool of FNR appears to represent a constant population, that is not significantly affected by transient changes in the expression level of FNR. Therefore the physiological function of this binding site might be to maintain a fixed pool of FNR, that is available to function during linear photosynthetic electron transport only. The thylakoid-associated pool of FNR can participate in the reduction of the intersystem chain, when redox-conditions allow. The electron donor for this reaction may either be reduced ferredoxin (or flavodoxin) or NADPH. The kinetics involving ferredoxin:plastoquinone oxidoreductase function are expected to be faster than the kinetics of NADPH:plastoquinone oxidoreductase, catalysed by FNR. The latter reaction may include a futile cycle, involving the photosyn-
thetic reduction of NADP⁺, and the subsequent oxidation of NADPH. Of course, also NADPH generated via the pentose phosphate pathway may contribute to reduction of the intersystem chain via FNR. However, a competitive binding site for NADPH is present in the thylakoid membrane (the NAD(P)H dehydrogenase complex). Moreover, this complex is more active than FNR as a NADPH:plastoquinone oxidoreductase. It remains to be determined if a binding site for ferredoxin, other than FNR, is present in the thylakoids. Overexpression of flavodoxin is observed in *Synechocystis* PCC 6803 during salt stress conditions. In addition, a flavodoxin deficient mutant essentially shows the same phenotype with respect to PSI dependent cyclic electron transport, as the mutant carrying a truncation of FNR (R. Jeanjean and M. Hagemann; personal communication). No induction of the cyclic pathway is observed as a result of salt stress in this mutant either. Therefore, flavodoxin is the most likely electron donor during reduction of the intersystem chain by FNR in *Synechocystis* PCC 6803.

It should be pointed out that plastoquinone reduction by FNR represents only one of several pathways, that have been shown to exist in *Synechocystis* PCC 6803. Next to the NAD(P)H dehydrogenase complex, an NDH2 complex may contribute to the NAD(P)H:plastoquinone oxidoreductase activity in this organism. In addition, its genome contains most genes encoding the enzymes that catalyse the citric acid cycle. Therefore the succinate dehydrogenase complex is likely to contribute to the reduction of the intersystem chain during the consumption of glycogen. Based on the reaction stoichiometries, this reaction is expected to constitute approximately 20% of the activity of respiratory oxidation of NADPH (synthesised via carbohydrate catabolism) by the NAD(P)H dehydrogenase complex.

A likely hypothesis for the function of the phycobilisome-association of FNR was that this complex provided a specific interaction, resulting in an increased energy transfer efficiency from the phycobilisomes to photosystem I. It was speculated that the removal of FNR from the phycobilisomes would result in a decreased energy transfer from the phycobilisomes to PSI. But before this could be examined it was necessary to describe the evidence for ‘direct’ excitation energy transfer from the phycobilisomes to PSI. In addition it was speculated that (phycobilisome-bound) FNR would contribute to ‘switching’ between light-states 2 and 1, via association and dissociation with PSI. Therefore we reported on the current views regarding state transitions in cyanobacteria, and found that still some widely divergent opinions are held (chapter 1).

The actual binding site of FNR in the phycobilisome complexes is to the phycocyanin hexamer proximal to the core, placing the reductase close to the thylakoid membrane surface (chapter 5). Taking into account the probable binding between phycobilisome-associated FNR and photosystem I, it is surprising that the presence of FNR in the phycobilisome complex does not (measurably) influence the energy transfer between the antenna and photosystem I (chapter 5). In cyanobacteria such as *Synechocystis* PCC 6803, the light harvesting function of the phycobilisomes for photosystem I is considerable (chapters 1 and 5). Possibly the average distance (and orientation) between the phycobilisome terminal emitters and the acceptor chromophores of photosystem I is such that the for-
mation of a transient super-complex phycobilisome: FNR: PSI does not reduce it further. Fluorescence emission spectra of red algae, taken at 77 K with excitation of the phycobilisomes, show emission from PSI as well as from PSII, as is the case with cyanobacteria (chapter 1). Since the phycobilisomes of red algae probably do not contain FNR, it was not expected that the removal of FNR from the phycobilisome complexes of Synechocystis PCC 6803 would result in a complete disruption of energy transfer from the phycobilisomes to PSI. Rather, an influence on the distribution of energy transfer between PSII and PSI could have been the result of the N-terminal truncation of FNR.

Two evolutionary aspects deserve further attention. First, since cyanobacteria are considered to be the ancestors of chloroplasts, an evolutionary event had to occur that caused the loss of phycobilisome complexes. From organisms such as red-algae and prochlorophytes, answers can possibly be found that help our understanding of what were the intermediate stages between the cyanobacteria and the chloroplasts. Red algae are interesting in this respect because they are eukaryotic organisms that contain phycobilisomes that are highly similar to cyanobacterial complexes. No FNR sequence from a red algae has been reported yet, but there is cause to believe that CpcD-homologous domains are not present in the petH genes in these organisms. Analysis of the polypeptide composition of purified phycobilisome complexes from red algae has shown the absence of proteins with a molecular mass of about 40-50 kDa, in all cases. Possibly, the N-terminal CpcD-homologous domain was lost from the petH genes, before the phycobilisomes were lost during the development of the chloroplasts. In this respect the Prochlorophytes are also interesting, since they are prokaryotic algae closely related to cyanobacteria, that do not contain phycobilisomes (with the exception of a Prochlorococcus strain that has been shown to contain a phycobiliprotein-encoding gene). We amplified an internal fragment of the petH gene of Prochlorothrix hollandica by PCR, and found the sequence to be highly similar to the petH sequence of Anabaena variabilis. The sequence of the entire chromosomal copy of the petH gene was not determined, however. The domain structure of the Prochlorothrix hollandica petH gene is therefore not yet known.

A second evolutionary aspect that relates to the subjects presented in this work, is the nature of cyclic photosynthetic electron transport in green sulfur- and heliobacteria. These organisms contain one single type of photosynthetic reaction center and perform anoxygenic -cyclic- photosynthesis. These reaction centers are similar to photosystem I reaction centers of oxygenic phototrophic organisms (with the exception of containing a homodimeric- in stead of a heterodimeric core), and contain Fe-S clusters at the acceptor side. Probably reduction of a soluble ferredoxin is part of the cyclic electron transport scheme. The cyclic electron transport scheme involves a cytochrome bc₁-type complex and menaquinone as the membrane associated electron carrier. The mechanism of menaquinone reduction is not known till date (for a review see Blankenship, 1995. Antonie van Leeuwenhoek 65: 311-329). However, it can not be excluded that these reaction centers contain a quinone binding site in addition to the Fe-S clusters at the acceptor side (R.E. Blankenship; personal communication). The current schemes for the occurrence of both photosystem I and photosystem II in
the first oxyphototrophs (cyanobacteria), include the assembly of genes encoding such Fe-S cluster-containing type I reaction centers as well as 'quinone' type II reaction centers. Possibly cyanobacteria inherited a FQR mechanism from the green sulfur- or heliobacteria. If this was the case, further study of the mechanism of menaquinone reduction in these organisms would generate opportunities for the analysis of the diverse pathways of plastoquinone reduction in cyanobacteria (and chloroplasts) as well.

Besides these matters, some other conclusion can be drawn from the experimental work presented here. In chapter 6 we describe that, depending on the light-regime history and the growth rate of the cultures, in Synechocystis PCC 6803 circadian transcriptional control is not always observed. This suggests that the circadian clock in cyanobacteria present in the environment is not always operative. In particular, the extent of the oscillations were found to be highest in cultures with a high growth rate. Possibly, circadian transcriptional control has a specific function in the physiology of rapidly dividing cyanobacteria (under conditions of 'blooming').

In chapters 3 and 5 we have successfully used Green Fluorescent Protein as a reporter molecule for binding of the N-terminal domain of FNR both to the thylakoids and the phycobilisomes. In chapter 7 we have investigated the photoconversion of a blue-light absorbing species into a green-light absorbing species of GFP. It was found that the protonation state of the chromophore, but not that of glutamic acid 222, changes after photoconversion of GFP. The experimental observation regarding the protonation state of this glutamic acid residue after photoconversion contrasted with models proposed on the basis of X-ray crystallography. Probably, Glu222 functions as a transient proton-acceptor, and the proton is released into the aqueous medium after photoconversion of the chromophore of GFP into the anionic species. These studies have prompted new questions regarding the mechanism of photoconversion of GFP. For example, is the proton that is released into the medium during photoconversion detectable? And can the change in hydrogen-bonding be observed by X-ray diffraction analysis of crystals of GFP, before and after photoconversion?
A second evolutionary aspect that relates to the subjects presented in this work is the nature of cyclic photosynthetic electron transport in green sulfur- and purple bacteria. These organisms contain the single type of photosynthetic reaction center and perform accessory cyclic phosphorylation. These reaction centers are similar in photosynthetic reaction centers of oxygenic photosynthetic bacteria (with the exception of containing a non-quinone electron pool), and contain P700 chlorophyll in the acceptor side. Productivity re-duction of a suitable electron is part of the cyclic electron transport scheme.

Cyclic electron transport involves a cytochrome bc₁-type complex and a reaction center in the cyanobacterial photosynthetic electron transfer. The mechanism of photosynthetic phosphorylation in the green chloroplast is unknown. The acceptor side of photosystem II contains a quinone-binding site in addition to the P700 acceptor in the acceptor side (Shi, Blankenship, personal communication). The acceptor side of photosystem I and photosystem II is located.