Ferrodoxin: NADP+ reductase and photosynthetic energy transduction in Cyanobacteria
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Synechocystis PCC 6803 ferredoxin:NADP$^+$ reductase is tightly bound to the core-proximal phycocyanin hexamer of the phycobilisome complex, yet does not significantly contribute to energy transfer to the reaction centers.
Summary

The phycobilisome complex of the cyanobacterium *Synechocystis* PCC 6803 binds approximately 2.4 copies of ferredoxin:NADP\(^+\) reductase (FNR). A mutant of *Synechocystis* PCC 6803 that carries an N-terminally truncated version of the *petH* gene, lacks the 9 kDa domain of its FNR that is homologous to the phycocyanin-associated linker polypeptide CpcD, and therefore assembles phycobilisome complexes that do not contain FNR. A phycocyanin deficient mutant, 4R (Plank *et al.*, 1995. *J Bacteriol* 177: 6798-6803) assembles allophycocyanin aggregates, that contain only a very small amount of FNR. Phycobilisome complexes from a mutant, expressing heterologous phycocyanin in the 4R background (R20; Plank and Anderson, 1995. *J Bacteriol* 177: 6804-6809), consist of the allophycocyanin core and only the core-proximal phycocyanin hexamers. These complexes contain a full complement of FNR, as compared to the wildtype strain, showing that the binding site of FNR is not the core-distal binding site that is occupied by CpcD, but in the core-proximal phycocyanin hexamer. Phycobilisome complexes of a mutant, expressing a fusion protein between the N-terminal domain of FNR and the *Aequorea victoria* Green Fluorescent Protein in the Δ*petH* (N-terminally truncated) background, contain this protein in tightly bound form. Calculations of the fluorescence resonance energy transfer (FRET) characteristics between GFP and acceptors in the phycobilisome complex indicate that the donor-acceptor distance is between 3 and 7 nm. Fluorescence spectroscopy at 77K, and measurements in intact cells of accumulated levels of P700\(^+\) induced by chlorophyll- and phycobilisome-absorbed light, indicate that the presence of FNR in the phycobilisome complexes does not influence the distribution of excitation energy of phycobilisome absorbed light between PSII and PSI. We conclude that FNR does not measurably contribute to energy transfer from the phycobilisomes to the reaction centers, and also does not affect the occurrence of 'light-state transitions'.

SYNECHOYSTIS FNR BINDS TO THE CORE-PROXIMAL PHYCOCYANIN HEXAMER
Introduction

Three cyanobacterial petH genes have been sequenced (Synechococcus PCC 7002; Schluchter and Bryant, 1992, Anabaena variabilis PCC 7119; Fillat et al., 1993, and Synechocystis PCC 6803; van Thor et al., 1998a). All three contain a domain at the N-terminus that is homologous to a polypeptide that plants and eukaryotic algae do not contain, in addition that the two domains that suffice for the enzymatic activity of FNR. This third, approximately 9 kDa domain, is highly homologous to the small phycocyanin rod-linker polypeptide CpcD (Schluchter and Bryant, 1992; Fillat et al., 1993; van Thor et al., 1998a). Homology to related linker polypeptides such as CpcC (the $L_R^{35}$ rod-linker) and ApcC (the small core linker $L_c^8$) is apparent, but is not as high as the homology to CpcD. Consequently, it was assumed that FNR binds to the phycobilisomes at the core-distal phycocyanin hexamer (Schluchter and Bryant, 1992), which is the position that CpcD is known to occupy (de Lorimer et al., 1990).

It is not directly obvious what the function is of this localisation of FNR at the outer position of the phycocyanin rods of the phycobilisome. One proposed function might be that this provides a 'localised' higher concentration of FNR, which may be necessary for catalysing maximal rates of $\text{NADP}^+$ reduction during linear photosynthetic electron transport with less copies of the FNR enzyme than would be required if its localisation was cytoplasmic (van Thor et al., 1998b). Alternatively, it could be a means of introducing heterogeneity in the pool of FNR, carrying the N-terminal domain, since free, cytoplasmic FNR will bind to the thylakoid membrane via this domain (van Thor et al., 1998d). Others have proposed that a phycobilisome-PSI (PSI-trimer) supercomplex is formed via the interaction of phycobilisome-bound FNR with PSI. In this model FNR is explicitly placed at the core-distal phycocyanin hexamer, and interaction with PSI is believed to be mediated via the stromal subunit PsaE of PSI (Bald et al., 1996). Formation of this complex could enhance transfer of excitation energy from the phycobilisomes to PSI specifically, as a result of the reduced distance between the chromophores from the antenna and the reaction centers.

Mutations in apcA or apcE, affecting the core of the phycobilisome complex, result in a significant uncoupling of the complexes from the reaction centers (Su et al., 1992, Shen et al., 1993). In the apcA mutant only a limited amount of phycocyanin is detected. As a result of an apcE mutation allophycocyanin is still produced, but does no longer couple functionally to the reaction centers. However, in both cases, some energy transfer from the remaining phycobilisomes specifically to PSI is still observed. Interestingly, a comparable situation develops in heterocysts of Anabaena variabilis, when allophycocyanin is degraded before phycocyanin. Also in these (PSII deficient) cells, with 77K fluorescence spectroscopy, some energy transfer from phycocyanin to PSI is observed (Peterson et al., 1981; Tyagi et al., 1981).

Here we show that the Synechocystis FNR is tightly bound to the core-proximal phycocyanin hexamer, via its N-terminal domain, with approximately two copies per complex. A mutant, truncated in the N-terminal domain of FNR, shows a decreased phycocyanin/allophycocyanin ratio. Truncation of the N-terminus of FNR was found not to influence the distribution of excitation energy absorbed by the
phycobilisome between PSII and PSI, but the overall energy transfer rate from the phycobilisomes to both PSII and PSI reaction centers may be decreased in the mutant, as compared to the wildtype. We used GFP, translationally fused to the N-terminus of FNR, in order to show that this latter domain directs binding of the protein to the phycobilisome complex. Radiationless energy transfer from GFP, to presumably both allophycocyanin and phycocyanin was observed. Dissociation of these complexes was used to estimate the distance between the chromophore of GFP and the phycocyanobilin chromophores.

**Materials and Methods**

**Strains and Growth Conditions**

*Synechocystis* PCC 6803 and mutants thereof were cultured in BG-11 medium (Rippka et al., 1979). The genetic construction of the ΔpetH truncation mutant SM8 (lacking 75 residues at its N-terminus, due to a 225 bp deletion), and the construction of a strain expressing a fusion protein between GFP and the N-terminal domain of FNR were described earlier (van Thor et al., 1998d). An ndhB deficient mutant ‘M55’, lacking a functional NAD(P)H dehydrogenase complex, was obtained from Prof. Ogawa (Ogawa, 1991). A double mutant (i.e., ‘M55’ containing the ΔpetH truncation), ‘DM4’, was also described earlier (van Thor et al., 1998d). A mutant with a deletion of the apcE gene was kindly provided by Prof. Vermaas (Shen et al., 1993). Mutants carrying the ΔpetH gene were cultured in medium containing streptomycin (10 μg/ml), the GFP-expressing strains and the ndhB deficient strains were cultured with kanamycin present (50 μg/ml), and the ApcE deficient single mutant was selectively grown in medium containing erythromycin (10 μg/ml). A double mutant, carrying both the ΔpetH gene of strain SM8 and a deletion of apcE was constructed as described earlier for the single apcE mutant (Shen et al., 1993) using a construct kindly provided by Dr. Ashby, encoding kanamycin resistance. A phycocyanin deficient mutant, ‘4R’ (Plank et al., 1995a) and a mutant, ‘R20’ expressing cpcA and cpcB genes from *Synechocystis* PCC 6701 (Plank et al., 1995b) were kindly provided by Prof. Anderson. Mutant ‘R20’ was cultured in the presence of kanamycin (50 μg/ml).

**Isolation of Phycobilisome Complexes**

Phycobilisomes were isolated according to the method of Glazer (1988). This procedure relies on the dissociation of the phycobilisome complexes from the thylakoid membranes with Triton X-100 (ultrapure, Sigma), and their subsequent isolation with sucrose density gradient ultracentrifugation, using 0.75 M phosphate buffer, pH 8.0. The entire isolation procedure was performed at room temperature. The structural integrity of isolated complexes was assayed with fluorescence spectroscopy and SDS-PAGE analysis.

**Fluorescence Spectroscopy**

Fluorescence spectra at 77K were recorded with a laboratory built fluorimeter with a spectral resolution of 0.16 nm (Figures 3 and 5). The sample, cooled to 77K,
was excited with light from a tungsten-halogen lamp, filtered through either a 440 or 574 nm bandpass filter. Fluorescence was collected in a 90° geometry with a CCD detector, shielded with a 620 nm high-pass filter, and corrected for instrument response. Spectra recorded at 77 K with an Aminco Bowman Series 2 fluorimeter (Figure 4) were corrected for instrument response with a build-in photodetector, and had a spectral resolution of 1 nm. Fluorescence spectra of intact cells, taken at 77K, required samples with low optical density (absorption below 0.05 at relevant wavelength). Also, glycerol can not be used in such samples, since this denatures phycobiliproteins and partially uncouples energy transfer from the phycobilisomes to the reaction centers. Measurements of fluorescence spectra and fluorescence polarisation at room temperature were recorded with the Aminco Bowman Series 2 fluorimeter, equipped with polarizers.

**Analytical techniques**

Absorption spectra were recorded with an Aminco DW2000 spectrophotometer. Phycocyanin and allophycocyanin concentrations were determined according to Demidov and Mimura (1995) and Bryant (1979). SDS-PAGE was performed with 15% acrylamide mini-gels (Biorad). A monoclonal mouse-α-GFP antibody (Clontech) was used at 1:20,000 dilution for the detection of GFP, in Western blots, with chemiluminescent detection of the HRP-labeled secondary antibody using SuperSignal (Pierce). FNR was quantified by assaying the diaphorase activity of the phycobilisome preparations, as described previously (van Thor et al., 1998a). Recombinant GFP (mutant ‘GFPuv’) was isolated as described previously (van Thor et al., 1998e) and used as a standard for quantification of GFP fusion protein in recombinant phycobilisome complexes.

Measurements of absorption changes at 830 nm in intact cells, reflecting the formation of P700+, were performed using a modulated fluorescence measurement system MKII, equipped with the P700+ detector and emitter system (Hansatech).

**Results**

**FNR IS BOUND TO THE CORE-PROXIMAL PHYCOCYANIN HEXAMER**

Phycobilisomes, purified by sucrose density gradient ultracentrifugation, are isolated as high molecular weight aggregates that contain only small amounts of impurities. Yet substantial amounts of ferredoxin:NADP+ reductase are copurified with these complexes (Figure 1). Since even two-domain (N-terminally truncated) FNR's have been shown to copurify or associate in vitro with the cytochrome b_{6}f complex (Clark et al., 1984) and with PSI via the PsaE subunit (Andersen et al., 1992; van Thor et al., 1998b), it was necessary to test whether a two-domain FNR, lacking the CpcD-homologous domain, does not copurify with the phycobilisomes. For example, co-purification of chloroplast FNR with the cytochrome b_{6}f complex has been shown to be an artifact of the isolation procedure (Coughlan et al., 1985).
A

**Figure 1. SDS-PAGE analysis of wildtype, mutant and recombinant purified phycobilisome complexes from Synechocystis PCC 6803 and mutants.**

Phycobilisome complexes isolated from the wildtype (lane 2), ΔpetH truncation mutant SM8 (lane 3), double mutant SM8 containing the GFP-fusion protein (lane 4), phycocyanin deficient mutant '4R' (lane 5), and strain R20 expressing heterologous phycocyanin in the 4R background (lane 6). Western detection of the GFP fusion protein present in lane 4 is shown separately. GFP, FNR, phycocyanin and allophycocyanin concentrations were determined as described in Materials and Methods.

A mutant ‘SM8’ was constructed that carries a truncated ΔpetH gene, via the in frame deletion of 225 nucleotides downstream of the initiation codon of the wildtype petH gene. This mutant expresses a functional two-domain FNR (replacing the original three-domain FNR) that is competent in linear photosynthetic electron transport, with activities comparable to the wildtype protein (van Thor et al., 1998b, 1998a). When phycobilisomes are isolated from this mutant, no FNR is copurified with the complexes, as expected (Figure 1).

A phycocyanin deficient mutant ‘4R’ assembles allophycocyanin-containing core-complexes that are functional in thylakoid association and excitation energy transfer to the reaction centers (Plank et al., 1995; Plank and Anderson, 1995). These aggregates can be isolated by a modified sucrose density gradient ultracentrifugation protocol, in spite of their reduced molecular mass. However, these complexes contain some high molecular weight impurities (Figure 1). Surprisingly, these complexes were found to contain sub-stoichiometric amounts of FNR. Given the reduced purity of these samples, this does not unequivocally...
prove that FNR has affinity for allophycocyanin. This latter conclusion can only be drawn after a double mutant, carrying the ΔpetH gene and the phycocyanin-deficient genotype, has been tested for this copurification phenomenon.

The ‘R20’ strain, expressing *Synechocystis* PCC 6701 phycocyanin in the phycocyanin deficient *Synechocystis* PCC 6803 genetic background ‘4R’, assembles phycobilisome structures with a reduced molecular mass, as compared to the wildtype. This is due to the (near) absence of core-distal phycocyanin hexamers in the rods, since only the 27 kDa rod-core linker is detected in substantial amounts. The L<sub>R</sub><sup>35</sup> phycocyanin linker polypeptide was detected only in very small amounts, whereas the L<sub>r</sub><sup>33</sup> linker polypeptide, that is associated with the core-distal phycocyanin hexamer, is completely absent (Figure 1). In addition, quantification of the phycocyanin:allophycocyanin ratio indicated that these complexes contain about 3 phycocyanin hexamers per core. In contrast to expectations, wildtype levels of FNR were detected in these isolates (Figure 1). The small rod-linker CpcD that shows the highest homology with the N-terminus of FNR, is present in the wildtype complexes exclusively in the core-distal hexamers (de Lorimier et al., 1990). In the absence of these core-distal hexamers FNR is still copurified with the complex. We therefore assume that FNR is bound to the core-proximal phycocyanin hexamer.

Since both the complexes from strains ‘4R’ and ‘R20’ contain FNR, it is suggested that FNR binds the core-proximal hexamer at a position between the core-structure and the phycocyanin hexamer. Most probably the N-terminal domain of FNR competes with the 27 kDa rod-core linker for binding sites in the phycobilisome complex. However, based on this data it can not be ruled out that binding of the domain to phycocyanin is on the distal side of this hexamer; the binding site of the 35 kDa rod-linker that is the product of *cpcC*. Surprisingly, the N-terminal domain of FNR seems to possess affinity for allophycocyanin. It is not known if binding to allophycocyanin is possible only in the absence of phycocyanin, or if some copies in wildtype structures are bound to allophycocyanin, in stead of to phycocyanin exclusively. When the amino acid sequence of the N-terminal domain of FNR (75 residues) was compared to the amino acid sequences of the phycobilisome linker polypeptides of *Synechocystis* PCC 6803, the highest homology was found for the L<sub>R</sub><sup>35</sup> small phycocyanin-associated linker polypeptide, the product of *cpcD* (van Thor et al., 1998a). When these sequences were compared, 57% identity and 72% similarity was detected, for 61 residues. However, the homology with L<sub>R</sub><sup>35</sup> (the product of *cpcC*) and L<sub>C</sub><sup>8</sup> (the product of *apcC*) was also significant. For the L<sub>R</sub><sup>35</sup> linker polypeptide, associated with the core-proximal and ‘second’ phycocyanin hexamer, the identity was 40%, and the similarity was 68%, for 34 residues. For the small allophycocyanin-associated core linker L<sub>C</sub><sup>8</sup>, the identity was 45%, and the similarity was 66%, for 23 residues (Kaneko et al., 1996). In particular the homology with the L<sub>R</sub><sup>35</sup> linker polypeptide is suggestive, since this linker is associated with the core-proximal phycocyanin hexamer. The homology with the core linker L<sub>C</sub><sup>8</sup> is suggestive in light of the apparent affinity of FNR for allophycocyanin.

A translational fusion between the 75 N-terminal residues of the wildtype three-domain FNR, and a mutant form of green fluorescent protein (GFPuv), was
expressed both in the wildtype background and in the ΔpetH mutant SM8, via transformation of a construct designed to integrate at the petH promoter region. As a result, the GFP fusion protein copurified with the phycobilisome complexes of both strains. The GFP fusion protein in the phycobilisomes was detected with coomassie staining after SDS-PAGE, as well as with immunodetection using an anti-GFP monoclonal antibody (Figure 1). Furthermore, the fluorescence emission of GFP was detected in both types of recombinant complexes. The complexes isolated from the wildtype background contained both FNR (comparable to wildtype levels), and the GFP fusion protein (approximately 50% of the copies present in the complexes isolated from the FNR-free SM8 background (not shown)). This illustrates that FNR and the GFP fusion protein, most probably, both are bound to core-proximal phycocyanin hexamers in the intact complexes, since they seem to compete for the same binding site(s).

**Fluorescence Resonance Energy Transfer between GFP and the Allophyocyanin-and Phycocyanin Chromophores**

Recombinant phycobilisome complexes from the SM8-GFPtranslat strain, contain the GFP-fusion protein and no FNR (Figure 1). These complexes shows a detectable but relatively weak fluorescence emission maximum at 510 nm, with a corresponding excitation maximum at 395 nm. However, the emission peak from GFP is well separated from phycocyanin and allophyocyanin emission. A second excitation maximum, at 480 nm, originating from the anionic form of GFP, could not be detected with this signal intensity. This allowed measurement of the polarisation of the fluorescence emission, arising from excitation of the neutral form of GFP at 395 nm. Strikingly, this polarisation was found to be higher, i.e. 0.37, than the corresponding fluorescence polarisation that was determined for the monomeric form of GFPuv, 0.195, in a low-ionic strength buffer at pH 8.0. For the free monomeric recombinant form of GFP it is assumed that the fluorescence lifetime is 3.3 ns (Perozzo et al., 1988). In addition, the fluorescence quantum yield for excitation of the neutral species of the chromophore of GFP at 395 nm was determined to be approximately 0.8, using sodium-fluorescein at pH 8 as a standard. This increased polarisation indicates that the excited state of the form of GFP, that is copurified with the phycobilisomes, is shorter lived, and/or its rotational motion is decreased, relative to the monomeric form of GFP.

The phycobilisomes, when isolated, require specific buffer- and temperature-conditions, in order to maintain their proper energy transfer characteristics (Gantt et al., 1979). Upon dilution into, for instance, 50 mM phosphate buffer, pH 8.0, these complexes dissociate within minutes (Gantt et al., 1979; Maxson et al., 1989). With wildtype phycobilisome complexes from Synechocystis PCC 6803 the dissociation of phycocyanin from allophyocyanin, apparent from the increase of fluorescence emission at 650 nm, proceeds at this timescale as well.

Upon transfer of the recombinant complexes, containing the GFP fusion protein, into 50 mM phosphate buffer, the fluorescence intensity at 510 nm increased, until a maximum was reached 20 minutes later (Figure 2A). The degree of polarisation of this fluorescence emission rapidly decreased, with kinetics that could be fitted with a mono-exponential function (Figure 2B). After 20 minutes
the polarisation was close to the value of the polarisation of the monomeric form of GFP, that was determined at the same temperature and buffer conditions. Based on the assumption that the GFP-fusion protein was completely dissociated from the phycobilisomes, and energy transfer was therefore disrupted, the rotational correlation times were calculated from these data (Figure 2C). At t = 0, the rotational correlation time was calculated to be 1.5 ns. This could be accurately determined, since both the fluorescence intensity, and the fluorescence polarisation could be measured under steady-state conditions, using intact complexes in a high ionic strength buffer. In addition, measurements of fluorescence intensity and -polarisation of GFP emission in intact recombinant phycobilisome complexes was reproducible when different isolated were compared. The fluo-

**Figure 2. Fluorescence characteristics of GFP-fusion protein upon dissociation of the recombinant complex**

A. Fluorescence intensities were determined by integration of the entire emission band, from 450 nm to 600 nm, with excitation at 395 nm. It was verified that during this experiment photoconversion of GFP (van Thor et al., 1998) did not contribute to the amplitude that was determined, by measuring the relative excitation maximum at 480 nm and 395 nm before and after the completion of the experiment. In addition, the signal was corrected for the changes in scattering of the sample at the relevant wavelengths.

B. Fluorescence polarisation measurements were taken with excitation at 395 nm for the emission at 510 nm, with slit widths of 4 nm. The polarisation at time zero was taken from a measurements in 0.75 M phosphate buffer for the intact complex. The measurements were fitted with a mono-exponential function. The dotted line represents the polarisation of monomeric recombinant GFP at identical buffer and temperature conditions.

C. Calculated rotational correlation time-constants (ns) for the GFP fusion protein, based on the assumption that at t = 20 minutes \( \tau = 3.3 \) ns (energy transfer is disrupted). The rotational correlation time constant \( \tau_{rot} \) was calculated according to the Perrin equation, \( \frac{1}{A} = \frac{1}{A_0} \left( 1 + \frac{2}{\tau_{rot}} \right) \) that is valid for isotropic rotational motions (based on the assumption that the intrinsic anisotropy for the fluorescence emission for monomeric GFP, \( A_0 = 0.4 \), i.e. the maximal polarisation \( P_0 = 0.5 \)).
rescence polarisation that was measured during the dissociation process showed more scatter in the data (Figure 2B). Therefore, values taken from the mono-exponential fit were used for the determination of the rotational correlation times, for the other time points. The rotational correlation time rapidly increased to about 2.5 ns directly after dilution, suggesting that transfer to a low ionic strength buffer induced a decrease in the rotational motion of the GFP fusion protein. Possibly, electrostatic interactions induced the formation of a larger complex, that resulted in reduced molecular motion. Alternatively, the mode of rotational diffusion changed upon transfer into low ionic strength buffer, from an anisotropic- to an isotropic motion. The Perrin equation, that was used in order to calculate the rotational correlation times presented in Figure 2C, is valid only in the case of isotropic motions. Most likely the fusion protein was fully dissociated from the complex 20 minutes after dilution, since significant uncoupling of energy transfer had occurred, and the mode of rotational diffusion had become comparable to that of the recombinant, monomeric form of GFP. The residual polarisation, relative to the monomeric form of GFP, that was left after complete dissociation (Figure 2B), may indicate that the rotational velocity of the 30 kDa protein is affected by the addition of the 9 kDa linker at the N-terminus. The alternative, less likely, possibility is that still some energy transfer may have occurred.

Taking into account the probable binding-site for the GFP-fusion protein, positioned between the allophycocyanin core and the first phycocyanin hexamer, a possible scenario for a ‘staged’ dissociation process could be envisioned, based on these measurements and calculations. Within the first minute, the phycocyanin rods are released from the core, energy transfer from GFP to allophycocyanin is disrupted, and the GFP fusion protein gains an isotropic mode of rotational motion. Dissociation from the phycocyanin rods then takes place within the next 19 minutes, resulting in the further uncoupling of energy transfer. During this process no additional rotational freedom is gained. Presumably, the N-terminus of the recombinant protein contains a flexible region that allows free movement on the ns timescale independent of the association of that domain to even larger molecules. This scenario would also fit the observation that dissociation of phycocyanin from the allophycocyanin core is the first stage of complex-dissociation in low ionic strength buffers (Gantt et al., 1979; Maxson et al., 1989).

The fluorescence intensity of the GFP-fusion protein, 20 minutes after dissociation of the complex, was quantified by comparing the signal to a standard dilution series of recombinant GFP under identical buffer and temperature conditions. Using the calculated extinction coefficient for phycobilisome complexes of SM8, 27,000 mM⁻¹ cm⁻¹, a ratio of 0.26 GFP:phycobilisome was found. The band representing the recombinant GFP-fusion protein present in these complexes, stained significantly less intense with coomassie, than the band of 47 kDa FNR in the wildtype complexes, for which a ratio of 2.4:phycobilisome was determined (Figure 1). This also suggests that 20 minutes after dilution most energy transfer was disrupted. In addition it was observed that wildtype FNR, bound to the phycobilisomes, is fully released from the complexes in a low ionic strength
A. Förster radii and donor-acceptor radii assuming exclusive energy transfer to the presented acceptors.

The total energy transfer rate that was determined experimentally (1.16 \times 10^9 s^{-1}) was assigned to the respective phycocyanobilin chromophores separately, assuming exclusive energy transfer to these acceptors. For these separate cases Förster radii and donor-acceptor radii were calculated for several values of the orientation factor $\kappa^2$. The refraction index $n$ was taken as 1.567 (Grabowski and Gantt, 1978).

B. Förster radii and donor-acceptor radii assuming a 'staged' dissociation of the complex.

Exclusive disruption of energy transfer to allophycocyanin is assumed to proceed in the first minute after dissociation (Figure 1). The energy transfer rate $2.78 \times 10^8$ s^{-1} at $t=1$ min is then assigned exclusively to the three phycocyanin chromophores, as in Table 1A.

buffer, and subsequently associates with the thylakoid membranes (van Thor et al., 1998a). This was observed for the GFP fusion protein as well, indicating that complete dissociation was likely to have occurred.

**Förster energy transfer calculations**

These conclusions are based on the assumption that excitation energy transfer from GFP to phycocyanobilin chromophores proceeds with rates that are high enough to compete with the fluorescence decay rate. These rates are a function of the spectral overlap integral that contains the integral of the donor emission spectrum and the acceptor absorption spectrum (Förster, 1960). The emission maximum of GFP is at 510 nm whereas the $\beta$–155 chromophore of phycocyanin, that absorbs maximally at 590 nm (Demidov and Mimuro, 1995), is the most likely acceptor. The Förster overlap integrals for GFP as the donor and all possible acceptors were calculated, using the deconvoluted absorption spectra of the individual phycocyanin chromophores (Demidov and Mimuro, 1995), and the absorption spectrum of the allophycocyanin core, isolated from mutant 4R, assuming an extinction coefficient of 235 mM^{-1}.cm^{-1} at 650 nm for the $\alpha\beta$ monomer (Bryant et

### Table 1

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The spectral overlap integrals for GFP and the possible acceptors are respectively: \( 2.08 \times 10^{-13} \text{ cm}^3 \text{ M}^{-1} \) for the \( \beta-155 \) chromophore of phycocyanin, \( 5.08 \times 10^{-14} \text{ cm}^3 \text{ M}^{-1} \) for the \( \beta-84 \) chromophore of phycocyanin, \( 1.09 \times 10^{-13} \text{ cm}^3 \text{ M}^{-1} \) for the \( \alpha-84 \) chromophore of phycocyanin, and \( 1.95 \times 10^{-13} \text{ cm}^3 \text{ M}^{-1} \) for the \( \alpha-84 \) chromophore of allophycocyanin. The overlap integral for the \( \beta-84 \) chromophore of allophycocyanin was neglected, since its absorbance maximum is red-shifted significantly. Excitation energy transfer from phycocyanobilin chromophores to GFP was neglected as well: both in intact phycobilisomes, when excitation energy is transferred to the terminal emitters with very high efficiency, and for dissociated complexes, fluorescence emission of the phycocyanin and allophycocyanin chromophores is in the red region of the spectrum, in which the chromophore of GFP does not absorb.

From the observed changes in fluorescence quantum yield in the presence of the acceptor and the known fluorescence lifetime in absence of the acceptor, the total rate of energy transfer from GFP to the acceptors (Figure 2; time 0) was calculated to be \( 1.16 \times 10^9 \text{ s}^{-1} \). In order to calculate the energy transfer characteristics, the orientation of the donor and acceptor chromophore dipoles has to be considered. The rotational correlation time for GFP in the complex is in the ns range, as is the fluorescence lifetime (Figure 2C), and the extent of the movements of GFP in the complex is unknown. Therefore, the assumption that the orientation of the chromophore of GFP is averaged in the sample can not realistically be made. The squared orientation factor \( \kappa^2 \) can in principle vary between 0 and 4, depending on the relative orientations of the donor emission dipole and the acceptor absorption dipole, and becomes 2/3 for randomly oriented dipoles. In practice a value of 0 is seldom observed (van Grondelle, 1985). Therefore values for \( \kappa^2 \) of 1/3, 2/3, 1 1/3 and 4 were used to calculate the Förster radii, reflecting unfavourable, average, good and optimal orientation of the dipoles. Table 1A presents the \( R_0 \) and \( R_{DA} \) values for all acceptor chromophores. The \( R_{DA} \) values were calculated, assigning the total energy transfer rate \( 1.16 \times 10^9 \text{ s}^{-1} \) to each individual chromophore exclusively (Table 1A). Assuming a ‘staged’ dissociation scenario involving a dissociation of the GFP fusion protein from allophycocyanin within the first minute (see previous section), \( R_{DA} \) values were calculated assigning the total energy transfer rate for the first minute exclusively to the APC-\( \alpha-84 \) chromophore (Table 2B). From these calculations it is concluded that the minimal distance between GFP and the phycocyanobilin acceptor chromophore(s) in the complex is 3 nm, and 7 nm maximally (Tables 1A and 1B).

**FNR does not significantly contribute to energy transfer from the phycobilisomes to the reaction centers**

Since FNR is bound to the first phycocyanin hexamer, it may be positioned in close proximity to other constituents in the thylakoid membrane. In particular, contact between FNR and photosystem I would be possible, given the dimensions of the core of the phycobilisome and the stroma-exposed region of PSI. The stromal-exposed subunits PsaE, -D and -C are predicted to protrude about 30 Å into stromal space (Schubert et al., 1997). The distance between the thylakoid surface and FNR that is bound to the phycobilisomes may be between 0 Å and
Wildtype (solid line) and ΔpetH mutant SM8 (grey line) cells were dark adapted before being frozen in liquid N$_2$. Emission spectra were recorded with either 440 nm or 574 nm excitation. The emission spectra recorded with 440 nm excitation are normalized at 725 nm, whereas the emission spectra recorded with 574 nm excitation are normalized at 665 nm.

Fluorescence spectroscopy at 77K of intact cyanobacterial cells was used in order to determine the pathway of energy transfer in wildtype Synechocystis PCC 6803 and mutants derived thereof. The emission maxima associated with PSII, 685 nm and 695 nm, PSI, 725 nm, phycocyanin, 650 nm and allophycocyanin, 665 nm, were identified in emission spectra upon excitation of chlorophyll $a$, with 440 nm light, and of phycobilisomes, with 574 nm light (for a review see: van Thor et al., 1998c). Figure 3 shows the emission spectra, taken with either 440 nm or 574 nm excitation of cells of the wildtype and ΔpetH mutant ‘SM8’ cells. The emission spectra taken with 440 nm excitation of both strains were almost iden-
tical. The emission maxima at 685 nm and 695 nm, originating from PSII, are somewhat more intense in the mutant, possibly indicating a slightly increased PSII:PSI ratio. The emission spectrum, taken with 574 nm excitation, of the mutant SM8 shows a significant decrease of the emission at 650 nm, associated with phycocyanin, relative to the emission at 665 nm, associated with allophycocyanin. Since a decrease in the phycocyanin:allophycocyanin ratio results from the removal of FNR from the phycobilisome complex, the relative decrease of phycocyanin emission is most likely the consequence of the decreased phycocyanin content of these cells, and not of a change in the energy transfer characteristics between phycocyanin and allophycocyanin. Interestingly, upon excitation with 574 nm light, emission from both PSII (i.e. 685 nm and 695 nm maxima) and PSI (the 725 nm emission maximum), are decreased in the mutant, relative to the emission amplitude of allophycocyanin, at 665 nm. This may indicate that the \( \Delta \text{petH} \) mutation affects energy transfer from phycobilisomes to both reaction centers. No increase in the emission from the phycobilisome terminal emitters, at 685 nm, is observed. Therefore, the rate of energy transfer from allophycocyanin to the reaction centers is decreased in the mutant, for the total pool of antenna complexes, rather than a complete uncoupling of a sub-population of phycobilisome complexes from the reaction centers. This may be the result of either an increase in the number of phycobilisome complexes relative to the number of reaction centers, provided that all complexes contribute to energy transfer to the reaction centers both in the mutant and the wildtype, or an increase in the distance between the complexes and the thylakoid-bound acceptors. A third possibility is that a change in the orientation of the relevant donor

**Figure 4.** 600 nm fluorescence emission spectra of apcE single mutant and apcE/SM8 double mutant cells recorded at 77K. 

apcE single mutant (open symbols) and apcE/SM8 double mutant (closed symbols) cells were dark adapted before being frozen. Emission spectra were recorded with 600 nm excitation, and normalized at 665 nm.
and acceptor chromophores results in a decreased energy transfer rate.

Mutations in \textit{apcA} and \textit{apcE} affect the core of the phycobilisome. Deletion of \textit{apcA}, encoding the allophycocyanin \(\alpha\) subunit, resulted in mutant \textit{Synechocystis} PCC 6803 cells that completely lack allophycocyanin (Su \textit{et al.}, 1992). Some phyco-
cyanin was still present in these mutant cells, but was largely uncoupled from PSII, as judged from 77K fluorescence spectra (Su \textit{et al.}, 1992). In the \textit{apcE} mutant, lacking the large core-linker polypeptide and one of the terminal emitters, allophycocyanin is present, but appears not to couple to the remaining terminal emitters (Shen \textit{et al.}, 1993). Interestingly, some energy transfer from phycocyanin to PSI may still occur in both mutants (Su \textit{et al.}, 1992; Shen \textit{et al.}, 1993). It was sug-
gested that FNR may bind phycocyanin to PSI and result in the fluorescence emis-
sion from PSI upon excitation of phycocyanin in this mutant (Su \textit{et al.}, 1992).

The emission spectrum, taken with 620 nm excitation, of a ‘single’ \textit{apcE} mutant showed a prominent maximum at 665 nm, that was assigned to allophycocyanin (Figure 4). A single maximum at 625 nm was present in the excitation spectrum, obtained with 665 nm emission, indicating that only phycocyanin contributes to this emission band (not shown). A second maximum, at 725 nm, was assigned to PSI in this emission spectrum. No emission from PSII at 685 nm nor 695 nm was observed, in agreement with previous observations (Shen \textit{et al.}, 1993). The excita-
tion spectrum of the 725 nm emission showed three maxima. Two maxima, at 445 nm and 685 nm, were assigned to the Soret band and the \(Q_Y\) transition of chloro-
phyll \(a\), respectively (not shown). The amplitude of the third maximum, at 625
nm, was comparable to the amplitude of the Soret band. Approximately 50% of
the emission at 725 nm originates from PSI, with excitation at 600 nm, judging
from the 600 nm emission spectra from an \textit{apcE} single mutant and an \textit{apcE}/PSI-
deficient double mutant (Shen \textit{et al.}, 1993). Therefore phycocyanin is expected to
contribute to the excitation spectrum of the 725 nm emission also in the absence
of excitation energy transfer. However, the fluorescence quantum yield at 77K of
allophycocyanin has to exceed that of PSI-associated chlorophyll \(a\) significantly
in order to explain the intensity of the excitation maximum at 625 nm if excita-
tion energy transfer from phycocyanin to PSI does not occur.

In order to determine if FNR is responsible for the possible energy transfer
from phycocyanin to PSI, observed in the \textit{apcE} and \textit{apcA} mutants, a double mu-
tant was constructed carrying both the \textit{ApetH} truncation and a deletion of \textit{apcE}.
The emission spectra, recorded with 625 nm excitation at 77K, are nearly identi-
cal for the \textit{apcE} single mutant and the \textit{apcE}/SM8 (\textit{apcE}/\textit{ApetH}) double mutant, featuring both the 665 nm and the 725 nm emission maxima (Figure 4). The fluo-
rescence excitation spectra for both the 665 nm and the 725 nm emission bands
were also very similar (not shown). This clearly indicates that the N-terminal
domain of FNR is not responsible for the possible energy transfer from phycocy-
anin to PSI in the \textit{apcE} deficient background.

\textit{Phycobilisome-bound FNR does not affect the phenomenon
of ‘light-state transitions’}. 

Cyanobacteria, like plants, are able to respond to changes in their light climate by
redirecting pathways of excitation energy transfer. Such light-state transitions
FIGURE 5. 574 NM FLUORESCENCE EMISSION SPECTRA TAKEN AT 77K OF WILDTYPE AND ΔpetH TRUNCATION MUTANT ‘SM8’ CELLS FROZEN IN LIGHT-STATE 2 AND LIGHT-STATE 1
A. Wildtype cells were dark adapted for light-state 2 (solid line) and incubated for 5 minutes in far-red light (6710 nm; +/- 12 mW) for light-state 1. Fluorescence emission spectra recorded with 574 nm excitation were normalized at 665 nm to 30,000 counts (A.U.).
B. Cells of ΔpetH truncation mutant SM8 were adapted to light-states 2 and 1 and emission spectra were recorded and normalized as in A.

can be characterized by fluorescence measurements and other techniques (for a review see: van Thor et al., 1998c). Emission spectra at 77K of intact cells were recorded to detect the extent of fluorescence changes upon transition between light-states 2 and 1. Figure 5 presents the emission spectra, recorded with phycobilisome excitation at 574 nm, of cells of the wildtype and the SM8 mutant, frozen in either light-state. All spectra were normalized to 30,000 A.U. at 665 nm. It was observed that emission bands assigned to both PSII and PSI were more intense in the wildtype than in the mutant, when the spectra of the same light-states were compared (compare Figure 6A and 6B). This confirmed that the earlier results (Figure 3) also hold for a light-state 1. After induction of a light-state 1, it was seen for both wildtype and mutant cells that emission from PSII increased whereas emission from PSI decreased, when the spectra were normalized at 665 nm. This indicates that the N-terminus of FNR is not directly involved in the mechanism of transitions between different light-states.

Fluorescence spectroscopy at 77K has been extensively used to quantify the fluorescence yield of PSII in particular, in different light states. For several reasons, however, this technique may be less reliable when changes in the energy transfer efficiency from the phycobilisome complexes to PSI are of interest. In particular, it has to be performed at low temperature, since the fluorescence yield of PSI is very low at ambient temperatures. In addition, ‘spill-over’ of excitation energy from PSII to PSI may occur more frequently, since not only the bulk of the PSI-associated chlorophyll a is red-shifted by several nanometers compared to the bulk of PSII-associated chlorophylls, but PSI also contains several pig-
FIGURE 6. STEADY STATE LEVELS OF P700+ ACCUMULATED IN WHITE AND GREEN LIGHT OF WILDTYPE AND MUTANT CELLS OF SYNCHOCYSTIS PCC 6803

In vivo pulse-modulated measurements of the increase in absorbance at 830 nm, reflecting the accumulation of P700+. Cells were deposited on a filter and illuminated with light from a tungsten-halogen lamp, filtered through neutral density filters and a broad-band 560 nm band-pass filter in the case of orange-green actinic light. Levels of P700+ are given as the percentage of the maximal amplitude, that was reached at 400 μmol photons m⁻² s⁻¹ white light.

A. Wildtype Synechocystis PCC 6803 cells. B. Mutant M55, lacking the NADPH dehydrogenase complex. C. ΔpetH truncation mutant SM8. D. Double mutant DM4 (M55/SM8), carrying both the ΔpetH truncation and lacking the NADPH dehydrogenase complex.

ments that are even more red-shifted. Consequently, in the absence of sufficient thermal energy, transfer from PSI to PSII will significantly decrease. From the literature it is known that after transition to a light-state 1, the fluorescence yield of PSII has increased, whereas a decrease of PSI emission is not always observed (van Thor et al., 1998c).

Therefore methods have been employed in order to probe the phycobilisome-mediated absorption cross-section of PSI at room temperature, during state transitions. Flash-induced photo-oxidation of P700 was measured at room temperature in intact cells, using phycobilisome-absorbed light. Flash-yield studies indicated an efficient coupling between the phycobilisome and PSI, and it was found
that in light-state 1 the phycobilisome-antenna size for PSI decreased in *Synechococcus* PCC 6301 (Mullineaux, 1992). Such experiment require the accurate measurement of the yield of P700 photo-oxidation, as a function of the light intensity that is used for flash-excitation of the phycobilisomes, in whole cells.

An alternate approach was taken, in order to measure the distribution of phycobilisome-absorbed light between PSII and PSI, at room temperature. The level of P700$^+$ that accumulates, at a certain light intensity and wavelength, is the result of the light harvesting capacity and the distribution of excitation energy between PSII and PSI. In addition, electron transport capacity is limited by the turnovers that the components in the intersystem chain can make, as well as the turnovers that the reaction centers can make. The result of these effects is a saturation curve, and accumulation of P700$^+$ is reached at a certain light intensity. We used this method in order to determine a change in the balance of excitation energy distribution, depending on the light-state of the cells. This method, however, does not easily allow cells to be pre-incubated under conditions that induce a state 1. Therefore, advantage was taken of the fact that ndhB deficient mutant M55, lacking a functional NAD(P)H dehydrogenase complex, is in light-state 1 due to the over-oxidation of the plastoquinone pool (Schreiber *et al.*, 1995; van Thor *et al.*, 1998c, 1998d). The construction of the double mutant DM4, containing both the ΔpetH mutation of strain SM8 and a deletion of ndhB, was described previously (van Thor *et al.*, 1998d). These strains allow the comparison of excitation energy distribution of strains that either do, or do not contain FNR in the phycobilisomes, both in light-state 2 and in light-state 1. However, the electron transport capacity of strains carrying either or both mutations, is affected, when compared to the wildtype (van Thor *et al.*, 1998d). This is predicted to affect the accumulation of P700$^+$ as well.

Cells of wildtype *Synechocystis* PCC 6803 showed accumulation of P700$^+$ in white light, as well as in green light, to approximately the same extent (Figure 6; panel A). This indicates that both phycobilisome-absorbed light and chlorophyll-absorbed light results in photosynthetic activity of both PSII and PSI, at room temperature. The titration of green light reflects the resulting accumulation of P700$^+$ in state 2, since cells, illuminated with phycobilisome absorbed light, tend to remain in light-state 2. The titration with white light, with these intensities presumably reflects excitation energy distribution of cells that are still in light-state 2. High intensities of white light, however, can induce cells to reach a light-state 1. Previous results have indicated, however, that photochemistry of PSII is saturated at lower intensities, and saturation of PSI activity, and therefore oxidation of the plastoquinone pool via 'Emerson enhancement', inducing a light-state 1, proceeds only at intensities higher than 50 μE. m$^{-2}$ s$^{-1}$ in the case of *Synechocystis* PCC 6803 (van Thor *et al.*, 1998d). Therefore, the titration of cells of wildtype *Synechocystis* PCC 6803 with both white and green light intensities below 50 μE. m$^{-2}$ s$^{-1}$ is proposed to reflect light-state 2.

Cells of ndhB deficient mutant M55 show significant accumulation of P700$^+$ with white light already at relatively low light intensities (Figure 6; panel B). This is interpreted as the result of over-oxidation of the plastoquinone pool of this mutant. From 15 μE.m$^{-2}$ s$^{-1}$ to 30 μE.m$^{-2}$ s$^{-1}$ the titration curve is flattened. This is
interpreted as an indirect effect of the ‘permanent’ light-state 1: the imbalance of excitation energy distribution, resulting in the accumulation of electrons in the intersystem chain. In this mutant is observed with chlorophyll- as well as with phycobilisome-absorbed light at these light intensities (Schreiber et al., 1995; van Thor et al., 1998a). It was observed that the concentration of P700+ that accumulated with phycobilisome-absorbed light was notably lower in the case of the mutant M55. The clear difference that was observed between green- and white-light titrations between this mutant and the wildtype, demonstrates that the changes in excitation energy distribution are the effect of a light-state transition. In light-state 1 phycobilisome absorbed light is distributed in favour of PSII, compared to light-state 2. This resulted in lower concentrations of P700+, when white and green light at the same intensity were compared, for cells of mutant M55.

Cells of ΔpetH truncation mutant SM8 and cells of double mutant DM4 (SM8/M55) show accumulation of P700+ in white- and green actinic light, that are not significantly different from the wildtype and mutant M55 cells, respectively (Figure 6; panels C and D respectively). Therefore it is concluded that, also at room temperature, the distribution of phycobilisome absorbed light between PSII and PSI is not measurably different, when light-states 2 and 1 are compared between strains that either do, or do not contain FNR bound to the phycobilisomes.

Discussion

It was found that FNR binds to the phycobilisomes at a different site than was predicted, based on the sequence similarity of the binding domain with linker polypeptide sequences. The homology with the small core linker Lc8 may explain why FNR can bind to the core as well. Of course, copurification of FNR with core-particles from 4R can be considered an artefact due to the lower molecular mass of the complex. On the other hand, only few impurities are present in such preparations and the presence of FNR is suggestive when the sequence homology with the small core-linker Lc8 is considered. An argument can be made that FNR binds to the core-proximal phycocyanin hexamer in the structures from mutant ‘R20’, only in the absence of core-distal rods. A precedent for this is found in a mutant of Synechococcus PCC 7942, lacking the Lr33 protein. In these phycobilisomes Lr30 could occupy the position of Lr33, which induced proper spectral tuning and was functional in maintaining the stability of the rods in vivo and in vitro (Bhalerao et al., 1991). However, a strong indication that indeed FNR is bound in wildtype structures to the core-proximal hexamer as well, is found in the composition of particles obtained from partially dissociated phycobilisomes from Synechococcus PCC 6301 (Yamanaka et al., 1982). Although at that time it was not known that the 45 kDa protein that was copurified with these complexes was in fact FNR, it was observed that this polypeptide was present exclusively in fractions that contained both phycocyanin and allophycocyanin, and no Lc75 core linker (ApcE). Importantly, the product of cpcD, Lr40, was mostly enriched in a fraction that contained most phycocyanin and therefore represented the com-
position of the peripheral rods. Only a small amount of this $L_R^{10}$ rod-linker polypeptide was observed in the fraction that contained all 45 kDa polypeptide (Yamanaka et al., 1982). Thus, FNR is localised at a more core-proximal position than $L_R^{10}$ in the phycobilisomes of *Synechococcus* PCC 6301 as well.

Recombinant phycobilisome complexes, containing a tightly bound fusion protein between the N-terminal domain of FNR and the *Aequorea victoria* Green Fluorescent Protein were dissociated in order to disrupt fluorescence resonance energy transfer (FRET) between GFP and acceptors in the complex. Energy transfer rates, Förster radii and donor-acceptor radii were calculated for all possible acceptors and a range of orientation factors, based on the assumption that energy transfer became fully disrupted, 20 minutes after dilution of the complexes into low ionic strength buffer. The fluorescence polarisation that was determined under those conditions suggests that the fluorescence lifetime of the dissociated GFP fusion protein was comparable to the fluorescence lifetime of monomeric recombinant GFP. The donor-acceptor radii that were calculated are minimally 3 nm, and maximally 7 nm, depending on the identity of the acceptor chromophore(s) and the (average) orientation of the GFP chromophore. The rotational correlation time that was calculated from the intensity and the polarisation of the fluorescence, was 1.5 ns for the fusion protein bound to the complex, and 2.5 ns rapidly after dilution into low ionic strength. Since the fluorescence lifetime presumably increased from 0.7 ns to 3.3 ns during the experiment, it is concluded that the orientation of the chromophore of GFP can not be taken as an averaged value. Therefore the conclusion remains that the uncertainty in the donor acceptor distance is approximately 4 nm.

It is concluded from the results obtained with fluorescence spectroscopy and P700$^+$ determinations, that the presence of FNR in the phycobilisome does not affect the balance of excitation energy distribution with respect to PSII and PSI, in light-state 2 as well as in light state 1. Possibly, a decrease in the energy transfer rate from the phycobilisome terminal emitters to both reaction centers may result from the absence of FNR in the phycobilisomes. However, an increase in the number of phycobilisome light-harvesting complexes in the mutant may also account for the observed experimental data.

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