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Localization and function of ferredoxin:NADP$^+$ reductase bound to the phycobilisomes of *Synechocystis*

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Each phycobilisome complex of the cyanobacterium *Synechocystis* PCC 6803 binds ~2.4 copies of ferredoxin:NADP$^+$ reductase (FNR). A mutant of this strain that carries an N-terminally truncated version of the *petH* gene, lacking the 9 kDa domain of FNR that is homologous to the phycocyanin-associated linker polypeptide CpcD, assembles phycobilisome complexes that do not contain FNR. Phycobilisome complexes, consisting of the allophycocyanin core and only the core-proximal phycocyanin hexamers from mutant R20, do contain a full complement of FNR. Therefore, the binding site of FNR in the phycobilisomes 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 of the N-terminal domain of FNR and green fluorescent protein (GFP) contain this fusion protein in tightly bound form. Calculations of the fluorescence resonance energy transfer (FRET) characteristics between GFP and acceptors in the phycobilisome complex indicate that their donor–acceptor distance is between 3 and 7 nm. Fluorescence spectroscopy at 77K and measurements in intact cells of accumulated levels of P700$^+$ indicate that the presence of FNR in the phycobilisome complexes does not influence the distribution of excitation energy of phycobilisome-absorbed light between photosystem II and photosystem I, and also does not affect the occurrence of 'light-state transitions'.

Keywords: ferredoxin:NADP$^+$ reductase/fluorescence resonance energy transfer/green fluorescent protein/photosystem I/phycobilisome

Introduction

Three cyanobacterial *petH* genes have been cloned and 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 encode a protein composed of three domains. The two C-terminal domains suffice for the enzymatic activity of FNR. The third, ~9 kDa N-terminal domain, is homologous to the small phycocyanin rod-linker polypeptide CpcD (Schluchter and Bryant, 1992; Fillat *et al*., 1993; van Thor *et al*., 1998a). Similarity to the related linker polypeptides CpcC (the L_R^{35} rod-linker) and ApcC (the small core-linker L_C^8) is detectable, but less than to CpcD. Purified phycobilisome complexes contain significant amounts of FNR. Based on these two considerations, it was assumed that FNR binds to phycobilisomes at the core-distal phycocyanin hexamer (Schluchter and Bryant, 1992), which is the position that CpcD is known to occupy (de Lorimier *et al*., 1990).

It is not directly obvious what the function is of this localization of FNR at the outer position of the phycocyanin rods of the phycobilisome. One possibility is that this provides a 'localized' higher concentration of FNR, which may be necessary for catalysing maximal rates of NADP$^+$ reduction during linear photosynthetic electron transport, with less copies of FNR than would be required if its localization was cytoplasmic. It could also be a means of introducing heterogeneity in the pool of FNR, since free, cytoplasmic FNR binds to the thylakoid membrane via this domain (J.J.van Thor, R.Jeanjean, M.Havaux, F.Joset, K.A.Sjollema, K.J.Hellingwerf and H.C.P.Matthijs, in preparation).

Others have proposed that a phycobilisome–photosystem I (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 has been proposed to be mediated via the stromal PsaE subunit of PSI. Formation of this supercomplex is proposed to specifically enhance transfer of excitation energy from the phycobilisomes to PSI, as a result of the reduced distance between the chromophores of the antennae and the reaction centres (Bald *et al*., 1996).

Mutations in *apcA* or *apcE*, affecting the core of the phycobilisome complex, result in a significant uncoupling of the phycobilisomes from the reaction centres (Su *et al*., 1992; Shen *et al*., 1993). In the *apcA* mutant, only a limited amount of phycocyanin is detected; an *apcE* mutant still produces allophycocyanin, but no longer couples this functionally to the reaction centres. However, in both the *apcA* and *apcE* mutants, some energy transfer from the remaining phycobilisomes specifically to PSI might still be observed. Interestingly, a comparable situation develops in heterocysts *of Anabaena variabilis*, when allophycocyanin is degraded before phycocyanin. Also in these (PSII-deficient) cells, some energy transfer from phycocyanin to PSI has been observed with 77K fluorescence spectroscopy (Peterson *et al*., 1981; Tyagi *et al*., 1981).

Here we show that *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

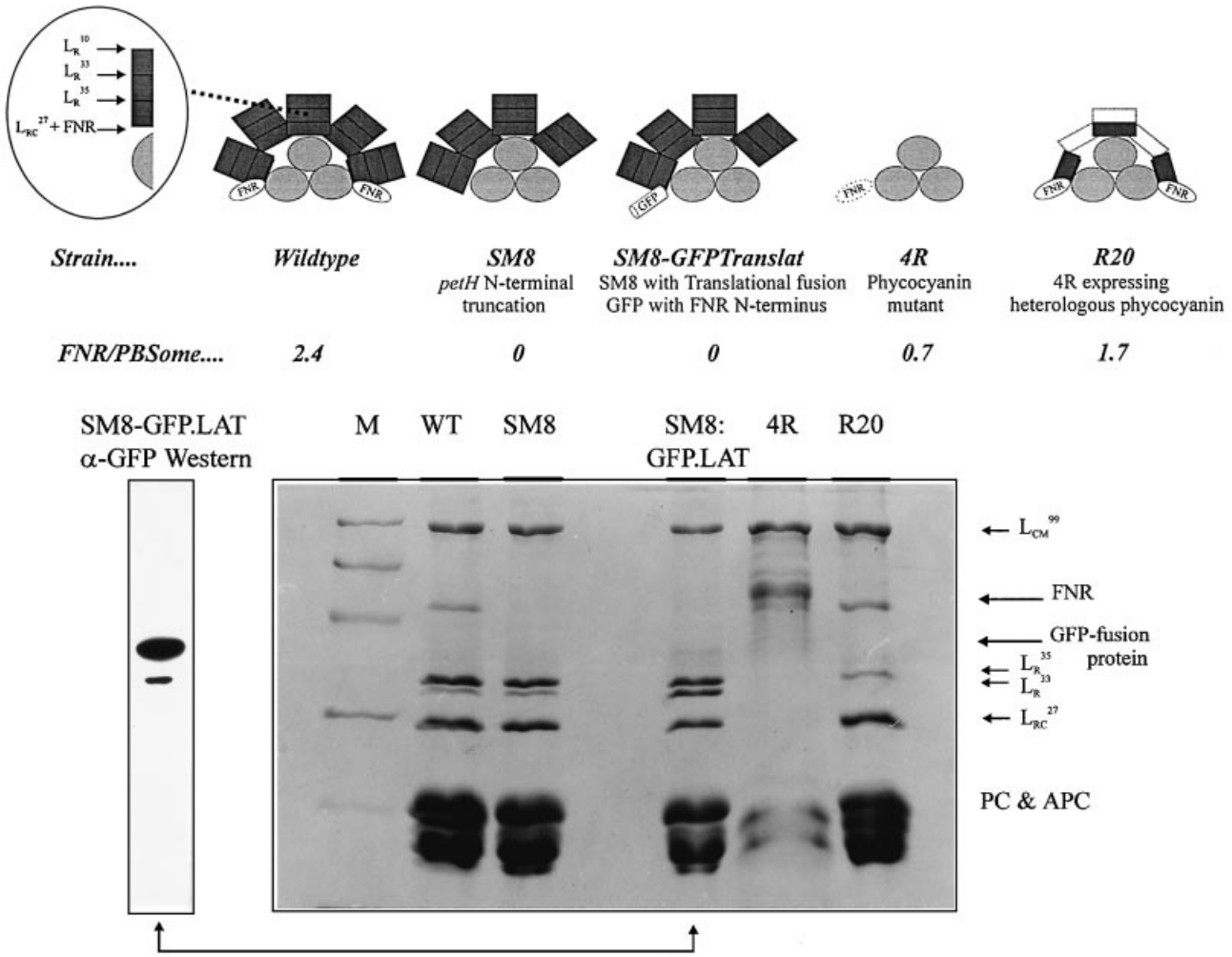

Fig. 1. SDS–PAGE analysis of wild-type, mutant and recombinant purified phycobilisome complexes from *Synechocystis* PCC 6803 and mutants. Phycobilisome complexes isolated from the wild type (lane 2), Δ*petH* truncation mutant SM8 (lane 3), double mutant SM8 containing the GFP fusion protein (lane 4), phycocyanin-deficient mutant 4R (lane 5) and strain R20 expressing heterologous phycocyanin in 4R background (lane 6) were analysed. 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. The inset shows the localization of the rod-associated linker polypeptides.

overall energy transfer rate from the phycobilisomes to both PSII and PSI reaction centres may be decreased in the mutant, as compared with the wild type. We fused GFP translationally to the N-terminus of FNR to show that this latter domain directs binding of the protein to the phycobilisome complex. Radiationless energy transfer from GFP, presumably to 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.

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) FNRs have been shown to copurify or associate *in vitro* with the cytochrome b_6f complex (Clark *et al*., 1984) and with PSI via the PsaE subunit (Andersen *et al*., 1992), it was nessecary to test whether a two-domain FNR lacking the CpcD-homologous domain copurifies with the phycobilisomes. Copurification of chloroplast FNR with the cytochrome b_6f complex has been shown to be an artefact of the isolation procedure (Coughlan *et al*., 1985).

A *Synechocystis* mutant (SM8) was constructed that carries a truncated Δ*petH* gene, via the in-frame deletion of 225 nucleotides downstream of the initiation codon of the wild-type *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 activites comparable with the wild-type protein (J.J.van Thor, R.Jeanjean, M.Havaux, F.Joset, K.A.Sjollema, K.J.Hellingwerf and H.C.P.Mattijs, in preparation). When phycobilisomes are isolated from this mutant, no FNR is copurified with these 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 centres (Plank and Anderson, 1995; Plank

et al., 1995). These aggregates can be isolated by a modified sucrose density gradient ultracentrifugation protocol, in spite of their reduced molecular mass, albeit with some high molecular weight impurities (Figure 1). These complexes were found to contain sub-stoichiometric amounts of FNR. However, given the impurities observed in these samples, this does not unequivocally prove that FNR has affinity for allophycocyanin.

The 'R20' strain, heterologously 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 with the wild type. This is due to the near absence of core-distal phycocyanin hexamers in the phycobilisome rods. The 27 kDa rod–core linker is the only phycocyanin-associated linker that is detected in substantial amounts in this mutant. The L_R^{35} phycocyanin linker polypeptide was detected only in very small amounts, whereas the L_R^{33} linker polypeptide, which is associated with the core-distal phycocyanin hexamer, is completely absent (Figure 1). In addition, the small rod-linker L_R^{10} was not detectable in the mutant phycobilisomes (not shown). Therefore, the occurrence of L_R^{35} associated phycocyanin hexamers is low, and no core-distal hexamers are present in these complexes. Quantification of the phycocyanin:allophycocyanin ratio indicated that these complexes contain about three phycocyanin hexamers per core. In contrast to expectations, near wild-type levels of FNR were detected in these isolates (Figure 1). The presence of FNR, and the absence of L_R^{10} (CpcD) in the phycobilisomes of R20 are fully in agreement with our conclusion that these proteins occupy different binding sites (Figure 1). The small rod-linker CpcD, which shows the highest homology with the N-terminus of FNR, is present in the wild-type complexes exclusively in the core-distal hexamers (de Lorimier *et al*., 1990). In the absence of these core-distal hexamers, FNR still copurifies with the phycobilisome complexes. We therefore propose that FNR is bound to the core-proximal phycocyanin hexamer.

Since the complexes from both strains, 4R and R20, contain FNR, we propose that FNR binds the core-proximal hexamer at a position between the core structure and the phycocyanin hexamer. Most probably, FNR binds to the same location as the 27 kDa rod–core linker in the phycobilisome complex. However, based on this data it cannot be ruled out that binding of FNR to phycocyanin occurs on the distal side of this hexamer, the binding site of the 35 kDa rod-linker CpcC. Surprisingly, the N-terminal domain of FNR seems to possess affinity for allophycocyanin. It is not known whether binding to allophycocyanin is possible only in the absence of phycocyanin, or whether also in wild-type structures some copies of FNR are bound to allophycocyanin, rather than to phycocyanin exclusively. When the amino acid sequence of the N-terminal domain of FNR (75 residues) was compared with the amino acid sequence of the phycobilisome linker polypeptides of *Synechocystis* PCC 6803, the highest similarity found was to the L_R^{10} small phycocyanin-associated linker polypeptide, the product of *cpcD* (van Thor *et al*., 1998a; 57% identity and 72% similarity, for 61 residues). However, the similarity to L_R^{35} (the product of *cpcC*) and L_C^8 (the product of *apcC*) was also significant. For the L_R^{35} linker polypeptide, associated with the core-proximal and 'second' phycocyanin hexamer, the identity was 40%, and the similarity was 68%, for a stretch of 34 residues. For the small allophycocyanin-associated core linker L_C^8, the identity was 45%, and the similarity 66%, for 23 residues (Kaneko *et al*., 1996). In particular the similarity to the L_R^{35} linker polypeptide is suggestive, since this linker is associated with the core-proximal phycocyanin hexamer. The similarity to the core linker L_C^8 may be significant because of the apparent affinity of FNR for allophycocyanin.

A translational fusion between the 75 N-terminal residues of FNR and a mutant form of green fluorescent protein (GFPuv) was expressed both in the wild-type background and in the Δ*petH* 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 Blue staining after SDS–PAGE, as well as with immunodetection using an anti-GFP monoclonal antibody (Figure 1). Furthermore, fluorescence emission of GFP was detected in both types of recombinant complexes. The complexes isolated from the wild-type background contained both FNR (at wild-type levels), and the GFP fusion protein [~50% of the level present in the complexes isolated from the FNR-free SM8 background (not shown)]. This suggests that FNR and the GFP fusion protein compete for the same binding site(s) in the core-proximal phycocyanin hexamers.

Fluorescence resonance energy transfer (FRET) between GFP and the allophycocyanin 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 relatively weak fluorescence emission maximum at 510 nm, with a corresponding excitation maximum at 395 nm. However, this emission peak from GFP is well separated from phycocyanin and allophycocyanin emission. The fluorescence anisotropy of the phycobilisome-bound GFP was higher than the value that was determined for free, monomeric, recombinant GFP (Table I), indicating that its excited state is shorter lived- and/or the rotational motion is decreased. The measured fluorescence anisotropy for recombinant GFP (Table I) agreed well with the published value (Swaminathan *et al*., 1997). Possibly, the rotational correlation time-constant (τ_{rot}) for the phycobilisome-bound GFP fusion protein is larger than for free monomeric GFP. For the phycobilisome-bound GFP, τ_{rot} is calculated to be ~50 ns. A minimal value of 13 ns is estimated within the obtained accuracy of these measurements (Table I). This could indicate that the GFP fusion protein, when bound to the phycobilisomes, moves by anisotropic rather than by isotropic rotational diffusion.

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). We observed the dissociation of wild-type phycobilisome complexes from *Synechocystis*

Table I. Fluorescence and rotational diffusion characteristics of monomeric GFP and the phycobilisome-bound GFP fusion protein

	A	τ_f (ns)	τ_{rot} (ns)
30 kDa recombinant GFP	0.325 ± 0.002	3.3	$13.8 > \tau_{rot} > 14.8$
39 kDa fusion–GFP (PBS-bound)	0.40 ± 0.02	0.7	$\tau_{rot} >> 13$
39 kDa fusion–GFP (dissociated)	0.33 ± 0.02	3.3	$11 > \tau_{rot} > 23$

The fluorescence anisotropy (A) was determined for the free, monomeric recombinant form of GFP, the phycobilisome-bound form in high salt conditions, and the dissociated form (20 min after dilution into low ionic strength conditions). The fluorescence lifetimes (τ_f) were calculated assuming a lifetime of 3.3 ns for the wild type (Perozzo *et al*., 1988). The rotational correlation times (τ_{rot}) were calculated using the Perrin equation $\frac{1}{A} = \frac{1}{A_0}(1 + \frac{\tau_f}{\tau_{rot}})$, assuming that the intrinsic anisotropy $A_0 = 0.4$.

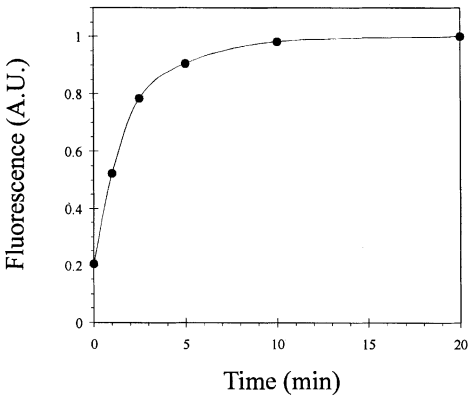

Fig. 2. Fluorescence amplitude of the GFP fusion protein during dissociation of the recombinant phycobilisome complex. Fluorescence intensities were determined by integration of the entire emission band, from 450 to 600 nm, with excitation at 395 nm. It was verified that during this experiment, photoconversion of GFP (van Thor *et al*., 1998c) did not contribute to the amplitude that was determined by measuring the relative excitation maximum at 480 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.

PCC 6803 on this time scale as well, apparent from the increase of fluorescence emission at 650 nm.

Upon transfer of the recombinant phycobilisome complexes containing the GFP fusion protein, into 10 mM phosphate buffer, the fluorescence quantum yield of the emission at 510 nm increased, until a maximum was reached 20 min later (Figure 2). A decrease of the fluorescence anisotropy was measured after dissociation of the phycobilisome complex, until a value was reached comparable with recombinant monomeric GFP (Table I). Most probably energy transfer from GFP to phycobilisome-associated chromophores was completely disrupted within 20 min after dilution, and rotational diffusion of the GFP fusion protein had gained an isotropic mode, comparable with monomeric GFP.

Taking into acount 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, based on these measurements and calculations is the following: within the first minute, the phycocyanin rods are released from the core, and energy transfer from GFP to allophycocyanin is disrupted. The GFP fusion protein presumably gains an isotropic mode of rotational motion already during the first minute of dissociation, since the fluorescence anisotropy declines most significantly during this period (not shown). Dissociation from the phycocyanin rods then takes place within the next 19 min, resulting in the further uncoupling of energy transfer. This scenario would also fit the observation that dissociation of phycocyanin from the allophycocyanin core is the first stage of complex dissociation under low ionic strength conditions (Gantt *et al*., 1979; Maxon *et al*., 1989).

The fluorescence intensity of the GFP fusion protein, 20 min after dissociation of the complex, was quantified by comparing this signal to a standard dilution series of recombinant GFP under identical buffer and temperature conditions. Using the calculated extinction coeficient for phycobilisome complexes of SM8, 27.000/mM/cm, a ratio of 0.26 GFP:phycobilisome was found. The band representing the recombinant GFP fusion protein present in these complexes stained significantly less intensely with Coomassie Blue, than the band of 47 kDa FNR in the wild-type complexes, for which a ratio of 2.4 FNR: phycobilisome was determined (Figure 1). This also suggests that most energy transfer was disrupted (i.e. the GFP fusion protein has a high fluorescence quantum yield after dissociation) 20 min after dilution. In addition, it was observed that wild-type FNR, bound to the phycobilisomes, is fully released from the complexes in a low ionic strength buffer, and subsequently associates with the thylakoid membranes. This was observed for the GFP fusion protein as well, indicating that complete dissociation was likely to have occurred.

Förster energy transfer calculations

The observed changes of the fluorescence quantum yield of the GFP fusion protein during dissociation of the phycobilisome complexes were interpreted as changes in the rate of resonance energy transfer. This interpretation rests upon the assumption that excitation energy transfer from GFP to phycocyanobilin chromophores can proceed 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 β-155 chromophore of phycocyanin, which 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),

Table II. Förster radii and donor–acceptor radii assuming exclusive energy transfer to the presented acceptors

Energy transfer rate for GFP k_{ET}^{DA} (s^{-1})	Acceptor chromophore	Spectral overlap integral (cm^3/M)	R_0 (Å)				R_{DA} (Å)			
			$\kappa^2 = 1/3$	$\kappa^2 = 2/3$	$\kappa^2 = 4/3$	$\kappa^2 = 4$	$\kappa^2 = 1/3$	$\kappa^2 = 2/3$	$\kappa^2 = 4/3$	$\kappa^2 = 4$
1.16×10^9	PC-β-155	2.08×10^{-13}	46	52	58	70	36	40	45	54
1.16×10^9	PC-α-84	1.09×10^{-13}	42	47	52	63	32	36	40	49
1.16×10^9	PC-β-84	5.1×10^{-14}	37	41	46	55	28	32	36	43
1.16×10^9	APC-α-84	1.95×10^{-13}	46	51	58	69	35	40	45	53

The total energy transfer rate that was determined experimentally (1.16×10^9/s) 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 κ^2. The refraction index n was taken as 1.567 (Grabowski and Gantt, 1978).

Table III. Förster radii and donor–acceptor radii assuming a 'staged' dissociation of the complex

Energy transfer rate for GFP k_{ET}^{DA} (s^{-1})	Acceptor chromophore	Spectral overlap integral (cm^3/M)	R_0 (Å)				R_{DA} (Å)			
			$\kappa^2 = 1/3$	$\kappa^2 = 2/3$	$\kappa^2 = 4/3$	$\kappa^2 = 4$	$\kappa^2 = 1/3$	$\kappa^2 = 2/3$	$\kappa^2 = 4/3$	$\kappa^2 = 4$
2.78×10^8	PC-β-155	2.08×10^{-13}	46	52	58	70	45	51	57	69
2.78×10^8	PC-α-84	1.09×10^{-13}	42	47	52	63	41	46	51	62
2.78×10^8	PC-β-84	5.1×10^{-14}	37	41	46	55	36	40	45	54
8.82×10^8	APC-α-84	1.95×10^{-13}	46	51	58	69	37	42	47	56

Exclusive disruption of energy transfer to allophycocyanin is assumed to proceed in the first minute after dissociation (Figure 2). The energy transfer rate 2.78×10^8/s at $t = 1$ min is then assigned exclusively to the three phycocyanin chromophores, as in Table II.

and the absorption spectrum of the allophycocyanin core, isolated from mutant 4R, assuming an extinction coefficient of 235/mM/cm at 650 nm for the αβ monomer (Bryant *et al*., 1979). The spectral overlap integrals for GFP and the possible acceptors are: 2.08×10^{-13} cm^3/M for the β-155 chromophore of phycocyanin, 5.08×10^{-14} cm^3/M for the β-84 chromophore of phycocyanin, 1.09×10^{-13} cm^3/M for the α-84 chromophore of phycocyanin and 1.95×10^{-13} cm^3/M for the α-84 chromophore of allophycocyanin, respectively. The overlap integral for the β-84 chromophore of allophycocyanin was neglected, since its absorbance maximum is red-shifted significantly. Excitation energy transfer from phycocyanobilin chromophores to GFP was neglected: 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 significantly.

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 rate of energy transfer from GFP to the acceptors (Figure 2; time 0) was calculated to be 1.16×10^9/s. In order to calculate the energy transfer characteristics, the orientation of the donor and acceptor chromophore dipoles must be considered. Rotational diffusion of the GFP fusion protein bound to the phycobilisome complex proceeds at a longer time scale than the lifetime of the excited state. However, the extent of the movements and the orientation of the chromophores are unknown. Therefore, values for the squared orientation factor κ^2 of 1/3, 2/3, 4/3 and 4 were used to calculate the Förster radii, reflecting unfavourable, average, good and optimal orientation of the dipoles, respectively. Table II presents the R_0 and R_{DA} values for all acceptor chromophores. The R_{DA} values were calculated, assigning the total energy transfer rate 1.16×10^9/s to each individual chromophore exclusively (Table II). 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-α-84 chromophore (Table III). If simultaneous energy transfer from GFP to more than one acceptor chromophore occurs in the complex, the Förster radii and donor–acceptor radii will become somewhat larger. 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 II and III).

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

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 PSI would be possible, given the dimensions of the core of the phycobilisome and the stroma-exposed region of PSI. The stroma-exposed subunits PsaE, -D and -C are predicted to protrude ~30 Å into stromal space (Schubert *et al*., 1997), whereas the distance between the thylakoid surface and FNR bound to the phycobilisomes may be up to 300 Å, given the specific rod it is attached to. Phycobilisome-bound FNR may interact with PSI via the PsaE subunit (Andersen *et al*., 1992), promoting energy transfer from the antennae to the reaction centres.

Fluorescence spectroscopy at 77K of intact cyanobacterial cells was used in order to determine the pathway of energy transfer in wild type and mutants of *Synechocystis* PCC. The emission maxima associated with PSII, 685 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. Figure 3 shows the emission spectra, taken with either 440 or 574 nm excitation of cells of the wild-type and Δ*petH* mutant 'SM8' cells. The emission

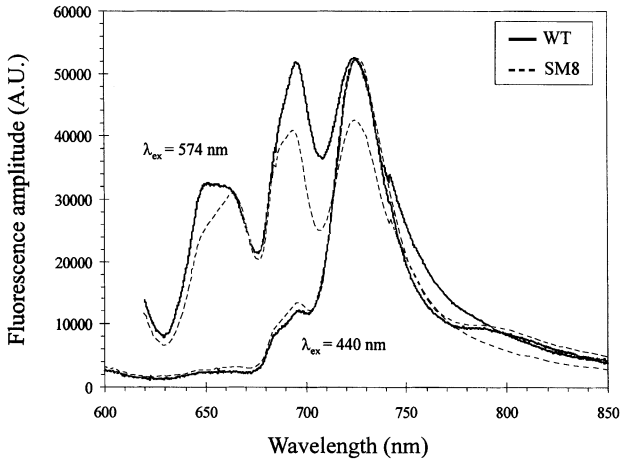

Fig. 3. Fluorescence emission spectra of wild-type and Δ*petH* truncation mutant 'SM8' cells recorded at 77K, with phycobilisome and chlorophyll excitation. Wild-type (solid line) and Δ*petH* mutant SM8 (dashed line) cells were dark adapted before being frozen in liquid N_2. Emission spectra were recorded with either 440 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.

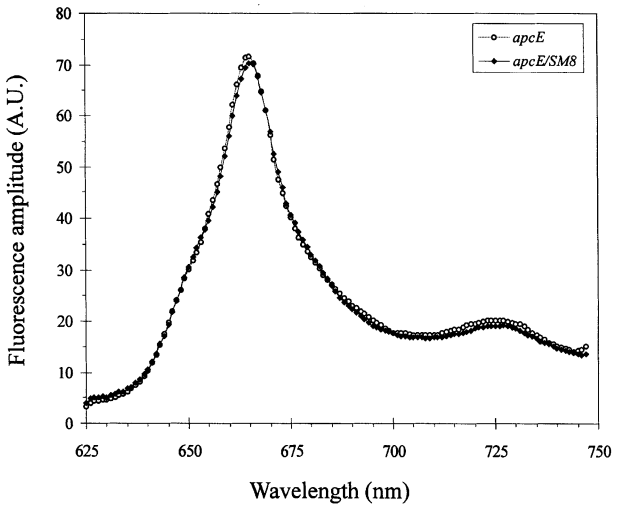

Fig. 4. Fluorescence emission spectra (600 nm) of *apcE* single mutant and *apcE*/SM8 double mutant cells recorded at 77K. *apcE* single mutant (open symbols) and *apcE*/SM8 double mutant (closed symbols) cells were dark adapted before being frozen. Emission spectra were recorded with 600 nm excitation and normalized at 665 nm.

spectra taken with 440 nm excitation of both strains were almost identical. The emission maxima at 685 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 in 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. Upon excitation with 574 nm light, emission from both PSII (i.e. 685 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 is probably the result of an increase in the number of phycobilisome complexes relative to the number of reaction centres in mutant SM8.

Mutations in *apcA* and *apcE* affect the core of the phycobilisome. Deletion of *apcA*, encoding the allophycocyanin α subunit, resulted in mutant *Synechocystis* PCC 6803 cells that completely lack allophycocyanin (Su *et al*., 1992). Some phycocyanin was still present in these mutant cells, but this was largely uncoupled from PSII, as judged from 77K fluorescence spectra (Su *et al*., 1992). In the *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 *et al*., 1993). Interestingly, in both *apcA* and *apcE* mutants, some energy transfer from phycocyanin to PSI may still occur. Su *et al*. (1992) suggested that FNR may link phycocyanin to PSI and give rise to the fluorescence emission from PSI, upon excitation of phycocyanin in the *apcA* mutant.

The emission spectrum, taken with 600 nm excitation, of a 'single' *apcE* mutant showed a prominent maximum at 665 nm, which was assigned to allophycocyanin (Figure 4). A second maximum at 725 nm was assigned to PSI in this emission spectrum. No emission from PSII at 685 or 695 nm was observed, in agreement with previous observations (Shen *et al*., 1993). Approximately 50% of the emission at 725 nm originates from PSI, judging from the 600 nm emission spectra from an *apcE* single mutant and an *apcE*/PSI-deficient double mutant (Shen *et al*., 1993). It was estimated that the fluorescence quantum yield at 77K of allophycocyanin must exceed that of PSI-associated chlorophyll *a* by ~10-fold in order to explain the intensity of the excitation maximum at 600 nm for the 725 nm emission, if excitation energy transfer from phycocyanin to PSI does not occur.

In order to determine whether FNR is responsible for the possible energy transfer from phycocyanin to PSI observed in the *apcE* and *apcA* mutants, a double mutant was constructed carrying both the Δ*petH* truncation and a deletion of *apcE*. The emission spectra, recorded with 600 nm excitation at 77K, are nearly identical for the *apcE* single mutant and the *apcE*/SM8 (*apcE*/Δ*petH*) double mutant, featuring both the 665 nm and the 725 nm emission maxima (Figure 4). This clearly indicates that the N-terminal domain of FNR is not responsible for the possible energy transfer from phycocyanin to PSI in the *apcE*-deficient background.

Phycobilisome-bound FNR does not affect '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 can be characterized by fluorescence measurements and other techniques (for a review see van Thor *et al*., 1998b). Emission spectra at 77K of intact cells were recorded with excitation of phycobilisomes at 574 nm in order to detect the extent of fluorescence changes upon transition between light-states 2 and 1. After induction of light-state 1 by illumination with far-red light for 5 min ($\lambda > 710$ nm; ±12 mW), it was seen for both wild-type and mutant cells that emission from PSII increased, whereas emission from PSI decreased, when the

spectra were normalized at 665 nm (not shown). This suggests that the N-terminus of FNR is not directly involved in the mechanism of transitions between the different light states.

However, migration of excitation energy at 77K does not necessarily reflect the pathways that are operative at room temperature (van Thor *et al*., 1998b). In particular, 'spill-over' of excitation energy from PSII to PSI might occur at cryogenic temperatures. An experimental approach was therefore designed to measure the distribution of phycobilisome-absorbed light between PSII and PSI at room temperature, in both light-state 2 and light-state 1. 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 number of turnovers that the components in the intersystem chain can make, as well as those the reaction centres can make. The result of these effects is a saturation curve: $P700^+$ will accumulate with increasing 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 is not compatible with pre-incubation of the cells 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 its plastoquinone pool (Schreiber *et al*., 1995; van Thor *et al*., 1998b). The construction of the double mutant DM4, containing both the Δ*petH* mutation of strain SM8 and a deletion of *ndhB*, is described elsewhere (J.J.van Thor, R.Jeanjean, M.Havaux, F.Joset, K.A. Sjollema, K.J.Hellingwerf and H.C.P.Matthijs, in preparation). These strains allow the comparison of excitation energy distribution of strains that either do or do not contain FNR in the phycobilisomes, in both light-states 2 and 1.

Cells of wild-type *Synechocystis* PCC 6803 showed accumulation of $P700^+$ in white light, as well as in green light, to approximately the same extent (Figure 5A). This indicates that both phycobilisome- 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 intensities up to 50 $\mu E/m^2/s$ presumably reflects excitation energy distribution of cells that are still in light-state 2.

Cells of *ndhB*-deficient mutant M55 show significant accumulation of $P700^+$ with white light already at relatively low light intensities (Figure 5B). This is interpreted as the result of over-oxidation of the plastoquinone pool of this mutant. From 15 to 30 $\mu E/m^2/s$, 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*., 1998b). It was observed that the concentration of $P700^+$ that accumulated with phycobilisome-absorbed light was notably lower in the case of mutant M55. The clear difference that was observed between green- and white-light titrations between this mutant and the wild type, 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 with 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 Δ*petH* truncation mutant SM8 and of double mutant DM4 (SM8/M55) show accumulation of $P700^+$ in white and green actinic light, which is not significantly different from the wild-type and mutant M55 cells, respectively (Figure 5C and D). 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 phycobilisomes.

Discussion

It was found that FNR binds to the phycobilisomes at a different site from that predicted based on the sequence similarity of the binding domain with sequences of linker polypeptides. The homology with the small core-linker L_C^8 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 L_C^8 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. This is unlikely, since L_R^{10} (CpcD) does not seem to be present in the phycobilisomes in the absence of core-distal phycocyanin hexamers. An example of non-specific binding of linker polypeptides to phycocyanin is found in a mutant of *Synechococcus* PCC 7942 lacking the L_R^{33} protein. In these phycobilisomes, L_R^{30} could occupy the position of L_R^{33}, 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, another strong indication that FNR is indeed also bound to the core-proximal hexamer in wild-type phycobilisomes, 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 FNR copurified with phycobilisome complexes, a protein with a molecular mass of 45 kDa can be observed in their gels, representing the composition of a particle that contained both phycocyanin and allophycocyanin, and no L_{CM}^{75} core-linker (ApcE). Importantly, the product of *cpcD*, L_R^{10}, was mostly enriched in another particle that contained most phycoyanin and therefore presumably contained the peripheral rods (Yamanaka *et al*., 1982). Thus, FNR is also localized at a more core-proximal position than L_R^{10} in wild-type phycobilisomes of *Synechococcus* PCC 6301.

Recombinant phycobilisome complexes, containing a tightly bound fusion protein composed of the N-terminal domain of FNR and the *Aequorea victoria* GFP were dissociated in order to disrupt 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

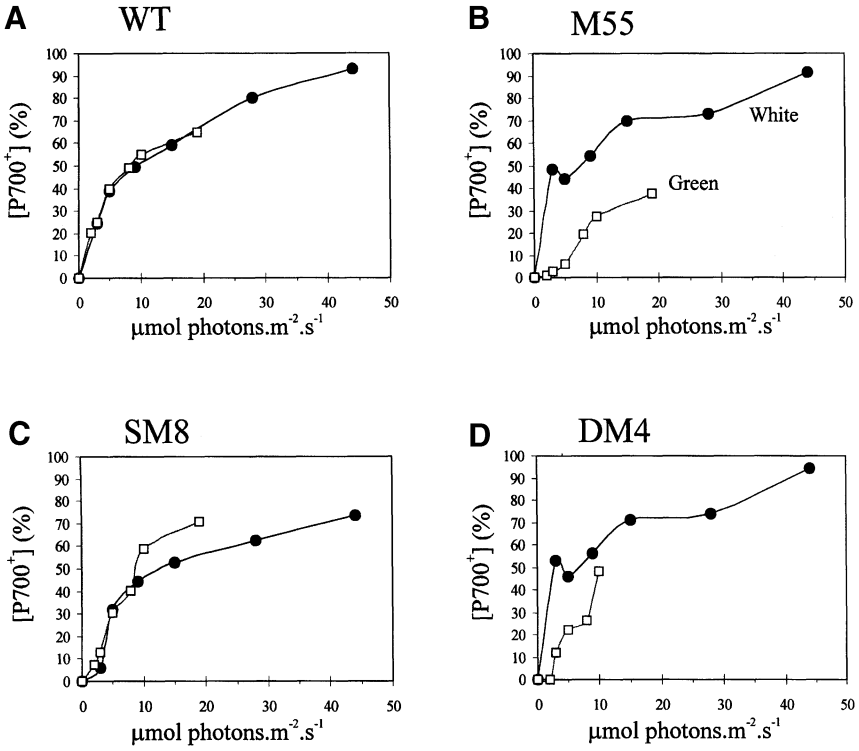

Fig. 5. Steady-state levels of $P700^+$ accumulated in white and green light of wild-type and mutant cells of *Synechocystis* PCC 6803. *In vivo* pulse-modulated measurements of the increase in absorbance at 830 nm, reflecting the accumulation of $P700^+$. Cells were deposited on a filter and illuminated 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 reached at 400 µmol photons/m^2/s white light. (**A**) Wild-type *Synechocystis* PCC 6803 cells. (**B**) Mutant M55, lacking the NADPH dehydrogenase complex. (**C**) Δ*petH* truncation mutant SM8. (**D**) Double mutant DM4 (M55/SM8), carrying both the Δ*petH* truncation and lacking the NADPH dehydrogenase complex.

assumption that energy transfer became fully disrupted within 20 min of dilution of the complexes into low ionic strength buffer. The fluorescence anisotropy that was determined under those conditions suggests that the fluorescence lifetime of the dissociated GFP fusion protein was comparable with 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.

It is concluded from the results obtained with fluorescence spectroscopy and measurements of $P700^+$ accumulation, 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-states 2 and 1. The technique presented here, measuring $P700^+$ accumulation, has shown clearly that the phycobilisomes have a light-harvesting function for PSI at room temperature, particularly in light-state 2.

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 Δ*petH* 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 elsewhere (J.J.van Thor, R.Jeanjean, M.Havaux, F.Joset, K.A.Sjollema, K.J.Hellingwerf and H.C.P.Matthijs, in preparation). An *ndhB*-deficient mutant 'M55', lacking a functional NAD(P)H dehydrogenase complex, was obtained from T.Ogawa (Ogawa, 1991). A double mutant (i.e. 'M55' containing the Δ*petH* truncation), 'DM4', will also be described elsewhere (J.J.van Thor *et al*., in preparation). A mutant with a deletion of the *apcE* gene was kindly provided by W.F.Vermaas (Shen *et al*., 1993). Mutants carrying the Δ*petH* 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 grown in medium containing erythromycin (10 µg/ml). A double mutant, carrying both the Δ*petH* 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 M.Ashby, encoding kanamycin resistance. A phycocyanin-deficient mutant, '4R' (Plank *et al*., 1995) and a mutant, 'R20' expressing *cpcA* and *cpcB* genes from *Synechocystis* PCC 6701 (Plank and Anderson, 1995) were kindly provided by L.K.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.

Fluorescence spectroscopy

Fluorescence spectra at 77K were recorded with a laboratory built fluorimeter with a spectral resolution of 0.16 nm (Figure 3). The sample, cooled to 77K, was excited with light from a tungsten–halogen lamp, filtered through either a 440 or a 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 built-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 cannot be used in such samples, since this denatures phycobiliproteins and partially

uncouples energy transfer from the phycobilisomes to the reaction centres. Measurements of fluorescence spectra and fluorescence polarization at room temperature were recorded with the Aminco Bowman Series 2 fluorimeter, equiped 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 *et al.* (1979). SDS–PAGE was performed with 15% acrylamide mini-gels (Bio-Rad). 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-labelled 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*., 1998c) and used as a standard for quantification of GFP fusion protein in recombinant phycobilisome complexes. The fluorescence quantum yield for excitation of the neutral species of the chromophore of GFP at 395 nm was determined to be ~0.8, using sodium fluorescein at pH 8 as a standard. Therefore it was assumed that the fluorescence lifetime was 3.3 ns (Perozzo *et al*., 1988).

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).

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