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Structure and energy transfer of the phycobilisome in a linker protein replacement mutant of cyanobacterium *Synechococcus* 7942

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The role of the linker proteins in the biogenesis and energy transfer of the phycobilisome rod was monitored by making insertional inactivation in the *cpcI* gene coding for the core-proximal 33 kilodalton (kDa) protein in the cyanobacterium *Synechococcus* 7942. The insertion leaves the *cpcH* gene coding for the core-distal 30 kDa protein intact and functional. Analysis of the phycobilisome protein composition of the *cpcI* mutant shows that the 30 kDa protein is present in normal amounts in the rod, indicating that the 30 kDa linker protein can replace the 33 kDa protein in the biogenesis and structural integrity of the rod. The absorption and fluorescence characteristics of the mutated phycobilisome is almost indistinguishable from that of the wild-type of the same rod length. The fluorescence kinetics from the *cpcI* mutant show that the dominating decay component has a lifetime from phycocyanin of 69 ps as compared to 72 ps found for the wild-type phycobilisome with the same rod length. The results show that replacing the 33 kDa for the 30 kDa linker in the rod does not alter the energy harvesting or the energy transfer characteristics of the rod in contrast to what has been concluded from data obtained from in vitro experiments. We conclude that the linker polypeptides have only a minor influence on the energy transfer characteristics of the rod but are mainly involved in determining the length of the rod in response to changing environmental light conditions.

Introduction

The phycobilisome serves as the light harvesting antennae in cyanobacteria [1–4]. It harvests photons and transfers the energy to the photosynthetic reaction centre of Photosystem II [1–4]. The phycobilisome, which looks like a hemispheric disc in the electronmicroscope, is a multi-protein complex with a highly ordered structure consisting both of chromophorylated polypeptides as well as non-chromophorylated structural linker proteins [2–4]. It is built up as a core component attached to the thylakoid membrane with rods bound to the core, the rods pointing outwards [4]. The chromophoric proteins in the rod have their light absorption maxima at a shorter wavelength than the chromophoric proteins in the core thereby focusing the excitation energy efficiently down to the chlorophyll molecules in the reaction centre.

The phycobilisomes of the cyanobacterium *Synechococcus* 6301/7942 (also denoted *Anacystis nidulans* 625/R2) has an over-all absorption maximum at approx. 625 nm [5–7]. It contains the chromophoric proteins allophycocyanin (APC) and phycocyanin (C-PC) in its core and rod, respectively. Each protein consists of two subunits, α and β. The rod length can be changed in a highly regular manner in accordance with changing environmental conditions [8,9]. In its fully extended form it contains three discs, each of which is a double trimer of phycocyanin, $(\alpha_3\beta_3)\times 2$ [5]. Four non-chromophorylated linker polypeptides are positioned at specific places in the rod, thus it is more complex than the rods found in the cyanobacterium *Agmenellum quadruplicatum* turn [10]. A linker protein

Abbreviations: PBS, phycobilisome; C-PC, cyanobacterial phycocyanin; APC, allophycocyanin; kb, kilobase pairs; kDa, kiloDalton.

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with the size of 27 kDa anchors the first hexamer in the rod to the core. A second linker, 33 kDa in size, is located between the first and second hexamer, while a 30 kDa linker anchors the outermost third hexamer. A small 9 kDa linker is believed to be positioned at the tip of each rod. The linker polypeptides are thought not only to contribute to the ordered structure but also to the precise energy transfer characteristics within the phycobilisome rod as well as from the rod to the core [4,5].

In a number of papers, we and other groups have investigated the energy transfer in different protein aggregates of C-PC [8,11–13]. In the basic hexameric unit, which is the most important building-block of the phycobilisome rods, a fast energy transfer process of around 10 ps, which leads to an equally fast equilibration of the excitation energy among the 18 C-PC chromophores within a hexameric unit, has been observed. Furthermore, it was shown in a series of papers [8,11–13] that the length of the rods in phycobilisomes of *Synechococcus* 6301 influenced the observed rate of energy transfer from the rods to the core of the phycobilisome. The data collected for rod lengths of one and three hexameric C-PC units indicated that the rate was roughly inversely proportional to the size of the rod. One important question still to be resolved is whether the linker proteins contribute to the energy transfer process between adjacent hexameric units or if they mainly have a function to anchor the hexamers to each other. It has been suggested from in vitro reconstitution experiments that a red shift is induced in the rod by the linker polypeptides which would be of decisive importance for the efficiency of the energy transfer to the core [5].

The genes coding for most phycobilisome rod components in *Synechococcus* 6301/7942 have been cloned and sequenced [14,15]. The genes coding for α- and β-phycocyanin are located close to each other with the β-phycocyanin gene positioned upstream from the one coding for α-phycocyanin. The phycocyanin genes are in addition duplicated in *Synechococcus* 6301/7942, the two gene pairs are separated by approx. 2.5 kb [16]. The genes, *cpcH*, *cpcI* and *cpcD*, coding for the rod linker proteins 30 kDa, 33 kDa and 9 kDa, respectively, are located in the region between the two phycocyanin gene pairs [15]. The gene order of the *cpcH* and *cpcI* genes is, thus, inverse to the order found in the phycobilisome rod. Both phycocyanin gene pairs are transcribed and the linker genes are co-transcribed with the upstream phycocyanin gene pair [16].

Synechococcus 7942 can easily be transformed with exogenous DNA. Shuttle vectors have been developed that can replicate as autonomous plasmids in both *Escherichia coli* and *Synechococcus* 7942 [17]. Also, a platform system by which cloned genes can be integrated at known places into the chromosome of *Synechococcus* 7942 has been developed [17]. *Synechococcus* 7942 thus offers an elegant system by which mutated phycobilisome genes could be transferred from *Escherichia coli* to *Synechococcus* 7942 in order to monitor the precise function of its phycobilisome rod polypeptides. The technique has been used in *Synechococcus* 7942 to transfer mutated *psbA*-genes, coding for the D1 polypeptide, and *cytA* genes, coding for cytochrome *c*-553, to probe their function [18,19].

We have probed the function of the phycobilisome rod linker polypeptides by producing phycobilisomes of *Synechococcus* 7942 with the 33 kDa linker exchanged for the 30 kDa linker using the insertional inactivation technique. Time-resolved fluorescence measurements of both C-PC and APC showed that the novel phycobilisomes have a normal energy transfer between the two C-PC hexamers and to the APC-core.

Materials and Methods

Culture conditions

The cyanobacterium *Synechococcus* sp. PCC 6301/7942 (formerly designated *Anacystis nidulans* 625R2) was used in this study [20]. *Synechococcus* 6301/7942 was grown at the steady state at 38 °C in flat culture flasks minimizing self shading as has been described before [10]. The wild-type was grown in incandescent light at an intensity of 160–170 $\mu E/m^2$ per s to produce a rod of two hexamers. The *cpcI* mutant was grown at a light intensity of 50 $\mu E/m^2$ per s also to produce two hexamers per rod.

DNA manipulations

DNA was isolated from *Synechococcus* 6301/7942 as has been described before [14]. Plasmid DNA isolation from *Escherichia coli* and DNA manipulations were performed according to standard techniques [21]. Southern blot transfer and hybridization were performed according to established protocols [21,22].

DNA transformation

Synechococcus 7942 cells were transformed with exogenous DNA according to Ref. 17.

Phycobilisome preparation and analysis

Phycobilisomes were prepared according to Ref. 23. The polypeptide composition of purified phycobilisomes was analyzed on Coomassie-stained polyacrylamide gels. The gel system used was 20% homogeneous SDS-polyacrylamide gel using the Pharmacia (Sweden) PHAST system operated according to the instructions of the vendor.

Probes

The genetic organization of the phycocyanin operons as well as DNA fragments used as probes is shown

in Fig. 1A. The *Cla*I-*Cla*I shown in Fig. 1A was used to probe the linker genes.

Spectroscopic analysis

Steady-state emissions at 77 K were obtained by a house-built instrument [24] and at room temperature a Spex fluorolog 211 spectrofluorimeter was used. Absorption spectra were recorded on a Beckman DU-70 spectrophotometer.

A cavity-dumped dye laser synchronously pumped by a mode-locked Ar^+-laser (Spectra-Physics) was used as excitation source for the time-resolved fluorescence work. The laser dye used was Rhodamin 110 and the laser pulse repetition rate was 820 kHz with a power of approx. 10 mW at 590 nm and a pulse length of 7–10 ps. The main elements of the single-photon counting apparatus was a fast microchannel plate detector (Hamamatsu RI 564-U-01) for the emitted fluorescence (start-pulse) and a fast photodiode for the stop pulse. Constant fraction discriminators (Tennelec TC 455) were used for both the start and stop pulses that were fed into the time-to-amplitude converter (Ortec 567). The multi-channel analyzer (Nucleus PCA-8000 AT) had 8000 channels and the time resolution of the system in this work was 4.76 ps/channel. The fluorescence was detected at the magic angle to the vertical polarization of the excitation light in order to avoid false kinetics due to anisotropy relaxation. The fluorescence kinetics were analyzed by a deconvolution program (Edinburgh Instruments) admitting three exponential decays to be resolved. The fluorescence cuvette had an optical path length of 3 mm and the absorbance of the sample at 620 nm was 0.1–0.2. All measurements were done at room temperature (23–25 °C) on freshly prepared phycobilisomes in 0.75 M KP-buffer at pH 7.5. Typically, the light intensity at the sample was less than 10^{11} photons/pulse per cm^2 in order to avoid excitation annihilation effects that otherwise might distort the kinetics.

Results

Construction and genetic analysis of cpcI insertion mutant

The genetic organization of the gene cluster coding for phycobilisome rod components in *Synechococcus* 7942/6301 is shown in Fig. 1A. The first rod-rod linker, 33 kDa in size, is coded for by the *cpcI* gene, the second gene, while the core distal linker, 30 kDa in size, is coded for by the first gene, the *cpcH* gene.

A DNA cassette containing the gene conferring kanamycin resistance, Km^r, was inserted into a unique *Bal*I-site found in the *cpcI* gene using standard techniques (Fig. 1B). A DNA fragment containing the interrupted *cpcI* gene fragment was transferred back to *Synechococcus* 7942 by traditional transformation. Km^r resistant *Synechococcus* colonies were selected for. DNA as well as the phycobilisomes were isolated from Km^r resistant cells to check the status of the

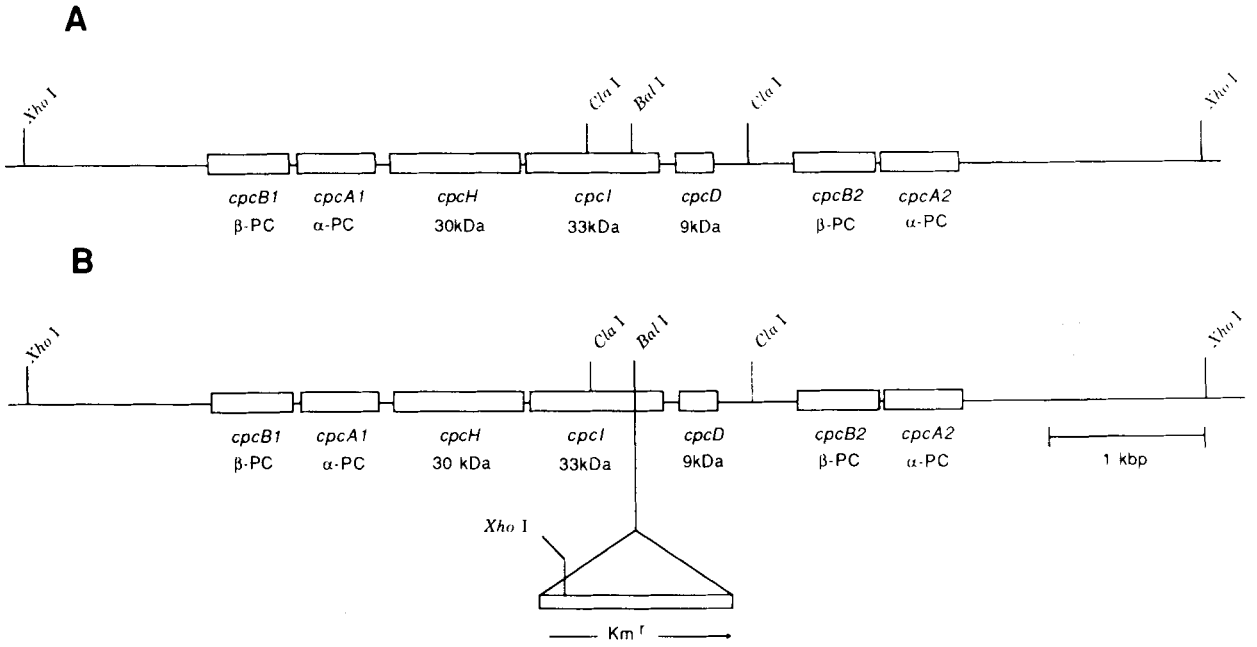

Fig. 1. Genetic structure of the phycocyanin rod operon in *Synechococcus* 6301/7942 wild-type (A) and *cpcI* mutant (B). The genes coding for phycocyanin α- and β-units are shown as well as the genes coding for the different rod-rod linker polypeptides. Different restriction endonuclease sites used in the article are indicated. The position and size of the Km^r antibiotic resistance cassette used to inactivate the *cpcI* gene is shown as well as the *Cla*I fragment used as probe in the Southern blot. The size of the genes are shown by the bar denoted 1 kb (kilobases).

integrated DNA fragment as well as the polypeptide composition of the phycobilisome rod, respectively.

Southern blot hybridizations of total DNA isolated from Km^r resistant cells were probed with a DNA fragment containing the linker genes to show that the Km^r cassette was inserted in the middle of the intergenic region in the *cpcI* mutant (Figs. 1B and 2). The genomic DNA was cut with the enzyme *Xho*I. The digested DNA was run on agarose gels and transferred to nylon membranes. The membrane was probed with the *Cla*I-*Cla*I fragment shown in Fig. 1A and the result is shown in Fig. 2. The wild-type DNA should show a fragment 7.6 kb in size while DNA from the IMcpcI mutant should show two fragments, 4.1 and 5.0 kb in sizes due to the extra *Xho*I site in the inserted Km^r cassette. The Southern blot clearly confirms the predicted result and also shows that no rearrangements have taken place during the transformation and integration events. The result also shows that the Km^r cassette is only present in one place on the cyanobacterial genome.

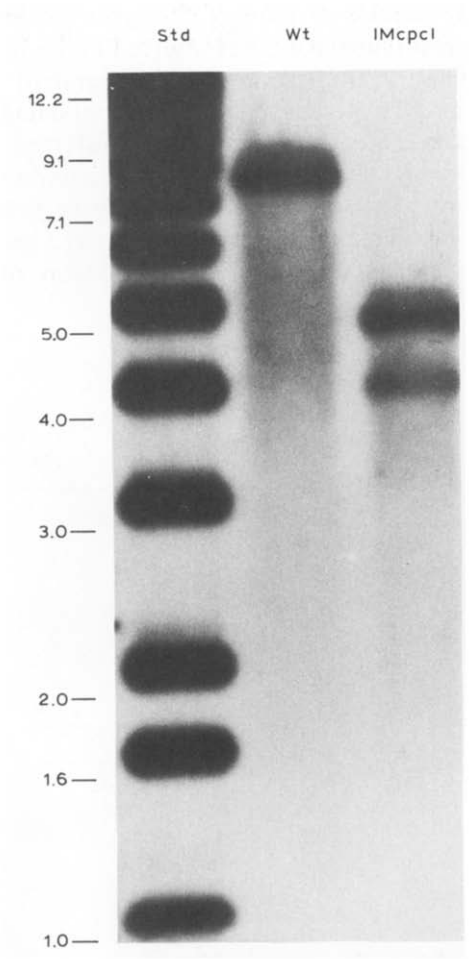

Fig. 2. Southern blot of genomic DNA from *Synechococcus* 7942 wild-type and mutant *cpcI* DNA. The prepared DNA was cut with the restriction endonuclease *Xho*I. The resulting blot was probed with the *Cla*I-*Cla*I fragment shown in Fig. 1. The ladder indicates the size of the DNA fragments.

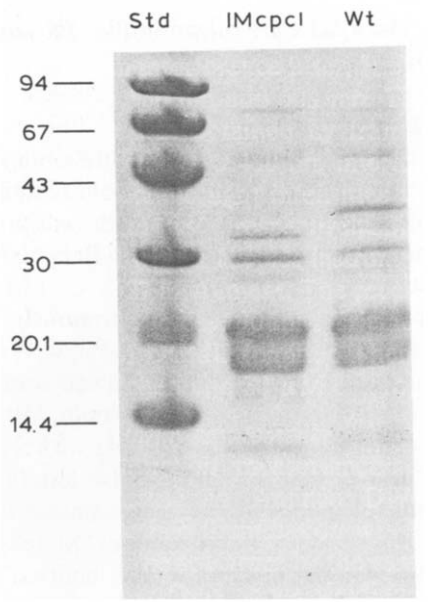

Fig. 3. SDS-PAGE of polypeptides from purified phycobilisomes. Phycobilisomes were isolated from *Synechococcus* 7942 wildtype and *cpcI* mutant cells. The molecular weight markers are shown on the left side of the figure.

Protein composition of phycobilisome rod

Phycobilisomes were isolated from the *cpcI* mutant from cultures grown under low light conditions. The protein composition of the isolated phycobilisome was analyzed on SDS-polyacrylamide gels (Fig. 3). Under those light conditions, the phycobilisome of wild-type *Synechococcus* 6301/7942 cells contains all three linker polypeptides, the 27 kDa, the 33 kDa and the 30 kDa linker [8,9]. The phycobilisome from the *cpcI* mutant grown under low light conditions contains no traces of the 33 kDa linker. However, the 30 kDa linker is present in almost equal amounts as compared to the 27 kDa core-rod linker (Fig. 3). Under all growth conditions, free phycocyanin can be found in the cells (data not shown). This indicates that the amounts of the linker proteins available in the cells determines the amount of phycocyanin that can be assembled into the rod and thus the length of individual rods.

The result clearly shows that there is no incorporation of the 33 kDa linker protein in the *cpcI* mutant due to the insertional inactivation of the gene coding for the 33 kDa protein. More interestingly, the result also clearly shows that the 30 kDa linker protein can structurally replace the 33 kDa protein in the rod. In addition, we find that a maximum of one rod-rod linker protein can be integrated on average into each rod also in the *cpcI* mutant as evident when looking at the ratio between the 30 kDa and 27 kDa proteins from low light adapted phycobilisomes which is always below 1.

Steady-state fluorescence and absorption spectra of isolated phycobilisomes

The low-temperature fluorescence curve of *cpcI* excited at 578 nm has a maximum at 682 nm, which shows that the phycobilisomes are functional what the energy transfer concerns (Fig. 4A and B). Fluorescence at room temperature excited at 580 nm gave an emission maximum at 675 nm, with a shoulder at 660 nm (Fig. 5A and B)

These results clearly show two things. Firstly, there is an efficient energy transfer from the C-PC rods to the APC core. Secondly, at 77 K the emission is from the APC-B chromophore, while at room temperature some of the emission comes from APC. This indicates a distribution of the excitation energy in the core which is temperature dependent.

We have measured the absorbance ratio at 624 and 654 nm of the two phycobilisome preparations with two hexamers per rod (Fig. 5C and D). This ratio was found to be 1.89, this is close to the highest value of 1.9 reported [8,25] for the AN135 mutant, which also should contain a maximum number of two hexamers per rod.

Time-resolved fluorescence

The fluorescence kinetics at 630/640 and 680 nm of mutant *cpcI* phycobilisomes as well as wild-type phycobilisomes with two hexamers excited at 590 nm are shown in Fig. 6. In the same figures the fit to a sum of three experiments is shown with the residual plots. The corresponding lifetimes are displayed in Table I. The most prominent signal at 630/640 nm is a decay with a lifetime of 72 and 59 ps, which carries large amplitudes (57 and 65%) for the wild-type and mutant *cpcI* phycobilisomes, respectively. There are also 1.57 and 1.81 ns emission components whose amplitudes are 19 and 28% and a smaller component with a lifetime of 209 and 185 ps for the wild-type and mutant *cpcI* phycobilisomes, respectively. In the corresponding fluorescence kinetics at 680 nm determined for the wild-type phycobilisome, a fast risetime of 65 ps is observed along with a decay of 1.81 ns which well matches the decay component of 72 ps at 640 nm. The same is true for the mutant phycobilisome. The fast risetime of 70 ps at 680 nm is matched by the dominant decay of 59 ps at 640 nm. Minor components has a lifetime of 479 and 423 ps at 680 nm. The results clearly show that the mutant

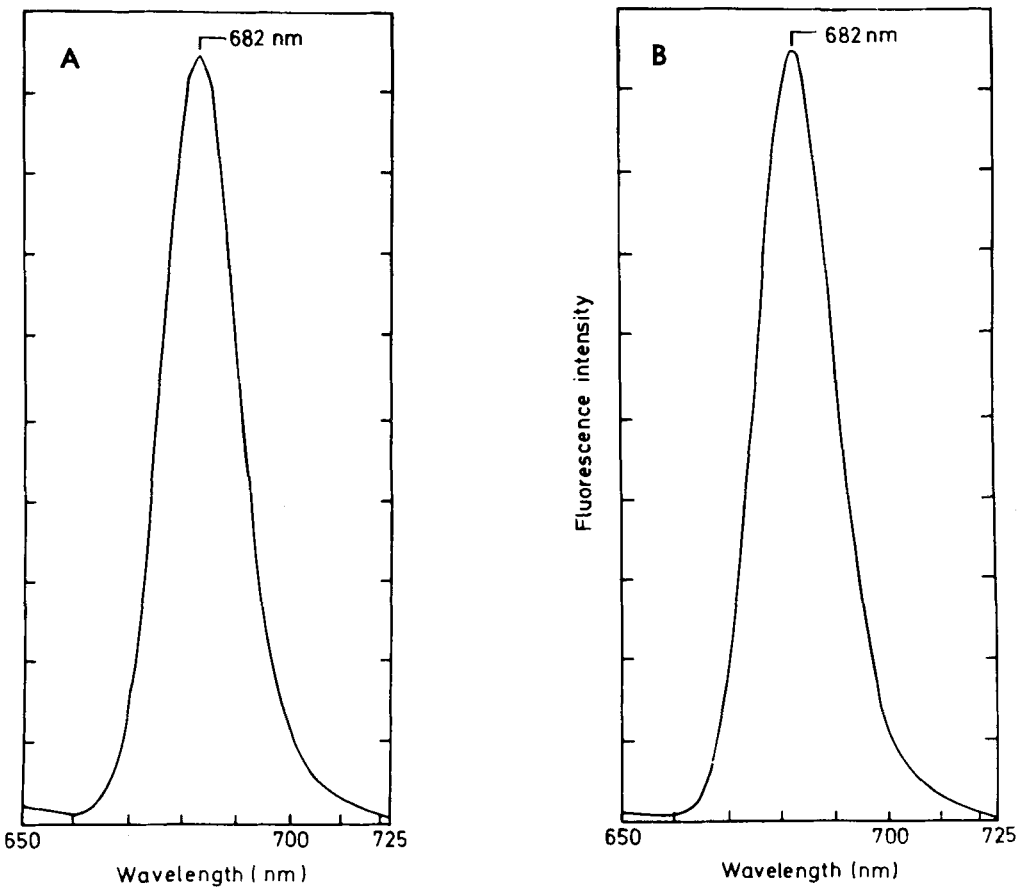

Fig. 4. (A) 77 K fluorescence spectra of wild-type phycobilisomes with two hexamers. (B) 77 K fluorescence spectra of mutant *cpcI* phycobilisomes with two hexamers.

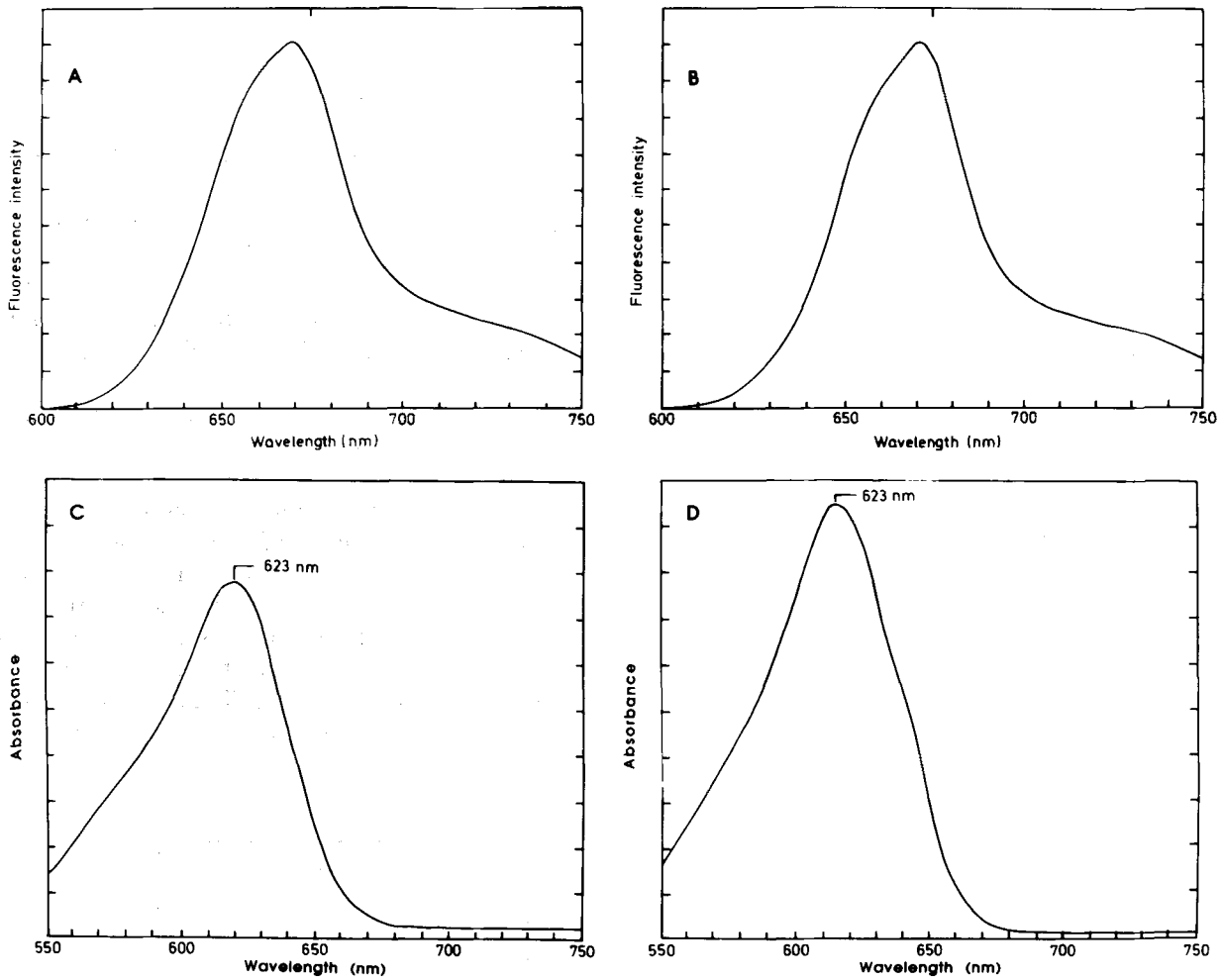

Fig. 5. (A) Room temperature fluorescence spectra of wild-type phycobilisomes with two hexamers. (B) Room temperature fluorescence spectra of mutant *cpcI* phycobilisomes with two hexamers. (C) Absorption spectra of wild-type phycobilisomes with two hexamers. (D) Absorption spectra of mutant *cpcI* phycobilisomes with two hexamers.

phycobilisome is structurally intact and transfers energy just as efficiently as the wild-type phycobilisome.

Discussion

In this paper we report on the characterization of a phycobilisome mutant of *Synechococcus* 7942 that can no longer produce the 33 kDa rod-rod linker polypeptide. The mutant phycobilisome was used to study (i) the kinetics of the energy transfer within the phycobilisome rod using time-resolved spectroscopy, (ii) the polypeptide composition of the rod. The results of the study enable us to address the following questions: (1) Are the linker polypeptides involved in optimizing the efficiency of energy transfer from one hexamer to the adjacent one in the rod? (2) Are the linker polypeptides inserted in a precise and regulated order during the biogenesis of the phycobilisome rod?

In previous papers, it has been shown that the energy transfer from the C-PC rods to the core in *Synechococcus* 6301 varied with the number of hexameric units in the rods [11,12,26]. The transfer rate varied from approx. 40 ps^{-1} to about 90 ps^{-1} when going from one to three hexamers per rod. In this model it is assumed that there is a fast equilibration (within 10 ps) of excitation energy within the rod chromophores and the last transfer step from C-PC to APC in the core is rate-limiting and, thus, the rate of transfer would be proportional to n^{-1}, where n is the number of chromophores. In the case of two hexamers as in the *cpcI* mutant phycobilisomes studied in this work we would expect a rate of approx. 65 ps^{-1} which is very close to and within the experimental error of the observed value. The obtained value is just within the expected range if the equilibration of energy is fast between the two hexamers connected by the 30 kDa linker. We conclude that exchanging the 33 kDa linker with the 30 kDa does not prevent or hamper the energy transfer to any significant extent. If indeed a prevention of the energy transfer had occurred, a 40 ps transfer lifetime from the innermost hexamer and a much longer lifetime with a high amplitude from dis-

TABLE I

Fluorescence kinetics on isolated wild-type and cpcI mutant phycobilisomes

The phycobilisomes were isolated as described under Materials and Methods. The isolated phycobilisomes were excited at 590 nm.

Fluorescence wavelength	Lifetime		Amplitude	
	wild-type two hexamers	*cpcI* mutant	wild-type two hexamers	*cpcI* mutant
630/640 nm	72 ps	59 ps	57%	65%
	209 ps	185 ps	24%	7%
	1.57 ns	1.81 ns	19%	28%
680 nm	65 ps	70 ps	neg.	neg.
	479 ps	423 ps	9%	5%
	1.62 ns	1.81 ns	91%	95%

connected distal hexamers would have been expected. The conclusion of the time-resolved and steady-state fluorescence measurements must therefore be that the *cpcI* phycobilisomes are fully functional and structurally intact.

Lundell et al. [27] demonstrated in in vitro experiments that purified linker polypeptides from *Synechococcus* 6301 could form a complex with one phycocyanin hexamer or even higher forms of aggregates composed of several linker molecules with several phycocyanin hexamers. It was also shown that these linker phycocyanin hexamer complexes had distinct spectroscopic properties. The in vitro assembled 27 kDa-PC hexamer complex was red shifted both in terms of absorption maximum and fluorescence emission maximum compared to the 33 kDa linker containing hexamer. Similar results were obtained comparing the 33 kDa linker and 30 kDa linker hexamer complexes; the former being red shifted compared to the latter. Assuming a similar situation to occur in vivo within the rod of the phycobilisome, the data from the in vitro reconstitution experiments were interpreted to explain the highly efficient energy transfer from one hexamer

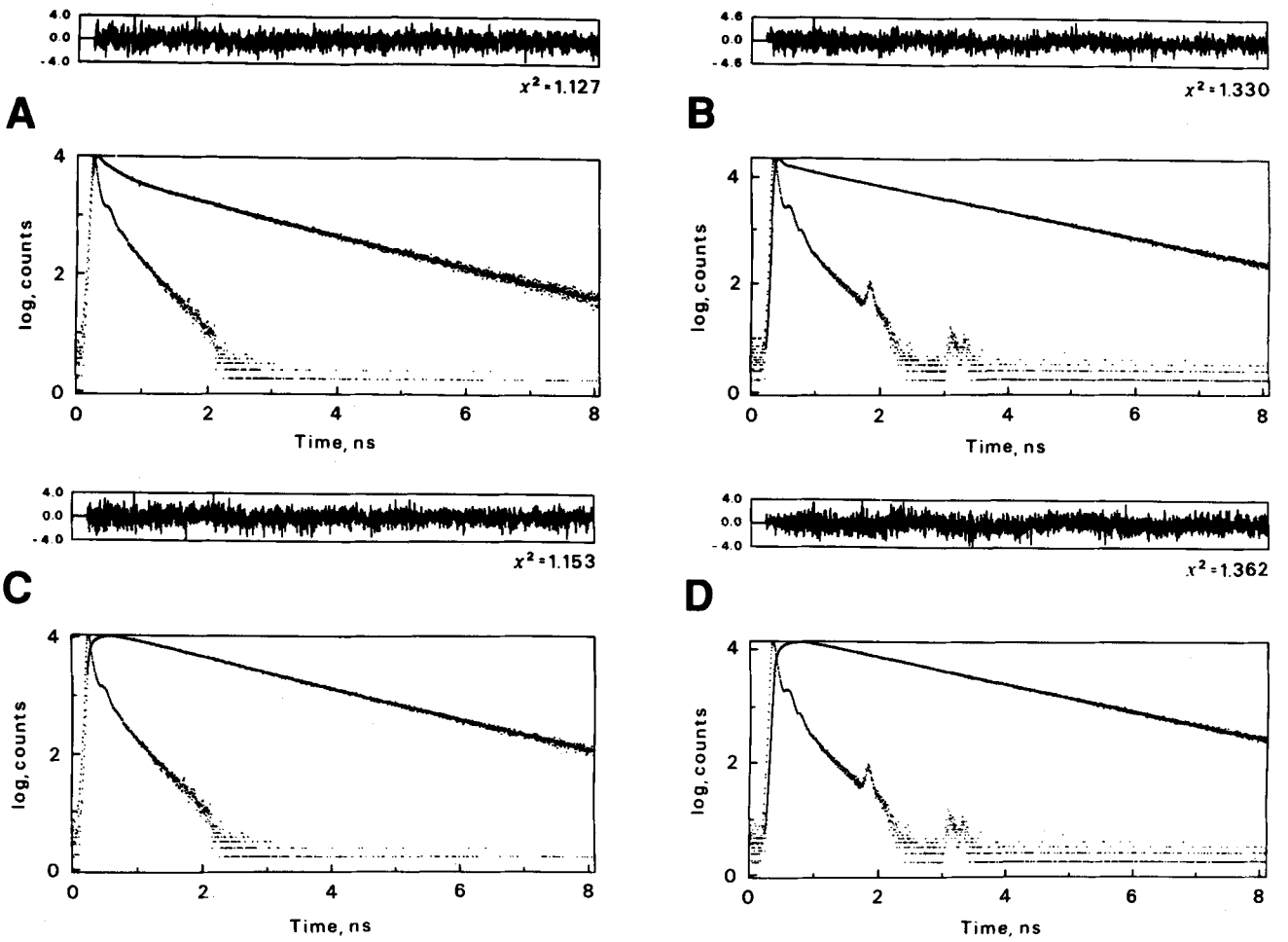

Fig. 6. (A) Isotropic fluorescence decay of wild-type phycobilisomes with two hexamers detected at 630 nm, λ_{exc} = 590 nm. In this figure (A–D), plots of the weighted residuals for this fits are also shown. The lifetimes and amplitudes of the three fluorescence components can be found in Table I. (B) Isotropic fluorescence decay of mutant *cpcI* phycobilisomes with two hexamers detected at 640 nm, λ_{exc} = 590 nm. (C) Isotropic fluorescence decay of wild-type phycobilisomes with two hexamers detected at 680 nm, λ_{exc} = 590 nm. (D) Isotropic fluorescence decay of mutant *cpcI* phycobilisomes with two hexamers detected at 680 nm, λ_{exc} = 590 nm.

to an adjacent hexamer (tip-PC-30-PC-33-PC-27-core) within the rod as one moves from the tip of the rod towards the core [5]. The hypothesized stepwise red shift of both absorption and fluorescence emission maxima in each successive linker hexamer complex would ensure a greater degree of spectral overlap between the adjacent hexamers and minimize the random walk of energy. It could thus be argued that a phycobilisome rod with a scrambled order of rod-rod linker polypeptides would function less efficient in energy transfer. Our results clearly contradict the hypothesis that the order of the linker proteins in the rod should influence the rate of energy transfer. Our results indicate that the red shift found in the in vivo system is only of minor importance for the effecient energy transfer from one disc to an adjacent within the rod of the phycobilisome.

It is well established in a wild-type cell, that the rod-rod linker polypeptides are found in a defined order in a fully extended rod, i.e., one having three hexamers. The order of the linkers going from the core to the tip being the 33 kDa, the 30 kDa and finally 9 kDa linker polypeptides [8,9]. It is thus very interesting to find that the 30 kDa linker polypeptide can replace the 33 kDa in the *cpcI* mutant. However, the questions why the 33 kDa linker is preferred over the 30 kDa linker in the rod following the 27 kDa linker, how the rod length is controlled and how these processes determine the structure and function of the rod in vivo remains unanswered.

Several mutations in phycobilisome genes have been made lately [28–30]. A mutant was produced in *Synechococcus* 7002 (*Agmenellum quadruplicatum* PR-6) that lacked the small, 8 kDa, core linker polypeptide, coded for by the *apcC* gene [28]. The *apcC* mutant produced normal but less stable phycobilisomes. No or minute differences were found in the steady-state and time-resolved fluorescence indicating that also this core linker contributes only to a minor extent to the energy transfer characteristics of a phycobilisome. Also, mutants in the *cpcC* and *cpcD* genes coding for the 33 kDa and 9 kDa linkers, respectively, in *Synechococcus* 7002 have been produced by insertional inactivation [29,30]. These are the only rod linkers present in these cyanobacteria. It was concluded that the linker polypeptide may contribute to the 4 nm wavelength difference found between pure C-PC and C-PC bound to the 33 kDa linker. In contrast, the phycobilisome of *Synechococcus* 7942 used in this study presents a more complex system with a rod composed of three hexameric discs and three rod-rod linker with very precise positions in the rod. Future studies using mutants in phycobilisome genes in *Synechococcus* 7942 will increase our knowledge about the structural and functional properties of light antennae.

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