FEBS Letters 553 (2003) 68–72 FEBS 27636

# Effects of glycerol and high temperatures on structure and function of phycobilisomes in *Synechocystis* sp. PCC 6803

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Received 7 March 2003; revised 30 April 2003; accepted 30 April 2003

First published online 9 September 2003

Edited by Richard Cogdell

**Abstract The effects of glycerol and high temperatures on structure and function of phycobilisomes (PBSs) in vivo were investigated in a *chlL* deletion mutant of the cyanobacterium *Synechocystis* sp. PCC 6803. When the mutant was grown under light-activated heterotrophic growth conditions, it contained intact and functional PBSs, but essentially no chlorophyll and photosystems. So the structural and functional changes of the mutant PBSs in vivo can be handily detected by measurement of low temperature (77 K) fluorescence emission spectra. High concentration glycerol induced an obvious disassembly of PBSs and the dissociation of phycocyanins in the rod substructures into their oligomers and monomers. PBSs also disassembled at high temperatures and allophycocyanins were more sensitive to heat stress than phycocyanins. Our results demonstrate that the *chlL*$^-$ mutant strain is an advantageous model for studying the mechanisms of assembly and disassembly of protein complexes in vivo.**



*Key words:* Cyanobacterium; Energy transfer; Glycerol; Fluorescence emission; High temperature; Phycobilisome

## 1. Introduction

In cyanobacteria and red algae, the phycobilisomes (PBSs) serve as the major light-harvesting antenna [1,2]. Light energy is absorbed by PBSs on the cytoplasmic surface of the thylakoid membrane, then transferred and apportioned to the photosystem reaction centers. The structural and energy transfer properties of PBSs have been the subject of extensive investigation. As a supramolecular complex, PBS is primarily composed of phycobiliproteins (PBPs), a brilliantly colored family of water-soluble proteins that contain covalently linked, open-chain tetrapyrroles known as phycobilins. In addition, PBS also contains smaller amounts of linker polypeptides, which are vital for proper assembly and functional organization of the complex. PBS has two distinct regions, a core composed mainly of allophycocyanins (APCs) and peripheral rods composed of mainly phycocyanins (PCs) and/or phycoerythrins. Light energy absorbed by PC is transferred to the slightly longer wavelength absorbing core APC, through the terminal energy emitter, then onto reaction centers [2].

The structures of some PBPs are known to high resolution (for review see [3,4]). Each PBP is composed of two polypeptide subunits (α and β). The αβ heterodimer (referred to as a monomer) assembles into disc-like trimers $(\alpha\beta)_3$, which possess a central cavity. The linker polypeptides bind to the central cavity of PBP discs, aid to assemble the discs into the core and rod substructures of PBS. The light-harvesting and conduction functions of the PBS depend strongly on the three-dimensional arrangement of the complexes. However, in structural terms we know little about the details of the association between the core and rod substructures in the entire PBS. In *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis* 6803), the PBS core is made up of three cylinders, each containing four APC trimers. Six rods, each composed of three stacked PC hexamers, radiate from the core [5].

To keep in the intact and energetically coupled state, isolated PBSs require a buffer containing sodium and/or potassium phosphate at a concentration ranging from 0.6 to 1.0 M, a pH of 7–8, and a temperature of 18–23°C [6,7]. This implies that the protein–protein interaction in the PBSs should be significantly weaker than those in other protein complexes and raised a question about how the PBSs retain their molecular association in the cytosol without such a concentration of phosphate and comfortable temperature. Low temperature (77 K) fluorescence measurement is a powerful tool for the study of the structural and functional changes of PBSs and photosystems in vivo. However, it is difficult to investigate the detailed information on the excitation energy transfer from PC to the terminal emitter in vivo because of the strong overlap of the emission spectrum of the PBSs with that of photosystem (PS) II near 686 nm.

*Synechocystis* 6803 contains both light-dependent and light-independent chlorophyll *a* biosynthesis pathways [8]. Deletion of the *chlL* gene, which encodes one of the components of the light-independent protochlorophyllide oxidoreductase, results in a lack of chlorophyll synthesis in darkness [9]. Chlorophyll availability affects the translation, stability, and assembly of chlorophyll binding proteins, most of which are part of PS II and PS I [10–13]. When the mutant is propagated under light-activated heterotrophic growth conditions (LAHG, in darkness except for 15 min/day of weak light) for 2 weeks, essentially neither chlorophyll nor functional photosystems are detectable [9,14]. Since the synthesis and assembly of PBSs and

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*Abbreviations:* APC, allophycocyanin; PC, phycocyanin; PBP, phycobiliprotein; PBS, phycobilisome

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doi:10.1016/S0014-5793(03)00973-6

photosystems are independently regulated [15,16], this mutant strain provides an advantageous model for the investigation of the structural and functional characteristics of PBSs in vivo.

In this work, we investigated the effects of glycerol and high temperature treatments on structure and function of PBSs in a *chlL* deletion mutant strain of *Synechocystis* 6803 by measurements of low temperature fluorescence emission spectra. In recent decades, glycerol has been widely used as cryoprotectant in low temperature fluorescence measurement. However, it has been found to alter the emission spectrum of cyanobacterial cells and tends to functionally uncouple PBSs from photosystems [15,17]. Our results demonstrate that high concentration glycerol can induce an obvious disassembly of PBSs in vivo. In addition, PBSs also disassemble at high temperatures and APC is more sensitive to heat stress than PC.

## 2. Materials and methods

Both wild-type and mutant strains of *Synechocystis* 6803 was cultured at 30°C in BG-11 inorganic liquid medium [18] supplied with 20 mM HEPES/NaOH (pH 7.5) and 5 mM glucose. Under LAHG conditions, the cells were kept in complete darkness except for one 15-min light period (white light at 50 μE/m$^2$/s) every 24 h. Aeration was provided through bubbling. The cells were harvested during the late logarithmic growth phase (around $8\times10^7$ cells/ml) by centrifugation at $6000\times g$ for 7 min and resuspended in fresh medium.

Cell suspensions were mixed with either culture medium or glycerol to designated concentrations of glycerol. The final cell concentrations were $4\times10^7$ cells/ml. Re-absorption of emitted fluorescence was negligible at this cell concentration. In darkness, the samples were incubated at room temperature for 10 min and frozen in liquid nitrogen. For the high temperature treatments, cell suspensions ($4\times10^7$ cells/ml) were incubated at the designated temperatures for 10 min in the dark and then quickly dipped into liquid nitrogen.

77 K fluorescence emission spectra were recorded in a Hitachi 850 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Excitation was performed at 590 nm for PC excitation. The slit widths for the excitation and emission were 10 and 3 nm, respectively.

## 3. Results

### 3.1. Fluorescence emission characteristics of PBSs in the chlL$^-$ mutant strain

The growth rate of *chlL*$^-$ mutant cells was identical to that of the wild-type strain under LAHG conditions; the doubling time during logarithmic growth in both strains was about 23 h. This indicates the deletion of *chlL* does not impact the growth and viability of *Synechocystis* cells. After growth under LAHG conditions for 2 weeks, chlorophyll content per mutant cell was about 4% of that per wild-type cell [9].

Fig. 1 shows the 77 K fluorescence emission spectra of the dark-adapted wild-type and *chlL*$^-$ mutant strains. Cell samples were excited at 590 nm mainly absorbed by PC. In the spectrum of wild-type cells, the peak at 645–665 nm derives from PC and APC. The shoulder at 686 nm comes from the terminal emitter and the antenna CP 43 of PS II. The fluorescence emission around 695 nm mainly results from the antenna CP 47 and the reaction center of PS II whereas the peak at 720 nm originates from PS I [17,19,20]. The intensive fluorescence emission from PS I and PS II reflects high efficiency of energy transfer through PBSs to photosystems. In the spectrum of the *chlL*$^-$ mutant, there is only one emission maximum around 686 nm corresponding to the terminal emitter, in the absence of obvious fluorescence emission from PC

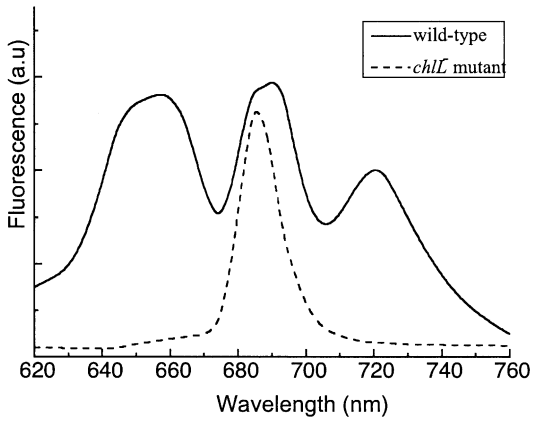


Fig. 1. 77 K fluorescence emission spectra of the wild-type and *chlL*$^-$ mutant strains grown under LAHG conditions. The excitation wavelength was 590 nm. The spectra were normalized at the emission maxima.

(648 nm) and APC (665 nm). No fluorescence emission from chlorophyll associated with photosystems was detected at 695 or 725 nm, in line with the lack of chlorophyll in the mutant grown under LAHG conditions. It should be noted that the 77 K fluorescence emission spectrum of the *chlL*$^-$ mutant strain is identical with that of isolated intact PBSs in vitro [21], indicating the mutant cells contain intact PBSs with tight energy coupling between the PBPs.

### 3.2. Effects of glycerol on PBSs in the chlL$^-$ mutant strain

Fig. 2 shows the effects of various concentrations of glycerol on 77 K fluorescence emission spectra of the *chlL*$^-$ mutant cells. The fluorescence emission maximum of the control cells was predominantly at 686 nm. After the mutant cells were treated with glycerol at various concentrations lower than 20% (v/v) for 10 min, their 77 K fluorescence emission spectra were almost unaffected (data not shown). In the spectrum of the mutant cells treated with 30% glycerol, a weak fluorescence emission from PC was detected around 650 nm, indicating the energy transfer efficiency from PC to APC decreased. This PC fluorescence emission increased markedly and became the maximum emission in the cells incubated with 40% glycerol, suggesting that the rod substructures were partially dissociated from the cores of PBSs. There was also a shoulder around 665 nm that indicated the glycerol treatment leads to decreased energy transfer from APC to the terminal emitter. However, the structure of the core primarily remained intact, because main energy absorbed by APC gave rise to the fluorescence characteristic of the terminal emitter. With the higher concentrations of glycerol, the fluorescence emission from APC increased whereas that from the terminal emitter decreased relatively, suggesting the interruption of energy transfer in PBSs. The spectrum of cells treated with 80% glycerol exhibited two major emission peaks at 647 and 665 nm and a shoulder at 682 nm, suggesting energy transfer from APC to the terminal emitter was greatly reduced.

The effects of glycerol on room temperature emission spectra of the *chlL*$^-$ mutant cells were also determined. Although the emission bands are much broader at room temperature, these emission spectra can provide reliable information on fluorescence intensity changes of different PBS components.

The effects of glycerol on 77 K emission spectra reported above are confirmed by these room temperature emission spectra (data not shown).

It should be noted that the emission peaks around 650 and 686 nm are significantly blue-shifted upon the increase of glycerol concentration. The PC emission peak stayed at 650 nm with glycerol at concentrations lower than 60%, and shifted to 647 nm in the presence of 80% glycerol. The emission peak wavelength of the terminal emitter seemed more sensitive to glycerol. When the concentration of glycerol increased from 30% to 80%, it shifted from 686 nm to 682 nm accordingly. However, no marked blue shift in the APC emission peak was observed upon the addition of glycerol.

### 3.3. *Effects of high temperature treatments on PBSs in the* chlL$^-$ *mutant strain*

Denaturation of PBPs induced by high temperature treatments can be detected by absorbance decrease of whole cells around 620 nm [22]. The 620 nm absorbance remained almost constant at a temperature ranging from 30°C to 58°C. However, it decreased largely at 60°C, indicating the majority of PBPs were damaged (data not shown). Fig. 3 shows fluorescence emission spectra at 77 K of the *chlL*$^-$ mutant cells incubated at various temperatures for 10 min. Treatment of the cells at 56°C caused a shoulder around 665 nm and a slight emission around 650 nm, which originated from APC and PC, respectively. Fluorescence intensities from APC and PC increased greatly and the 665 nm emission became the maximum peak at 58°C, indicating the PBSs were disassembled and energy transfer from APC to the terminal emitter in the core substructures was interrupted. At 60°C, the 686 nm fluorescence was largely diminished with an increase of PC emission, implying the energy transfer in PBSs was badly damaged. The APC fluorescence peak shifted from 665 nm to 663 nm. However, the emission peaks from PC and the terminal emitter were not blue-shifted obviously upon heat treatments.

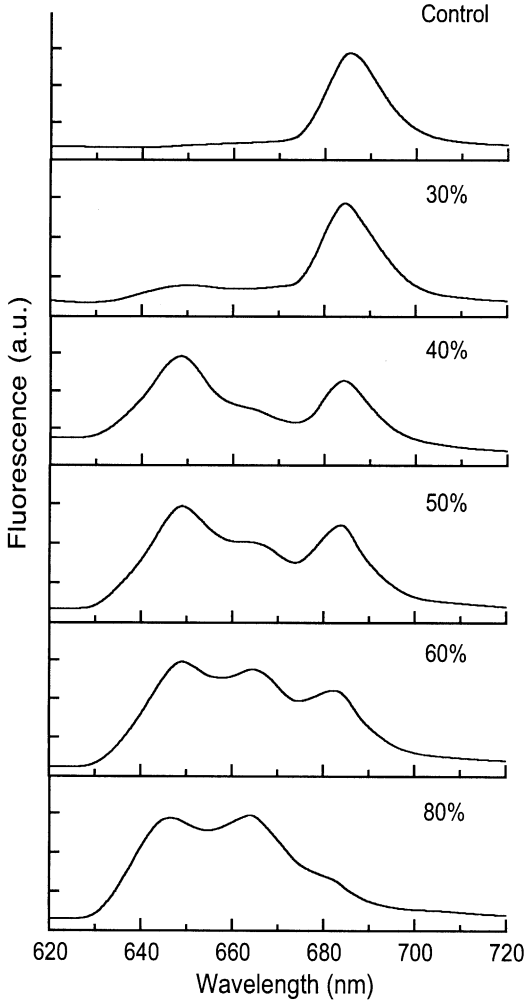


Fig. 2. Effects of glycerol on 77 K fluorescence emission spectra of PBSs in the *chlL*$^-$ mutant strain grown under LAHG conditions. After treatment with various concentrations of glycerol at room temperature for 10 min, the samples were frozen in liquid nitrogen. The excitation wavelength was 590 nm. The spectra were normalized at the emission maxima.

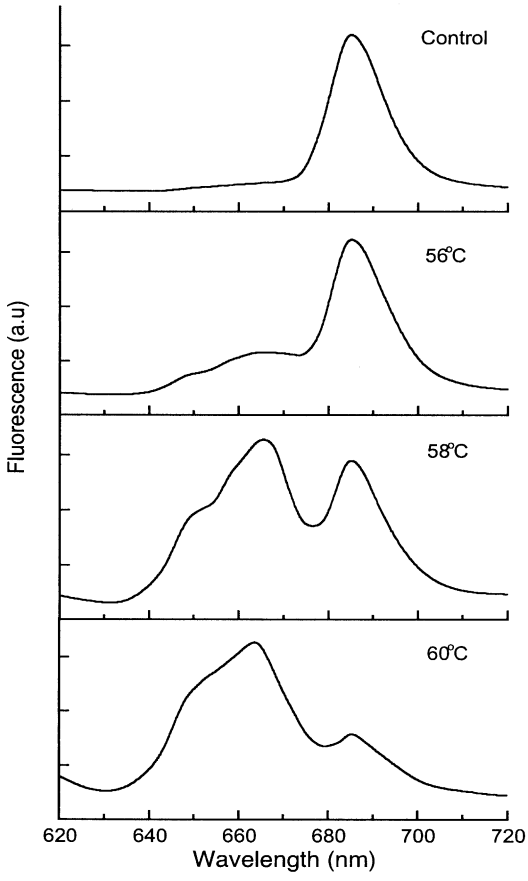


Fig. 3. Effects of high temperature treatments on 77 K fluorescence emission spectra of PBSs in the *chlL*$^-$ mutant strain grown under LAHG conditions. After incubation at the indicated temperatures for 10 min, the samples were quickly frozen in liquid nitrogen. The excitation wavelength was 590 nm. The spectra were normalized at the emission maxima.

## 4. Discussion

Isolated PBSs require a high concentration of phosphate and a temperature about 20°C to keep in their intact state [6,7]. However, in vivo PBSs can maintain their structure and function without such conditions. Moreover, cooling the mutant cells at 0°C for 4 h did not result in significant

change in the 77 K fluorescence emission spectrum (data not shown). This indicates the structural and functional stability of PBSs in vivo is higher than that of isolated PBSs in vitro. Taking into account the hydration of protein molecules with 2.3 volumes of water, the existence of PBPs at a high level (10–20% of cell dry weight) would enhance the local concentration of solutes in the free water around the thylakoid membrane [7]. The local high concentration of solutes may interact with the PBSs to offer them additional stability and be crucial to the intactness of PBSs in vivo.

When the *chlL*$^-$ mutant is grown under LAHG conditions, PBPs are present in approximately normal amounts in the absence of chlorophyll [14]. The 77 K fluorescence emission spectra demonstrate they are fully assembled into intact and functional PBSs (Fig. 1). In wild cyanobacterial cells, PBSs associate with the photosystems and transfer light energy to chlorophyll in reaction centers [2]. PBSs in the *chlL*$^-$ mutant remain membrane-associated in the absence of photosystems, since detergent treatment is still required to isolate intact PBSs from the mutant cells, indicating a strong interaction between the PBSs and the thylakoid membrane [14]. Mullineaux et al. proposed that the PBSs may interact with lipid head groups at the membrane surface [23].

In *Synechocystis* 6803, PBS rods are composed of PC hexamers held together with colorless linker polypeptides. The latter serve variously to assemble PC trimers into hexameric discs and assemble the discs into rods [1]. They also fine-tune the spectral properties of the PC phycocyanobilins so that the PC at the rod periphery can absorb higher energy than the PC next to the core [24,25]. These small differences in the absorption of the PC phycocyanobilins insure the directional energy transfer from the rod to the core. When the rods dissociate into PC oligomers (hexamers or trimers) and monomers, the emission maximum from PC is blue-shifted [26]. In vivo, the PC rods and oligomers have similar spectra, with a maximum at 650 nm [27,28]. Therefore the significant blue shift in PC emission must indicate dissociation of the PC oligomers into monomers (Fig. 2). 30% glycerol induced a small fluorescence emission from PC, suggesting initial dissociation of the PC rods from the PBSs. Higher concentrations of glycerol led to an increase in the relative intensity of PC emission, in a concentration-dependent manner. We suppose that the dissociated PC further disassembled into $(\alpha\beta)_6$ and $(\alpha\beta)_3$ upon the increase of glycerol concentration. The blue shift in PC emission caused by 80% glycerol is indicative of the dissociation of PC oligomers into monomers.

In addition to common APC, the PBS core of *Synechocystis* 6803 also contains three longer wavelength PBPs, only two copies of each per PBS. These PBPs, ApcE (the Lcm or anchor polypeptide), ApcD (the α-subunit of APC B), and ApcF (the $\beta^{18}$ polypeptide) [29], are located in the core along with the APC. The PBS core has three cylinders. Two basal cylinders are in close contact with the thylakoid membrane, while the third cylinder is located in the upper furrow of the two basal cylinders. The three longer wavelength PBPs, one copy of each per basal cylinder, are all exposed on the PBS surface [30]. The emission maxima of APC trimers containing the longer wavelength PBPs are red-shifted about 20 nm relative to those of pure APC trimers [31]. ApcE appears to contain the most red-shifted phycocyanobilin in the core and serves as the main terminal emitter [21,32], transferring most of the excitation energy harvested by the PBSs to reaction centers. The understanding of the organization of the PBS core is less complete, but features similar to those seen in the rods govern the directional transfer of energy [33]. The fluorescence emission characteristics of PBPs are very dependent upon the environment of their chromophores. For example, deletion of the PB loop in ApcE resulted in a 1 nm blue shift in terminal emitter emission, a consequence of a slight distortion of ApcE around the linked phycocyanobilin [34]. Since pure APC trimers in vivo have an emission maximum at 665 nm [28], the blue shift of the 665 nm fluorescence peak induced by high temperature treatments may indicate structural changes in the APC trimers (Fig. 3). In a similar way, the blue shift in the terminal emitter emission induced by glycerol (Fig. 2) could imply structural changes in the APC trimers containing the longer wavelength PBPs and a consequential reduced efficiency of energy transfer from the bulk APC to ApcE.

Glycerol contains hydrocarbon chains with three hydroxyl groups that allow hydrogen bonding with water. Priev et al. [35] suggested that glycerol may alter the protein in a number of ways. The addition of glycerol decreases the volume of the protein core by 8% resulting in a reduction in the radius of an equivalent sphere by only 2.6%, but also increases hydration at the particle surface. Larger molecules, such as proteins, show a net decrease in volume because they have a large core. In addition, compression of the protein core may alter the extent to which different amino acids are exposed or buried in the presence of glycerol. Changes in protein size or surface characteristics can have large effects on protein interactions in PBSs, resulting in dissociation of the PBSs. The glycerol sensitivity of PBPs may be partly attributed to their spatial positions in the PBSs. Low concentrations of glycerol have significant effects on both the PC rods and the terminal emitter, which are located on the PBS surface and are prone to approach glycerol molecules (Fig. 2). However, half of APC are located in the upper cylinders of the cores and covered by the rods and basal cylinders, and an obvious APC emission peak only appears in the presence of higher concentrations of glycerol. Our results also demonstrate that the concentration of glycerol used as cryoprotectant should be lower than 20%.

High temperatures cause structural changes of plasma membranes and result in leakage of various ions through the membranes [22,36]. When *Synechocystis* 6803 is treated at around 50°C for 5 min, its plasma membranes become leaky [36]. However, incubation of the *chlL*$^-$ mutant at 54°C for 10 min did not cause any significant changes in fluorescence emission spectrum (data not shown). This implies that the PBS fluorescence changes induced by high temperatures in our experiments could be attributed to thermodynamic effects of heat stress. Fluorescence emission of APC was more sensitive to high temperature treatments, as compared with that of PC (Fig. 3). At 60°C, APC emission was blue-shifted about 2 nm, whereas the PC emission peak remained at 650 nm. We thus conclude that PC is more stable to heat stress than APC in *Synechocystis* 6803. This is in agreement with results obtained in the thermophilic cyanobacterium *Synechococcus vulcanus* [22].

To perform their biological function numerous biological macromolecules are arranged in complex interaction systems. PBSs are very good examples for explaining these interactions because their function depends strongly on the three-dimensional structure of the complexes. Moreover, changes in PBS

structure and function can be conveniently followed by fluorescence emission spectra. Our results demonstrate that the *chlL*$^{-}$ mutant strain can be used as a simplified model for studying the mechanisms of assembly and disassembly of supramolecular complexes in vivo.

*Acknowledgements:* This work was supported by the State Key Basic Research and Development Plan (G 1998010100) and a grant from the National Natural Science Foundation (39890390) of the People's Republic of China.

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