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Specific bleaching of phycobiliproteins from cyanobacteria and red algae at high temperature in vivo

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Abstract. Exposure of blue-green or red algal cells to temperatures exceeding 60–65° C for several minutes resulted in bleaching of all phycobilin absorption in the visible range, with virtually no alteration in chlorophyll or carotenoid absorption. Difference spectra of non-bleached vs bleached cells appeared identical to absorption spectra of purified phycobilisomes isolated from the same cell culture in high phosphate medium. All phycobilin chromophores were bleached at approximately the same rate during heating. There were no changes in apparent molecular weights or relative amounts of the phycobilisome apoproteins during chromophore bleaching. Phycobilisomes in cell extracts from *Anacystis nidulans* resisted bleaching when suspended in medium of high phosphate concentration, but were bleached at 60–65° C within a few minutes when placed in diluted medium. The results indicate that phycobilisomes in vivo are stabilized by a mechanism other than high osmotic and ionic strength. This represents a rapid and quantitative method to characterize the phycobiliprotein content of cyanobacteria and red algae in vivo.

Key words: Algal heating damage – *Anacystis nidulans* – Cyanobacterial pigments – Phycobilin bleaching – Phycobilisomes

Phycobilisomes are macromolecular complexes bound extrinsically to the photosynthetic membranes of cyanobacteria and red algae, serving as the major antennae for collecting light energy to be delivered to Photosystem II (Gantt 1980; Glazer 1987). The specific structure of individual chromophores and their associated proteins (phycobiliproteins), as well as their greater organization within the phycobilisome, varies among species. However, the general flow of energy is in the order: [PE] → [PC] → [APC] → [Chl]. Accordingly, PE (when present) occurs at the distal part of the phycobilisome, while APC is most proximal to the membrane (Glazer 1989). The phycobilisomes are functionally stable within intact cells, and not subject to damage under mild environmental stress. Yet their specific composition, in particular the ratio of the different phycobiliprotein components, may vary greatly as the cells adapt to various environmental conditions (Cohen-Bazire and Bryant 1982). Disruption of cells results in the release of the phycobiliproteins into the medium. The soluble phycobiliproteins retain their chromophores but lose most of their macromolecular organization unless isolated and maintained in appropriate medium of high osmotic and ionic strength, such as 0.75 M phosphate (Gantt et al. 1979).

Many kinds of studies utilizing metabolically active cyanobacteria and red algae, such as chromatic adaptation and physiological effects of environmental stress, require determinations of the specific pigment composition of cells. This is difficult in whole cells because of overlapping absorption bands of various pigments. Phycobiliproteins can be separated from broken cell extracts for analysis, but this is time-consuming and usually not quantitative. We previously noted (Brand 1977) that brief heating of *Anacystis* cells results in rapid bleaching of phycobilin pigments. Here we describe conditions which result in specific bleaching of phycobilin chromophores and demonstrate the general applicability of this method to the analysis of the pigment composition of cyanobacteria and red algae.

Materials and methods

Anacystis nidulans TX20 (PCC 6301) was obtained from J. Myers at the Univ. of Texas and *Synechocystis* PCC 6714 (UTEX 2470) was obtained from S. E. Stevens at the Pennsylvania State U. *Phormidium fragile* UTEX 2426, *Fremyella diplosiphon* UTEX 481 (Calothrix PCC 7601) and *Porphyridium creuntum* UTEX 161 were obtained from the Culture Collection of Algae at the University of Texas at Austin. *A. nidulans* and *Synechocystis* were maintained in continuous culture turbidostats in Medium C (Kratz and Myers 1955) as described previously (Becker and Brand 1985). *P. fragile* was illuminated continuously at 23° C in ES-enrichment seawater (Starr and Zeikus 1987) with Sylvania F20T12-CW lamps at a photon flux of 30 $\mu E \cdot m^{-2} \cdot s^{-1}$. *P. cruentum* was illuminated exactly like *P. fragile*, in half-strength ES-enrichment seawater at 23° C. *F. diplosiphon* was cultured in Medium C aerated with 1.2% CO_2-enriched air at 32° C, illuminated either with the F20T12-CW lamps at 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ or with red light obtained by wrapping the cultures with red cellophane (cut-off below 600 nm) and

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Abbreviations: Chl, chlorophyll; APC, allophycocyanin; PC, phycocyanin; PE, phycoerythrin; SPM medium, 0.2 M sucrose, 15 mM $MgCl_2$, 0.75 M Na/KPO_4, pH 7.8

illuminating with tungsten lamps to provide a photon flux of 50 μE · m^{-2} · s^{-1} to the cells. Light intensities were determined with a LiCor photon meter as the average photon flux density of photosynthetically active radiation (400–700 nm) delivered to the culture surface.

Cell cultures were collected in log phase growth and diluted with culture medium to a density equivalent to a Chl concentration of 5.0 μg · ml^{-1}. Cultures were subjected to heating treatment in glass test tubes 18 mm diameter while continuously shaken, to insure that the culture rose to within one degree of the treatment temperature within 20 s. After the desired heating time the tube was placed in chilled water, or an aliquot of the culture was removed with a pipette to a pre-chilled test tube, to assure that the temperature dropped to near ambient within a few s. Filaments of *P. fragile* and *F. diplosiphon* in normal culture settled quickly to the bottom of any container not continuously agitated. These filaments were fragmented immediately prior to heating by first homogenizing in a Teflon pestle tissue grinder (Thomas Scientific), then subjecting the culture to 4 of 1-s bursts of ultrasonic shock (Branson 3200) in a standard 18 mm diameter test tube. Individual cells were not disrupted or morphologically altered by this treatment, nor did filament fragmentation affect the ability of the culture to respond to exposure to high temperatures.

Cell extracts of *Anacystis* were prepared from exponentially growing cultures. All procedures were at room temperature unless otherwise indicated. The cells were pelleted by centrifugation at 5,000 × *g* for 10 min, and resuspended in SPM medium at a cell concentration equivalent to 0.25 mg Chl·ml^{-1}, while maintaining approximately ambient temperature. Solid lysozyme (egg white, Sigma Chemical Co. Grade I) was added to a final concentration of 1.0 mg · ml^{-1}, and the suspension was stirred at 30° C for 1 h. Cells were pelleted as before and resuspended in SPM medium at a concentration equivalent to 0.3 mg Chl·ml^{-1}. DNAase I (Sigma Chemical Co., D4263) was added from a concentrated solution to a final concentration of 2.0 μg · ml^{-1}, and the suspension was forced at 100 MPa through a French Press at approximately 2 drops · s^{-1}. Triton X-100 at a final concentration of 0.2% (w/v) was added slowly to this broken cell suspension while it was rapidly mixed. The suspension was then immediately centrifuged at 4,000 × *g* to remove particulate and aggregated matter along with any unbroken cells. The supernatant was diluted to a Chl concentration of 10 μg · ml^{-1}, either in unmodified SPM medium or in SPM medium modified by altering the concentration of the phosphate component.

Cell extracts were prepared for electrophoresis by first precipitating the proteins in 85% acetone, then resuspending in a solution containing 0.5% (w/v) sodium dodecyl sulfate, 1% (v/v) beta-mercaptoethanol and 50 mM HEPES (N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid)/NaOH, pH 6.8. Electrophoresis was performed according to the method of Laemmli (1970), except that 4.0 M urea was included in the gels. Chl concentration was determined at 663 nm after extraction into 80% acetone (MacKinney 1941).

Intact phycobilisomes were isolated from *Anacystis* in high phosphate medium as described previously (Grossman and Brand 1983). Phycobilisomes from *F. diplosiphon* were isolated by the method of Rosinski et al. (1981).

Absorption spectra of whole cells and subcellular preparations were determined with a Cary 219 twin beam spectrophotometer. Matched translucent scattering plates 3 mm thick were placed within the rectangular sample and reference cuvettes for all absorption measurements (Shibata 1958), which provided a light path through the sample of 7 mm, and attenuated the light by approximately 2 absorbance units. All spectra were produced with a slit width of 2.0 nm.

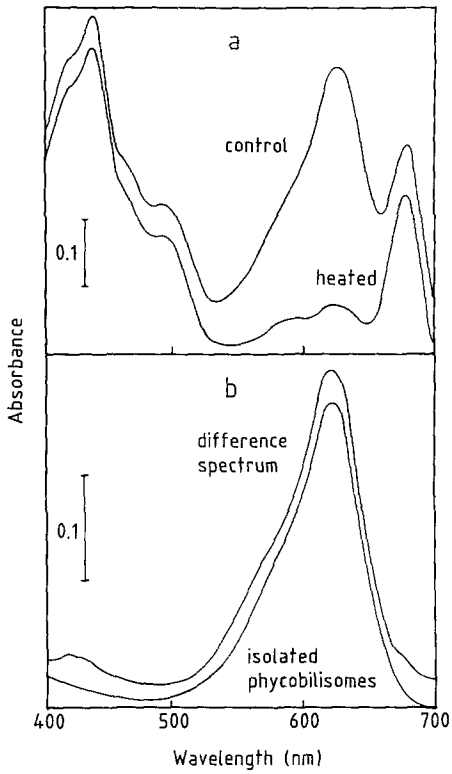

Fig. 1A, B. Absorption spectra of whole cells and phycobilisomes isolated from *Anacystis*. **A** Absorption spectra measured at room temprature of control (not heated) and heated (65° C for 8 min) cells, at a culture density equivalent to 5.0 μg Chl · ml^{-1}. Both spectra were measured against culture medium containing no inoculum. The baseline (A_{750}) of the spectrum of the "control" sample was shifted up by 0.08 absorbance units with respect to that of the period "heated" sample. **B** The difference spectrum was generated by placing control cells in the sample cuvette and heat treated cells in the reference cuvette. The absorption spectrum of isolated, intact phycobilisomes in SPM medium (see Materials and methods) was recorded against pure SPM medium in the reference cuvette. The baseline of the "difference spectrum" was shifted up by 0.03 absorbance units with respect to the "isolated phycobilisomes"

Results

The phycobiliproteins of cyanobacteria or red algae in liquid cultures became bleached after a brief exposure to temperatures higher than 60° C. Figure 1A shows the visible absorption spectra of *Anacystis* cells before and after heating for 8 min at 65° C. The absorption band centered at 623 nm was greatly decreased following heating. The difference spectrum obtained by subtracting the absorbance of heated cells from the absorbance of unheated controls showed a large, asymmetrical peak centered at 623 nm (Fig. 1B). This difference spectrum appeared virtually identical to the absorption spectrum of intact phycobilisomes isolated from *Anacystis* (Fig. 2B). The small shoulder at 680 nm may reflect a slight

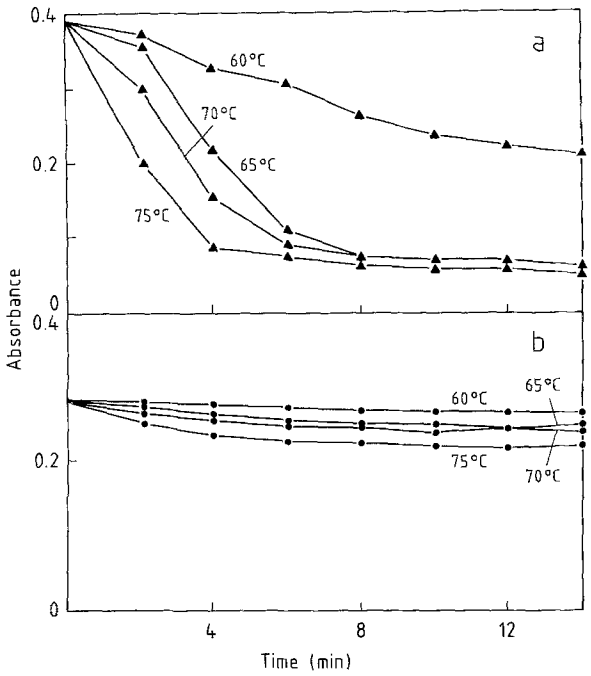

Fig. 2A, B. Change in absorption at 623 nm and at 680 nm of *Anacystis* cells as a function of time of heat treatment. Cells were prepared for heat treatment as described in Fig. 1. A batch of cell culture was placed at each temperature shown, and aliquots were removed at times indicated for measurements of A_{623} (**A**) and A_{680} (**B**)

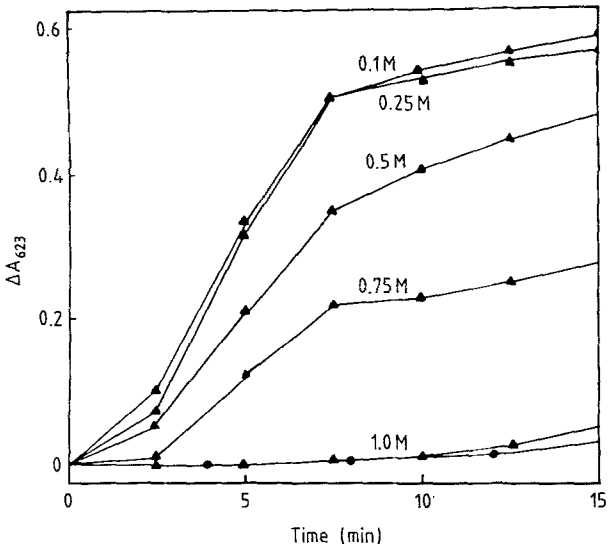

Fig. 3. Change in absorption at 623 nm of *A. nidulans* cell extracts in various concentrations of phosphate. Cells were broken in SPM medium, and portions of the extract were suspended in medium containing 0.2 M sucrose, 10 mM $MgCl_2$, and Na/KPO_4, pH 7.8, at concentrations indicated. Extracts were heated to 65°C, and aliquots were removed at 2.5 min intervals for measurements of A_{623} (triangles). The A_{623} at each heat treatment time was subtracted from the A_{623} prior to heat treatment in order to determine the Δ absorption. Phycobilisomes were purified from cell extract as described in Materials and methods, and suspended in medium containing only 1.0 M Na/KPO_4, pH 7.8. This suspension was heated to 65°C, and aliquots were removed at 2-min intervals for measurements of A_{623} (circles). The Δ absorption was determined as described above for cell extracts

(< 4%) bleaching of chlorophyll. We have extracted quantitatively the lipid-soluble pigments (chlorophyll *a* and carotenoids) from control and heat-treated cells. The difference spectrum of the extracts showed that there was virtually no loss in chlorophyll or carotenoid content during heat treatment (data not shown). Apparently the bleaching of phycobiliproteins was complete since the ratio of A_{623}/A_{680} in heat-treated cells was 0.26, which is in agreement with the data obtained from thylakoid membranes free of phycobiliproteins (Jones and Myers 1965; Myers et al. 1978).

Although cells were not viable after heat treatment, they did not lose any pigments into the medium, and they appeared morphologically the same before and after heating, as viewed by light microscopy.

Figure 2 shows the time course of bleaching of *Anacystis* during heat treatment at various temperatures. A_{623}, which reflects mostly PC absorption, was reduced to minimum within 8 min at temperatures $\geq 65°C$. At 60°C the A_{623} decreased much more slowly. A_{680}, which reflects mostly Chl absorption, was only slightly diminished by heat treatment. Most of the small decline of A_{680} at 65°C and 70°C is explained by the absorption of phycobiliproteins at that wavelength (Jones and Myers 1965; Myers et al. 1978), although a slight (2% ± 2%) decline in chlorophyll content was detected in acetone extracts prepared subsequent to heating. At $\geq 75°C$ Chl is gradually bleached. Illumination during heat treatment did not influence the bleaching pattern; cells heated in darkness or at a photon flux up to 2100 $\mu E \cdot m^{-2} \cdot s^{-1}$ showed the same rates of bleaching.

Anacystis cells were broken and the resulting cell extract was heated to 65°C. Figure 3 shows the absorption change at 623 nm when cell extracts were suspended in various concentrations of phosphate during heat treatment. Phycobiliproteins in the cell extracts were very stable at 65°C in medium containing 1.0 M phosphate. As the concentration of phosphate in the medium was decreased the phycobiliproteins became more susceptible to bleaching by heat treatment. Phycobilisomes purified from the cell extract were also very stable in 1.0 M phosphate at 65°C (Fig. 3). These intact phycobilisome chromophores were bleached significantly only at temperatures exceeding 80°C.

The heated and unheated cell extracts were analyzed by SDS-PAGE (Fig. 4, lanes 1 and 2). Several major electrophoretic bands of the cell extracts corresponded to apoprotein bands of isolated phycobilisomes (Fig. 4, lane 3). The only detected effect of heating was the disappearance of a small (approximately 5 kD) polypeptide (arrow, lane 1), although differences in minor bands of higher molecular mass might not be detected. The loss of the 5 kD polypeptide may not be related directly to bleaching since there is no corresponding polypeptide in isolated phycobilisomes (Lundell and Glazer 1983). We were unable to resolve this small polypeptide unless 4 M urea was included in the gel, and we do not know its origin in the cells.

Since nearly all visible absorption of phycobilisomes in *Anacystis* is due to PC, this organism could not be used to study the relative rates of heat-induced bleaching of different phycobiliproteins. *Fremyella diplosiphon*, in contrast, synthesizes a large proportion of PE under some growth conditions. A culture of *F. diplosiphon* containing PE as its major phycobiliprotein was placed in red growth light, which caused a gradual increase in the PC content. When absorp-

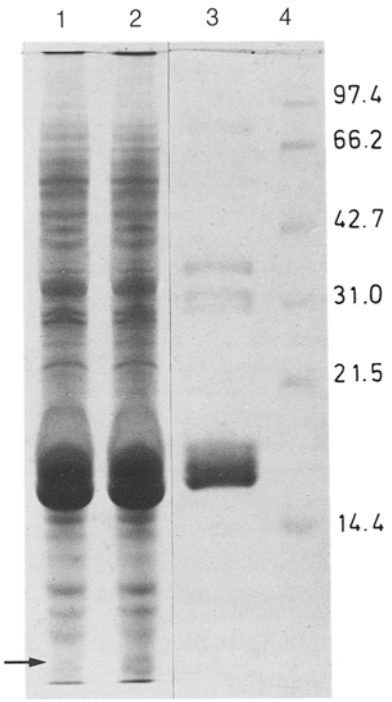

Fig. 4. Protein profiles of *A. nidulans* cell extracts before and after heating in comparison to purified phycobilisome proteins. Cell extracts in medium containing 0.2 M sucrose and 0.25 M Na/KPO_4, pH 7.8, were heated to 65°C for 8 min (*lane 1*) or left at room temperature (*lane 2*). Phycobilisomes were isolated in 0.75 M Na/KPO_4, pH 7.8 (*lane 3*). Samples were prepared for electrophoresis and resolved on SDS-PAGE as described in Materials and methods. *Lanes 1* and *2* were slightly overloaded in order to visualize the small polypeptides with Coomassie blue. The relative molecular weights (in kD) of polypeptide standards (*lane 4*) are indicated at right

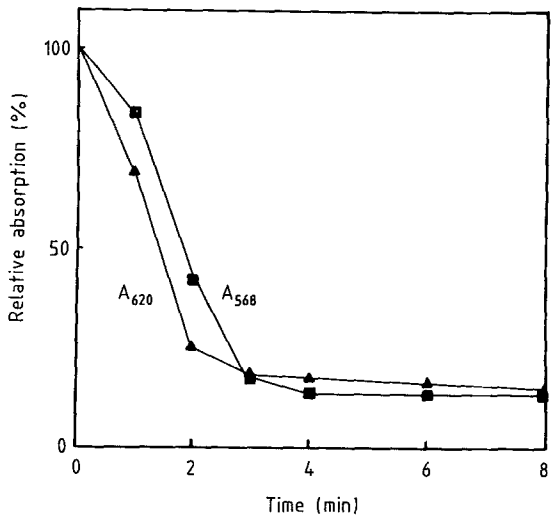

Fig. 5. Decrease in A_{568} and A_{620} of *F. diplosiphon* as a function of time at 65°C. Cells continously cultured in cool white fluorescent light were transferred to red light, then cultured for another 8 h prior to collecting for heating. The filaments were fragmented as described in Materials and methods. The culture was then heated to 65°C. At each time indicated an aliquot of the culture was cooled for measurement of A_{568} and A_{620}. Absorbances at each heating time are expressed as a percentage of the absorbance prior to heating. The initial A_{568} was 0.42; the initial A_{620} was 0.39

Table 1. Minimum heating conditions required for complete bleaching of phycobiliproteins in various strains of cyanobacteria and red algae

Algal Strain	Measuring Wavelength (nm)	Treatment Temperature (°C)	Treatment Time (min)
Anacystis nidulans (UTEX 625)	623	65	8
Fremyella diplosiphon (UTEX 481)	620, 568	65	5
Phormidium fragile (UTEX 2426)	568	60	5
Synechocystis PCC 6714 (UTEX 2470)	620	65	5
Porphyridium creutum (UTEX 161)	565	60	5

The absorption spectrum of each strain was measured prior to heating to determine which wavelength(s) represent phycobiliprotein absorbance peaks. For each strain a series of heating experiments were conducted as shown for *Anacystis* in Fig. 2. The minimum time and temperature combination required for complete bleaching is shown.

tion peaks of PE and PC became approximately equal, the cells were collected and heated to 65°C. Aliquots were collected for absorption measurements at times indicated (Fig. 5). A_{568} (PE) and A_{620} (PC) decreased at approximately the same rates. Also, in all other organisms examined each of the phycobilin chromophores appeared to be bleached at approximately the same rate.

We examined several other cyanobacteria and red algae to determine the heating conditions which caused bleaching of phycobilin pigments. Phycobilins were completely bleached within 8 min at 60–65°C in all organisms tested (Table 1). Higher temperatures bleached phycobilin pigments even faster, but also resulted in gradual bleaching of Chl and carotenoids.

In each organism examined we confirmed by difference spectroscopy that the decreased absorbance which occurred upon heating was due only to bleaching of phycobilins. The difference spectra of control minus heated cells are shown in Fig. 6 for three representative species. *P. fragile* produces an abundance of PE, which was reflected in the difference spectrum. Note that under our growth conditions this organism produced very little PC. The unicellular red alga, *P. cruentum*, also produced phycobilisomes with a high PE/PC ratio under our growth conditions. *Synechocystis* does not produce PE; however, it contains a higher APC content than does *A. nidulans*, as shown in the difference spectrum (compare Fig. 6 with Fig. 1 B). The occurrence of clearly resolved peaks for PC, PE and APC by the heating method, as illustrated in Fig. 6, makes for easy identification of pigment compositional changes as an organism is adapted to various environmental conditions. This is shown in Fig. 7 for an organism chromatically adapted to white or red light prior to heating for spectral analysis.

In all strains tested, cells did not survive heating which caused bleaching of phycobiliproteins. Apparently the heating treatment causes damage beyond the immediate effect on photosynthesis, because *Synechocystis* did not grow heterotrophically in darkness following heating. Hetero-

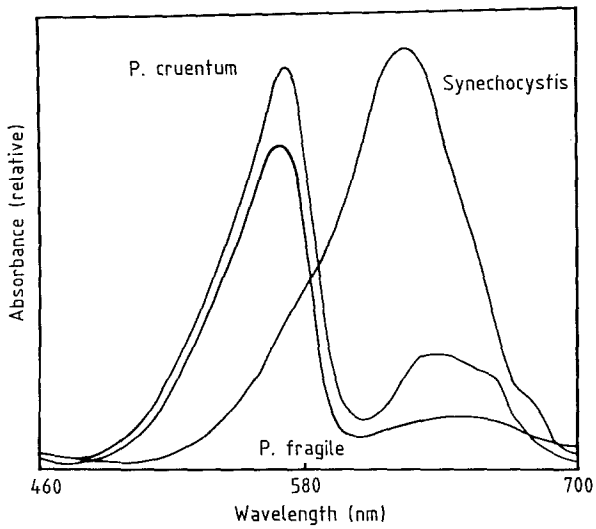

Fig. 6. Difference spectra of several phycobilisome-containing organisms (control minus heat-treated). Each strain was heated as described in Table 1. Difference spectra were obtained as described in Materials and methods

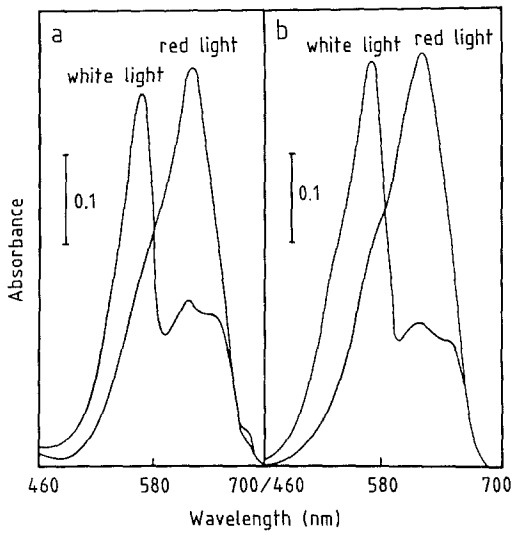

Fig. 7. Difference spectra (control minus heated) of *F. diplosiphon* chromatically adapted to two different light conditions. The cultures were grown in white fluorescent or in red light. **B** Absorption spectra of purified phycobilisomes from cultures of *F. diplosiphon* grown in white fluorescent or red light. The experiments illustrated in panels A and in B used the same cultures

trophic growth of *Synechocystis* in darkness was supported in control (unheated) cells by 1% (w/v) glucose (Rippka et al. 1979).

Discussion

Phycobilisomes can be isolated intact only in medium of high osmotic and ionic strength (Gantt et al. 1979). These conditions result in low water activity, which has been proposed as the actual stabilizing influence on phycobilisomes in vivo (Katoh 1988). The sensitivity of phycobilisomes to bleaching in cell-free extracts at high temperature decreases as the phosphate concentration in the suspension medium is increased (Fig. 3). Thus, phycobilisomes in vitro are apparently much more resistant to bleaching at high temperature when intact than when dissociated. In the presence of a high concentration of phosphate the phycobilisomes in cell-free extracts were not significantly affected by heating until the temperature was raised to at least 80° C. In contrast, phycobilisomes were completely bleached in vivo at 65° C within 8 min in all species examined (Table 1). Phycobilisomes in cell free extracts at low osmotic and ionic strength were bleached at essentially the same heating temperatures as temperatures which caused bleaching in whole cells. We conclude that phycobilisomes may be stabilized in vivo by some mechanism other than high ionic and osmotic strength (low water activity), and the stabilizing forces are insufficient to protect the phycobilisomes from bleaching at temperatures higher than 60 – 65° C in mesophilic species.

Within a given organism each of the phycobilin chromophores were bleached at essentially the same rate at any specified heating temperature (e.g. Fig. 5). Yet, the heating temperature and time required to induce bleaching varied among species (Table 1), and in thermophilic species such as high temperature *Synechococcus* (Yamaoka et al. 1980) the cells maintain normal, functional phycobilisomes at temperatures exceeding 60° C. Then what is the mechanism of phycobilin bleaching by elevated temperatures? Heating apparently leads to a direct (probably covalent) modification of the bilin chromophores, rather than simply changing their environment, since the visible absorption bands totally disappear rather than undergoing spectral shifts (e.g. Fig. 1). We see no evidence for any change in the apparent molecular weights of the phycobilisome proteins following heating to induce bleaching (Fig. 4). Yet, the aromatic tetrapyrrole chromophore would be expected to be resistant to non-enzymatic degradation at the temperatures (60 – 65° C) which we employed (Arciero et al. 1988). Thus, perhaps one of the proteins of phycobilisomes can cause a pronounced change in the chromophores at elevated temperatures. Preliminary results demonstrate that phycocyanin fluorescence emission (655 nm) in whole *A. nidulans* cells increases during the first two minutes of heating at 65° C, followed by a decrease, which approximately parallels the decrease in absorbance shown in Fig. 2A. This indicates that the phycobilisomes become incapable of energy transfer prior to their bleaching, perhaps by their dissociation. That might be expected since isolated phycobilisomes are much more sensitive to bleaching when in the dissociated form (Fig. 3).

Controlled heating of whole cells followed by difference absorption spectroscopy represents a very rapid, convenient and accurate method to measure quantitatively the relative pigment composition of cells which contain phycobiliproteins as part of their photosynthetic antenna. In this paper we have demonstrated its effectiveness in characterizing phycobiliprotein content. Moderate heating does not appear to alter the transmittance of incident light except by eliminating phycobiliprotein chromophore visible absorption. Thus, accurate difference absorption spectra are obtained even without correcting for stray reflected and scattered light in turbid suspensions when the two spectrophotometric cuvettes contain identical samples except that one of the samples is heated to bleach the phycobilisomes. This method also allows for accurate measurements of the lipid soluble pigment content (Chl *a* and carotenoids) of phycobiliprotein-containing cells by measuring the absorption spectrum of the bleached cells directly against a blank

cuvette when the spectrum is corrected for scattering and other stray light which is not transmitted through the turbid sample.

The physiological significance of the selective bleaching of phycobiliproteins at moderate temperatures has not yet been identified. Apparently no temperature regime will bleach all of the bilin pigments, yet still allow the cells to remain viable. Perhaps this system participates in regulation of the phycobilisome chromophore content in a more subtle way at lower temperatures, which could then modulate the rate of energy flow to Photosystem II.

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