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FLUORESCENCE AND ENERGY TRANSFER IN PHYCOBILIPROTEIN-CONTAINING ALGAE AT LOW TEMPERATURE

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Summary

Fluorescence emission spectra of *Anacystis nidulans*, *Porphyridium cruentum* and *Cyanidium caldarium*, three phycobiliprotein-containing algae, were measured at temperatures between 4 and 120 K in the absence and in the presence of quinones as quenchers of chlorophyll fluorescence. In all species three major emission bands were observed in the chlorophyll *a* region, near 685 nm (*F*-685), 695 nm (*F*-695) and between 710 and 730 nm. Additional bands were observed at shorter wavelengths; these were preferentially excited by light absorbed by the phycobiliproteins and are presumably due to phycocyanins and allophycocyanins.

The amplitudes of *F*-685, *F*-695 and the long-wave emission showed a distinct increase upon cooling. For *F*-685 and *F*-695 the temperature dependence was similar to that earlier observed with spinach chloroplasts, for the long-wave emission it appeared to depend on the location of the emission bands, which was different for different species. All three bands were strongly quenched by quinones. These and other data suggest that the origin of these bands is the same as in higher plants, and that the fluorescence increase upon cooling can be explained by a lowering of the efficiency of energy transfer between chlorophyll molecules. It is concluded that at most a small percentage of the emission at 685 nm can be ascribed to allophycocyanin B, and that the efficiency of energy transfer between allophycocyanin B and chlorophyll *a* probably exceeds 99% both at 77 and 4 K. Experiments with isolated phycobilisomes suggest that energy transfer from allophycocyanin to allophycocyanin B occurs with an efficiency of about 90% at low temperature.

Abbreviations: DBMIB, dibromothymoquinone; Chl, chlorophyll.

The effect of quenchers can be understood by the assumption that the quenching is caused by the formation of non-fluorescent traps in the bulk chlorophyll. Of three quinones tested, the strongest quenching was observed with dibromothymoquinone, which quenched *F*-685, *F*-695 and the long-wave emission approximately equally. Menadione and 1,4-naphthoquinone, however, preferentially quenched the long-wave bands, indicating a stronger interaction with Photosystem I than with Photosystem II chlorophylls.

Introduction

It is well-known that the fluorescence yield of Chl *a* in vivo increases strongly upon cooling (e.g. [1,2]), whereas the fluorescence yield of a dilute Chl *a* solution is practically independent of temperature [3]. For chloroplasts and intact cells of algae and higher plants the most conspicuous effect of cooling to about 100 K consists in a strong increase in the long-wave region of the emission spectrum, whereas at still lower temperatures the emission at shorter wavelengths is also stimulated [4—8]. Experiments with chloroplasts and chloroplast preparations [6—8] indicate that these effects can be explained by a decrease of the rate of energy transfer between different Chl *a* 'pools' and between Chl *a* molecules within these pools. In addition, it has been possible to some extent to relate the low temperature emission bands to chlorophyll-protein complexes that have been isolated from the thylakoid membrane by means of detergent treatment [6,9].

In the present paper we report experiments with phycobilin-containing algae: the blue-green alga *Anacystis nidulans*, the red alga *Porphyridium cruentum* and *Cyanidium caldarium*, an algal species of uncertain taxonomic position [10]. The results indicate that the same mechanisms that cause the temperature dependence of Chl *a* fluorescence in higher plants do also operate in these algae. In addition some data will be presented concerning the effect of quenchers on the emission spectra and concerning the efficiencies of energy transfer between phycobiliproteins and from allophycocyanin B to Chl *a*.

Materials and Methods

Anacystis nidulans and *Porphyridium cruentum* were grown at 25°C in media described by Kratz and Myers (C medium) [11] and Jones et al. [12], respectively. *Cyanidium caldarium* was grown in a medium after Allen [13] at a temperature of 38°C. For *A. nidulans* the medium was supplied with nitrogen and 5% CO_2, for the other species air and 5% CO_2 was used. Phycobilisomes were prepared according to the method of Gantt et al. [14].

The samples were rapidly cooled in liquid nitrogen to avoid changes in energy transfer between phycobilisomes and Chl *a* which may occur if the algae are subjected for some time to temperatures between +5 and −10°C [15]. All measurements were done with crystalline samples contained in perspex vessels of 1 mm thickness. The suspensions of algae had an absorbance of 10^{-3}/mm at 680 nm. Glycerol, often used to obtain clear samples upon cooling, was found to cause a several times increased phycocyanin emission in *Anacystis*

and a much lower Chl *a* emission upon excitation at 562 nm, suggesting a decreased rate of energy transfer from phycocyanin to Chl *a*. Therefore, the measurements were done in the absence of glycerol.

The fluorescence measurements were performed with the apparatus described before [6,7]. Fluorescence was detected from the illuminated side of the cuvette. The excitation light was filtered by interference filters (Schott AL 442 or 562 nm) in combination with absorption filters (Corning CS 4-96 and Schott BG 18). Colored glass filters (CS 2-62 and Schott RG 610) were positioned in front of the monochromator to absorb scattered light. The monochromator was set at a bandwidth of 1.6 nm. Because of small differences in the position of the samples in the apparatus, the fluorescence intensities measured were reproducible within 10%. The spectra were corrected for the wavelength-dependent sensitivity of the apparatus and are plotted in arbitrary units proportional to Watts per wavelength interval.

Results and Interpretation

Emission spectra

Fluorescence emission spectra of *Anacystis nidulans* at different temperatures and with different wavelengths of excitation are shown in Fig. 1. Emission spectra for the same temperature range, but with less spectral resolution have been published by Cho and Govindjee [16]. As will be discussed below, above 680 nm the emission is probably mainly due to Chl *a*; fluorescence emitted below 670 nm comes from the phycobilisomes and is relatively weak upon excitation at 442 nm (Fig. 1A). In the chlorophyll region the spectra show the usual pattern with three dominant peaks, observed with various species of algae and with higher plant chloroplasts. The location of the first two of these bands, which are usually called *F*-685 and *F*-695, is remarkably independent of species; that of the long-wave maximum varies between 710 and 740 nm, depending on the organism [2]. In *Anacystis* the long-wave maximum was located near 715 nm at 4 K. We shall call this band *F*-715. The

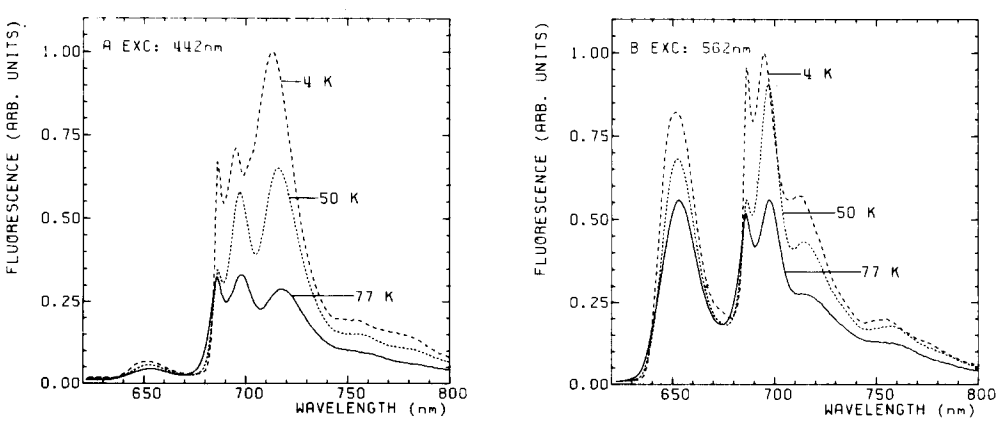
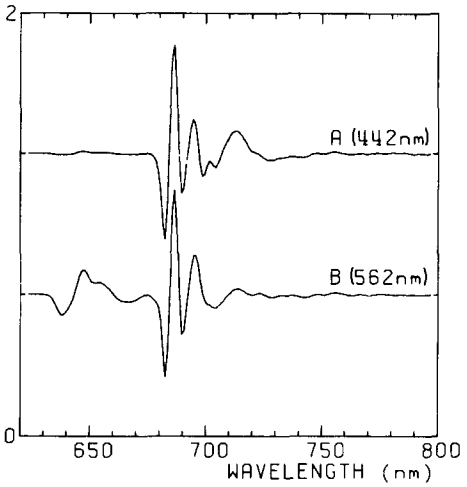


Fig. 1. Emission spectra of *A. nidulans* at three different temperatures upon excitation with 442 nm (A) and 562 nm light (B). The units applied for (A) and (B) are different.



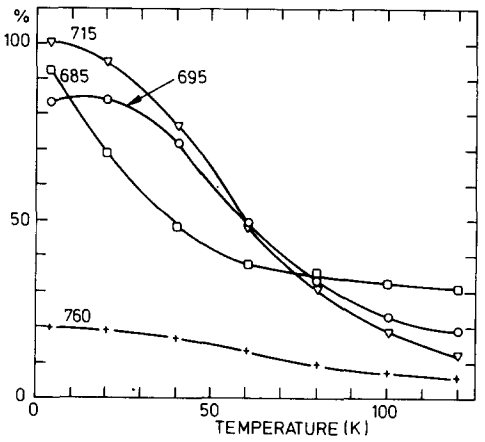


Fig. 2. Second derivatives (inverted) of the emission spectra recorded at 4 K shown in Fig. 1. A and B correspond to 442 and 562 nm excitation, respectively.

Fig. 3. Temperature dependence of the emission intensities at 685, 695, 715 and 760 nm of *A. nidulans* upon 442 nm excitation. The batch of algae was different from that used for Fig. 1.

F-680 band, observed in higher plant chloroplasts was absent in the emission spectra of *Anacystis*. This is in agreement with the assignment of this band to the light-harvesting Chl *a*/*b* complex [6].

In order to determine the number and location of the emission bands, we calculated the second derivatives of the emission spectra presented in this paper. Except for the bands beyond 750 nm, which were too broad to be measured in this way, the peak positions to be cited below refer to those measured at 4 K from the second derivative spectra. The peak positions of *F*-685, *F*-695 and *F*-715 in *Anacystis* (Fig. 2) were at 686—687, 695 and 715 nm upon excitation with 442 or 562 nm; the derivative spectrum showed also a maximum at 702 nm with blue light. As was noted earlier [2,16,17], *F*-715 was relatively weak upon excitation of the phycobilisome, indicating that it is mainly associated with pigment system I. In addition to the bands mentioned above, at least two additional bands are visible at longer wavelengths, with maxima at 757 and about 775 nm, both upon excitation with 442 and with 562 nm. The relative heights of the main emission bands at low temperature in *Anacystis* were quite variable, and appeared to depend on the culture conditions (see also Refs. 18 and 19). Compared to most other low temperature spectra published (cf. Refs. 2, 16) our spectra showed a high amplitude of *F*-685 and *F*-695 relative to *F*-715.

The temperature dependences of the amplitudes at various wavelengths are shown in more detail in Fig. 3. They showed a distinct increase in the intensity of all bands when cooling from 77 to 4 K. As noted for higher plant chloroplasts [6—8], *F*-685 did not increase between 77 and 50 K, but showed a strong enhancement below 50 K, whereas *F*-695 showed its main increase between 80 and 40 K. In contrast to *F*-735 in higher plant chloroplasts, *F*-715 continued to grow considerably below 80 K.

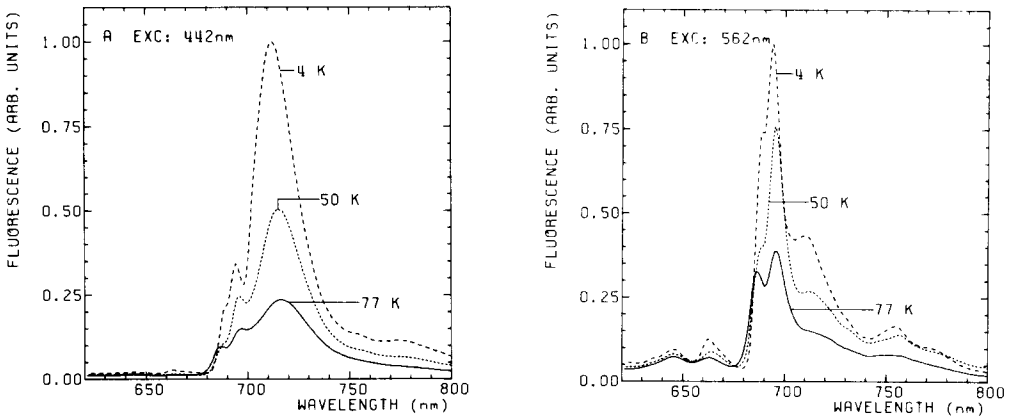


Fig. 4. Emission spectra of *P. cruentum*, obtained as for Fig. 1.

The intensity of phycobiliprotein emission near 650 nm increased by about 50% between 77 and 4 K (Fig. 1). This emission is composed of two bands, located at 646 and 654 nm (Fig. 2), which are probably due to C-phycocyanin and allophycocyanin [14,20]. In addition to this there was a weaker band at 676 nm, probably due to a long-wave emitting form of allophycocyanin.

Emission spectra of two other algal species that contain phycobiliproteins, *Porphyridium cruentum* and *Cyanidium caldarium*, are shown in Figs. 4 and 5. At 77 K, the spectra of *Porphyridium* resembled those obtained earlier for this organism [2,21]. The temperature dependences of *F*-685, *F*-695 and *F*-715 were very similar to those of *Anacystis*. *F*-685, *F*-695 and *F*-715 were located at 687, 694 and 712 nm, respectively. In addition, there was a clear band at 704 nm in the derivative spectrum upon blue excitation and there were long-wave bands at 755 and 775 nm.

In *Cyanidium*, *F*-685 and *F*-695, located at 689 and 695 nm, respectively, showed a similar temperature dependence as in the other species. The long-wave emission at 710—740 nm in this organism was clearly resolved in at

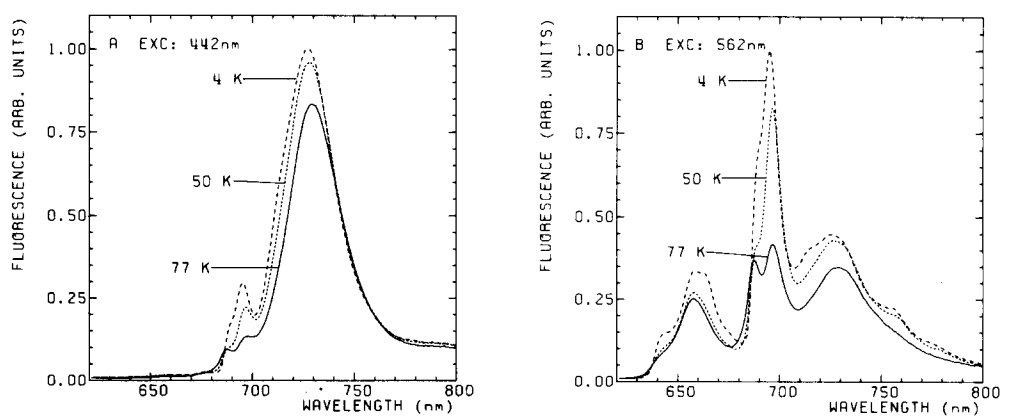


Fig. 5. Emission spectra of *C. caldarium*, obtained as for Fig. 1.

least two bands at 4 K, situated at 712 and 727 nm. The first one behaved like *F*-715 in *Anacystis* and *Porphyridium*, but the last one (*F*-730), like *F*-735 in higher plant chloroplasts [6—8], increased only little in intensity below 77 K. The emission band near 755 was most prominent upon excitation at 562 nm, and appears to be associated with Photosystem II, as was also concluded by Ley and Butler [22].

The main phycobiliprotein bands in *Porphyridium* were located at 646 and 662 nm, presumably representing R-phycocyanin and allophycocyanin emission [20]. *Cyanidium* showed bands at 641, 657 and 664 and probably near 676 nm, the last one perhaps being due to allophycocyanin B. Both species showed a clear increase in phycobiliprotein emission upon cooling. The emission intensities in the phycobiliprotein region, when calculated per equal number of quanta, were several times lower than for *Anacystis*.

Fluorescence quenchers

Fig. 6 shows the effect of the quenchers 1,4-naphthoquinone and DBMIB on the emission spectra of *Anacystis*. The quinones were added to the sample in ethanol solution (final ethanol concentration 2% v/v). The control spectra were likewise recorded in the presence of ethanol, the addition of which caused an increase of *F*-695 by about 15—20% relative to the other chlorophyll peaks, and an increase of the phycocyanin band at 646 nm relative to that at 654 nm. The total emission yield around 650 nm was little changed.

Both quinones (especially DBMIB) gave a strong quenching of the three major emission bands of Chl *a*. Within the limits of reproducibility of the measurements (see Materials and Methods) no effect on the intensity of phycobiliprotein emission at 654 nm could be detected. Therefore, the emission spectra were normalized at 654 nm in order to facilitate comparison. *F*-685 was somewhat less sensitive to quencher than *F*-695, and at high quinone concentration became the highests band in the Chl *a* region. 1,4-Naphthoquinone and manadione preferentially quenched *F*-715. Fig. 7 shows the concentration dependence for menadione quenching, measured at 695 and 715

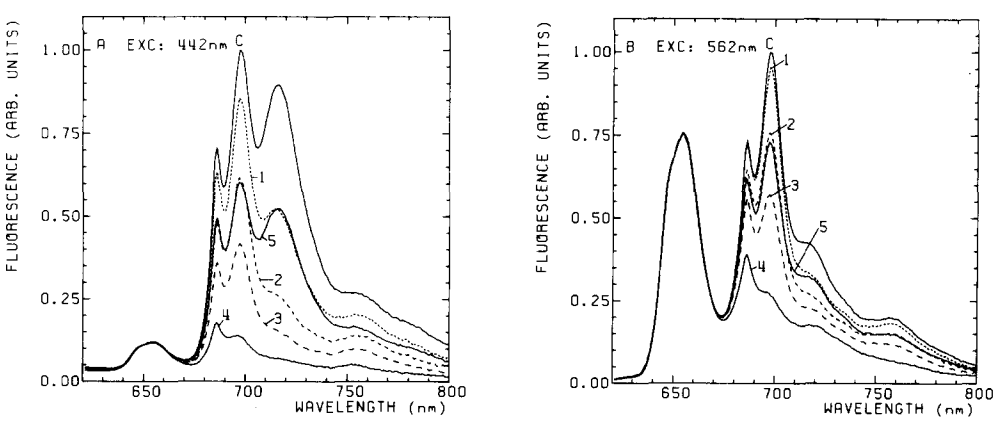


Fig. 6. Emission spectra of *A. nidulans* at 77 K upon excitation at 442 (A) and 562 nm (B). C, control; 1—4, in the presence of 1,4-naphthoquinone: (1) $2 \cdot 10^{-5}$ M, (2) $5 \cdot 10^{-5}$ M, (3) $2 \cdot 10^{-4}$ M, (4) $5 \cdot 10^{-4}$ M, (5) in the presence of 10^{-5} M DBMIB.

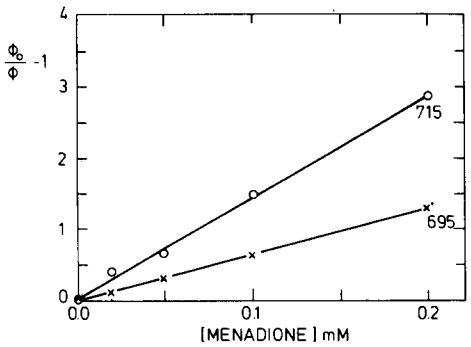


Fig. 7. Intensity of fluorescence emission at 695 and 715 nm in *A. nidulans* as a function of menadione concentration at 77 K. ϕ_0 and ϕ denote the relative fluorescence intensities without and with menadione, respectively.

nm. Up to a concentration of $2 \cdot 10^{-4}$ M, which gave about 70% quenching at 715 nm, the quenching obeyed a relation of the Stern-Vollmer type.

Fig. 8 shows the effect of DBMIB on the emission spectra of *Cyanidium*. Apart from the fact that higher concentrations were needed, the quenching pattern was similar as with *Anacystis*, with a somewhat smaller quenching of *F*-685 than of the other bands. No further quenching was observed above $5 \cdot 10^{-4}$ M. Similar results were obtained with *Porphyridium* (not shown). 1,4-Naphthoquinone and menadione again gave a preferential quenching of the long-wave bands (*F*-730 and *F*-715) for these species.

The temperature dependences of the various emission bands were similar in the presence and in the absence of quencher (not shown). In general, the extent of quenching decreased somewhat with decreasing temperature.

Phycobilisomes emission

Phycobilisomes of *Anacystis*, prepared by the method of Gantt et al. [14] showed two about equally strong emission bands, near 655 and 680 nm (Fig. 9). These bands have been ascribed to allophycocyanin, and allophycocyanin

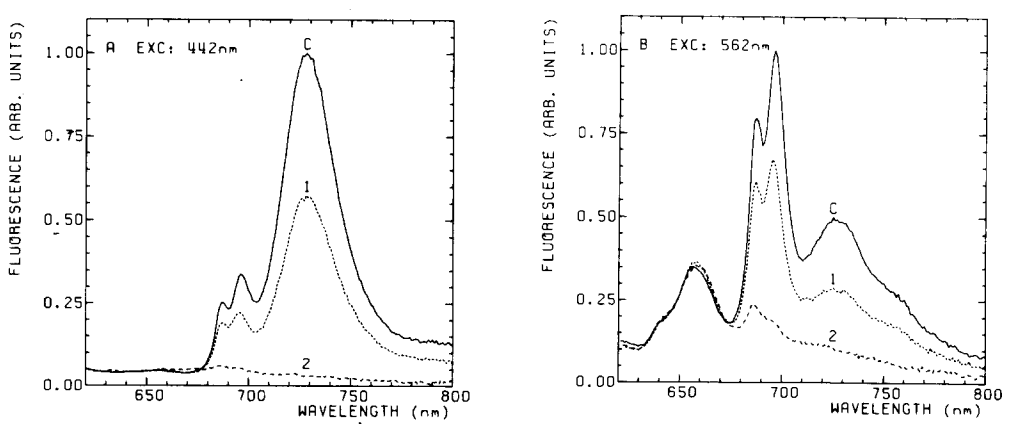


Fig. 8. Emission spectra of *C. caldarium* at 77 K upon excitation at 442 (A) and 562 nm (B). C, control; 1 and 2, in the presence of DBMIB (1, 10^{-4} M; 2, $5 \cdot 10^{-4}$ M).

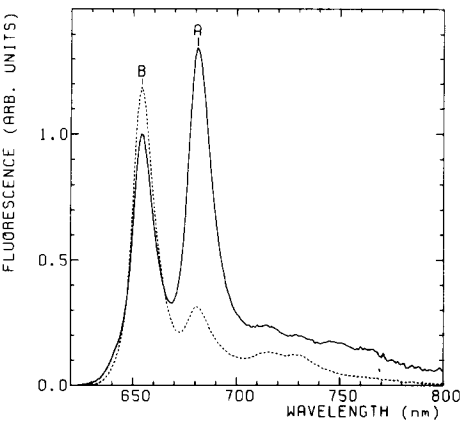


Fig. 9. Emission spectra of isolated phycobilisomes upon excitation with 562 nm light before (A) and after (B) treatment with lauryldimethylamine oxide (curve B is reduced by a factor of 4).

B, respectively [14]. The band at 655 nm was considerably higher, relative to that at 680 nm, than observed by Gantt and coworkers, indicating that energy transfer to allophycocyanin B was partially uncoupled in our preparations. In agreement with this, the fluorescence yield at 655 was 6—8 times higher than in intact cells. The slight shoulder at 640 nm is probably due to C-phycocyanin. Cooling from 77 to 4 K had practically no effect on the emission intensities of the three bands.

Incubation with 1% lauryldimethylamine oxide during 30 min removed almost all 680 nm emission and caused an increase of the 655 nm band by a factor of about 3—5 (Fig. 9). Again, the fluorescence yield was almost independent of temperature.

Discussion

Our data, together with earlier results, and those of others [6,7,15,23] strongly suggest that the origin of the three major emission bands in the species studied here is the same as in higher plant chloroplasts. *F*-715 and *F*-730, like *F*-735 in spinach chloroplasts are due to a long-wave emitting Chl *a* species associated with Photosystem I, whereas *F*-685 and *F*-695 are primarily associated with Photosystem II.

It was recently suggested [24] (see also Ref. 22) that *F*-685 or at least a considerable proportion of it would be due to allophycocyanin B emission, the main argument being that *F*-685 (and *F*-695) are drastically reduced in intensity when the phycobilisomes are removed from the membrane. However, since Chl *a* of Photosystem II receives most of its excitation from the phycobiliproteins, this experiment does not prove that *F*-685 is emitted by allophycocyanin B. Our emission spectra of *Anacystis*, which were obtained with a higher resolution than those published earlier, do not support this notion either. The maximum (686—687 nm) is very close to that obtained for *F*-685 in higher plant chloroplasts, whereas the emission maximum of allophycocyanin B is at 680—683 nm, both for isolated phycobilisomes (Fig. 9 and

Ref. 14) and in situ, as indicated by experiments with cold-treated cells [15]. Furthermore, the emission band of allophycocyanin B is fairly broad, with a half-width of 13—16 nm, whereas *F*-685 is distinctly narrower. The experiments with quenchers also support the notion that *F*-685 is emitted by Chl *a*, since this band, like in spinach chloroplasts, is strongly quenched by quinones, whereas there is no evidence for direct quenching of allophycocyanin B fluorescence by these substances [15]. It might be argued that the emission by allophycocyanin B is only quenched by quinones when attached to the membrane, where the quencher concentration is presumably highest. However, this hypothesis still would not explain the narrowness of *F*-685 and the fact that the quenching was at least as effective upon excitation at 442 as at 562 nm (Fig. 6).

No evidence for an additional band near 680—683 nm can be seen in the emission spectra of *Anacystis* (Fig. 1B). This suggests that the emission intensity of allophycocyanin B is at least 10 times lower than the emission near 650 nm. A similar estimate can be obtained from emission spectra in the presence of a high concentration of quencher. In isolated 'well-coupled' phycobilisomes the allophycocyanin B band is about 10 times higher than the emission near 652 nm [14]. Assuming that the yield at 652 nm is the same in these preparations as in intact cells, this indicates that the yield of allophycocyanin B emission is at least 100 times lower in the intact cells than in the isolated phycobilisome. This indicates that energy transfer from allophycocyanin B to Chl *a* occurs with an efficiency of at least 99%, both at 77 and 4 K.

The data obtained with lauryldimethylamine oxide-treated phycobilisomes allow an estimate of the transfer efficiency between allophycocyanin and allophycocyanin B. The high yield of allophycocyanin emission and the relatively weak emission near 680 nm in this preparation indicate practically complete cessation of energy transfer to allophycocyanin B, presumably because this pigment has been detached from the complex by lauryldimethylamine oxide treatment. By comparison of the yields of allophycocyanin fluorescence in this preparation and in the intact cells we calculated an efficiency of energy transfer from allophycocyanin to allophycocyanin B of about 95% at 77 K and of about 90% at 4 K in vivo. The relatively low yields of phycocyanin and allophycocyanin fluorescence in *Porphyridium* and *Cyanidium* suggest a higher efficiency of energy transfer within the phycobilisomes in these organisms.

According to Gantt et al. [21], the low temperature emission maximum of allophycocyanin B in phycobilisomes of *P. cruentum* is located at 685 nm, which is at distinctly longer wavelength than for other species of the same genus [14]. The peak thus would almost coincide with *F*-685. Nevertheless, the sensitivity to quenchers and the sharpness of the emission band in intact cells as e.g. evidenced by the relatively much lower emission intensity near 680 nm (Fig. 4B) than in isolated phycobilisomes [21] strongly suggests that, like in *Anacystis*, allophycocyanin B contributes only a small fraction to the total emission intensity at 685 nm. As far as we know emission spectra of allophycocyanin B from *Cyanidium* have not been published. However, the similar shape and quenching properties of *F*-685 suggest that this band, like in *Porphyridium*, is mainly due to Chl *a*. This is also strongly suggested by the

emission spectrum of *Cyanidium* mutant that did not contain phycobilisomes [23].

The temperature dependences of the three major emission bands in the algae examined show a general agreement with those observed in higher plant chloroplasts. They can be explained by a decrease in the efficiency of energy transfer between Chl *a* molecules in the same way as earlier discussed for higher plants [6] and purple bacteria [25]. The main difference is that *F*-715 continues to increase upon cooling below 80 K, whereas for higher plants the long-wave emission (*F*-735) remains approximately constant below this temperature. The strong increase of *F*-735 was explained by the assumption that back transfer from *F*-735 to bulk chlorophyll ceases upon cooling, which effect may be due to a decrease of the overlap integral [26] in the Förster equation. This explanation agrees with recently measured lifetimes of *F*-735 [27]. The same mechanism may operate for *F*-715, but in this case it is conceivable that the overlap integral decreases less rapidly with temperature because the emission band is located at shorter wavelength. An alternative hypothesis would be that energy transfer from the long-wave emitting chlorophylls to the reaction center is interrupted by cooling. However, as far as we know, there is no clear evidence for a close association between these long-wave chlorophylls with the reaction center, and in fact in isolated Photosystem I complexes the intensity of long-wave emission appears to decrease with increasing purity of preparation (see Ref. 6).

The effect of quencher on the amplitudes of the emission bands in the low temperature spectra can be understood by the assumption that the quenching is caused by the formation of non-fluorescent traps in the bulk chlorophyll [28]. The decrease in quenching activity upon cooling from 77 to 4 K may be explained by the decrease in energy transfer discussed above. For all three quinones tested, *F*-695 was only a little more strongly quenched than *F*-685, also below 50 K, where energy transfer from the *F*-685 to the *F*-695 chlorophyll appears to be irreversible [6]. This indicates that for Photosystem II most of the quenching takes place in the Chl *a* associated with *F*-685. For 1,4-naphthoquinone and menadione the quenching of the long-wave bands (*F*-715 and *F*-730) was stronger than for *F*-685 and *F*-695 in all three organism tested, suggesting a specific interaction of these quinones with Photosystem I chlorophylls. For all three species the emission spectra suggest a constant ratio of the amplitudes of *F*-695 and the emission band at about 760 nm. The same appears to be true for *F*-715 and the band near 775 nm in *Anacystis* and *Porphyridium*. This suggests that the bands near 760 and 775 nm may be due to vibrational subbands of *F*-695 and *F*-715, respectively.

Acknowledgements

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