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Functional assignment of chromophores and energy transfer in C phycocyanin isolated from the thermophilic cyanobacterium *Mastigocladus laminosus*

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The optical characteristics and pathway of energy transfer in the C phycocyanin trimer isolated from the thermophilic cyanobacterium *Mastigocladus laminosus* were investigated at steady state by absorption, circular dichroism, fluorescence and fluorescence polarization spectroscopy. Based on the comparison of optical data with the 3-dimensional structure of the C-phycocyanin trimer determined by X-ray analysis (Schirmer, T., Bode, W., Huber, R., Sidler, W. and Zuber, H. (1984) in Proceedings of the Symposium on Optical Properties and Structure of Tetrapyrroles, (Blauer, G. and Sund, M., eds.), pp. 445–449, Walter de Gruyter, Berlin, and (1985) J. Mol. Biol. 184, 257–277), the functional assignment of three types of chromophore was established. An α subunit has an s chromophore and the chromophores at the positions 84 and 155 in the amino acid sequence of the β subunit are assigned as f and s chromophores, respectively. In the C phycocyanin trimer energy transfer occurs from the α chromophore in one monomer to the β_f chromophore in an adjacent monomer, and from the β_s chromophore to the β_f chromophore in the same monomer. The direction of energy flow is from the outside to the inside of the trimer, where the locus for the binding of a colourless polypeptide is postulated. In the phycobilisomes the energy concentrated at the β_f chromophores might be transferred toward the allophycocyanin core mainly by the β_f chromophores in the phycocyanin rods.

Introduction

Phycobiliproteins, the light-harvesting pigment-protein complexes of cyanobacteria and red algae, form supramolecular aggregates, called phycobilisomes at the surface of the thylakoid membranes [1]. Light energy captured by the phycobiliproteins is efficiently transferred to Chl *a* in the thylakoid membranes [1,2]. The energy-transfer process occurs on the picosecond time scale, thus the development of picosecond spectroscopy should enable us to study the detailed transfer process [3–6].

The transfer times from the initially excited pigments in phycobilisome to Chl *a* are found to depend on the organism and type of phycobilisome. The apparent transfer time from phycoerythrin to Chl *a* in intact cells of the red algae *Porphyridium cruentum* [3,6,7] was shorter than that from phycocyanin to Chl *a* in the cyanobacterium *Anacystis nidulans* [6,7]. This kind of phenomenon was also observed in the isolated phycobilisome from both organisms [8–10]. However, the difference in phycobilisome structure and pigment composition did not make clear the elementary process responsible for the difference in the apparent transfer time. One of us (Mimuro, M. et al., see Refs. 7 and 11) measured the apparent

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Abbreviations: Chl, chlorophyll; CD, circular dichroism.

rate constants of energy transfer in the chromatically adapted cyanobacteria, *Tolypothrix tenuis* and *Fremyella diplosiphon*, whose pigment compositions varied depending on the growth conditions. However, in this case the hemidiscoidal phycobilisome structure was not changed. The observed difference in transfer time was attributable to the difference in the apparent rate constant of energy transfer at the phycocyanin level. We also found that, based on computer simulation data, the number of transfer steps is expected to be small in the phycocyanin rod. We proposed the idea of a special channel which facilitates one directional energy flow to the allophycocyanin core. The same proposal was also made by Suter et al. [10], based on the analysis of decay kinetics on isolated phycobilisome from *Synechococcus* 6301.

To determine the nature of this special channel, it is necessary to analyze the energy transfer among individual chromophores. For this purpose, we tried to describe the optical characteristics of individual chromophores and the occurrence of energy transfer among them in phycocyanin. In hemi-discoidal phycobilisome without phycoerythrin, phycocyanin rods consist mainly of three hexamers, each of which is associated with a specific colourless polypeptide. Each hexamer is composed of two trimers, one of which binds a colourless polypeptide [2]. This arrangement indicates that the trimer is a basic unit of the phycocyanin rod in phycobilisome. As a first step, we analyzed the optical characteristics of C phycocyanin in different association states from single subunits to trimers without colourless polypeptides. The results indicate that in the C phycocyanin trimer both α and β subunits have s chromophores (sensitizing chromophore) [12,13]. The energy transfer occurs from these chromophores to the f chromophore (fluorescing chromophore) of the β subunit.

Materials and Methods

The isolation procedure for C phycocyanin and its subunits and the analytical methods used were essentially the same as described by Füglistaller et al. [14].

Algal culture. Mastigocladus laminosus Cohn (Genus *Fischerella* phycocyanin C 7603) were cultured in Castenholz medium D [15] under continuous illumination by fluorescent lamps. The growth temperature was 58°C. After two weeks culturing, cells were harvested and stored at −25°C until use.

Isolation of C phycocyanin and its subunits. Isolated phycobilisome were used as starting material to avoid contamination by intracellular proteins and proteins of the thylakoid membrane or by degradation products of phycobilisome. To obtain pure C phycocyanin without colourless polypeptides, the isolation procedure [14] was slightly modified. C phycocyanin$_{620}$ fractions from Cellex-D chromatography were dialyzed against 0.9 M potassium phosphate (pH 7.0) to obtain C phycocyanin$_{620}$ crystals. The crystals were used without any further purification. Separation of C phycocyanin into its subunits was done with Bio-Gel P-60 (= 400 mesh) chromatography in 63 mM formic acid. The fractions which contained α or β subunit were dialyzed against 0.9 M potassium phosphate (pH 7.0) for at least two days at 4°C. By this treatment, the contaminating subunits could be removed as a precipitate. The α or β subunits thus obtained were pure according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, without contamination by the other subunits or colourless polypeptides.

Reconstitution of C phycocyanin trimer. The isolated subunits were dialyzed separately against 63 mM formic acid and mixed in the same solution in a molar ratio 1 : 1. Then, the mixture was dialyzed against 5 mM potassium phosphate (pH 7.0). After that, the trimers formed were separated by a sucrose density gradient.

Formation of 'treated' trimers. The α subunits in 5 mM potassium phosphate (pH 7.0) were first dialyzed against 63 mM formic acid and the chromophores were bleached in the same solution with 5 mM sodium dithionite. The reduction was carried out at room temperature until the absorption in the red region was no longer detectable. The solution was aerated by stirring for 1 h. The β subunits in 63 mM formic acid were added in a molar ratio of 1 : 0.8, because the subunit which was bleached with sodium dithionite sometimes partly precipitated. The mixture was dialyzed against 10 mM potassium phosphate (pH 7.0) for more than 20 h. The trimers were separated by a sucrose density gradient.

Sucrose density gradient. A linear gradient from 5 to 18% sucrose in 5 mM potassium phosphate (pH 7.0) was used for the separation. The gradient was run at 25000 r.p.m. ($115000 \times g$) for at least 20 h. Catalase (molecular weight, 240000) was always used as an internal standard of the sedimentation pattern. The molecular weight of each band on the gradient was estimated by the equation described by Martin and Ames [16].

Control of association state. The monomer of C phycocyanin was obtained be the addition of $NaClO_4$ up to 1.0 M to the trimer of C phycocyanin. Under this condition C phycocyanin is in the monomeric state, which was verified by analytical ultracentrifugation with a Beckman L8-70, equipped with an ultraviolet-absorption detecting system.

Optical measurements. Absorption, CD and fluorescence spectra were measured with a Bausch & Lomb spectronic 200 ultraviolet spectrophotometer, a JASCO J-500 spectropolarimeter and an Aminco–Bowman spectrofluorometer, respectively, at room temperature (20°C). The sample concentration for fluorescence measurements was always adjusted to less than 0.06 absorbance at its maximum. The spectral sensitivity of the apparatus was not corrected. The quantum yields of fluorescence relative to that of the α subunit were estimated from the area under the fluorescence spectrum up to 750 nm, under the normalized condition of the same amount of light absorption. For the fluorescence polarization spectrum a Glan prism polarizer was used. The correction factor for polarized light [17] was estimated separately using a fluorescein block, a rhodamine B solution and C phycocyanin. The wavelength of each photometer was calibrated with the minimum transmittance of a dedimium filter (572.5 and 585.5 nm), which had been calibrated against the line spectrum of a D_2 lamp at 656.1 nm. The difference in wavelength of each photometer was less than 1 nm.

The extinction coefficient of C phycocyanin in various association states was always estimated by using the extinction coefficient of the C phycocyanin solution in 8 M urea (pH 2.0) [18,19].

Results

α Subunit of C phycocyanin

The α subunit of C phycocyanin from *M. laminosus* has only one chromophore [20]. The absorption maximum was located at 618 nm, with an extinction coefficient of $1.08 \cdot 10^5\ M^{-1} \cdot cm^{-1}$ (Fig. 1a). A clear shoulder was observed around 570 nm. The excitation polarization spectrum of fluorescence, monitored at 670 nm (Fig. 1a), showed an almost constant value between 510 and 650 nm, indicating that the absorbtion of this wavelength region originates from only one electronic transition. The oscillator strength estimated from the absorption spectrum from 470 to 700 nm was 0.902, suggesting a semicyclic configuration of the chromophore [21,22]. The CD maximum was at the same wavelength as the absorption maximum (618 nm) and the molar ellipticity was $3.29 \cdot 10^5$ deg cm^2/decimol (Fig. 1b). A small shoulder was also observed at 570 nm. Between 250 and 450 nm the CD spectrum showed a negative ellipticity, indicating that these bands come from different electronic transitions than that of the red band. A good correspondence was observed between the bands in the absorption and the CD spectra in the 300–700 nm region.

On excitation at 550 nm the fluorescence maximum was located at 638 nm (Fig. 1c), and the emission polarization spectrum showed an almost constant degree of polarization (0.4), as in the case of the excitation polarization spectrum. This value was close to the theoretical maximum, 0.5, which strongly suggests that α-C phycocyanin is not in an associated state under our experimental conditions.

β Subunit of C phycocyanin

The β subunit of C phycocyanin from *M. laminosus* has two chromophores [20]. The absorption maximum was located at 606 nm, with an extinction coefficient of $1.72 \cdot 10^5\ M^{-1} \cdot cm^{-1}$ (Fig. 2a). There was no clear shoulder on the short-wavelength side of the maximum. The excitation polarization spectrum (Fig. 2a) showed a stepwise increase toward longer wavelengths. The value reached 0.42 at wavelengths longer than 650 nm, suggesting that the β subunit is not in any higher association state under our experimental conditions. This stepwise increase indicates that the energy transfer occurs between two chromophores; from one having an absorption maximum in the shorter-wavelength region (hereafter, this

chromophore is called β_s chromophore, i.e., the sensitizing chromophore of the β subunit) to the other one, in the longer-wavelength region (β_f chromophore, the fluorescing chromophore of the β subunit). The β_s chromophore is expected to have an absorption maximum at a wavelength shorter than 600 nm, where the degree of polarization was already larger than the value below 590 nm. This finding agrees with the data of Scheer [23]. The oscillator strength from 470 to 700 nm was 1.769. This value is about twice that of the α subunit, suggesting no strong interaction between two chromophores.

In the CD spectrum (Fig. 2b), the maximum ellipticity was observed at 582 nm with a molar ellipticity of $3.60 \cdot 10^5$ deg cm^2/decimol. Around 625 nm another band was clearly observed. These two bands might correspond to the absorption maxima of the β_s and β_f chromophores, respectively. The distinct difference in ellipticity of the two bands suggests that the configurations of the two chromophores are very different. Between 250 and 450 nm some negative bands, corresponding to the absorption bands in this wavelength region were observed, as in the case of the α subunit.

The fluorescence spectrum (Fig. 2c), excited by 550 nm light, showed the maximum at 643 nm. The fluorescence polarization spectrum showed a stepwise decrease toward the longer-wavelength region. This indicates that the energy transfer from the β_s chromophore to the β_f chromophore is not complete, and that at the blue edge of fluorescence, a weak fluorescence from the β_s chromophore overlaps a strong fluorescence from the β_f

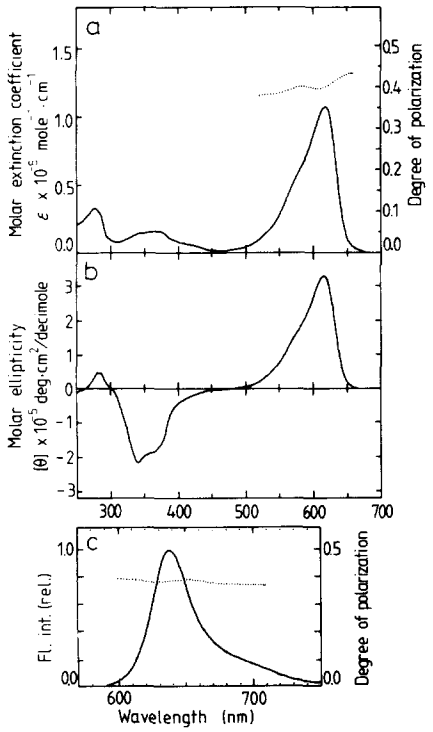

Fig. 1. Optical characteristics of α-C phycocyanin in 5 mM potassium phosphate (pH 7.0). (a) Absorption (———) and fluorescence excitation polarization spectra (· · · · · ·); (b) CD spectrum; (c) fluorescence emission (———) and emission polarization spectra (· · · · · ·). The slit width was 2 nm, 1 nm and 3 nm for the absorption, CD and fluorescence spectra, respectively. The excitation polarization spectrum was monitored at 670 nm (half-band width, 5.5 nm) and the fluorescence spectrum was obtained by the excitation at 550 nm (half-band width, 5.5 nm). For details, see text.

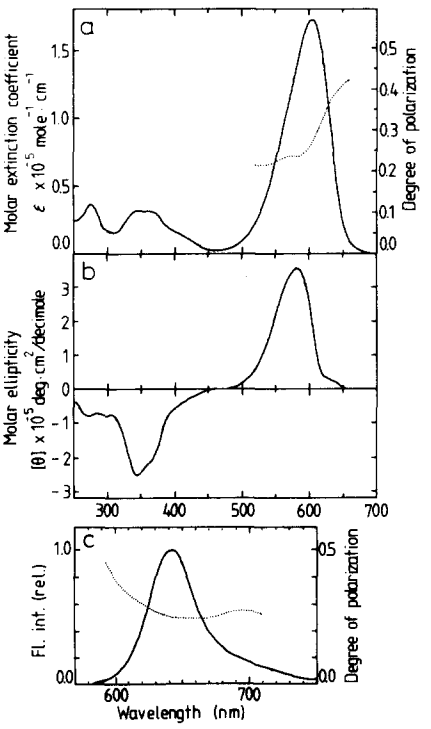

Fig. 2. Optical characteristics of β-C phycocyanin in 5 mM potassium phosphate (pH 7.0). (a) Absorption (———) and fluorescence excitation polarization spectra (· · · · · ·); (b) CD spectrum; (c) fluorescence (———) and emission polarization spectra (· · · · · ·). Measuring conditions were the same as in Fig. 1. For details, see text.

chromophore. The emission at 643 nm should originate from the β_f chromophore. Since the α chromophore show the absorption maximum at 618 nm and the corresponding fluorescence maximum at 638 nm (20 nm Stokes' shift), the absorption maximum of the β_f chromophore is expected to be located at a longer wavelength than 618 nm, assuming the same Stokes' shift. The 625 nm band observed as a shoulder in the CD spectrum probably corresponds to the absorption band of the β_f chromophore.

The quantum yield of fluorescence in the β subunit relative to that of the α subunit was about 0.65. The low yield is caused by the β_f chromophore. This finding agrees well with the faster decay constant of the β subunit than that of the α subunit isolated from the same organism as used in this experiment [24]. A low quantum yield of the β subunit is also found in the case of allophycocyanin [25].

The optical characteristics of isolated subunits were essentially the same as reported by Glazer et al. [26].

Separation of the absorption spectrum of the β subunit into the component spectra

The excitation polarization spectrum of the β subunits (Fig. 2a) clearly showed that the two chromophores have different absorption maxima. We therefore tried to resolve individual spectra by the polarization spectrum, as in the case of C phycoerythrin isolated from *Pseudoanabaena* W1173 (27]: where the mirror image of the fluorescence spectrum served as the absorption spectrum of the β_f chromophore. However, as shown by the emission polarization spectrum (Fig. 2c), the fluorescence spectrum of the β subunit is a mixture of the β_s and the β_f chromophore. Thus, we tried to resolve the components by a different method.

High concentrations of sucrose induce an increase in the absorbance of allophycocyanin [28]. The changes are assumed to be the modification of the tertiary structure of the proteins. We applied this method to the β subunit. Fig. 3a showed the difference absorption spectrum of the β subunit induced by the addition of sucrose (1.5 M). The difference maximum was located at 624 nm due to about 10% increase in absorbance. The excitation polarization spectrum and the CD spectrum were

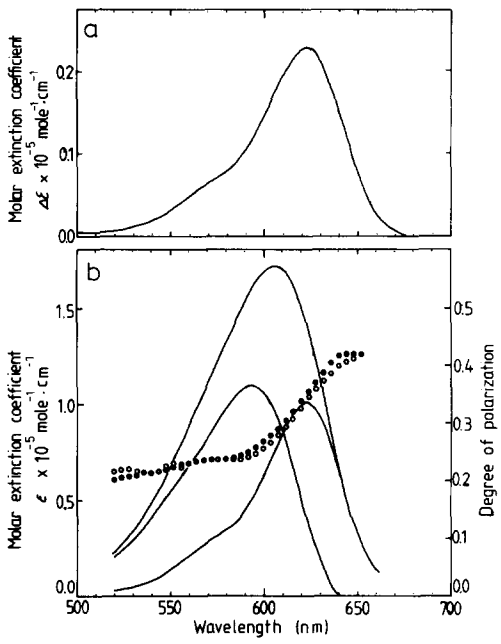

Fig. 3. Separation of the absorption spectrum of β-C phycocyanin into component spectra. (a) Difference absorption spectrum obtained by the addition of sucrose (1.5 M); (b) component spectra obtained by computer simulation of anisotropy. The spectrum with the maximum at shorter wavelength was the component spectrum of the β_s chromophore and at the longer wavelength, of the β_f chromophore. Open circles mean the observed degree of polarization and closed circles, simulated values. For details, see text.

not substantially changed by the addition of sucrose. Judged from the fluorescence polarization spectrum, there is little possibility of changes in the association state, which would be expected to affect the degree of polarization at the red edge of the absorption spectrum. Thus we consider the resulting difference spectrum to be induced by the modification of the tertiary structure of the protein. The location of the difference maximum was very close to that of the small band in the CD spectrum (Fig. 2b), and it is to be expected that, due to the small magnitude of the band, similar changes in the CD spectrum corresponding to the increase in absorbance should not be observed. Thus, we adopted this difference spectrum as the absorption spectrum of the β_f chromophore in the β subunit.

The sum rule of anisotropy is theoretically established [29]. We tried to separate the absorp-

tion spectrum of the β subunit into component spectra by a computer simulation based on a fitting of anisotropy. For this purpose, the observed degree of polarization was numerically converted to the anisotropy. Fig. 3b showed the absorption spectra of individual chromophores obtained. The fitting of the degree of polarization agreed reasonably well with the original data. The absorption maximum of the β_s chromophore was located at 594 nm, with a faint shoulder around 550 nm. On the other hand the β_f chromophore has its maximum at 624 nm, with a clear shoulder around 565 nm. The intensity of the shoulder relative to peak height is small in the β_f chromophore. The estimated locations of maxima were at slightly longer wavelengths than those reported for the β_s and β_f chromophores at 590 and 620 nm, respectively [29]. The oscillator strengths, calculated from each component spectrum, were 1.024 and 0.745 for the β_s and the β_f chromophore, respectively, and the mutual orientation of the dipole moments was 43°. A significant difference in oscillator strength between the two chromophores may be the reflection of the difference in configuration of the chromophores, which is clearly shown in the CD spectrum.

From this separation, the energy levels of each chromophore in the α and β subunits became clear: 618, 594 and 625 nm for α, β_s and β_f chromophores, respectively.

Monomer of C phycocyanin

The C phycocyanin monomer has three chromophores (α, β_s and β_f chromophores). The conversion to the monomeric form is only achieved by the addition of chaotropic reagents in high concentration (in this experiment, 1.0 M of $NaClO_4$). Under our experimental condition only one molecular species was found by analytical ultracentrifuge. The molecular weight of this species was 25 500, assuming the partial specific volume to be 0.731 [14].

The absorption maximum of the monomer was located at 611 nm with an extinction coefficient of $2.63 \cdot 10^5\ M^{-1} \cdot cm^{-1}$ (Fig. 4a). No shoulder was evident in the shorter-wavelength region. The oscillator strength estimated from 470 to 700 nm, was 2.511. This value is very close to the summed value of both subunits (2.671). The CD spectrum (Fig. 4b) showed a broad maximum at 596 nm and a small, but clear shoulder around 625 nm. A large negative band was clearly observed in the wavelength region from 250 to 450 nm, with a minimum at 342 nm. The maximum molar ellipticity was $6.19 \cdot 10^5$ deg cm^2/decimol.

Glazer et al. [26] have already reported that the absorption spectrum of monomer C phycocyanin can be recognized as the sum of the spectra of the

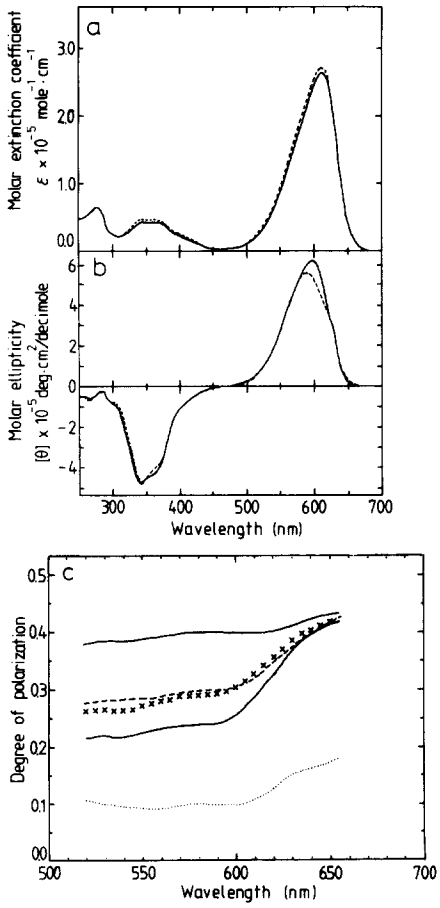

Fig. 4. Optical characteristics of the C phycocyanin monomer in 5 mM potassium phosphate (pH 7.0). To obtain the monomeric state, $NaClO_4$ (final concentration, 1.0 M) was added. In (a) (absorption spectra) and (b) (CD spectra), the spectra of monomer (———) and the summed spectra of both subunits (- - - - - -). In (c), the fluorescence excitation polarization spectra of the two subunits (———) (the upper for the α subunit and the lower for the β subunit), the monomer (- - - - - -) and the trimer (· · · · · ·). Crosses indicate the spectrum of the monomer calculated using the subunit spectra. Measuring conditions were the same as in Fig. 1. For details, see text.

α and the β subunit. This is also the case in *M. laminosus* C phycocyanin. The sum of the spectra of both subunits showed the maximum at 611 nm, the same wavelength as that of the monomer. However, the extinction coefficient of the summed spectrum was a little larger than that of the monomer in the wavelength region from 500 to 620 nm (Fig. 4a). The difference exceeded the experimental error (2.5% for the absorption spectrum). A small difference was also observed in the CD spectrum (Fig. 4b). The summed spectrum showed the CD maximum at 590 nm, with a shoulder around 625 nm. The magnitude of the two spectra is comparable, but the spectra were different. Generally, the CD spectrum is a more sensitive index which reflects the configuration and the environment of the chromophore. The above-mentioned difference thus indicates that monomer formation brings about a small modification of protein structure of each component subunit. However, the energy levels of each chromophore seemed to be identical: the absorption maximum of the monomer was identical with that of the summed spectrum of both subunits, and the band location of the β-subunit in the CD spectrum was very close to that of the shoulder in the monomer. On this basis, the conformational changes associated with the monomer formation do not seem to be large.

The fluorescence maximum was located at 642 nm (data not shown), and the quantum yield relative to the α subunit was 0.78. Both values were intermediate to those of the two subunits.

A striking feature was observed in the excitation polarization spectrum (Fig. 4c). The degree of polarization of the monomer was always higher than that of the β subunit, and reached the same value at the red end of the spectrum. This strongly suggests that, in the monomeric state, no energy transfer process other than that in each isolated subunit occurs. The monomer is also the sum of both subunits, even in the sense of energy transfer. This was confirmed by the simulation of the polarization spectrum with the assumption that the absorption spectrum of the monomer is the sum of those of each subunit.

The calculated excitation polarization spectrum agreed well with the observed spectrum (Fig. 4c). Furthermore, the fluorescence maximum and the quantum yield of the monomer were very close to the values estimated from each subunit. The expected values were 641 nm for the fluorescence maximum and 0.74 for the quantum yield. These findings clearly indicate that the energy transfer in the monomer is only from the β_s to the β_f chromophore, as in the single β subunit. The energy levels of each chromophore is identical to those of the subunits. The lack of energy flow from the β_s to the α chromophore and/or from the α to the β_f chromophore may be due to the long distance and/or the unsuitable orientations of these chromophores.

Trimer of C phycocyanin

The C phycocyanin trimer has nine chromophores (three α, three β_s and three β_f chromophores). The absorption maximum was shifted to 615 nm, and a clear shoulder was observed around 570 nm (Fig. 5a). The extinction coefficient was $8.83 \cdot 10^5\ M^{-1} \cdot cm^{-1}$ and the oscillator strength between 470 and 700 nm was 8.158. Both values are more than 3 times as high as those of the monomer. The difference spectrum due to trimer formation showed its maximum at 632 nm, suggesting the appearance of a new absorption band (Fig. 5a).

The CD spectrum of the trimer (Fig. 5b) showed two clear maxima at 598 and 633 nm, neither of which corresponds to the apparent absorption maximum. The latter band, however, agreed well with the band in the difference absorption spectrum. The difference CD spectrum caused by trimer formation showed two maxima at 634 and 598 nm, both of which agreed with the CD maxima. The 632 nm band, which was not observed in the monomer, was evident both in absorption and CD spectra. Since the negative band was observed in the difference absorption spectrum (Fig. 5a), the 632 nm band seems to be due to a shift of the component(s) rather than the formation of a new absorption band. There are two possibilities to explain such spectral changes: chromophore–protein interactions or chromophore–chromophore interactions. In the former case one can assume that the protein conformation is changed by the trimer formation, and in the latter case, some of the chromophores should interact more closely upon trimer formation (see Discussion).

On excitation with 550 nm light, the fluo-

rescence maximum was observed at 643 nm (Fig. 5c) and the quantum yield relative to the α subunit was 0.93. The maximum was the same as that of the β subunit, but the bandwidth was less. The emission polarization spectrum (Fig. 5c) showed a monotonous decrease toward the longer-wavelength region and reached a constant low value (0.15) at wavelengths longer than the fluorescence maximum. This suggests that a new component was not present, which corresponds to an absorption band and thus is expected to be located at a wavelength longer than the maximum of that of the β subunit. The 643 nm fluorescence thus most probably comes from the energy sink of the trimeric state. The smaller degree of polarization clearly indicates the energy transfer to the energy sink.

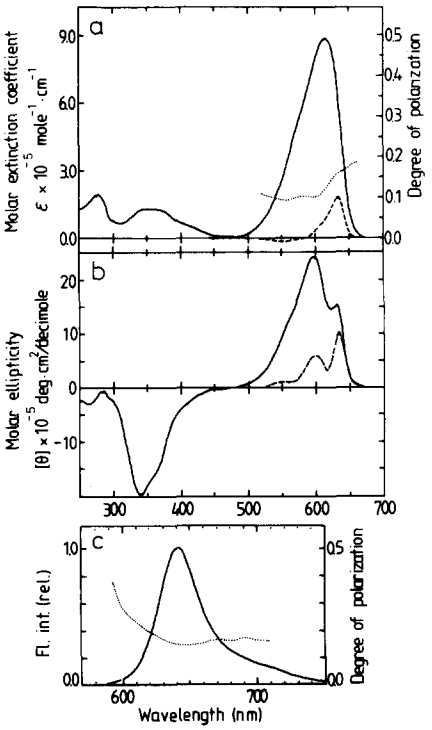

Fig. 5. Optical characteristics of the C phycocyanin trimer in 5 mM potassium phosphate (pH 7.0). measuring conditions were the same as in Fig. 1. In (a), absorption (———), fluorescence excitation polarization (· · · · · ·) and the difference spectra (- - - - - -), and (b), CD spectrum (———) and the difference CD spectrum (- - - - - -). The difference spectra due to the trimer formation were obtained by the subtraction of three times the monomer spectra from the trimer spectra. In (c), fluorescence (———) and emission polarization (· · · · · ·) spectra. For details, see text.

The occurrence of energy transfer was confirmed by the excitation polarization spectrum monitored at 670 nm (Fig. 5a). The degree of polarization of the C phycocyanin trimer is low (0.10) from 520 to 600 nm and increased to 0.18 at 650 nm. This spectrum showed the occurrence of energy transfer, which did not occur in the monomer, that is, the energy transfer from the α to the β_f chromophore and/or from the β_s to the α chromophore. The low degree of polarization at wavelengths longer than 650 nm clearly indicates that the final energy sink is one chromophore. If multiple chromophores (α and β_f chromophores) could act as the energy sink, a higher degree of polarization (about 0.4) is expected in the wavelength region where only the f chromophores absorb light, similar to the case of the β subunit and the monomer of C phycocyanin. Thus, the possible energy flow is from the α to the β_f chromophore and/or from the β_s to the β_f chromophore via the α chromophore. In the latter case the α-chromophore is expected to be located in a different monomer, because the energy transfer from the β_s to the α chromophore was not observed in the monomeric state (cf. Fig. 4c). The new pathway of energy transfer might be achieved by the close location of chromophore(s) of different monomers within the trimers, if a drastic conformational change does not accompany trimer formation.

Discussion

The three-dimensional structure of the C-phycocyanin trimer without colourless polypeptides in *M. laminosus* has already been determined by X-ray analysis with a resolution of 0.3 nm [31,32]. Each monomer, which has a crescent-like structure, and is oriented with a C_3 symmetry arrangement around a central axis (cyclic trimer with a central hole) (Fig. 7). The α chromophore in position 84 of the amino acid sequence [20] is located at the edge of each monomer outside of the cyclic trimer. In the β subunit, the chromophore in position 84 lies at the edge opposite to the α chromophore, but inside the cyclic trimer. The distance between these chromophores is greater than 7 nm. The chromophore in position

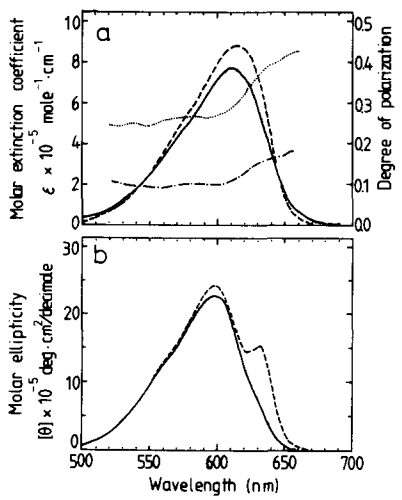

Fig. 6. Optical characteristics of treated trimer in 5 mM potassium phosphate (pH 7.0). (a) Absorption (———) and fluorescence excitation polarization (·····) spectra of treated trimer and absorption (- - - - - -) and fluorescence excitation polarization (·–·–·) spectra of the corresponding native trimer. In (b), CD spectrum of treated trimer (———) and of the native trimer (- - - - - -). Measuring conditions were the same as in Fig. 1. For details, see text.

155 of the β subunit lies at the outside of the cyclic trimer, but closer to the β-(84) chromophore (4 nm) than to the α-(84) chromophore (5.2 nm). Within the trimer the α and the β-(84) chromophore, although each in different monomers, exist with a center-to-center distance of 2.2 nm. The distance between the β-(155) chromophore in one monomer and the α chromophore in an adjacent monomer is about 4 nm, the same as that between the β-(84) and the β-(155) chromophore in the same monomer.

The energy transfer between these chromophores depends on the association state of the proteins. In the monomer, the transfer occurs only from the β_s chromophore to the β_f chromophore (center-to-center distance: 4 nm). In the trimer, an additional pathway was observed from the α to the β_f chromophore between adjacent monomers (2.2 nm and/or from the β_s to the β_f chromophore in the same monomer via the α chromophore in an adjacent monomer. When we compare the occurrence of the energy flow with the 3-dimensional structure, it is possible to assign three types of chromophore with the following roles in the energy transfer: the β-(84) chromophore as β_f chromophore, and the β-(155) chromophore as β_s chromophore. The energy flow in the trimer would be from the β_s chromophore to the β_f chromophore in the same monomer, and from the α chromophore in one monomer to the β_f chromophore in an adjacent monomer. Other pathways do not seem feasible, based on the results men-

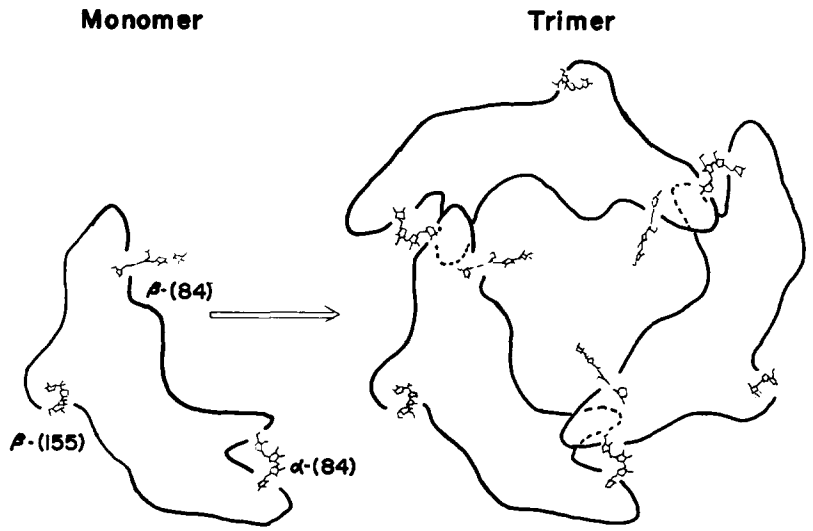

Fig. 7. Schematic arrangement of individual chromophores in the monomer (left) and the trimer (right) according to the Fig. 11 in Ref. 32. α-(84), β-(84) and β(155) mean the chromophores which are covalently bound to the cysteine residue in position 84 or 155 of α or β subunit, respectively. For details, see Discussion.

tioned below. Since the β_f chromophore is located in the inner part of the trimer, the energy transfer to the β_f chromophore in C phycocyanin trimer is likely from the outside to the inside. If a colorless polypeptide binds the inner side of the trimer and modifies the optical characteristics of the energy sink [2], then the transfer from the β_f chromophore would occur mainly between the β_f chromophores in different trimers or hexamers to the allophycocyanin core. Glazer et al. [26] had assigned this to the α-(84) chromophore as an f chromophore, whereas we assigned this to the β-(84) chromophore. Our assignment indicates the uniformity of the functional differentiation of the two subunits, that is, the β subunit has an f chromophore. This assignment has also been shown for phycoerythrocyanin [33,34] and allophycocyanin [25].

The one ambiguous pathway in the energy transfer of the trimer, i.e., from the β_s chromophore in one monomer to the α chromophore in an adjacent monomer, had been further considered below by checking the possible energy flow from the β to the α chromophore between adjacent monomers in the reconstitution experiments.

It has already been shown that C phycocyanin trimer can be reconstituted from subunits [18]. If the trimer is reconstituted using a subunit whose chromophore has a different energy level than the native one, then we can expect the energy flow in the trimer to be altered. The energy level of the chromophore was changed by using sodium dithionite to reduce the α-(84) chromophore [35,36]. Removal of this reagent results in restoration of the native electronic state. However, the trimer thus formed showed different optical characteristics from those of the native trimers, as shown below.

The C phycocyanin trimer which was formed after the treatment with sodium dithionite (see Materials and Methods), hereafter called 'treated' trimer, showed the same sedimentation pattern in the ultracentrifuge as the native C phycocyanin trimer. Both subunits were present in a nearly equal molar ratio, judged from the staining intensity on sodium dodecylsulfate-polyacrylamide gel electrophoresis (data not shown). The absorption maximum of the 'treated' trimer was located at 611 nm, which was the same as that of the monomer. A small change in the spectrum was observed only in the shorter wavelength region of the maximum (Fig. 6a vs. 4a). The absorbance ratio of its maximum (611 nm) to that at 278 nm was 3.99, which is comparable to the monomer (4.06). This indicates that the chromophore is restored completely with respect to the extinction coefficient by the removal of sodium dithionite. The 598 nm CD maximum had a clear shoulder at 555 nm (Fig. 6b), and the 633 nm band, present in the trimer, was not observed. There was only a red-shift of the band of the shortest wavelength component due to trimer formation. This component was independent of the treatment, thus it most probably comes from the β_s chromophore which showed the maximum at 594 nm in the monomer. The α and the β_f chromophore in the treated trimer seems to keep almost the same energy levels as in the monomer.

The excitation polarization spectrum of the treated trimer was almost the same as that of the monomer (Fig. 6a vs. 4c). This clearly indicates that the energy flow in the treated trimer is the same as in the monomer, and that the energy transfer between adjacent monomers does not occur. Furthermore, reconstitution of *C*-phycocyanin trimer, without chemical treatment, showed the same characteristics as those of the native trimer (data not shown). These results suggest that the energy transfer from the β_s chromophore to the α chromophore between adjacent monomers is not feasible even in the native trimer. The spatial arrangement of the chromophores in the treated trimer is expected to be similar to the native trimer. Therefore, the energy transfer in the native trimer is limited to only two pathways; one is from the α chromophore in one monomer to the β_f chromophore in an adjacent monomer, and the other pathway is from the β_s to the β_f chromophores in the same monomer. The energy once transferred to the inside of the trimer might be transferred to β_f chromophore in a different trimer more proximal to the center of the phycobilisome, and finally reach the allophycocyanin core. This transfer process results in the minimum number of transfer steps to the allophycocyanin core and most probably corresponds to the 'special channel' of the energy transfer in the phycocyanin rod, which is expected from the analysis of intact cells [6,7,11] and isolated phycobilisome [10].

The reconstitution experiment indicates the nature of the coupling between chromophores. The 632 nm band in the CD spectrum and in the difference absorption and CD spectra is evidently due to the trimer formation (Fig. 5a and 5b). Trimer formation brings about an increase in the extinction coefficient (12%) and the oscillator strength (8%). This increase is probably caused by changes either in the chromophore–protein interaction or in the chromophore–chromophore interaction. The spatial location of the α and the β_f chromophore in the trimer is close enough for direct coupling (2.2 nm).

The optical characteristics of the treated trimer is different from those of the native trimer in two points; one is the disappearance of the 632 nm band (Fig. 6a) and the other the lack of the energy transfer from the α to the β_f chromophore, as shown by the excitation polarization spectrum (Fig. 6b). The energy levels of the α and the β_f chromophore in the treated trimer were equal to those of the monomer. These results clearly indicate that the 632 nm band is formed by the direct interaction between the α and the β_f chromophore. It appears that in the treated trimer the polypeptide structure between two chromophores is changed such that the direct interaction can no longer occur (Mimuro, M. et al., unpublished results). The CD spectrum of the native trimer did not show any negative band in the red region. An intermediate coupling is the most probable, as in the case of allophycocyanin [37,38].

The β_f chromophore is a common acceptor of energy from the α and β_s chromophores. The interaction between the α and the β_f chromophore is stronger than that between β_s and the β_f chromophore. This suggests that in the analysis of fluorescence decay kinetics in the picosecond time region, we can expect at least three components corresponding to each chromophore. The transfer time of 10 ps [39] or 20 ps [10] may correspond to the transfer from the α to the β_f chromophore. However, the other decay components are not necessarily clearly assigned. The β-(155) chromophore is relatively isolated from the other two chromophores. Therefore, the decay time of the β-(155) chromophore is expected to be rather long. This indicates the possibility that the β-(155) chromophore shows characteristics like an f chromophore. The importance of this chromophore is suggested in the hexameric condition [32]. Thus, the functional assignment in the hexamer is also necessary to understand the energy flow in the phycobilisome.

The vibrational structure of absorption bands varies depending on chromophores in C phycocyanin. On the β_s chromophore, the energy difference between S_{10} and S_{11} was estimated to be 1300 cm^{-1} from the component spectrum in a single subunit. This difference was maintained in the trimer, judged from the CD spectrum (598 and 555 nm, 1300 cm^{-1}), and which was also the case in the α subunit in the monomer (1350 cm^{-1}). Compared with these values, the energy difference in the β_f chromophore in the monomer was about 1650 cm^{-1}. This large energy difference was also observed in the f chromophore of both subunits of C phycoerythrin [26]. The difference in the vibrational level reflects the difference in the nature of the bond of nitrogen atoms in the pyrrole rings, This might be the reason for the differentiation of phycocyanobilin into s and f chromophore in C phycocyanin.

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