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The microenvironment around the chromophores and its changes due to the association states in C-phycocyanin isolated from the cyanobacterium *Mastigocladus laminosus*

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The chromophore–protein interaction in C-phycocyanin was investigated as a function of the association state of the protein. Changes in the microenvironment around the chromophores were monitored by the following three indices: (1) the accessibility of a small molecule to the chromophore; (2) the fluorescence from aromatic amino acid residues; and (3) the effect of configurational changes of the chromophore on the conformation of the polypeptide chain. In the C-phycocyanin trimer, all the chromophores are shielded from the aqueous phase, probably by contact between subunits, and by a loop structure which surrounds the chromophores, even though that loop structure is not shown by X-ray analysis (Schirmer, T., Bode, W., Huber, R., Sidler, W. and Zuber, H. (1985) J. Mol. Biol. 184, 257–277). The polypeptide folding depends on the electronic structure of the chromophores; the oxidized chromophore of the α subunit inhibits the formation of the trimer and the reduced state of the chromophore of the α subunit allows the formation of trimers, in which the chromophores have the same electronic structure as in the monomers. The fluorescence from the aromatic amino acid residues showed that the conformational changes were induced by the reduced chromophore. These results indicate that the chromophore structure and the protein conformation affect each other. A definite configuration of the chromophore and also a definite conformation of the polypeptide are necessary for the intact energy transfer within C-phycocyanin.

Introduction

C-phycocyanin, one of the algal biliproteins, consists of two distinct subunits, α and β. The former contains one, and the latter two chromophores (phycocyanobilin), which are covalently bound to cysteine residues of the apoprotein through thio-ether linkages [1,2]. Monomeric C-phycocyanin is made up of one α and one β subunit, but this association state is not observed under physiological conditions; the trimer is known as the stable and the minimum functional unit [2].

The three-dimensional structure of the C-phycocyanin trimer without a linker polypeptide has been determined by X-ray analysis at 3 Å resolution [3]. Each monomer is arranged in C_3 symmetry around a central axis. In a crescent-like monomer, the α chromophore and the β (84) chromophore (i.e., the chromophore at position 84 in the amino acid sequence of the β subunit) are

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Abbreviation: s and f chromophore, sensitizing and fluorescing chromophore, respectively.

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located at the individual edges, and the β(155) chromophore is located on the outside of the monomer, about 40 Å from the β(84) chromophore. In the trimer, the α chromophore in one monomer and the β(84) chromophore in an adjacent monomer are in close contact, with the α chromophore outside and the β(84) chromophore inside the trimer ring.

The three types of chromophore (α, β(84) and β(155) chromophores) have different energy levels, which cause the functional differentiation in the energy-transfer sequence. Recently, we have assigned the following functions to these three chromophores [4]; the α and the β(155) chromophores are the sensitizing (s) chromo-phores (the latter chromophore is called β_s chromophore) [5,6] and the β(84) chromophore is the fluorescing (f) chromophore (β_f chromophore). The energy transfer occurs from the s chromophores to the f chromophore, or in terms of the spatial arrangement, from the outside to the inside of the trimer ring. Furthermore, we reported that the direct interaction between the α and β_f chromophores in adjacent monomers is formed in the trimer, whose center-to-center distance is about 22 Å [3]. The fast decay component in C-phycocyanin (10 ps [7] or 20 ps [8]) can be attributed to the decay of the α chromophore [4].

However, the direct interaction between these chromophores is not necessarily generated even when C-phycocyanin is in the trimeric state. This was shown by reconstitution of the C-phycocyanin trimer from the component α and β subunits, using the α subunit whose chromophores were reduced by sodium dithionite [4]. Trimer with unusual spectral properties were obtained. After removal of sodium dithionite by dialysis, the α subunit in the trimers restored its blue color, but the trimer showed almost the same absorption, circular dichroism (CD) and excitation polarization spectra as those of the monomer. This clearly indicates that the direct interaction between α and β_f chromophores does not occur in this trimer. Therefore, a close proximity of the chromophores is not necessarily enough for complete energy transfer; other structural features are required in this process. The microenvironment around the chromophores might play an important role in the native trimer. To clarify this, we carried out the following three experiments on different association states of the proteins: (1) the accessibility of a small molecule to the chromophore as a probe of the state of the polypeptide folding around the chromophores; (2) the fluorescence from the aromatic amino acid residues as a reflection of the spatial arrangement between the chromophores and the polypeptides; and (3) the effect of chemically modified chromophores on the folding of the polypeptide and on the association states of C-phycocyanin. The results indicate that the chromophore configuration and the polypeptide structure affect each other to give the most favourable structure for efficient energy transfer.

Materials and Methods

Isolation of C-phycocyanin and separation of its subunits. Cultivation of *Mastigocladus laminosus* cells and the isolation of phycobilisomes were carried out according to Füglistaller et al. [9].

C-phycocyanin was isolated from dissociated phycobilisomes by ion-exchange column and purified by crystallization. Phycobilisomes in 0.75 M potassium phosphate (pH 7.0) were dissociated by dialysis against distilled water and were applied to a Cellex-D (Bio-Rad, Richmond, CA) column. The column was developed with a linear gradient of potassium phosphate (10–200 mM, pH 7.0). CPC_{620} fraction (cf. Ref. 10), which was eluated by approx. 120 mM potassium phosphate was pooled, and dialyzed against 0.75 M potassium phosphate (pH 7.0) to get trimer crystals. Separation of C-phycocyanin into subunits was carried out by gel filtration. Crystallized C-phycocyanin was dialyzed against distilled water and formic acid was added (final concentration, 63 mM). The sample was applied to a Bio-Gel P-60 (up to 400 mesh) (Bio-Rad, Richmond, CA) column, and was developed by 63 mM formic acid. The dissolving medium was changed from 63 mM formic acid to 5 mM potassium phosphate (pH 7.0) by dialysis against about 1000-fold volume of solvent. The purity of each sample was determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis.

Control of various association states. The trimeric C-phycocyanin was obtained by dissolving crystallized C-phycocyanin into 5 mM potassium

phosphate (pH 7.0). Under this condition, only a trimer was found by sucrose density gradient. The monomer was obtained by adding $NaClO_4$ (final concentration, 1 M) to the trimer. Analytical ultracentrifugation proved the existence of only one component (cf. Ref. 4). Isolated α and β subunits are not in a higher association state, i.e., dimer, in 5 mM potassium phosphate (pH 7.0) (cf. Ref. 4).

Reduction of the α and β chromophores by sodium dithionite. The chromophore was reduced by adding solid sodium dithionite to the sample solution (10 μM of subunits) (final concentration of sodium dithionite, 10 or 50 mM) in the cuvette. The cuvette was immediately sealed with teflon and parafilm, placed in the photometer, and spectra were measured at various times.

Reconstitution of C-phycocyanin trimers. For the reconstitution of C-phycocyanin trimers, freshly separated subunits in 63 mM formic acid were used. The two subunits were mixed in the same solvent. After mixing, the formic acid was replaced with 5 mM potassium phosphate (pH 7.0) by dialysis for more than 20 h at 4°C.

Reconstitution experiments were carried out under four different conditions. (1) Reconstitution without any chemical treatment: both subunits, each in 63 mM formic acid were mixed in a molar ratio of 1 : 1. (2) Reconstitution after oxidation of the α chromophore with potassium ferricyanide: the oxidation was carried out in 63 mM formic acid with potassium ferricyanide (50 μM) for 2 h at room temperature. After the treatment, the reagent was removed by dialysis against 63 mM formic acid for 15 h at 4°C. The β subunit was added to the oxidized α-subunit in a molar ratio of 1 : 1. (3) Reconstitution after reduction of the α chromophore with sodium dithionite: the α subunit in 63 mM formic acid was reduced with sodium dithionite (5 mM) for 1 h at room temperature. After this treatment, sodium dithionite was removed by dialysis against 63 mM formic acid for 15 h at 4°C. The β subunit was added in a molar ratio of 1 : 1. (4) Reconstitution after reduction and partial removal of sodium dithionite: the α subunit in 63 mM formic acid was reduced with sodium dithionite (5 mM) for 1 h at room temperature in a cuvette with a teflon stop. After opening the stop, the solution was vigorously airated by stiring for 1 h at room temperature to reduce the concentration of sodium dithionite. The β subunit was added in a molar ratio of 1 : 1.

The mixture of the two subunits was applied to a sucrose density gradient (5–18% linear gradient), to separate the complexes of different association states, and centrifuged at $115000 \times g$ for at least 20 h at 15°C. Catalase (molecular weight, 240000) was used as an internal standard of the sedimentation. The molecular weight of the sample on the gradient was estimated using the equation of Martin and Ames [11].

Spectroscopic analysis. Absorption, CD and fluorescence spectra were measured with a Bausch & Lomb spectronic 200-UV spectrophotometer, a JASCO J-500 spectropolarimeter and an Aminco-Bowmen spectrofluorometer, respectively. Measurements were carried out at room temperature (22°C) (cf. Ref. 4).

Results

Accessibility of a small molecule to the chromophores

We determined the accessibility of a small molecule to the chromophores by using sodium dithionite to reduce the chromophores. This process can be monitored by spectroscopic analysis.

In 63 mM formic acid (pH 2.5) both α and β subunits were reduced by 5 mM sodium dithionite within 15 min to give a yellow-colored pigment with an absorption maximum at 414 nm (cf. Fig. 4-2). A similar degree of reduction was observed even in 2 mM sodium dithionite. This indicates that the chromophores are exposed to the aqueous phase, probably due to the partial unfolding of the polypeptide in 63 mM formic acid. When each subunit was dissolved in 5 mM potassium phosphate (pH 7.0), the reactivity of the chromophores was drastically reduced. Fig. 1A shows the time-course of chromophore bleaching by 10 mM sodium dithionite. Of the two subunits, the β subunit was more susceptible to the reductant. The monomer of C-phycocyanin showed a time-course of bleaching between those of the two single subunits. The trimer seemed to be insensitive to the reductant, except for an initial small decrease in the absorption spectrum.

The difference absorption spectra between the native and the reduced samples allows a more

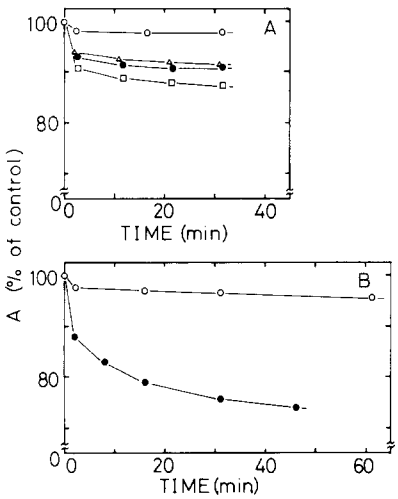

Fig. 1. Time-course of chromophore bleaching in various association states of C-phycocyanin. Samples were dissolved in 5 mM potassium phosphate (pH 7.0) and treated with 10 mM (A) or 50 mM (B) sodium dithionite. Absorbance was monitored at the maximum of the respective association state (618 nm for the α subunit, 606 nm for the β subunit, 611 nm for monomer and 615 nm for trimer). △, α subunits; □, β subunit; ●, monomer and ○, trimer. For details, see text.

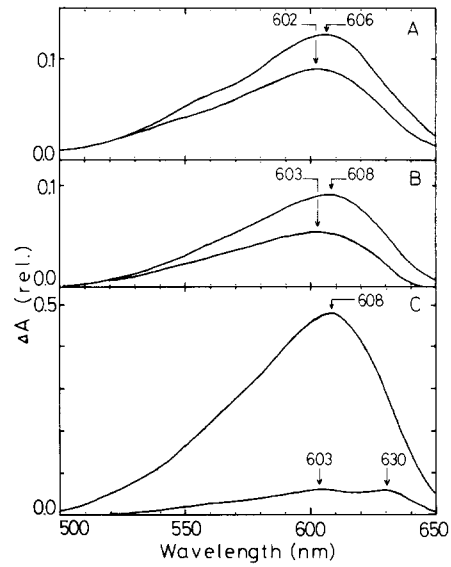

Fig. 2. Absorption difference spectra of C-phycocyanin induced by the addition of sodium dithionite in various association states. Difference spectra (control-minus-treated sample) were expressed after the normalization of the maximum absorbance of the control to 1.0. (A) Difference spectra of the β subunit. Lower, spectrum 70 s after the addition of sodium dithionite (10 mM), and upper, after 30 min. (B) Difference spectra of the monomer. Lower, the spectrum 70 s after the addition of sodium dithionite (10 mM), and upper, that after 30 min. (C) Difference spectra of monomer (upper) and the trimer (lower) 15 h after the addition of sodium dithionite (50 mM). For details, see text.

detailed analysis. The difference spectrum of the α subunit was identical with the absorption spectrum (data not shown). The difference maximum of the β subunit was located at 602 nm just after the addition of sodium dithionite; it shifted to a longer wavelength with time, and reached the same wavelength as that of the absorption maximum (606 nm) after 30 min (Fig. 2A). This indicates that the component with the shorter wavelength maximum (β_s chromophore) is more susceptible to the reductant. This feature was also observed in the monomer (Fig. 2B). The difference maximum at 603 nm (70 s after the addition) shifted to 608

TABLE I

OPTICAL CHARACTERISTICS OF C-PHYCOCYANIN IN VARIOUS ASSOCIATION STATES

The values for FL_{max} are uncorrected to the spectral sensitivity of the apparatus. All data are cited from Ref. 4. For the spectra, see Fig. 4.

	α subunit	β subunit	Monomer	Trimer
Absolute (apparent) maximum (nm)	618	606	611	615
of α chromophore	618	–	618	
of β_s chromophore	–	594	594	598
of β_f chromophore	–	624	624	632
CD_{max} (nm)	618	582	596	598
		625 [a]	625 [a]	633
FL_{max} (nm)	638	643	642	643
Energy transfer	none	$\beta_s \rightarrow \beta_f$	$\beta_s \rightarrow \beta_f$	$\beta_s \rightarrow \beta_f$
				$\alpha \rightarrow \beta_f$

[a] Shoulder

nm (30 min), whereas the absorption maximum was located at 611 nm. In the monomer, α, β_s and β_f chromophores have absorption maxima at 618, 594 and 624 nm, respectively (Table I). Therefore, in the monomer, the β_s chromophore is the most susceptible of the three chromophores, as in the case of the single β subunits. However, the difference spectrum was not identical with the absorption spectrum of the β_s chromophore. Any combination of two out of the three component spectra did not agree with the observed spectrum. Thus, it is concluded that in the monomer, all three chromophores are reduced, but their susceptibilities are not the same.

The low concentration of sodium dithionite (10 mM) did not induce substantial changes in the absorption spectrum of the trimer, so we examined the effect of a higher concentration (50 mM) of sodium dithionite (Fig. 1B). The reactivity of the trimer with reductant was much less than that of the monomer even in this concentration. The absorbance decreased by about 6% after 15 h incubation, whereas in the monomer absorbance decreased by about 50%. The difference spectrum of the trimer showed two maxima around 603 and 630 nm (Fig. 2C). The former is close to the absorption maximum of the β_s chromophore in the trimer (598 nm), and the latter to that of the β_f chromophore in the trimer (632 nm) (Table I). However, the band corresponding to the α chromophore (around 620 nm) was not observed. These results indicate that the two chromophores in the β subunit are a little sensitive to the reductant, but the α chromophore does not react with the reductant. The difference spectrum of the trimer is very different from that of the monomer (Fig. 2C). If the chromophores in the trimeric C-phycocyanin are reduced after dissociating to the monomer due to the shift of the equilibrium, we would expect the same difference spectrum as in the monomer. The reduction of chromophores in the trimer, therefore, occurred while in the trimeric form. This result indicates that trimer formation drastically reduces the reactivity of all chromophores.

The low reactivity of the chromophores with a small molecule in the trimer indicates the low accessibility of the small molecule to the chromophores. The main reason for the low accessibility seems to be steric hindrance by the polypeptide chain. There are two processes which drastically affect the accessibility; one which occurs at the subunit level is the conformational changes of the polypeptide chain due to the replacement of the solvent of 63 mM formic acid (pH 2.5) by 5 mM potassium phosphate (pH 7.0), and the other is the changes in the polypeptide conformation which accompany the changes in the association state from monomer to trimer. Formation of trimer produces an almost complete shielding of the chromophores from the aqueous phase.

Fluorescence from the aromatic amino acid residues

The changes in the polypeptide structure which occurred with changes in the association states was also confirmed by another method. In C-phycocyanin, fluorescence from the aromatic amino acid residues is hardly observed. This result is interpreted being due to quenching of the fluorescence by the chromophores, probably due to reabsorption. Thus, it is suggested that the fluorescence from aromatic amino acid residues can be used as a measure of the relative protein conformation, since the reabsorption of the light is closely related to the orientation and distance between the chromophores and the aromatic amino acid residues. It is postulated that the quenching of the fluorescence from aromatic amino acid residues occurs when the residues are located within 20 Å of the chromophores [12]. Thus the fluorescence from aromatic amino acids can be used to monitor the conformational changes in a larger area, compared with the information obtainable by the accessibility of a small molecule to the chromophores.

The α subunit has 11 tyrosine, 3 phenylalanine and 1 tryptophan residues, whereas the β subunit has 5 tyrosine and 4 phenylalanine, but no tryptophan residues [13]. Since the extinction coefficient and the quantum yield of phenylalanine are very small, the observed fluorescence originates mainly from tyrosine and tryptophan [14].

In 63 mM formic acid, under which conditions the polypeptide is partly unfolded, the α subunit showed a fluorescence maximum around 310 nm coming from tyrosine and a shoulder around 345 nm from tryptophan (Fig. 3A). The fluorescence maximum of the β subunit was located around 310 nm and no shoulder was observed in the

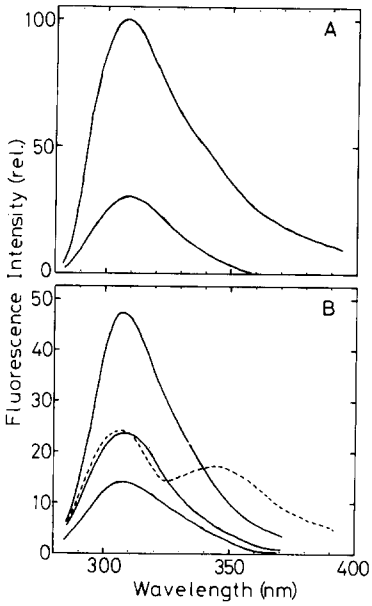

Fig. 3. Fluorescence spectra from aromatic amino acid residues for various association states of C-phycocyanin. (A) samples in 63 mM formic acid. Upper, α subunit and lower, β subunit. (B) samples in 5 mM potassium phosphate (pH 7.0). Upper, α subunit; middle, monomer; and lower, trimer. Dotted line, spectrum of the reconstituted trimer after reduction and partial removal of the reductant. Intensities are expressed relative to that of the α subunit in 63 mM formic acid. Excitation, 260 nm. For details, see text.

longer wavelength region (Fig. 3A). These spectra agreed well with the composition of aromatic amino acids in each subunit. In 5 mM potassium phosphate (pH 7.0), under which condition the polypeptide remains folded, the fluorescence intensity from the α subunit was less than that in 63 mM formic acid, and no shoulder was observed around 345 nm (Fig. 3B). This indicates that the conformational changes which occur on the changing the solvent induce the quenching of the tyrosine and tryptophan fluorescence. The intensity of fluorescence of the monomer was lower than that of the subunit with the maximum at 310 nm. The fluorescence of the trimer was about 10% of the sum of the fluorescence intensities of both subunits in 63 mM formic acid (Fig. 3B). These results indicate that the fluorescence from aromatic amino acid residues depends on the association state.

Conformational changes, as indicated by fluorescence intensity and accessibility of the chromophores to a small molecule, were induced by a change of the solvent (63 mM formic acid to 5 mM potassium phosphate (pH 7.0)). However, following monomer formation, only changes in the fluorescence intensity were observed. This suggests that the two methods reveal conformational changes in different areas of the environment of the chromophores. It has been proposed that quenching of the fluorescence from aromatic amino acids occurs in an area within 20 Å of the chromophores [12]. In the C-phycocyanin trimer, almost all aromatic amino acid residues are located within 20 Å of the chromophores (cf. Fig. 5). The observed fluorescence was about 10% of the sum of the two subunits in 63 mM formic acid, where the intensity was maximum. From the statistical point of view, it is possible to conclude that quenching occurs within 20 Å in the C-phycocyanin trimer.

Reconstitution of C-phycocyanin trimers

When reconstituted without any chemical treat-

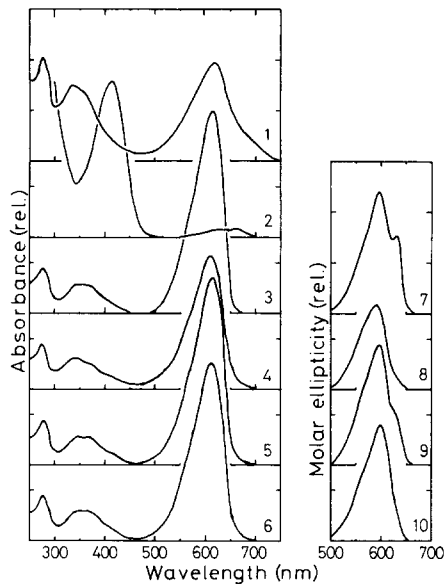

Fig. 4. Absorption (1–6) and CD (7–10) spectra of various association states of C-phycocyanin. (1) α subunit in 63 mM formic acid after oxidation with 50 μM potassium ferricyanide for 2 h; (2) α subunit in 63 mM formic acid after reduction with 5 mM sodium dithionite for 1 h; (3) and (7) native trimer; (4) and (8) reconstituted monomer after oxidation; (5) and (9) reconstituted trimer after oxidation; (6) and (10) reconstituted trimer after reduction and partial removal of the reductant. For details, see text.

ment the C-phycocyanin trimer showed the same spectroscopic characteristics as those of the native trimer (Fig. 4-3 and 4-7); the absorption maximum was located at 615 nm, and in the CD spectrum, two maxima were clearly observed at 598 and 633 nm (cf. Table I). The latter CD band is the characteristic band of the trimer [4]. Reduction of the chromophore before reconstitution gives a trimer whose optical characteristics are almost the same as those of the monomer [4]. This suggests that the modification of the electronic structure of the chromophore could influence the formation of higher association states. We checked this by oxidation and reduction of the chromophores, which would be expected to modify the electronic structure and/or the configuration of the chromophores.

Ferricyanide is known to oxidize the chromophore [15] and also to oxidize sulfur-containing amino acid residues (methionine and cysteine). However, the α subunit does not contain any methionine or free cysteine. Only one cysteine is bound to the chromophore via a thio-ether linkage [13]. Therefore, in the α subunit, the oxidation by ferricyanide is only expected to occur in the chromophore. Addition of ferricyanide (50 μM) to the α subunit in 63 mM formic acid induced bleaching of the chromophore, which was accompanied by an increase in the absorbance at 340 nm (Fig. 4-1). The oxidized product was chemically stable, since the absorption spectrum of the sample remained the same after dialyzing against 63 mM formic acid for more than 15 h. The reaction velocity was concentration dependent, but concentrations higher than 50 μM caused precipitation of the α subunit. The yield of oxidized product could not be increased with 50 μM ferricyanide and was about 50% after 2 h, as estimated by the absorption spectrum. Therefore, it is supposed that the sample for the reconstitution consists of intact and oxidized α subunits in a ratio of 1 : 1.

The C-phycocyanin reconstituted after oxidation exhibited two different association states which were separated on the sucrose density gradient. The lower fraction, whose molecular weight corresponded to the native trimer (molecular weight 110000), showed similar optical characteristics to the native trimer, except for a small shift of the absorption maximum from 615 to 614 nm and a slightly lower ellipticity at 630 nm in the CD spectrum (Fig. 4-5 and 4-9 and Table I). The absorption spectrum of the upper fraction whose molecular weight corresponded to that of the monomer (molecular weight, 35000), showed that this fraction contained the oxidized α subunit; the extinction coefficient was lower and the absorption spectrum was characteristic with the maximum at 340 nm (Fig. 4-4 and 4-8) as shown by the oxidized α-chromophore alone (cf. Fig. 4-1). This indicates that oxidation of the chromophore of the α subunit inhibits trimer formation but not monomer formation. The oxidized chromophore of the α subunit may not have the native configuration, so that the correct folding of the polypeptides is impaired. However, the altered protein conformation has no effect on the interaction of α and β subunits. Thus it is concluded that the α subunit within the trimer has two domains; one is essential for the monomer formation (binding between α and β subunits), and the other is important for trimer formation (binding between two monomers). Our experiments indicate that the chromophore of the α subunit is located at the binding site between the monomers. These findings are consistent with the structure obtained by X-ray analysis [3].

Reduction of the chromophores with sodium dithionite before reconstitution of the trimers resulted in the formation of different trimers, depending on the reconstitution conditions. In the first case the α subunit was reduced and dialyzed against 63 mM formic acid for more than 15 h. During this time, the reduced chromophore recovered its blue color. To this sample was added the β subunit in 63 mM formic acid and the mixture was dialyzed against 5 mM potassium phosphate (pH 7.0). The resulting trimers showed the same optical characteristics as the native trimer (absorption maximum at 615 nm and CD maxima at 598 and 633 nm) (Table I). In the second case, the trimer was reconstituted using α subunits that had been reduced and in which the reductant had been only partly removed before mixing with the β subunits. These trimers showed almost the same optical characteristics as the monomers (absorption maximum at 611 nm and CD maximum at 598 nm) (Fig. 4-6 and 4-10 and Table I). This means there is no direct interaction

between α and β_f chromophores of adjacent monomers. The only difference in the reconstitution experiments was that in the second case, the reductant had been partly removed, whereas in the first case, it was completely removed from the α subunit before mixing with the β subunit. Under the second conditions, the added β subunit was also partly reduced. Furthermore, in the course of dialysis against 5 mM potassium phosphate (pH 7.0), the mixture gave a temporary precipitate, but it was finally dissolved completely. This temporary precipitate was not observed in the first experiment. This indicates that in the second experiments the trimer temporarily showed the hydrophobic properties, probably due to a folding process different from that in the formation of the native trimer, consequently giving rise to a different conformation from the native trimer.

The changes in the polypeptide conformation in the reconstituted trimers were confirmed by the fluorescence from aromatic amino acid residues. The trimer obtained in the second experiments showed a higher fluorescence intensity than that of the native trimer, with a clear tryptophan fluorescence which was quenched in the native trimer (Fig. 3B). In this trimer, the direct interaction between the α and β_f chromophores is blocked. Therefore, it is reasonable to conclude that the changes in the polypeptide conformation bring about the inhibition of the direct interaction between the chromophores.

Discussion

The three-dimensional structure of the C-phycocyanin trimer without a linker polypeptide has been analyzed by X-ray diffraction [3]. The location of the chromophores and the backbone structure were clearly shown at 3 Å resolution. However, the folding of the loop region and the position of the side chain in the environment of the chromophore, which can affect the configuration and thus optical characteristics and reactivity of the chromophores, have not yet been determined.

Changes in the polypeptide conformation due to the changes in the association state were clearly shown by two different methods; the accessibility of a small molecule to the chromophores and the fluorescence from aromatic amino acid residues. In 63 mM formic acid (pH 2.5), both subunits were partly unfolded, but the configuration of the chromophores remained relatively intact. Therefore, the absorption spectra were different from those in 8 M urea (pH 2.0), under which conditions the chromophores adopt a cyclic configuration free from polypeptide interaction [15]. When formic acid (63 mM) was replaced with potassium phosphate (5 mM, pH 7.0), conformational changes occurred, which decreased the reactivity with a reductant (Fig. 1A) and caused quenching of the fluorescence from aromatic amino acids (Fig. 3A). However, the quantum yield of the fluorescence from the chromophore increased, indicating that the configuration of the chromophore was more fixed (data not shown). Restoration of the secondary and tertiary structure at neutral pH might be responsible for this. Formation of the monomer from the component subunits had only a minor effect on the polypeptide structure around the chromophore. Therefore, the time-course of chromophore bleaching of the monomer was in between the bleaching curves of the two subunits (Fig. 1A). These data are also supported by the fact that the absorption and CD spectra of the monomer are almost identical with the sum of both component subunits (cf. Ref. 4). However, following monomer formation the fluorescence from aromatic amino acids decreased. This may be because binding of the two subunits facilitates the energy transfer between aromatic amino acid residues. Formation of the trimer brings about an interaction with the other monomer. In this process, a clear reduction in the reactivity of the chromophore with a small molecule (Fig. 1B) and strong quenching of the fluorescence from aromatic amino acids (Fig. 3B) were observed. These phenomena can be explained by changes in the polypeptide conformation due to the interaction of the three monomers.

These data can be understood better on the basis of the three dimensional structure [3]. X-ray analysis has shown that the β_s chromophore is in the turn between the G and H helices of the β subunit, the β_f chromophore seems to protrude toward the central hole of the trimer (35 Å in diameter), and the α chromophore is located at the outside of the trimer. The β_s chromophore,

which is exposed to the aqueous phase in the monomer, is shielded in the trimer (cf. Fig. 1B). At the same time, the absorption maximum shifted from 594 to 598 nm due to trimer formation (Table I). This chromophore does not interact with the other subunits in the trimer. Thus conformational changes of the turn between the G and H helices to give a tight packing of the peptide induces the changes in the shielding and absorption characteristics of the chromophore. From the X-ray structure, the β_f chromophore seems to be exposed to the aqueous phase. However, our results indicate that in the trimer it is almost completely shielded from the aqueous phase. It is possible that a long loop structure around the β_f chromophore consisting of about 10 amino acid residues (between the F and G helices) is responsible for this shielding. This loop structure is not clearly visible in the X-ray structure because of its weak diffraction pattern. The possible role of the loop is to protect the β_f chromophore from the exposure to the aqueous phase (this type of shielding by the loop structure has been confirmed in the case of *Agmenellum quadruplicatum* [16]). Three other loop regions were found near the contact region of the monomers; two (between the f and g helices of the α subunit, and between the B and E helices of the β subunit) are between the α chromophore of one monomer and the β_f chromophore of an adjacent monomer, and the third one (between the b and e helices of the α subunit), is outside the α chromophore, that is, at the outermost position of the trimer structure. The complete shielding of the α chromophore in the trimer is partly due to the loop structure.

The oxidized or reduced chromophores have an electronic structure different from that of the native chromophores. In the course of the polypeptide folding, these modified chromophores might affect the polypeptide conformation especially around the chromophores themselves. These changes can inhibit the formation of the trimer, as in the case of oxidation (cf. Fig. 4-4 and 4-8), or the direct interaction between two chromophores even after trimer formation as in the case of the reduction (cf. Fig. 4-6 and 4-10). Particularly in the latter modification, the bleached chromophores regain their native optical characteristics following dialysis. Thus the changes in protein folding might occur in the initial refolding process when the bleached chromophore has an intermediate configuration. This refolding process induces a conformation which inhibits the direct interaction between the two chromophores. In this sense, the chromophore–protein interaction is the mutually affecting process. Small chromopeptides from C-phycocyanin show various CD spectra depending on the number of amino acids [17]. This might be another line of evidence for the mutually affecting chromophore–protein interaction.

The trimer reconstituted after reduction showed a higher fluorescence intensity than that of the native trimer, especially in the longer-wavelength region where the fluorescence originates mainly from tryptophan residues (Fig. 3B). This residue (α-126 tryptophan) is found in the g helix and very close to the α chromophore (Fig. 5). The increase in the fluorescence from tryptophan indicates that a small conformational change occurs in this helix, since the increase in tryptophan fluorescence is relatively small compared with the intensity in 63 mM formic acid. There is no direct evidence that the loop structure is changed. The

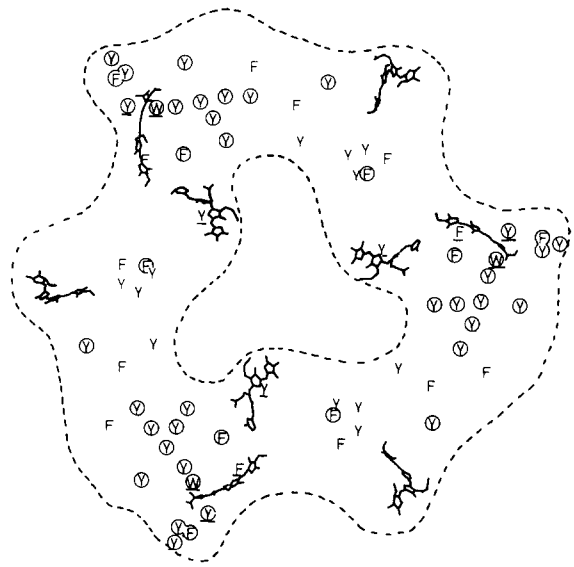

Fig. 5. Schematic presentation of the locations of aromatic amino acid residues in the C-phycocyanin trimer. Letters indicate the locations of aromatic amino acid residues in the backbone structure. Circled letters indicate the amino acid residues of the α subunit and letters without a circle, those of the β subunits. The underlined residues are those of the first group. For details, see Discussion.

loop structure is more flexible than the helix structure, thus the small changes in the helix structure might induce larger conformational changes of the loop structure. These changes may block the direct interaction between two chromophores. The direct interaction between chromophores can be formed only when each chromophore has a definite configuration, which requires the interaction with the polypeptide chain in a definite conformation.

In C-phycocyanin, there are 24 aromatic amino acid residues per monomer (Fig. 5). These residues can be classified into two groups depending on their locations. The first group consists of residues close to the chromophores (near α chromophore, α-65 tyrosine, α-126 tryptophan and β-60 phenylalanine, and near β_f chromophore, β-119 tyrosine). These residues most probably affect the electronic structures of the chromophores, as in the case of the bacteriochlorophyll polypeptide complex [18]. The other residues constitute the second group. The aromatic amino acids of the first group are conserved in the sequence of each subunit isolated from some different species [2]. On the other hand, some residues in the second group are variable depending on the species. This also suggests the importance of the first group.

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