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Subunit selective bleaching of C-phycocyanin from *Synechococcus* PCC 6301 strain AN 112 by Cu^{2+} and sodium perchlorate

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The bleaching of the monomer and the separated α and β subunits of C-phycocyanin from *Synechococcus* PCC 6301 Strain AN 112 by Cu^{2+} has been investigated. Upon the addition of Cu^{2+}, the visible absorption band of the $\alpha\beta$ monomer of phycocyanin was bleached and the maximum of the band shifted from 614 nm to 620 nm. The change in absorption induced by Cu^{2+} was accompanied by a decreased fluorescence emission intensity and a shift of the fluorescence maximum from 649 nm to 643 nm. The difference spectra showed negative peaks at 604 nm in absorption and at 653 nm in fluorescence when the concentration of Cu^{2+} was low. Spectroscopic titration curves showed biphasic behavior with respect to the concentration of Cu^{2+}. Comparison of these results with the properties of the separated α and β subunits indicates that the β-subunit has higher affinity for Cu^{2+} and is bleached preferentially over the α-subunit. This enables one to deduce the spectra of individual subunits from the monomer without separating them. This work also indicates that the β-subunit of phycocyanin is more labile than the α-subunit to denaturation with $NaClO_4$. Partial unfolding of the protein in the presence of $NaClO_4$ appears to decrease the protein–chromophore interaction, allowing binding of Cu^{2+} to the nitrogen atoms of the tetrapyrrole chromophores. This conclusion is supported by the results of a spectroscopic titration of biliverdin with Cu^{2+}.

Introduction

C-phycocyanin is the major light-harvesting pigment from cyanobacteria [1–3]. The basic unit of phycocyanin is the $\alpha\beta$ monomer which is composed of α and β subunits bearing one and two open-chain tetrapyrrole chromophores, respectively. The chromophores are linked via thioether bonds to cysteine residues of the polypeptides [4,5]. The properties of the individual chromophores in the native biliprotein play essential roles in harvesting and transferring light energy. To obtain information about these chromophores, the subunits were separated following urea denaturation, and spectroscopic characteristics of the subunits were examined and compared with those of the monomer [6–10]. Also, the absorption spectra of the individual chromophores of phycocyanin were simulated from the spectra of the $\alpha\beta$ monomer and its subunits [10,11]. These studies indicated that, although the three types of chromophore of phycocyanin are chemically the same, they differ considerably in their spectroscopic characteristics. These differences influence the directional flow of energy in the native biliprotein [10,12]. A reasonable explanation for the differences is that the chromophores have different conformations and/or are in different environments with respect to the protein-chromophore interactions [13,14].

Denaturation of phycocyanin results in a large change in its absorption spectrum. This observation was utilized in deducing the conformation of the chromophores and the protein–chromophore interactions [7,9,15–17]. Treatment of the phycocyanin from *Mastigocladus laminosus* with *p*-chloromercuribenzenesulfonate (PCMS) and the concomitant spectral changes allowed the authors to assign a chromophore (β1) to a specific binding site on the β-subunit [18–20]: we denote the β-chromophores absorbing at the longer and the shorter wavelengths as β1 and β2, which are β82 and β153 for the phycocyanin from *M. laminosus*, respectively.

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Abbreviations: PC, C-phycocyanin; PCMS, *p*-chloromercuribenzenesulfonate

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Schmidt et al. [21,22] showed that one of the three chromophores of phycocyanin from *M. laminosus* is photoisomerizable after denaturation. This chromophore was assigned to $\beta 2$, which differs in configuration from others in the native state [23] and thus can fall into the photochemically labile Z,Z,E-configuration during denaturation.

Cupric ion (Cu^{2+}) was reported to bleach the absorption of phycocyanin [24]. Because a difference in the environment of the chromophores of phycocyanin can result in a difference in their affinity for Cu^{2+}, it is possible to bleach a chromophore or chromophores selectively. This provides an opportunity for obtaining the spectra of the component chromophores from the intact assembly of the α and β subunits without prior separation. The partial and/or selective bleaching of chromophores should yield information about the environment of the chromophores and thus about the chromophore–protein interactions. In this paper, we present the results of such studies. The subunit selective bleaching of the phycocyanin *Synechococcus* PCC 6301 strain AN 112 by Cu^{2+} is demonstrated. Also, the difference in stability against $NaClO_4$ denaturation between the subunits of the phycocyanin, both in monomeric and separated forms, is presented.

Materials and methods

Algal culture, isolation of PC and its subunits

The AN112 mutant of *Synechococcus* sp. 6301 [25] was grown in modified Gorham's medium BG11 [26] at ambient temperature under approx. 5% CO_2/95% N_2 atmosphere and white light. The cells were harvested by centrifugation. Phycocyanin was isolated from the cells [27] and purified by column chromatography on DEAE-cellulose DE-52 (Whatman) using a linear gradient of phosphate buffer [28]. The phycocyanin fraction was dialyzed against water, and then against 10 mM cacodylate buffer (pH 6.0). A part of the phycocyanin solution was acidified with glacial acetic acid to pH 2.7 and applied on a column of Bio-Rex-70 cation-exchange resin; the subunits of phycocyanin were separated by a step gradient of urea according to Glazer and Fang [28]. Subunit fractions were dialyzed exhaustively against water and then against 10 mM cacodylate buffer.

Biliverdin

Biliverdin dihydrochloride was obtained from Sigma (St. Louis, MO, U.S.A.). A small amount of the sample was suspended in 10 mM cacodylate buffer overnight at 4°C. The undissolved solid was filtered off and the green filtrate was used for the spectroscopic studies.

Determination of chromophore concentrations

The concentrations of the phycocyanin and its subunits were determined from the absorption spectra of the chromoprotein solutions in 8 M urea at pH 2 [28]. The molar absorptivity at the visible absorption maximum, 662 nm, was taken to be 35000 $M^{-1} \cdot cm^{-1}$ per tetrapyrrole chromophore.

Spectral measurements

Absorption spectra were recorded on a Varian 2300 UV-Vis-NIR spectrophotometer. Steady-state fluorescence measurements were performed with a SPEX Fluorolog 2 model 212 spectrofluorimeter with an RCA C31034A photomultiplier tube. Both the excitation and emission spectra were corrected. Fluorescence polarization was measured by the same spectrofluorimeter and defined as

$$p = (P_r - 1)/(P_r + 1)$$

where the polarization ratio, P_r, defined as $I_{\parallel}/I_{\perp}$, was determined from $(I_{vv}/I_{hh})/(I_{vh}I_{hv})$ to correct for instrument polarization artifacts: 'v' refers to polarization vectors normal to the plane of the light paths, while 'h' refers to vectors in that plane; the first and second letters of the subscripts of fluorescence intensity (I) refer to the excitation and emission components, respectively.

All solutions for spectral measurements were prepared with 10 mM cacodylate buffer at pH 6.0. The use of sodium azide, mercaptoethanol, sodium thiocyanide and buffers bearing amino and/or carboxylate groups was deliberately avoided, because they can form complexes with Cu^{2+}. Solutions of phycocyanin or its subunits with the desired concentration of $NaClO_4$ were prepared and left overnight prior to the spectral measurements. Treatment of the solutions with Cu^{2+} was carried out by adding a stock solution of cupric sulfate with a microsyringe. Spectra of the solutions were taken after 15–30 min of the treatment with Cu^{2+}. Dilution of the solution upon the addition of Cu^{2+} was kept below 2%, and the change in absorbance upon the dilution was not corrected for.

Results

Spectroscopic titration of PC monomer with Cu^{2+}

The monomeric form ($\alpha\beta$) of the phycocyanin was induced by the addition of $NaClO_4$ (final concentration, 2.0 M) to the phycocyanin solutions [29]. Upon the addition of $NaClO_4$, the maximum of the visible absorption band of the phycocyanin shifted to 614 nm from 618 nm, and the absorbance of the band decreased by about 10%. These spectral changes accord with the reported trend [6] accompanying dissociation of phycocyanin aggregates to monomers, and reflect the monomeric state of the phycocyanin in 2 M $NaClO_4$.

Addition of Cu^{2+} to the solutions of phycocyanin monomer resulted in large changes in the absorption

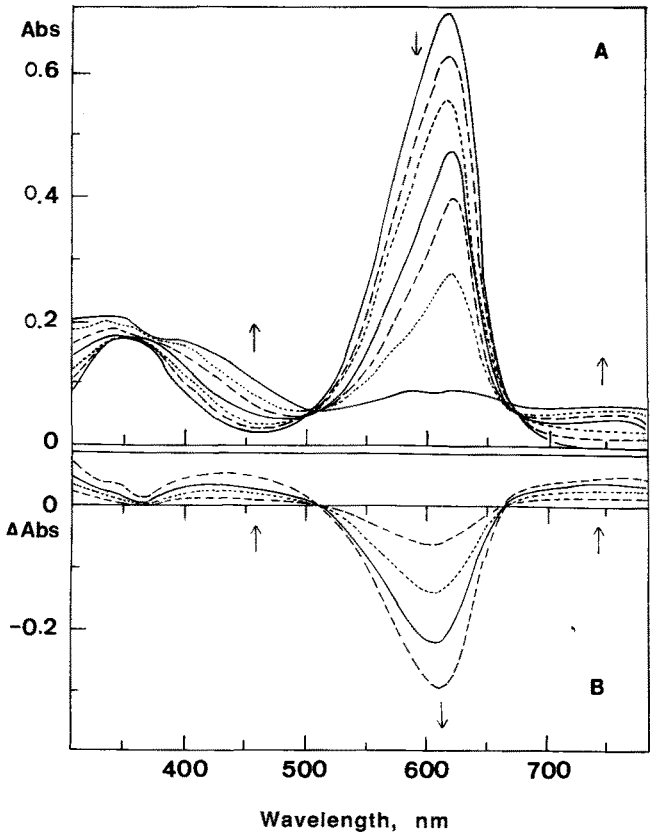

Fig. 1. Absorption (A) and difference absorption (B) spectra of the phycocyanin monomer with Cu^{2+}. The difference spectra were recorded for the phycocyanin solutions with Cu^{2+} against the PC solution without Cu^{2+} as reference. The phycocyanin monomer was induced by the addition of $NaClO_4$ (2 M) and the spectra were taken 15–20 min following the treatment with Cu^{2+}. The concentration of phycocyanin monomer was 3.0 μM. The concentrations of Cu^{2+} were 0.0, 1.5, 3.0, 5.0, 10, 23 and 100 μM for (A), and 1.5, 3.0, 5.0 and 10 μM for (B). Arrows point in the direction of increasing concentration of Cu^{2+}.

spectra (Fig. 1). As the concentration of Cu^{2+} increased, the absorbance of the visible absorption band was reduced, while the absorption above 660 nm and in the 360–500 nm region was enhanced. Isosbestic points were evident at 490 and 660 nm when the concentration of Cu^{2+} did not exceed 2–3-times of that of the phycocyanin monomer. With increasing concentration of Cu^{2+}, the absorption maximum of the visible band shifted gradually to longer wavelength, reaching 620 nm. The negative peak in the difference spectrum (Fig. 1B) was located at 604 nm and remained unchanged when the concentration of Cu^{2+} was low. For $[Cu^{2+}] \gg$ [phycocyanin], the visible absorption band was completely bleached and replaced by a diffuse band with little structure above 500 nm.

A fluorescence spectral titration of the phycocyanin monomer using various concentrations of Cu^{2+} is shown in Fig. 2, along with the difference fluorescence spectra. In the absence of Cu^{2+}, the phycocyanin solution showed a strong emission band with maximum at 649 nm. Added Cu^{2+} quenched the fluorescence emission and shifted the fluorescence maximum to the blue. The maximum was found to be at 644 nm at high concentration of Cu^{2+}. In the difference fluorescence spectrum, the negative band with peak at 655 nm grew with increasing concentration of Cu^{2+}; the shape of the band remained unchanged until the concentration of the metal ion became about 4-times the concentration of the phycocyanin monomer. At higher concentrations of Cu^{2+}, the difference peak moved to shorter wavelength and eventually located at 649 nm, which is the wavelength of the emission maximum of the phycocyanin monomer in the absence of Cu^{2+}. The fluorescence was almost completely quenched at a high concentration of Cu^{2+}. The excitation spectra (not shown), scanned in the 350–650 nm range, did not show any wavelength region where fluorescence intensity increases upon the addition of Cu^{2+}. These are in contrast to the absorption spectrum and suggest that the Cu^{2+} complex with the phycocyanin, which is responsible for the enhanced

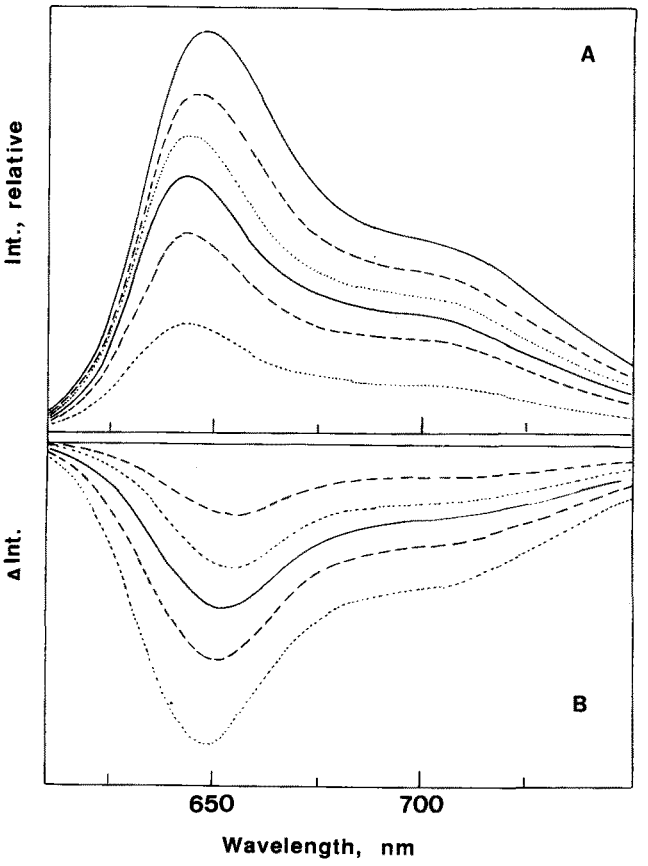

Fig. 2. (A) The fluorescence spectra of phycocyanin monomer solutions at different concentrations of Cu^{2+}. (B) The calculated difference fluorescence spectra: spectrum of phycocyanin solution with Cu^{2+} minus the spectrum of the solution without Cu^{2+}. The excitation wavelength was 600 nm. The concentration of phycocyanin monomer was 0.38 μM and those of Cu^{2+} were 0.0, 0.50, 1.0, 2.0, 5.0, and 10.0 μM for (A) and 0.5, 1.0, 2.0, 5.0 and 10.0 μM for (B), both from top to bottom. Solutions contained 2 M $NaClO_4$.

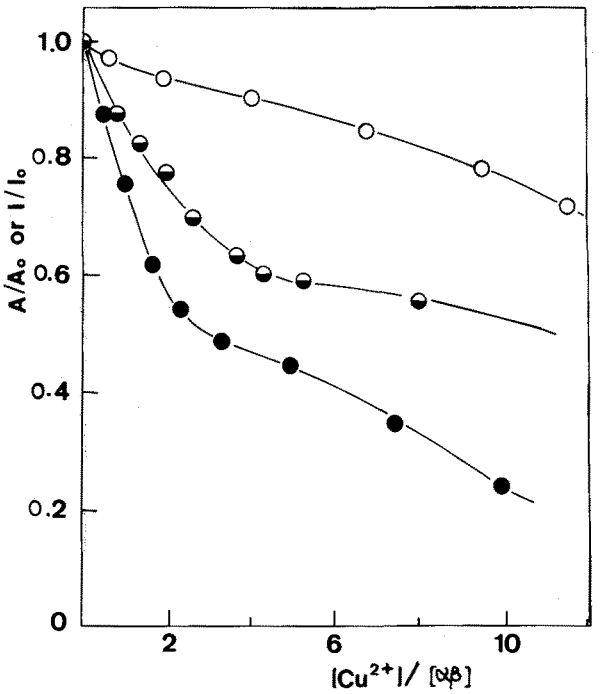

Fig. 3. Dependence of the relative absorbance at 600 nm (●) and fluorescence intensity at 650 nm (◒) on the ratio of concentrations of Cu^{2+} and $\alpha\beta$ monomer in 2 M $NaClO_4$. Conditions are the same as in Fig. 1 (for absorbance) and Fig. 2 (for fluorescence). The open circles (○) are absorbance data taken for solutions without $NaClO_4$.

absorption in the presence of the metal ion at 360–490 nm and above 660 nm, is essentially non-fluorescent at wavelengths shorter than 800 nm.

In Fig. 3, we plot the absorbance and fluorescence intensity of the phycocyanin monomer in 2 M $NaClO_4$ as a function of the concentration ratio between Cu^{2+} and the phycocyanin monomer ($\alpha\beta$). Interestingly, the titration curves of the solutions containing 2 M $NaClO_4$ showed a biphasic behavior. In the region of low concentration of Cu^{2+}, the absorbance and fluorescence intensity of the phycocyanin monomer decreased sharply with increasing amount of Cu^{2+}. The aforementioned gradual shift in absorption and fluorescence spectra occurred, while the peaks in difference spectra remained unchanged. At higher concentrations of the metal ion, where there was a much weaker dependence of the absorbance and fluorescence intensity on the concentration of Cu^{2+}, the peaks in the difference spectra shifted and the maxima of absorption and fluorescence remained unchanged. The boundary of the two regions is at $[Cu^{2+}]/[\alpha\beta] = 2$ to 4.

Because of the large difference in concentrations of the phycocyanin and Cu^{2+} employed in the two sets of experiments, and because of the complex nature of the chemistry of PC with respect to interactions with Cu^{2+} and $NaClO_4$, we were not able to compare and analyze the titration curves quantitatively. However, the close resemblance between the two titration curves indicates strongly that the decrease in the fluorescence emission from the phycocyanin upon the presence of Cu^{2+} is due mainly to the bleaching of the phycocyanin by the metal ion. In fact, in the low concentration region of Cu^{2+}, the relative fluorescence quantum yield (I/A) increases as phycocyanin is bleached by Cu^{2+}. This indicates that the remaining chromophores experience less quenching than those that are bleached.

The biphasic pattern of the titration curves of the phycocyanin shown in Fig. 3 is suggestive that there are at least two different types of chromophore with respect to bleaching by Cu^{2+}. The first is bleached mainly at low concentration of Cu^{2+} and the second requires higher concentration of Cu^{2+} to be bleached. The spectral change of the phycocyanin with Cu^{2+} at low concentration of Cu^{2+} reflects the spectrum of mainly the first type of chromophore. Therefore, the absorption maximum of the initially bleached chromophore is expected to be at about 604 nm, which was revealed in the difference spectrum. After the bleaching of the first type of chromophore, the remaining spectrum of the phycocyanin represents that of the unbleached chromophores, with an absorption maximum at 620 nm. By the same reasoning, we conclude that the fluorescence maximum of the remaining chromophores is at 644 nm. Because of the excitation energy transfer among the chromophores in phycocyanin monomer [10–12,30], locating the fluorescence maximum of the initially bleached chromophore is less straightforward. However, observation of the difference peak at 655 nm, not at 649 nm where the peak position is in the absence of Cu^{2+}, indicates that energy transfer between the two types of chromophore is inefficient and the initially bleached chromophores have their fluorescence maximum at about 655 nm.

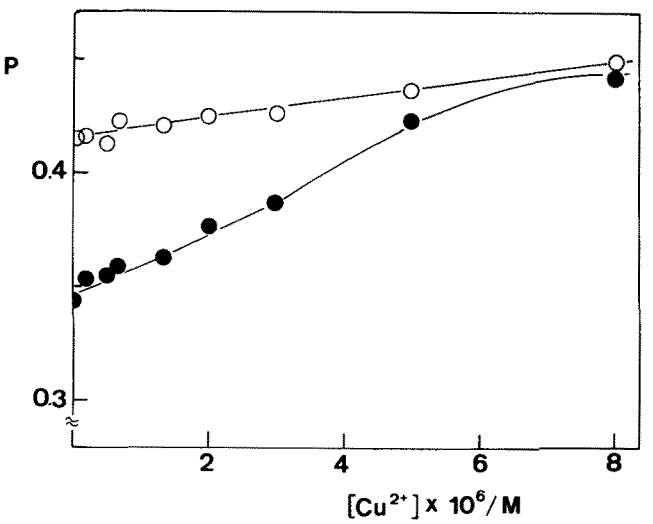

Fig. 4. Fluorescence polarization of phycocyanin monomer: (●), λ_{ex} = 550 nm; (○), λ_{ex} = 620 nm. The fluorescence was monitored at 650 nm. The monomeric state of phycocyanin was induced by the presence of 2 M $NaClO_4$. $[\alpha\beta] = 0.38\ \mu M$.

Fluorescence polarization

The fluorescence polarization of the phycocyanin monomer solutions was measured for fluorescence emission at 650 nm using excitation wavelengths of 550 and 620 nm. The results are presented in Fig. 4 as functions of Cu^{2+}.

In the absence of Cu^{2+} but in 2 M $NaClO_4$, the polarization of the phycocyanin solution was 0.35 for $\lambda_{ex} = 550$ nm and 0.42 for $\lambda_{ex} = 620$ nm. The dependence of the polarization on excitation wavelength is consistent with the presence of considerable excitation energy transfer from 'sensitizing' to 'fluorescing' chromophores in the phycocyanin monomer [30].

The polarization increases upon the addition of Cu^{2+}, more so for $\lambda_{ex} = 550$ nm than for $\lambda_{ex} = 620$ nm. Polarization values at the two wavelengths were almost identical at high concentrations of Cu^{2+}. This indicates that the partial bleaching of the phycocyanin monomer by Cu^{2+} results in less efficient energy transfer in the chromoprotein, although the dependence of the fluorescence maximum on the concentration of Cu^{2+} indicates little energy transfer between the two types of chromophore distinguished based on the affinity for the metal ion. Mimuro et al. [9] concluded that the major excitation transfer in phycocyanin monomer from *M. laminosus* is from the 'sensitizing' βs chromophore to the 'fluorescing' βf chromophore in the same β-subunit and there is little energy transfer between the chromophores in different subunits of the phycocyanin monomer. If this holds also in the present system, the polarization data imply that the chromophores in the β-subunit are preferentially bleached by Cu^{2+}.

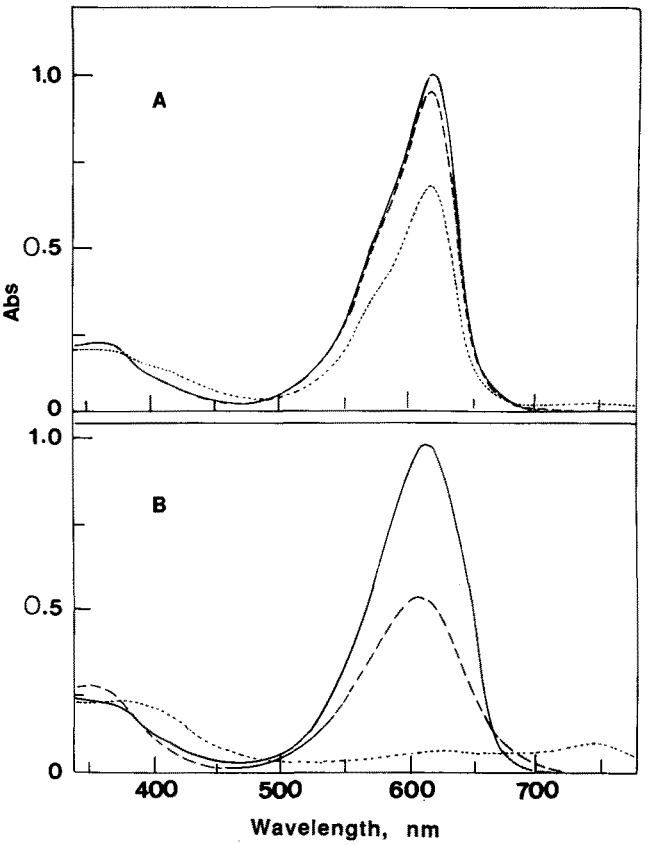

Fig. 5. Absorption spectra of α-subunit (A) and β-subunit of phycocyanin (B) in 10 mM cacodylate buffer at pH 6.0: ———, without $NaClO_4$; - - - - - -, in the presence of 2 M $NaClO_4$; ·····, in the presence of 2 M $NaClO_4$ and $1.0 \cdot 10^{-5}$ M Cu^{2+}. The concentration in each case was $1.0 \cdot 10^{-5}$ M based on the tetrapyrrole chromophore unit.

Studies with subunits

To examine the correlation between the subunits of the phycocyanin monomer and the two types of bleaching of chromophores in the phycocyanin monomer, we separated the subunits and studied their spectroscopic properties. The absorption spectra of the subunits under different conditions are shown in Fig. 5.

In agreement with a previous report [6], the absorption spectra of the α- and β-subunits differ significantly from each other with respect to the shape and the position of the long-wavelength maxima. The separated α and β subunits in 10 mM cacodylate buffer without added $NaClO_4$ and Cu^{2+} exhibit visible absorption maxima at 620 and 614 nm, respectively. The molar absorptivities of the visible bands, calculated at absorption maxima of the visible bands using the concentrations determined from the absorbances of the urea denaturated solutions, were $0.99 \cdot 10^5$ for the α and $1.81 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$ for the β-subunit. Marked differences in behavior between the subunits were exhibited in terms of the spectral change upon the successive addition of $NaClO_4$ and Cu^{2+} (Fig. 5). The presence of 2 M $NaClO_4$ with the α-subunit gave only about 5% absorbance decrease without noticeable shift of the spectrum. However, the same treatment of the β-subunit resulted in as much as 45% absorbance decrease and a shift of the absorption maximum from 614 to 608 nm. For both subunits, the decrease in the absorbance of the visible band was accompanied by a small increase in the ultraviolet band located near 350 nm.

The subsequent addition of Cu^{2+} to the solutions of the subunits in 2 M $NaClO_4$ resulted in bleaching of their visible absorption bands. Spectra taken at equimolar concentration of Cu^{2+} to that of the tetrapyrrole chromophore are shown in Fig. 5. The overall pattern of the spectral change induced by Cu^{2+} is similar to that observed in the phycocyanin monomer. Added Cu^{2+} bleached the visible band and increased absorbance at both sides of the band for each subunit. However, in contrast to the monomer, no appreciable shift in absorption maximum was observed for either subunits.

In $NaClO_4$ and Cu^{2+}-free solutions, both the α and β subunits are strongly fluorescent, with maxima of the emission spectra located at 644 and 653 nm, respectively. The addition of $NaClO_4$ quenched the fluores-

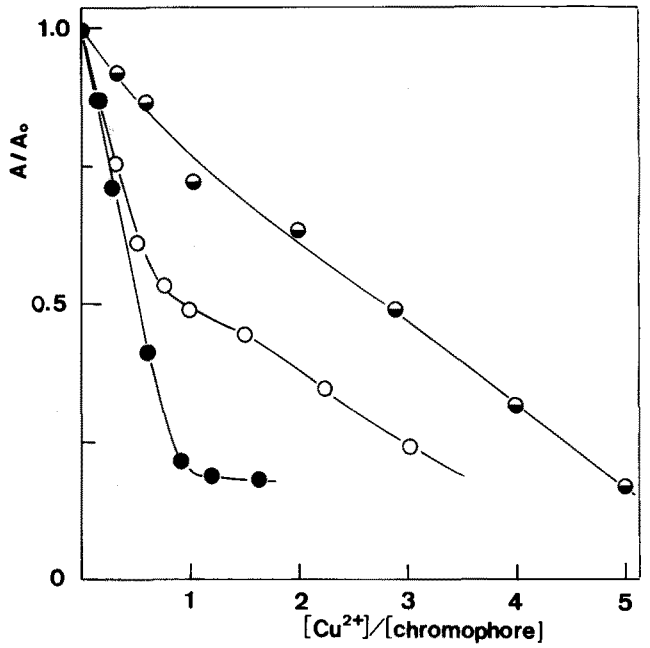

Fig. 6. Changes of the relative absorbances of α-subunit (◒), β-subunit (●), and αβ-monomer (○) of phycocyanin with the $[Cu^{2+}]$/[chromophore] ratio. The solutions contained 2 M $NaClO_4$.

cence from the α-subunit by about 10%, which was the magnitude of the absorbance change. However, the fluorescence intensity of the β-subunit was reduced by as much as 90%, despite a decrease of only 45% in absorbance, upon the addition of 2 M $NaClO_4$. Thus, the fluorescence quantum yield of the β-subunit in 2 M $NaClO_4$ is lower than that in the absence of the chaotropic agent.

The results of the absorbance titrations of the subunits in 2 M $NaClO_4$ with Cu^{2+} are presented and compared with that of the PC monomer in Fig. 6. For the β-subunit, the absorbance of the visible band decreases linearly with increasing concentration of Cu^{2+} until the molar concentration ratio between Cu^{2+} and the tetrapyrrole chromophore becomes 1, at which point the visible absorption band of the β-subunit is completely bleached. This implies that the reaction between Cu^{2+} and the β-subunit is essentially quantitative in 2 M $NaClO_4$, and the stoichiometry of the reaction is 1 : 1 with respect to the metal ion and the tetrapyrrole chromophore. The dependence of the absorbance on the concentration of Cu^{2+} is much weaker for the α-subunit, which requires about 5-fold excess of Cu^{2+} for near completion of the bleaching. Comparison of titration curves of subunits with that of the PC monomer makes it apparent that the chromophores of the phycocyanin monomer bleached at low concentrations of Cu^{2+} are those in the β-subunit and the remaining chromophore which requires a higher concentration of Cu^{2+} is the one in the α-subunit. Both the difference absorption and fluorescence spectra taken during the earlier part of the titrations match well with the corresponding spectra of the separated β-subunit. Also the residual spectra after partial bleaching accord with those of α-subunit. These results are consistent with the fluorescence polarization data, and with an earlier finding that the absorption spectrum of phycocyanin monomer does not significantly differ from the sum of the spectra of the α- and β-subunits [6,10]. It is also significant that the β-subunit, both in monomer and in separated form, has greater affinity for Cu^{2+} than does the α-subunit in the presence of 2 M $NaClO_4$.

Biliverdin

Biliverdin, an open-chain tetrapyrrole, is an analog of the isolated chromophore of PC [15]. Fig. 7 shows the absorption spectra of biliverdin with and without addition of Cu^{2+}. In the absence of Cu^{2+}, biliverdin showed two major absorption bands, with maxima at 370 and 660 nm; the vis-to-UV absorbance ratio (A_{vis}/A_{uv}) is 0.3, which is much smaller than the value of about 4 shown in phycocyanin monomer and its subunits. Upon the addition of Cu^{2+}, the absorbance of both bands was decreased and the absorbance in the 400–500 nm region and above 720 nm increased. The absorbance titration of the compound with Cu^{2+} showed a linear relation between the absorbance and the concentration of Cu^{2+} to an endpoint, beyond which significant absorbance change was not observed

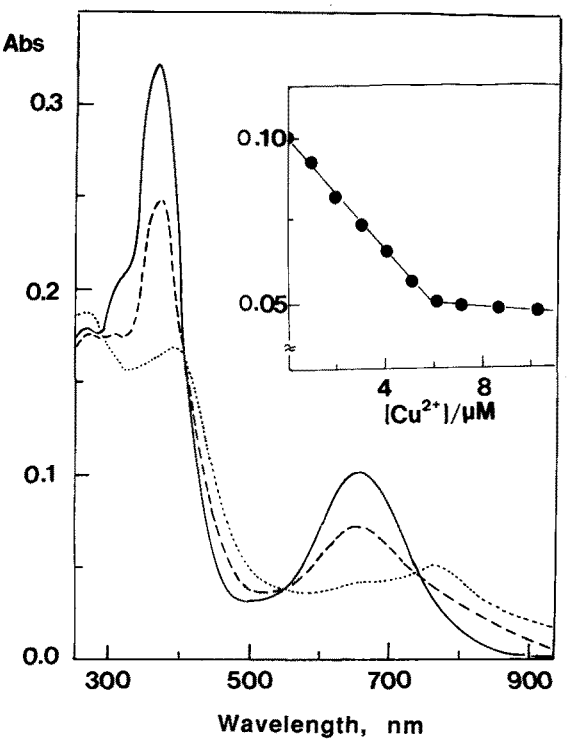

Fig. 7. Spectra of biliverdin in 10 mM cacodylate buffer (pH 6.0) without added Cu^{2+} (———), with 3.0 μM (- - - - -), and 10.0 μM Cu^{2+} (· · · · · ·). Inset is the absorbance titration data followed at 660 nm.

by the further addition of the metal ion (see, inset of Fig. 7). This is an indication that the magnitude of the association constant between biliverdin and Cu^{2+} is greater than 10^8 M^{-1}. Assuming a 1:1 stoichiometry for the titration, the concentration of biliverdin is estimated to be $6 \cdot 10^{-6}$ M. This gives a molar absorptivity of biliverdin at 660 nm of 1700 $M^{-1} \cdot cm^{-1}$, which agrees well with the value calculated from the spectrum of biliverdin taken in aqueous medium at pH 10.5 [31].

The effect of Cu^{2+} on the spectral properties of biliverdin is very similar to that on phycocyanin monomer and its subunits. This strongly implies that the change in spectral characteristics of the chromoproteins is the result of the *direct* interaction between Cu^{2+} and the tetrapyrrole chromophore. This differs from the effects of denaturing agents [15–17] and PCMS [18–20], which modify the spectrum of phycocyanin by affecting the conformation of protein and thus the chromophore–protein interaction. The latter is manifested as the spectral change.

Both carboxylate and ring nitrogen atoms of the tetrapyrrole moiety can serve as the binding site for Cu^{2+}. Ca^{2+} and Mg^{2+}, which have substantial affinity for the carboxylate group did not yield any noticeable effect on the spectral properties of biliverdin or on the chromoproteins. Given the drastic spectrum change caused by Cu^{2+}, this implies that Cu^{2+} is preferentially chelated by nitrogen atoms of the pyrrole rings. A fresh solution of biliverdin with excess Cu^{2+} showed an absorption maximum at 770 nm (see Fig. 7), which is replaced by a similar sized peak at 650 nm as the solution is aged. A plausible explanation for this is that the two nitrogen atoms of the central pyrrole rings chelate with Cu^{2+} at the first stage of complex formation and that further chelation of the remaining two nitrogen atoms is kinetically slow. The possibility of the chelation of four nitrogen atoms with a cupric ion is supported by a report of the similar complex in zinc-biliverdin from crystallographic work [32]. Another possible explanation for the spectral change upon aging the biliverdin/Cu^{2+} solution is the oxidation of the tetrapyrrole by O_2. It has been suggested that metal ion (Cu^{2+}, Zn^{2+}, Cd^{2+}) complexes of biliverdin are oxidized by O_2 in alkaline medium [31]. The change in the 770 nm band upon aging the solution was less pronounced in the chromoproteins, probably due to the inhibition of the additional chelation by the polypeptide chains.

Effect of $NaClO_4$

The bleaching of the phycocyanin monomer and its subunits by Cu^{2+} was less effective without $NaClO_4$, as illustrated in Fig. 3. This suggests that, in addition to dissociating phycocyanin to monomer [27], $NaClO_4$ may facilitate the accessibility of Cu^{2+} to the chromophores. To examine the role of the chaotropic agent on the metal ion binding, we took the spectrum of the phycocyanin at various concentrations of $NaClO_4$ (Fig. 8). As the concentration of $NaClO_4$ increased, the absorbance of the visible band decreased. The absorption maximum was shifted from 618 nm to 614 nm at 1–2 M $NaClO_4$ and then to 620 nm at 4 M $NaClO_4$. The fluorescence intensity as well as the absorbance-to-fluorescence intensity ratio (I/A) decreased upon increasing the concentration of $NaClO_4$. The change in the phycocyanin spectrum using less than 2 M $NaClO_4$ can be attributed to the dissociation of the phycocyanin aggregates into monomers [6]. The spectral change at a higher concentration of $NaClO_4$ resembles the effect of Cu^{2+} on the phycocyanin in 2 M $NaClO_4$: a minor difference was that the increase in absorbance above 660 nm and in the 350–500 nm range was more pronounced with Cu^{2+}.

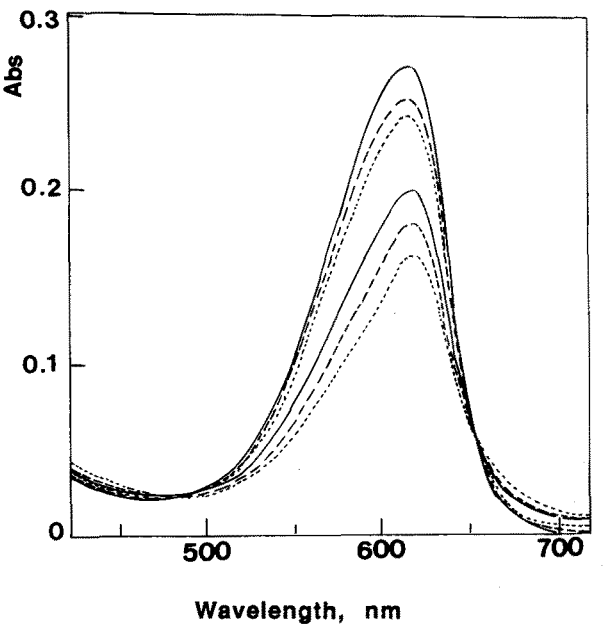

Fig. 8. Absorption spectra of phycocyanin at (from top to bottom) 0.0, 1.0, 2.0, 2.5, 3.0 and 4.0 M (from top to bottom) $NaClO_4$. Phycocyanin was in 10 mM cacodylate buffer (pH 6.0).

It is generally recognized that denaturation of phycocyanin results in a decrease of the visible absorption, and that the fluorescence quantum yield of the denatured state is lower than that of the native state. If both subunits of the phycocyanin monomer undergo the same extent of denaturation at a given concentration of $NaClO_4$, we would not usually expect a shift in spectral maximum upon the addition of $NaClO_4$. Therefore, the spectral change in phycocyanin induced by $NaClO_4$ can be ascribed to the preferential denaturation of one subunit by $NaClO_4$. Obviously, the β-subunit whose absorption maximum is located at 604 nm appears to be the preferred one. The large decrease in absorbance of the β-subunit (Fig. 5), as compared to the α-subunit, upon the addition of 2 M $NaClO_4$ supports this interpretation. This behavior is quite analogous to the pref-

erential bleaching of the subunit by Cu^{2+} in the presence of 2 M $NaClO_4$.

Discussion

Various amino acid residues bearing amine, sulfhydryl, or carboxylate side chains and the tetrapyrrole chromophores in phycocyanin constitute potential binding sites for Cu^{2+}. Among these, we conclude that the nitrogen atoms of the tetrapyrrole chromophores are the preferred sites for binding and that the binding leads to bleaching of the visible absorption band of the chromoproteins. These conclusions are supported by the low concentration of Cu^{2+} required to produce a noticeable spectral change, a sharp break at 1:1 stoichiometric ratio of Cu^{2+} to tetrapyrrole chromophore in the β-subunit of PC, the similarity in pattern of the spectral changes between the chromoproteins and biliverdin, and the absence of any noticeable effect of Ca^{2+} and Mg^{2+} on the spectrum of biliverdin. A similar 1:1 chelation of tetrapyrrole chromophores with a metal ion and concomitant bleaching of the visible absorption band was recently reported in the P_{fr} form of a phytochrome using Zn^{2+} [33].

The conformation/configuration of the tetrapyrrole chromophores in native PC is known to be *Z-anti, Z-syn, Z-anti*, with the exception of $\beta 2$ of which the configuration of C-15 is almost midway between *Z* and *E* [23]. On the other hand, the fully denatured chromoprotein and isolated chromophores including biliverdin adopt the macrocyclic helical *Z-syn, Z-syn, Z-syn* conformation [34,35]. The strained conformation of the chromophore in its native state in the chromoprotein is stabilized by interactions between the tetrapyrrole and the protein: the nitrogen atoms of pyrroles B and C of the chromophores are hydrogen-bonded to one of the carboxylate oxygens of an aspartate residue, and the propionic sidechains of the chromophores form salt bridges with arginine and lysine [23]. These chromophore–protein interactions protect the chromophores from deexcitation via conformational change [21] and decrease non-radiative decay rates following absorption of light. Furthermore, they make the nitrogen atoms of pyrroles inaccessible to Cu^{2+}. Apparently, $NaClO_4$ decreases or eliminates the chromophore–protein interactions through the denaturation of the protein. This results in a decrease of the visible absorption, which reflects changes of the chromophore conformation. Also, the weakening of the chromophore–protein interaction enables Cu^{2+} to bind to nitrogen atoms of the chromophores effectively.

Scheer and Kufer [15] proposed that the ratio of the visible to UV absorbance (A_{vis}/A_{uv}) of biliproteins is a good measure of the conformation of the tetrapyrrole chromophores: the extended conformation present in the native state has a higher ratio than does a cyclic conformation. This was supported by theoretical calculations (Ref. 36 and references therein). The subunits of phycocyanin in $NaClO_4$-free solutions showed the absorbance ratio in the range 4.4–4.8, which is very close to the value observed in native phycocyanin. This indicates that the chromophores in the isolated subunits also adopt the extended conformation, probably owing to strong chromophore–protein interactions. The effectiveness of 2 M $NaClO_4$ in perturbing the spectra of the α and β subunits differs, probably reflecting the difference in the stability of protein of the two subunits against denaturation, which in turn results in a difference in affinity for Cu^{2+} and in the extent of bleaching induced by the metal ion in the presence of $NaClO_4$. The increase in the apparent fluorescence quantum yield (I/A) during early stages of the titration with Cu^{2+} supports the conclusion that the chromophores in the β-subunit are those which interact with protein less strongly, and thus exhibit lower fluorescence quantum yield, and are the preferential binding sites for the metal ion.

Each β-subunit of phycocyanin bears two tetrapyrrole chromophores which differ in their spectroscopic properties [10–12,18–20] and, possibly, in configuration [21–23]. We were not able to bleach the individual β-subunit chromophores selectively, due to the lack of site-specificity of $NaClO_4$. It was shown that PCMS binds to the free cysteine group of the β-subunit of phycocyanin from *M. laminosus*, resulting in spectral changes in the neighboring $\beta 2$ chromophore [18–20]: there is only one free cysteine group in the $\alpha\beta$ monomer of the phycocyanin. Although the spectral changes, about 10% decrease in absorbance, allowed the authors to assign the chromophore, the changes are too complex to yield the exact position, shape and absorptivity of the chromophore. For example, the difference spectra for the phycocyanin monomer and the β-subunit exhibit a positive band at about 655 nm and a negative band, about twice as large as the positive one, near 615 nm [18–20], whereas the simulated absorption spectrum of the $\beta 2$ chromophore shows a maximum at 622–624 nm [10,11,19]. Information about spectroscopic properties of chromophores is important for understanding the mechanism of energy transfer in PC aggregates [11,12]. The use of Cu^{2+} with PCMS or other chemicals, which have site-specific binding properties and thus influence the chromophore–protein interactions, may lead to chromophore-selective bleaching of phycocyanin, enabling one to obtain the spectra of the individual chromophores in the β subunit. Investigations along this line are currently underway.

The present studies can also be regarded as an example of subunit-specific chelation of Cu^{2+} with tetrapyrroles in phycocyanin. It should also be possible to achieve the selective chelation using other metal ions with phycocyanin, resulting in complexes that exhibit

high absorptivity and luminescence quantum yield in the visible wavelength region. This will be helpful in elucidation of the energy transfer mechanism in phycocyanin and in designing semi-artificial light harvesting systems.

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