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Spectroscopic changes for C-phycocyanin and phycoerythrin 545 produced by ferric ion

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Abstract

Biliproteins are light-harvesting pigments in photosynthesis. Their chromophores are open chain tetrapyrroles, which are not complexed to metal ions. Using visible absorption and circular dichroism (CD) spectroscopy, the effects of ferric and zinc ions were studied for two biliproteins. These biliproteins – C-phycocyanin and phycoerythrin 545 – were selected because they vary in their source, structure and spectroscopic properties. While zinc ions had no effect, ferric ions at pH 5.0 change the absorption and CD spectra in the wavelength range of the first excited state of the chromophores of both biliproteins. Concerning the relationship between chromophore topography and the function of light-harvesting pigments, the possibility of exciton splitting is a topic of current debate. For phycoerythrin 545, the effect of ferric ions reveals that two different pairs of chromophores are linked in their behavior, and this may mean that the pairs are involved in exciton splitting. Exciton splitting would extend the energy range for light harvesting and establish exciton-migration routes within the protein. For C-phycocyanin, the effect of ferric ions on the absorption spectra was primarily focused on the high-energy chromophores, but CD at higher ferric ion to protein ratios showed that the lower-energy chromophores were also affected. At a C-phycocyanin concentration of 0.050 g/l, the end point of the ferric effect in the high-energy region of the spectrum was reached at about $1.7 \cdot 10^{-4}$ M ferric ion. C-Phycocyanin was treated with ferric ions at three concentrations of protein (0.20, 0.10, 0.050 g/l). These same protein concentrations were examined without ferric ions by gel filtration chromatography showing that as the concentration of C-phycocyanin was lowered the trimeric protein dissociated to more monomer. The effect of the protein denaturant, urea, was also investigated with C-phycocyanin. The effects of urea and ferric ion were shown to be quite different.

Keywords: C-Phycocyanin; Phycocyanin; Biliprotein; Phycoerythrin 545; Ferric ion; Circular dichroism

1. Introduction

Biliproteins and chlorophylls are light-harvesting pigments active in photosynthesis. These pigments harvest solar radiation and then channel the excitation energy to the reaction centers, where it is transduced to chemical energy. The chromophores of biliproteins and chlorophylls are very similar in that biliproteins have open chain tetrapyrroles and chlorophylls have cyclic tetrapyrroles (porphyrins). One way in which they differ is that the chlorophyll chromophores have a metal ion complexed to them whereas biliproteins are metal-free [1–7].

In the study of certain model membrane systems [8], C-phycocyanin and several other biliproteins were shown to possess electron-directing capabilities across lipid bilayers. In these systems, ferric ions were also present. The observation suggested a possible relationship between biliproteins and the metal ion, at least in these artificial systems. Visible absorption and circular dichroism (CD) studies on solutions of ferric ions and two different biliproteins, C-phycocyanin and phycoerythrin 545, were therefore carried out. These studies would allow observation of any ferric-induced changes for the biliprotein chromophores. A change in the visible spectra could demonstrate either direct interaction of metal ions with tetrapyrroles, or binding of metal ion to apoprotein resulting in a structural change that indirectly affects the spectra of the chromophores. CD measurements were employed since these experi-

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ments should better determine which of the tetrapyrroles were affected.

Zinc ions were used to investigate whether any ferric ion effects might be specific, or examples of a general phenomenon. Zinc ions will complex to these chromophores, when they are not blocked by protein, to give characteristic spectra [9,10]. The selection of zinc ions as a control was based on their strong affinity for these tetrapyrroles and the ability to detect a complex by absorption spectroscopy.

2. Materials and methods

C-Phycocyanin was isolated from the cyanobacterium *Phormidium luridum*. The alga was grown in the laboratory, harvested and stored frozen. Cells were mixed with pH 6.0, 0.1 ionic strength, sodium phosphate buffer and treated with lysozyme. The biliproteins were extracted, precipitated with 50% saturated ammonium sulfate, and solutions further purified by chromatography at pH 7.0 on a column of hydroxyapatite using a potassium phosphate gradient. Allophycocyanin remained near the top of the column under these conditions. Solutions having an A_{620}/A_{280} above 4.0 were considered purified. Purity was checked by high performance liquid chromatography through a gel filtration column (the system will be described below). The photodiode array detector was used to identify the spectra of the protein eluting from the column. Only C-phycocyanin was detected, and the spectrum of allophycocyanin would be shown by the photodiode array detector. Purified protein was dialyzed into doubly-distilled water, lyophilized, and stored in a freezer.

Phycoerythrin 545 was isolated from the cryptomonad, *Rhodomonas lens*. The alga was grown in the laboratory, harvested by centrifugation, and stored frozen. Two cycles of freezing and thawing lysed the cells into pH 6.0 buffer. The protein was precipitated with 80% saturated ammonium sulfate and purified by two types of gel filtration chromatography as described previously [11,12]. An A_{545}/A_{280} above 8.0 was considered purified protein. The purified protein was dialyzed to distilled water, lyophilized and stored in a freezer.

Absorption measurements were performed on a Beckman DU640 spectrophotometer, and CD spectra were obtained on a JASCO J-720 spectropolarimeter. All spectra were obtained at 23.0°C; the temperature of samples in the DU640 was controlled by a Peltier device, and for the J-720 sample temperature was maintained by water circulating from a Neslab RTE-110 refrigerated circulator. The absorption measurements were done in a 1-cm light path, and the CD measurements were performed at light paths of 5 and 10 mm. Several CD scans were averaged to obtain the final spectrum in order to improve the signal-to-noise ratio. The spectrum of solvent was obtained in the same manner and subtracted from each CD spectrum of protein. The spectra were then smoothed using a Savitzky-Golay filter.

All the metal ion and urea studies were done in pH 5.0, 0.010 M, sodium acetate buffer. The concentration of phycoerythrin 545 was 0.10 g/l and those of C-phycocyanin were 0.050, 0.10, or 0.20 g/l. Ferric ion was studied at all protein concentrations, but zinc ion was not studied at 0.050 g/l. Ferric ion solutions were prepared fresh the same day as needed in the pH 5.0 buffer, and the pH readjusted to 5.0 after adding ferric chloride with a dilute solution of sodium hydroxide. Ferric ions were soluble under the conditions employed. Metal ions or urea were mixed with a protein solution and refrigerated overnight (20–29 h) or longer before measurements were made. A Hanna pH meter was used.

The zinc chloride was from Fisher Scientific, certified A.C.S. and the ferric chloride was ferric chloride hexahydrate from Sigma Chemical.

Samples of C-phycocyanin in pH 5.0 buffer at 0.20, 0.10 and 0.050 g/l were analyzed by high performance liquid chromatography using a Waters 525 liquid chromatography system. Samples were injected from an autosampler (Waters 717 plus) on to an Ultropac TSK G3000SW column (LKB). The column (8.0 mm × 300 mm) was equilibrated in pH 5.0 buffer and run under isocratic conditions. A photodiode array detector (Waters 996) was used to obtain spectra of the C-phycocyanin bands. The gel filtration column was calibrated with molecular mass standards of 1350, 17000, 44000, 158000 and 670000, which were vitamin B-12, myoglobin, ovalbumin, γ-globulin, and thyroglobulin, respectively (Bio-Rad, standards).

The CD spectrum of phycoerythrin 545 in pH 5.0 buffer was deconvoluted using the PeakFit program (Jandel Scientific). Four band types were tried either alone or in couples: Gaussian, Lorentzian, Voight and Pearson VII. The method used a Levenberg-Marquardt interactive fitting. Phycoerythrin 545 has eight chromophores located on a dimeric protein structure. Therefore, four bands would be the minimum required to fit the spectra. The actual fits may require more than four bands if exciton coupling existed between chromophores that ordinarily would have identical spectra, i.e., if two appropriate chromophores interacted across the monomer-monomer interface.

3. Results and discussion

3.1. Effects of metal ions

Ferric ions at pH 5.0 were seen to change the visible absorption spectra (Fig. 1) of C-phycocyanin. Results

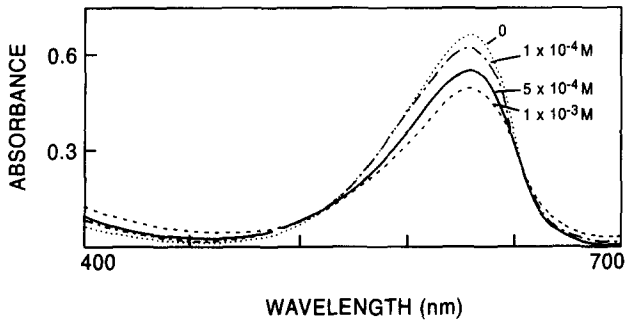

Fig. 1. Absorption spectra of C-phycocyanin and C-phycocyanin with ferric ions. Protein concentration was 0.10 g/l at pH 5.0, 23°C and a 1-cm light path. Ferric ion concentrations were: 0, $1 \cdot 10^{-4}$, $5 \cdot 10^{-4}$, and $1 \cdot 10^{-3}$ M.

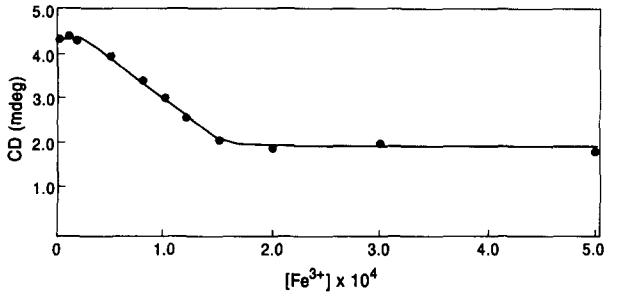

Fig. 3. A plot of CD at 600 nm of C-phycocyanin against ferric ion concentrations. Protein concentration was 0.050 g/l at pH 5.0, 23°C and a 1-cm light path.

were similar at 0.050, 0.10, and 0.20 g/l of protein, and are shown for 0.10 g/l (Fig. 1).

The changes in the CD spectra in the visible region were extensive (Fig. 2). At 0.050 g/l of protein, there was an extensive loss of rotational strength at the blue side of the maximum and some gain in intensity on the red side (Fig. 3). The half-point of the interaction occurs at $0.9 \cdot 10^{-4}$ M ferric ion for 0.050 g/l of protein. The protein at 0.050 g/l would be $4.76 \cdot 10^{-7}$ M based on 105 000 trimer molecular mass, having nine chromophores per trimer. Under these conditions, there are about 21 ferric ions for each chromophore, or 32 per polypeptide in these solutions, suggesting a weak interaction. At 0.10 and 0.20 g/l of protein, the effect of ferric was to lower blue-side intensity, but no gain in the red region was observed (Fig. 4).

Zinc ions possess the ability to complex with the chromophores of phycoerythrin and C-phycocyanin when their tetrapyrroles are isolated from the protein or on denatured protein as shown by absorption spectroscopy. However, it has been reported that zinc ions will not bind to these chromophores on native protein [9,10]. It appeared that the chromophores were situated on the protein so that they were inaccessible to the zinc and presumably other metal ions. An exception is phycourobilin, which could complex zinc ions on both native and denatured R-phycoerythrin [10].

Although previous work had shown no binding of zinc ions to these proteins, we repeated these experiments under our experimental conditions and using CD. Zinc ions at pH 5.0 were used in the same concentration range as with ferric ions. The results for both spectroscopic methods showed no significant changes with zinc ions (Fig. 4).

There are some differences in the results for 0.050 g/l of C-phycocyanin compared with the higher con-

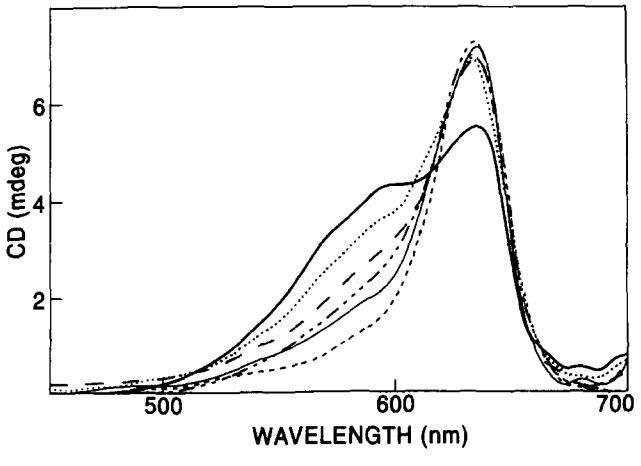

Fig. 2. CD spectra of C-phycocyanin and C-phycocyanin with ferric ions. Protein concentration was 0.050 g/l at pH 5.0, 23°C and a 1-cm light path. Ferric ion concentrations in order of high-to-low rotational strength at 600 nm: 0 (solid line), $0.5 \cdot 10^{-4}$ (dots), $0.8 \cdot 10^{-4}$ (long dashes), $1 \cdot 10^{-4}$ (dots dash), $1.2 \cdot 10^{-4}$ (thin solid), and $3 \cdot 10^{-4}$ (short dashes) M.

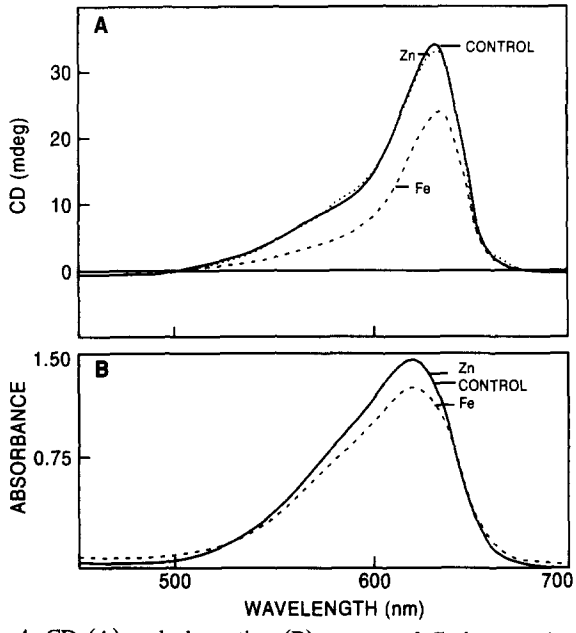

Fig. 4. CD (A) and absorption (B) spectra of C-phycocyanin and C-phycocyanin with ferric or zinc ions. Protein concentration was 0.20 g/l at pH 5.0, 23°C and a 1-cm light path. The control had no metal ion, and the zinc and ferric ion concentrations were $1 \cdot 10^{-3}$ M.

Table 1
The effect of protein concentration on the properties of C-phycocyanin at pH 5.0 measured by CD and chromatography

Protein concentration (mg/ml)	CD633 nm/ CD600 nm	Percentage of monomers
0.20	2.3	45
0.10	1.9	58
0.05	1.4	77

centrations. The first difference is observed from the protein in the absence of ferric ions (Figs. 2 and 4). The shape of the CD spectra shows a shift to higher energies as the protein concentration is lowered. These three concentrations of C-phycocyanin were further investigated using gel-filtration column chromatography (Table 1). All three concentrations showed the same two different molecular mass bands, but the percentage of the lower molecular mass form increased as the concentration decreased. The two forms were shown to have molecular masses indicating that they are monomer ($\alpha\beta$) and trimer ($\alpha_3\beta_3$) aggregates of C-phycocyanin. Secondly, the effect of ferric ion on the CD differs at 0.050 g/l of protein in that the low-energy chromophores are affected as well as the high-energy chromophores (Fig. 2). This effect may be related to the first, in that monomers of protein may react with ferric ions more extensively than with the larger aggregate, and could also be explained by the higher ferric ion-to-protein ratios achieved at the lowest protein concentration.

The effects of ferric and zinc ions were examined with phycoerythrin 545 at pH 5.0 (Figs. 5–7). Ferric ions affected the visible absorption and CD spectra and zinc ions did not. The protein concentration was 0.10 g/l and the metal ion concentration was varied from $1 \cdot 10^{-4}$ to $1 \cdot 10^{-3}$ M. The protein was mostly $\alpha_2\beta_2$ at this concentration. Spectra were obtained one day after solutions were prepared.

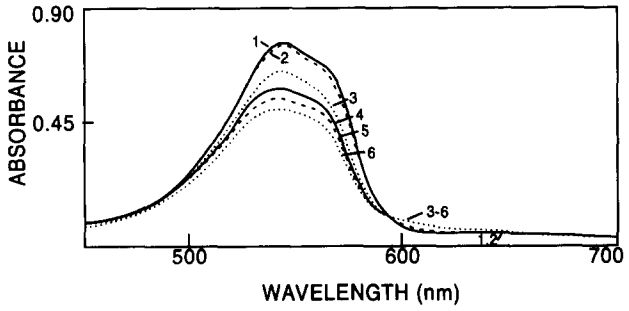

Fig. 5. The absorption spectra of phycoerythrin 545 and phycoerythrin 545 with ferric ions. Ferric concentrations were: 1. 0; 2. $1 \cdot 10^{-4}$; 3. $3 \cdot 10^{-4}$; 4. $5 \cdot 10^{-4}$; 5. $7.5 \cdot 10^{-4}$; 6. $1 \cdot 10^{-3}$ M. Number on each spectrum refers to ferric ion concentration. Protein concentration was 0.10 g/l at pH 5.0, 23°C and a 1-cm light path.

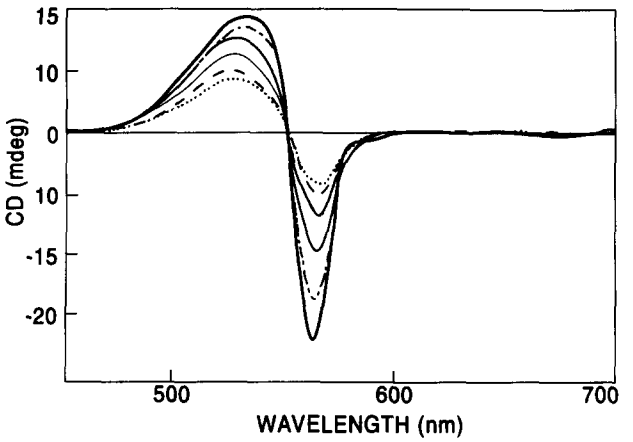

Fig. 6. CD spectra of the effects of ferric ions on phycoerythrin 545. The experimental conditions were the same as in Fig. 5. Ferric ion concentrations increased as rotational strength decreased.

3.2. Effect of urea

Urea in high concentrations is a protein denaturant. Its effect on C-phycocyanin has previously been studied [13]. In order to compare the effect of ferric ion with a substance of known capabilities, the study of urea and C-phycocyanin was repeated but using the same experimental conditions as applied with ferric ions. In a low-to-moderate concentration range, urea produced a shift to higher energy in the visible CD and absorption spectra (Fig. 8). This shift resembles the

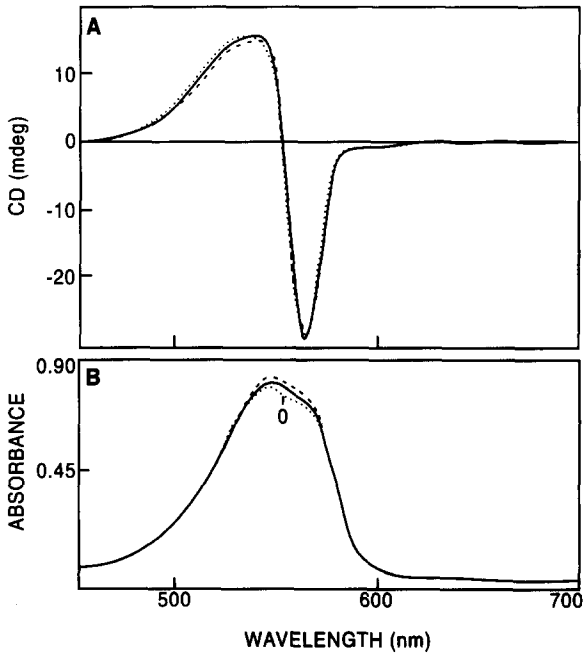

Fig. 7. CD (A) and absorption (B) spectra of the effects of zinc ions on phycoerythrin 545. The experimental conditions were the same as in Fig. 5. Zinc ion concentrations were the same as ferric ion concentrations in Fig. 5. Most spectra overlap extensively, and the zero metal ion concentration is shown as a dotted line.

one observed when the concentration of C-phycocyanin was lowered, and that effect was shown by chromatography to be caused by dissociation to monomers (Table 1). The spectroscopic shift was very different from the effect found with ferric ions (Figs. 1 and 2).

3.3. Chromophore organization

Biliproteins are found in three types of organisms – cyanobacteria, red algae and cryptomonads. The biliproteins in the cyanobacteria and red algae are arranged as organelles, phycobilisomes. C-Phycocyanin is located in the rod portion of the phycobilisomes and structurally serves as a nexus between the allophycocyanin core and any high-energy biliproteins that are present. In several cases, C-phycocyanin is the high-energy biliprotein, while in other organisms it is phycoerythrin or phycoerythrocyanin. The position of the chromophores of C-phycocyanin are known from X-ray crystallographic studies [14–17]. The three-dimensional structure of B-phycoerythrin has also been determined by X-ray crystallographic methods [18,19]. The knowledge of chromophore position has led to substantial progress in the study of C-phycocyanin and the related protein, allophycocyanin [20–28]. A monomer of C-phycocyanin is composed of two different polypeptides and has three chromophores of different energies. Calculations have shown that the two higher-energy chromophores can rapidly transfer their excitation energies

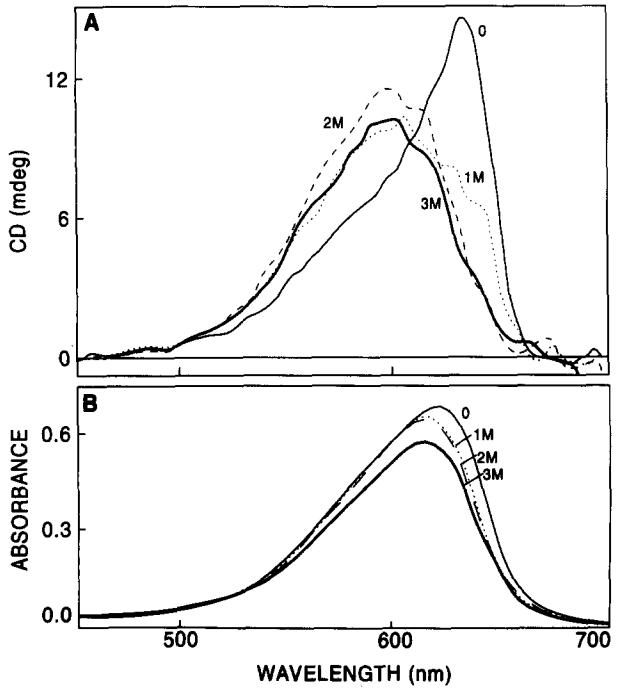

Fig. 8. CD (A) and absorption (B) spectra of C-phycocyanin and C-phycocyanin with urea. Protein concentration was 0.20 g/l at pH 5.0, 23°C, and a 1-cm light path.

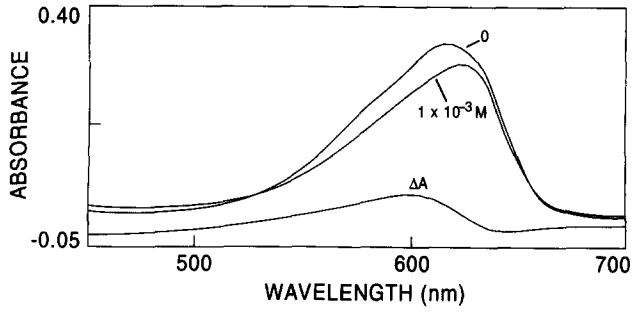

Fig. 9. Difference spectrum of the absorption of C-phycocyanin minus C-phycocyanin plus $1 \cdot 10^{-3}$ M ferric ions. C-Phycocyanin concentration was 0.050 g/l at pH 5.0, 23°C in a 1-cm light path. Spectra are shown for 0 and $1 \cdot 10^{-3}$ M ferric ion concentrations. The difference spectrum is labeled Δ A.

to the lowest-energy chromophore [23]. The positions of the absorption maxima for the three chromophores has been found at 600, 624, and 628 nm [29], and at somewhat different maxima by others who used a different algal source [30,31]. Ferric ions especially influence the absorption of the high-energy chromophores (Fig. 1). An absorption difference spectrum for C-phycocyanin with and without ferric ions illustrates this effect (Fig. 9). The maximum in the difference spectrum is at 600 nm, exactly where the highest-energy chromophore is located. Clearly, even the affected chromophores lose only a small quantity of absorption compared with a large change in chirality (Fig. 2).

Phycoerythrin 545 has been purified from a cryptomonad. Cryptomonad biliproteins do not form phycobilisomes [32]. The cryptomonads biliproteins are isolated as $\alpha_2\beta_2$ polypeptide structures, which usually have eight total chromophores [11,12], although there is at least one exception to the chromophore count [33]. The topography of the chromophores is not known, and there has been no detailed X-ray diffraction study on crystals of any cryptomonad biliprotein. The results of these experiments with ferric ions, however, have presented an excellent chance to evaluate some of the organization of these chromophores. The opportunity is forthcoming from a unique faculty of CD spectroscopy. If two chromophores are sufficiently close, their excitation dipoles will interact and the absorption spectra of the chromophores will be split into high- and low-energy forms. In general, the splitting may typically be very small and is detected as only very small changes in the absorption spectrum. For CD, however, exciton splitting has a salient appearance. Exciton splitting results in a CD spectrum that is conservative, i.e., it is composed of a positive and a negative band of equal rotational strengths. The rotational strengths of bands will be increased by exciton splitting.

When a difference CD spectrum for phycoerythrin 545 is generated by subtracting the spectrum in $3 \cdot 10^{-4}$ M ferric ions from the spectrum without ferric ions, the

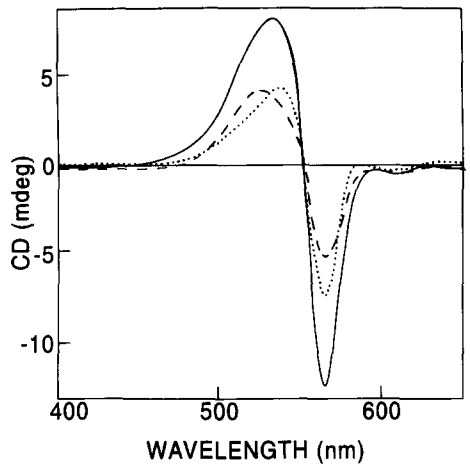

Fig. 10. Difference CD spectrum of phycoerythrin 545 minus phycoerythrin 545 plus ferric ions. The difference spectrum is shown in dots. Ferric ion concentration was $1 \cdot 10^{-3}$ M. Protein concentration was 0.10 g/l at pH 5.0, 23°C, and a 5-mm light path. Spectra were taken two days after sample preparation. The CD spectra of phycoerythrin 545 (solid line) and phycoerythrin 545 plus $1 \cdot 10^{-3}$ M ferric ion (dashed line) are shown for comparison.

result is a spectrum where the shape is fairly similar to that expected from exciton coupling (Fig. 10). Furthermore, the spectrum in $3 \cdot 10^{-4}$ M ferric ions still exhibits the characteristic CD band pattern of exciton splitting, but the spectrum is shifted from that of the ferric-sensitive pair of coupled chromophores (Fig. 10). An interpretation of this effect may be that there are two different pairs of chromophores both producing exciton splitting with each pair having slightly different spectra. The positive bands for the proposed exciton splitting are particularly different, about 12–13 nm apart, when the ferric-sensitive spectrum is compared with the spectrum of the more stable pair (Table 2). The greater splitting for the residual excition couple perhaps suggests that the chromophores in this pair may be closer together than in the pair affected by urea. The results shown in Table 2 suggest that bands having maxima of 527–529 and 539–542 nm may exist in the CD spectrum of phycoerythrin 545. A deconvolution of the CD spectrum of phycoerythrin 545 was carried out using four bands – the number of chromophores on a monomer ($\alpha\beta$). The maxima for the components were at 564, 541, 528 and 490 nm, and thus strongly support the validity of the results shown in Table 2. The fit had for the highest-energy band a Pearson, and the three lowest-energy bands were Gaussian. Although these CD results possibly are evidence for exciton splitting, additional experimentation is required before more analysis is warranted. There are several other possible phenomena that can contribute to a CD spectrum in addition to exciton splitting.

Table 2
The CD maxima of phycoerythrin 545 with ferric ions[a] and of the difference spectra of phycoerythrin 545 minus phycoerythrin 545 plus ferric ions[b]

[Ferric]·10^4 (mol/l)	CD maxima (nm) [a]		ΔCD maxima (nm) [b]	
	–	+	–	+
3.0	564	529	562	542
5.0	564	528	562	541
7.5	565	527	562	539
10.0	566	527	563	539

4. Conclusion

Ferric ions are shown to change the visible absorption and CD spectra of two quite different biliproteins, C-phycocyanin and phycoerythrin 545. The interaction of ferric ions with these proteins is established, but its function, if any, is unknown. Since zinc ions apparently do not bind, it is possible that the interaction of ferric ion and biliproteins is specific. It is not apparent that ferric ions would have a role in the primary function of biliproteins – light harvesting and energy migration.

The interaction of ferric ions with phycoerythrin 545 has proven useful in demonstrating that exciton splitting may occur for their chromophores. Exciton splitting will extend the energy range of light harvesting and will establish the intraprotein exciton migration patterns. Energy transfer between high- and low-energy bands formed by exciton splitting will be designated to occur by internal conversion, and energy transfer between other chromophores will be by very weak dipole-dipole coupling [34].

For C-phycocyanin and other biliproteins, studies on model membranes showed that the protein had a pivitol effect on the direction of electron flow across chlorophyll-containing lipid bilayers [8]. The systems were prepared with various concentrations of ferric ion in the aqueous solution with the C-phycocyanin. These solutions were placed on one side of a chlorophyll-containing lipid bilayer and a solution without the protein was on the other side. These spectroscopic studies show that interactions between ferric ions and the protein would have occurred in the solutions used for the membrane studies.

What are some of the possibilities for ferric-biliprotein interaction? For the protein transferrin, the binding site is a ferric ion octahedrally complexed to an aspartic acid, a histidine, two tyrosines and two positions to a carbonate anion [35,36]. Bicarbonate is present because it aids in the incorporation of ferric ion into the protein. For the nitrogenase molybdenum-iron protein, there are many iron atoms that are coordinated to a number of sulfur atoms in tetrahedral or trigonal symmetry [37]. For biliproteins, the tetrapyrroles are another option, as only the lack of

coordination to zinc ions argues against them. Park and Sauer [38] have demostrated that cupric ions will bind to the tetrapyrroles of monomeric C-phycocyanin. The changes in the chromophore absorption resulting from cupric binding were evaluated.

Recently, CD results for several biliproteins have been published [39–42]. The unfolding of C-phycocyanins from a mesophilic and a thermophilic organism were studied in detail [42].

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