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The route of exciton migration in phycocyanin 612

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The biliprotein, phycocyanin 612, was purified from the cryptomonad, *Hemiselmis virescens*, and its chromophore content confirmed by an analysis of its tryptic peptides. Chromatography of a tryptic digest indicated that the isolated protein has three different phycocyanobilin chromopeptides and a single cryptoviolin chromopeptide. Spectroscopic data indicated that each different chromopeptide occurs twice for a total of eight chromophores on each oligomer. The tryptic digest data and the results from earlier experiments suggest that the protein may have two identical sets of four chromophores that comprise an energy-transfer unit within the protein. CD spectroscopy on phycocyanin 612 in the visible region of the spectrum was used to obtain information on the topography of these chromophores. Two chemicals, potassium permanganate and sodium thiocyanate, were used to alter the CD spectrum. The effects of both these chemicals suggested a close relationship between two of the four observed components in the visible CD spectrum of this biliprotein. This chromophore relationship is assigned to strong coupling of the dipoles of the two lowest-energy chromophores and produce exciton delocalization between these chromophores. Each dimeric protein has two identical delocalized pairs that produce spectral splitting and are the fluorescence emitters of the isolated protein. Based on this evidence and data from the literature, a tentative model is presented that shows the exciton migration route for phycocyanin 612. After excitation of the highest-energy chromophore, cryptoviolin, excitons are transferred by very weak dipole coupling to the highest-energy phycocyanobilin and from there by the same mechanism to the strongly coupled pair of phycocyanobilins. The identical process occurs on both halves of the protein.

Introduction

Biliproteins are light-harvesting and excitation-energy-transfer photosynthetic pigments found in blue-green, red and cryptomonal algae (for a review, see Ref. 1). Biliproteins have been shown to transfer excitation energy to Photosystem II. This migration occurs first among the bilins (linear tetrapyrrole chromophores) on a particular biliprotein aggregate and then to other neighboring biliproteins. Phycocyanin 612 is one of the six spectral types of biliprotein that have been isolated so far from the cryptomonads (*Cryptophyceae*). Its quaternary protein structure ($\alpha_2\beta_2$) and chromophore content have been studied [2]. It has been established that the α subunit of many, and perhaps all, cryptomonad

Abbreviation: TFA, trifluoroacetic acid.

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biliproteins is heterogeneous and results from at least two gene products [3–6].

Together with the structural data, both steady-state and ultrafast time-resolved spectroscopy have been used to study the functions of phycocyanin 612 [2,7–9]. Spectral deconvolution studies suggest that the chromophores are grouped into four separate energy states [9]. The questions asked by this research are how the four energy states are formed, their topography, and how the excitons migrate through them. CD spectroscopy can greatly aid in this task because of its salient ability to detect chromophores that are close enough to produce exciton delocalization and spectral splitting. In this study, the visible CD spectrum of phycocyanin 612 is measured in the presence of varying concentrations of either sodium thiocyanate or potassium permangate. The ensuing data are analyzed to enable our formulation of an exciton migration model for the pigment. Analysis of the number of chromopeptides after trypsin digestion of the biliprotein is used to assay its chromophore content and to determine if any identical chromophore binding sites occur on the protein.

Experimental

Hemiselmis virescens (Plymouth number 157, Culture Centre of Algae and Protozoa, Cambridge, England, LB 984/5) was grown, harvested and lysed, and its phycocyanin 612 purified as described previously [2]. The purification protocol included ammonium sulfate precipitations and chromatography on Sepharose 6B (Pharmacia) and Ultrogel AcA54 (LKB). The purified protein was exhaustively dialyzed into distilled water and lyophilized. The protein was then stored in the cold until needed.

Experiments were carried out after dissolving the protein in pH 6.0, 0.1 ionic strength, sodium phosphate buffer. The protein solution is freed of any undissolved protein by a brief, low-speed centrifugation. Solutions were prepared by mixing the appropriate proportions of three solutions: a concentrated phycocyanin 612 solution; a concentrated solution in the identical pH 6.0 buffer of either sodium thiocyanate (reagent grade, Matheson, Coleman & Bell), or potassium permanganate (reagent grade, Fisher); and the necessary volumes of additional pH 6.0 buffer. The thiocyanate and permanganate solutions were allowed to remain at room temperature for about 1 h and then stored in the cold overnight. The next morning the solutions were allowed to warm to room temperature and spectroscopic measurements taken.

CD spectra were taken using a Cary model 61 spectropolarimeter and absorbance measurements were made on a Perkin-Elmer model 320 spectrophotometer. All measurements were made in a 1-cm light path at room temperature. Experiments were performed on solutions at about 0.09 mg/ml. Fluorescence spectra were taken on a Perkin-Elmer model 44A spectrofluorometer. These solutions had absorbance in a 1-cm light path of 0.1 at 612 nm to avoid reabsorption of emission.

Peptides of phycocyanin 612 were obtained by treating the protein with *N*-tosylamido-L-phenylethylchloromethylketone trypsin (Cooper Biomedical) as follows: 50 mg phycocyanin 612 and 1.0 mg trypsin (2% w/w) were mixed in 4.0 ml of 0.02 M HCl and, after 10 min, 39.5 mg NH_4HCO_3 were added and the pH adjusted to 8.0 with 2 M NaOH. This solution was placed under nitrogen and shaken in the dark at 36° for 17 h. To test the reaction for completion, some solutions were run for longer periods of time. Other experiments were performed using larger quantities of trypsin, 2.6 and 3.0% w/w. The reaction was terminated by adding 2.6 ml of glacial acetic acid. Portions of this solution were analyzed by high performance liquid chromatography (HPLC) on a Beckman model 342 chromatograph with a model 165 dual-wavelength detector using a Bakerbond Wide-Bore C18 column and a linear gradient from 0.1% trifluoroacetic acid (TFA) in H_2O to 0.1% TFA/CH_3CN (1/4) at a flow rate of 1.0 ml/min. Elution from the column was monitored at both 660 and 590 nm. These solutions were then lyophilized and stored in the cold.

Results

Salts, such as sodium thiocyanate and sodium perchlorate, are known agents for biliprotein dissociation [10,11]. When sodium thiocyanate was mixed with solutions of phycocyanin 612, the CD (Fig. 1) and absorbance (data not shown) spectra were observed to decrease in intensity as the salt

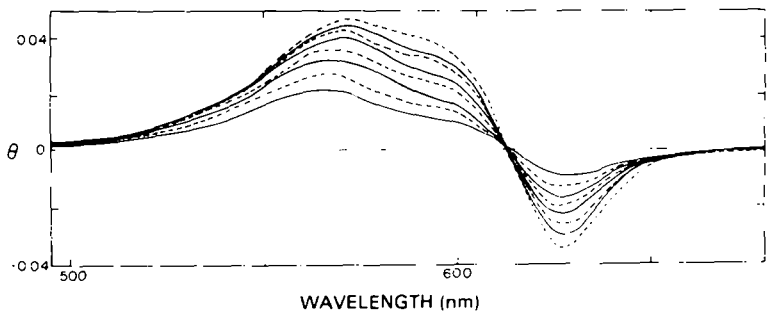

Fig. 1. Effect of sodium thiocyanate on the CD spectra of phycocyanin 612. Spectra were taken in pH 6.0 buffer, in a 1-cm light path, and at room temperature. NaSCN concentrations are 0, 0.10, 0.20, 0.30, 0.40, 0.50, 0.60 and 0.70 M from top to bottom.

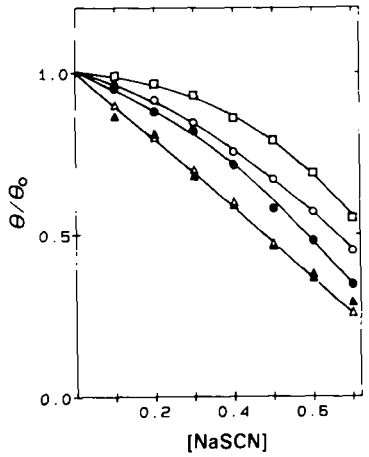

Fig. 2. Plot of the decline of the CD bands versus sodium thiocyanate concentration. ○ represents the decay of the rotational strength at 572 nm, while ● represents the actual 572-nm component calculated by subtracting the contribution of the 553-nm component; □, the 553-nm component; △, the 605-nm component; ▲, the 624-nm component. θ/θ_0 is the percentage change against the solution with no thiocyanate.

concentration increased. There were also changes in the shape of the visible CD spectra (Fig. 1). The variation in salt effect as a function of wavelength is shown for four selected wavelengths (Fig. 2). These wavelengths corresponded to the absorption maxima of the four components in the spectral deconvolution of phycocyanin 612 [9].

Identical experiments were also carried out with potassium permanganate. As with sodium thiocyanate, permanganate treatment produced a lowering of CD intensity at all wavelengths together with a change in the shape of the CD spectra (Fig. 3). The same four wavelengths are used to produce an intensity versus salt concentration plot (Fig. 4) for the permanganate effect.

Fluorescence excitation spectra (uncorrected) have been obtained for phycocyanin 612 solutions in pH 6.0 buffer as a function of emission wavelength between 640 and 720 nm. The excitation spectra were identical to each other in all cases (Fig. 5).

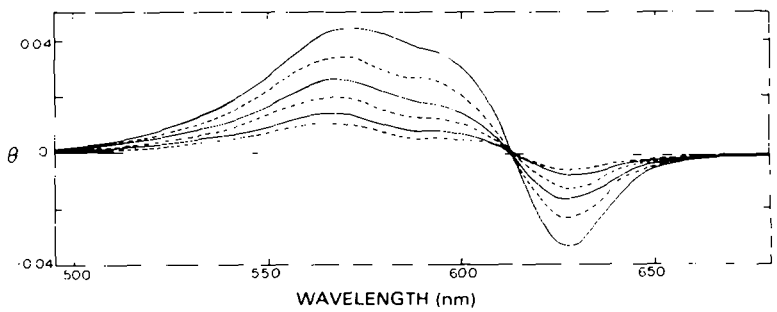

Fig. 3. Effect of potassium permanganate on the CD spectra of phycocyanin 612. Spectra were taken in pH 6.0 buffer, a 1-cm light path, and at room temperature. $KMnO_4$ concentrations are 0, 3.8, 7.6, 11.4, 15.2 and $19.0 \cdot 10^{-6}$ M from top to bottom.

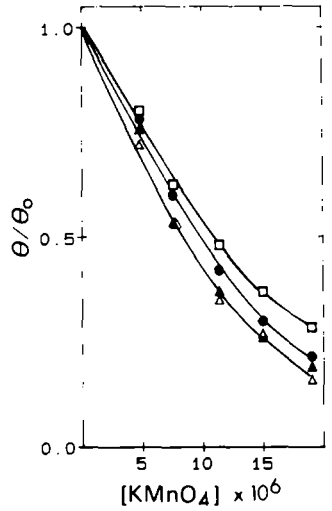

Fig. 4. Plot of the decline of the CD bands of phycocyanin 612 vs. potassium permanganate concentration. ●, the 572-nm component; □, the 553-nm component; △, the 605-nm component, ▲, the 624-nm component. θ/θ_0 is the percentage change against the solution with no premanganate.

HPLC results are shown at 660 and 590 nm for a particular experiment (Fig. 6). Phycocyanobilin has an absorption maximum at about 660 nm and cryptoviolin has a maximum at 590 nm. The two wavelengths are monitored for the same sample using two recorders simultaneously. In this sample a smaller second cryptoviolin band is observed at a slightly shorter retention time than the central phycocyanobilin band (Fig. 6). This minor band is absent in experiments using longer digestion times. The areas under each phycocyanobilin band are nearly identical. Longer digestion times do not affect this elution profile. The results shown are for a 2.0% w/w trypsin to phycocyanin 612 ratio, and additional experiments were done at 2.6 and 3.0%. At these high ratios the slowest eluting chromopeptide, containing phycocyanobilin (Fig. 6, 660 nm), is progressively shifted to a slightly faster elution time (data not shown). The other major chromopeptides peaks are unchanged by the increase in trypsin. The minor peaks in these elution profiles (Fig. 6) are too small to represent significant entities and are considered to be partially proteolyzed fragments that resist complete trypsin cleavage.

Discussion

Spectral measurements on phycocyanin 612 in acidic urea have been used to calculate its chromophore content [2]. For each $\alpha\beta$ unit of protein, there were three phycocyanobilins and one cryptoviolin chromophore. This result depended on the absorptivity of the bilins and bilin stability in acidic urea. These factors are not rigorously known and an independent check on these results is necessary. An exhaustive trypsin digestion was therefore, performed and the chromopeptides analyzed by HPLC (Fig. 6).

If the protein is exhaustively digested by trypsin and the peptides containing each chromophore are unique, the number of chromophores may be obtained. When 660 nm is monitored, there are three main peaks eluted from the HPLC colums and, at 590 nm, and additional peak is observed.

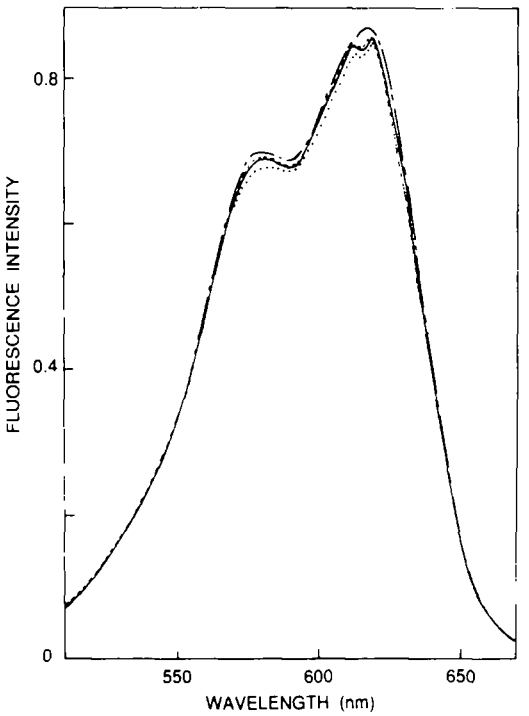

Fig. 5. Fluorescence excitation spectra (uncorrected) of phycocyanin 612 as a function of emission wavelength. In this case, emissions at 720, 700, 680 and 640 nm were used to generate excitation spectra. Repeating this experiment demonstrated that there is essentially no difference among the various spectra: 720 nm (- — - — -), 660 nm (· · · · · ·), 700 nm (———), 640 nm (- - - - - - -).

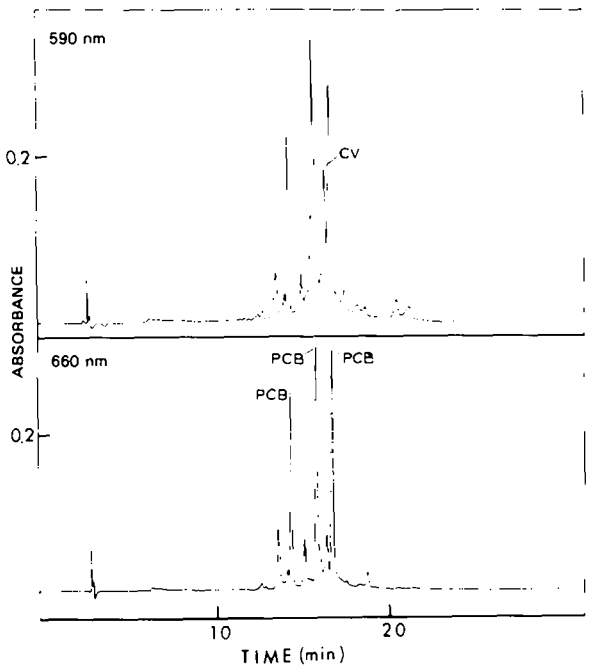

Fig. 6. Separation of the chromopeptides of phycocyanin 612 by HPLC after tryptic digestion. CV is cryptoviolin and PCB is phycocyanobilin. Cryptoviolin has an absorption maximum at 590 nm and phycocyanobilin absorbs maximally at about 660 nm in acidic solvents and when not part of the native protein.

The three 660-nm bands corresponds to the spectroscopic result of three phycocyanobilins and the additional band at 590 nm correspond to the previous finding [2] of a single cryptoviolin on each half of the protein. In addition, this peptide analysis supports the idea that the two $\alpha\beta$ halves of the protein are virtually identical. The detection of four unique chromopeptides, also, agrees very well with the deconvolution results which showed that the visible absorption and CD spectra each consist of four components [9]. The complete protein ($\alpha_2\beta_2$) then has a total of six phycocyanobilins and two crytoviolins as suggested by the spectroscopy experiments [2].

The shape of the visible CD spectrum of phycocyanin 612 shows a group of positive bands and a negative band (Fig. 1). Previously, we have shown that this CD spectrum can be deconvoluted into four components, having wavelength maxima at 553, 572, 605 and 624 nm [9]. The eight bilins of the protein reduce to four spectral components because the protein may be composed of two virtually identical halves. When two chromophores are close together and properly oriented, they can interact in a way that produces spectral splitting and delocalization of excitation between them (strong coupling of dipoles). Such a delocalization phenomenon results in a characteristic sigmoidal CD spectrum having a positive and a negative band. The question is whether the chromophores of phycocyanin 612 have a pair of chromomophores that share delocalized excitation and, if so, which of the three positive components is involved.

The strategy argues that, if a pair of bands is really the result of band splitting from shared excitation, what happens to one band must happen identically to the other band. This concept was first used in a study of the effect of potassium permanganate and mercuric acetate on another biliprotein [12]. We have also used potassium permanganate, which probably operates on phycocyanin 612 by oxidizing its tetrapyrrole chromophores. The chemical nature of bilins permits them to be readily oxidized. In addition, we have selected sodium thiocyanate because its mode of action must be entirely different from that of permanganate. In this case, sodium thiocyanate, a chaotropic salt, acts to produce subunit dissociation and unfolding of the protein. The chromophores then experience changes in environment and conformation that can affect the oscillator strengths of their energy levels. Bilins are held in a linear conformation by the apoproteins. This conformation causes a strong absorbance in the visible region and when biliproteins dissociate or partially unfold the bilins take on a more cyclic conformation which has a lower visible absorbance (for a review, see Ref. 1). By employing two entirely different tactics for chromophore perturbation, it was thought that more cogent arguments concerning the chromophores could be constructed. Similar spectral changes from both reagents would then suggest the response is a characteristic of the bilin arrangement.

A comparison of the effects of these chemicals on the CD spectra of phycocyanin 612 shows an important similarity between the activities of permanganate and thiocyanate (Figs. 2 and 4). In both cases, the rotational strengths at 624 and 605 nm decline identically when calculated on a per-

centage decrease from the control solution. The identity of behavior is, of course, our criterion for a delocalized pair. Moreover, as required, one of the bands of this pair is the negative CD band. The remaining two bands, which decline independently, are deemed not to be parts of delocalization events.

The deconvolution components of the CD spectrum of phycocyanin 612 show a complication regarding the 572-nm band. At its maximum, this band is strongly overlapped by the CD band of the higher-energy component [9]. Therefore, the decline at 572 nm is really a joint effect of two components. Since the deconvolution shows the proportion of the two components at 572 nm [9] and the percentage decline of the pure 553-nm component is known (Figs. 2 and 4), it is possible to calculate the amount of pure 572-nm component and its percentage decrease. When this correction is made, it is still clear that this component is behaving independently of the delocalized pair (Figs. 2 and 4).

Having proposed that two chromophores of phycocyanin 612 are close enough to form a delocalized pair with bands having absorption maxima at 603 and 622 nm, these data and those from the literature [2,7] can be used to formulate a tentative model for the bilin-to-bilin flow of excitons through the dimeric protein as follows.

(1) Results from spectroscopic analysis of urea denatured protein [2] and the tryptic peptide results (Fig. 5) suggest that there are six phycocyanobilins and two cryptoviolins per dimer.

(2) Results from the deconvolution of the spectrum [9] and the number of tryptic peptides (Fig. 6) both show four components and, in addition, the protein subunit structure is divided into two halves ($\alpha\beta$ and $\alpha'\beta$). These findings suggest that the eight bilins are divided into two identical groups of three phycocyanobilins and one cryptoviolin each.

(3) Picosecond time-resolved spectroscopy of phycocyanin 612 [7] has indicated that the 553-nm band (cryptoviolin) transfers excitons in less than 7 ps to the phycocyanobilin with an absorption maximum at 572 nm. The excitation of this highest-energy phycocyanobilin decays in 10 ps and gives rise to the excitation of the fluorescing components.

(4) CD studies using $KMnO_4$ and NaSCN treatments of phycocyanin 612 show the two lowest energy phycocyanobilins are coupled (Figs. 1–4). We assume this coupling is indicative of exciton delocalization because of the sigmoidal shape of the CD spectrum of these two bands.

(5) Steps 1 and 2 above suggest that we can reduce the problem of understanding of exciton flow from 8 to 4 different bilins. The ultrafast spectroscopy results clearly show the processes occurring for the two highest energy bilins. Since the lowest energy bilin must serve as the fluorescing chromophore, the role of one bilin – the one having a maximum at 605 nm – was unknown after the kinetics studies. This research has now answered the question and the fluorescing chromophore is in fact a pair of bilins. A model can thus be constructed for the topography of the chromophores and the flow of excitons (Fig. 7). It is also quite possible that energy is exchanged between the two pairs of emitters on either half of the protein.

The model (Fig. 7) suggests that there is only one type of emitter for the protein. This assignment can be tested by fluorescence spectroscopy. The excitation spectra of phycocyanin 612 in pH 6.0 buffer, in which the dimeric form is stable, have been determined as a function of emission wavelength. These excitation spectra are found to be independent of emission wavelength (Fig. 5) as is expected for a single type of emitter. The rates of excitation energy transfer between pairs of bilins is quite fast (7 and 10 ps) compared to the normal emission lifetime of over 1000 ps. Therefore, very little emission from these high-energy bilins will be observed in the steady-state spectra. Recent calculations on another biliprotein, C-phycocyanin, are important in this regard [13]. They find that there is an equilibrium between two spectrally distinct chromophores. They show that 80–85% of the emission may come from one chromophore and the rest from a second, higher energy chromophore. This type of mechanism may be operative in phycocyanin 612 but current evidence supports the delocalized pair as the emitter. When this protein is part of the intact photosynthetic system, this excitation on the strongly coupled pair is transferred with high efficiency to the next pigment in the chain instead being emitted as

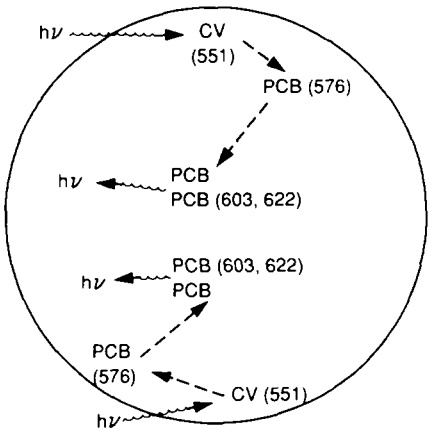

Fig. 7. A model of the flow of excitons among the chromophores of phycocyanin 612. The scalloped arrows are photons. The dashed arrows are excitation energy-transfer events that occur by very weak coupling of dipoles, which does not involve band splitting. The pairs of chromophores that have been shown by CD measurements to be involved in exciton delocalization are drawn close together, and have absorption maxima at 603 and 622 nm. The maxima in the CD spectrum for the delocalized pair are 605 and 624 nm [9]. An important criterion in energy transfer by very weak coupling of dipoles is the amount of spectral overlap between the fluorescence emission of the donor and absorption of the acceptor. This model adheres very well to this requirement. There is, also, a smaller amount of spectral overlap between the cryptoviolin chromophore and the delocalized pair of absorption bands. Although not shown in the diagram, a certain amount of excitation energy could be transferred directly from the cryptoviolin to the delocalized pair depending on certain other factors, the distance between the chromophores and the dipole-to-dipole orientation.

fluorescence. For C-phycocyanin, it has also been suggested that a bilin may serve as a sensitizing chromophore in one system, but is has a new route for energy transfer in a more complex situation [13]. This type of behavior may possibly also occur for phycocyanin 612 and therefore both fluorescing and sensitizing bilins may be considered candidates for transferring energy out of a dimer, but no data exist on this topic as yet. Additional studies on the excition migration in phycocyanin 612 could include time-resolved spectroscopy on the femtosecond level, X-ray crystallography, and calculations on the chromophore orientations in the delocalized pair.

A key feature of our hypothesis for exciton flow within the isolated phycocyanin 612 molecule is that it mixes both delocalization of excitation between two closely spaced pairs of phycocyanobilins and exciton transfer by very weak coupling (Förster inductive resonance) in the other cases (Fig. 7). Excitation energy transfer by very weak coupling of dipoles [14] has frequently been discussed for biliproteins [13,15,16].

Acknowledgement

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