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RESEARCH NOTE

BILIPROTEINS FROM THE BUTTERFLY *Pieris brassicae* STUDIED BY TIME-RESOLVED FLUORESCENCE AND COHERENT ANTI-STOKES RAMAN SPECTROSCOPY

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Abstract—The fluorescence decay time of the biliverdin IXγ chromophore present in biliproteins isolated from *Pieris brassicae* is determined to be 44 ± 3 ps. This value suggests a cyclic helical chromophore structure. The vibrational frequencies determined by CARS-spectroscopy are compared with those of model compounds. The data confirm that the chromophore in the protein-bound state adopts a cyclic-helical, flexible conformation.

INTRODUCTION

Biliproteins with biliverdin-type chromophores are found in plants and algae as well as in insects (Kayser, 1985). There are, however, considerable differences in both apoprotein and chromophore structure of chromoproteins found in plant bile pigments (Scheer, 1986; Rüdiger, 1971) and those isolated from insects like the butterfly *Pieris brassicae*. In the latter case the chromophore, namely biliverdin IXγ (Rüdiger, 1971), is not covalently linked to the apoprotein. From absorption, emission and circular dichroism studies Scheer and Kayser (1988) concluded that the chromophore should adopt a cyclic helical structure in the protein-bound state, whereas in phycobiliproteins from e.g. blue-green algae, the chromophore is held in an extended conformation by specific chromophore–protein interaction (see Fig. 1 for chromophore structures). In this note, we present additional experimental evidence for the above mentioned hypothesis on chromophore geometry.

MATERIALS AND METHODS

Details on the preparation of the biliprotein are given elsewhere (Kayser and Zipfel, 1988). Absorption and stationary emission spectra were recorded before and after each of the described measurements to check for photoinduced deterioration; the absorption spectra were identical to those shown by Scheer and Kayser (1988).

Polarized fluorescence decay curves were recorded by means of a Hamamatsu synchroscan streak camera. For excitation in the red absorption band the output from a synchronously pumped dye laser was used. Details on the apparatus and the data handling have been described previously (Hefferle *et al.*, 1984).

Resonance-enhanced Coherent Anti-Stokes Raman Scattering (CARS)‡ was recorded with an apparatus described by Schneider *et al.* (1987). Two excimer laser-pumped dye lasers generated the pump and Stokes beam, respectively, with wavelengths λ_p (fixed) and λ_s (variable). The intensity of the generated anti-Stokes radiation (wavelength λ_{as}) depends non-linearly on both the solvent background and the resonance-enhanced solute contribution, for which reason rather complex line-shapes can occur (for more details see e.g. Schneider *et al.*, 1987a and references therein).

RESULTS

The red absorption band of the native biliprotein extends from about 700 to 590 nm (broad maximum around 660 nm, shoulder around 600 nm). The excitation wavelength was kept constant at 620 nm for all time-resolved fluorescence measurements, i.e. excitation occurred between short wavelength shoulder and absorption maximum.

Fluorescence was monitored under magic angle either without or with spectral selection by interference filters (716 nm and 743 nm). In addition fluorescence decay curves were recorded with the analyzer parallel (I_p) and orthogonal (I_s) to the polarization of the excitation light. Since all decay curves looked alike, only one example is shown in Fig. 2 to demonstrate the achieved *S/N* ratio, the time resolution and the experimental curves on the basis of a single-exponential decay law (decay time 44 ± 3 ps). It is noteworthy that even the polarized decay curves could be fit by mono-exponentials with the same lifetime. The ratio of the amplitudes $I_p(t=0)/I_s(t=0)$ is found to be 3:1 (under otherwise

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‡*Abbreviations*: CARS, Coherent Anti-Stokes Raman Scattering

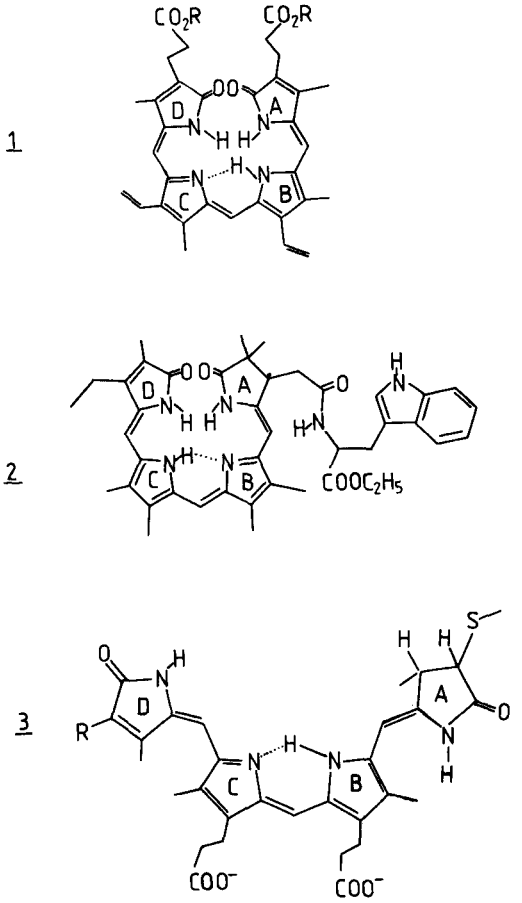

Figure 1. Structures of chromophores. (**1**) Biliverdin IXγ in biliprotein of *Pieris brassicae*. (**2**) Tryptophan substituted 2,3-dihydrobilatriene (model chromopeptide). (**3**) Phycocyanobilin (extended geometry).

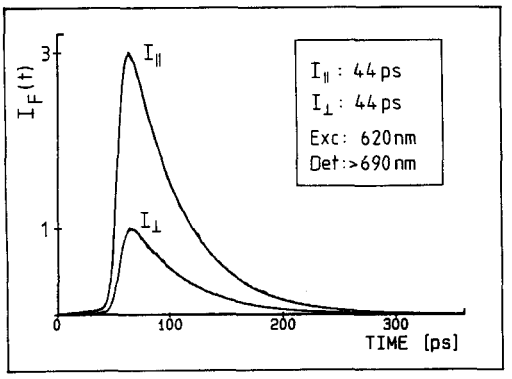

Figure 2. Isotropic fluorescence decay curves of biliprotein isolated from *Pieris brassicae*. Excitation 620 nm; detection 743 nm. Solid line is fit curve with τ = 44 ps.

identical conditions). From this, the limiting value for the fluorescence anisotropy $R(t=0)$ is derived to 0.4.

The pump wavelength for recording the CARS-spectra was chosen to be λ_p=640 nm in order to get a strong resonance enhancement. By tuning the Stokes laser beam from 685 to 720 nm, the difference covers the interesting vibrational frequency region between 1100 and 1700 cm^{-1} (Fig. 3). Absorption spectra recorded before and after a CARS-scan were nearly identical ($\Delta OD < 1.5\%$). As CARS-spectra recorded with different sample concentrations produced the same spectra, a perturbation of the sample composition by the pump and Stokes laser can be excluded. The overall appearance of the CARS-spectrum is similar to that of phycocyanin trimers from *Mastigocladus laminosus* (Schneider *et al.*, 1987). There are, however, distinct differences in the two spectral regions in which the CC-single and double bond stretching frequencies and the CH bending vibrations, respectively, appear. Only two intense vibrations are found in the finger print region: λ_1 = 1230 cm^{-1} and λ_2 = 1260 cm^{-1}. Above 1600 cm^{-1} also two bands appear, a stronger one around 1620 cm^{-1} and a weaker one around 1630 cm^{-1}. In addition, there are fairly strong bands located at 1592, 1505, 1463, 1436, 1412 and 1350^{-1}. The positions marked in Fig. 3 refer to the maxima in the spectrum. Due to the above mentioned complex line shape function, the molecular vibration frequencies can—depending on actual line-shape—differ from the peak position by as much as one linewidth.

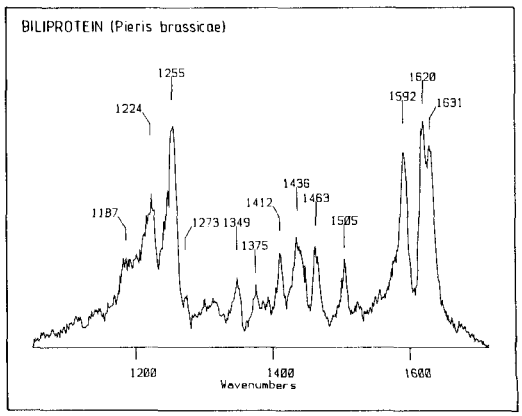

Figure 3. CARS-spectrum of biliprotein of Fig. 1. λ_p = 640 nm; 685 < λ_s < 780 nm.

DISCUSSION

The most intriguing features of the fluorescence decay curves of the insect biliprotein, especially when compared to the results of algal pigments, are the following:

(i) Although absorption and emission spectra are rather broad, there is no indication that the fluorescence originates from more than one emitting species (mono-exponential decay at all observation wavelengths). The distance between like chromophores must be much larger than the radius for efficient energy transfer (Förster radius) and the orientational relaxation time must be much larger than the electronic lifetime since no fluorescence depolarization is observed (the time course of the

polarized fluorescence $I_p(t)$ and $I_s(t)$ is identical within experimental error). Additional evidence for this conclusion is provided by the fluorescence anisotropy calculated for $t = 0$. The derived value of 0.4 corresponds to the theoretical limit, when absorbing and emitting transition dipole moments are parallel to each other. This is in agreement with the presence of one chromophore per apoprotein (mol. wt. = 25 000 KDa) and the absence of aggregation (Kayser and Zipfel, 1988).

(ii) In biliproteins isolated from blue-green algae, the geometry of the chromophore in the native state is an extended one, which is rigidly fixed by interactions with the protein (see e.g. Scheer, 1982; Schirmer *et al.*, 1985, 1987) exhibiting an excited state lifetime between 1 and 2 ns (for a review see e.g. Scheer, 1986; Holzwarth, 1987). Upon denaturation, the chromophore adopts a cyclic helical and much more flexible structure, its lifetime is reduced greatly. In chromopeptides, where this situation is also dominant the excited state lifetimes of the tetrapyrrol chromophores were found to vary between 25 and 50 ps (Schneider *et al.*, 1986; Schneider *et al.*, 1988c). The lifetime found in the *Pieris* biliprotein is similar and thus confirms the conclusion drawn by Scheer and Kayser from the shape of the absorption band: in this pigment, the chromophore in its native state adopts a cyclic helical structure, which has a similar internal mobility as free chromophores have.

The attempt to draw conclusions about molecular geometry from the CARS spectra is presently impeded by the lack of normal mode analysis with high reliability and/or accuracy. Therefore, the hypothesis of cyclic helical chromophore structure must rely largely on a comparison of this spectrum with those of similar compounds, for which the geometry has been determined by independent methods. As the biliprotein from *Pieris brassicae* is currently under x-ray studies, a further refinement of the preliminary data, published recently (Huber *et al.*, 1987), will make possible a direct correlation of the bilin conformation with spectroscopic results.

It can be stated as a general rule that phycocyanins originating from blue-green algae exhibit strong bands around 1650, 1273 and 1234 cm^{-1}, respectively, if the chromophore is held in an extended conformation by interaction with the native protein (Schneider *et al.*, 1987, 1988a,b; Szalontai *et al.*, 1985). Upon denaturation of the apoprotein, which causes the phycocyanobilin chromophore to adopt a more cyclic helical geometry, the above mentioned bands disappear and a new, strong band appears in the 1620–1630 cm^{-1} region (Szalontai *et al.*, 1987; Margulies and Toporowicz, 1984). That the chromophore's vibrational frequencies in the insect biliprotein approximate those of denatured phycocyanin can be taken as additional evidence for the cyclic helical structure. In addition, it was found (Schneider *et al.*, 1988c) that the model chromopeptide (Fig. 1, **2**) mentioned above (Falk *et al.*, 1985) yields a similar CARS-spectrum with bands around 1260, 1610 and 1630 cm^{-1} respectively. Despite the fact that the influence of the substituents on the various normal modes can not be predicted without a numerical analysis, we feel that the overall pattern in the finger print and double bond region is striking evidence for the difference in chromophore geometry in the biliproteins from algae and insects, respectively.

CONCLUSION

Both fluorescence lifetime and CARS spectrum (vibrational frequencies) support the hypothesis that the biliverdin IXγ chromophore found in the biliprotein of *Pieris brassicae* is bound in a cyclic helical conformation with high degree of internal flexibility. In view of the importance, which the knowledge of chromophore structure of native pigments in room temperature solutions has, with respect to the understanding of their various biological functions, work is under way to establish a force field for tetrapyrrol chromophores with the aim to correlate spectral information and chromophore geometry.

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REFERENCES

Falk, H., G. Kapl and W. Medinger (1985) Zur Chemie der Pyrrolpigmente, 62 Mitt., *Monatsh. Chemie* **116**, 1065–1085.

Hefferle, P., W. John, H. Scheer and S. Schneider (1984) Thermal denaturation of monomeric and trimeric phycocyanins studied by static and polarized time-resolved fluorescence spectroscopy. *Photochem. Photobiol.* **39**, 221–232.

Holzwarth, A. R. (1987) Picosecond fluorescence spectroscopy and energy transfer in photosynthetic antenna pigments. In *The Light Reactions* (Edited by J. Barber), pp. 95–157. Elsevier Science Publishers B.V., Amsterdam.

Huber, R., M. Schneider, O. Epp, I. Mayr, A. Messerschmidt, J. Pflugrath and H. Kayser (1987) Crystallization, crystal structure analysis and preliminary molecular model of the bilin binding protein from the insect *Pieris brassicae*. *J. Mol. Biol.* **195**, 423–434.

Kayser, H. (1985) In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Edited by G. A. Kerkut and L. I. Gilbert), pp. 367–415. Pergamon Press, New York.

Kayser, H. and D. Zipfel (1988) Manuscript in preparation.

Margulies, L. and M. Toporowicz (1984) Resonance Raman study of model compounds of the phytochrome chromophore. 2. Biliverdin dimethyl ester. *J. Amer. Chem. Soc.* **106**, 7331–7336.

Rüdiger, W. (1971) Gallenfarbstoffe und Biliproteide. *Fortschr. Chem. Org. Naturstoffe* **29**, 60–139.

Scheer, H. (1982) Phycobiliproteins: molecular aspects of photosynthetic antenna system. In *Light Reaction Path of Photosynthesis* (Edited by F. K. Fong), pp. 7–45. Springer, Berlin.

Scheer, H. (1986) Excitation transfer in phycobiliproteins. In *Encyclopedia of Plant Physiology*, Vol. 19, Photosynthesis 3 (Edited by L. A. Staehelin and C. J. Arntzen), pp. 327–337. Springer, Berlin.

Scheer, H. and H. Kayser (1988) Conformational studies of biliproteins from the insects *Pieris brassicae* and *Cerura vinula*. *Z. Naturforsch.* **43c**, 84–90.

Schirmer, T., W. Bode and R. Huber (1987) Refined three-dimensional structures of two cyanobacterial C-phycocyanins at 2.1 and 2.5 Å resolution. *J. Mol. Biol.* **196**, 677–695.

Schirmer, T., W. Bode, R. Huber, W. Sidler and H. Zuber (1985) X-ray Crystallographic structure of the light-harvesting biliprotein C-phycocyanin from the thermophilic cyanobacterium *Mastigocladus laminosus* and its resemblance to globin structures. *J. Mol. Biol.* **184**, 257–277.

Schneider, S., F. Baumann, P. Geiselhart, S. Siebzehnrübl, R. Fischer and H. Scheer (1988b) Resonance CARS and time-resolved fluorescence studies of native and chemically modified phycocyanin trimers. In *Proceedings of the Second European Conference on the Spectroscopy of Biological Molecules* (Edited by E. D. Schmidt, F. Siebert and F. Schneider). Wiley, Chichester.

Schneider, S., F. Baumann and U. Klüter (1987) CARS investigation of changes in chromophore geometry of C-phycocyanin from *Mastigocladus laminosus* induced by titration with p-chloromercury benzene sulfonate. *Z. Naturforsch.* **42c**, 1269–1274.

Schneider, S., F. Baumann, W. Steiner, R. Fischer, S. Siebzehnrübl and H. Scheer (1988a) Coherent anti-Stokes Raman spectroscopy of phycobilisomes, phycocyanin and allophycocyanin from *Mastigocladus laminosus*. In *Photosynthetic Light-Harvesting Systems* (Edited by H. Scheer and S. Schneider), p. 317–322, Walter de Gruyter, Berlin.

Schneider, S., P. Geiselhart, F. Baumann, H. Falk and W. Medinger (1988c) Studies on biliprotein model compounds. CARS, picosecond time-resolved absorption and emission spectroscopy of a tryptophan-substituted 2,3-dihydrobilatrien. *J. Photochem. Photobiol.* Part B, Accepted.

Schneider, S., E. Köst-Reyes, C. Scharnagl and P. Geiselhart (1986) Investigation of chromopeptides from C-phycocyanin by UV-vis absorption, emission and circular dichroism spectroscopy. *Photochem. Photobiol.* **44**, 771–777.

Szalontai, B., Z. Gombos and V. Csizmadia (1985) Resonance Raman spectra of phycocyanin, allophycocyanin and phycobilisomes from blue-green alga *Anacystis Nidulans*. *Biochim. Biophys. Res. Commun.* **130**, 358–363.

Szalontai, B., Z. Gombos, V. Csizmadia and M. Lutz (1987) The chromophore structure and chromophore–protein interactions in C-phycocyanin as studied by Resonance Raman spectroscopy. *Biochim. Biophys. Acta* **893**, 296–304.

Note added in proof: The high-resolution x-ray structure (2.0A) was published after this manuscript had been prepared [Huber, R., M. Schneider, I. Mayr, R. Müller, R. Deutzmann, F. Suter, H. Zuber, H. Falk, and H. Kayser; *J. Mol. Biol.* **198**, 499–513 (1987)].