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Original Communications

Conformational Studies of Biliproteins from the Insects *Pieris brassicae* and *Cerura vinula*

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Chromophore conformation and protein secondary structure of biliproteins from the butterfly, *Pieris brassicae*, and the moth, *Cerura vinula*, have been investigated by absorption, circular dichroism and fluorescence spectroscopy. The chromophore of the *P. brassicae* protein, biliverdin IXγ, has probably a cyclic-helical structure similar to that of free bile pigments of the biliverdin type. Though achiral by structure the chromophore displays strong optical activity in the native protein-bound state, but becomes inactive after urea denaturation of the protein. A minor biliprotein from *P. brassicae* shows absorption, circular dichroism and fluorescence spectra identical to the main biliprotein. In the biliprotein from *Cerura vinula* the structure of the pigment is still unknown. It has a semi-open conformation intermediate between that of the *Pieris* proteins and that of the phycobiliprotein, C-phycocyanin, and it retains optical activity after urea denaturation. The band widths and the size of the Stokes shifts of the fluorescence spectra indicate a high degree of conformational flexibility of the chromophores in the two *Pieris* pigments, and a decreased flexibility in the one from *Cerura*. In the biliproteins from both insects the polypeptides are low in α-helix content compared to that of phycobiliproteins. From these and earlier data, insect and algal biliproteins seem to be related only distantly if at all, but there exist also considerable differences among insect biliproteins from different species.

Introduction

Insect biliproteins are different from those of other organisms in several respects [1]. Unlike the phycobiliproteins, antenna pigments from several algal phyla [2], their chromophores are non-covalently bound to the apoproteins. Unlike all mammalian and plant bile pigments, they also contain chromophores of the IXγ-series which are derived from cleavage of the porphyrin precursors at the C-15 (formerly γ) methine bridge [3a]. The function of insect bile pigments is presently still unclear [1]. In combination with yellow carotenoids they provide strongly green, but non-fluorescent and thus non-phototoxic colors for camouflage. In addition more active roles have recently been suggested [3b] which depend on their polychromic properties [2] influenced *e.g.* by conformation, state of protonation, etc.

Here, we wish to present further results [4] from absorption and circular dichroism studies, which indicate that both the chromophore conformation and protein secondary structure show distinct differences among different insect biliproteins, but that they are at the same time profoundly different from the well characterized phycobiliproteins [2, 5a]. To date no information on these points had been obtained from insect biliproteins (see note). The pigments under study were two isoforms from the butterfly, *Pieris brassicae*, [5b] and a very different biliprotein from the moth, *Cerura vinula* (Kayser, unpublished).

Materials and Methods

Preparations

Details of the preparation of the biliproteins and their apoproteins from *Pieris brassicae* L. will be published elsewhere [5b]. Briefly, the holoproteins were extracted from whole adult insects with 0.2 M potassium phosphate pH 7.2. The purification procedure involved chromatofocussing on a PBE 94 (Pharmacia) column with Polybuffer 74 adjusted to pH 5.0 as eluant. This step yielded a main biliprotein I and a

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minor fraction, biliprotein II, in essentially pure form as judged from their absorptions at 383 and 280 nm. After removal of Polybuffer on a phenyl-sepharose column, and of salt by ultrafiltration the proteins were lyophilized. The apparent molecular weight was determined by SDS-polyacrylamide gel electrophoresis to 22.5 kDa. The following extinction coefficients were obtained: Holoprotein: 280 nm (64200), 383 nm (64200), 675 nm (36300 $cm^{-1}M^{-1}$). Apoprotein: 280 nm (48100 $cm^{-1}M^{-1}$). The chromophore-apoprotein stoichiometry is 1:1 in both biliproteins from *Pieris*.

The biliprotein from *Cerura vinula* L. was obtained from larval haemolymph by chromatography on DEAE-cellulose (Kayser, unpublished results). On the basis of SDS-PAGE its molecular size was estimated to 80–85 kDa and its purity to 95%.

Absorption spectra were recorded with a model 320 spectrophotometer (Perkin-Elmer, Überlingen), fluorescence spectra with a model fluorolog spectrofluorometer (Spex) with a data acquisition system. Circular dichroism spectra were measured with a dichrograph V (Jobin-Yvon, Unterhaching). The optical density for CD measurements was ≤ 0.5, and the pathlength 1 cm in the visible and near-UV, 0.1 cm in the far-UV. Secondary structure of the proteins was estimated from an analysis of the far-UV CD spectrum using the program of Provencher and Glöckner [6].

Results

Absorption spectra

The absorption spectrum of the *Pieris* biliprotein I (the main fraction) has a broadened visible absorption band indicative of two superimposed component bands (Fig. 1a). The near UV band is structured, too. The intensity ratio of the maximum absorption $Q^{A}_{vis/uv}$ of the visible to that of the near UV band is 0.56. The maximum absorptivity of the aromatic amino acids (280 nm, not shown) is the same as that of the near UV band. The absorption spectrum of the minor biliprotein II is identical to that of the main fraction (not shown).

The absorption spectrum of the *Cerura* biliprotein (Fig. 2a) is considerably different. The visible band is structured, too, but has only a pronounced short-wavelength shoulder and is much more increased in intensity relative to that of the near UV band ($Q^{A}_{vis/uv}$ = 1.22). The intensity of the band arising from the aromatic amino acid residues is 7–8 times that of the near UV band.

Circular dichroism spectra

The CD spectrum of the *Pieris* protein I (Fig. 1b) has a positive visible and a negative near UV band, whose structure follows closely that of the absorption spectrum (Fig. 1a). The intensity ratio (maximum ellipticities) in the CD spectrum is slightly smaller (0.49) than in the absorption spectrum. In the biliprotein II no difference to protein I was observed.

The same correspondences with the absorption spectra are seen in the CD spectrum of the *Cerura* pigment. The fine structure is again very similar to that of the absorption spectrum, and the intensity ratio of the visible and near UV band is also smaller (0.89).

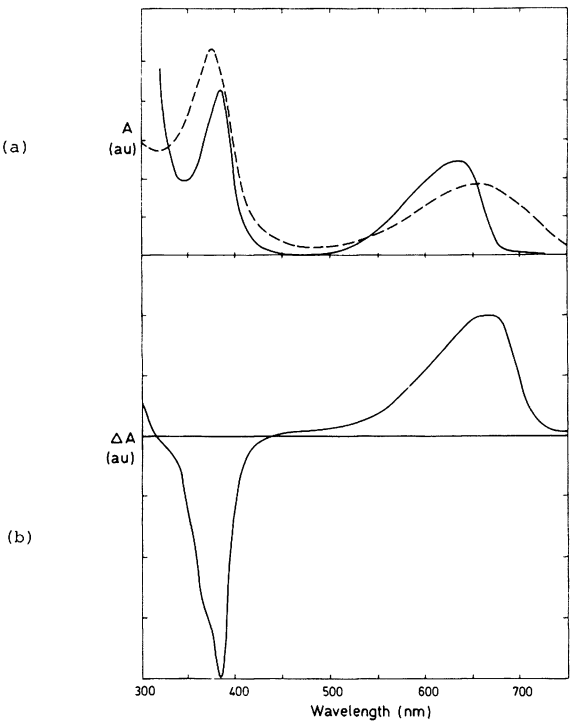

Fig. 1. Absorption (a) and circular dichroism spectra (b) of the biliprotein I from *Pieris brassicae*. The spectra of the native pigment (50 mM potassium phosphate buffer, pH 6.9, solid lines) are compared to those of the denatured pigments in 8 M urea solution (dashed lines). See experimental part for molar extinction coefficients and ellipticities.

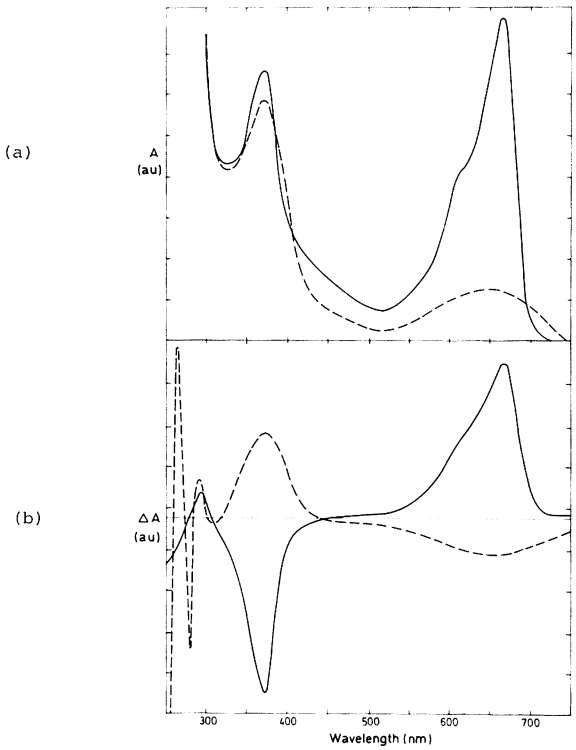

Fig. 2. Absorption (a) and circular dichroism spectra (b) of the biliprotein from *Cerura vinula*. Other details as in Fig. 1.

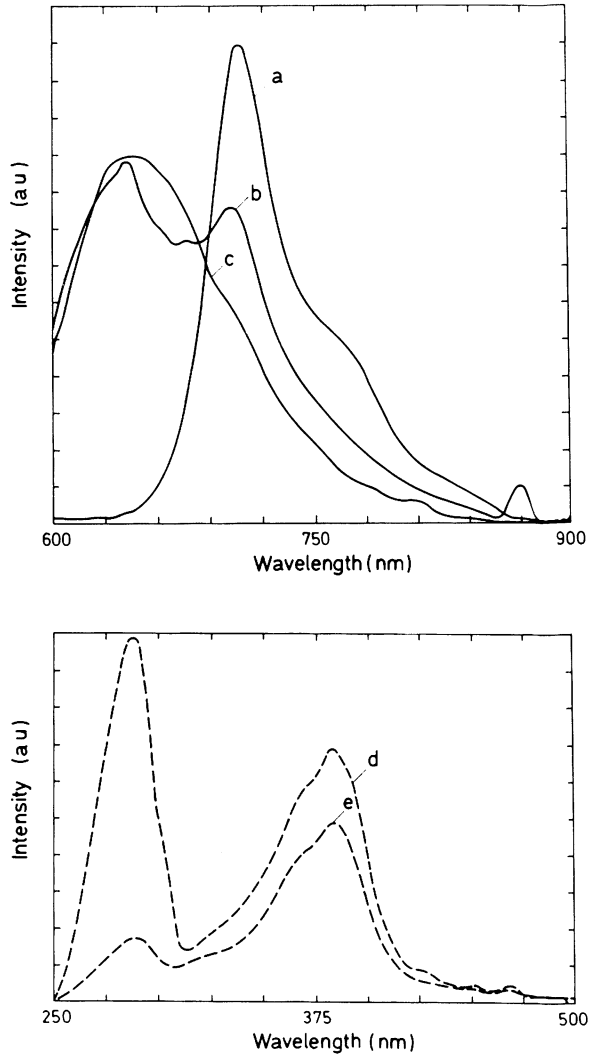

Fig. 3. Fluorescence spectra of the biliprotein I from *Pieris brassicae*. Emission spectra (solid lines): excitation at 382 (a), 290 (b) and 270 nm (c). Excitation spectra (dashed lines): emission at 675 (d) and 705 nm (e). All spectra are uncorrected.

Fluorescence spectra

The main emission of the *Pieris* biliprotein I is at 705 nm (Fig. 3). This maximum is independent of the excitation wavelength and corresponds to a Stokes shift of appx. 50 nm, with the exact value depending on the assumed position of the rather flat-topped absorption band. If the excitation wavelength is shifted below 320 nm a second emission band arises at 641 nm, which becomes most prominent at excitations below 280 nm. This indication of two emitting species being present, is supported by the excitation spectra. Whereas the excitation spectrum with observation at 705 nm is dominated by a structured near UV band (λ_{max} = 384 nm), an excitation band at 288 nm becomes more prominent at shorter observation wavelengths. The properties of the biliprotein II from this species are again very similar.

The main emission of the *Cerura* biliprotein (Fig. 4) is located at 691 nm corresponding to a Stokes shift of only 25 nm. Here, too, a shorter wavelength emission (λ_{max} = 632 nm) is observed upon excitation below 300 nm (λ_{max} = 297 nm).

Protein conformation and stability

The protein secondary structure was estimated from the far UV-CD spectrum by an analysis based on a set of standard proteins of known secondary structure composition [6]. The data for biliprotein I from *Pieris* and for the *Cerura* protein are shown in

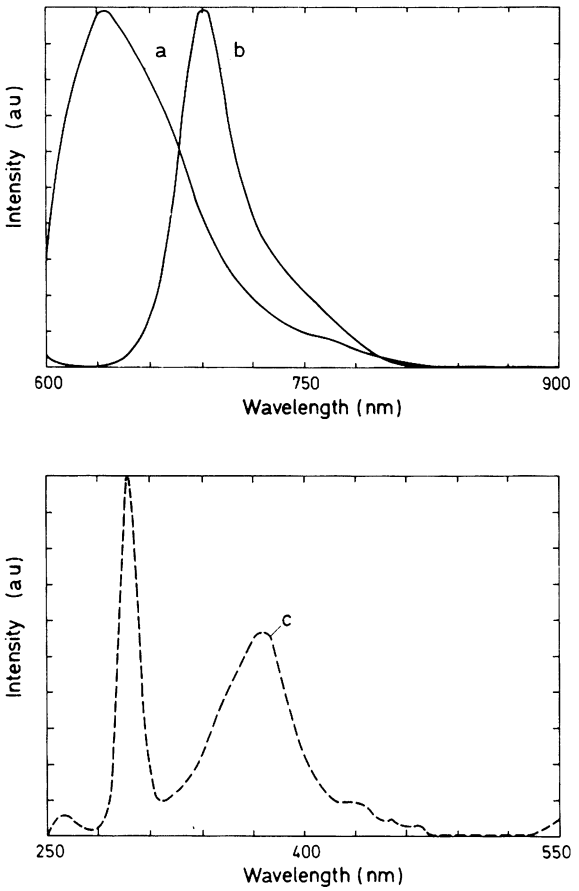

Fig. 4. Fluorescence spectra of the biliprotein from *Cerura vinula*. Emission spectra (solid lines) with excitation at 370 (a) and 256 nm (b), excitation spectrum (dashed line) with emission at 691 nm (c).

Table I together with those for the phycobiliprotein, C-phycocyanin from *Mastigocladus laminosus*. Although the precision of the method is limited by the relatively poor signal-to-noise ratio in the case of the insect biliproteins, it is clear that the α-helix content is much smaller in both pigments than in the phycobiliproteins. A comparison of the *Pieris* chromoprotein with the apoprotein also indicates a decreased α-helix content in the latter.

Upon titration of the *Pieris* protein I with urea, the far-UV CD band decreases monotonously and also shifts to longer wavelengths (Table I, Fig. 5). The analysis suggests a rapid loss of α-helix structure, whereas the estimates for β-sheet remain high up to 8 M urea. The process has been monitored, too, by observation of the absorption and CD spectra in the chromophore region. In the absorption spectrum, the most pronounced change is a loss of fine structure in both the visible and the near UV bands (Fig. 1 and 5). By contrast, the intensity ratio and position of the two bands remain nearly the same. The optical activity decreases to less than 1% of its original value in the native pigment during unfolding. In comparison to the monotonous decrease of the far-UV CD-band, the decrease in the visible and near-UV is largest between 0 and 2 M and above 4 M urea, whereas the change between 2 and 4 M urea is only very small. This behavior has been reproduced in three different titrations.

Due to the optical activity being so sensitive to the native state of the protein, it was also used to test the stability towards acid and base. Whereas the bilipro-

Table I. Secondary structures of insect biliproteins. Estimated from the far-UV CD spectra by the method of Provencher and Glöckner [6]. We thank the authors for a copy of the CONTIN program. The data used are the ones shown in Fig. 2 and 5.

(Chromo)protein	Secondary structure [%]		
	α-helix	β-sheet	others
Pieris brassicae (I) Holoprotein	23	47	30
Pieris brassicae (I) Holoprotein, 2 M urea	5	57	35
Pieris brassicae (I) Holoprotein, 4 M urea	6	42	52
Pieris brassicae (I) Holoprotein, 6 M urea	0	50	50
Pieris brassicae (I) Holoprotein, 8 M urea	7	42	51
Pieris brassicae (I) Apoprotein	13	55	32
Cerura vinula Holoprotein	18	58	24
Mastigocladus laminosus Phycocyanin	85	7	8

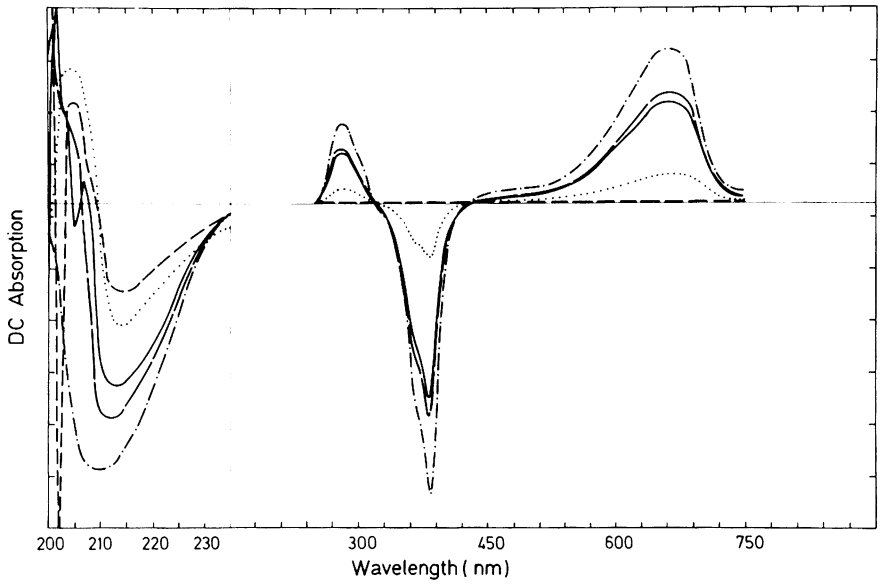

Fig. 5. Unfolding of the polypeptide chain of the *Pieris* chromoprotein I with urea as monitored by CD spectroscopy. The ordinate in the left part (≤240 nm) is decreased by a factor of ten relative to that of the right part (≥280 nm). See Table I for molar ellipticities. (···) = Original solution, as in Fig. 1: (—·—) = 2 M urea, (——) = 4 M urea, (·····) = 6 M urea, (-----) = 8 M urea.

tein I from *Pieris* was quite stable towards acid, treatment with base led to unfolding of the protein (Table II). In this respect, incubation at pH 9.5 was equivalent to a urea concentration of appx. 4 M.

Table II. pH Stability of biliprotein I from *Pieris brassicae*. Optical activity of the chromophore absorption bands (in % relative to pH 7), and ratios of the CD signal maximum amplitudes $Q^{CD}_{vis/nuv}$.

pH	$\delta\bar{A}_{vis}$	$\delta\bar{A}_{nuv}$	$Q^{CD}_{vis/nuv}$
2.9	98%	88%	0.50
6.4	100%	100%	0.45
9.5	60%	56%	0.48

The changes in the *Cerura* pigment upon unfolding of the protein, have been investigated less extensively due to the limited availability of the material. The absorption and CD spectra of the fully denatured pigment (8 M urea) are shown in Fig. 2. Two points are noteworthy: Firstly, the absorption spectrum of the denatured pigment has a markedly changed $Q^{A}_{vis/uv}$, which is decreased by a factor of 6 to 0.22, with a concomitant blue-shift of the visible band. Secondly, the circular dichroism remains considerable after unfolding, but the sign of both bands is inverted as compared to the native state of this pigment.

Discussion

Pieris brassicae biliproteins

The spectra of bile pigments are sensitive to a variety of factors. The most important one seems to be the geometry of the chromophore. This has been born out of a series of molecular orbital calculations performed in different laboratories [7–11], and more recently corroborated experimentally [3, 5a, 12–15]. Whereas the cyclic-helical chromophores typical for bile pigments in solution have a low ratio $Q^{A}_{vis/nuv}$ of the intesities of the visible and near-UV absorption bands, this ratio is high in extended chromophores like the ones in native phycocyanins [5a], and in the neobiliverdins found first in insects [3], and more recently in other invertebrates [16].

As judged from this criterion, the chromophores of the *Pieris* biliproteins I and II are present in a cyclic-helical conformation which is quite similar to that of the respective free chromophore, which is present *e.g.* after denaturation of the polypeptide (Fig. 1). The main difference is, that the conformational freedom [2] seems to be somewhat more restricted in the native biliprotein, leading to the structured absorption bands. However, the large Stokes shift (50 nm) is still indicative of a mobile chromophore. Recent kinetic fluorescence data also indicate a high degree of similarity between the native *Pieris* pigment and free bile pigments as far as

conformation and conformational freedom is concerned [17]. In the phycobiliproteins, partial loosening of the interactions with the proteins, *e.g.* by partial denaturation of the latter, leads to photochromic behavior resembling the primary reactions of phytochrome, which is again lost when the protein is fully uncoupled [4]. Similar studies with the *Pieris* biliprotein I did not show any photochemistry besides bleaching upon prolonged irradiation, which would also support that the chromophore reactivity is similar to that of free biliverdins.

Binding to the protein does induce, however, a strong optical activity. Since the chromophore, biliverdin IXγ, is achiral, this optical activity must be induced by the protein. Probably, a similar mechanism is operative as in the complexes of bile pigments to serum albumin [9, 18], where it has been rationalized by preferential binding of one enantiomeric form of the helical chromophore to the chiral protein. This situation is quite different from the phycobiliproteins, *e.g.* phycocyanin [2, 5a], where inherently optically active chromophores are bound covalently in extended conformations to the apoproteins.

The secondary structure of the apoprotein is considerably different, too, from that of apophycocyanin. The latter is related to the globins [5a] and contains a large proportion of α-helix structure. In the case of C-PC from *Mastigocladus laminosus*, the x-ray results give α-helix contents of 65 and 62% for the α- and β-subunits, respectively. When estimated [6] from the far-UV CD spectrum, an even larger value of 85% is obtained (Table I). Similarly high contents are also obtained by CD analysis of another biliprotein, the plant photomorphogenetic pigment phytochrome [19]. The shape of the far-UV CD spectrum of the *Pieris* pigments with a single, unstructured negative band in the 200–225 nm region, is already indicative of a low content in α-helix. The curve resolution [6] yields a content of only 23% α-helix, about 50% β-sheet, and a high amount of "other" secondary structures.

In summary, the two *Pieris* biliproteins are thus so different from the phycobiliproteins that a relationship seems very unlikely. Their chromophore, biliverdin IXγ, differs fundamentally from the that of the algal proteins in its molecular structure, binding situation and conformation, and the secondary structure of the apoproteins is also basically different from that of the phycobiliproteins. However, no significant differences were found between the two isoproteins from *Pieris*.

Cerura biliprotein

The biliprotein from *Cerura* has been included in this study in order to compare the *Pieris* pigments to another insect biliprotein. It is less well characterized to date (Kayser, unpublished) and in particular is the chromophore molecular structure yet unknown. The spectral data presented here show, that the chromophores of these two insect biliproteins and their binding situation are likely to be quite different. They indicate the following structural elements: The chromophore does not appear to belong to the class of neobilins [3]. In these pigments, the additional bridges between the pyrrole rings lead to a fixation of more extended conformations, which are characterized by an increased $Q^{A}_{vis/uv}$ value. In the denatured *Cerura* pigment, this ratio is rather similar to that of classical biliverdins and thus indicative for the absence of additional bridges.

The moderately strong CD signal in the denatured protein indicates furthermore, that the chromophore is optically active, *e.g.* that it contains an asymmetric C-atom or another chiral element. Induced optical activity by the protein should be negligible under these conditions. Strong optical activity can be induced in bile pigments by an intramolecular induction mechanism first proposed by Moscowitz *et al.* [20]. It originates from the fact that bile pigments in cyclic-helical conformations are present in two enantiomeric forms differing in the sense of their helice. Since these are inherently dissymmetric, a small shift in the distribution of these two enantiomeric forms by asymmetric centers on the molecule can produce large anisotropies. In contrast to the asymmetric induction by the matrix, *e.g.* a binding protein (see above), this intramolecular asymmetric induction persists after the unfolding of the protein, as shown for the phycobiliproteins [21].

The binding site of the *Cerura* protein also seems to be different from that of the *Pieris* proteins. The increased ratio $Q^{A}_{vis/uv}$ suggests a more extended conformation for the chromophore, which is intermediate between the latter and C-phycocyanin. Due to the comparably small increase, the situation is less clear-cut, however, than in the phycobiliproteins. Principally, the increased intensity of the visible absorption band can be rationalized as well by a proto-

nation of the chromophore [22]. However, protonation is accompanied by a pronounced red-shift of this band, which does not seem to be present in the *Cerura* pigment (Fig. 2a). The biliprotein from *Cerura* would then be the first example of a biliprotein with an (at least partly) extended chromophore besides the plant- and phycobiliproteins.

The secondary structure of the *Cerura* biliprotein is again similar to the one from *Pieris* in its low α-helix content. Curve resolution of the far-UV CD spectrum yields values of 13% α-helix, 55% β-sheet and 32% "others". In view of the relatively poor signal-to-noise and the unknown precise molecular weight, these values should be regarded as rough estimates only. Nonetheless is the secondary structure of the *Cerura* protein, like that from *Pieris*, quite distinct from that of the well characterized phycobiliproteins.

Conclusions

The data suggest that insect biliproteins have several distinct conformational differences which set them apart from the plant and phycobiliproteins and render a phylogenetic relationship unlikely. The secondary structure of the polypeptides of the three insect pigments investigated, seems to be more closely related. However, the pigments from *Pieris* and *Cerura* differ markedly in the molecular structure and the native conformation of their chromophores. Together with the large difference in molecular weight, this suggests a considerable variability among the insect biliproteins.

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Note: After preparation of this manuscript, the x-ray structure analysis and a preliminary molecular model for the biliprotein I (termed bilin binding protein, BBP) from *Pieris brassicae* has been published (R. Huber, M. Schneider, O. Epp, I. Mayr, A. Messerschmidt, J. Pflugrath, and H. Kayser: J. Mol. Biol. **195**, 423–434, 1987). The data support and detail the conformational assignments as given in our present work and also the large differences between insect and cyanobacterial biliproteins.

[1] H. Kayser, in: Comprehensive Insect Physiology, Biochemistry, and Pharmacology (G. A. Kerkut and L. I. Gilbert, eds.), chapter 11, pp. 367–415, Pergamon Press, New York 1985.
[2] H. Scheer, in: Light reaction path of photosynthesis (F. K. Fong, ed.), chapter 2, pp. 7–45, Springer Verlag, Berlin 1983.
[3] a: M. Bois-Choussy and M. Barbier, Heterocycles **9**, 677–690 (1978).
b: M. Barbier, Comp. Biochem. Physiol. **84b**, 619–621 (1986).
[4] W. John, R. Fischer, S. Siebzehnrübl, and H. Scheer, in: Antennas and Reaction Centers of Photosynthetic bacteria (M. E. Michel-Beyerle, ed.), pp. 17–25, Springer Verlag, Berlin-Heidelberg 1985.
[5] a: T. Schirmer, W. Bode, R. Huber, W. Sidler, and H. Zuber, J. Mol. Biol. **184**, 257–277 (1985).
b: H. Kayser and D. Zipfel, manuscript in preparation.
[6] S. W. Provencher and J. Glöckner, Biochemistry **20**, 33–37 (1981).
[7] M. I. Burke, L. H. Pratt, and A. Moscowitz, Biochemistry **11**, 4025–4031 (1972).
[8] Q. Chae and P. S. Song, J. Am. Chem. Soc. **97**, 4176–4179 (1975).
[9] G. Wagnière and G. Blauer, J. Am. Chem. Soc. **98**, 7806–7810 (1976).
[10] T. Sugimoto, M. Kikushima, M. Saito, and H. Suzuki, J. Phys. Soc. Jpn. **53**, 873–881 (1984).
[11] H. Scheer, H. Formanek, and S. Schneider, Photochem. Photobiol. **36**, 259–272 (1982).
[12] H. Falk, K. Grubmayr, E. Haslinger, T. Schlederer, and K. Thirring, Monatsh. Chem. **109**, 1451–1473 (1978).
[13] H. Nesvabda and A. Gossauer, J. Am. Chem. Soc. **109**, 6545–6546 (1987).
[14] H. Falk and K. Thirring, Tetrahedron **37**, 761–766 (1981).
[15] S. E. Braslavsky, A. R. Holzwarth, and K. Schaffner, Angew. Chem. **95**, 670–689 (1983); Angew. Chem. Int. Ed. **22**, 656–675 (1983).
[16] E. Benedikt, Ph. D. Thesis, Universität München 1987.
[17] S. Schneider, F. Baumann, P. Geiselhart, H. Kayser, and H. Scheer, Photochem. Photobiol., submitted for publication (1987).
[18] G. Blauer and G. Wagnière, J. Am. Chem. Soc. **97**, 1949–1955 (1975).
[19] E. M. Tobin and W. R. Briggs, Photochem. Photobiol. **18**, 487 (1973).
[20] A. Moskowitz, W. C. Krueger, I. T. Kay, G. Skews, and S. Bruckenstein, Proc. Natl. Acad. Sci. USA **52**, 1190–1194 (1964).
[21] H. Lehner and H. Scheer, Z. Naturforsch. **38c**, 353–358 (1983).
[22] H. Scheer, Z. Naturforsch. **31c**, 413–417 (1976).