Studies on Plant Bile Pigments, IV [1]:

Conformational Studies on C-Phycocyanin from *Spirulina platensis*

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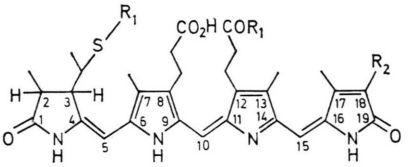
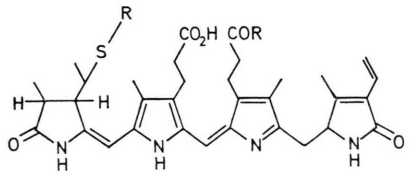
Bile Pigments, Protein Interaction, Conformation, Denaturation

The chromophore-protein interactions of C-phycocyanin (C-PC) from *Spirulina platensis* have been studied by following the partial and complete denaturation with UV-Vis spectroscopy. From comparison with published MO calculations, an elongated conformation of the chromophore is suggested for native C-PC, a cyclic one for denatured C-PC. By means of partial denaturation, a stepwise unfolding of the protein has been demonstrated. The presence of at least two sets of spectroscopically different chromophores is suggested from the partial denaturation and low temperature experiments.

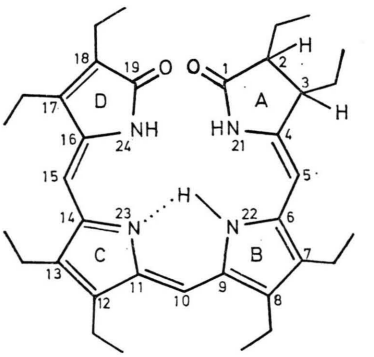
Phycocyanins and phycoerythrins are photosynthetic light harvesting pigments of blue-green, red and cryptophytan algae, which contain bile-pigment chromophores covalently bound to proteins [2]. The absorption of the various types of these pigments cover the spectral range between about 500 and 670 nm practically completely. In spite of this spectral variety, however, the phycobiliproteins contain with only few exceptions just two chemically distinct chromophores: the blue phycocyanobilin (**1a**) and the red phycoerythrobilin (**2**) *, [2, 3]. Uninfluenced by the protein, the free bases of the two chromophores absorb around 600 and 530 nm, respectively, in the visible spectral region, and around 350 and 320 nm in the near UV. The spectra of the free chromophores are quite dissimilar to those of the native pigments, too, in terms of intensity and shape



1a: R_1 = Protein; $R_2 = C_2H_5$
1b: R_1 = Protein; $R_2 = C_2H_3$



2: R = Protein



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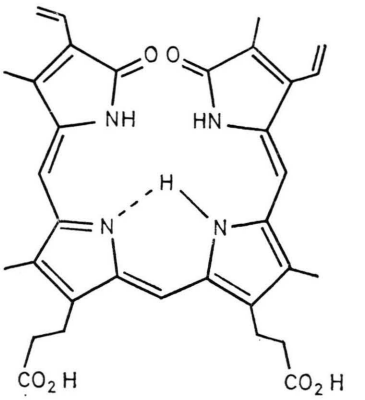

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* The terms phycocyanobilin and phycoerythrobilin are used for compounds with the molecular structure of **1a** and **2**, which are characterized by a substituted ethylgroup at C-3. For a critical discussion, see ref. 2.

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of the two main absorption bands (vide infra), and of their fluorescence. The specific chromophore-protein interactions which underly these profound optical changes are hitherto only little understood.

These interactions render the chromophore an intrinsic and sensitive indicator for the state of the biliproteins. Qualitatively, this built-in probe has been widely used to test any alterations of biliproteins during their isolation and purification. Brown *et al.*[4] have demonstrated, that the renaturation of allophycocyanin subunits, depending on the buffer system used, yields pigments absorbing at either 650 nm (like allophycocyanin) or 620 nm (like C-PC). The influence of protein aggregation and isotope substitution on the chromophore absorption has been studied by several groups[2, 3, 5–9]. The rearrangement of the protein induced by the initial photochemical reaction of the chromophore is also believed to be responsible for the sequence of intermediates identified during the phytochrome (**1b** = P_r) interconversions[10, 11].

The influence of the protein moiety in biliproteins can be abolished completely by denaturation with 8 M urea, 6 M guanidinium chloride, or heat. Spectral comparison of the thus exposed chromophores with free bile pigments of known structure has been widely used as a sensitive and very mild method to obtain structural information on biliproteins which are less accessible or unstable[12, 13].

To obtain more detailed information on the chromophore-protein interaction, we have studied by UV-Vis spectroscopy the controlled (partial) denaturation and renaturation of C-phycocyanin (C-PC) from *Spirulina platensis*. To exclude aggregation effects, these studies were performed under conditions where C-PC is monomeric[5–9]. Spectra have been measured in the range between 320 and 700 nm. Thus, spectral changes have not only followed for the long wavelength band of the chromophore, but also for the less studied short wavelength band. The results indicate a stepwise denaturation of C-PC, the presence of at least two chromophore populations, and a predominant influence of the protein *via* conformational modifications of the flexible bile pigment chromophore.

Materials and Methods

Frozen cells of *Spirulina platensis* (110 g) were thawed, and broken in a beaker-type cell mill with glass beads (350 g, 0.25 mm ϕ). The pigments were extracted twice with sodium phosphate buffer (0.1 M, pH 7.0) containing NaN_3 (10^{-3} M) and EDTA potassium salt (10^{-3} M). The crude extract was freed from chlorophyll by centrifugation for 1 h at 78 000 × *g*, and the pigments precipitated by 50% saturation with $(NH_4)_2SO_4$. Purification on Ultrogel AcA22 (LKB, Sweden) and subsequently DEAE cellulose Servacel CM23 (Serva, W. Germany) yielded C-PC with a E_{620}/E_{280} value of 4.1.

Spectra were recorded on a DMR 22 spectrophotometer (Zeiss, Germany) equipped with a pair of cryostats model 600 (Thor, England) and a temperature controller (Kisch, Germany). Gelchromatographic determinations of molecular weights were carried out on thermostated Sephadex G-200 columns (1.5 × 60 cm at 4 °C, and 2.5 × 50 cm at 39 °C) calibrated with Dextran blue, catalase, bovine serum albumin, and cytochrome c. Sedimentation velocity (S_{20}) determinations were run in a Beckmann E analytical ultracentrifuge (UV analyzer at 350 nm), and corrected for temperature changes, solvent viscosity, solvent density and the partial molar volume of the protein. For the denaturation experiments a stock solution of C-PC in potassium phosphate buffer (0.05 M, pH 7.5) containing NaN_3, EDTA and sodium ascorbate (5×10^{-4} M each) was prepared. In the case of heat denaturation, a fresh sample (~50 µl) of this solution was added to the prethermostated cell containing 2.0 ml of buffer to yield a final concentration of 0.25×10^{-6} M, and the spectrum followed in time. In the case of urea denaturation, aliquots were added to buffer containing increasing amounts of urea. The low temperature experiments were carried out in a 1:1 buffer:glycerol mixture, the sedimentation experiments in a buffer without ascorbate, and the Sephadex MW determinations in a Tris buffer (0.01 M, pH 7.8) containing KCl (0.1 M), NaN_3 (10^{-3} M) and EDTA potassium salt (10^{-3} M). All spectroscopic measurements were performed under N_2.

Results

Complete denaturation

The UV-Vis spectral changes of C-PC upon complete denaturation by either heat (Fig. 1) or urea at pH 7.5 * are similar. The intensity (ε_{max}) of the long-wavelength band is decreased by a factor of 4.5, that of the near UV-band is increased by a factor of 2. The red band is shifted by about 20 nm

* For denaturation at different pH values leading to either cations or anions, or denaturation in the presence of Zn^{2+}, *c. f.* ref. 12.

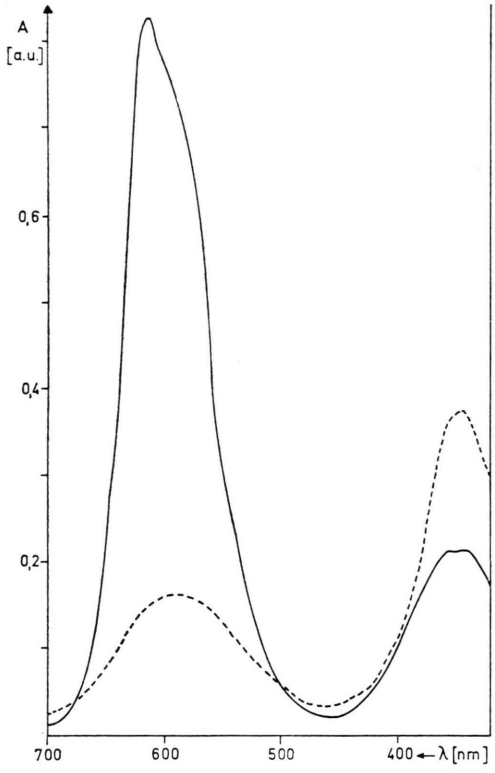

Fig. 1. UV-Vis absorption spectra of C-phycocyanin: —— native, at 4 °C and pH 7.5, — — heat denatured at 71 °C.

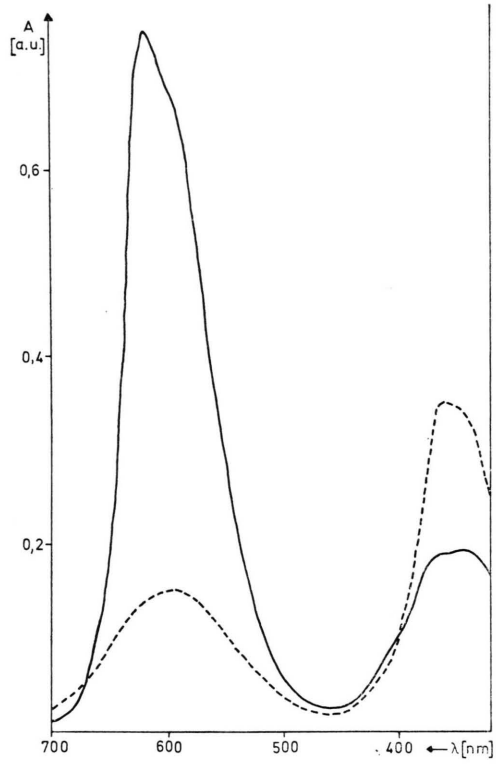

Fig. 2. UV-Vis absorption spectra of C-phycocyanin: —— native, solution in sodium phosphate buffer at 4 °C and pH 7.5, — — denatured with 8 M urea.

to 600 nm, the near UV-band remains at roughly the same position. The fine structure of both bands is diminished. The long-wavelength band of native C-PC has a pronounced peak at 620 nm, with a broader shoulder to shorter wavelengths (see below), the near UV-band is just resolved into two bands of about equal intensity at 360 and 347 nm. The respective bands in denatured C-PC are considerably broadened. Except for a slight redshift due to the different substituents, the spectrum is similar to that of the free base of the synthetic A-Dihydrobilin (**3**)[1a]. Like in other bilins, the spectrum probably constitutes but an envelope of various conformers of the flexible bilin chromophore. On this basis, the comparably narrow bands in the native pigments indicate an increased rigidity, and the presence of only few conformers of the tetrapyrrole skeleton. Increased rigidity is evidenced, too, by the strong fluorescence of C-PC **.

However, the spectral changes upon denaturation do not only indicate an increased flexibility of the free bilin chromophore, but also a profound change in the average chromophore conformation. MO calculations of several groups [14–16] agree that the relative intensity of the two main bands is an indicator for the chromophore conformation. The results predict a weak long-wavelength and a more intense near UV-band for cyclic "porphyrin-type" conformations. This situation is gradually reversed upon stretching the chromophore (*c.f.* Fig. 3). The trends predicted from these calculations [15] gain strong support from the recent x-ray analysis of the dimethylester of biliverdin (**4**)[17]. A cyclic "porphyrin-type" structure has been found for **4**, and its UV-Vis spectrum is indeed similar to that in Fig. 3 a ($E_{655}/E_{370} \approx 1:4$). The presence of cyclic conformations in bile pigment solutions, possibly among other conformations, had already been suggested in 1964 by Moscowitz *et al.*[18] from chiroptical data ***. In the cyclic conformation, the two terminal O-atoms overlap thus rendering the

** This rigidity is a prerequisite, too, for the efficient photoreactivity of P_r. In both cases, the radiationless, energy degrading decay by internal conversion is slowed down sufficiently by conformational fixation.

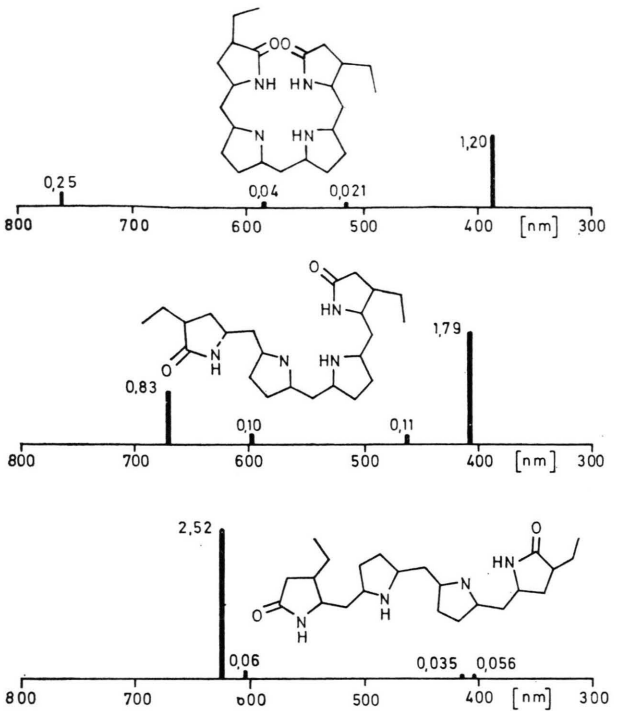

Fig. 3. Calculated oscillator strengths for the biliverdin chromophore in three different conformations: a: cyclic, "porphyrine type", b: semicyclic, c: elongated, "polyene type" (modified from Chae and Song, 1975).

chromophore inherently dissymmetric. The predominance of only one helicality in bilins containing asymmetric α-pyrrole C-atoms then leads to unusually large Cotton effects ****.

Thus, the intensity ratio calculated by Chae and Song [15] for the cyclic conformation(s) is well supported by experimental evidence. Based on the same calculations, the intensity ratio observed in the spectra of native biliproteins would then suggest the chromophores to be in a linear, polyene-type conformation (Fig. 3c). The conformation shown in Fig. 3c is chosen from the set used by Chae and Song [15] with regard to minimal steric hindrance at the β-pyrrolic substituents. Conformations like the ones used in formulas **1** and **2** are sterically much more hindered, but the relief of steric strain by twisting the methine bridges is not expected to change the intensity ratio significantly [14]. Although other influences cannot be ruled out, no presently published calculations indicate similar pronounced influences, *e. g.* of electric fields [16a]. However, these factors may be responsible for the large shifts observed among native biliproteins with the same chromophore **1a**.

Partial denaturation

At 4 °C the red absorption band of C-phycocyanin from *S. platensis* (0.25×10^{-6} M) is distinctly asymmetric with a narrow peak at 620 nm, which is superimposed to a broader band centered around 615 nm. At 40 °C the red band exhibits no longer this asymmetry, and the maximum absorption is reduced by about 20% (Fig. 4a). If the decrease of the long wavelength absorption maximum is followed as a function of temperature (Fig. 4b), there is a distinct transition between 20 and 40 °C, and after a plateau a second decrease above 50 °C. The second decrease corresponds to the complete unfolding of the protein chain [5b]. The first decrease must then be related to a conformational change from the "low temperature" form, which is stable below 20 °C, to a "high temperature" form stable between 35 and 55 °C. A two-step unfolding is observed, too, by adding C-PC to increasing concentrations of urea, a denaturing agent (Fig. 5). There is again a distinct transition between 1.5 and 4 M, followed by a plateau and a second decrease above 5 M which again corresponds to the onset of complete unfolding of the peptide chain. As long as the temperature does not exceed 50 °C, and the urea concentration is kept below 5 M, respectively, these spectral changes are completely reversible by cooling to 4 °C, and by dialyzing the urea out of the solution.

That these spectral changes are due to conformational changes in the monomeric protein, rather than to aggregation, is suggested from molecular weight measurements. On calibrated Sephadex columns, PC has an apparent molecular weight of 44,000 and 47,000 daltons at 4 °C and 40 °C, respectively. Similarly, ultracentrifugation experiments yield sedimentation coefficients of 2.99 S at 5 °C and 3.15 S at 35 °C, comparable to the 3 S found for monomeric C-PC [5–9].

*** "Porphyrin type" structures have been found for tripyrrins, too, both in the crystal [19a] and in solution [19b].

**** A more elongated structure for 4 has been proposed recently from comparison of MO results with low temperature absorption and fluorescence data [15]. However, biliverdin and other bilins are protonated at low temperatures in ethanol (−196 °C) [20]. The increase of the long-wavelength band in bilin cations, as compared to the free bases, would then simulate a more elongated structure.

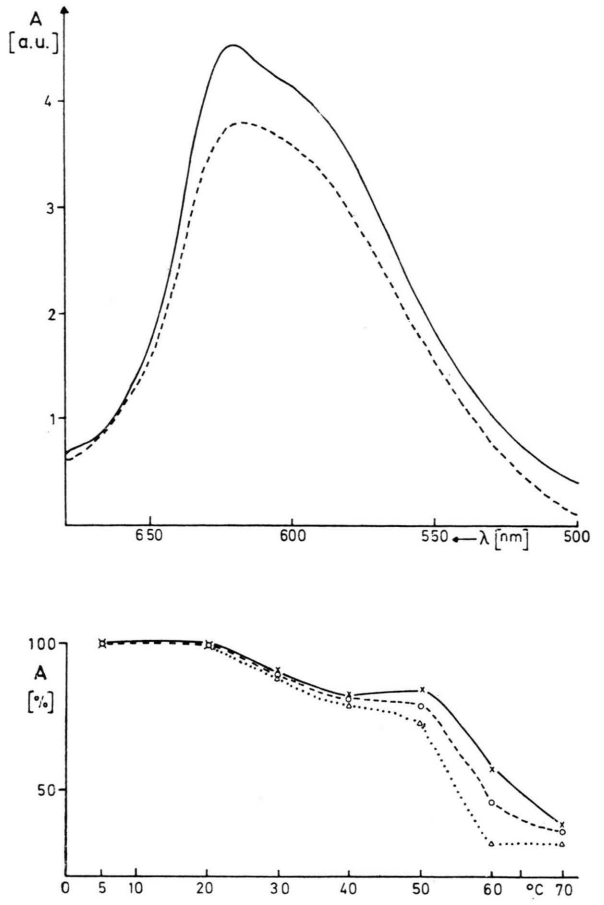

Fig. 4. Partial denaturation of C-phycocyanin by heat
a) long wavelength band of the "low temperature" conformation at 5 °C (——), and of the "high temperature" conformation at 40 °C (— —).
b) Decrease of the long wavelength absorption maximum relative to $E^{5°}_{620} = 100\%$. The three points at each temperature correspond to a time of 1, 3 and 15 min, respectively. Prolonged treatment, especially at higher temperatures, leads to irreversible side reactions.

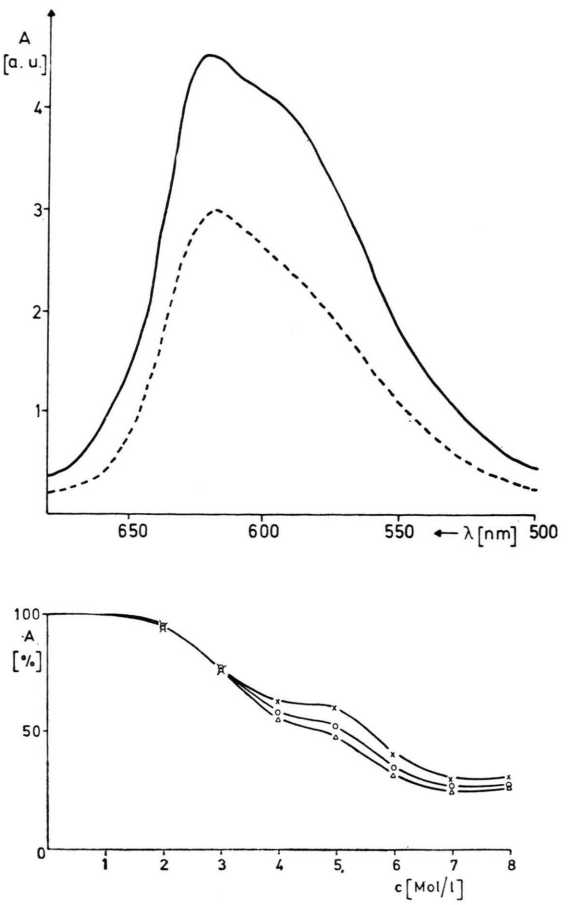

Fig. 5. Partial denaturation of C-phycocyanin by urea.
a) Long wavelength band of the conformation stable in the absence of urea at 4 °C (——), and in the presence of 4 M urea (— —);
b) decrease of the long wavelength absorption band ($E^{OM}_{620} = 100\%$). The three points correspond to denaturation times of 3, 15 and 30 min.

Spectroscopic differences between the two intermediate forms of C-PC, *viz.* the "high temperature" form obtained by heating to 40 °C, and the form obtained by adding 4 M urea, respectively, indicate that the two forms are somewhat different from each other. With urea, the intermediate still has an asymmetric red band, although the maximum is shifted to 617 nm. In the spectrum of the "high temperature" intermediate, such fine structure is no longer discernible at all, but the peak originally present at 620 nm is decreased in intensity and/or shifted to shorter wavelengths. These differences would indicate the presence of (at least) two sets of chromophores in C-PC. The two sets are affected to a different degree by heat, but they are less discriminated by partial denaturation with urea. Monomeric C-PC generally contains three phycocyanin chromophores, one in the light (or α), and two in the heavy (or β) subunit [2, 3]. The spectral changes upon partial heat denaturation may then reflect the preferential unfolding of the peptide chain in the environment of one chromophore. Two different chromophore populations for C-PC had been suggested, too, from fluorescence data [21, 22]. Both sets are efficiently coupled to allow excitation energy transfer from the chromophore(s) absorbing at shorter wavelengths (termed sensitizers, "s"), to the one(s) absorbing at longer wavelengths (termed fluorescers, "f"). Experiments

identical to the ones described above, but utilizing fluorescence rather than absorption spectroscopy as a probe [5b], are under way.

The presence of two different chromophore populations is supported, too, by low temperature absorption spectroscopy of C-PC. The UV-Vis spectrum of a solution of C-PC (0.3×10^{-4} M) in a 1 : 1 mixture of buffer and glycerol at 4 °C is similar to that in buffer. In particular, the long-wavelength band is again asymmetric (*c. f.* Fig. 1). If this solution is cooled, both components of the band gradually sharpen, and eventually split into two bands centered at 630 and 595 nm below -100 °C (Fig. 6)[23]. The end spectrum (-196 °C) is the same, irrespective of the time required for cooling down (15 – 180 min), and upon warming up to 4 °C the original spectrum is retained. Presently, it cannot be excluded that both bands observed at low temperatures are due to a single chromophore in a defined conformation. This is unlikely, however, in view of the low temperature spectra of free bilins. If care is taken to avoid protonation during cool-down (see above), the spectra still remain broad, and only little fine structure appears down to -196 °C. There is only one type of bile pigment known to have a two-peaked long wavelength absorption maximum. Purpurins are characterized by spectra with two comparably narrow bands centered around 540 and 500 nm. This spectrum has been interpreted, too, as to arise from two distinct forms present in solution [1b].

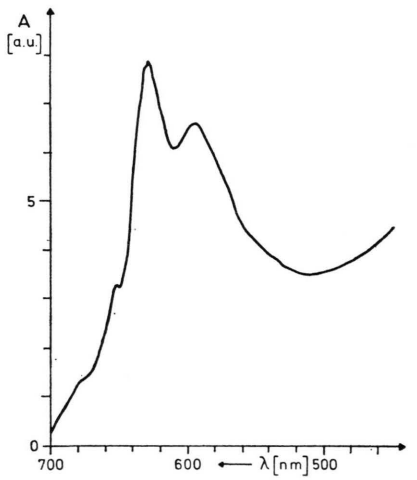

Fig. 6. Low temperature UV-Vis absorption spectrum (-196 °C) of C-phycocyanin in a 1 : 1 mixture of sodium phosphate buffer, pH 7.5, and glycerol.

The results indicate, that the bilin chromophore is held in an elongated conformation in the interior of biliproteins. They also demonstrate, that partial denaturation is a sensitive method to study the interaction between the bile-pigment chromophores and the protein in biliproteins, and to differentiate between distinct chromophore populations.

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[1] a: No. 1; H. Scheer, Z. Naturforsch. **31 c,** 413 [1976]; b: No. 2: H. Scheer, U. Linsenmeier, and C. Krauss, Hoppe-Seyler's Z. Physiol. Chem. **358,** 185 [1977]; c: No. 3: H. Scheer and C. Krauss, Photochem. Photobiol. **25,** 311 [1977].
[2] W. Rüdiger, Fortschr. Chem. Org. Naturst. **29,** 60 [1971]; W. Rüdiger, Ber. Dt. Bot. Ges. **88,** 125 [1975].
[3] L. Bogorad, Ann. Rev. Plant Physiol. **26,** 369 [1975].
[4] A. S. Brown, J. A. Foster, P. V. Voynow, C. Franzblau, and R. F. Troxler, Biochemistry **14,** 3581 [1975].
[5] a: D. S, Berns, Subunits in Biological Systems, part A (S. N. Timasheff and G. D. Fasman, ed.), p. 105, Marcel Dekker, New York 1971; b: D. S. Berns, H. L. Crespi, and J. J. Katz, J. Amer. Chem. Soc. **85,** 8 [1963].
[6] A. Hattori, H. L. Crespi, and J. J. Katz, Biochemistry **4,** 1225 [1965].
[7] G. J. Neufeld and A. F. Riggs, Biochim. Biophys. Acta **181,** 234 [1969].
[8] C. Vernotte, Photochem. Photobiol. **14,** 163 [1971].
[9] B. H. Gray, J. Cosner, and E. Gantt, Photochem. Photobiol. **24,** 299 [1976].
[10] C. J. P. Spruit, R. E. Kendrick, and R. J. Cooke, Planta **127,** 121 [1975].
[11] E. M. Tobin, W. R. Briggs, and P. K. Brown, Photochem. Photobiol. **18,** 497 [1973].
[12] H. P. Köst, W. Rüdiger, and D. J. Chapman, Justus Liebigs Ann. Chem. **1975,** 1582 [1975]; S. Grombein, W. Rüdiger, and H. Zimmermann, Hoppe-Seyler's Z. Physiol. Chem. **356,** 1706 [1975].
[13] A. N. Glazer and C. S. Hixson, J. Biol. Chem. **250,** 5487 [1975].
[14] G. Blauer and G. Wagniere, J. Amer. Chem. Soc. **97,** 1949 [1975].
[15] Qu. Chae and P. S. Song, J. Amer. Chem. Soc. **97,** 4176 [1975].
[16] a: T. Sugimoto, K. Ishikawa, and H. Suzuki, J. Phys. Soc. Japan **40,** 268 [1976]; b: M. J. Burke, D. C. Pratt, and A. Moscowitz, Biochemistry **11,** 4025 [1975]; J.-H. Fuhrhop, P. K. W. Wasser, J. Subramaniam, and U. Schrader, Justus Liebigs Ann. Chem. **1974,** 1450 [1974].
[17] W. S. Sheldrick, J. Chem. Soc. Perkin Trans II, p. 1457 [1976].
[18] A. Moscowitz, W. C. Krueger, I. T. Kay, G. Skewes, and S. Bruckenstein, Proc. Nat. Acad. Sci. U.S. **52,** 1190 [1964].
[19] a: D. Cullen and K. M. Smith, private communication,

1976; b: H. Falk, S. Gergely, K. Grubmayr, and O. Hofer, Z. Naturforsch. **32 b**, 299 [1977].
[20] H. Scheer and W. Kufer, to be published.
[21] R. E. Dale and F. W. J. Teale, Photochem. Photobiol. **12**, 99 [1970].
[22] C. Vernotte and I. Moya, Photochem. Photobiol. **17**, 245 [1973].
[23] This split has been observed for C-PC from other organisms by: D. Frackowiak and J. Grabowski, Photosynthetica **5**, 146 [1971].