Circulardichroism of C-Phycocyanin: Origin of Optical Activity in Denatured Biliproteins and Evidence for an Intermediate during Unfolding

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Z. Naturforsch. **38 c,** 353–358 (1983); received January 17, 1983

Phycocyanin, Phycoerythrin, Phytochrom, Circulardichroism, Denaturation

The circulardichroism spectra (cd) of native and (partially) urea-denaturated C-phycocyanin (PC) in the spectral range 700–210 nm are presented. The large ellipticities observed in the chromophoric region of native PC are retained in the fully denatured state (8 M urea). This is similar to the behavior of the red form of phytochrome (P_r), but in contrast to C-phycoerythrin (PE). These differences are rationalized in terms of epimeric equilibria between P- and M-helix shaped chromophores. Depending on the number and location of the chirality centers present in the tetrapyrrol moities *a priori*, the excess populations of the inherently chiral P- and M-helices differ, thus accounting for large (PC, P_r) or small (PE) ellipticities in the denatured pigments. Hence, the large optical activity observed for the former is generated by an excess population of the P-helix induced by the asymmetric C-2, C-3 and C-3′. In PE the additional chirality center at C-16 counteracts the influence of the others. The excess population of the inherently chiral species is therefore lowered, in agreement with the nearly vanishing cd reported for denatured PE.

The cd has also been studied at intermediate urea concentrations. Unfolding of PC with urea can be interpreted from these data as a stepwise process. Monitoring the urea induced unfolding of PC by cd at different wavelengths ($\lambda = 220$, 345, and 610 nm); the "melting point" of the apoprotein (4–5 M urea) coincides with the extrema of the titration curves obtained in the chromophore region (345, 610 nm). These results give direct evidence for the existence of an intermediate species whose population reaches a maximum at 4–5 M urea.

Introduction

Phycobiliproteins are light harvesting pigments of certain algae (for some recent reviews, see *e.g.* refs. 1–4).

They contain chromophores like **1** covalently bound to the apoproteins. Whereas the denatured pigments have properties typical to free bile pigments of similar structure, *e.g.* **3,** these properties are conspicously changed in the native pigments, in an adaptation to their function. From UV-Vis studies and complementary MO-calculations it has been concluded, that these changes are in part due to a conformational transition from a cyclic helical (*e.g.* **1a**) to a more extended conformation (*e.g.* **1b,** for leading references, see refs. 4), and that this process involves at least one intermediate state [5]. The denaturation of PE [6] and phytochrome P_r and P_{fr} [7] has been studied in detail by a combinatory evaluation of absorption, fluorescence, and circulardichroism spectroscopy. It could be shown that these techniques are complementary in exhibiting different sensitivities to the processes involved during denaturation. As an example in PE both the fluorescence and the cd-signals of the chromophores [4] decrease to almost zero upon complete unfolding of the protein [6]. On the other hand, the optical activity of phytochrome (P_r, **2**) is retained upon unfolding of the protein [7].

We here wish to report the corresponding cd spectra for the urea-induced denaturation of PC. The results obtained permit conclusions with respect to the origin of optical activity of biliproteins in general, especially in their denaturated states. Moreover, more direct evidence has been obtained for a stepwise unfolding of PC proposed earlier from UV-Vis data [5].

Abbreviation: PC, C-phycocyanin; PE, C-phycoerythrin; P_r, red absorbing form of phytochrome; cd, circulardichroism; UV-Vis, absorption spectroscopy in the ultraviolett and visible range.

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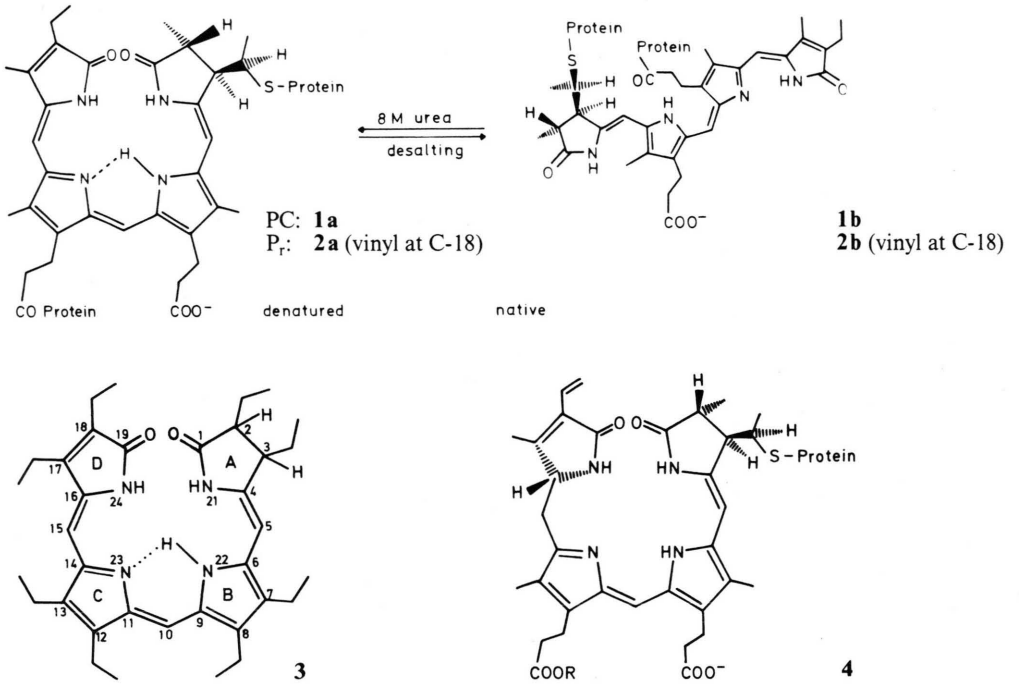

Experimental part

PC from *Spirulina platensis* was prepared by the method reported earlier [8]. Urea was reagent grade (Merck, Darmstadt) or ultrapure (Schwarz & Mann, New York). In the spectroscopic studies, stock solutions of PC ($\approx 20\,\mu$M) in potassium phosphate buffer (0.05 M, pH 7) were mixed with this buffer, and with buffer containing urea (8 M) in the appropriate ratios. UV-Vis and fluorescence spectra were recorded on a model DMR 22 (Zeiss, Oberkochen) spectrometer equipped with thermostated cell holder, at 20 °C. Cd-spectra were recorded on a dichrograph Mark III (Jobin-Yvon, France) using thermostated (20 ± 1 °C) cylindrical quartz cuvettes with variable path length (10 − 0.1 mm). The base lines were obtained by measuring the appropriate buffer-urea solutions under the same conditions.

Results

The cd of native PC (Fig. 1) has a positive long-wavelength band peaking at 632 nm and a negative near-uv cd-band peaking at 342 nm, which are due to the chromophores. The bands below 280 nm arise primarily from the apoprotein. Both chromophore bands are structured in a similar fashion as the absorption bands (Fig. 1), although there are small but distinct variations between the cd- and UV-Vis maxima (*vide infra*).

PC contains three chromophores of identical molecular structure (**1**), but in different protein environments (see refs. 1 − 4). The fine structure of

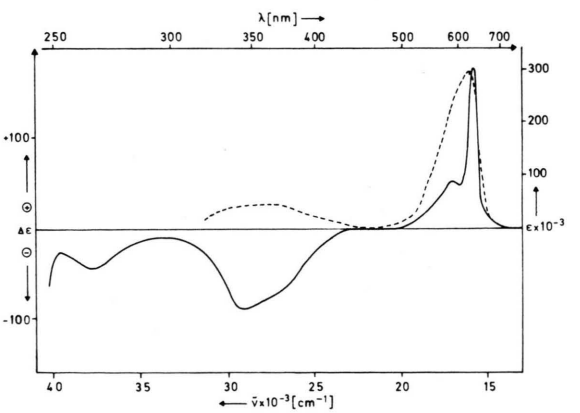

Fig. 1. Cd (——) and UV-Vis absorption spectrum (------) of PC (4×10^{-6} M) in the chromophoric region in phosphate buffer (0.05 M, pH 7) at room temperature. Molar extinction coefficients (ε) and $\Delta\varepsilon$-values are given for the monomer containing three chromophores.

the long-wavelength cd-band is roughly similar to that of the absorption band. In the latter, the major band corresponds to two chromophores, one each on the α- and β-chain. The shoulder at shorter wavelengths which is resolve as a second band at low temperatures [9] arises from the second chromophore on the β-chain. This assignment is likely from the spectra of the isolated subunits (Scheer *et al.*, unpublished). The similarity of the cd-band shape to this pattern indicates a similar differentiation. Upon denaturation, the long-wavelength cd-band ($\lambda_{max} = 615$ nm) exerts a hypsochromic shift by

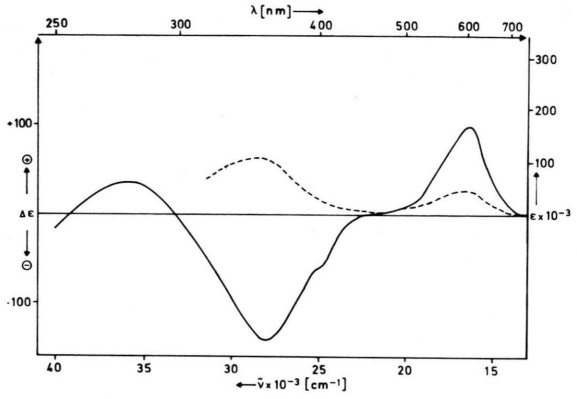

Fig. 2. Cd (——) and UV-Vis absorption spectrum (------) of PC (4×10^{-6} M) in phosphate buffer (0.05 M, pH 7) containing urea (8 M) at room temperature. See Fig. 1 for molar extinction coefficients and $\Delta\varepsilon$-values.

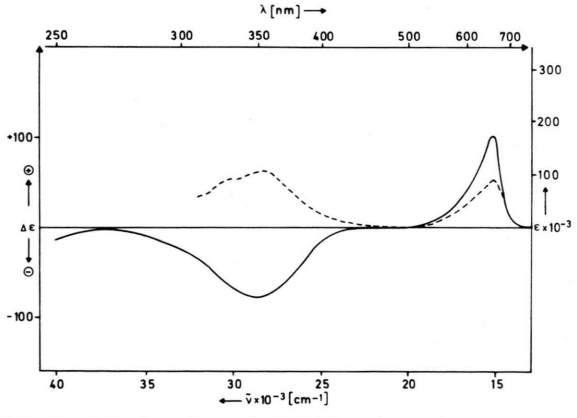

Fig. 3. Cd (——) and UV-Vis absorption spectrum (------) of PC (4×10^{-6} M) in aqueous urea (8 M) titrated with HCl to pH 1.5, at room temperature. See Fig. 1 for molar extinction coefficients and $\Delta\varepsilon$-values.

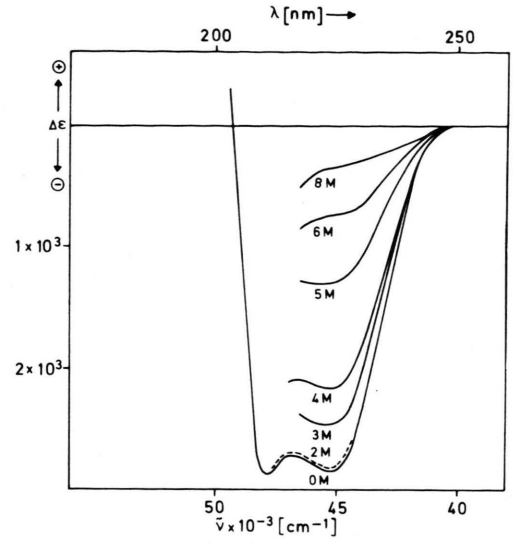

Fig. 4. Cd spectra of PC (4×10^{-6} M) in the peptide absorption region at various urea concentrations (0 M, 2 M, 3 M, 4 M, 5 M, 6 M, and 8 M) in 0.05 M phosphate buffer at pH 7; room temperature.

17 nm, and the $\Delta\varepsilon$-value is decreased only by about 40% (Fig. 2). The changes in the negative band at 342 nm are less pronounced, there is even a slight increase of the ellipticities. Both bands loose their fine structure with increasing urea concentrations.

For a comparison, the cd of PC in 8 M urea at pH 1.5 is given in Fig. 3. The chromophhore is present in its protonated form under these conditions. In the UV-Vis spectrum, this leads to an increased absorption and a concomitant red-shift of the long-wavelength band [10], whereas the near-uv band remains almost unchanged. The red-shift is matched by the cd-band. The anisotropy factor of both bands is decreased by a factor of two.

The region of the peptide cd-bands (200–250 nm) shows a steady decrease with increasing urea concentrations (Figs. 4, 5), with an inflection at about 4.5 M urea. The cd-titration curves at wavelengths $\geqq 300$ nm (corresponding to chromophore absorptions) do not show similarly steady changes but rather irregularities which are most pronounced in the region between 4 and 5 M urea. Two such curves are shown in Fig. 5, and phenomenologically similar curves are obtained at any other wavelength corresponding to chromophore rather than peptide absorptions.

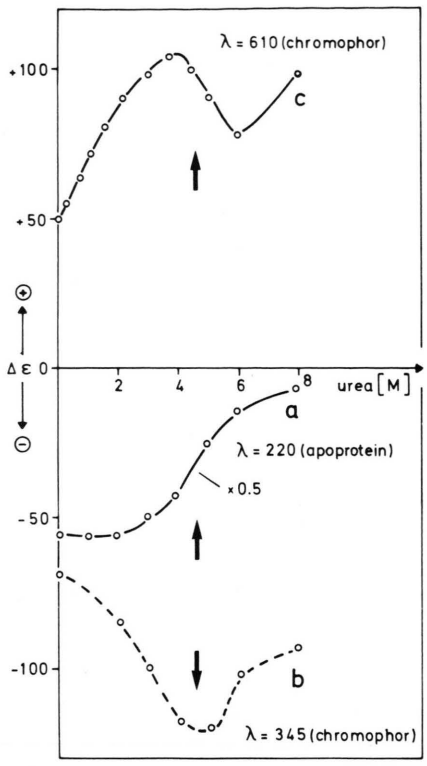

Fig. 5. Titration curves of PC with urea at three wavelengths: a: 220, b: 345 and c: 610 nm, monitored by cd.

Discussion

a. Noncoincidences between cd- and UV-Vis absorption extrema

Whereas the cd fine structure in the native pigment can be interpreted like the electron absorption spectra by the three chromophores present in different environments (see refs. 1–4), the bathochromic shift of the cd-spectra with respect to the electron absorption spectra in the chromophoric region needs further discussion.

Principally, it may arise from several origins: i) A dynamic equilibrium between different conformers of each chromophore, with the populations absorbing more to the red exhibiting a stronger optical activity. ii) Each of the three chromophores is in a rather distinct environment, but the one absorbing mostly to the red has a proportionally low absorption and high optical activity. This situation is similar to (i), but represents a frozen rather than a dynamic state. iii) At least two of the chromophores are close enough and appropriately oriented to each other to produce an exciton couplet with a positive band at the long-wavelength side.

Whereas several lines of evidence (see ref. 4) render the possibility (i) unlikely, both (ii) and (iii) are compatible with results assigning each chromophore type to a rather distinct and rigid site. Exciton coupling (*e.g.* iii) seems at least possible from the following arguments: In native PC the long-wavelength cd-maximum occurs at 632 nm, which is shifted by 306 cm^{-1} (12 nm) to the red as compared to the long-wavelength absorption maximum. A corresponding negative extremum would be expected around 608 nm. The pronounced dip in the cd-curve at 602 nm is indicative of this and may arise from a superposition of the negative part of the couplet with the positive band due to the "s"-chromophore. The opposite changes around 630 (negative) and 600 nm (positive) upon denaturation would also support this explanation. Excition splittings have earlier been proposed for a cryptophytan PC [11] and several allophycocyanins [12], and also discussed for PE [6], but only in the former case there exists more than circumstantial evidence.

b) Origin of optical activity in native and denatured biliproteins

The spectral properties and the conformation of the prosthetic groups of native biliproteins are strongly influenced by the corresponding apoproteins to which they are covalently bound. Thus, the chromophores exhibit an extended conformation which is chiral and rigid due to non covalent interactions with the apoprotein. On destroying the influence of the apoprotein by denaturation with urea, the conformation of the tetrapyrrol chromophores changes to a cyclic, helical arrangement (see ref. 4 for leading references). Hence, denatured biliprotein-chromophores predominantly adopt a cyclic helical conformation (**2a** and **1a** respectively). In open-chain tetrapyrrols lacking any chiral influence the P- and M-helices are enantiomeric and thus equipopulated. Since the inversion barrier of the tetrapyrrol-helix is low (42 kJ · mol^{-1} in biliverdin [13]) and inversion fast at ambient temperature, the P/M-helix ratio can be changed by exerting a diastereoisomeric relationship on it. Since such

helices represent inherently chiral chromophores large optical activity is to be expected even if the excess population is small. An example for an external chiral influence represents the cd of achiral bile pigments complexed with the protein, serum albumin [14] or dissolved in chiral, optically active solvents [15–17].

In the denatured state of biliproteins the optical activity observed, is governed mainly by internal chiral influences, viz. of the chirality centers present in the chromophores. The PC chromophores possess chirality centers at C-2, C-3 and C-3[1] inducing a twist of the C-5 methine bridge, which is energetically favoured in one distinct direction. Hence, the large optical activity of even denatured PC may be rationalized in terms of an excess population of the P-helical arrangement being diastereoisomeric to the less populated M-helix. This interpretation is further corroborated by recent Ramachandran-type calculations [18, 19] indicating a twist of the C-5 methine bridge due to steric hindrance of the asymmetrically substituted ring A with rings B and D. Accordingly, the P-helical form would be favoured, in agreement with both the sign and the strong optical activity of denatured PC.

This interpretation likewise accounts for both the low optical activity observed for denatured PE (chromophore **4**) [6] and the large $\Delta\varepsilon$-values reported for denatured red-absorbing phytochrome (P_r, chromophore **2**) [7]. The chromophores of P_r (**2**) and PC (**1**) are constitutionally and configurationally closely related. The reason for the high optical activity in the denatured state of P_r may then likewise be attributed to the influence of the chirality centers at C-2, C-3 and C-3[1], in generating an excess population of the P-type tetra-pyrrol-helix. In PE (**4**), the additional chirality center at C-16 leads to a pertubation at the C-15 methine bridge as well. However, this influence counteracts the one excerted by C-2, C-3 and C-3[1]. In a situation similar to that in the i(=inactive)-urobilins with asymmetric C-4 and C-16 [20] these opposing effects in PE account for the extremly low optical activity observed for denatured PE [6].

For a more relevant comparison of PC with P_r we have also measured a cd-spectrum of the urea-unfolded PC under acidic conditions (Fig. 3). The cd-spectrum of protonated PC (Fig. 3) possesses the same phenotype as that of protonated P_r. It is positiv in the long wavelength band, negative in the band around 360 nm. Even the $\Delta\varepsilon$-values are very similar: P_r: 660 nm, $\Delta\varepsilon = +20$; 375 nm, $\Delta\varepsilon = -15$; PC (values for *one* chromophore): 658 nm, $\Delta\varepsilon = +101/3 = +33$; 350 nm, $\Delta\varepsilon = -75/3 = -25$. In the native state, by contrast, the cd-spectra of phytochrome P_r [7] and of PC (Fig. 1) are (expectedly) quite different indicating the varying influence of the two different proteins on the chromophores.

The close resemblance of the cd-spectra of the denatured PC (Fig. 2, 3) and phytochrome in the P_r-form [7] with that of a model pigment, 2,3-dihydrooctaethylbilindion (**3**), in chiral, optically active solvents [17] further corroborates the arguments made above. The cd-maxima of the latter at 610/348 nm [17] are only slightly shifted as compared to denatured PC at 615/358 nm (Fig. 2). Moreover, the similar ratios of $\Delta\varepsilon$-values (0.7 for dihydrooctaethylbilindion **3** and 0.8 for PC in 8 M urea) indicate a similar pitch for the helix of the two chromophores.

c. Stepwise unfolding of PC during denaturation

In an earlier study of PC unfolding [5] an intermediate during the denaturation process of PC with urea has been postulated to account for the singular behavior of the UV-Vis absorption spectra. Fig. 5 nicely shows that the cd-titration curves yield more direct evidence for this assumption. The titration curve of the apoprotein ($\lambda = 220$ nm, curve **a** in Fig. 5) taken from the data of Fig. 4 has an inflection point between 4 M and 5 M urea. This point roughly represents the "melting point" of the apoprotein of PC. However, monitoring the chromophoric region by cd, *e.g.* at 380 nm and 610 nm (curves **b** and **c** in Fig. 5), curves are obtained whose maxima approximately coincide with the inflection point of curve **a** for the peptide region (arrows). In a mathematical sense, curves **b** and **c** are derivatives of **a**. This means, that at least three species of PC are involved in the denaturation process: Between 0 M and 2 M urea the native species dominates. With increasing urea concentration, the concentration of this species decreases and an intermediate, new species appears reaching a maximum at the "melting point" of the apoprotein (4 M–5 M urea). Higher urea concentrations then favour the completely randomized, denatured PC and the concentration of the intermediate vanishes again at 8 M urea.

Acknowledgements

H. L. thanks the "Hochschuljubiläumsstiftung der Stadt Wien" and the "Österreichischer Fonds zur Förderung der wissenschaftlichen Forschung" (project 3033) for support, and H. S. acknowledges support of this work by the Deutsche Forschungsgemeinschaft, Bonn.

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