Circular Dichroism of C-Phycoerythrin: A Conformational Analysis

Elisabeth Langer, Harald Lehner,

Institut für Organische Chemie der Universität Wien, Währingerstraße 38, A-1090 Wien

Wolfhart Rüdiger and Barbara Zickendraht-Wendelstadt

Botanisches Institut der Universität München, Menzinger Straße 67, D-8000 München 19

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An extensive study of the chiroptical properties of C-phycoerythrin and the α- and β-subunits in the spectral region from 700–200 nm is presented.

Based on the VIS-circular dichroism inherently chiral conformations are proposed for the covalently linked chromophores.

By means of mean residue ellipticities and the experimental circular dichroism spectra in the region of the $n \rightarrow \pi^*$ peptide transition the α-helix contents of the apoproteins of the α- and β-subunits are estimated to amount to 60% and 40%, respectively. The circular dichroism spectrum of native C-phycoerythrin is congruent with a linear superposition of the α- and β-subspectra, in the whole spectral region studied. Since α- and β-subunits are associated in native C-phycoerythrin as revealed by sedimentation analysis the interactions between the subunits in the native chromoprotein are not accompanied by substantial conformational changes. In the temperature range 0 °–40 °C the thermally induced changes of the chromophores in native C-phycoerythrin are not associated with changes of the secondary structure of the apoprotein. Unfolding occurs at 60 °–70 °C but slowly leads to irreversible denaturation.

Protein unfolding starts at 3 M urea. The random coil secondary structure of the apoproteins is reached at 8 M urea. At this concentration the absorbance and the optical activity of the chromophores are reduced by a factor 3 and 10, respectively. The conformational changes in the peptide with increasing denaturant concentration are not synchronous with those induced in the chromophore indicating that a multistep process is operative during unfolding. The CD results on denaturation are supplemented by absorption and emission spectroscopy.

Introduction

Phycoerythrins are antenna pigments located in the phycobilisomes of Rhodophyceae and Cyanobacteria. They are conjugated proteins containing tetrapyrrol moieties as prostethic groups [1]. The chromophore content of the subunits varies with their origin. C-PE from *Pseudanabaena* W 1173 consists of two subunits, called α- and β-chain, which comprise two and three chromophores, respectively [2]. Each subunit carries one fluorescing (f-) chromophore absorbing at longer wavelengths whereas the chromophores absorbing at shorter wavelengths are termed sensitizing (s-) chromophores [3]. The chromophores are covalently bound to the apoproteins by a thioether linkage. A second (ester-) linkage was found for one of the α-chain chromophores [4]. Cleavage of all chromophores from the protein furnishes exclusively PEB which contains an ethylidene group instead of the thioether bond [1, 2, 5]. The total synthesis of PEB-dimethylester was recently described and the absolute configuration of its chirality centers determined to be 2R, 16R [6, 7]. As the isolated chromophore is homogeneous, the different spectroscopic behaviour of the five prosthetic groups in native C-PE is governed by the protein and indicates inhomogeneity of conformational and/or tautomeric nature. Thus, for the state of the chromophores the interactions with the protein play an important role. However, little is known in detail about that subject. Therefore we have initiated a study of the CD of C-PE and its subunits in the chromophore and peptide electronic absorption region to gain more insight into the inter- and intramolecular interactions and to elucidate qualitatively the conformation and conformational behaviour of the prosthetic groups and apoproteins.

Abbreviations: CD, circular dichroism; C-PE, C-phycoerythrin; PEB, phycoerythrobilin; C-PC, C-phycocyanin.

Reprint requests to Prof. Dr. W. Rüdiger.

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Materials and Methods

For isolation of native C-PE from *Pseudanabaena* W 1173 (obtained from R. Wagenmann, München)

and the separation of the α- and β-subunits by ion exchange chromatography *cf.* refs. [2, 3].

For all solutions prepared degassed 0.025 M (for CD) or 0.1 M (for UV-VIS and fluorescence spectra) phosphate buffer pH 7 (Na_2HPO_4/KH_2PO_4 in bidistilled water) was used. For CD spectra 50 µl of concentrated stock solutions (appr. 4 to 8×10^{-5} M) of the biliproteins prepared immediately prior to use were injected into 2 ml of buffer resulting in C-PE solutions of 1 to 2×10^{-6} M. The concentrations were checked by absorption spectroscopy [2]. In the case of denaturation experiments 2 ml of urea solutions (1 M, 2 M, 3 M, 4 M, 4.5 M, 5 M, 5.5 M, 6 M, 6.5 M, 8 M and 9 M) were used instead. Due to a loss of optical activity fivefold concentrated stock solutions were employed for the measurements in 8 M and 9 M urea. Urea (ultra pure) was purchased from Schwarz/Mann (Orangeburg, N. Y.). All handlings and operations were performed under anaerobic conditions in the dark.

CD spectra were taken within one hour after preparation although no significant changes of the spectra were observed even after several hours. The CD spectra were run on a Jobin Yvon Mark III instrument (slit width: 10 Å, scan speed: 0.1 nm/sec) using thermostated (± 1 °C) 0.5 cm or 1 cm cylindrical quartz cuvettes. The base lines were obtained by measuring the appropriate solvents under the same conditions. Temperature was controlled by a copper/constantan thermoelement. The absorption spectra were taken on a Cary 15 or Zeiss DMR 22 instrument. Fluorescence spectra were recorded with the Zeiss DMR 22 spectrophotometer equipped with accessories for fluorescence investigations. Sedimentation coefficients were determined in a Beckman E analytical ultracentrifuge, equipped with scanner optics (UV analyser) as described in ref. [3]. 2R-, 16R-PEB dimethylester kindly supplied by H. Scheer was measured in spectrograde methanol, CH_2Cl_2 (Uvasol, Merck) and a 1:1 mixture of methanol and an aqueous 8 M urea solution at 20 °C and $c = 5 \times 10^{-5}$ M.

Results and Discussion

The subunits

Prosthetic groups

The chromophore responsible for the CD spectrum in the 700–250 nm region of C-PE and its subunits (and PEB dimethylester) involves the conjugated system comprising rings A, B and C of the tetrapyrrol moiety. Although ring D and the steric requirements of its substituents bear a pronounced influence on the overall conformation of the molecule our spectral data do not permit direct conclusions concerning the relative orientation of ring D. Therefore all statements concerning the chromophore refer to the conjugated part of the molecule which should be distinguished from the prostetic group per se. The CD spectrum of the α-chain exhibiting two maxima with positive rotational strengths in the VIS absorption region and a smaller weaker band at 307 nm (32570 cm^{-1}) (*cf.* Table I) bears a striking resemblance with the corresponding absorption spectrum showing maxima at 566 and 543 nm (17670 and 18420 cm^{-1}, respectively) [3]. As has recently been concluded from the absorption spectra [3], these bands can be attributed to two individual chromophoric moieties in conformationally or tautomerically different states. This inhomogeneity is further corroborated by the different (reversible) temperature response of the two bands: only the Cotton effect at $\lambda_{max} = 574$ nm (17420 cm^{-1}) is affected significantly being enhanced on lowering the temperature as outlined in Table I.

Table I. CD-spectra of C-PE subunits at 20 °C and 0 °C. (The changes observed are reversible).

Subunit	T [°C]	λ_{max} [nm]	ν'_{max} [cm^{-1}]	$\Delta\varepsilon_{max}$ [$M^{-1}\,cm^{-1}$]	θ_{max} $\times 10^{-5}$
α	0	577	17 331	+ 198	+ 6.53
		545	18 349	+ 67	+ 2.21
		307	32 573	− 67	− 2.21
		222	45 045	− 1046	− 34.52
		210	47 619	− 932	− 30.76
α	20	574	17 422	+ 131	+ 4.32
		545	18 349	+ 77	+ 2.54
		307	32 573	− 54	− 1.78
		222	45 045	− 995	− 32.84
		210	47 619	− 892	− 29.44
β	0	567	17 637	− 393	− 12.97
		546	18 315	+ 265	+ 8.75
		520 s	19 231	+ 147	+ 4.85
		307	32 573	− 69	− 2.28
		220	45 455	− 917	− 30.26
		209	47 847	− 950	− 31.35
β	20	570	17 544	− 336	− 11.09
		549	18 215	+ 255	+ 8.42
		520 s	19 231	+ 137	+ 4.52
		307	32 573	− 74	− 2.44
		222	45 045	− 898	− 29.63
		209	47 847	− 928	− 30.62

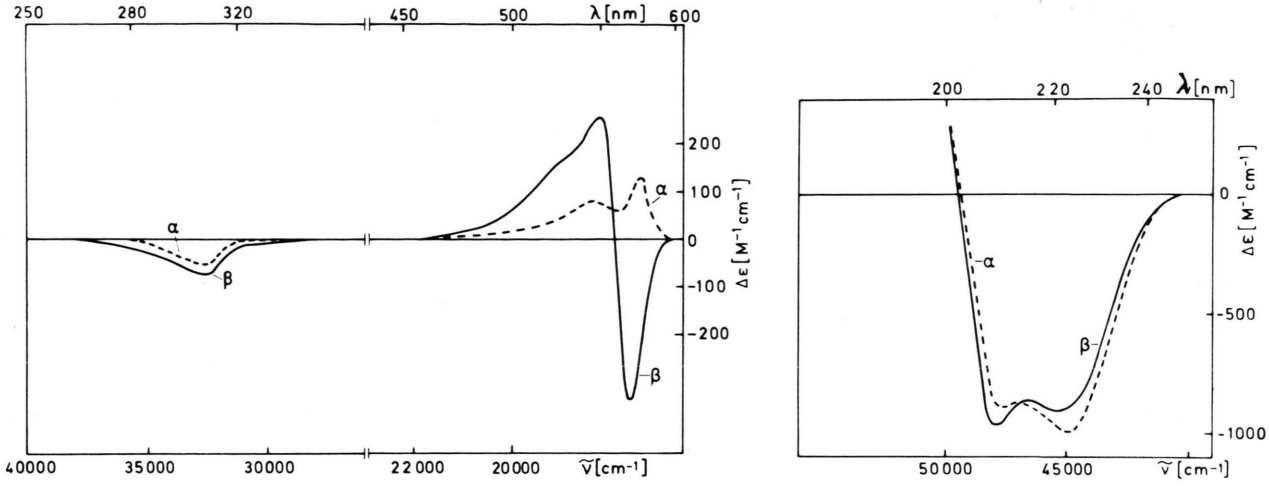

Fig. 1. CD-spectra of α- and β-subunits of C-PE a) (left) in the chromophoric region and b) (right) in the peptide absorption region at 20 °C in 0.025 M phosphate buffer pH 7 (2×10^{-6} M).

The CD spectrum of the β-chain in the VIS absorption region is quite different. It exhibits a bisignate Cotton effect ($\lambda_{max} = 570$ and 549 nm; 17540 and 18220 cm^{-1}, respectively, $\Delta\nu = 680\,cm^{-1}$) with a shoulder around 520 nm (19230 cm^{-1}). The absorption maximum (556 nm; 17990 cm^{-1}) corresponds roughly to the point of inversion ($\Delta\varepsilon = 0$, $\lambda =$ 559 nm; 17890 cm^{-1}) of the CD curve. A shoulder at 520 nm is also evident from the absorption spectrum [3]. Only small changes are observed upon cooling the solution to 0 °C (*cf.* Table I).

Investigations of centrochiral tetrapyrrols furnished evidence that their chiroptical properties are mainly governed by the asymmetric carbons next to the meso positions [8–11]. Thus, the optical activity of PEB-dimethylester isolated from native C-PE is largely determined by the R-configuration at C-16 inducing an inherently chiral conformation of the chromophore [6].

In native C-PE and in renatured subunits, the influence exhibited by the protein on the prosthetic groups is superimposed. The exceedingly strong positive Cotton effects of the two α-chain chromophores ($\Delta\varepsilon = +131$ and $+77$, *cf.* Table I and Fig. 1) can be understood in terms of strongly twisted conformations.

The negative and positive Cotton effects observed at 570 and 549 nm respectively in the CD spectrum of the β-chain can be attributed to the f- and one of the s-chromophores for which an energy difference of approximately 400 cm^{-1} has been calculated [3]. Thus, a priori two interpretations can be offered: the bisignate bands can either be due to an exciton splitting arising from a proximate arrangement of the two chromophores involved. Lacking further evidence about their relative orientation a conclusion concerning either helical sense cannot be drawn. Alternatively, the same phenomenon could be brought about by two independent Cotton effects arising from chromophores of opposite chiral sense. Hence, the conformation of the f-chromophore in the β-chain should differ from the other four chromophores of C-PE by its helicity. However, assuming this interpretation to be valid, a mutual attenuation of the two energetically close lying bands should be operative. The remaining third β-subunit prosthetic group (of s-type [3]) is responsible for the positive shoulder at 520 nm in the CD spectrum, so that it exhibits the same helical sense as the two α-chain chromophores.

Without attempting a final decision between the two explanations for the inversion of sign in the CD of the β-subunit chromophores the arguments given above for the α-chain hold with respect to the rotational strengths observed: in the undeconvoluted β-subunit all three chromophores exist in strongly twisted conformations.

The apoproteins

The UV-CD spectra of the C-PE subunits comprise two maxima between 250 and 200 nm due to

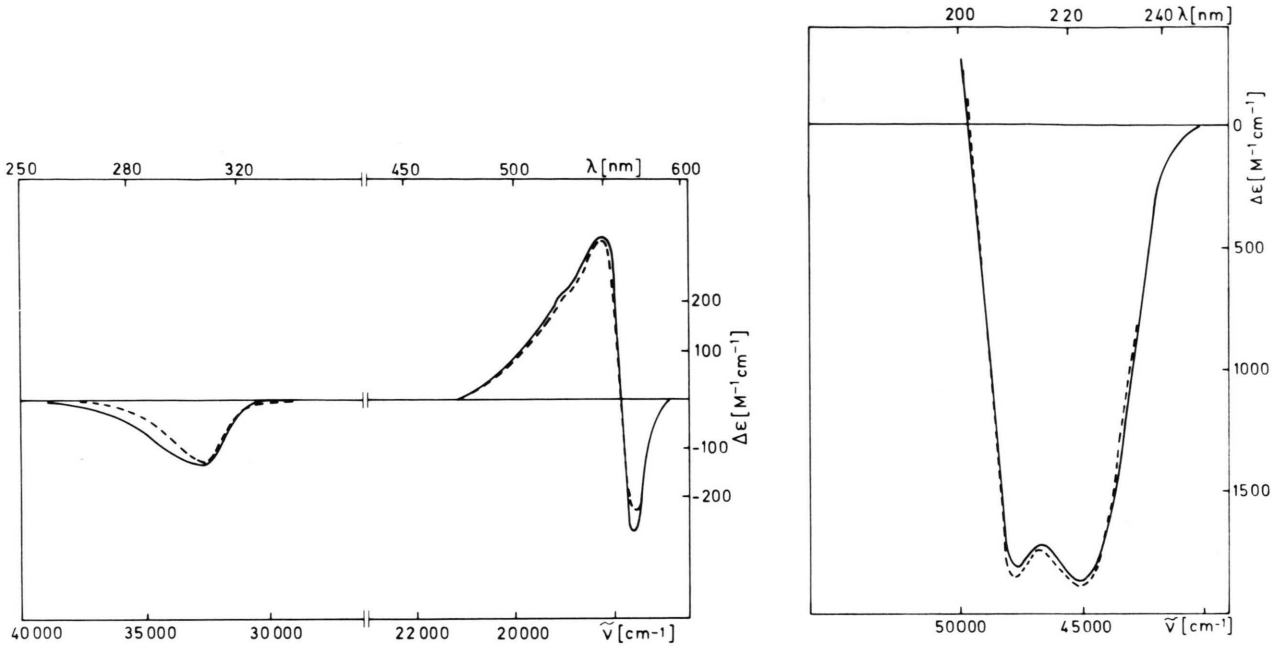

Fig. 2. CD-spectra of native C-PE (——) and the spectrum obtained by a linear superposition of α-chain and β-chain (- - - -) a) (left) in the chromophoric region and b) (right) in the peptide absorption region at 20 °C in 0.025 M phosphate buffer pH 7 (10^{-6} M).

the peptide chains (α-chain: $\lambda_{max} = 222$ and 210 nm, β-chain: $\lambda_{max} = 222$ and 209 nm, *cf.* Fig. 1 b).

Because of the remarkably high ellipticities observed for both proteins (*cf.* Table I) the α-helix content can be expected to be large in both cases. Since standard values exist for the mean residue elipticities of α-helix, pleated sheet and random coil peptides [13–17] the secondary structure can be estimated. However, the contribution of the α-helix to the optical activity is much higher than that of any other conformation. Thus, the accuracy for the proportion pleated sheet/random coil is poor and can only be given qualitatively. Mean residue ellipticities for two selected wavelength-pairs (222/210 nm and 228/216 nm) were taken from Chen *et al.* [16, 17]. 157 and 188 amino acids were assumed for the α- and β-chain, respectively [18]. In this way a helix content of approximately 60% is estimated for the α-subunit and about 40–50% for the β-chain. A similar helix contribution (~ 60%) has been determined for phycocyanin and allophycocyanin by the same method [19]. This rough estimation does not reveal any pleated sheet secondary structure in the α-chain, while a contingent of 10–20% is obtained for the β-subunit. A more significant contribution of pleated sheet protein structure in the β-chain is already evident from an inspection of the phenotype of the CD spectrum. This result is comparable with data from another biliprotein. Zuber [20] has calculated the following values from the primary structure of a phycocyanin: α-chain 41% α-helix and 17% pleated sheet, β-chain 29% α-helix and 30% pleated sheet.

Native C-PE

Molecular weight determinations show that C-PE is monomeric [α β] in the concentration range up to 0.6×10^{-5} [3]. This means that we do not deal with a mixture of the monomeric subunits (*cf.* Table III).

The CD spectrum of native monomeric C-PE is shown in Fig. 2. Within experimental error the linear superposition of the α- and β-subunit CD spectra is equivalent to that of native C-PE in the whole spectral region accessible. A similar additivity has been found for the VIS absorption spectra [3]. Hence, under the conditions employed the intermolecular interactions between the α- and β-subunits being responsible for the association do not give rise to a mutual change of the secondary

structure of the proteins or states of the chromophores.

It should be mentioned that our VIS-CD spectra for native C-PE and the α-subunit respectively strongly resemble those for "single" and "double peaked" PE given by Fujimori *et al.* [12]. However, in contrast to the highly associated PE studied in ref. [12], our investigations clearly show that there are no CD detectable interactions between chromophores of different subunits in monomeric C-PE.

Denaturation

Denaturation of biliproteins has been described by means of heat and chemical agents [2, 4, 21–23]. The thermally induced denaturation of C-PE as monitored by UV-VIS spectroscopy and by CD spectroscopy is shown in Figs. 3 and 4 and Table II. Increasing temperature leads to a decrease of the oscillator strength and a hypsochromic shift of the visible absorption maximum (Fig. 3). Furthermore, the longwavelength absorption at 565 nm (*i. e.* the absorption of f-chromophores) is decreased prior to the absorption of s-chromophores (determined at 540 nm) and prior to the main protein unfolding process (Fig. 4). The latter can be deduced at 60 to 70 °C from absorbance increase at 280 nm and from the change of the chromophore absorption spectrum

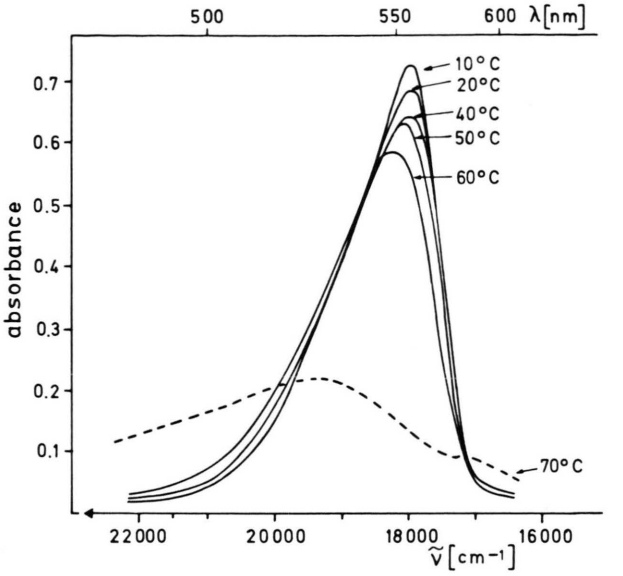

Fig. 3. Visible absorption spectrum of C-PE in 0.1 M sodium phosphate buffer, pH 7, kept for 15 min at different temperatures. All spectra are completed within 1 to 2 min.

which closely resembles that in 7–8 M urea [24]. The fluorescence quantum yield gradually decreases with increasing temperature from 0.75 at 10 °C to 0.2 at 50 °C. This can be interpreted as increasing flexibility of f-chromophores. This decrease is paralleled by an attenuation of the optical activity at 569 nm,

Table II. CD-spectra of native C-PE at 0 °C, 20 °C and 42 °C and *after recooling to 20 °C. The data in brackets are obtained by summation of the pertinent α- and β-chain values.

T [°C]	λ_{max} [nm]	ν'_{max} [cm^{-1}]	$\Delta\varepsilon_{max}$ [M^{-1} cm^{-1}]	$\theta_{max} \times 10^{-5}$
0	568	17 606	− 334 (− 310)	−11.02 (−10.23)
	549	18 215	+ 375 (+ 330)	+12.38 (+10.89)
	520	19 231	+ 176 (+ 170)	+ 5.81 (+ 5.61)
	307	25 573	− 125 (− 140)	− 4.13 (− 4.62)
	222	45 045	−1818 (−1900)	−59.99 (−62.7)
20	568	17 699	− 259 (− 250)	− 8.55 (− 8.25)
	546	18 315	+ 331 (+ 320)	+10.92 (+10.56)
	520	19 231	+ 176 (+ 170)	+ 5.81 (+ 5.61)
	306	32 680	− 127 (− 130)	− 4.19 (− 4.29)
	222	45 045	−1858 (−1900)	−61.31 (−62.7)
42	569	17 575	− 199	− 6.57
	548	18 248	+ 289	+ 9.54
	520	19 231	+ 156	+ 5.14
	306	32 680	− 120	− 3.96
	222	45 045	−1709	−56.40
20*	569	17 575	− 230	− 7.59
	550	18 182	+ 285	+ 9.41
	306	32 680	− 105	− 3.47
	222	45 045	−1800	−59.40

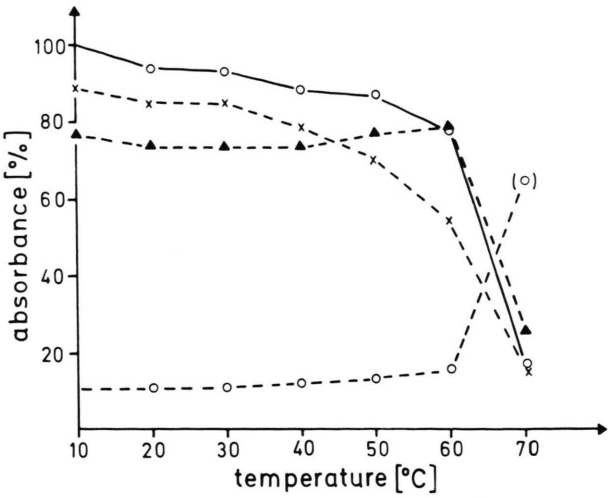

Fig. 4. Decrease of absorbance of C-PE during thermal treatment. Conditions see Fig. 3. Absorbance at (–○–○–) 558 nm, (-x- -x-) 565 nm, (-▲- -▲-) 540 nm, (-○- -○-) 280 nm.

Table III. Sedimentation constants of C-PE and its subunits.

Protein	Protein concentration [mg/ml]	Solvent	$s^{\circ}_{20,w} \times 10^{13}$
C-PE	0.51	SPB *, 5 °C	4.6
C-PE	0.40	SPB *, 20 °C	5.4
C-PE	0.15	SPB *, 20 °C	4.0
C-PE	0.40	SPB *, 40 °C	4.8
C-PE	0.11	SPB *, 40 °C	4.3
C-PE	0.44	Tris **, 4 M urea	2.4
C-PE	0.10	Tris **, 4 M urea	2.2
α-subunit [3]	0.48	SPB *	2.42
β-subunit [3]	0.43	SPB *	2.62

* SPB = 0.1 M sodium phosphate buffer, pH 7.0.
** Tris = Tris-HCl buffer, pH 7.0, ionic strength 0.1, temperature 20 °C if not otherwise stated.

which is more pronounced than the decrease at shorter wavelengths (Table II). In the temperature range investigated by CD (0–40 °C) no significant changes of optical activity in the peptide region can be seen (Table II). This means that no change of protein secondary structure occurs. This confirms the conclusion that the conformation of the f-chromophores is affected prior to the conformation of the bulk of the apoprotein. A further confirmation is obtained from the determination of the sedimentation constants (Table III) which clearly show that no dissociation of C-PE occurs up to 40 °C. This corresponds to the behaviour of monomeric C-PC [21] whereas C-PC from some blue green algae may show dissociation [25] or association [26] with increasing temperature.

Whereas thermally induced absorbance changes are reversible up to 15 min, prolonged heating leads to irreversible changes in the chromophoric region. Therefore the VIS-CD values after recooling are not identical with the original values (Table II). Prolonged heating at 60–70 °C leads to turbidity of the solution and finally to precipitation of denatured protein.

The stepwise denaturation with urea has been investigated by CD, visible absorption and fluorescence spectroscopy. The decreasing peptide optical activity with increasing urea concentration is shown in Fig. 5. Peptide unfolding is initiated by urea solutions > 3 M. Complete deconvolution is reached at 8 M urea and no further changes are detected if the concentration of urea is further increased. Accordingly, the experimental $\Delta\varepsilon$ value for C-PE in 8 M urea is in agreement with the value calculated from mean residue ellipticities [16, 17] assuming the whole peptide in a random coil state (observed: $\Delta\varepsilon_{220} = -280$, calculated: $\Delta\varepsilon_{220} = -320$).

In the chromophore region of the CD spectra (Fig. 6), no spectral changes are observed below concentrations of 3 M urea. However, while the 307 nm CD band turns out to be insensitive towards larger urea concentrations up to 5 M, the VIS Cotton effects exhibit considerable changes, *i. e.* a variation of the positive and negative bands and a gradual loss of optical activity with increasing urea concentration.

At 8 M urea (complete protein deconvolution) the bisignate Cotton effect stemming from the β-chain has disappeared and the remaining positive optical activity in this region is mainly determined by the absolute configuration at C-16 of the chromophores. A small contribution of the C-PE zinc complex at about 590 nm cannot be excluded [24]. For a comparison the CD of 2R-, 16R-PEB dimethylester was recorded in a 1:1 mixture of methanol and 8 M aqueous urea and a value $\Delta\varepsilon(540) = +3.5$ determined. This is in reasonable agreement with one fifth of the optical activity in the chromophoric region of completely denatured C-PE (*cf.* Fig. 6).

The VIS-absorption spectrum (Fig. 7) is mainly affected at > 4 M urea the final decrease being arrived at 8 M urea. According to the sedimentation

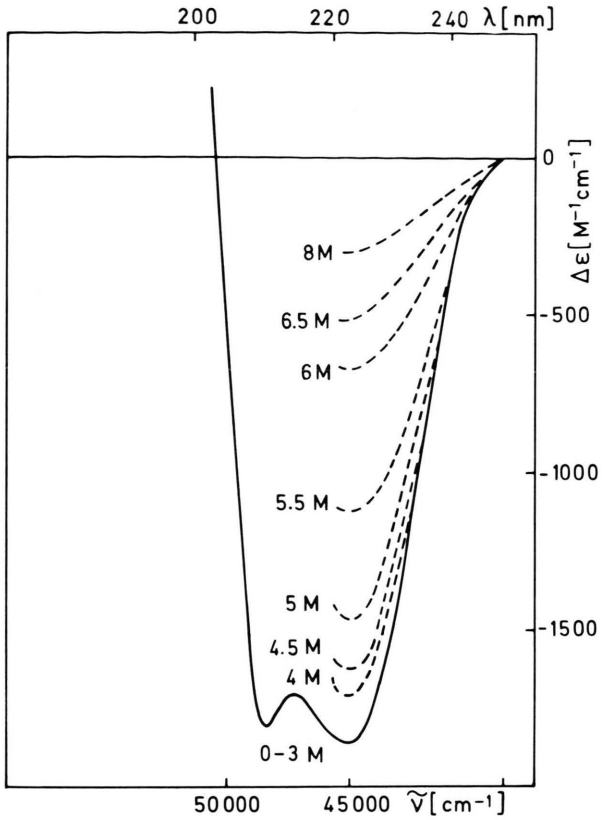

Fig. 5. CD-spectra of C-PE at 0, 1, 2, 3, 4, 4.5, 5, 5.5, 6, 6.5 and 8 M urea in the peptide absorption region at 20 °C.

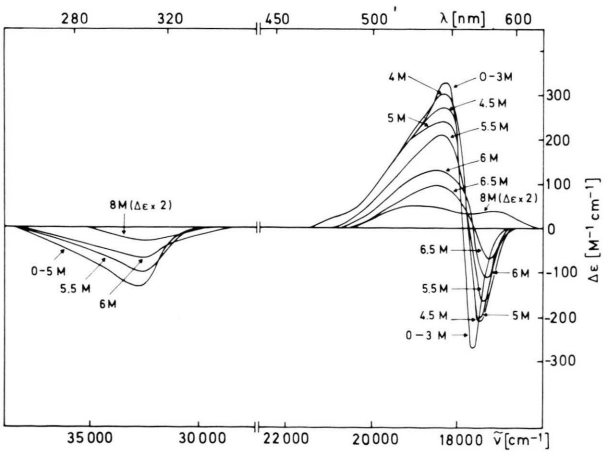

Fig. 6. CD-spectra of C-PE at 0, 1, 2, 3, 4, 4.5, 5, 5.5, 6, 6.5 and 8 M urea of the chromophoric region at 20 °C. The $\Delta\varepsilon$ scale has been enlarged by a factor 2 for 8 M urea.

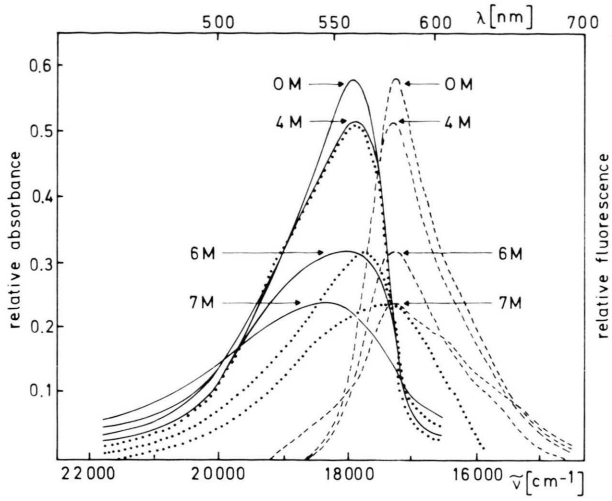

Fig. 7. Visible absorption, fluorescence excitation and emission spectra of C-PE at various urea concentrations at 20 °C. Excitation wavelength 535 nm, emission wavelength 578 nm. (——) Absorption, (- - - - -) fluorescence emission, (· · · · ·) fluorescnce excitation.

constants (Table III) the dissociation of C-PE into the subunits is completed at 4 M urea. Because no significant changes of absorbance or optical activity in the range 0–3 M urea are seen the negligible influence of the dissociation upon these properties is again confirmed. The drastic decrease of absorbance at urea concentrations > 6 M corresponds to the denaturation by increased temperature (see above). The fluorescence excitation spectra are identical with the absorption spectra up to 4 M urea, the fluorescence emission is identical with that of native C-PE (Fig. 7). At further increasing urea concentrations, increasing uncoupling of s-chromophores can be seen, this means no contribution to fluorescence or energy transfer. Finally, at 7 M urea a red shift in the excitation spectrum and a shoulder in the emission spectrum at 600–610 nm is observed. This is typical for the zinc complex of the chromophore which easily forms in such solutions with metal ion traces dissolved from the glass [24].

In Fig. 8, urea titration curves for unfolding of the peptide chain (measured by CD of the peptide region), for fluorescence and for absorbance of the chromophores are compared with each other. Increasing urea concentrations affect the fluorescence first, then the absorbance and finally the optical activity of the peptide. This points to the possibility that the unfolding is a stepwise process which affects

Table IV. Relation of absorbance of C-PE, native and denatured.

	λ^1_{max} [nm]	Relative absorbance [%]	λ^2_{max} [nm]	Relative absorbance [%]	absorbance λ^2_{max} / absorbance λ^1_{max}
buffer, pH 7.0 (native)	558	100	305	100	1 : 6.68
8 M urea, pH 7.0 (denatured)	525 – 540	31	305	166	1 : 1.25
8 M urea, pH 3.0 (denatured)	555	55	305	184	1 : 1.99

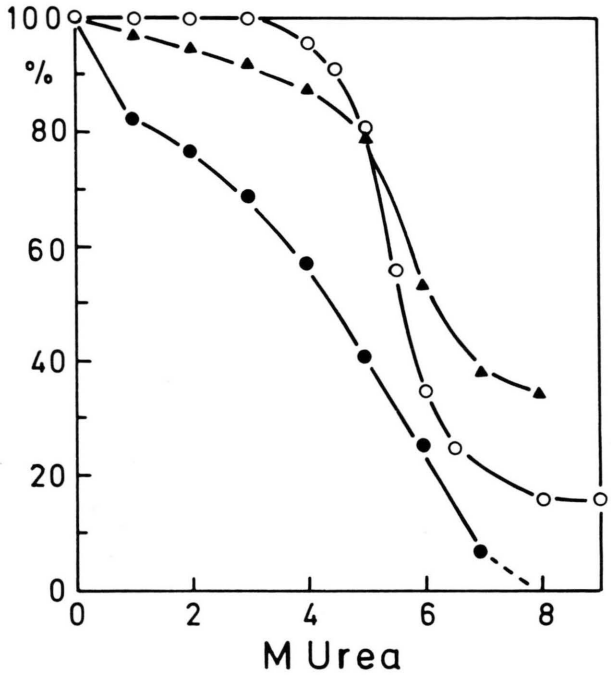

Fig. 8. Urea denaturation of C-PE at 20 °C monitored by CD at $\lambda = 222$ nm (–○–○–), absorbance at 560 nm (–▲–▲–), fluorescence emission at 578 nm (–●–●–).

different properties at different steps defined by different urea concentrations. We consider these different steps as being well defined by urea concentrations. Furthermore renaturation of C-PE is possible even from solutions in 8 M urea yielding "native" C-PE in 80–82% yield as judged from UV-VIS spectra and quantum yield of fluorescence.

In Table IV the UV-VIS absorption band ratios of native and denatured C-PE are compiled. Although the chromophore UV band of C-PE at 305 nm has only a small absorbance compared to the visible band at about 560 nm changes obtained by denaturation are nevertheless significant (Table IV).

Based on model calculations, changes of oscillator strength ratios have been used to correlate experimental data of phycocyanins and bilatrienes with conformational changes [27–32]. If this criterion is also valid for phycoerythrin (and biladienes) the spectral changes observed would indicate a more "closed" conformation of the chromophores in the denatured state of the protein. On the other hand Cole *et al.* [9] pointed out that in native PE the globular apoprotein might furnish a hydrophobic cavity for the embedded chromophores exhibiting a "helical" (closed) conformation. From this point of view and bearing in mind the change of the medium an inverse conformational change of the chromophoric moieties during the protein unfolding process from a more coiled to a more extended topology would be likely.

This interpretation is in accord with the decrease of $\Delta\varepsilon$-values observed for 2R-, 16R-PEB dimethylester in solvents with increasing hydrogen bonding power: CH_2Cl_2 (neutral): $\Delta\varepsilon\,(537) = +\,11.5$, methanol: $\Delta\varepsilon\,(540) = +\,5.0$, methanol/8 M aqueous urea: $\Delta\varepsilon\,(540) = +\,3.5$.

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