Chromophore Content of C-Phycoerythrin from Various Cyanobacteria

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Phycobiliproteins, Phycobilins, Cyanobacteria

The molar extinction coefficient for phycoerythrobilin (**1 a**) was calculated by two independent methods. It is different from that of the cleaved chromophore, phycobiliviolin (**2**). By unfolding with urea or tryptic digestion, the chromophore absorption of C-phycoerythrin (PE) was determined free of any protein influence. The chromophore content of PE from various Cyanobacteria was determined with these data to be either 5 or 6, depending on the organism. This corresponds to a chromophore distribution over phycoerythrin subunits $\alpha:\beta$ of either 2:3 or 2:4. The phylogenetic significance of varying chromophore content is discussed.

C-phycoerythrin (PE) and C-phycocyanin (PC) are light harvesting chromoproteins for photosynthesis in Cyanobacteria (blue-green algae), acting as antenna pigments of photosystem II [1, 2]. These chromoproteins are composed of subunits (α and β peptide chains) in a molar ratio 1 : 1 which both carry covalently linked chromophores, either phycocyanobilin (PCB) or phycoerythrobilin (PEB) [3–5]. Whereas it has been established that the α subunit of PC carries one and the β subunit two PCB chromophores [6], this question has not yet been settled in the case of PE. For PE from *Fremyella diplosiphon*, densitometry of alkylated subunits separated by acid urea gel electrophoresis indicated a 1 : 2 ratio for the chromophore content of α and β subunits [4]. The assumption of one and two PEB chromophores [4] was supported by isolation of the PE subunits from the same organism and determination of the molar extinction coefficients in acid urea as 25 549 for the α subunit and 48 456 for the β subunit [7]. However, these low extinction coefficients are inconsistent with the coefficient of *Aphanocapsa* sp. PE in acid urea determined as 260 000 per $\alpha\beta$ unit [8]; six PEB chromophores were deduced from this finding with a molar extinction coefficient of 43 300 per chromophore, but no distribution over the subunits was given. In a recent work on Rhodophytan phycoerythrins from *Porphyridium cruentum* [9], 2 PEB chromophores per α subunit and 4 PEB chromophores per β subunit were calculated, however, only 2.58 or 3.28 chromophores per isolated β subunit were determined instead of the calculated 4. This fact was explained by rapid rate of either isomerization or hydrolytic cleavage of one of the bilin moieties during the isolation procedure.

These discrepancies prompted us to determine the molar extinction coefficient of PEB and the chromophore number and distribution over the subunits of PE from various Cyanobacteria.

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Abbreviations: PE, C-phycoerythrin; PC, C-phycocyanin; PEB, phycoerythrobilin; PCB, phycocyanobilin.

Experimental

C-Phycoerythrin was isolated from the following cyanobacterial strains: *Pseudanabaena* W 1173 (obtained from R. Wagenmann, München), *Tolypothrix tenuis*, *Pseudanabaena catenata* B 1464-1, *Nostoc muscorum* A B 1453-12b (obtained from Dr. W. Koch, Göttingen), *Fremyella diplosiphon*, *Phormidium persicinum* (obtained from Prof. D. J. Chapman, Los Angeles), and *Phormidium autumnale* C-5 (obtained from Prof. W. Nultsch, Marburg). Cultivation of these strains is described elsewhere [10].

Frozen algal cells (10 g fresh weight) were disrupted by grinding with glass powder (7 g, ϕ 0.25 mm) in a Vibrogen Zellmühle (Bühler, Tübingen) for 2–5 minutes (*P. persicinum:* only 0.5 min). All subsequent procedures were performed at 0–4 °C. Pigments were extracted with 0.1 M sodium phosphate buffer (3 × 5 ml, pH 7.0); after centrifugation, PE was precipitated with ammonium sulfate (50% saturation), dissolved in 1–2 ml 0.1 M Tris-HCl-buffer (pH 7.0) and at first applied to gel chromatography (Sephadex G-100, elution with 0.01 M Tris-HCl-buffer, pH 7.0, containing 0.1 M KCl, 1 mM EDTA and 1 mM NaN_3). The main PE fraction was then applied to ion exchange chromatography (Servacel DEAE 23SS, equilibrated with 0.1 M Tris-HCl-buffer, pH 8.0, elution with the same buffer and 0–0.3 M KCl gradient). Fractions with

$E_{560}/E_{280} > 6.3$ were pooled, PE precipitated with ammonium sulfate (40 – 60% saturation). The precipitate was dissolved in 0.001 M Tris-HCl-buffer (pH 7.0) and desalted by dialysis for 12 – 14 hours against 0.001 M Tris-HCl-buffer and subsequently for 3 – 4 hours against bidistilled water. PE thus obtained was pure according to disc gel electrophoresis (7.5% polyacrylamide gel, Tris-glycine-buffer, pH 8.8, electrophoresis for 8 h at 4 °C and 150 – 200 V) and SDS gel electrophoresis [3].

Trypsin digestion was performed by incubation of pure PE (2 mg) in 10 ml 0.1 M Tris-HCl-buffer (pH 8.0) with 40 µg trypsin at 37 °C under nitrogen. Extinction at 560 nm dropped at first rapidly, but remained nearly constant after 60 – 90 min incubation. Spectra were recorded after acidification with HCl to pH 2. Isolation and analysis of single chromopeptides from the digest is described elsewhere [13].

UV-vis spectra were recorded with a DMR-22 spectrophotometer (Zeiss, W. Germany), gels were scanned with a densitometer TLD 100 with recorder UR 400 and integrator (Vitratron, W. Germany), slit width 0.25 mm, filter 559 nm (for chromophore determination) or 609 nm (for protein bands stained with coomassie-blue).

Results and Discussion

Molar extinction coefficient of PEB

The PEB chromophore (structure **1a**) [1, 2] has the same system of conjugated double bonds as mesobilirhodin (structure **1b**) [11]. It is, therefore, likely that not only the wavelength of absorption [12] but also the molar absorption of **1a** is identical with or similar to that of **1b**. Saturated side chains of bile pigments exhibit only a very minor effect on the spectrum if any. The molar absorption of the cation of **1b** in methanol has been determined as 52,800 [11]. **1b** is not soluble in water whereas the biliproteins have to be measured in aqueous solution. For a calculation of the solvent influence we compared bilipeptides prepared from PE (see below) in methanol/HCl and water/HCl; the ratio $E^{\max 550}_{\text{methanol}} : E^{\max 550}_{\text{water}} = 1.16$ was determined. With this factor, a probable extinction coefficient for the PEB chromophore of 52,800 : 1.16 = 45,500 was calculated.

To confirm this value, spectral properties of a chromopeptide isolated from a tryptic digest of *Pseudanabaena* W 1173 PE [13] were determined. Amino acid analysis of the chromopeptide [13] reveiled the amino acids Arg, Gly, Pro, Ser, Cys in the molar ratio 1.0 : 1.0 : 1.0 : 0.94 : 0.76. This means that each of these amino acids is present once in the pentapeptide; consequently, it should contain one PEB. An aquous solution of this chromopeptide at pH 3.0 (Fig. 1) exhibited the expected maximum at 550 nm with a molar extinction coefficient of 47,100 (based on the amino acid analysis).

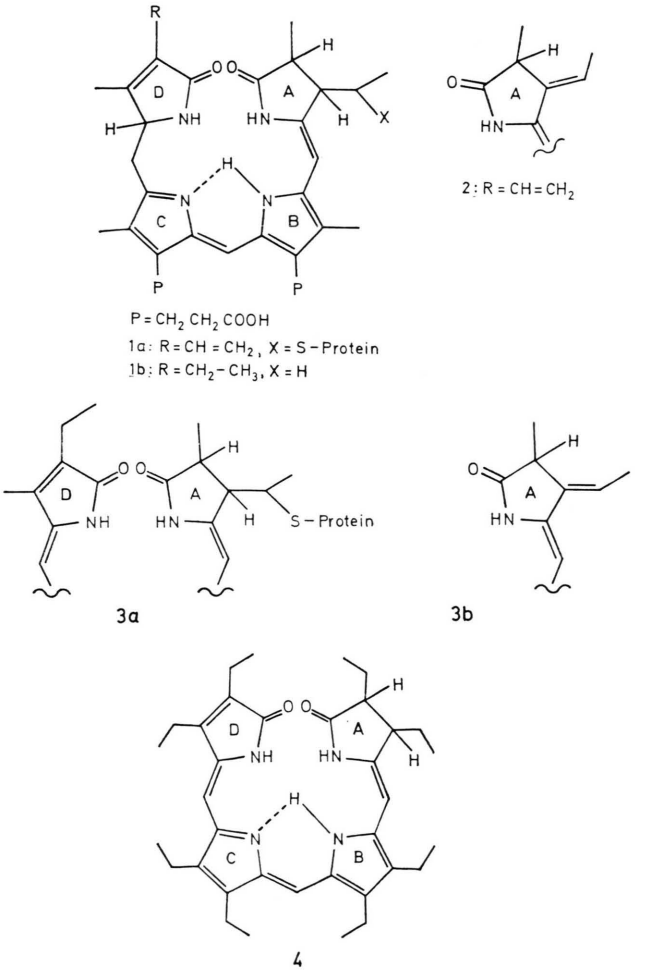
These values 45,500 and 47,100 agree reasonably with the value 43,300 calculated by Glazer and Hixson [8] for the PE chromophore. The lower value of Takemoto and Bogorad [7] (25,549 fort he α subunit) must be due either to bleaching of chromophores during the isolation (14 hours incubation

Table I. Maxima of absorption and molar extinction coefficients of phycobilins and related bilins.

Bilin	Solvent	λ_{max} [nm]	ε	Reference
phycobiliviolin (**2**)	5% HCl/methanol	591 326	25,200 15,800	14
mesobilirhodin (**1b**)	methanol/HCl	560	52,800	11
	water/HCl	560	45,500	
phycoerythrobilin (**1a**)	water/HCl	560	45,500	this
PE-chromopeptide	water/acetic acid, pH 3	550 307	47,100 25,500	paper
1a in unfolded PE	8 M urea/pH 2	555 308	43,300 18,300	8
phycobiliverdin (**3b**)	5% HCl/methanol	690 374	37,900 47,900	15
A-dihydrobiliverdin (**4**)	methanol/HCl	665 351	34,000 36,500	16
phycocyanobilin (**3a**) in unfolded PC	8 M urea/pH 3	662,5 352	35,500 34,700	6

with formic acid was applied, the material was lyophilized as PE and again as subunit which both causes bleaching, see below) or to the presence of urea in the lyophilized subunits.

Table I summarizes the extinction coefficients. It should be pointed out that the chromophore which has been split off from the biliprotein, namely phycobiliviolin (structure **2**), has a chromophore system extended by the ethylidene double bond and exhibits spectral properties (including extinction coefficients) different from those of **1a** (Table I). The same situation exists between PCB (structure **3a**) and phycobiliverdin (structure **3b**). Spectral properties of **3a** are more similar to those of the model compound A-dihydrooctaethylbiliverdin (structure **4**) than to those of **3b** (Table I) although the saturated side chains differ in **3a** and **4**. It is, therefore, impractical to use the same terms PEB for **1a** and **2** or PCB for **3a** and **3b** without any differentiation. The calculation of the chromophore content of PC with the extinction coefficient of **3b** [6] or of PE with the extinction coefficient of **2** [8] can give erroneous results.



Spectrum of PEB in situ

Spectral properties of bilins are strongly influenced by the protein moiety in native phycobiliproteins; this has been explained by a change of the chromophore conformation in the case of PC [16]. The influence of the protein can be eliminated by unfolding of the peptide chain without splitting of covalent linkages between chromophores and protein [1,2]. Unfolding with acid urea has been used for measurement of qualitative [12] and quantitative [6, 8, 9] chromophore spectra. For quantitative purpose, complete unfolding of the peptide chain without bleaching of the chromophore has to be checked carefully. Glazer and coworkers [6, 8, 9] assumed this without control. We checked this by

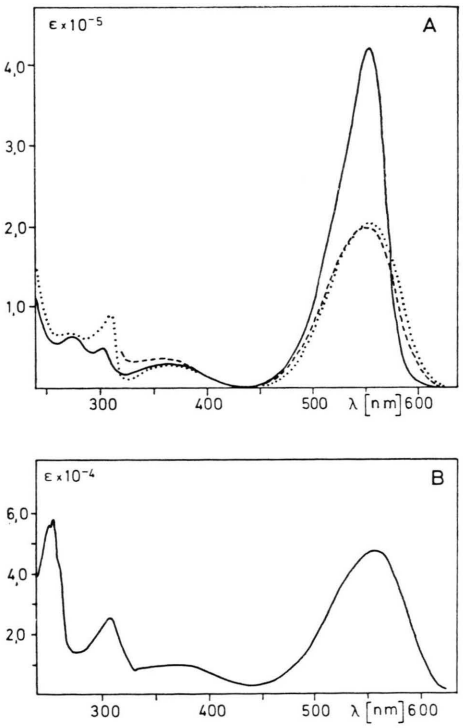


Fig. 1. Electronic spectra of C-phycoerythrin from *Pseudanabaena* W 1173 and tryptic peptides. A —— PE in 0.1 M Tris-HCl-buffer pH 8.0; ···· PE in 8 M urea-HCl, pH 2.0; ------ PE after tryptic digestion in Tris-HCl-buffer pH 8.0 for 2 hours (= mixture of all chromopeptides) acidified with HCl to pH 2.0. — B —— isolated chromopeptide (containing Arg, Gly, Pro, Ser, Cys) in dilute acetic acid pH 3.0.

Table II. Molar extinction coefficients and calculated chromophore content of various phycoerythrins. Aliquots of a PE stock solution (PE content determined by dialysis and lyophilization) are taken for the native spectrum in 0.1 M Tris-HCl at pH 7, for the spectrum in 8 M urea at pH 2, for the spectrum after trypsin digestion and acidification to pH 2. Molar extinction coefficients are given per [$\alpha\,\beta$] unit. PEB chromophore content is calculated with the extinction coefficient 45,500.

Phycoerythrin from	$\varepsilon^{560}\cdot 10^5$ native	$\varepsilon^{550}\cdot 10^5$ 8 M urea pH 2	$\varepsilon^{550}\cdot 10^5$ trypsin digest pH 2	PEB content	
				calculated	next
Pseudanabaena W 1173	4.22	2.03	2.04	4.5	5
Phormidium persicinum	5.21	2.61	2.62	5.7	6
Fremyella diplosiphon	4.83	2.51	—	5.5	6
Aphanocapsa sp. [8]	4.90	2.60	—	5.7	6
Porphyridium cruentum B-PE [9]	—	2.39	—	(5.25)*	6
Porphyridium cruentum b-PE [9]	—	2.45	—	(5.4)*	6

* Glazer and Hixson [9] calculated the values 5.45 and 5.72 with $\varepsilon = 42{,}800$.

comparison with a trypsin digestion; complete transformation of chromophore regions into chromopeptides should lead to the same spectral effect as complete unfolding.

Fig. 1 shows that *unfolding by urea and trypsin digestion lead to the same decrease of absorption* of native PE. After unfolding or complete digestion, the further decrease of absorption makes up only 2% per hour under the applied conditions. Therefore, the bleaching of chromophores is of no importance if spectra are immediately recorded.

Table II compares the molar extinction coefficients of PE from various algae. By use of the above determined PEB extinction coefficients, a content of 6 PEB chromophores can be calculated in most cases. This includes *F. diplosiphon* PE. The previously assumed content of only 3 PEB chromophores for PE from this organism [4, 7] is thus disproved. A lower chromophore content, namely 5 PEB, is, however, found for *Pseudanabaena* W 1173 PE. The value 5 PEB for PE from this organism in contrast to 6 PEB for PE from other Cyanobacteria is confirmed by determination of the chromophore distribution over the subunits.

Chromophore content of subunits

Subunits of PE were separated either by ion exchange chromatography with urea [17] or by SDS gel electrophoresis [3]. Contrary to the method of Glazer *et al.* [8, 9, 17], we used a linear urea gradient because stepwise elution with 6, 8, and 9 M urea can give rise to artefacts. The elution profile (Fig. 2) exhibits 2 peaks. Control by SDS gel electrophoresis shows that the first peak contains the α subunit and the second peak the β subunit. In the case of *Pseudanabaena* W 1173 PE (Fig. 2), integration of the peaks at E_{550} gives the ratio 2.0 : 3.2 for $\alpha : \beta$ subunit. The isolated subunits give the same ratio, 2.0 : 3.0 for E_{550} based on the protein content 1 : 1 (determined as E_{280}). In the case of *Phormidium persicinum* PE (not shown), variable ratios of the elution peaks are obtained. This is due to partial proteolysis during biliprotein isolation which preferably affects the β subunit. However, the isolated subunits exhibit a constant ratio 1 : 2 (or 2 : 4) for E_{550} based on the protein content. This corresponds to the 1 : 2 ratio determined in similar experiments with *Fremyella diplosiphon* PE [7].

A rapid and convenient method for separation of the subunits is the SDS gel electrophoresis which has extensively been used for molecular weight determination of subunits [1–5]. Whereas the 1 : 1 stoichiometry of the subunits has been determined by gel scanning after staining with coomassie-blue [3–5], scanning of gels for determination of chromophore content has not been used so far. A

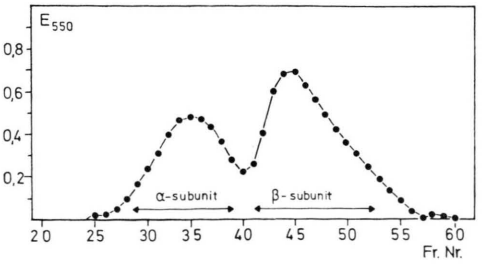


Fig. 2. Separation of subunits of C-phycoerythrin from *Pseudanabaena* W 1173. Column with Bio-Rex 70 equilibrated with 5 M urea at pH 2.9 18 mg PE were applied, elution with a gradient 5 to 7 M urea at pH 2.9. Fractions of 1 ml were collected, α-subunit fractions 27–39, β-subunit fractions 41–55.

Table III. Densitometry of SDS gels of PE subunits from various Cyanobacteria. Chromophore ratio determined in 6 M urea at pH 3 with filter 559 nm, protein ratio determined after staining with coomassie-blue with filter 609 nm.

Phycoerythrin from	Chromophore ratio		Protein ratio	
	α-chain : β-chain		α-chain : β-chain	
Pseudanabaena W 1173	2.0 : 2.80		1.02 : 1.0	
Tolypothrix tenuis	2.0 : 2.90		1.22 : 1.0	
Pseudanabaena catenata	2.0 : 4.18		1.15 : 1.0	
Fremyella diplosiphon	2.0 : 4.54		1.12 : 1.0	
Fremyella diplosiphon	2.0 : 3.60–3.74[4]		1.03–1.07 : 1.0[4]	
Phormidium persicinum	2.0 : 3.90		1.01 : 1.0	
Phormidium autumnale	2.0 : 4.20		1.16 : 1.0	
Nostoc muscorum	2.0 : 3.90		1.10 : 1.0	

direct scanning of SDS gels gives misleading results because the absorption maxima of α and β subunits on SDS gels are different. The α chain of PE exhibits a red colour, the β chain a bluish-violet tint. Different colours have also been reported for α and β subunits for Cryptomonad PE and PC after SDS gel electrophoresis [18]. This means that SDS does not break all protein-chromophore-interactions of PE and PC subunits. We found, however, that incubation of SDS gels with 6 M urea at pH 3 for 3–4 hours gives an uniform colour of separated subunits. Scanning in this solution has furthermore the advantage that the incubated gel and the solution have about the same refraction index so that any irregularity at the gel surface does not interfere with densitometry.

Table III shows the result of the densitometry of separated PE subunits from various Cyanobacteria. The previously reported 1 : 1 ratio of the subunits is confirmed. Staining with coomassie-blue is stronger with the α-subunits than with the β-subunits; this is possibly due to the higher lysin content of the α-subunit which can be responsible for intensity of staining [19]. Based on the 1 : 1 stoichiometry of the subunits, two groups of PEs can be distinguished according to their chromophore content: those with a 2 :3 PEB ratio on α : β subunits, found in *Pseudanabaena* W 1173 and *T. tenuis*, and those with a 2 : 4 PEB ratio on α : β subunits, found in all other investigated Cyanobacteria. These ratios confirm the value of either 5 or 6 PEB per biliprotein found by independent methods.

It is remarkable that PE from *Pseudanabaena* W 1173 carries only 5 chromophores whereas PE from the closely related organism *P. catenata* has 6 chromophores instead. This behaviour corresponds to the immunological difference between PEs from these two organisms [20]. Glazer [21] has suggested that evolution of biliproteins proceeded from those with a lower to those with a higher chromophore content. If this turns out to be true, the 2 PE groups found here could reflect biliproteins of different stages of evolution: PE with 5 PEBs should be more ancient than PE with 6 PEBs.

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