

# Unfolding of C-phycocyanin followed by loss of non-covalent chromophore–protein interactions

## 1. Equilibrium experiments

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Received 28 June 2007; received in revised form 19 October 2007; accepted 25 October 2007  
Available online 1 November 2007

### Abstract

Optical spectroscopic properties of the covalently linked chromophores of biliproteins are profoundly influenced by the state of the protein. This has been used to monitor the urea-induced denaturation of C-phycocyanin (CPC) from *Mastigocladus laminosus* and its subunits. Under equilibrium conditions, absorption, fluorescence and circular dichroism of the chromophores were monitored, as well as the circular dichroism of the polypeptide. Treatment of CPC trimers ( $\alpha\beta$ )<sub>3</sub> resulted first in monomerization ( $\alpha\beta$ ), which was followed by a complex unfolding process of the protein. Loss of chromophore fluorescence is the next process at increasing urea concentrations; it indicates increased flexibility of the chromophore while maintaining the native, extended conformation, and a less compact but still native-like packing of the protein in the regions sampled by the chromophores. This was followed by relaxation of the chromophores from the energetically unfavorable extended to a cyclic-helical conformation, as reported by absorption and CD in the visible range, indicating local loss of protein structure. Only then is the protein secondary structure lost, as reported by the far-UV CD. Sequential processes were also seen in the subunits, where again the chromophore–protein interactions were reduced before the unfolding of the protein. It is concluded that the bilin chromophores are intrinsic probes suitable to differentiate among different processes involved in protein denaturation.  
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**Keywords:** Biliprotein; Protein folding; Protein mobility; Protein dissociation; Chromophore–protein interaction; Optical spectroscopy

### 1. Introduction

Folding of proteins encompasses an enormous reduction of the conformational space: from a multitude of largely unstructured macroscopic conformations in the unfolded state, the protein is transformed to a single, sometimes few, highly structured conformer(s) that exhibit only local structural fluctuations. Folding is cooperative, and can in the extreme be treated by a two-state model of the folded structure and the equilibrated ensemble of unfolded conformations. Folding intermediates have been characterized, however, for many proteins both under

equilibrium conditions and in kinetic experiments (see [1–4]). In many cases, folding to the correct native conformation does not require any cofactors, even though it is *in vivo* frequently assisted by helper proteins [5,6].

Folding and unfolding of proteins can be followed by the use of local intrinsic or extrinsic probes, more global structural parameters can be probed by e.g. the far-UV circular dichroism (CD) [7,8]. The majority of detailed data on protein folding and unfolding is derived from a relatively small number of proteins, notably globular, water-soluble proteins. In the case of heme proteins, e.g. cytochrome *c* (Cyc), a wealth of information has been obtained from spectral changes of the covalently bound heme-chromophore. High-resolution optical and Mössbauer spectroscopy also served to substantiate and critically evaluate the conformational landscape of folded proteins [9–12]. Due to the short lifetime of heme excited states, optical bands are broad, however, and high-resolution spectra require removal or replacement of the central Fe [13].

**Abbreviations:** CD, circular dichroism; CPC, C-phycocyanin; Cyc, cytochrome *c*; Gua, guanidinium hydrochloride; PCB, phycocyanobilin

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Phycobiliproteins consist of a central globin domain to which the chromophores are bound, and a two-helix domain that mediates aggregation of  $\alpha$ - and  $\beta$ -subunits [14–17]. The cofactors are covalently bound via thioether bonds as in Cys, but they are, in contrast to the macrocyclic Fe-porphyrin, metal-free linear tetrapyrroles. C-phycocyanin (CPC) carries three phycocyanobilin (PCB) chromophores in the heterodimeric ( $\alpha\beta$ ) protomer, at cysteines  $\alpha$ -84,  $\beta$ -84 and  $\beta$ -155 [18]. Except for a small red shift due to the loss of one conjugated double-bond, covalent binding to the protein does not affect *per se* the spectral properties of the chromophore: the absorption, fluorescence and CD spectra of denatured biliproteins resemble those of free bilins, with a flexible, cyclic-helical conformation [19,20]. However, absorption, fluorescence and CD of the chromophores are strongly modified in the native protein. The fluorescence yield is increased by three orders of magnitude, the absorption in the visible range by a factor of 5–8, and the intensity of the CD signal about doubled; there are also both batho- and hypsochromic shifts in the maxima spanning nearly 100 nm [21]. These properties, which prevail only in the native protein, render the biliproteins excellent light-harvesting pigments in cyanobacteria, red-algae and cryptophytes [18,22–24].

By the same token, the chromophores can be seen as sensitive probes for the state of the protein [25–31]. CPC is reversibly unfolded with urea or guanidinium chloride. This process can be monitored not only by far-UV spectroscopy, but very sensitively by absorption, fluorescence and CD in the visible range, using the native chromophore as a probe. The situation is complicated, however, by the aggregation of the  $\alpha$ - and  $\beta$ -subunits to monomers (heterodimers,  $\alpha\beta$ ) and trimers (heterohexamers,  $\alpha_3\beta_3$ ), in the presence of linkers even to higher aggregates [27]. In order to separate these effects, we have carried out a characterization of the spectral properties of CPC from *Mastigocladus laminosus* (*Fischerella* PCC7603) in the trimeric, monomeric and denatured states, and of the subunits in the native and denatured states. This basis set was then used to follow the unfolding of the protein with urea under equilibrium conditions. The data indicate a sequential process by which first the rigidity of the chromophore is decreased, then its energetically unfavorable conformation relaxed, and only in the last step the secondary structure of the protein was lost. They serve, at the same time, to identify spectral fingerprints by which the aggregation state can be monitored spectroscopically.

## 2. Materials and methods

### 2.1. Phycocyanin and subunits

*M. laminosus* (*Fischerella* PCC7603) was cultivated in Castenholz Medium [32] at 50 °C under green light, harvested after ~4 weeks with a Padberg centrifuge; the cells were stored at –20 °C. CPC was isolated by adaptation of the methods of [33–35]. Thawed cells were washed twice with potassium phosphate buffer (KPB, 900 mM, pH 7) and centrifuged at 1100 \*g for 10 min. The washed cells were resuspended in the same buffer ( $\approx 1$  ml/g wet cells), and broken by 2 passages through a French Press ( $13.8 \times 10^6$  Pa). The suspension was incubated with *N,N*-dimethyldodecylamin-*N*-oxide (LDAO, 2%, 1 h, ambient temperature) and centrifuged twice (47,000 \*g, 17 °C, 20 min; then 150,000 \*g, 17 °C, 30 min). The resulting supernatant was applied to a sucrose step-gradient (15%/25% w/v) in KPB (900 mM, pH 7) and centrifuged (300,000 \*g, 17 °C, 18 h). The

phycobilisome (PBS) pellet was washed with KPB (900 mM, pH 7; 22,000 \*g, 17 h), and then resuspended in distilled water for disaggregation. The resulting phycobiliprotein solution was desalted (Sephadex G25, KPB (5 mM, pH 7)), and fractionated over a DEAE-cellulose column (DE52, Whatman, KPB 5–200 mM, pH 7). Fractions were pooled according to their UV–Vis absorption spectra, precipitated by  $(\text{NH}_4)_2\text{SO}_4$  (70% w/v), and stored at 6 °C. For removal of linker proteins, the suspension was centrifuged (26,000 \*g, 30 min), the pellet resuspended in distilled water, and desalted over Sephadex G25. After addition of solid urea (4 M), the solution was kept for 1 h at room temperature, chromatographed over a Sepharose (Amersham Pharmacia Biotech) column (KPB, 50 mM, pH 7), and then desalted and precipitated as before.  $\alpha$ - and  $\beta$ -subunits were isolated by isoelectric focusing according to [36]. Purity of the proteins was controlled by SDS-polyacrylamide gel electrophoresis [37], gels were stained first with  $\text{Zn}^{2+}$  for fluorescence detection of proteins with covalently bound bilins [38], and subsequently with Coomassie blue.

### 2.2. Spectroscopy

UV–Vis spectra were recorded with a model UV-2401 PC spectrophotometer (Shimadzu, Duisburg), CD spectra with a model CD6 (Jobin Yvon/Horiba, München) spectropolarimeter, and fluorescence spectra with a model Spex Fluorolog 1680 (Jobin Yvon/Horiba, München).

**Extinction coefficients:** The extinction coefficient of native CPC was calculated relative to the known one in acidic urea ( $35.4 \text{ mM}^{-1} \text{ cm}^{-1}$  at 660 nm [39,40]. Aliquots from a stock solution were diluted 1:9 with either KPB (50 mM, pH 7.7), or with an acidic urea solution (9 M, pH 1.9) in KPB (50 mM), and the absorption spectra recorded. Based on the extinction coefficient of native CPC, those in 1–8 M urea were then determined by 1:9 dilution of the stock solution with the appropriate aliquots of KPB (50 mM, pH 7.7) and the same buffer containing urea (9 M).

### 2.3. PC dissociation and denaturation under different parameters

**Buffer concentration:** The CPC stock solution ( $1.7 \times 10^{-5}$  M) in KPB (1 mM, pH 7) was diluted 1:10 with appropriate aliquots of KPB (1000 mM, pH 7) and distilled water to final KPB concentrations of 1–900 mM. Spectral changes were followed by absorption, CD and fluorescence spectroscopy.

**Protein concentration:** The CPC stock solution in distilled water was diluted 1:9–1:21. Spectral changes were followed by Vis-CD derivative spectroscopy.

**Urea:** Stock solutions of integral CPC or its subunits were diluted 1:9, but with KPB of different concentrations (3, 50 or 300 mM, pH 7) to final urea concentrations of 0–8 M. Spectral changes were followed by absorption, CD and fluorescence spectroscopy.

**Data were analyzed** with Origin V 7.0 (OriginLab Co., Northampton). Spectra were smoothed by multiple point averaging. The native fraction of the protein,  $n_{\text{native}}$ , during titrations with urea was then calculated according to  $n_{\text{native}} = (A_0 - A_x) / (A_0 - A_8)$ , where  $A_0$  is the amplitude of the native protein (0 M urea),  $A_8$  is the amplitude in 8 M urea, and  $A_x$  is the amplitude in  $x$  M urea. The amplitudes were taken at the absorption maximum, the positive extremum of the Vis-CD at  $\lambda \sim 600$  nm, the emission maximum at  $\lambda \sim 635$  nm, and the UV CD at 222 nm. The resulting normalized titration curves were fitted with the logistic function:  $y = (A_0 - A_8) / (1 + (x/x_0)^p)$ , where  $x_0$  is the inflexion point, and  $p$  the slope at the inflexion point. The dissociation of  $\text{CPC}_{\text{tri}}$  to  $\text{CPC}_{\text{mono}}$  was followed by Vis-CD difference or derivative spectroscopy (see text). Secondary structure was calculated from the UV CD spectra using CDPPO software (Version July 2001) [41–43]. The fluorescence decrease during monomer formation was fit using the exponential decay function  $y = y_0 + Ae^{(-x-x_0)/t}$ , where  $y_0$  is the offset,  $A$  the amplitude at the urea concentration  $x_0$  the average of  $x$ , and  $t$  the decay constant. Free enthalpies for folding in water,  $\Delta G^\circ_{\text{w}}$ , were obtained from the titration curves according to Schellman [44].

## 3. Results

### 3.1. Trimer–monomer equilibrium

At high phosphate concentrations, linker-free CPC is present as trimers ( $\alpha\beta$ )<sub>3</sub> [25,45]. Dilution of the buffer to 30 mM results

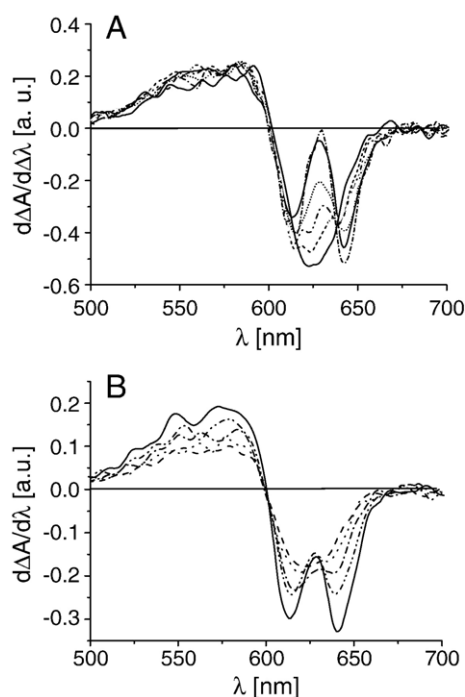


Fig. 1. Monomerization of phycocyanin trimer by reducing concentration of buffer (A) and pigment (B). A: 1st derivative of the long-wavelength CD band of phycocyanin (1.71  $\mu$ M) in KPB (pH 7) of 900 mM (—), 30 mM KPB (---), 6.1 mM KPB (.....), 3.1 mM KPB (- - - - -), 1.1 mM KPB (- - - - -), and of CPC<sub>mono</sub> in KPB (900 mM, pH 7) containing urea (4 M) (—). B: 1st derivative CD spectra of phycocyanin in distilled water at concentrations of 1.75  $\mu$ M (—), 1.3  $\mu$ M (- - - - -), 1.06  $\mu$ M (- - - - -), 0.9  $\mu$ M (.....) and 0.75  $\mu$ M (- - - - -).

only in insignificant changes, they become more distinct at lower concentrations. There is in particular a long-wavelength shoulder of the positive CD band ( $\lambda_{\text{max}}=627$  nm) that is characteristic for the trimer (see below). It disappears upon dilution, which can be monitored with high sensitivity by derivative spectroscopy (Figs. 1 and 2), by the increase at the maximum (Fig. 2), and by difference spectra against the 900 mM solution (not shown). In all cases, the changes are negligible down to 30 mM, but then increase sharply. Most

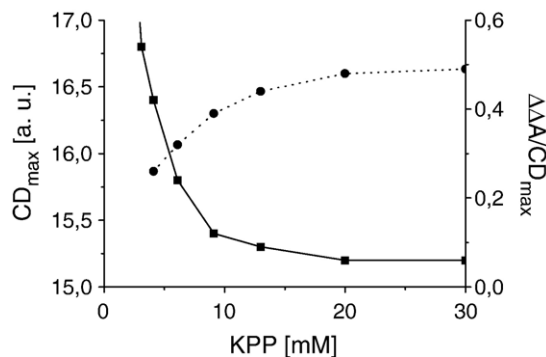


Fig. 2. Monomerization of phycocyanin trimer by reducing buffer concentration. CD intensity of the red maximum (■, —), and normalized amplitude ( $\Delta\Delta A/CD_{\text{max}}$ ) of the S-shaped CD difference spectrum (PC<sub>tri</sub> minus CPC<sub>mono</sub>) (●, .....), see inset Fig. 5) in dependence of the buffer concentration (KPB, pH 7).

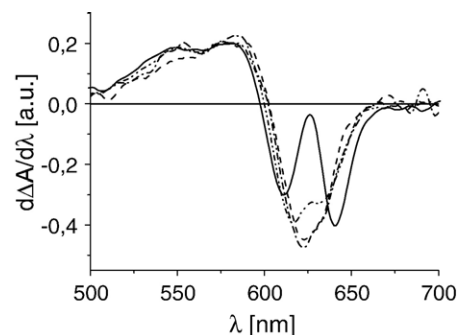


Fig. 3. Monomerization of phycocyanin trimer with urea. 1st derivative of the long-wavelength CD band of phycocyanin (17  $\mu$ M) in KPB (50 mM, pH 7) containing urea at concentrations of 0 M (—), 3 M (---), 4 M (.....) and 5 M (- - - - -).

spectra in the following were obtained in 50 mM KPB, where dissociation is still negligible.

The dissociation does not saturate at KPB concentrations down to 1.1 mM, there is e.g. still a structured derivative spectrum (Fig. 1A). Reduction of the buffer concentration is therefore not sufficient to induce complete dissociation of the trimer ( $\alpha\beta$ )<sub>3</sub> to the monomer ( $\alpha\beta$ ). This is even true if also the CPC concentration is reduced to 0.75  $\mu$ M by dilution with water, where the spectra become already very noisy (Fig. 1B). In order to induce complete dissociation, increasing amounts of urea were added to trimeric CPC in KPB (50 mM, pH 7). Under these conditions dissociation is complete at  $c_{\text{urea}}=4-5$  M, if judged from the CD derivative spectrum (Fig. 3). Since unfolding begins at urea concentrations >4 M, a full characterization of the monomer was done at 4 M urea. Both higher and lower buffer concentrations destabilize the trimer (Fig. 4): dissociation is complete already with 3 M urea in 3 mM KPB, and almost complete in 300 mM KPB, while nearly 30% remain

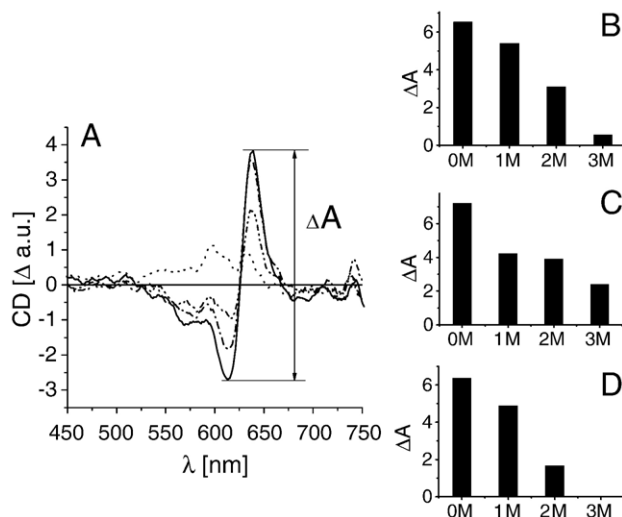


Fig. 4. Influence of urea and buffer concentration on the Vis-CD spectrum of CPC. A: Difference spectra (x minus monomer) in KPB (300 mM, pH 7) containing 0 M (—), 1 M (---), 2 M (.....) and 3 M urea (.....). Amplitudes of the difference signal at KPB concentration of 300 mM (B), 50 mM (C) and 3 mM (D).

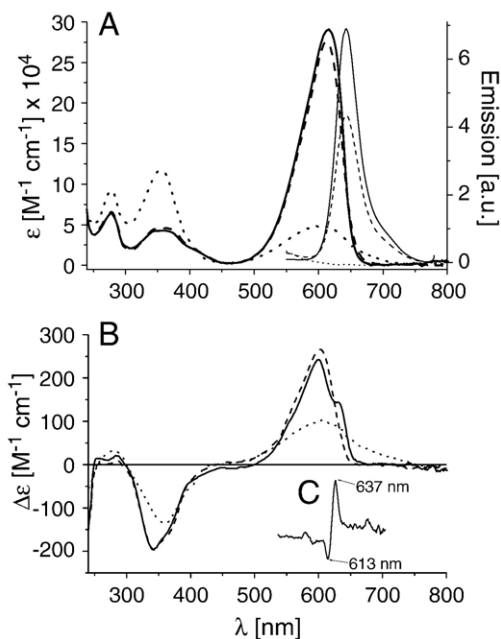


Fig. 5. Absorption, fluorescence and CD spectra of trimeric, monomeric and denatured phycocyanin. CPC<sub>tri</sub> (—) in KPB (50 mM, pH 7), CPC<sub>mono</sub> (---) in the same buffer containing urea (4 M), and CPC<sub>denatured</sub> (•••••) in the same buffer containing urea (8 M). A: Absorption (heavy lines) and fluorescence spectra (thin lines,  $\lambda_{em}=400$  nm), the fluorescence of CPC<sub>tri</sub> is normalized to the absorption of CPC<sub>tri</sub>, the fluorescence of CPC<sub>mono</sub> and CPC<sub>denatured</sub> is scaled relative to the fluorescence of CPC<sub>tri</sub>. B: Vis-CD spectra. All amplitudes refer to a single chromophore. The inset shows the CD difference spectrum (PC<sub>tri</sub> minus CPC<sub>mono</sub>) in the region of the long-wavelength band.

aggregated at this urea concentration in 50 mM KPB (Fig. 4), the concentration used in all subsequent studies. It should be noted that the spectra of monomers obtained with urea show small, but distinct qualitative differences from those obtained in its absence at very low CPC and buffer concentrations. The CD maximum is red-shifted (613 vs. 610 nm in 4 M urea), and the band is broader in 4 M urea. These differences cannot be explained by incomplete dissociation, if judged from the CD spectra, but rather reflect an influence of urea on the monomer spectra [46–50]. Spectral changes induced at low urea concentrations have been reported, too, for another CPC [30]. In the context of this work, these are second order effects, however, which were not investigated further.

### 3.2. Spectral characterization of CPC

Based on the results described in the previous section, quantitative absorption spectra, emission spectra, and quantitative CD spectra were obtained for trimeric and monomeric CPC in KPB (50 mM, pH 7) (Figs. 5–7, Table 1). The absorption spectra of CPC<sub>tri</sub> and CPC<sub>mono</sub> are very similar, there is only a slightly reduced absorbance and a minor hypsochromic shift of the red band in the latter, both are comparable to the changes reported for another CPC [30]. There are larger qualitative and quantitative differences in the CD spectrum, most notably the long-wavelength shoulder of the red band that is characteristic for the trimer and has already been used to follow dissociation

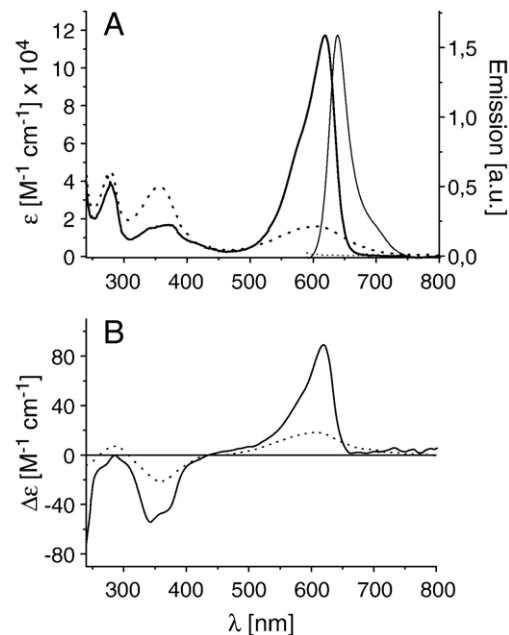


Fig. 6. Spectroscopic characterization of the  $\alpha$ -subunit of phycocyanin in the native state,  $\alpha_{native}$  (—) in KPB (50 mM, pH 7), and in the denatured state  $\alpha_{denatured}$  (•••••) in the same buffer containing urea (8 M). A: Absorption (heavy lines) and fluorescence spectra (thin lines,  $\lambda_{em}=400$  nm), the fluorescence of  $\alpha_{native}$  is normalized to the absorption of the native subunit, the fluorescence of  $\alpha_{denatured}$  is scaled relative to that of  $\alpha_{native}$ . B: Vis-CD spectra.

(see above). As shown by the difference spectrum (inset Fig. 5), it is part of an S-shaped signal that is superimposed, in the trimer, on the featureless band of the monomer. The  $\alpha$ -84 and

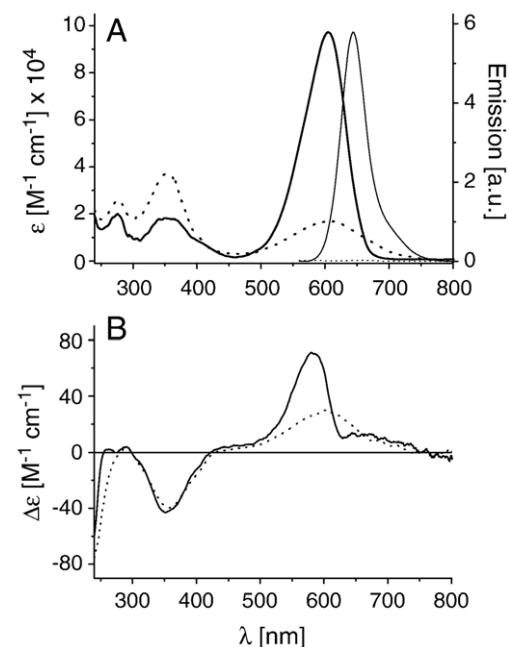


Fig. 7. Spectroscopic characterization of the  $\beta$ -subunit of phycocyanin in the native state,  $\beta_{native}$  (—) in KPB (50 mM, pH 7), and in the denatured state,  $\beta_{denatured}$  (•••••) in the same buffer containing urea (8 M). A: Absorption (heavy lines) and fluorescence spectra (thin lines,  $\lambda_{em}=400$  nm), the fluorescence of  $\beta_{native}$  is normalized to the absorption of  $\beta_{native}$ , the fluorescence of  $\beta_{denatured}$  is scaled relative to that of  $\beta_{native}$ . B: Vis-CD spectra. All amplitudes refer to a single chromophore.



Table 1  
Spectral characteristics of integral CPC and the  $\alpha$ - and  $\beta$ -subunits in the native and urea-unfolded states

Protein	Absorption					Vis-CD				Emission
	Maximum <sub>Vis</sub>		Maximum <sub>nuv</sub>		Q (A <sub>Vis</sub> /A <sub>nuv</sub> )	Positive extremum		Negative extremum		
	$\lambda_{\max}$ [nm]	$\epsilon$ [M <sup>−1</sup> cm <sup>−1</sup> ]	$\lambda_{\max}$ [nm]	$\epsilon$ [M <sup>−1</sup> cm <sup>−1</sup> ]		$\lambda_{\max}$ [nm]	$\Delta\epsilon$ [M <sup>−1</sup> cm <sup>−1</sup> ]	$\lambda_{\min}$ [nm]	$\Delta\epsilon$ [M <sup>−1</sup> cm <sup>−1</sup> ]	
PC <sub>tri</sub> <sup>a</sup>	616–618	97.300	361	14.300	6.8	600–602	81	342–345	−65	643–650
PC <sub>mono</sub> <sup>a</sup>	611–613	90.700	362	15.300	6.0	601/602	88	341/342	−65	642–647
PC <sub>denatured</sub>	604/605	16.200	354–357	39.100	0.4	605/606	33	358–360	−35	–
$\alpha$ <sub>native</sub>	619	117.300	375	16.800	7.0	619	93	341	−53	639
$\alpha$ <sub>denatured</sub>	602–599	16.100	355/356	37.000	0.4	606	18	359	−23	–
$\beta$ <sub>native</sub>	604/605	97.300	354/356	18.300	5.3	583	71	353	−43	644
$\beta$ <sub>denatured</sub>	608/609	11.300	353/354	37.200	0.5	602	35	357	−40	–

All spectra in KPB (50 mM, pH 7), the spectra are shown in Figs. 5–7. All quantitative values refer to a single chromophore.  
<sup>a</sup> The difference maxima of the CD spectra (monomer *minus* trimer) are located at 613 (+) and 637 nm (–).

$\beta$ -84 chromophores of adjacent monomers approach each other in the trimer at a center-to-center distance of  $\sim 20$  Å [14]. We take the S-shaped band as an indication for weak excitonic coupling between these chromophores in the trimer that is lost after dissociation [51].

Large quantitative differences between CPC<sub>tri</sub> and CPC<sub>mono</sub> are also seen in the emission spectra; the fluorescence intensity is reduced by 37% in the latter, with a concomitant blue shift ( $\Delta\lambda \sim 2$  nm) (Fig. 5, Table 1). A major factor for fluorescence in linear tetrapyrroles is the extent of conformational freedom: when the latter is high, the fluorescence is low, and *vice versa* [21,52,53]. The reduced fluorescence yield therefore indicates an increased conformational freedom in the monomer, where the chromophore becomes partly exposed to the solvent. An additional factor might be local structural changes of the protein near the chromophore(s) that do not significantly affect the secondary structure (see below, analysis of the far-UV CD).

The third set of spectra shown in Fig. 5 was obtained in 8 M urea. The loss of the far-UV CD signal, and of secondary structure, saturates at  $c_{\text{urea}} \geq 7$  M (see below), this is in agreement with previous studies [19,54] that CPC from various cyanobacteria, including *M. laminosus*, is fully denatured at 8 M urea. Pronounced changes of all spectra are seen under these conditions, as compared to the native state, that have been discussed already in some detail [21]. They include a nearly complete loss of fluorescence, a strong absorption decrease in the red spectral region, a moderate absorption increase in the near-UV region, and a  $\approx 50\%$  reduction of the chromophore-related CD bands.

### 3.3. Steady-state spectra of subunits

PC consists of two subunits that were separated by isoelectric focusing [36]. The  $\alpha$ -subunit carries a single chromophore at cysteine-84, the  $\beta$ -subunit two chromophores at cysteines-84 and -155. Full sets of spectra for the native and denatured  $\alpha$ -subunit are shown in Fig. 6, they have been obtained in KPB (50 mM, pH 7) in the absence of urea, and in the presence of 8 M urea, respectively. The same set of spectra of the  $\beta$ -subunit is given in Fig. 7. The general features of the native vs. the denatured spectra are similar among the subunits and to those of monomeric vs. denatured CPC. Upon denaturation, there is

always a large decrease of the red absorption, a moderate increase of the near-UV absorption, a reduction of the Vis-CD band, and a loss of fluorescence as well as of the far-UV CD. Notable differences of the  $\alpha$ - vs. the  $\beta$ -subunit are a red shift and a narrowing of the long-wavelength absorption, fluorescence and CD bands of the former, and a larger decrease of absorption of this band upon denaturation. A particular difference is the structured CD of the long-wavelength band of the  $\beta$ -subunit (Fig. 7), that is absent in the  $\alpha$ -subunit (Fig. 6), this feature has been reported before [55,56]. While the three chromophores share extended geometries, the detailed conformations are different, in particular that of the one at cysteine- $\beta$ 155 [14–17], thus giving rise to different spectra [56,57]. Residual secondary structure at 8 M urea has been reported for the  $\beta$ -subunit of an R-phycoerythrin, as evidenced by residual fluorescence [31], this is absent in all samples studied here, and may reflect different protein stabilities.

### 3.4. Unfolding with urea

Using the spectra of the native and denatured CPC samples as a base, the spectral changes were followed during stepwise addition of urea using selected spectral features as probes. In the case of integral CPC, only that part is shown where  $c_{\text{urea}} \geq 3$  M, corresponding to unfolding of the monomer. For the subunits, the data are shown from  $c_{\text{urea}} = 2$ –8 M, to avoid interference from (dissociation) processes at lower concentrations ([31], see also Discussion). The “characteristic traits” monitored in Fig. 8 were the amplitudes of the absorption, fluorescence and CD signals of the red band, and of the far-UV CD signal at 222 nm. In each case, these amplitudes were normalized, and the data points were then fitted by a sigmoidal function (see Materials and methods). The fit parameters are summarized in Table 2. In each of the three proteins, the changes seen for the chromophore occur at lower concentrations of urea than the changes of the secondary structure of the protein (far-UV CD), this effect is most pronounced in integral CPC. Secondary structure analysis [41] of the latter indicates a decrease from the original 60%  $\alpha$ -helix, maintained up to  $\sim 5$  M urea, to zero during this transition (Fig. 9). With the exception of the  $\beta$ -subunit, there are also distinct differences among the spectral traits of the chromophores, viz. absorption, fluorescence and CD. In integral CPC,

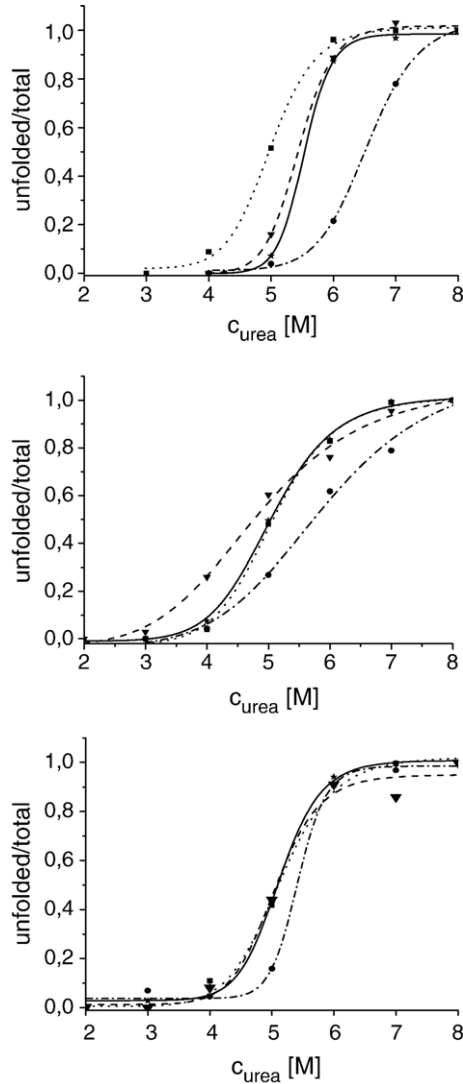


Fig. 8. Unfolding of CPC<sub>mono</sub> (top), α-CPC (center) and β-CPC (bottom) in KPb (50 mM, pH 7) by urea. Normalized changes of absorption (★, —), fluorescence (■, ●, ●, ●, ●) and CD (▼, — — —) of the long-wavelength band, and of the far-UV CD (●, — — — —). Symbols indicate measured values, and lines the sigmoidal fits.

the fluorescence decreases already at lower urea concentrations than the absorption and CD, whereas the CD change precedes the fluorescence and CD in the α-subunit. In the latter, the transitions are particularly broad, indicating sample heteroge-

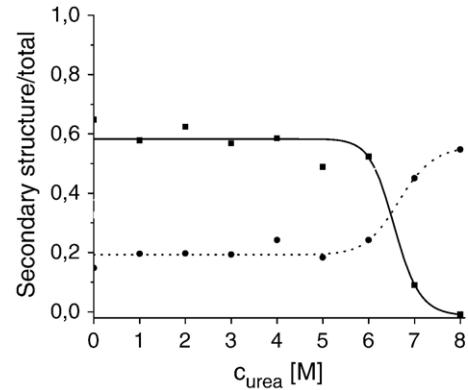


Fig. 9. Influence of urea on the secondary structure of CPC in KPb (50 mM, pH 7). Fraction of α-helix (■, —) and of random structure (●, ●, ●, ●, ●), as analyzed by SELCON [39–41]. Symbols indicate experimental values, and lines the sigmoidal fits.

neity, and/or lower cooperativity than in the β-subunit and CPC<sub>mono</sub>.

Free enthalpy changes for protein folding in water ( $\Delta G_w^\circ$ ) were obtained from the individual titration curves assuming the linear relationship  $\Delta G_{urea}^\circ = \Delta G_w^\circ + a \cdot c_{urea}$  [44], where the  $\Delta G_{urea}^\circ$  are obtained from the equilibrium concentrations of native and unfolded CPC at the different  $c_{urea}$ . For the α-subunit, the  $\Delta G_w^\circ$  for a folding range from  $-30.3$  (absorption monitored) to  $-18.9$  kJ/mol (Vis-CD monitored), the values for the β-subunit are in the same range; the folding enthalpies of the monomer are consistently larger (Table 3). The latter can be compared to values of  $\Delta G_w^\circ$  of  $-10$  to  $-36.6$  kJ/mol for a series of CPC from cyanobacteria of various ecotypes [58]. Those from thermophilic cyanobacteria were in the upper range; *M. laminosus* belongs to this ecotype. Much smaller  $\Delta G_w^\circ$  were obtained for the trimer. They are not included in Table 3, however, because the multi-step titration curve clearly violates the assumption of a two-state model.

## 4. Discussion

### 4.1. Monomer–trimer equilibrium

The unfolding of multimeric proteins has been investigated under equilibrium conditions by titration with urea or guanidinium chloride [47]. Multi-step processes have generally been observed, in favorable cases dissociation of the subunits and

Table 2  
Sigmoidal fit parameters for urea titration curves monitored with different spectral parameters, shown in Fig. 8

Protein	CPC <sub>mono</sub>		α-subunit		β-subunit	
	$x_0$	$p$	$x_0$	$P$	$x_0$	$p$
Fit parameter	$c_{urea}$ [M]				$c_{urea}$ [M]	
Vis-CD <sub>max</sub>	5.44±0.06	19.7±2.1	4.83±0.16	5.41±0.96	5.04±0.11	14.0±5.6
Absorption <sub>vis-max</sub>	5.53±0.05	25.1±2.6	5.05±0.05	9.68±0.85	5.12±0.05	15.1±2.5
Emission <sub>max</sub>	4.99±0.05	13.9±2.6	5.07±0.08	10.1±1.6	5.13±0.08	11.7±2.3
UV CD <sub>222 nm</sub>	6.54±0.05	16.0±1.3	5.88±0.29	6.31±1.7	5.42±0.07	24.0±3.5

See Materials and methods for details of fitting.

Table 3  
Extrapolated free energy of folding in water,  $\Delta G^\circ_{\text{w}}$ , of monomeric CPC, and of the  $\alpha$ - and  $\beta$ -subunits

	Absorption	Fluorescence	Vis-CD	UV CD
$\alpha$ -subunit	$30.3 \pm 3.1$	$32.2 \pm 2.8$	$18.9 \pm 2.0$	$21.5 \pm 2.8$
$\beta$ -subunit	$28.2 \pm 3.1$	$31.3 \pm 3.2$	$19.9 \pm 6.9$	$22.3 \pm 5.3$
Monomer	$42.0 \pm 13.1$	$33.7 \pm 2.8$	(50.1)	$35.9 \pm 2.8$

Values were extrapolated from the urea titration curves, assuming a 2-state model [44].

unfolding can be separated [3,59,60]. Such a process seems to be valid in phycocyanin, where the first process at  $c_{\text{urea}} \leq 4$  M shows the spectral fingerprints of dissociation, evidenced in particular by the loss of the exciton couplet in CD, with no indication of a concomitant loss of secondary structure (Fig. 9). While disulfide bonds are absent in CPC [14–17], the case is complicated by the presence of monomers ( $\alpha\beta$ ) that can further aggregate to trimers, hexamers and dodecamers [61]. Aggregation studies with CPC from *M. laminosus* that was freed of linker proteins gave trimers as largest aggregates [62,63]; all CPC samples used in the present study had been freed from linkers [64]. Trimers are therefore considered in the following as largest aggregates, and the changes at  $c_{\text{urea}} < 4$  M assigned to their dissociation. The clearest spectral fingerprint for this dissociation process is the loss of the S-shaped exciton couplet in the CD spectrum (Figs. 1 and 5). Aggregation of monomers to trimers brings chromophores  $\alpha$ -84 and  $\beta$ -84 on different monomers in close proximity (center-to-center distance of 20–22 Å), where weak excitonic coupling is expected [25,57,65–67].

Dissociation of biliproteins is favored at low ionic strength of the buffer (see [18,27,68–71]). However, while our trimer spectra saturate at buffer concentrations  $\geq 50$  mM, no pure monomer spectra could be obtained even in dilute solution in water, as judged from the presence of a residual S-shaped CD-feature which only vanishes in the presence of urea. Very high buffer concentrations also seem to destabilize the aggregates, as indicated by the dissociation studies shown in Fig. 4 with urea at different buffer concentrations.

While the CD monitors well the loss of trimers,  $(\alpha\beta)_3$ , at  $c_{\text{urea}} = 4$  M, none of the spectroscopic methods used is capable of monitoring the dissociation of monomers,  $\alpha\beta$ , into the isolated subunits (see [31] for an alternative). Steady-state absorption, fluorescence and CD spectra of the subunits of CPC [26] and the related phycoerythrocyanin [54] add up to the spectra of the respective monomers. Evidence for the lack of dissociation into subunits comes, however, from preparative studies: both isoelectric focusing [36], and chromatography [40,72,73], require 8 M urea to isolate subunits. We therefore conclude that in the range of 0–4 M urea, at a buffer concentration of 50 mM, the predominant process is the dissociation of trimers into monomers.

The other major spectral change seen in this process is the drop of fluorescence; the absorption shows only a minor shift. Since the absorption is very sensitive to conformational changes of the chromophores, which in turn are strongly coupled to the conformation of the protein, the mean secondary structure must remain unchanged during the process.

Dissociation can then be seen as a process that neither affects significantly the protein nor the chromophore structure. The only parameter that cannot be rationalized on this basis is the reduction in fluorescence (Fig. 5, Table 1). In bile pigments, restricted conformational dynamics is a major factor for the high fluorescence [36,53,74], it has also been implicated in the high fluorescence of native CPC [21,29]. Its reduction upon dissociation then indicates that the chromophore gains conformational freedom. This is not accompanied by a change in conformational equilibrium structure, as judged from the minor changes in the absorption and, besides the loss of the exciton couplet, in the CD spectra. The  $\alpha$ -84 chromophore, from which a large fraction of the fluorescence originates [25,54], is in the contact zone between monomers and involved in inter-monomer H-bonds in CPC<sub>tri</sub> [14–17]. At least in part, these contacts then seem to contribute to the (functionally important) reduced conformational freedom.

Aggregation also occurs with isolated subunits. Biliprotein subunits dimerize readily, and monomers can only be stabilized transiently [77]. In the  $\beta$ -subunit, monomerization is accompanied by a change on the long-wavelength side of the VIS-CD band [75] and in fluorescence polarization [76]. None of the four parameters tested indicates, however, a multi-step unfolding with dissociation that precedes unfolding (Fig. 8), with the possible exception of fluorescence of the  $\alpha$ -subunit. Aggregation most likely involves the N-terminal X- and Y-helices protruding from the globin-type structure, which are also responsible for subunit aggregation to monomers (=heterodimers) [75]. There is relatively little quantitative information on the subject. Judged from a recent study with R-phycocyanin  $\beta$ -subunit [31], homo aggregation is weaker and dissociation occurs already at  $c_{\text{urea}} < 2$  M. In summary, these data indicate that at the onset of unfolding, CPC is present as a heterodimer ( $\alpha\beta$ ), and the subunits as homodimers ( $\alpha_2$  or  $\beta_2$ ).

#### 4.2. Unfolding

In 8 M urea, the subunits of CPC are separated [36] and the protein is unfolded [19] (see Fig. 9). The spectral changes observed during the treatment with urea at concentrations between 4 and 8 M then reflect two processes, dissociation of subunits and unfolding. The individual curves for the four spectral signatures, Vis absorption, fluorescence and CD, and UV CD, could all be fitted well by a single-step sigmoidal function (Fig. 8). These differ, however, in the urea concentrations at the transition points: that of the far-UV CD signal, which reflects the secondary structure of the protein, is at considerably higher urea concentrations than those of signals related to the native chromophore, and the latter also differ among the different spectral signatures.

Absorption, fluorescence and CD monitor different properties of the chromophore: loss of fluorescence is caused by increased conformational dynamics of the chromophore, irrespective of its conformation [21,74,78,79], while *vice versa* absorption and CD are sensitive to conformational changes and are less affected by conformational dynamics as long as the average conformation is retained [53,55,80]. Using

these criteria, the loss of fluorescence at the lowest urea concentrations relates to increased conformational dynamics of the chromophores, the subsequent absorption and CD changes in the visible region relate to conformational changes of the chromophore, likely together with conformational changes of the nearby parts of the protein that do not affect significantly the secondary structure; the latter only collapses at a further increase of  $c_{\text{urea}}$  by 1 M (Fig. 9).

One of the unfolding steps may reflect subunit separation. This is, however, unlikely for two reasons. Firstly, the fluorescence yield of the subunits is only moderately reduced compared to the monomer, while it decreases to zero already in the first step of the urea titration. Secondly, the transition concentrations for the fluorescence signal and UV CD signals are also 1 M apart in the  $\alpha$ -subunits. The process then includes changes that are not directly related to subunit separation. However, since the subunits unfold already at lower concentrations than the monomer, the interactions then seem to persist up to the loss of secondary structure monitored by the UV CD signal (Figs. 8 and 9). We therefore conclude that neither the loss of fluorescence, nor the subsequent relaxation of the chromophore to the cyclic-helical one characteristic for free bilins [21,55,56], reflects subunit dissociation, but rather processes in individual subunits.

The different transition points are indicative of the presence of two intermediates in the equilibrium unfolding of CPC. Both have certain characteristics of the molten globule [2,81]. The low-fluorescence, high-absorption intermediate that is formed at low urea concentrations is reminiscent of the near-native molten globule described for cytochrome *c* or for anion-stabilized apo-myoglobin at low pH [2,82–84]. The unchanged absorption and Vis-CD then indicate that the extended conformation of the chromophores and tertiary contacts are retained. The low fluorescence indicates, on the other hand, that high-frequency motions are allowed in this state that allow rapid intersystem crossing. A similar softening of the protein, while maintaining the chromophore conformation, has recently been reported for the  $\beta$ -subunit of an R-PC [31].

In the next state, the chromophore absorption spectrum indicates that it has relaxed from the extended to a cyclic-helical conformation that is characteristic of denatured biliproteins [21,52,53]. Indirectly, this indicates a loss of tertiary structure in the regions sampled by the chromophores, while at the same time the secondary structure (UV CD) is still near-native. This situation is reminiscent of the relatively unstructured molten globule states that have been characterized for apo-myoglobin or lactalbumin (reviewed in [2]), or a pre-molten globule ([85], reviewed in [4]).

This sequence is also maintained, albeit shifted to somewhat lower urea concentrations, in the  $\alpha$ -subunit, it is not seen for the  $\beta$ -subunit, indicating a tighter correlation among the different processes. The first step, occurring already at  $<2$  M urea, has been ascribed to a conformational change coupled to dissociation [31]. We also noticed a small change in fluorescence which may be related to dissociation. It was not studied in detail; but in order to avoid interference the data were evaluated only at  $c_{\text{urea}} \geq 2$  M. At these concentrations, the separation of softening

and unfolding seems absent in the  $\beta$ -CPC studied here. It may relate to the different  $\beta$ -155 chromophores, to the protein, or possibly even to the slightly different pH used in the two studies. The presence of more than one folding intermediate has been reported for other proteins [4,85], the particularly favorable situation in CPC is that the bilin chromophore appears suitable to monitor separately dynamic and equilibrium-conformational changes.

## Acknowledgements

Work was supported by a grant from the Deutsche Forschungsgemeinschaft, Bonn (SFB 533 'Light-induced dynamics of biopolymers', project A1). We thank C. Bubenzer and B. Strohmann for their technical assistance.

## References

- [1] O.B. Ptitsyn, The molten globule state, in: T.E. Creighton (Ed.), *Protein Folding*, W.H. Freeman and Co, New York, 1992, pp. 243–300.
- [2] K. Maki, K. Kamagata, K. Kuwajima, Equilibrium and kinetically observed molten globule states, in: J. Buchner, T. Kiefhaber (Eds.), *Protein Folding Handbook*, vol. 2, Wiley-VCH, Weinheim, 2005, pp. 856–883.
- [3] R. Jaenicke, H. Lilie, Folding and association of oligomeric and multimeric proteins, *Adv. Protein Chem.* 53 (2000) 329–401.
- [4] V.N. Uversky, Protein folding revisited. A polypeptide chain at the folding–misfolding–nonfolding cross roads: which way to go? *Cell. Mol. Life Sci.* 60 (2003) 1852–1871.
- [5] F.U. Hartl, M. Hayer-Hart, Molecular chaperones in the cytosol: from nascent chain to folded protein, *Science* 295 (2002) 1852–1858.
- [6] P. Lund, *Molecular Chaperones in the Cell*, Oxford University Press, Oxford, 2001.
- [7] P.E. Wright, R.L. Baldwin, *Mechanisms of protein folding*, 2nd Ed., Oxford University Press, Oxford, 2000.
- [8] J. Buchner, T. Kiefhaber, *Protein Folding Handbook*, Wiley-VCH, Weinheim, 2005.
- [9] A. Ostermann, R. Waschipky, F.G. Parak, G.U. Nienhaus, Ligand binding and conformational motions in myoglobin, *Nature* 404 (2000) 205–208.
- [10] F.G. Parak, Proteins in action: the physics of structural fluctuations and conformational changes, *Curr. Opin. Struct. Biol.* 13 (2003) 552–557.
- [11] J. Schlichter, J. Friedrich, M. Parbel, H. Scheer, New concepts in spectral diffusion physics of proteins, *Photo. Sci. News* 6 (2000) 100–111.
- [12] H. Frauenfelder, S.G. Sligar, P.G. Wolynes, The energy landscapes and motions of proteins, *Science* (1991) 1598–1603.
- [13] H. Lesch, J. Schlichter, J. Friedrich, J.M. Vanderkooi, Molecular probes: what is the range of their interaction with the environment? *Biophys. J.* 86 (2004) 467–472.
- [14] T. Schirmer, W. Bode, R. Huber, Refined three-dimensional structures of two cyanobacterial C-phycocyanins at 2.1. and 2.5 Å resolution — a common principle of phycobilin–protein interaction, *J. Mol. Biol.* 196 (1987) 677–695.
- [15] B. Stec, R.F. Troxler, M.M. Teeter, Crystal structure of C-phycocyanin from *Cyanidium caldarium* provides a new perspective on phycobilisome assembly, *Biophys. J.* 76 (1999) 2912–2921.
- [16] X.Q. Wang, L.N. Li, W.R. Chang, J.P. Zhang, L.L. Gui, B.J. Guo, D.C. Liang, Structure of C-phycocyanin from *Spirulina Platensis* at 2.2 Å resolution: a novel monoclinic crystal form for phycobiliproteins in phycobilisomes, *Acta Crystallogr., D Biol. Crystallogr.* 57 (2001) 784–792.
- [17] N. Adir, R. Vainer, N. Lerner, Refined structure of C-phycocyanin from the cyanobacterium *Synechococcus vulcanus* at 1.6 Å: insights into the role of solvent molecules in thermal stability and co-factor structure, *Biochim. Biophys. Acta* 1556 (2002) 168–174.
- [18] W.A. Sidler, Phycobilisome and phycobiliprotein structures, in: D.A. Bryant (Ed.), *The Molecular Biology of Cyanobacteria*, Kluwer, Dordrecht, 1994, pp. 139–216.



- [19] H. Lehner, H. Scheer, Circular dichroism of C-phycocyanin: origin of optical activity in denaturated biliproteins and evidence for an intermediate during unfolding, *Z. Naturforsch.* 38c (1983) 353–358.
- [20] E. Köst-Reyes, H.-P. Köst, The protein chromophore bond in B-phycoerythrin from *Porphyridium cruentum*, *Eur. J. Biochem.* 102 (1979) 83–91.
- [21] H. Scheer, Phycobiliproteins: molecular aspects of photosynthetic antenna systems, in: F.K. Fong (Ed.), *Light Reaction Path of Photosynthesis*, Springer, Berlin, 1982, pp. 7–45.
- [22] H. Scheer, The pigments, in: B. Green, W. Parson (Eds.), *Light-Harvesting Antennas in Photosynthesis*, Kluwer, Dordrecht, 2003, pp. 29–81.
- [23] K.E. Apt, J.L. Collier, A.R. Grossman, Evolution of phycobiliproteins, *J. Mol. Biol.* 248 (1995) 79–96.
- [24] A.N. Glazer, Adaptive variations in phycobilisome structure, *Adv. Mol. Cell Biol.* 10 (1994) 119–149.
- [25] M.P. Debrecezy, K. Sauer, J. Zhou, D.A. Bryant, Comparison of calculated and experimentally resolved rate constants for excitation energy transfer in C-phycocyanin. 2. Trimers, *J. Phys. Chem.* 99 (1995) 8420–8431.
- [26] M.P. Debrecezy, K. Sauer, J. Zhou, D.A. Bryant, Comparison of calculated and experimentally resolved rate constants for excitation energy transfer in C-phycocyanin. 1. monomers, *J. Phys. Chem.* 99 (1995) 8412–8419.
- [27] D.S. Berns, R. Maccoll, Phycocyanin in physical–chemical studies (review), *Chem. Rev.* 89 (1989) 807–825.
- [28] M. Mimuro, R. Rübels, P. Füllstaller, H. Zuber, The microenvironment around the chromophores and its changes due to the association states in C-phycocyanin isolated from the cyanobacterium *Mastigocladus laminosus*, *Biochim. Biophys. Acta* 851 (1986) 447–456.
- [29] H. Scheer, Biliproteins, *Angew. Chem., Intl. Ed.* 20 (1981) 230–250.
- [30] K.L. Thoren, K.B. Connell, T.E. Robinson, D.D. Shellhamer, M.S. Tammaro, Y.M. Gindt, The free energy of dissociation of oligomeric structure in phycocyanin is not linear with denaturant, *Biochemistry* 45 (2006) 12050–12059.
- [31] Y. Ma, J. Xie, C. Zhang, J. Zhao, Three-stage refolding/unfolding of the dual-color  $\beta$ -subunit in R-phycocyanin from *Polysiphonia urceolata*, *Biochem. Biophys. Res. Commun.* 352 (2007) 787–793.
- [32] R.W. Castenholz, Laboratory culture of thermophilic cyanophytes, *Schweizer. Z. Hydrol.* 35 (1970) 538–551.
- [33] W. Reuter, Spektrale, Strukturelle und Biochemische Charakterisierung des Phycobilisomenzentrums und Einiger Konstituenten der Phycobilisomenperipherie von *Mastigocladus laminosus* unter Berücksichtigung der Funktion, Philipps-Universität, Marburg/Lahn, 1989.
- [34] M. Nies, W. Wehrmeyer, Isolation and biliprotein characterization of phycobilisomes from the thermophilic cyanobacterium *Mastigocladus laminosus* Cohn, *Planta* 150 (1980) 330–337.
- [35] G. Wiegand, A. Parbel, M.H. Seifert, T.A. Holak, W. Reuter, Purification, crystallization, NMR spectroscopy and biochemical analyses of alpha-phycoerythrocyanin peptides, *Eur J Biochem* 269 (2002) 5046–5055.
- [36] E. Köst-Reyes, S. Schneider, W. John, R. Fischer, H. Scheer, H.-P. Köst, Fast preparative isoelectric focusing of phycocyanin subunits in layers of granulated gels, *Electrophoresis* 8 (1988) 335–336.
- [37] U.K. Lämmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227 (1970) 680–685.
- [38] T. Berkelman, J.C. Lagarias, Visualization of bilin-linked peptides and proteins in polyacrylamide gels, *Anal. Biochem.* 156 (1986) 194–201.
- [39] A.N. Glazer, S. Fang, Chromophore content of blue-green algal phycobiliproteins, *J. Biol. Chem.* 248 (1973) 659–662.
- [40] W.M. Schluchter, D.A. Bryant, Analysis and reconstitution of phycobiliproteins: methods for the characterization of bilin attachment reactions, in: A.G. Smith, M. Witty (Eds.), *Heme, Chlorophyll, and Bilins*, Humana Press, Totowa, NJ, USA, 2002, pp. 311–334.
- [41] N. Sreerama, R.W. Woody, A self-consistent method for the analysis of protein secondary structure from CD, *Anal. Biochem.* 209 (1993) 32–44.
- [42] N.W.R.W. Sreerama, Estimation of protein secondary structure from CD spectra: comparison on CONTIN, SELCON, and CDSSTR methods with an expanded reference set, *Anal. Biochem.* 287 (2000) 252–260.
- [43] N. Sreerama, R.W. Woody, Estimation of the number of  $\alpha$ -helical and  $\beta$ -strand segments in proteins using circular dichroism spectroscopy, *Prot. Sci.* 8 (1999) 370–380.
- [44] J.A. Schellman, Solvent denaturation, *Biopolymers* 17 (1978) 1305–1322.
- [45] L. Gottschalk, R. Fischer, F. Lottspeich, H. Scheer, Origin of the red-shifted absorption in phycocyanin 632 from *Mastigocladus laminosus*, *Photochem. Photobiol.* 54 (1991) 283–288.
- [46] A.M. Labhardt, Folding intermediates studied by circular dichroism, *Methods Enzymol.* 131 (1986) 126–135.
- [47] N.C. Pace, Determination and analysis of urea and guanidine hydrochloride denaturation curves, *Methods Enzymol.* 131 (1986) 266–280.
- [48] S. Lapanje, Survey of experimental results obtained by various methods in protein-denaturation studies, in: S. Lapanje (Ed.), *Physicochemical Aspects of Protein Denaturation*, New York, 1978.
- [49] D. Guard-Friar, R. Maccoll, Spectroscopic properties of tetrapyrroles on denatured biliproteins, *Arch. Biochem. Biophys.* 230 (1984) 300–305.
- [50] A. Möglich, F. Krieger, T. Kiefhaber, Molecular basis for the effect of urea and guanidinium chloride on the dynamics of unfolded polypeptide chains, *J. Mol. Biol.* 345 (2004) 153–162.
- [51] K. Sauer, H. Scheer, P. Sauer, Förster transfer calculations based on crystal structure data from *Agmenellum quadruplicatum* C-phycocyanin, *Photochem. Photobiol.* 46 (1987) 427–440.
- [52] S.E. Braslavsky, A.R. Holzwarth, K. Schaffner, Solution conformations, photophysics, and photochemistry of bili-pigments — bilirubin and biliverdin dimethylesters and related linear tetrapyrroles, *Angew. Chem., Int. Ed. Engl.* 22 (1983) 656–674.
- [53] H. Falk, *The Chemistry of Linear Oligopyrroles and Bile Pigments*, Springer, Wien, New York, 1989.
- [54] A. Parbel, K.H. Zhao, J. Breton, H. Scheer, Chromophore assignment in phycoerythrocyanin from *Mastigocladus laminosus*, *Photosynth. Res.* 54 (1997) 25–34.
- [55] C. Scharnagl, S. Schneider, UV–visible absorption and circular dichroism spectra of the subunits of C-phycocyanin. II. A quantitative discussion of the chromophore–protein and chromophore–chromophore interactions in the  $\beta$ -subunit, *J. Photochem. Photobiol. B.* 8 (1991) 129–157.
- [56] M.P. Debrecezy, K. Sauer, J. Zhou, D.A. Bryant, Monomeric C-phycocyanin at room temperature and 77 K: resolution of the absorption and fluorescence spectra of the initial chromophores and the energy-transfer rate constants, *J. Phys. Chem.* 97 (1993) 9852–9862.
- [57] K. Sauer, H. Scheer, Excitation transfer in C-phycocyanin. Förster transfer rate and exciton calculations based on new crystal structure data for C-phycocyanins from *Agmenellum quadruplicatum* and *Mastigocladus laminosus*, *Biochim. Biophys. Acta* 936 (1988) 157–170.
- [58] C.H. Chen, D.S. Berns, Comparison of the stability of phycocyanins from thermophilic, mesophilic, psychrophilic algae, *Biophys. Chem.* 8 (1978) 191–202.
- [59] R.S. Gokhale, S.S. Ray, H. Balaram, P. Balaram, Unfolding of *Plasmodium falciparum* triosephosphate isomerase in urea and guanidinium chloride: evidence for a novel disulfide exchange reaction in a covalently cross-linked mutant, *Biochemistry* 38 (1999) 423–431.
- [60] J.A. Hornby, J.K. Luo, J.M. Stevens, L.A. Wallace, W. Kaplan, R.N. Armstrong, H.W. Dirr, Equilibrium folding of dimeric class mu glutathione transferases involves a stable monomeric intermediate, *Biochemistry* 39 (2000) 12336–12344.
- [61] R. MacColl, D. Guard-Friar, *Phycobiliproteins*, CRC Press, Boca Raton, FL, 1987.
- [62] L. Gottschalk, F. Lottspeich, H. Scheer, Reconstitution of allophycocyanin from *Mastigocladus laminosus* with isolated linker polypeptide, *Photochem. Photobiol.* 58 (1993) 761–767.
- [63] A. Parbel, H. Scheer, Model for the phycobilisome rod with interlocking disks based on domain-weighted linker-polypeptide sequence homologies of *Mastigocladus laminosus*, *Int. J. Photoenergy* 2 (2000) 31–40.
- [64] A. Parbel, Charakterisierung Von Phycobiliprotein-Linkerkomplexen Aus Dem Phycobilisom Von *Mastigocladus Laminosus*. Vergleich Nativer Und Überexprimierter Linker, Ludwig-Maximilians-Universität, München, 1997.
- [65] A.V. Sharkov, I.V. Kryukov, E.V. Khoroshilov, P.G. Kryukov, R. Fischer, H. Scheer, T. Gillbro, Femtosecond spectral and anisotropy study of excitation energy transfer between neighbouring alpha-80 and beta 81 chromophores of allophycocyanin trimers, *Biochim. Biophys. Acta* 1188 (1994) 349–356.

- [66] K. Csatorday, S. Cambell, B.A. Zilinska, Exciton interactions in phycoerythrin, *Photosynth. Res.* 10 (1986) 209–215.
- [67] M. Hücke, G. Schweitzer, A.R. Holzwarth, W. Sidler, H. Zuber, Studies on chromophore coupling in isolated phycobiliproteins. 4. Femtosecond transient absorption study of ultrafast excited state dynamics in trimeric phycoerythrocyanin complexes, *Photochem. Photobiol.* 57 (1993) 76–80.
- [68] E. Gantt, Phycobilisomes, *Ann. Rev. Physiol.* 32 (1981) 327–347.
- [69] W. Reuter, G. Wiegand, R. Huber, M.E. Than, Structural analysis at 2.2 Å of orthorhombic crystals presents the asymmetry of the allophycocyanin-linker complex, AP L<sub>c</sub>(7.8), from phycobilisomes of *Mastigocladus laminosus*, *Proc. Natl. Acad. Sci. U. S. A.* 96 (1999) 1363–1368.
- [70] W. Reuter, C. Nickel-Reuter, Molecular assembly of the phycobilisomes from the cyanobacterium *Mastigocladus laminosus*, *J. Photochem. Photobiol., B* 18 (1993) 51–66.
- [71] M. Glauser, W. Sidler, H. Zuber, Isolation, characterization and reconstitution of phycobiliprotein rod–core linker polypeptide complexes from the phycobilisome of *Mastigocladus-laminosus*, *Photochem. Photobiol.* 57 (1993) 344–351.
- [72] A.N. Glazer, S. Fang, Formation of hybrid proteins from the alpha- and beta-subunits of phycocyanins from unicellular and filamentous blue-green algae, *J. Biol. Chem.* 248 (1973) 663–671.
- [73] P. Füglistaller, F. Suter, H. Zuber, The complete amino-acid-sequence of both subunits of phycoerythrocyanin from the thermophilic cyanobacterium *Mastigocladus laminosus*, *Hoppe Seyler's Z. Physiol. Chem.* 364 (1983) 691–712.
- [74] J.A. De Groot, R. Vandersteen, J. Lugtenburg, Synthesis and reactivity of 22,23-methylene-,2,3,17,18-tetramethyl-23-hydro-1,19-(21H,24H)-bilindion-10-enium trifluoroacetate, *Rec. Trav. Chim.* 101 (1982) 263–266.
- [75] J. Gottstein, Der Einfluß Von Grenzflächenaktiven Substanzen Auf Die Selbstaggregation Von Chlorophyllen Und Phycocyaninen., Dissertation, Universität München. (1990).
- [76] R. Fischer, J. Gottstein, H. Scheer, P. Geiselhart, S. Schneider, Picosecond time-resolved fluorescence of phycobiliproteins: subunits of phycocyanin from *Mastigocladus laminosus*, *J. Photochem. Photobiol., B* 5 (1990) 151–165.
- [77] S. Böhm, α-Phycoerythrocyanin: Dynamik Des Reversiblen Photochromismus Und Lyase-Katalysierte Holoproteinassamblierung, Dissertation, Ludwig-Maximilians-Universität, München (2006).
- [78] W. Kufer, H. Scheer, A.R. Holzwarth, Isophorcarubin — a conformationally restricted and highly fluorescent bilirubin, *Isr. J. Chem.* 23 (1983) 233–240.
- [79] J.J.G.S. Van Es, J.H. Koek, C. Erkelens, J. Lugtenburg, Synthesis and spectroscopic study of 1,1'-methylene-2,2'-pyromethen-5(1H)-one and its 3,4-dihydro and 2,3,4,6-tetrahydro derivate, *Rec. Trav. Chim.* 105 (1986) 360–367.
- [80] H. Scheer, H. Formanek, S. Schneider, Theoretical studies of biliprotein chromophores and related bile pigments by molecular orbital and Ramachandran type calculation, *Photochem. Photobiol.* 36 (1982) 259–272.
- [81] O.B. Ptitsyn, Molten globule and protein folding, *Adv. Protein Chem.* 47 (1995) 83–229.
- [82] M. Jamin, R.L. Baldwin, Two forms of the pH 4 folding intermediate of apomyoglobin, *J. Mol. Biol.* 276 (1998) 491–504.
- [83] Y. Goto, N. Takahashi, A.L. Fink, Mechanism of acid-induced folding of proteins, *Biochemistry* 29 (1990) 3480–3488.
- [84] S.N. Loh, M.S. Kay, R.L. Baldwin, Structure and stability of a second molten globule intermediate in the apomyoglobin folding pathway, *Proc. Natl. Acad. Sci. U. S. A.* 92 (1995) 5446–5450.
- [85] A.F. Chaffotte, J.I. Guijarro, Y. Guillou, M. Delepierre, M.E. Goldberg, The “pre-molten globule”, a new intermediate in protein folding, *J. Protein Chem.* 16 (1997) 433–439.