Dissociating effect of chromophore modifications on C-phycocyanin heterohexamers

R. Fischer and H. Scheer†

Botanisches Institut der Universität München, Menzingerstr. 67, W-8000 München 19 (Germany)

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Abstract

The bilin chromophores of the α or β subunit of C-phycocyanin (PC) from *Mastigocladus laminosus* were modified, and subsequently recombined with the respective complementary unmodified chromophores. The modifications consisted of photobleaching (350 nm) or reversible reduction of the verdin- to rubin-type chromophore(s). Recombination led to heterodimers $(\alpha\beta)_1$, but the heterohexameric aggregation state $(\alpha\beta)_3$ could not be obtained with the modified chromophores. Autoxidation of the reduced α-84 chromophore in such a hybrid, which occurred on standing under aerobic conditions, induced reaggregation to heterohexamers. Chemical re-oxidation of the reduced chromophores did not produce reaggregation, and it was not promoted by a 22 kDa linker peptide fragment (Gottschalk *et al., Photochem. Photobiol., 54* (1991) 283), which in unmodified samples stabilized heterohexameric aggregates. Binding of the mercurial *p*-chloromercury-benzenesulphonate to the single free cysteine of PC near (approximately 0.4 nm) the β-84 chromophore had only a moderately destabilizing effect on the heterohexamer $(\alpha\beta)_3$. It was concluded that the intact chromophore structure is an important factor determining the quaternary structure of biliproteins. The tendency of heterohexamer destabilization is related to the situation in phycoerythrocyanin, where photoisomerization of the violobilin chromophore of the α subunit near the heterodimer–heterodimer contact region is also responsible for aggregate destabilization (Siebzehnrübl *et al., Photochem. Photobiol., 46* (1989) 753).

Keywords: Photosynthesis, energy transfer, antenna, phycobilisome, *Mastigocladus laminosus*, biliprotein, phycocyanin, phycoerythrocyanin, chromophore modification, photobleaching, borohydride.

1. Introduction

C-phycocyanin (PC) is one of the main constituents of phycobilisomes, the extramembraneous photosynthetic antenna complexes of cyanobacteria and red algae [1–3]. *In situ*, PC is highly aggregated as part of the phycobilisome rods. *In vitro*, in buffers of low ionic strength, the phycobilisomes dissociate, and PC can be isolated and crystallized as ring-shaped heterohexamers $(\alpha\beta)_3$ (*e.g.* in *Mastigocladus laminosus* [4, 5]) composed of three α and three β subunits in alternating order, or heterododecamers $(\alpha\beta)_6$ (*e.g.* in *Fremyella diplosiphon* [6] or *Agmenellum quadruplicatum* [5]). The α subunit carries one covalently linked cyanobilin chromophore (α-84), and the β subunit carries

†Author to whom correspondence should be addressed.

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two (β-84 and β-155), numbered according to the positions of the binding cysteine residues [4, 7]. *In situ*, these heterohexamers or heterododecamers are aggregated with so-called linker peptides, which function as structural proteins, but are also important for energy transfer by fine tuning of the chromophore spectra [8–12].

Energy transfer in phycobiliproteins is still only partly understood [13–18]. Some main features of excited state kinetics have been modelled by Förster transfer [13], but there is experimental [19, 20] and theoretical [13, 17, 18] evidence for exciton coupling, in particular among the proximate α-84 and β-84 chromophores of different $(\alpha\beta)_1$ heterodimer ("monomer") units within the heterohexamers or heterododecamers. There are also several reports on more complicated excited state kinetics which are unaccounted for by the simple model, and which have been taken as evidence for microheterogeneity [17, 18, 21, 22].

In order to investigate the role of individual chromophores in the process, we have begun to modify distinct chromophores within energy transferring chromophore ensembles. Particularly relevant are the α-84 and β-84 chromophores. They are proximate (approximately 2 nm) to each other at the contact site of the PC heterodimers $(\alpha\beta)_1$ within the heterohexamer $(\alpha\beta)_3$, whereas they are much further apart (approximately 5 nm) in the heterodimer. It is also likely that the β-84 chromophore is conformationally modified by the association of linker polypeptides. During such studies, we have observed that chromophore modifications also have a profound influence on protein–chromophore interactions and chromoprotein aggregation. It is this aspect which is documented here.

2. Materials and methods

2.1. Preparation of PC and its subunits

All work with native biliproteins was performed under subdued light, and with denatured biliproteins under very dim light or in the dark. PC was isolated from *Mastigocladus laminosus* as reported in ref. 23. Subunits of PC were prepared via isoelectric focusing of denatured PC and subsequent renaturation, as described previously [21]. Concentrations were determined spectrophotometrically with the extinction coefficients reported previously [24]. The coefficient of the heterohexamer was taken as three times that of the heterodimer ($\epsilon = 286000$, see ref. 25).

2.2. Chromophore modifications by reduction and photobleaching

2.2.1. Reduction with borohydride

PC, α-PC and β-PC were portioned (2.35, 1.0 and 1.35 $OD_{620} \times ml$ respectively) into 1.5 ml tubes and precipitated by ammonium sulphate (70%). In this form they could be stored deep frozen. For reduction, the pellets were dissolved in 1 ml potassium phosphate (PP) buffer (0.9 M) (pH 7.3) containing 8 M urea (DB2). A freshly prepared borohydride solution (140 μl) (Merck, 1.3 M) in the same buffer was added at room temperature, corresponding to a ratio of borohydride to chromophore of between 8000 and 25000. The reduction was generally complete after 45 min (loss of the band around 610 nm, Fig. 1). The pH value increased to 8.3 during this time. Unreacted borohydride was then removed by adding solid glucose (0.3–0.4 g) until generation of gas bubbles stopped. Finally, the sample was checked again by absorption spectroscopy, since the addition of glucose sometimes caused a slight re-oxidation of the chromophores. In the case of subunits, the reduction was followed by recombination with the complementary, untreated subunit. For renaturation, mercaptoethanol (20 μl) (diluted with

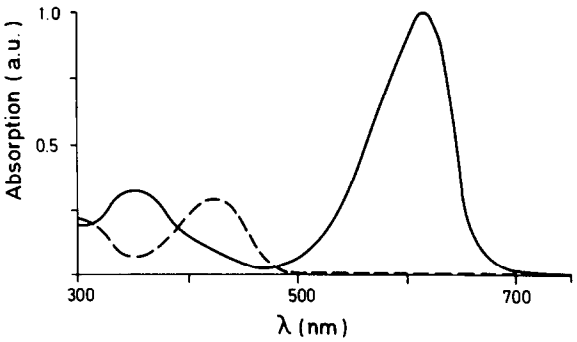

Fig. 1. Absorption spectra of PC before (——) and after (– – –) reduction with borohydride in 0.9 M PP buffer containing 8 M urea. The starting pH was 7.3, the final pH 8.3.

water) was added to a final concentration of 5 mM, and the sample was dialysed against 0.1 M PP buffer, pH 7 (NB) first for 4 h at room temperature and subsequently overnight at 4 °C with fresh buffer.

Some experiments were carried out with higher protein concentrations. In one experiment the procedure described above was followed, but the protein concentration was increased two to threefold. Reduction was achieved by adding 210 μl of the borohydride solution, and before renaturation the product was dialysed against DB2. In a second experiment the modification products were concentrated by ammonium sulphate precipitation, followed by dialysis of the dissolved pellet against NB, or by covering the sample in dialysis tubing with 'Aquacide I' (Pharmacia). Subsequent dialysis was necessary when the concentration of the sample was increased several fold.

2.2.2. Experiments with a linker peptide fragment

In one experiment PC632 was used. The (22 kDa) PC linker complex (PC632 [12]) was portioned (2.8 OD ml), analogous to PC and the subunits, by ammonium sulphate precipitation (see above). The reconstitution method described above for PC was changed in the following way. The recombined subunits (2.35 $OD_{620} \times ml$), when still in the denatured state (DB2), were treated with a linker PC pellet (2.8 $OD_{280} \times ml$) dissolved in 1 ml of the same buffer. Renaturation was then performed in the standard way.

In a second experiment preparations enriched in linker fragment were used. The β subunit (two pellets) was reduced, excess borohydride was removed by glucose, and the sample was dialysed for 4 h against PP buffer (0.1 M) (pH 7.5) containing 8 M urea (DB1). The pellet enriched in linker fragment (with a ratio of α subunit to linker $\approx 0.5:1$ and β subunit to linker $\approx 0.25:1$) dissolved in DB1 (0.75 ml) was then added such that the final ratio of β subunit to linker was approximately 3:1. In the control experiment the same procedure was used, but with unmodified β subunit.

2.2.3. Re-oxidation of the borohydride reduced chromophores

In some experiments autoxidation was performed. The following procedures were used: (i) the renatured hybrid samples were kept in the dark at 4 °C for up to 2 days (subunits) or 6 days (integral or recombined PC); (ii) renatured hybrid samples were precipitated with 70% ammonium sulphate and kept in the dark at room temperature for 1 (regular time) to 4 weeks; (iii) renatured hybrid samples were kept at room

temperature in the presence of PMSF (1–5 mM) and/or NaN_3 (5–10 mM) for up to 1 week. In the case of isolated subunits, the re-oxidized samples were pelleted by 70% ammonium sulphate. This pellet was then dissolved in 1 ml of PP buffer containing urea (8 M), in which an appropriate amount of the complementary subunit had been dissolved. The PP buffer was either strongly denaturing (0.1 M, pH 7.5) or weakly denaturing (0.9 M, pH 7.3).

In other experiments re-oxidation with quinones was performed. After degradation of the excess of borohydride with glucose (see above), 50 μl of an aqueous solution of sodium-1,2-naphthoquinone-4-sulphonate (1.2 mM) was added. After a reaction time of 10 min re-oxidation was checked by absorption spectroscopy. For a sixfold increased protein concentration, 100 μl of the naphthoquinone solution was added.

2.2.4. Photobleaching

The following light sources were used: 600 nm, 'Intralux 150H' cold light (Volpi), tungsten halogen lamp (150 W) with light guide, interference filter (Seitner, type 600 S-NOY 11-1; halfwidth, 9.6 nm; 93% maximum transmission), highest intensity level; 350 nm, light reactor (Southern New England Ultraviolet Company) equipped with 8–12 lamps (75 W each, type R.P.R. 3500 Å); 615 nm, reactor as before, but with cylindrical fluorescent screen (approximately 70% quantum conversion of the incident 350 nm light) placed between lamps and sample.

Bleaching experiments were performed with integral PC, and with the α subunit at room temperature. Air- or nitrogen-saturated samples were used with a chromophore concentration of about 8 μM in DB1. Every 30 min, an absorption spectrum was recorded, and when using the light reactor ('hot' light), the sample was cooled on ice for 5 min. The total irradiation time was up to 2.5 h. After complete bleaching of a subunit, the complementary subunit was added, and the samples renatured as above.

2.2.5. Indirect chromophore modification with p-chloromercury-benzenesulphonate (PCMS)

Reaction with PCMS was performed by titration of heterohexameric PC $(\alpha\beta)_3$ in a 1.1–1.2-fold excess [26].

2.2.6. Characterization of the modification products

The products were analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (see below) and spectroscopy (absorption, experiments with PCMS also by fluorescence), and were tested on sucrose gradients (see below) and by gel filtration (see below) to determine the heterodimer–heterohexamer equilibrium. Quantitative evaluation was, in both cases, performed by absorption spectroscopy. UV–visible absorption spectra were recorded with a model Lambda 2 (Perkin–Elmer) or model 8451 diode array (Hewlett Packard) spectrophotometer. Fluorescence spectra were measured with a model 221 Fluorolog (Spex) fluorometer.

SDS-PAGE was performed with the Laemmli system [27] with 3% stacking and 10%–25% gradient separation gel. In some cases, the samples were cross-linked before SDS-PAGE in the following way. The samples ($OD_{max}=0.5$–2) were filtered (0.2 μm steril filter (FP 030/3, Schleicher & Schüll)) and fresh or deep-frozen stored glutaraldehyde was added to a final concentration of 0.5%. The samples were kept for 2 h in the dark at room temperature and were shaken every 10 min. The excess of glutaraldehyde was removed by dialysis against NB at approximately 8 °C.

Gel filtration was performed on Sephadex-G-75 (Serva, Heidelberg) columns (20 cm$\times$0.5 cm) with a maximal probe volume of 0.6 ml, operated under hydrostatic

pressure at ambient temperature. For direct comparison, columns with identical dimensions and flow rates were used in parallel.

The sedimentation velocity was determined according to Martin and Ames [28]. The sample (100–200 μl) was layered on a 5 ml sucrose gradient (7%–17%) in NB buffer. The runs were performed at 20 °C for 15 h at $238000 \times g_{av}$ in a swingout rotor. Bands were identified by their blue colour and red fluorescence (excitation at 360 nm), marked, retrieved from the gradient, and characterized and quantitated by absorption spectroscopy. For reference, 'imaginary' bands in the heterohexameric $(\alpha\beta)_3$ or heterodimeric $(\alpha\beta)_1$ sedimentation range were also retrieved, and checked in the same way. Native molecular weight markers were heterohexameric PC ($(\alpha\beta)_3$: 112.2 kDa), its dimeric subunits (α2: 36 kDa, β2: 38.8 kDa [21, 25]), myoglobin (horse heart, Sigma, 17.5 kDa) and cytochrome *c* (horse heart, Sigma, 12.4 kDa).

3. Results

3.1. Experiments with reduced chromophores

3.1.1. Reduction

The chromophores of denatured or partly denatured PC can be reduced with $NaBH_4$ at the central methine bridge to yield A-dihydrobilirubin [29] (Fig. 2). High-molar phosphate buffer (0.9 M, pH 7.3 containing 8 M urea) was used in the present experiments to prevent a strong pH increase by the reagent. Under these high-phosphate conditions, 8 M urea causes only a moderate denaturation as shown by the absorption spectra (Fig. 1). There is a maximum 15% decrease in the red band compared with 80% or more on complete denaturation, correlated with a small hypsochromic shift of 3 nm or less, the exact amount being dependent on the amount of remaining

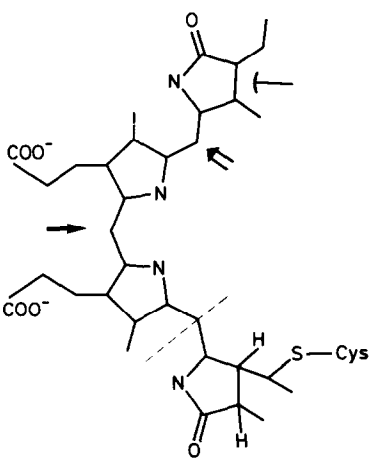

Fig. 2. Schematic representation of the chromophore modifications in PC: →, reduction with borohydride; this reaction changes the chromophore geometry and electronic structure; ⇒, *Z*/*E* isomerization in phycoerythrocyanin (PEC) (ref. 30); this reaction effects indirectly the chromophore geometry; ¦, photochemical bleaching, leading to loss of absorption at wavelengths longer than 300 nm, and hence to cleavage at all methine bridges. Protein conformational changes are a probable direct result of *p*-chloro-mercury-benzene-sulfonate (PCMS) modification (⊣) and indirectly possible in all other cases.

ammonium sulphate. The reduction of chromophores proceeds to completion (Fig. 1). By contrast, there is only little (see also ref. 29) or no reaction in the native state (in 0.1 M PP buffer).

In order to determine the saturating reduction, experiments were also carried out with up to a threefold increased concentration of borohydride. The borohydride concentration necessary for reduction was not proportional to the biliprotein concentration. As an example, a threefold increased biliprotein concentration required only a 1.5-fold increased borohydride concentration for saturating reduction of the chromophore(s). For renaturation, an additional dialysis against DB2 was required before dialysis against NB to avoid protein precipitation.

Assuming no side reactions, the extinction coefficient of reduced PC in DB2 was determined spectrophotometrically to be $\epsilon = 24100$ per chromophore. This is based on $\epsilon = 286000$ for the native $\alpha\beta$ protomer (three chromophores), using an appropriate correction for dilution, and can be estimated as $\epsilon = 26\,000 \pm 2000$ for the renatured product dissolved in NB. Kufer and Scheer [29] found similar values: $\epsilon = 21\,900$ for the reduced chromophore in the native apoprotein of PC from *Spirulina platensis* and $\epsilon = 24\,200$ in denaturing buffer (NaPP, 50 mM, pH 7.5, containing 8 M urea).

The value given is a lower limit, because the generation of byproducts to a minor extent was indicated by the following observations (Fig. 3). If reduction is performed with higher borohydride concentrations (170–420 mM) or renaturation occurs in the presence of borohydride (17–170 mM), an additional absorption peak at 560–565 nm is observed in the re-oxidized pigment (see below), most probably due to the formation of violin chromophores [31]. In addition, if reduction is incomplete, a spectral component in this range ($\lambda_{max} \approx 570$ nm) is detected. This indicates that double bonds other than Δ10, 11 can be reduced. The latter reduction products should oxidize more slowly than the rubins. In the reduced or re-oxidized samples, no protein cleavage is detectable by SDS-PAGE. However, other modifications of the protein cannot be excluded completely, and no other tests have been performed.

The aggregation state of native PC at concentrations of 3 μM or greater is heterohexameric $(\alpha\beta)_3$. With all the modified samples tested, it was not possible to obtain aggregates larger than (hetero)dimers (Table 1). This was true for complexes in which either one or both of the subunits were modified. Protein cross-linking with

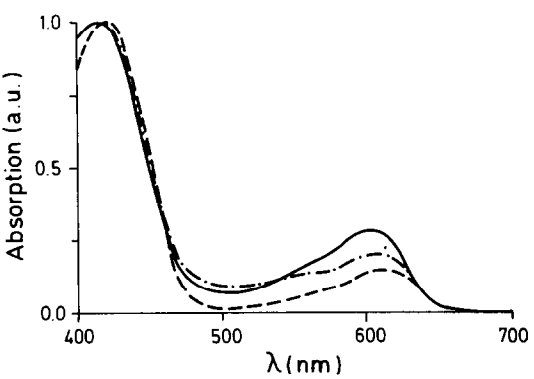

Fig. 3. Absorption spectra of incompletely reduced PC in DB2 (——) and of fully reduced and subsequently partly re-oxidized (autoxidized) PC in 0.1 M PP buffer, pH 7. Reduction was carried out with 170 mM (– – –) or 420 mM (– · –) borohydride.

TABLE 1

(Photo)chemical chromophore modifications of PC and effect on protein aggregation

Chromophore modification	Recombination	Aggregation
Reduction by $NaBH_4$	$\alpha_{red}+\beta$	Heterodimer
	$\alpha+\beta_{red}$	Heterodimer
	$\alpha_{red}+\beta_{red}$	Heterodimer
+linker	$\alpha_{red}+\beta$	Heterodimer
	$\alpha+\beta_{red}$	Heterodimer
	$\alpha_{red}+\beta_{red}$	Heterodimer
+'auto-re-oxidation'	$\alpha+\beta_{red/auto\text{-}reox}$	Heterodimer
	$\alpha_{red/auto\text{-}reox}+\beta$	Heterohexamer
+'quinone re-oxidation'	$\alpha_{red/reox}+\beta$	Heterodimer
	$\alpha_{red/reox}+\beta_{red/reox}$	Heterodimer
Bleaching by UV	$\alpha_{bleach}+\beta$	Heterodimer
	$\alpha_{bleach}+\beta_{bleach}$	Heterodimer
No modification	$\alpha+\beta$	Heterohexamer
+linker	$\alpha+\beta+$linker	Heterohexamer +linker

Red, reduced; reox, chemically re-oxidized; auto-reox, autoxidized; bleach, photobleached.

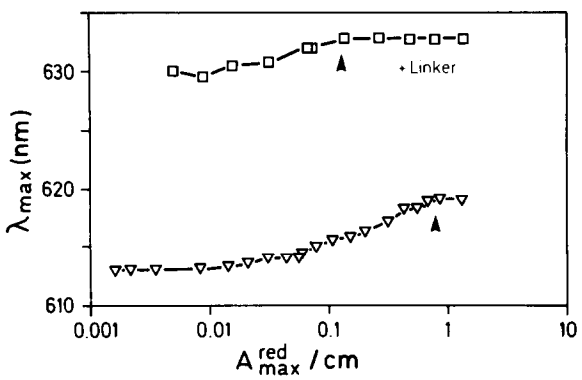

Fig. 4. Position of the red absorption maxima of PC (bottom) and the PC–22 kDa polypeptide complex (top) as a function of the protein concentration. Dilution series in 0.1 M PP buffer with measurement of the absorption 3 min after (further) dilution. The arrows denote the 'critical concentration' where dissociation starts to occur on decreasing the protein concentration.

glutaraldehyde and subsequent SDS-PAGE indicates that the dimers formed are heterodimers. It should be noted that unmodified heterohexameric PC was also only linked to heterodimers. The interheterodimer contact zone is obviously not suitable for linking by glutaraldehyde under the conditions used. *In vivo* and *in vitro*, PC aggregates are stabilized by linker polypeptides. To test the influence of such a linker on modified PC, a functionally active 22 kDa linker peptide fragment [12] was used. This polypeptide could not stabilize a heterohexamer with rubin-type chromophores, in contrast with unmodified PC (Fig. 4).

3.1.2. Re-oxidation and 'bleaching'

The reduced chromophores were not stable over a long period under aerobic conditions. Re-oxidation ('autoxidation') could be observed in partly denatured (DB2) protein and especially in the native (*i.e.* renatured) state. In the latter re-oxidation was more than four times faster than in the partly denatured state (Table 2, see also ref. 29). These re-oxidized products were also used for recombination experiments (see below). Re-oxidation was accompanied by a second process. This was a light-independent bleaching, which was dependent on the protein state and strongly accelerated with increasingly denatured protein (Fig. 5). The rates of bleaching showed relatively strong variations, but maximum bleaching of about 80% was observed within 20 h for

TABLE 2

Autoxidation (percentage determined spectrophotometrically) after reduction with $NaBH_4$ and removal of the excess borohydride

PC	Time (days)	Autoxidation (%)	
		Condition I	Condition II
α-PC	1	6–7	
	2	13–14	
	3	23	
	12		21–25 (first 2 days condition I: 14)
β-PC	1	2–3	
	2	4–5	
	7		13 (first 1 day condition I: 2.5)
	27		16–18 (first 1 day condition I)
PC	1–7	3–6	
	3/10	6/7	
	8	13	

Condition I, 0.1 M PP buffer, pH 7, 6–8 °C.
Condition II, >70% $(NH_4)_2SO_4$; 0.1 M PP buffer, pH 7, room temperature.

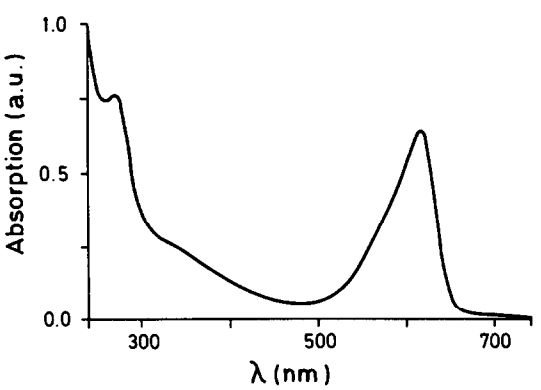

Fig. 5. Example of bleaching of the reduced cyanobilin chromophores: recombination product (1:1) of α-PC and reduced β-PC in 0.1 M PP buffer, pH 7. The absorption band around 420 nm of the reduced chromophores is lacking. (For more details, see text.)

the fully denatured protein and of 20%–30% within 7 days for the native polypeptide.

Autoxidation and bleaching could be arrested for at least a few days by adding 10% ascorbate or 170 mM borohydride to the sample and dialysis buffer. When the latter was used, destruction of the excess borohydride by glucose after the reduction procedure was omitted. Bleaching alone could be prevented in the presence of about 1% ascorbate or 3 mM mercaptoethanol. Autoxidation was slowed down to about one-half with ascorbate (1%) but was hardly influenced by mercaptoethanol (3 mM).

Chemical re-oxidation was tested with several quinones (1,4-benzoquinone, menadione, 2-methyl-anthraquinone, sodium-1,2-naphthoquinone-4-sulphonate), but naphthoquinone-sulphonate was found to be the most suitable as demonstrated by the generation of only few byproducts visible in the absorption spectrum.

3.1.3. Aggregation state after re-oxidation

In all cases of pigments re-oxidized by quinones, the recombination products were heterodimeric $(\alpha\beta)_1$ and not aggregated further to heterohexamers $(\alpha\beta)_3$. This result was obtained despite extensive variations in the modification and renaturation conditions, involving a variety of equilibration buffers, and variations in the concentration of mercaptoethanol.

By contrast, slow autoxidation in the presence of air promoted reaggregation to heterohexamers in the case of hybrids containing the reduced α subunit. When partly re-oxidized samples were analysed by ultracentrifugation, the heterohexamer band was free of modified chromophores as shown by absorption spectroscopy, and all modified pigments remained in the heterodimer band. This suggests that the chromophore modification, and not an irreversible side reaction of the polypeptide with borohydride, is responsible for the blocked aggregation. However, protein modification, which should be reversible via autoxidation, cannot be excluded completely, although there are no –S–S– bridges in PC, which has only one free cysteine in the β subunit. For the 'modified' β subunit, autoxidation did not lead to reaggregation; the reason(s) for this is (are) not yet clear.

3.2. Experiments with photobleached chromophores

Bleaching with UV light (350 nm, Fig. 6) was considerably more effective than with red light. The absorption of the red band decreased by about 20% within 2 h of red light irradiation; however, under UV light nearly complete bleaching (greater

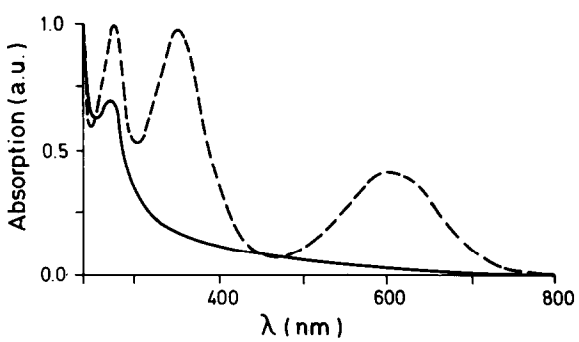

Fig. 6. Absorption spectra of PC in DB1 (– – –) and after bleaching with 350 nm light (2.5 h) in the presence of air, followed by "renaturation" (dialysis against 0.1 M PP, pH 7) (——), see text for details.

than 95%) was achieved within 30 min. In general, bleaching occurred more slowly in the air-saturated samples, which indicates an involvement of triplets, which are quenched by oxygen [32]. The bleaching product of the chromophores was not characterized in more detail, but from the lack of absorption at $\lambda > 320$ nm a conjugated π system extending over more than one five-membered heterocyclic ring no longer exists. In every case SDS-PAGE excluded protein cleavage. In addition, this type of modification prevents reaggregation of PC to heterohexamers (Table 1).

3.3. Indirect chromophore modification with mercurials

The reaction of PC with mercurials selectively affects cysteine-β111 (cys-β111) and also indirectly the neighbouring β-84 chromophore [26]. While the (photo)chemical modifications mentioned above completely prevent heterohexamer formation, modification with PCMS, which binds to the single free cysteine cys-111 located at the β subunit near the β-84 chromophore, causes only a moderately destabilizing effect on this aggregate. Over the concentration range in which the 'natural' pigment consists of both heterodimers $(\alpha\beta)_1$ and heterohexamers $(\alpha\beta)_3$, the heterodimer portion is increased in samples treated with PCMS in slightly more than molar (PCMS: $(\alpha\beta) = 1{:}1$) amounts.

From sedimentation experiments (Table 3), an aggregation constant $k_{agg} = 1.3 \times 10^{11}$ was determined, which is about two orders of magnitude smaller than that of unmodified PC ($k_{agg} = 1.2 \times 10^{13}$). A destabilization of aggregates was also concluded from gel filtration experiments. The latter method gave consistently lower concentration of dimeric aggregates. Both methods are subject to possible errors, caused by the high pressure in the ultracentrifuge [33] and dilution in the molecular sieve column. However, it should be noted that independent experiments gave no indication of a pressure-dependent dissociation in PC [34]. There was a slowly increasing dissociation detected with increasing time. Variations in both the absorption and fluorescence spectra over the time scale of several hours (not shown here) indicate a more complicated time-dependent situation, which points to 'secondary' changes of the modification product.

4. Discussion

PC of *M. laminosus* shows a very sensible disaggregation response to all the chromophore modifications studied. Chromophore reduction and photobleaching prevent

TABLE 3

Influence of the protein-bound mercurial PCMS on the heterodimer–heterohexamer ($(\alpha\beta)_1$–$(\alpha\beta)_3$) equilibrium of PC in 0.1 M PP buffer, pH 7. Ratios are based on the absorptivities at the red maximum

Absorbance	$(\alpha\beta)_1/(\alpha\beta)_3$	
	PC	PC+PCMS
0.25	93/7	92/8
0.5	–	68/32
0.9	–	52/48
1.2	12/88	45/55
2.0	8/92	32/68
3.9	–	16/84

the formation of aggregation states higher than dimers. The 'autoxidation' phenomenon after reduction and the correlated ability to reaggregate to the original state (experiments with modified α subunit) demonstrate that PC is a structurally well-defined and optimized chromophore–protein system, in which the chromophores have a structural function. For the α-84 chromophore, this disaggregating effect can be readily rationalized by its location at the interface and its probable involvement in hydrogen-bonding interactions between heterodimers. A more subtle effect must be postulated for modified β chromophores. β-84 is separated from the interface by the loop connecting helix B and E of the β subunit [4–6], and β-155 is even more remote. Removal of the chromophores by site-directed mutagenesis of the binding amino acids (cysteine–serine) has recently corroborated their stabilizing influence on the PC structure. The apoprotein is monomeric and rather unstable, and even loss of the β-155 chromophore destabilizes aggregates [30].

Modification of PC from the mesophilic blue–green alga *Spirulina platensis* by reduction of the chromophores with borohydride has been reported by Kufer and Scheer [29]. In contrast with the modification procedure described here, the alkalizing effect of borohydride was not buffered with high-molar phosphate. A partial reduction of the cyanobilin to rubin chromophores was observed in the native chromoprotein, and a complete and more rapid reduction after unfolding; it was concluded that a thermodynamic equilibrium exists between the natural verdinoid and rubinoid chromophores which is dependent on the state of the protein. In the present work, using high ionic strength buffer, at least a partial denaturation is a precondition for reduction. Using dithionite (also a small molecule), the reduction of *S. platensis* PC was not possible in the native state and, when reduced under denaturing conditions, the rubinoid product was converted back to the original state during renaturation. Under these conditions, using PC from *M. laminosus*, it cannot be decided whether accessibility or thermodynamics is decisive. Two factors may be responsible for a higher stability of the chromophore towards reduction: (i) the higher buffer concentration, which also leads to increased aggregation and stabilizes the protein (see above); (ii) the thermophilic nature of the organism, leading to a higher rigidity of the protein at ambient temperatures.

PCMS modification, although only showing a moderate effect on aggregation, confirms the observation that PC is an optimized protein–chromophore system, which cannot tolerate conformational or geometric variations of the chromophores in the heterohexamer. In this context, a comparison with the heterohexamer–heterodimer equilibrium shift in phycoerythrocyanin is interesting, which is induced by a green–orange reversible photochemical reaction (presumably a *Z–E* isomerization) of a violobilin chromophore located on the α subunit. The function of the photochemistry in the cell is unclear, but 'phycochrome-type' pigments such as phycoerythrocyanin may react as sensory photoreceptors, *e.g.* as photomorphochromes [35].

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