PERSPECTIVES IN PHOTOSYNTHESIS

PROCEEDINGS OF THE TWENTY-SECOND JERUSALEM SYMPOSIUM ON QUANTUM CHEMISTRY AND BIOCHEMISTRY HELD IN JERUSALEM, ISRAEL, MAY 15–18, 1989

Edited by

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INFLUENCE OF CHROMOPHORES ON QUARTERNARY STRUCTURE OF PHYCOBILIPROTEINS FROM THE CYANOBACTERIUM, *Mastigocladus laminosus*

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ABSTRACT. Chromophores of C-phycocyanin and phycoerythrocyanin have been chemically modified by reduction to rubins, bleaching, photoisomerization, or perturbation with bulky substituents. Pigments containing modified chromophores, or hybrids containing modified and unmodified chromophores in individual protomers have been prepared. All modifications inhibit the association of the (αß)-protomers of these pigments to higher aggregates. The results demonstrate a pronounced effect of the state of the chromophores on biliprotein quaternary structure. It may be important in phycobilisome assembly, and also in the dual function of biliproteins as (i) antenna pigments for photosynthesis and (ii) reaction centers for photomorphogenesis.

1. INTRODUCTION

Cyanobacteria, red algae and cryptophytes have specialized antenna pigments which enable them to harvest light efficiently in the green spectral region where chlorophylls have only poor absorption. These are the phycobiliproteins, extra-membraneous proteins carrying covalently bound open-chain tetrapyrrolic chromophores (Gantt, 1986, Glazer, 1983; MacColl and Guard-Friar, 1983; Scheer, 1982; Schirmer *et al.*, 1987; Wehrmeyer, 1983; Zuber, 1986). They are characterized by a remarkable degree of spectral adaptations covering the range from 480 to 670 nm. The spectral variation is based on the usage of only four different chromophore types, each of them being further modulated in its absorption properties by non-covalent interactions with the apoproteins.

In cyanobacteria and red algae, the phycobiliproteins show a high degree of organisation: Together with the linker

J. Jortner and B. Pullman (eds.), Perspectives in Photosynthesis, 121–131.

polypeptides, which are often devoid of chromophores, they form self-organizing microscopic structures, the phycobilisomes, which in turn form ordered arrays on the outer thylakoid membrane surface (Gantt, 1986; Glazer, 1983; Mörschel and Schatz, 1988; Wehrmeyer, 1983). By a combination of energetic and spatial ordering, and low internal conversion rates of the chromophores, the phycobilisomes are an antenna system working with quantum efficiencies approaching 100%.

The factors controlling tuning of the absorption, photochemistry and aggregation in biliproteins are presently becoming understood in considerable detail. This is based on an increasing body of structural data (Bishop *et al.*, 1987; Bryant, 1988; Dürring and Huber, 1989; Glazer, 1983; Schirmer *et al.*, 1987; Tandeau de Marsac, Zuber, 1986) with theoretical (Sauer and Scheer, 1988; Schneider *et al.*, 1988, Scheer, 1987) and spectroscopic studies (Fischer *et al.*, 1988; Glazer, 1983; MacColl and Guard-Friar, 1987; Mimuro *et al.*, 1986a,b, Scheer, 1982; Schirmer *et al.*, 1987). They involve conformational changes of the chromophores imposed by non-covalent interactions with the apoproteins (e.g., point charges, and π-π-interactions, and -in higher aggregates- excitonic interactions among chromophores (MacColl and Guard-Friar, 1983; Sauer and Scheer, 1988; Schneider *et al.*, 1988).

The assembly of phycobilisomes is a complex, and to a large extent autonomous process believed to be controlled by the presence of appropriate components. The factors controlling the aggregation of monomers ($\alpha\beta$) to oligomers ($\alpha\beta$)$_n$ have hitherto mainly been located on the proteins. Here we want to present data which show a pronounced influence of the chromophores, too, on the aggregation. The results may also be important for an understanding of a the second function of biliprotein, e.g. as photomorphogenetic reaction center pigments (Björn and Björn, 1980; Rüdiger and Scheer, 1983; Scheer, 1982; Song, 1988).

2. MATERIALS AND METHODS

C-phycocyanin (PC) and phycoerythrocyanin (PEC) were isolated from the cyanobacterium, *Mastigocladus laminosus*, as described before (Fischer *et al.*, 1988). Subunits were prepared by isoelectric focusing (Schmidt *et al.*, 1988).

Chromophores were bleached by irradiation with 350 nm light (Scheer, 1987).

Reduction of cyanin to rubin chromophores was done by a modification of the method of Kufer and Scheer, 1982). Denatured PC or isolated subunits (8-25μM) in potassium phoshphate buffer (0.9 M, pH = 7) were treated with NaBH$_4$ (final concentration $\approx$170 mM) for 45 min at ambient temperature. The reaction was followed spectrophotometrically (decrease at $\approx$600, increase at $\approx$420 nm). After completion,

excess reagent was destroyed by addition of glucose. Modified PC was renatured by dialysis against phosphate buffer (100 mM, pH = 7) at ambient temperature. Hybridization to native protomers with modified chromophores on only one of the subunits, was done by combining stoichiometric amount of the appropriate subunits in buffer containing urea (8M), followed by dialysis against decreasing amounts of urea and finally urea-free buffer.

Cystein-111 of PC was modified by treatment with p-chloro-mercurybenzenesulfonate (PCMS) (Siebzehnrübl *et al.*, 1987).

Photochemistry was induced with a cold light source (150W) equipped with light-guide and suitable interference filters (≈10 nm fwhh). Spectra were recorded with lambda2 (Perkin-Elmer) or ZWSII (sigma) spectrophotometers. Aggregation state of samples was determined by ultracentrifugation (Martin and Ames, 1961) with myoglobin and trimeric phycocyanin as reference.

3. RESULTS AND DISCUSSION

3.1. Effect of reduced chromophores on aggregation

Chromophores of PC were completely reduced with $NaBH_4$ in the presence of urea (8M) to rubins, which show no absorption at wavelengths >500nm. After renaturation, the modified chromophores are unstable in the native protein environment and are slowly oxidized to cyanin chromophores within several days. The process can be followed quantitatively by monitoring the absorption increase at 620nm.

The aggregation state of phycocyanin containing reduced chromophores on both subunits is monomeric. There is only a single, yellow band present after centrifugation of freshly prepared "phycorubin". Under uv-light, this band is non-fluorescent. After prolonged standing of the samples, they become slowly green due to re-oxidation of the chromophores. Ultracentrifugation of such samples shows generally two bands: a greenish one at the position of monomers, and an additional one at the position of the PC trimer. The latter exhibits the characteristic absorption and fluorescence of PC trimer, and lacks the 420 nm band of the rubin chromophore(s) ($\lambda_{max} \approx 420$ nm). It is at first only visible by its red fluorescence, and at increasing re-oxidation times becomes concentrated enough to become visible to the eye too. We, therefore, conclude that the lower band contains trimeric PC containing the re-oxidized native chromophores. If the aggregation pattern is followed through the reoxidation, the trimer band is always fluorescent and blue (if visible), whereas the monomer band is yellow or green. This shows clearly that modified PC bearing rubin chromophores can no longer aggregate.

In order to test if the inhibition of aggregation is due to specific chromophore(s), similar experiments were carried out with hybrids in which the α-subunit was bearing a modified rubin chromophore and the ß-subunit cyanin chromophores. For this purpose, isolated α-subunits were modified, and then hybridized with the respective complementary unmodified subunits. The results can be summarized in one sentence: Whenever there was a rubin chromophores present in the hybrid, the hybrids were monomeric; and they did only aggregate when the chromophores became re-oxidized.

3.2. Effect of cystein-111 modification on aggregation

The single free cystein residue in PC, e.g. ß-111 in the immediate vicinity of chromophore ß-84, was modified with PCMS, and the aggregation studied at different protein concentrations. At low concentrations (<0.3 μM), both PCMS-modified and unmodified PC were monomeric. Increasing concentrations shifted the equilibrium to trimers in unmodified PC, which at concentrations ≥3 μM PC was mostly present as trimer. The PCMS-modified PC remained monomeric up to the highest concentrations investigated, e.g. 10 μM.

This inhibition of aggregation was reversible, too. Treatment of PCMS-modified PC with an excess of dithiothreitol, removes the mercurial reagent from the chromoprotein as shown by the blue-shift of the spectrum. The difference spectra are mirror-images of the reaction with the mercurial, and the final spectrum of the recovered PC showed again the spectral characteristics of trimeric PC.

3.3. Effect of aggregation on photochemistry

3.3.1. Phycocyanin

Long-lived photoproducts have been observed in several phycobiliproteins. In phycocyanin, a small but measurable photochemistry is found only upon partial denaturation and/or disaggregation of the native trimers. This photochemistry was originally studied in phycocyanin treated with urea at concentrations of 3-5M, which are known to change aggregation and to some extent also the tertiary structure. The difference spectra are characterized by a decrease around 622nm, and only a minor increase in the 500nm range (John *et al.*, 1985). A similar photochemistry (if judged from absorption difference spectra) has now been observed under a variety of mildly denaturing conditions. Its magnitude (defined by the ratio of the amplitude of the difference spectrum, to the maximum absorption) can be as high as 60% (in the presence of 20% mercaptoethanol, Schmidt *et al.*, 1988). From comparison with the photoreactions of phycocyanin in which the native 15Z-configured (Schirmer *et al.*, 1987) chromophores have been partially converted to the 15E-isomers (Schmidt *et al.*, 1988), it is most likely due to a Z/E interconversion of the chromophore(s) at the C-15,16 double-bond. Under such condi-

Figure 1: Difference spectrum of phycoerythrocyanin (pre-irradiated with green light, 500 nm), and the same sample after orange irradiation (600 nm).

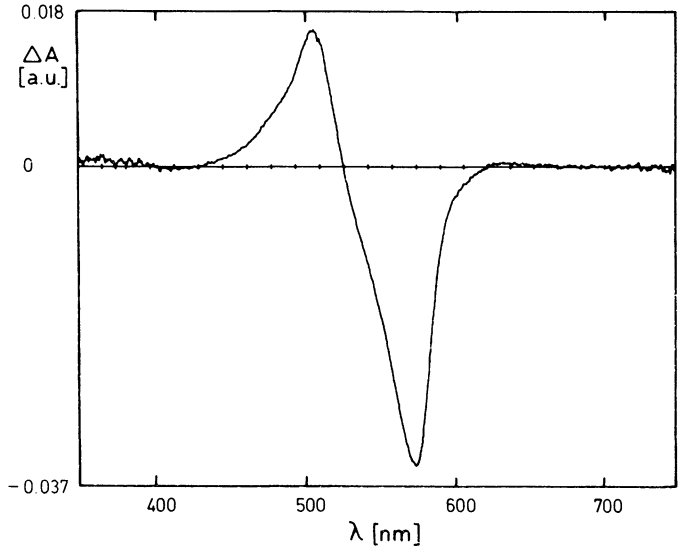

Table 1 : Absorption difference extrema and amplitudes of different PEC samples or samples subjected to different pre-treatments.

Sample	C_{urea} [M]	pH	Difference extrema [nm] orange	green	ΔΔA [%]
PEC	0	7.0	570	503	18
PEC	1	7.1	569	503	32
PEC	4	7.3	567	502	35
PEC	6	7.5	600	510	12
PEC	8	7.0	599	515	6
PEC	8	3.0	598	507	13
PEC-monomer	c)	7.0	565	504	36
α-Subunit	0	7.0	569	504	50
phycobilisome	d)	7.0	567	502	0.31
phycobilisome, diss	e)	7.0	567	504	2.82

a) Samples in potassium phosphate buffer (100 mM, pH = 7), if not indicated otherwise.
b) ΔΔA is the amplitude of the s-shaped difference spectrum from minimum to maximum, divided by the maximum absorbance of the sample (see Fig.1).
c) 1 M KSCN, no urea.
d) Coupled phycobilisomes, in phosphate buffer (0.9 M).
e) Dissociated phycobilisomes, in distilled water.

tions, the chromophores are apparently capable to perform the same type of photochemistry as that of phytochrome, but the subsequent changes -if there are any- must of course be different (as are the proteins).

3.3.2. Phycoerythrocyanin

Phycocyanin shows photochemistry only under partially denatured conditions and is photochemically "silent" in the native as well as in the fully denatured state. A closely related pigment, phycoerythrocyanin (PEC), shows a pronounced photochemistry under all conditions from fully native (Fig. 1) to fully denatured (Siebzehnrübl et al., 1989). There is nonetheless a pronounced effect of aggregation on the amplitude of the photochemical difference spectrum. It is very small in phycobilisomes, increases upon dissociation to trimers and further to monomers, and then decreases again in denatured PEC. Thus, the basic pattern is similar in PC and PEC, but the amplitudes of the difference spectra are always much larger in PEC (Table 1) than in PC (John *et al.*, 1985).

The α-subunit of PEC had been linked previously to photochromic activities in cyanobacterial extracts, and possibly to photomorphogenesis (Björn and Björn, 1980; Kufer, 1988). This pigment, which is structurally very similar to PC (Bryant, 1982; Dürring and Huber, 1989) carries a rare phycoviolobilin chromophore (alternatively also called PXB or cryptoviolin) at cystein α-84 (Bishop *et al.*, 1987), which replaces the common phycocyanobilin chromophore present at the same location in PC. Being a component of the phycobilisome, it is commonly regarded a light-harvesting pigment. A distinct difference from other phycobiliproteins, is however its pronounced photochemistry in the native state. It involves probably a Z/E-isomerization at the C-15 methine bridge similar to the photoreaction of the chromphore in phytochrome (Thümmler *et al.*, 1983), but this still has to be demonstrated. This reaction would require a decreased rigidity in the environment of α-84, which (contrary to preliminary indications) is not obvious in the crystal structure of (trimeric) PEC (Dürring and Huber, 1989).

3.4. Effect of chromophore α-84 photochemistry in PEC on aggregation

During photochemical studies with PEC, we noticed that it shows not only increased photochemistry upon disaggregation, but that there is also a reciprocal dependence of biliprotein aggregation on photochemistry. When PEC is alternately irradiated with orange (600nm) and green light (500nm), the α-subunit becomes enriched in one (15E) and the other form (15Z), respectively. Ultracentrifugation analysis showed, that at the same time there occurs a photoreversible change in aggregation: The amount of trimer increased each time the last irradiation was performed with green light, and decreased each time it was

performed with orange light. This means, that the configuration of α-84 influences aggregation, a fact which can be rationalized from the x-ray structure: α-84 is located very close to the contact surface of monomers in trimers (Dürring and Huber, 1989).

4. Discussion

In many cyanobacteria, profound alterations in phycobilisome structure (and other cyanobacterial activities) can be induced by changes in the environmental light conditions. The photomorphogenetic receptors (adaptachromes, photomophochromes) are hitherto unknown, but action spectra suggest that they also belong to the biliproteins (Björn and Björn, 1980; Scheer, 1982). Pigment(s) of the biliprotein type, albeit with very different apoproteins, are functional in higher plants for the same purpose. The green-plant photomorphogenetic reaction center pigment, phytochrome, has a chromophore which is very similar to the ones of the light harvesting pigment, phycocyanin (Rüdiger and Scheer, 1983). This multiple function of bile pigment chromophores is an aspect of biliproteins which is still poorly understood. The first event in photomorphogenetic pigments, is generally believed to be a structural change of the chromophore(s). This signal can then be propagated by induced structural changes of the protein, and beyond. In phytochrome, the primary reaction has been shown to involve a photoreversible Z/E-isomerization and possibly a protonation/deprotonation of the chromophore, while the induced changes on the protein are less understood (Rüdiger and Scheer, 1983; Song, 1988; Thümmler *et al.*, 1983).

The multiple function of biliproteins containing very similar chromophores, is reminescent of the multiple functions performed by chlorophylls of the same molecular structure in photosynthesis. In both cases, the photochemical and photophysical properties of the chromophores are diverse. The relative contributions of different de-excitation pathways must be regulated by specific interactions between the chromophores and the proteins. In analogy to interactions among living systems, these interactions can be termed as "molecular ecology".

5. SUMMARY

The results obtained by different modifications of the chromophores or their immediate vicinity, demonstrate an involvment of the biliprotein chromophores not only in energy transfer and photochemistry of biliproteins, but also in their quaternary structure. This effect may well be at the origin of a signal chain leading eventually to photomorphogenesis. In a more general context, it is an example for the intricate interplay of proteins with their cofactors, which leads to the stunning variety of properties of pigments with the same or very similar molecular structures.

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