Intermediates of reversible photochemistry of phycoerythrocyanin α-subunit from *Mastigocladus laminosus* probed by low temperature absorption and circular dichroism spectroscopy

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ABSTRACT. The reversible photochemistry of the α-subunit of phycoerythrocyanin (α-PEC) has been measured by low temperature absorption and circular dichroism in the range of 125 K to 295 K. Below 185 K, the photochemistry is nearly silent; above 205 K, the photochemistry increases gradually without an indication of intermediates, and between 185 to 205 K spectral changes in absorption and circular dichroism indicate an intermediate and/or changes in the interaction(s) between the chromophore and its environment.

1. INTRODUCTION

Phycobiliproteins are a class of brilliantly colored, intensely fluorescent chromoproteins, which consist of apoproteins and covalently bound linear tetrapyrroles (phycobilins). In cyanobacteria and red algae, the phycobiliproteins are arranged in highly ordered complexes, the phycobilisomes (PBsome). They absorb light and transfer the excitation efficiently to the photosynthetic reaction centers, predominantly of photosystem II (Gantt, 1986; Glazer, 1985; MacColl and Guard-Friar, 1987; Wehrmeyer, 1983). Phycoerythrocyanin (PEC) is an integral component of the PBsome antenna in several species of cyanobacteria (Bryant, 1982). Unlike other phycobiliproteins, however, isolated PEC shows a remarkable photochemistry (Bjoern, 1979; Bjoern and Bjoern, 1980; Hong *et al.,* 1993; Kufer and Bjoern, 1989; Siebzehnrübl *et al.,* 1989; Zhao and Scheer, 1995). PEC is photoreversibly photochromic, *i.e.*, it undergoes a phototransformation, which is photochemically reversible. This reactivity has been related to the unusual phycoviolobilin-chromophore (PVB) (This chromophore is also referred to as PXB (Bishop *et al.,* 1987)) bound to cys-84 on the α-subunit *via* a thioether linkage (Füglistaller *et al.,* 1983; Bishop *et al.,* 1987; Zhao *et al.,* 1995). The photochemistry of PEC and its α-subunit (α-PEC) is quite variable. There are two distinct types of photoreactions, type I (Siebzehnrübl, 1990) and type II (Hong *et al.,* 1993; Zhao and Scheer, 1995). Recent studies gave increasing evidence for a basic similarity of this type I photochemistry with that of the plant sensory photoreceptor, phytochrome. Both involve the 15Z/E-isomerization of the bilin chromophore (Zhao *et al.,* 1995), and the optical properties (absorption, fluorescence, circular dichroism) are largely comparable (Maruthi Sai *et al.,* 1993). PEC and other biliproteins have also been implicated in photomorphogenesis of cyanobacteria (Kufer and Bjoern, 1989; Maruthi-Sai *et al.,* 1992, Braune *et al.,* 1988), but direct proof for this is still lacking.

The molecular basis of the photochemistry of α-PEC has been identified as a 15Z/E isomerization of PVB (Zhao *et al.,* 1995). Generally, the initial Z/E isomerization of chromophores in photoreceptors like rhodopsin and phytochrome is followed by series of intermediates (Schaffner *et al.,* 1990; Rüdiger and Thümmler, 1991). This prompted us to search for intermediates in the PEC photoconversion by low temperature spectroscopy.

2. MATERIALS AND METHODS

Mastigocladus laminosus was cultivated in Castenholz (1970) medium in 300 l batches at the Gesellschaft für biotechnologische Forschung, Stöckheim (Germany) and stored at $-20\,^{\circ}$C.

PEC was prepared by chromatography on DEAE cellulose, similar to the method of Füglistaller *et al.,* (1981). α-PEC of type I photochemistry (α-PEC$_{\mathrm{i}}$, see next par. for the definition of type I and type II photochemistries) was isolated from PEC by isoelectric focusing (Köst-Reyes *et. al.,* 1987; Schmidt *et al.,* 1988), and subsequent treatment with mercaptoethanol (Zhao *et al.,* 1993). α-PEC of type II photochemistry (α-PEC$_{\mathrm{II}}$) was obtained by modification of the latter with PCMS (Zhao and Scheer, 1995).

Photochemistry was initiated by irradiation with a cold light source (Lumilux 150 W, Volpi, Denzlingen, Germany) equipped with suitable interference filters (10 nm fwhm). Each irradiation lasted 3 minutes. The irradiation light was from the top by an optical fiber. The reversible photochemistry of PEC was characterized by difference absorption spectra, and quantitated by the $\Delta\Delta A_{\mathrm{I}}$ and $\Delta\Delta A_{\mathrm{II}}$ values for type I and II reactions, respectively (Siebzehnrübl *et al.,* 1989). $\Delta\Delta A_{\mathrm{I}}$ and $\Delta\Delta A_{\mathrm{II}}$ were defined as before (Hong *et al.,* 1993). Briefly, the amplitudes of the light-induced difference signals at 505 and 565 nm (type I) and at 565 and 595 nm (Type II) were normalized to the maximum absorbance of the 500 nm irradiation-saturating form. All $\Delta\Delta A_{\mathrm{I}}$-values are given in %.

Absorption and circular dichroism (cd) spectra were simultaneously measured on a Dichrograph VI (ISA). The spectral bandwidth was 0.25 nm, the scan speed 5 nm/s. For these measurements, very narrow slit widths and rapid scan speed were utillized to minimize isomerization of the samples during the measurement. Low temperature was realized by the ISA accessory cryostat, combined with a temperature-controlling device. The sample was placed in a brass cuvette with quartz windows (optical path length 10 mm).

3. RESULTS

Dependence of α-PEC photochemistry on temperature. α-PEC_I and α-PEC_{II} were irradiated at temperatures of 125 to 295 K, then warmed to ambient temperature in the dark, and checked for reversible photochemistry (Table 1). The amplitudes of both type I and II reversible photochemistries decrease continously upon reducing the irradiation temperature. At ≈ 185 K, the amplitude of the difference absorption spectrum, ΔΔA, is reduced to only about 5%, and it is no longer detectable below, this should be compared to the value of 127% at ambient temperature. This trend is supported by the corresponding cd difference spectra: no apparent photochemistry took place below 185 K, and above this threshold, its amplitude increased with increasing temperature.

Table 1. *Dependence of reversible photochemistry of α-PEC on temperature.*
Sample in 100 mM KPP buffer (pH 7.0) containing 66% (v/v) glycerol. Reversible photochemistry started from P570 at various temperatures.

Sample	Temperature (K)	$\Delta\Delta A_I$ (%)	$\Delta\Delta A_{II}$ (%)
α-PEC_I	288	104*	-§
	288	76	–
	263	69	–
	243	51	–
	223	38	–
	203	16	–
	183	5	–
α-PEC_{II}	288	11	11
	273	11	10
	243	9	8
	223	7	6
	203	4	–
	183	0	–

∗ α-PEC_I in 100 mM KPP buffer (pH 7.0).
§ A'-' means that there is no 600 nm extremum representing type II photochemistry.

The low temperature spectra were recorded in 100 mM KPP buffer (pH 7.0) containing 66% (v/v) glycerol. In this medium and at ambient temperature, the reversible photochemistries of α-PEC_i and α-PEC_{II}, are qualitatively similar in this medium to those in pure KPP buffer. For example, α-PEC_i absorbed maximally at 505 nm after saturating irradiation with 570 nm light in the glycerol buffer, and at 570 nm after saturating irradiation with 500 nm light. As before (Zhao and Scheer, 1995), these forms of α-PEC are called P505 and P570, respectively. However, the amplitudes of the difference signals were reduced in both cases (Table1 and Figure1). The mixture of 100 mM KPP buffer with glycerol is more viscous than pure KPP buffer. In the more viscous solvent, the apoprotein of α-PEC moves more difficultly, which could explain the reduced photochemistry. This corresponds to decrease of the photochemistry of α-PEC with decreasing temperature; again the increasing viscosity reduces the capabilty of the α-PEC apoprotein to move.

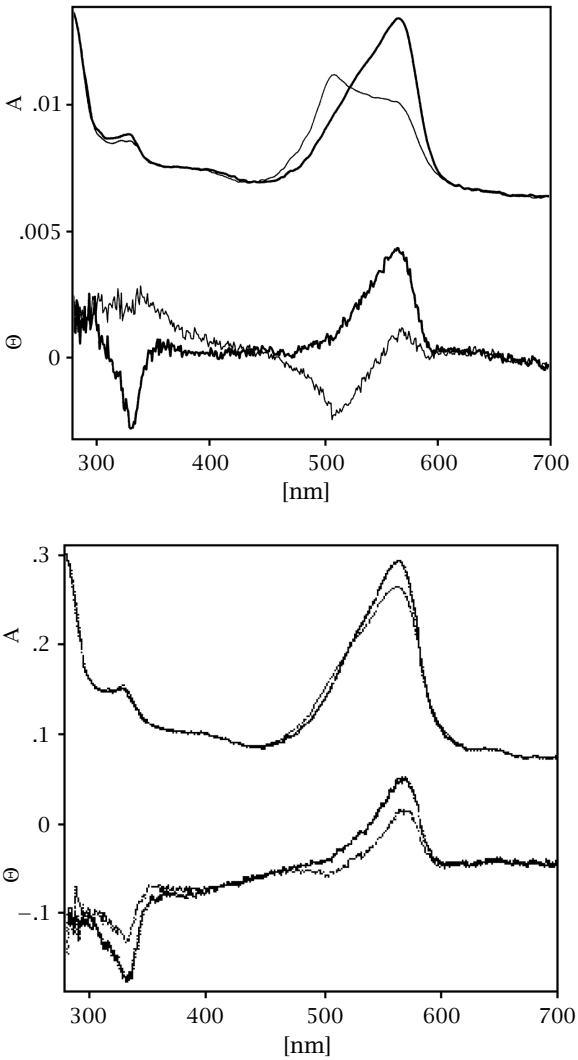

Figure 1. *Absorption (upper) and cd (lower) spectra of* α-PEC_I *(A) and* α-PEC_{II} *(B) in 100 mM KPP buffer (pH 7.0) containing 66% glycerol (v/v), measured at 295 K. Thick curves correspond to spectra of P570, and thin ones to P505.*

Intermediates during Z → E ***photoconversion.*** In order to search for intermediates, the samples were irradiated and the difference spectra (after irradiation *minus* before irradiation) taken at the same temperature. Because of the inhibition of α-PEC photochemistry below

185 K, all intermediate signals are small. Furthermore, the light intensity during measurement in the dichrograph had to be kept low in order to avoid any further transformation during measurement. The absorption and circular dichroism spectra of α-PEC measured under these conditions in a mixture of KPP buffer and glycerol, depends on the temperature.

No difference signal was detectable after irradiating P570 with 570 nm light below 185 K, indicating that any photochemistry ceases at this temperature 185 K. Above 205 K, the same type of signal was recorded as at ambient temperature, but with smaller amplitude. Obviously, the full photochemical conversion takes place above this threshold limit, albeit to a different degree, and no intermediate is stabilized. In the intervening temperature range around 195 K, an intermediate signal, could be recorded both in absorption and in cd (Figure 2). It had the same spectral signature irrespective of using α-PEC_I or α-PEC_{II} and was termed I_{570} according to its absorption difference maximum. Interestingly, this absorption difference spectrum is similar to that at ambient temperature, and only the cd difference deviates from that at room temperature. We therefore assign the signal to a Z → E photoisomerization, but with the chromophore stuck in an intermediate state of conformation and/or of interactions with the environment.

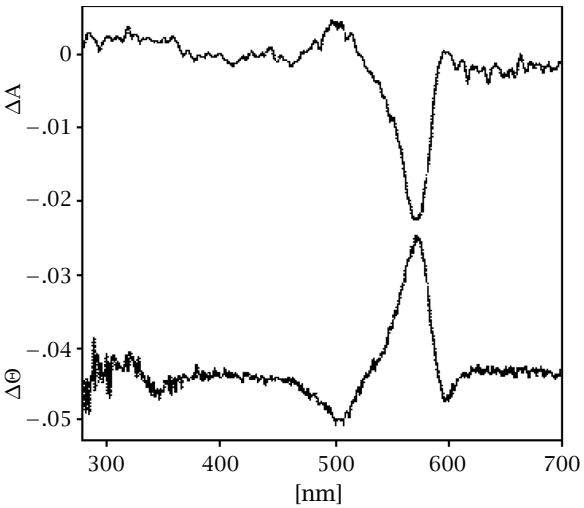

Figure 2. *Difference absorption (upper) and cd (lower) spectra of* α-PEC_I *in 100 mM KPP buffer (pH 7.0) containing 66% glycerol (v/v), after irradiating P570 with 570 nm light at 195 K, which characterizes* I_{570} *from* Z → E ($\Delta\Delta A_I$ = 10%). α-PEC_{II} *has an very similar spectra, but of smaller magnitude* ($\Delta\Delta A_{II}$ = 2%, *spectra not shown).*

Intermediates during E → Z ***photoconversion.*** The E → Z photo-conversion was started by irradiation of the P505 form of α-PEC_I or the P565 form of α-PEC_{II} with 500 nm light, again at various temperature. As for the Z → E reaction, the net photochemistry ceased below 185 K, where no difference signal could be detected after warming the sample back to ambient temperature. Also, the same absorption and cd signal difference signals were seen above 205 K as at ambient temperature, but with smaller magnitudes. Both the forward and reverse reactions therefore show the same temperature dependencies.

However, an intermediate signal was observed with α-PEC_I at around 165 K by 500 nm irradiation (Figure 3). This intermediate state absorbed maximally at *ca.* 535 nm, and it was therefore termed I_{535}. For α – PEC_{II}, no such intermediate signal was detected. From the results presented above, and from the relaxed difference spectrum in Figure 3, it is clear that I_{535} can not convert eventually to P570 upon warming, but instead reverts to the original state and hence no net E → Z photo-conversion takes place at 165 K.

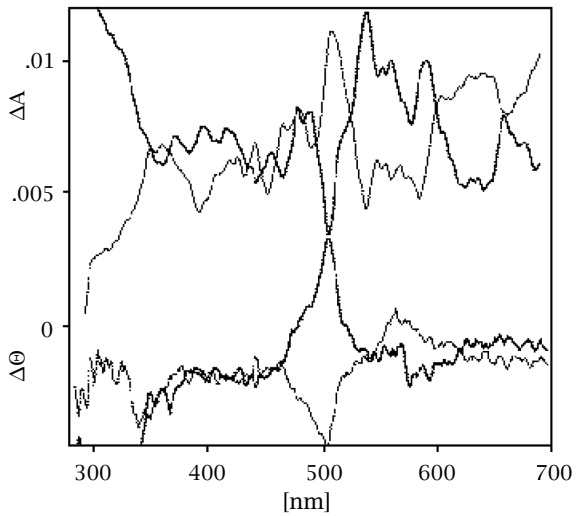

Figure 3. *Difference absorption (upper) and cd (lower) spectra* α-PEC_I *in 100 mM KPP buffer (pH 7.0) containing 66% glycerol (v/v), after irradiating P505 with 500 nm light at 165 K, which characterizes* I_{535} *resulting from* E → Z *photoconversion. For* α-PEC_{II}, *no such signal was observed. The thin curves give the dark relaxed difference spectra of* I_{535} *at 165 K after 12 min.*

Yet another intermediate was found for α-PEC_I at ≈ 195 K, which according to its difference maximum at 550 nm (Figure 4A) was termed I_{550}. After rapidly thawing the sample to ambient temperature, $\Delta\Delta A$ is 10%. Taking the maximum $\Delta\Delta A$ of 127% (Zhao and Scheer, 1995), this corresponds to a conversion of only 8%.

α-PEC_I and α-PEC_{II} differ by the state of cys-99 and/or cys-100 of the apoprotein (Zhao and Scheer, 1995). Low temperature spectra are sensitive to this change in the state of the apoprotein. For α-PEC_{II}, an intermediate was again seen at ≈ 195 K (Figure 4B), which has an absorption difference maximum at 600 nm. The 600 nm signal relaxed in the dark, to produce the normal type II difference spectrum, which always contains a 600 nm component (Hong *et al.,* 1993; Zhao and Scheer, 1995). This may relate to a state which is accessible at low temperature, and still remains partly populated at ambient temperature.

4. DISCUSSION

The molecular basis of α-PEC reversible photochemistry is a 15Z/E isomerization of the phycoviolobilin chromophore bound to cys-α84, which is similar to the isomerization of the phytochromobilin chromophore of phytochrome (Thümmler *et al.,* 1983; Zhao *et al.,* 1995). If judged by a comparison of the spectra of P_r and phycobiliproteins with their chromophores in 15Z-configuration, in this state the interactions between chromophore and apoprotein appear very similar.

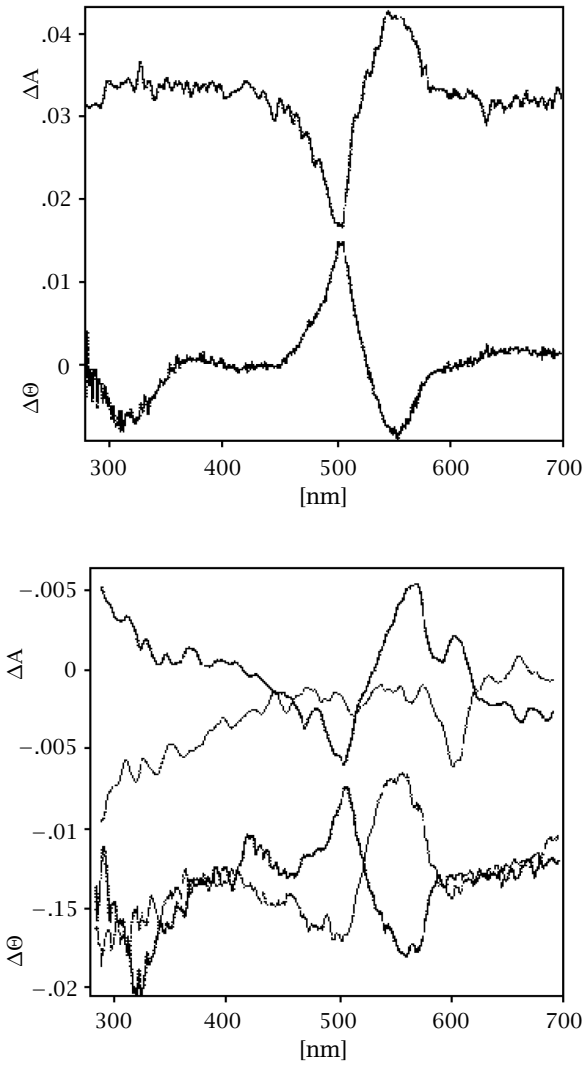

Figure 4. *Difference absorption (upper) and cd (lower) spectra of* α-PEC_I *(A), and* α-PEC_{II} *(B, obtained by dark modification of P505* α-PEC_{II} *with PCMS) in 100 mM KPP buffer (pH 7.0) containing 66% glycerol (v/v), after irradiating P505 with 500 nm light at 195 K, which characterizes* I_{550} *for* α-PEC_I*, and* I_{565} *for* α-PEC_{II} *both resulting from* E → Z *photoconversion. The thin curves are dark relaxed difference spectra of* I_{565} *at 195 K after 6 min.*

However, the 15E-isomer is blue-shifted in the former, and red-shifted in the latter, as compared to the respective Z-isomers. Since the blue shift is typical for E-isomer in free bilins, the red shift in phytochrome has been explained by more extensive pigment-protein interactions of unknown origin in P_{fr}. The low temperature photochemistry also reveals considerable differences between these two pigments.

In this work, one intermediate state, I_{570} was detected for Z → E interconversion of α-PEC. The E → Z, back reaction is more complex, with two states I_{535} and I_{550} for α-PEC_I, and one intermediate I_{565} for α-PEC_{II}. In all intermediates except one, the isomerization of the 15,16-double bond is complete if judged from the completion of the interconversion upon warming. The exception is I_{535}, which can not represent a complete Z/E isomerization, because it reverts back to the starting state after warming (Figure 3). It possibly represents a strongly twisted conformation of the 15,16-bond, intermediate between the Z- and E-states, but with a lower activation barrier in the backward than in the forward direction. This relatively simple photochemistry contrast from the considerably more complex one of phytochrome. The latter shows several intermediates in both directions, which is indicative of a more complex interaction with the apoprotein (see *e.g.*, Schaffner *et al.,* 1990; Rüdiger and Thümmler, 1991). This difference in interaction with the protein is also supported by vibrational spectroscopy of PEC (Kneip *et al.,* 1998).

A lesser interaction with the protein, and a stronger influence of the solvent environment, is also indicated by the fact that the photochemistry of α-PEC decreases with increasing solvent viscosity and decreasing temperature, freezing out at $\approx$ 185 K (see results). It is obvious from X-ray diffraction data of trimeric PEC that there is no sufficient space for a Z/E isomerization of PVB without some movement of the protein (Dürring *et al.,* 1990). The chromophore-pocket is expected to be more open in α-PEC, which may add to the strong influence of the solvent. By contrast, photochemistry of phytochrome hardly depends on solvent viscosity (Ruzsicska *et al.,* 1985), but rather appears to depend more on internal modes of the protein. It already shows changes at 205 K (Kendrick and Spruit, 1977), but even below 175 K, phytochrome can be photo-driven effectively (> 50%) to the first intermediate (Eilfeld and Rüdiger, 1985), which has probably already undergone Z/E isomerization (Eilfeld and Eilfeld, 1988). In phytochrome, the phytochromobilin chromophore is probably located in an apoprotein pocket (Rüdiger and Thümmler, 1991) and the reversible photochemistry of the chromophore is mainly assisted by near-by groups of apoprotein (Schaffner *et al.,* 1990).

In summary, these data support that PEC is intermediate in its chromophore-protein interactions between light-harvesting biliproteins like phycocyanin, and the phytochromes, and that it can therefore serve as a useful link in understanding the different photochemical and photophysical behavior of these two pigment classes.

Abbreviations. cd = circular dichroism,
KPP = potassium phosphate buffer,
M. = *Mastigocladus*,

PBsome = phycobilisome,
PCMS = *p*-chloromercury-benzene-sulfonate,
PEC = phycoerythrocyanin,
α-PEC= α-subunit of phycoerythrocyanin,
α-PEC$_I$ and α-PEC$_{II}$ denote two interconvertible forms of α-PEC characterized by type I and type II photochemistry, respectively (see Hong *et al.,* 1993 and Materials and Methods section for definition),
PVB = Phycoviolobilin (this chromophore is alternatively also termed PXB in the literature, (This chromophore is also referred to as PXB (Bishop *et al.*, 1987).))

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