Z,E isomerization of the α-84 phycoviolobilin chromophore of phycoerythrocyanin from *Mastigocladus laminosus* investigated by Fourier-transform infrared difference spectroscopy

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Abstract The photoreaction of the phycoviolobilin (PVB) chromophore-containing part of phycoerythrocyanin (PEC) from *Mastigocladus laminosus* was investigated by Fourier-transform infrared spectroscopy (FT-IR). Difference spectra between the parent states P566 and P507 were obtained in ^1H_2O and ^2H_2O for the first time. The spectra are generally characterised by large changes in the range between 1710 and 1590 cm^{-1} and by a strong difference band around 1270 cm^{-1}. In order to study the influence on the PVB chromophore upon aggregation, spectra of the α-subunit and the $(\alpha\beta)_3$ trimer are compared, showing distinct differences which may be of relevance for the chromophore-protein and protein-protein interactions. The difference spectra demonstrate many similarities to the spectra recently obtained for the $P_r \rightarrow$ meta-R_c transition of phytochrome [Foerstendorf et al. (1996) Biochemistry 35, 10793–10799]. In particular, a band around 1710 cm^{-1}, which was tentatively assigned to the C = O stretch of ring D is also observed in the spectra of PEC. It strongly supports this identification and the deduced molecular interpretation on the protonation state of the chromophore.

Key words: FT-IR spectroscopy; Photoreaction; Phycobiliprotein; Phycoerythrocyanin; Phycoviolobilin; Phytochrome

1. Introduction

Phycoerythrocyanin (PEC) is a protein in the light-harvesting complex (phycobilisome) of several cyanobacteria [1]. Each 'monomer' (i.e. (αβ)-heterodimer) contains one phycoviolobilin (PVB) chromophore at position α-84 (Fig. 1a) and two phycocyanobilin chromophores (PCB) at positions β-84 and β-155 (Fig. 1b). The chromophores are covalently linked via a thioether bond to cysteine residues. All phycobiliproteins absorb light and transfer the excitation energy with high quantum efficiency to the photosynthetic reaction centre. In contrast to all other phycobiliproteins, the α-chromophore of PEC can undergo in its native state a reversible photoreaction. The visible absorption maximum is shifted from ≈566 to ≈507 nm during the photoconversion, and the corresponding stable states are therefore referred to as P566 and P507, respectively [2]. Recently, a second type of photoreaction of the α-subunit was found which only shows line broadening of the absorption band around 560 nm [3]. The switch between the two types of reactions is supposed to be controlled by the oxidation state of cysteine residues CysA-98 and CysA-99 [2]. Optical spectroscopy and more directly NMR experiments show that in both types of photoreaction the α-chromophore undergoes a *Z,E* isomerization of the $C_{15}=C_{16}$ double bond between rings C and D [2,4]. Although the exact physiological role of the photoreaction is not known, it has been speculated that PEC can act as a photoreceptor similarly as phytochrome [5]. More specifically, there is evidence that photochemistry and the aggregation state of PEC are linked to each other [6]. From this behaviour one can deduce that the photoreaction must be accompanied by conformational changes of the protein and their deduction appears to be extremely interesting. Because of similarities between the chromophores of the PEC α-subunit and of phytochrome, the former can be used with respect to the chromophore and its interactions with the protein as a model system for the latter.

Vibrational spectroscopy has been successfully applied to the study of several biliproteins and model chromophores [7–19]. However, due to the lack of either precise structural data on the chromophore and/or spectral data including those on the influence of isotopic labels, the interpretation of the spectra in terms of structure and molecular interactions is not unequivocally possible as yet. The antenna pigments PC and PEC offer the advantages that the structure of the chromophore is known from X-ray crystallographic analysis [20,21] and that resonance Raman (or preresonance FT-Raman) [22] spectra can be obtained. PEC, due to its photoreaction, offers the additional possibility of studies with infrared difference spectroscopy enabling further information on vibrational spectra.

Both the interest in the structural changes occurring during the photoreaction and the possibility of using it as a well-characterised model system for photoactive biliproteins in general prompted us to study PEC with infrared difference spectroscopy. In this work we provide the Fourier-transform infrared (FT-IR) difference spectra between the P566 form (15*Z* conformation) and the P507 form (15*E* conformation) of the α-subunit and the $(\alpha\beta)_3$ trimer of PEC in ^1H_2O and ^2H_2O, respectively.

2. Materials and methods

2.1. Sample preparation

PEC from *Mastigocladus laminosus* was isolated as described earlier [23]. Subunits were separated according to Köst-Reyes et al. [24]. All preparations were further purified by chromatography [25]. For the infrared measurements the lyophilised samples were dissolved in ^1H_2O containing 0.3 vol.% glycerol or ^2H_2O (0.3 vol.% d_3-glycerol). The solutions were deposited on a ZnSe infrared window and the water was evaporated under a gentle stream of nitrogen. The obtained homogeneous film was rehydrated by adding either 1–2 μl of ^1H_2O or

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^2H_2O (after 3-fold ^2H_2O exchange for the latter) into the infrared cuvette which was sealed by another ZnSe window. Glycerol retained photoreversibility after rehydration of the film. The amount of PEC of a infrared probe was about 80 and 120 μg for measurements in ^1H_2O and ^2H_2O, respectively.

2.2. FT-IR measurements

The spectra were measured using a Bruker IFS 28 Fourier-transform infrared spectrophotometer equipped with a MCT detector. All spectra were measured at 0°C which was achieved by a water-cooled cuvette holder.

For the conversions band-pass filters with peak transmissions at 596 nm (illumination of the P566 form) and 498 nm (illumination of the P507 state) and a half-width of 9 nm were used. Irradiation time was 4 min which was sufficient to saturate the difference signals. A slide projector (150 W lamp) and fiber optics in the spectrometer were used.

Parallel to the infrared studies, difference spectra were recorded in the UV-Vis region serving as a control for undistorted photoreaction. As our results are in good agreement with published spectra shown in [2] we can assume that the photoconversion of the protein is not seriously affected by the state of the sample necessary for FT-IR difference spectroscopy (data not shown). In the rare cases where reduced photoreactivity was observed, the samples were discarded.

Each FT-IR difference spectrum shown represents a sum of several (approx. 10–20) difference spectra which were obtained from single-beam spectra recorded before and after illumination. For each single-beam spectrum, 256 scans were accumulated with a resolution of 4 cm^{-1}. The samples are characterised by an absorbance at 1650 cm^{-1} (amide I and water) ranging between 0.7 and 1 absorbance units. The noise level can be deduced from the baseline above 1750 cm^{-1}, where no bands could be identified. Approximate absorbance scale in the difference spectra is given in the respective figure legends.

3. Results and discussion

The light-induced FT-IR difference spectra of the α-subunit (α-PEC) and of the trimer ($(\alpha\beta)_3$-PEC) are shown in Fig. 2a and b, respectively. The corresponding spectra obtained for in ^2H_2O are shown in Fig. 3a and b. Starting from P566, positive bands are caused by the P507 form, negative ones by the P566 form.

The spectra for measurements in ^1H_2O are generally characterised by large changes in the region between 1710 and 1590 cm^{-1}. From our previous investigation of tetrapyrrolic model compounds, bands between 1610 and 1650 cm^{-1} have been assigned to the C=C stretching mode of the methine groups of the chromophore [9]. The spectral range between

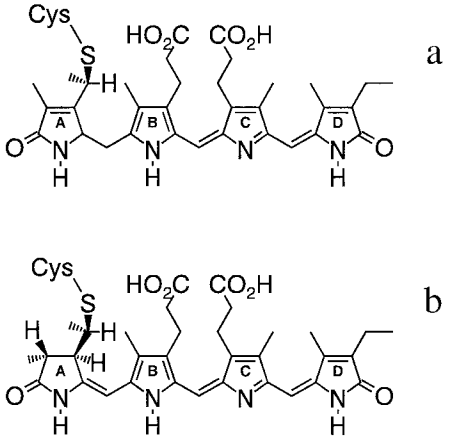

Fig. 1. Chromophores of phycoerythrocyanin (PEC) from *Mastigocladus laminosus*. Numbering of the pyrrole rings from left to right (A–D). (a) Phycoviolobilin (PVB) chromophore of the α-subunit (α-84). (b) Phycocyanobilin (PCB) chromophore of the β-subunit (β-84, β-155).

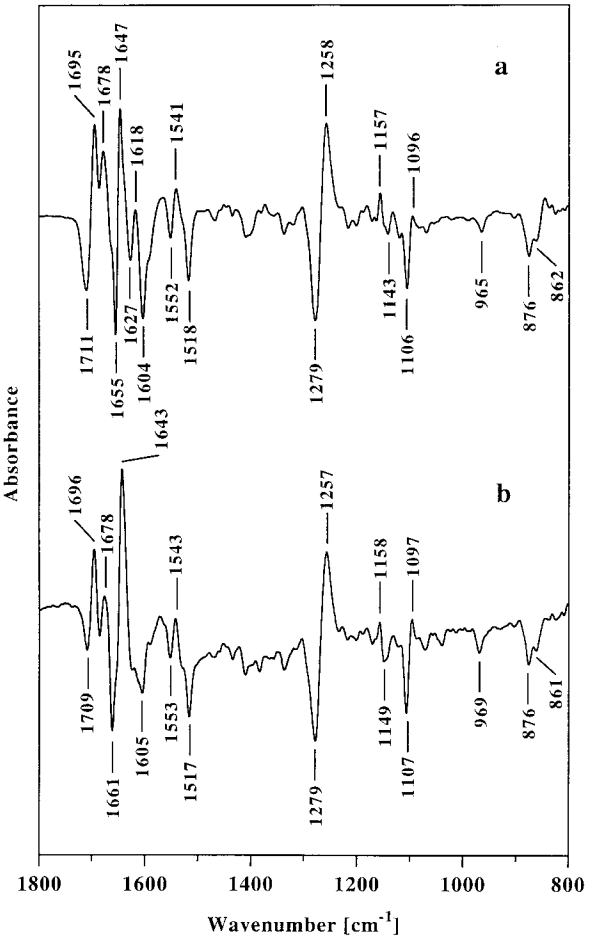

Fig. 2. Light-induced FT-IR difference spectra of the P566→P507 interconversion of PEC in a medium of ^1H_2O (T=0°C). (a) Spectrum of the α-subunit. (b) Spectrum of the $(\alpha\beta)_3$ trimer. Positive bands represent the P507 form, negative bands P566. The band intensities can be estimated from the difference band around 1270 cm^{-1} in Fig. 3a which is about 0.0007 absorbance units. In order to correct in the other spectrum the intensities for different samples the spectra were normalized to this band.

1700 and 1620 cm^{-1} is also characteristic of amide I bands of the peptide backbone. Therefore, we are not able to distinguish unequivocally between bands representing either the protein or the chromophore. A superposition of chromophore and protein bands occurs also in the range of the amide II bands of the peptide backbone (1580–1480 cm^{-1}). Since they represent a coupled mode between the NH bending and C-N stretching vibrations, they are strongly red-shifted by deuteration of the NH groups. This can be observed in the spectra measured in ^2H_2O (Fig. 3) where almost no bands between 1590 and 1500 cm^{-1} are found. However, the shifted bands cannot be detected. Due to the uncoupling from the NH bending mode, the amide II vibration now mainly represents the C-N stretching mode which is less sensitive to conformation changes of the peptide backbone. In case of the amide I bands such a distinction is not possible, since both chromophore and protein bands undergo small shifts. All spectra show a significant band around 1270 cm^{-1}. This is the range were amide III bands are observed. However, since the intensity of this band is of the same magnitude as of those in the amide I range, this assignment can be excluded (amide III bands have considerably lower intensities). Contributions

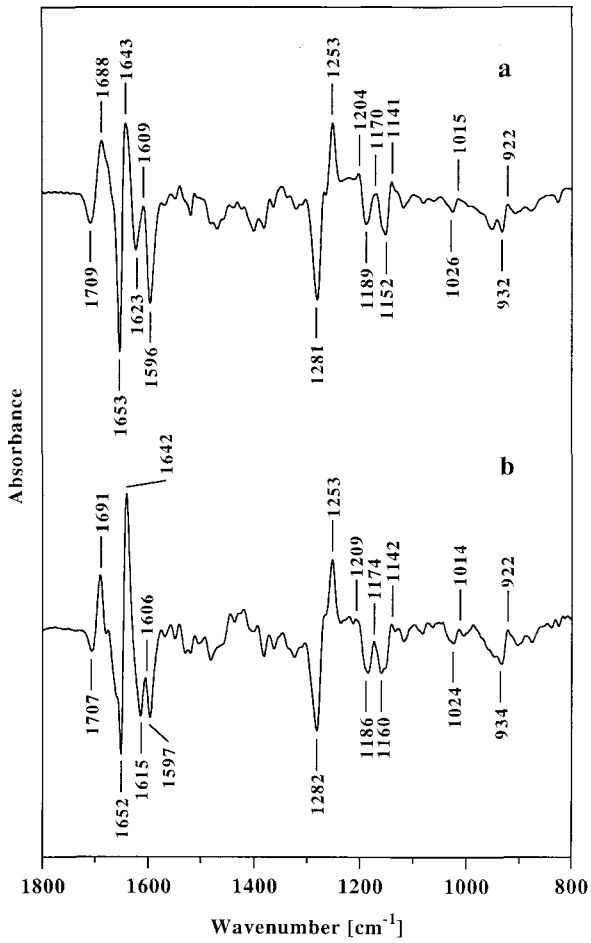

Fig. 3. Light-induced FT-IR difference spectra of the P566→P507 interconversion of PEC in a medium of ^2H_2O (T=0°C). (a) Spectrum of the α-subunit. (b) Spectrum of the $(\alpha\beta)_3$ trimer. Positive bands represent the P507 form, negative bands P566. The band intensities can be estimated from the difference band around 1270 cm^{-1} in Fig. 3a which is about 0.002 absorbance units. In order to correct in the other spectrum the intensities for different samples the spectra were normalized to this band.

from amino acid side chains are also to be unlikely. From FT-IR difference spectra of other photoreceptors we know that bands of such groups show much smaller intensities [26]. Therefore, this band must be assigned to the chromophore. Our studies of model compounds do not yet allow an assignment of this band to a distinct vibrational mode of the chromophore [9,12]. ^2H_2O causes a small upshift of the negative band and a downshift of the positive one. Since the NH group is the only part of the chromophore having exchangeable hydrogens, these opposite isotope effects may be due to a changed coupling behaviour with the NH mode which is again caused by structural alterations of the chromophore or its environment.

A comparison of the spectra between the α-subunit and trimer reveals small but distinct differences in intensities and positions of the bands. In the α-subunit, the negative band at 1655 cm^{-1} shows a weak shoulder around 1660 cm^{-1} (Fig. 2a). In contrast, in the spectrum of the $(\alpha\beta)_3$ trimer (Fig. 2b) the negative band is found at 1661 cm^{-1} with a shoulder around 1655 cm^{-1}. The positive part at 1647 cm^{-1} exhibits a downshift and intensity increase in the trimer. Another clear difference can be seen around 1610 cm^{-1}. In the spectra of the $(\alpha\beta)_3$ trimer a broad negative band is observed at 1605 cm^{-1} (^1H_2O) and 1597 cm^{-1} (^2H_2O). Additionally, in the case of the α-subunit a difference band at 1618(+)/1604(−) cm^{-1} is superimposed on this broad band. This is also true for the spectrum measured in ^2H_2O, where the band is now located at 1609(+)/1596(−) cm^{-1}. This spectral feature can probably assigned to the chromophore, since it is outside of the amide I/II spectral regions. Thus, the spectra indicate that the α-chromophore is influenced by aggregation. This is plausible since the three-dimensional structure of PEC trimer positions ring D in an aromatic pocket formed by a histidine residue of the α-subunit, and two residues of the β-subunit, i.e. a phenyalanine (PheB-76) and a histidine (HisB-78) [21]. This hydrophobic environment does not exist in the monomeric α-subunit. Influences of state of aggregation on biliprotein chromophore vibrations have also been detected by CARS and UV-resonance Raman spectroscopy [7,27].

All spectra show a difference band around 1700 cm^{-1} which is shifted down by ^2H_2O and is concomitantly reduced in intensity. It is also modified by the state of aggregation (spectra a vs. spectra b in Figs. 2 and 3). In this region the C=O stretching vibrations of carbonyls and protonated carboxyl groups show up. Based on investigations of model compounds and our recent studies of the intermediates of the photoreaction of phytochrome [9,18] we assign these bands to the carbonyl groups of the outer rings. Considering the *Z,E* isomerization around the $C_{15}=C_{16}$ double bond between rings C and D during the photoreaction, large changes of the environment of ring D of the chromophore are expected. Therefore, it seems reasonable to assign the band at 1700 cm^{-1} to the C=O group of ring D. The refined three-dimensional structures of PEC [21] and other phycobiliproteins [20] show as a common principle that the inner rings B and C of the chromophores arch around a central aspartate residue and that the nitrogen atoms of these rings are within hydrogen-bonding distance of one of the carboxylate oxygens, which is also interacting with ArgA-86 [21]. This strongly suggests that the chromophores are protonated, and that the carboxylate represents the required negative counterion. Our studies of model compounds have shown that the C=O stretching vibration of those carbonyl groups of the outer rings which are included in the conjugated π-electron system are located around 1690 cm^{-1} [9], i.e. too low for the position of 1710 cm^{-1}. Semiempirical force-field calculations of model compounds qualitatively indicate an upshift of this band upon protonation of the chromophore [28]. In the case of dihydrooctaethylbiliverdin and phycocyanobilin dimethyl ester we have directly demonstrated an upshift of about 10–20 cm^{-1} upon protonation (data not shown). Thus, we interpret the high position of 1710 cm^{-1} as a strong indication for a protonated α-chromophore in P566. The ^2H_2O dependence of this carbonyl band can be explained by coupling to the bending mode of the neighbouring NH group. The coupling will increase when the frequency of the NH group is closer to the frequency of the C=O group. It depends on the molecular environment as well as on the geometry of the chromophore. Upon $^1H/^2H$ exchange this coupling is removed. Therefore, the larger downshift observed for the P507 form indicates a larger coupling between these two functional groups in the 15*Z* chromophore. The position around 1695 cm^{-1} would be compatible with a deprotonated chromophore in P507. It should be noted that a downshift of the C=O stretching vibration of a protonated chromophore can also be caused

by hydrogen bonding to the carbonyl group. A discrimination between the two possibilities must await future experiments.

Only few bands are observed in the region below 1000 cm^{-1}. In the case of retinal proteins, this spectral range is characteristic of hydrogen-out-of-plane (HOOP) bending vibrations, in both resonance Raman and infrared difference spectra [29,30]. Such modes have also been detected in preresonance Raman spectra of phytochrome and have been interpreted in terms of local twists of the methine group(s) [17]. In the spectra of PEC mainly negative bands are observed in this region, which possibly indicate that the PVB chromophore exists in a more twisted conformation in the P566 form than in P507. However, it has to be emphasised that an unequivocal identification of these bands as HOOP bending vibrations is still lacking for tetrapyrrolic chromophores. However, after an assignment of these bands to specific normal modes they will be useful to resolve changes in geometry of the chromophore occurring upon photoisomerization.

The major spectroscopic difference between phytochrome and PEC is the red-shift of the absorption maximum upon $Z \rightarrow E$ isomerization of the phytochrome chromophore, which is also in contrast to 15*E* isomers of other bile pigments [31]. From our spectra of the photoreactions of phytochrome [18] and PEC (this work), we conclude that the transition from P566 to P507 in α-PEC, which was proved to be a *Z,E* isomerization of ring D of the chromophore [4], is in homology to the $P_r \rightarrow P_{fr}$ transition in phytochrome. Thus, P_r and P566 obviously represent the 15*Z* conformation whereas P_{fr} and P507 the 15*E* conformation of the respective tetrapyrrole chromophore. The 'unusual' behaviour of P_{fr} cannot be due to a simple deprotonation of the chromophore, since this would cause a blue-shift of the absorption maximum. One possible explanation for the larger red-shift of the absorption maximum of P_{fr} vs. P_r could be that it is caused by an altered environment resulting in a reduced shielding of the positive charge of the chromophore (e.g. altered interaction with the negatively charged counterion or with other residues).

4. Conclusions

We have shown that reliable FT-IR difference spectra can be obtained for the photoreaction of the PVB-chromophores of the α-subunit of PEC. Distinct bands around 1700, 1610, and 1270 cm^{-1} were assigned to chromophore vibrations. Similarly to other infrared investigations of biliproteins [8,13,17,18], significant and numerous bands were found in the protein amide I/II spectral region, where strong chromophore bands are also expected [9,12,28].

A comparison of the spectra of this study with the spectra of the late intermediate of the $P_r \rightarrow P_{fr}$ pathway, meta-R_c, of phytochrome [18] shows surprising similarities. In particular, bands at 1707(−)/1691(+)cm^{-1}, 1555(−)/1543(+)cm^{-1}, around 1515 cm^{-1}, and 970 cm^{-1} are observed in the spectra of PEC and phytochrome. Upon $^1H/^2H$ exchange the bands around 1700 and 1610 cm^{-1} are downshifted while the bands around 1550 cm^{-1} and 970 cm^{-1} show considerably decreased intensities in both biliproteins. This strongly corroborates that these bands represent modes of the chromophores since they show similar behaviour in very different proteins. The appearance in the spectra of PEC of a strong difference band around 1700 cm^{-1} assigned to the C=O stretching vibration of ring D strongly supports our previous conclusions on the protonation state of the chromophore in phytochrome for which a similar band has been observed [9,18]. The observation of such carbonyl vibrations of the chromophore, which are difficult to detect in resonance or preresonance Raman investigations, will be helpful for a molecular description of the events occurring upon photoisomerization.

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