Studies on a bacterial photosensor
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Chapter 4
Structural events associated with the photocycle of a xanthopsin


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Evidence for trans-cis isomerization of the p-coumaric acid chromophore as the photochemical basis of the photocycle of photoactive yellow protein

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Abstract. Analysis of the chromophore p-coumaric acid, extracted from the ground state and the long-lived blue-shifted photocycle intermediate of photoactive yellow protein, shows that the chromophore is reversibly converted from the trans to the cis configuration, while progressing through the photocycle. The detection of the trans and cis isomers was carried out by high performance capillary zone electrophoresis and further substantiated by \textsuperscript{1}H NMR spectroscopy. The data presented here establish the photo-isomerization of the vinyl double bond in the chromophore as the photochemical basis for the photocycle of photoactive yellow protein, a eubacterial photosensory protein. A similar isomerization process occurs in the structurally very different sensory rhodopsins, offering an explanation for the similar isomerization process occurs in the structurally very different sensory rhodopsins, offering an explanation for the similar isomerization process.

Key words: Capillary electrophoresis; \textsuperscript{1}H-NMR; p-Coumaric acid; Photo-isomerization (trans-cis); Photoactive yellow protein; Photocycle; Bacterial phototaxis

1. Introduction

Photosensory systems are present in all three domains of life. In the Eukaryota rhodopsin and phytochromes have been studied in detail, while the Archaea also contain sensory rhodopsins. These photosensory proteins have a common photochemical basis: light-induced isomerization of a chromophore double bond. Until recently, knowledge on the functioning of photosensors in Bacteria was largely lacking.

Photoactive yellow protein (PYP) from the purple sulfur bacterium \textit{Ectothiorhodospira halophila} is the first eubacterial photoreceptor to be characterized in detail and has recently been shown to contain a new chromophoric group: thiol ester linked p-coumaric acid [1,2]. This is the first report of a physiological role for p-coumaric acid in prokaryotes, a compound previously identified in higher plants, where it plays a central role in the phenylpropanoid metabolism [3]. The crystal structure of PYP has recently been re-determined down to 1.4 \textAA{} resolution and shows that the protein has an \textit{a} or \textit{b} fold similar to that of eukaryotic proteins involved in signal transduction [4]. Evidence has been obtained indicating that PYP functions as the blue-light photoreceptor for a new type of negative phototactic response [5].

After absorption of a blue photon, the ground state of PYP (pG, \( \lambda_{\text{max}} = 446 \text{ nm} \)) enters a photocycle in which a red-shifted intermediate, pR (\( \lambda_{\text{max}} = 465 \text{ nm} \)), and a blue-shifted intermediate, pB (\( \lambda_{\text{max}} = 355 \text{ nm} \)), are formed sequentially, followed by the reformation of the ground state [6,7]. This photocycle strongly resembles the photochemistry of the archaebacterial sensory rhodopsins. These latter photoreceptors function in phototaxis in halobacteria. Their signaling is triggered by all-trans/13-cis isomerization of their retinal chromophore, followed most probably by deprotonation of the Schiff base [8-10]. Also for PYP, evidence was presented that proton uptake and release is associated with the photocycle [11]. In the ground state pG, the chromophore of PYP is in the \textit{trans} configuration (referring to the vinyl protons) and in the deprotonated state (the \textit{p}-hydroxy group), as was indicated by \textsuperscript{1}H NMR [1] and resonance Raman spectroscopy [12], respectively. It has been proposed that also in the case of PYP light-induced chromophore photo-isomerization occurs [1,28].

Here we report, using high-performance capillary zone electrophoresis [13,14], that \textit{trans} \textit{cis} isomerization of \textit{p}-coumaric acid occurs during the photocycle of PYP. This result contributes to the understanding of the similarity in photochemistry of photoactive yellow protein and sensory rhodopsins, in spite of their great structural differences, in the protein (\textit{a} or \textit{b} fold vs. seven transmembrane \textit{a}-helices) as well as in the chromophore (\textit{trans} \textit{p}-coumaric acid vs. all-\textit{trans}-retinal).

2. Materials and methods

PYP from \textit{E. halophila} was isolated as previously described [15] with minor modifications [7]. A sample of 1 ml, containing 17 \textmu{}M purified PYP, was exposed for 15 s to a Schott KL1500 150 W halogen lamp containing a long-wavelength band-pass filter (50% cut-off at 430 nm), to accumulate pB. The light intensity in the blue region of the spectrum was estimated with use of a Licor LI-180A quantum sensor and a Schott narrow-band interference filter. Illumination was performed at low pH, to lower the rate constant of the last (recovery) reaction in the photocycle of PYP [16,28], causing an even more dominant accumulation of pB. To avoid low-pH induced bleaching of pP in the dark, however, the pH was not decreased below pH 4. Sodium dodecyl sulfate (SDS), 2% (w/w), final concentration, was added during the last 5 s of exposure to denature pB. This procedure prevents recovery of the ground state pG. During all subsequent steps, the sample was kept in the dark to prevent photo-isomerization of \textit{p}-coumaric acid by ambient light. The pH of the sample was adjusted to 4 and the sample was incubated for 15 min at room temperature to

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Fig. 1. (A) $^1$H NMR spectrum of 60 mM trans-p-coumaric acid in CD$_3$OD. Assignment of protons (ppm): a 6.28, b 7.60, c 7.46, d 6.81, e not visible due to exchange of H with deuterium present in the solvent. Vertical numbers indicate the peak areas. (B) $^1$H NMR spectrum after 3 h of UV irradiation, showing the resonances of the trans as well as of the cis isomer of p-coumaric acid. Assignment of cis-p-coumaric acid protons (ppm): a' 5.76, b' 6.74, c' 7.61, e' not visible.

Hydrolyze the thiol-ester bond between the chromophore and apo-PYP. After hydrolysis, the pH was re-adjusted to 4 and p-coumaric acid was extracted with 4 vols. of ethyl acetate. The organic phase was washed five times with 1 vol. of deionized water and dried in air. The extraction of the chromophore from pG was performed according to the same procedure, without exposure of PYP to light. The reversibility of the photo-isomerization of the chromophore in PYP was investigated by also extracting the chromophore from pG, after exposure of PYP for 15 s to the halogen lamp, followed by 60 s of recovery in the dark. As a positive control for extraction of trans-p-coumaric acid, the procedure was carried out with p-coumaric acid instead of purified PYP as starting material.

In order to study the photochemistry of p-coumaric acid, 10 mg of the trans isomer (Sigma, St. Louis) was dissolved in 1 ml of H$_2$O.
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Fig. 2. (A) Electropherogram of trans-p-coumaric acid, recorded at 284 nm. (B) Electropherogram of the p-coumaric acid isomer mixture after 3 h of UV irradiation. (C) Electropherogram of the isomer mixture after addition of trans-p-coumaric acid. The retention time is plotted on top of each eluted peak.

atom% CD$_3$OD (Aldrich Chemical Co.). This solution was irradiated for 3 h in a Rayonet preparative photochemical reactor (The Southern New England Ultraviolet Co., CT), containing RUL-350 nm lamps, covering the ultraviolet spectral region from 320 to 400 nm. Before and after irradiation, proton nuclear magnetic resonance spectra (H NMR) were determined using a Bruker ARX 400 (400 MHz) spectrometer. Chemical shifts (δ) are given in ppm downfield from tetramethylsilane.

Air-dried samples, containing p-coumaric acid isomers were dissolved in demineralized water and injected into a 50 µm fused silica capillary TSP050375 (Composite Metal Services Ltd), with an injection time of 0.2 min (unless stated otherwise) and injection pressure of...
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40 mbar. The sample was subjected to electrophoresis at room temperature in 60 mM Tris/30 mM valeric acid, pH 8.2, through a capillary with an effective length of 55 cm, at 25 kV and approx. 12 μA. On-column detection was performed at 284 and 265 nm, the wavelengths of maximal absorbance of trans- and cis-p-coumaric acid, respectively [17].

3. Results and discussion

3.1. Photo-isomerization of p-coumaric acid in aqueous solution

1H NMR spectra unambiguously show that, after irradiation of trans-p-coumaric acid with UV light, trans-cis isomerization occurs, as was previously demonstrated for this and other cinnamic acid derivatives [17-19]. Fig. 1A shows the 1H NMR spectrum of trans-p-coumaric acid and the assignment of its protons. The scalar coupling constant of the trans protons of the vinyl double bond J_HA-HB, present at chemical shifts of 6.28 and 6.81 ppm, respectively, equals 15.9 Hz. In intact PYP this coupling constant has been determined to be 16 Hz [1]. After UV irradiation, additional resonances are present in the spectrum, as a result of the formation of the cis isomer (Fig. 1B). The coupling constant of the cis protons J_HA'-HB' at 5.76 ppm and approx. 6.74 ppm, equals 12.8 Hz. This is in agreement with the finding that the coupling between trans protons is always greater than the coupling between cis protons in olefinic systems [20]. The ratio of trans:cis isomers after 3 h of UV irradiation, determined from the peak areas at 6.28 and 5.76 ppm, equals 1:1.66 (i.e. 62% cis). After 72 h, 66% of p-coumaric was in the cis configuration, representing the steady state under the conditions described, since no further changes in this ratio were observed in 1H NMR spectra upon increasing the UV-exposure time (data not shown).

trans-p-Coumaric acid was subjected to capillary electrophoresis as described in section 2, allowing the elution of a single, sharp peak (Fig. 2A). The retention time of trans-p-coumaric acid equals approx. 10 min under the conditions selected, but is slightly variable: an average of 10.55 min (± a standard deviation of 0.15 min) was calculated from a set of 25 representative experiments. However, since all relevant components are eluted from the capillary as a very sharp symmetrical peak (with a width at half-height of less than 0.1 min), identification of unknown compounds can be accomplished through co-injection analysis (when necessary at multiple conditions with respect to pH and ionic strength).

Electrophoresis of the p-coumaric acid isomer mixture, obtained after exposure to UV irradiation, which was redissolved in H2O after 1H NMR analysis, resulted in the elution of two distinct components, as shown in the electropherogram in Fig. 2B. Co-injection of the isomer mixture with trans-p-coumaric acid showed enrichment of the first peak (Fig. 2C), indicating that the relative electrophoretic mobility of the cis isomer is larger than that of the trans isomer. This could be rationalized by the more spherical structure of the cis isomer, which causes less frictional forces against the solvent during electrophoresis. It should be noted that the trans:cis ratio in the electropherogram in Fig. 2B cannot be calculated from the ratio of the peak area of each isomer, because of their widely different extinction coefficients in the UV region. The molar extinction coefficient at 284 nm is significantly lower for the cis isomer, as compared to trans-p-coumaric acid [17]. Using the proper extinction coefficients, the ratio of the two isomers, as present in the mixture analyzed in the experi-

Fig. 3. Determination of the isomeric state of p-coumaric acid in photocycle intermediates of PYP. Upper part: electropherograms of p-coumaric acid extract from pG, recorded at λ_{max} of the trans isomer, 284 nm (A) and the cis isomer, 265 nm (B); the injection time is 0.1 min. Lower part: electropherograms of p-coumaric acid extract from pH, recorded at 284 nm (C) and at 265 nm (D); the injection time is 0.2 min. See text for details.
The isomeric state of p-coumaric acid in the blue-shifted photocycle intermediate pB of PYP

Previous capillary electrophoretic analysis of the chromophore extracted from the ground state of PYP, pG, has contributed to the identification of trans-p-coumaric acid as the chromophore of PYP [11]; see also Fig. 3A,B), which was subsequently confirmed by 1H NMR spectra of intact PYP [1] and X-ray crystallography [4]. In order to be able to extract the chromophore from the blue-shifted photocycle intermediate, pB [6,7], a solution of PYP was illuminated at acidic pH (i.e. pH 4, see section 2), because under the latter conditions the last step of the photocycle of PYP (the recovery of the ground state pG) is decelerated and consequently, the intermediate pB accumulates to a larger extent than at neutral pH [16]. To prevent the recovery of pG after illumination, SDS was added during the last seconds of light-exposure resulting in the denaturation of the accumulated pB. Chromophore extraction of this mixture, followed by capillary electrophoresis, revealed cis-p-coumaric acid as the main component (74 mol%) in the electropherogram at 10.9 min (Fig. 3C,D). No cis-p-coumaric acid could be detected in a chromatophore extract, obtained from a sample in which the pH was directly increased to release the chromophore during illumination, without the addition of SDS. (see also section 2). Apparently, the recovery of pG is a faster process than thiol-ester lysis at high pH.

The relative amount of pB can be calculated under the selected illumination conditions using a simplified two-state model for the photocycle with the following equation, which is valid during the steady state (see also [24]):

\[ k_{4}[^{pG}]/k_{2}[^{pB}] = 0 \]  

The rate constant \( k_{1} \) refers to the rate of light-induced pG excitation. It equals 2.303 \( \cdot \) \( \Phi_{446} \cdot e_{446} \cdot \lambda_{446} \), with \( \Phi_{446} \) and \( e_{446} \), representing the quantum yield and molar extinction coefficient of pG and \( \lambda_{446} \) the intensity of illumination at 446 nm. The factor 2.303 results from the conversion of the napierian to the decimal absorption coefficient. \( \Phi_{446} \) equals 0.35 [25], \( e_{446} \) is 4550 m\(^2\) mol\(^{-1}\) [26] and \( \lambda_{446} \), as determined with a quantum sensor, is \( \sim 3.0 \times 10^{9} \) mol photons m\(^{-2}\) s\(^{-1}\). Consequently, \( k_{1} \) equals 1.1 s\(^{-1}\). The rate constant \( k_{2} \), referring to the rate of pG recovery, was determined to be 0.05 s\(^{-1}\) at pH 4 [16]. From Eq. 1, it follows that under the light intensity used \([pB]/[pG] = 0.03 \) at pH 4 [24]. From Eq. 1, it follows that under the light intensity used \([pB]/[pG] = 0.03 \) at pH 4 [24]
which proves that the proteinaceous environment of the chromophore (i.e. apoPYP) considerably facilitates the recovery of the ground state conformation of the chromophore. Because of the many details that we now know about this latter reaction at the level of structure, kinetics, and thermodynamics, PYP may become an excellent model system to reveal the atomic details of enzyme catalysis [28].

In conclusion, this paper shows that for the domain of Bacteria photosensing also can occur by light-induced isomerization of a chromophore double bond. This is also true for the domains of Archaea and Eukarya, where photo-isomerization of the chromophore in rhodopsins and phytochromes is involved in light sensing. It is interesting to note that also in plants, \textit{trans/cis} isomerization of cell wall bound \textit{p-coumaric} acid has been proposed to play a role in sensing of UV and blue light [29]. Thus, all three domains of life show a common photochemical basis for sensing the ambient light climate.

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References

trans/cis (Z/E) photoisomerization of the chromophore of photoactive yellow protein is not a prerequisite for the initiation of the photocycle of this photoreceptor protein

(4-hydroxyphenylpropionic acid)/7-hydroxycoumarin-3-carboxylic acid/low-temperature spectroscopy/Fourier-transform infrared spectroscopy/locked chromophore

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ABSTRACT The chromophore of photoactive yellow protein (PYP) (i.e., 4-hydroxyphenylpropionic acid) has been replaced by an analogue with a triple bond, rather than a double bond (by using 4-hydroxyphenylpropionic acid in the reconstitution, yielding hybrid I) and by a “locked” chromophore (through reconstitution with 7-hydroxycoumarin-3-carboxylic acid, in which a covalent bridge is present across the vinyl bond, resulting in hybrid II). These hybrids absorb maximally at 464 and 443 nm, respectively, which indicates that in both hybrids the deprotonated chromophore does fit into the chromophore-binding pocket. Because the triple bond cannot undergo cis/trans (or E/Z) photoisomerization and because of the presence of the lock across the vinyl double bond in hybrid II, it was predicted that these two hybrids would not be able to photocycle. Surprisingly, both are able. We have demonstrated this ability by making use of transient absorption, low-temperature absorption, and Fourier-transform infrared (FTIR) spectroscopy. Both hybrids, upon photoexcitation, display authentic photocycle signals in terms of a red-shifted intermediate; hybrid I, in addition, goes through a blue-shifted intermediate, with very slow kinetics. We interpret these results as further evidence that rotation of the carbonyl group of the thioester-linked chromophore of PYP, proposed in a previous FTIR study and visualized in recent time-resolved x-ray diffraction experiments, is of critical importance for photocactivity of PYP.

The primary importance of light-induced cis/trans (or E/Z) isomerization of the chromophore of biological photoreceptors, such as rhodopsins, phytochromes, and xanthophylls, for the initiation of signal transduction is generally accepted, although this issue has long been controversial (1-4), almost since the discovery of the molecular basis of vision by Wald in 1963 (5). As an alternative for the now well-accepted concept of light-induced cis/trans isomerization, mechanisms have been proposed, such as light-induced proton transfer (2), that subsequently were largely rejected.

The importance of cis/trans isomerization has been studied not only with several forms of transient spectroscopy but also in experiments in which the retinal chromophore of bacteriorhodopsin was replaced through reconstitution by an analogue, equipped with a covalent “bridge” (i.e., forming a five- to eight-membered ring across the double bond), which was anticipated to prevent isomerization (6, 7). Following this approach for bacteriorhodopsin (Brh), Delaney et al. (8) reported that a six-membered ring across C=C(9,10) or C=C(11,12) of retinal does allow formation of the intermediate J and K, albeit with slower kinetics than in Brh containing unmodified retinal. However, even a five-membered ring across C=C(13,14) blocks this primary photochemistry only partially (4). Very recently, it was reported that, by using atomic force microscopy, conformational changes can be detected in the microsecond time domain in Brh hybrids, reconstituted with a modified chromophore that would not be expected to allow primary photochemistry (9). This observation led these authors to conclude that “our data question the current working hypothesis which attributes all primary events in retinal proteins to an initial trans-cis isomerization.”

The interpretation of these experiments, however, is complicated because isomerization across one of the double bonds of retinal, neighboring the one that is locked or modified, may allow for alternative isomerization pathways in the rhodopsins containing a retinal analogue. We therefore have addressed the point of the importance of chromophore isomerization for photoreceptor activation by studying the photoactive yellow protein (PYP) (10). This is a photoreceptor from the purple bacterium Ecctotiorhodospirx halophilis (11), which shows many similarities with the archael sensory rhodopsins (12, 13), although PYP contains 4-hydroxyphenylpropionic acid as its chromophore (14, 15) and is water soluble. Activation of PYP function is supposed to proceed through light-induced cis/trans isomerization of the 7,8-vinyl bond of its chromophore (16, 17). The apo form of this photoreceptor can be produced heterologously in Escherichia coli (18), and then can be converted to functional holoprotein through reconstitution with the endogenous chromophore (19) or with analogues, resulting in the formation of hybrids (20).

Here we report reconstitution of PYP with chromophores in which (i) the vinyl double bond of 4-hydroxyphenylpropionic acid is replaced by a triple bond (by using 4-hydroxyphenylpropionic acid in the reconstitution; the resulting holoprotein is referred to below as hybrid I) or (ii) the vinyl double bond of the chromophore is locked against isomerization by the presence of a covalent “bridge” over the vinyl bond (by using 7-hydroxycoumarin-3-carboxylic acid in the reconstitution; the resulting holoprotein is referred to below as hybrid II).
results obtained show that in both hybrids authentic photocycle signals can be observed. These experiments therefore lead to the conclusion that isomerization across the double bond of the chromophore of the PYP photoreceptor is not a strict prerequisite for photoactivation of PYP.

MATERIALS AND METHODS

Materials. ApoPYP was produced heterologously in _E. coli_ as described previously (18, 20) and was used without prior removal of its polyhistidine tail. This method results in slightly slower kinetics of the recovery step of the photocycle of reconstituted holoPYP (see Fig. 2).

7-Hydroxycoumarin-3-carboxylic acid was obtained from Molecular Probes. 4-Hydroxyphenylpropionic acid (21) was synthesized from 4-4-butylidimethylsilylethylbenzaldehyde, via 4-4-butylidimethylsilylethyl-2-acetylacetamide. Purity of the final product was checked with 'H NMR (20) and demonstrated to be better than 98%. A small fraction of the contaminants consisted of 4-hydroxycinnamic acid (see Results). All other materials were reagent grade and obtained from commercial sources.

Reconstitution. Reconstitution of holoPYP and hybrid II was carried out by means of formation of the anhydride derivative of their chromophore, according to Imamoto et al. (19). Hybrid I, because of a more limited availability of its chromophore, was formed with the procedure described by Genick et al. (22), which makes use of chromophore activation via derivatization with carbonyldimimidazole in tetrahydrofuran.

Spectroscopy. Spectroscopic experiments were routinely carried out in 10 mM Tris-HCl, pH 7.0. UV/Vis (visible) static and transient absorption spectra were recorded with model 8453A and model 8452A Hewlett Packard diode array spectrophotometers, which have a time resolution of 0.1 and 0.5 sec, respectively. To measure light-induced transient absorption spectra, actinic flashes were provided with a conventional photoflash, with an output intensity of 25 W sec.

Low-temperature spectra were recorded as described by Hoff et al. (23). Samples were illuminated with a conventional slide projector, equipped with a 24-V/150-W tungsten lamp and fiber optics, through a narrow-band interference filter with maximal transmission at 460 nm and a bandwidth of 9 nm. The intensity of the output beam was 0.16 mW cm⁻².

Fourier-transform infrared (FTIR) spectra were recorded with home-made sandwich cells of 13, 26, or 52 μm thickness, constructed from polyethylene spacers and CaF₂ plates. Samples for FTIR measurements were concentrated to between 2.5 and 5 mM, with a 30-Ka cut-off Millipore NMWL filter spin column, at room temperature, in 10 mM Tris-HCl, pH 7.0. FTIR difference spectra of white-light photoconverted minus dark-adapted samples were measured on a Bio-Rad FTS-60A IR spectrophotometer and corrected for drift and H₂O vapor. Data manipulation was performed with software provided by the manufacturer. Visible light from a 150-W Osram (Strattford, CT) xenon lamp and fiber optics were used to illuminate the sample in the IR spectrometer.

RESULTS

The three proteins formed with the chromophores displayed in Fig. 1—i.e., holoPYP, hybrid I, and hybrid II—all show a major optical transition in the visible part of the spectrum, with maximal absorbance at 446, 464, and 443 nm, respectively. This indicates that each reconstituted protein contains a highly tuned chromophore, most likely inserted properly into the chromophore-binding pocket, with the phenolic hydroxyl group deprotonated and in hydrogen-bonding contact with Glu-46 (cf. refs. 24 and 25). Nevertheless, some differences are noticeable: Both hybrids have a more symmetrical main absorption band in the visible part of the spectrum (which indicates that they have less pronounced high-energy sidebands, characteristic for holoPYP (23); see further below). Hybrid II has a much narrower visible absorption band than either holoPYP or hybrid I (full width at half-maximum: 35 nm). Furthermore, its extinction coefficient appears to be slightly higher than the one for holoPYP (455 mM⁻¹ cm⁻¹ (26)), thus significantly higher than the extinction coefficient of free 7-hydroxycoumarin-3-carboxylic acid, which absorbs maximally at 388 nm, with an extinction coefficient of 32 mM⁻¹ cm⁻¹ (27). Hybrid II has a much more red-shifted absorption band than hybrid I, but also a considerably lower extinction coefficient than holoPYP. Because we have not been able to ascertain that reconstitution of this hybrid was complete, we cannot yet precisely calculate its extinction coefficient. Hybrids II and II also fluoresce, with a quantum yield that has increased in hybrid II and decreased in hybrid I, as compared with holoPYP. Furthermore, the Stokes shift of fluorescence is considerably decreased in hybrid II, which is in agreement with a more limited flexibility of the latter chromophore.

Next, it was tested whether hybrids I and II would also display photocycle characteristics, by assaying the recovery step of the photocycle with transient absorption spectroscopy (Fig. 2), using holoPYP as a control, and excitation with a conventional photoflash. Trace a of Fig. 2A shows the result of this control experiment. The polyhistidine tail slightly retards the recovery
A spectrum of holoPYP at least three (sub)maxima can be found in the absorption band, making it more evident than in the holoPYP spectrum. This observation is indicative for the formation of a blue-shifted (pB)-like state upon partial bleaching. In the difference spectra of holoPYP, a fine structure on the high-energy side of the main (sub)maximum is discerned, with a spacing of approximately 1500 cm⁻¹. Such a spacing is typical for the absorption of many conjugated polyene systems and is related to the excited-state vibrational frequency of a C=C double bond in such a system.

Interestingly, this fine structure becomes somewhat more pronounced upon partial bleaching. In the difference spectrum of holoPYP at least three (sub)maxima can be discerned, with a spacing of approximately 1500 cm⁻¹. Such a spacing is typical for the absorption of many conjugated polyene systems and is related to the excited-state vibrational frequency of a C=C double bond in such a system.

In hybrid I (Fig. 3B), the visible absorption band also sharpens and undergoes a slight red shift at 77 K, but no clear fine structure of the absorption band can be discerned. Bleaching of this hybrid occurs very efficiently. Its bands are sharpened, demonstrating that also for hybrid I this band is inhomogeneously broadened and that spectral selectivity of bleaching can be achieved. For hybrid II (Fig. 3C) again the sharpening and slight red-shift occur at 77 K, but in this case the change in bands is due to the presence of pR (see Discussion).
Whereas hybrid I (Fig. 3B) and hybrid II (Fig. 3C) both display bleaching of the main absorption band by actinic illumination, with neither hybrid was evidence for the formation of a red-shifted intermediate obtained, in contrast to holoPYP, which clearly shows this intermediate with an absorbance maximum at ~490 nm. The small increase observed in the absorbance of hybrid II in the 500-nm region does not show the typical characteristics of a specific absorption band. However, we interpret these low-temperature (difference) spectra as evidence that a pR-like state is formed in both hybrids, but that this latter state absorbs at a wavelength similar to that of its corresponding pG state, albeit with a considerably decreased extinction coefficient. This interpretation is fully in line with the results of room-temperature nanosecond transient absorbance measurements. In these latter experiments also, hybrid I does not show the increase in absorbance in the 500-nm region in the nanosecond to microsecond time scale (typical for pR formation in holoPYP), prior to formation of the blue-shifted intermediate, as shown in Fig. 2. Hybrid II shows only a transient bleaching of the ground state in the micro- to millisecond time scale; signals from a pB-like state could not be detected in the latter hybrid.

To obtain further evidence that authentic photocycle signals had been obtained, particularly from hybrid I, light – dark (i.e., pB – pG) FTIR difference spectra were recorded in solution, again using holoPYP as a control (Fig. 4). Steady-state accumulation of pB was accomplished by illumination of the sample in the IR spectrophotometer. For holoPYP, because of the relatively low light intensity available, a maximal conversion of 15% of the pigment was achieved. For hybrid I, at least in part because of its much slower recovery rate, a maximal conversion of 40% of the pigment into the pB-like state was achieved during steady-state illumination. For hybrid I, this level of photocconversion was calculated from the detection of photocconversion in the FTIR cell, directly after removal of the sample from the FTIR spectrometer, and from the complete conversion, obtained with much higher light intensities than obtainable within the FTIR set-up, outside the spectrometer. Similar difference spectra were obtained from samples of holoPYP and hybrid I incubated at pH 5.0.

The FTIR difference spectrum, here displayed from 1400 to 1000 cm\(^{-1}\), has a suboptimal signal-to-noise ratio, in part because of the relatively inefficient photocconversion conditions used in this experiment (Fig. 4). Nevertheless, this figure clearly shows two of the most typical features of the pB – pG difference spectrum, characteristic for holoPYP (ref. 30; A. Xie, W. D. Hoff, and K. J. H., unpublished experiments) at 1163 and 1203 cm\(^{-1}\). Both features are characteristic for phenolates. This interpretation has been documented for (para-substituted) phenols, tyrosine, etc. (in both their neutral and anionic forms) by FTIR and resonance Raman spectroscopy (31–33).

In the nomenclature of vibrational modes derived for benzene (34), these features have been assigned to the Y9a (C–H in-plane bending) and Y7b (predominantly C–O stretch) modes of the phenolate group, respectively. For hybrid I, a very similar difference spectrum was obtained, with the corresponding main peaks at 1172 and 1279 cm\(^{-1}\), indicating that in this hybrid also a protonated chromophore is transiently formed. The shift from 1163 to 1172 cm\(^{-1}\), which was predicted on the basis of pH-titration studies of ester model compounds of the chromophore of hybrid I, the rate of formation and decay of the signal at 1172 cm\(^{-1}\) was recorded in a series of measurements in which a smaller number of transients was averaged, to allow for time resolution of the FTIR measurements. The pB state of hybrid I was populated with a rate of ~0.01 sec\(^{-1}\), which is dependent on the intensity of actinic illumination. For the recovery of the ground state of hybrid I, a rate constant of 3.2 (±0.5) × 10\(^{-4}\) sec\(^{-1}\) was obtained (Fig. 4B), in close agreement with the rate of recovery of the ground state of this pigment, measured by using visible absorbance measurements (Fig. 2A).

Additional typical characteristics of the pB – pG FTIR difference spectra, such as the signals from Glu-46 at 1728 cm\(^{-1}\), were recorded, but are not shown in Fig. 4. FTIR, as a technique, was selected for these measurements because it was anticipated to be able also to detect the C=C triple bond of hybrid I, of which the stretch vibration is expected to absorb at ~2200 cm\(^{-1}\), as was concluded from measurements of derivatives of 4-hydroxyphenylpropiolic acid at neutral and alkaline pH. This is a region with little absorbance originating from the apoprotein. However, this band was not detected in the spectra. The explanation for this lack of detection may be related to the relatively large width of this latter band, as compared with the Y9a marker band at 1172 cm\(^{-1}\).

Because bleaching of hybrid II with continuous actinic illumination at room temperature could not be accomplished (presumably because of its very rapid recovery rate; see also Fig. 2A), no FTIR measurements with this hybrid have been performed. With hybrid I, care should be taken to allow full relaxation of the pigment to the ground state before measurements are started, because ambient light already causes significant accumulation of the pB-like intermediate.

\(^{13}H\) NMR analysis (see Materials and Methods) indicated that a small fraction of the impurities in 4-hydroxyphenylpropiolic acid consisted of 4-hydroxyaminoc acid. Because apoPYP might selectively recognize this contaminant in the recombinant experiments, we have carried out chromophore reconstruction experiments. These, however, have not allowed us to make an estimate of the maximal level of contamination of the recombinant chromophore in hybrid I, because only very small (far less than stoichiometric) amounts of 4-hydroxyphenylpropiolic acid were released by alkaline hydrolysis. This limited release may be
H

We have not succeeded in reconstituting any functional or acid was below the level of detection of the techniques used in conclusion that the level of contamination by 4-hydroxycinnamic Nevertheless, the results presented in Figs. 2A

The role of cis/trans isomerization as the chemical basis of the primary reaction of photoperception in sensory transduction in the rhodopsin-based visual process is under intense discussion. Understanding this system, however, is complicated by the polyenic character of retinal, which makes it necessary to consider isomerization across alternative bonds when this process across one particular bond is prohibited by chemical denaturation of the chromophore. Furthermore, both visual rhodopsin and Brh hold their chromophore—i.e., retinal—bound in a distorted conformation, which may significantly affect its basic photochemistry (35, 36) and thus complicate comparisons with model compounds.

We have therefore decided to study the role of chromophore isomerization in a member of the xanthopsin protein family (18), PYP, from Ectothiorhodospira halophila (10). The 4-hydroxycinnamic acid chromophore of this photoreceptor contains a unique double bond, which is subject to isomerization upon photoactivation (16, 17). On the basis of FTIR measurements, it has been proposed that this isomerization process involves a rotation across two bonds: the 7,8-vinyl bond of the chromophore and the S=C single bond through which the chromophore is linked to the apoprotein (37). Very recently, the atomic displacements during nanosecond laser activation of PYP have been revealed through subjecting crystals of this photoreceptor to time-resolved x-ray diffraction experiments (38). These experiments clearly demonstrated that the change in the position of the aromatic ring of the chromophore of PYP is very small; it can best be described as a rotation of 6°, within the plane in which it is clamped in its binding pocket, between the aromatic amino acid side chains (i.e., Phe-62 and Phe-96) on one side, and the hydrogen bonding Arg-52 and Tyr-98 on the other. The largest atomic displacement that is seen in these diffraction experiments, however, is the trans-to-cis displacement of the vinyl bond of the chromophore and—in particular—the rotation of the carbonyl group of the chromophore along the long axis through the chromophore and Cys-69.

This carbonyl group is therefore subject to a crankshaft-like motion, in which the initial hydrogen bond to the backbone-N of the cysteine is disrupted and a new hydrogen bond to Tyr-98 is transiently formed (38). Subsequent recovery of the ground state thus includes nonphotochemical cis/trans reisomerization of the double bond of the chromophore. In the absence of the apoPYP protein this reisomerization process displays a very high activation barrier (the isolated 4-hydroxycinnamic acid chromophore is thermally stable in both the cis and the trans forms). His-108 of the apoprotein may be of critical importance in the catalysis of the latter process by the apoprotein.

We have investigated the (in)dispensability of trans/cis isomerization of the chromophore for activation of the PYP photoreceptor (i) by replacing the double bond of the endogenous chromophore by a triple bond and (ii) by using a chromophore with a locked double bond. Both modified chromophores have no possibility to undergo trans/cis isomerization across the double bond that corresponds with the 7,8-vinyl bond of the authentic chromophore. Nevertheless, the resulting hybrid pigments do show absorption characteristics and photoactivity that are typical for holoPYP. Of the two, hybrid II is most restricted in its photochemistry. Formation of a pB-like intermediate could be recorded (see Figs. 2 and 4). These latter results strongly argue that the pB-like intermediate of hybrid I also contains a protonated chromophore (20, 24, 25, 30, 37)

These observations can be interpreted within the framework of a model that assumes that photoactivation induces rotation of the carbonyl group of the chromophores (see above). In both hybrids the carbonyl function of the chromophore is part of an extended conjugated system, ranging from the phenolate oxygen to the sulfur atom of Cys-69, as in holoPYP. After photoactivation, demonstrated at 77 K, albeit the pB-like intermediate formed does not show a shift in its absorption maximum with respect to its corresponding pG. Hybrid I behaved similarly in this respect; however, from the latter also authentic signals originating from a pB-like intermediate could be recorded (see Figs. 2 and 4). These observations are unequivocally demonstrated that trans/cis isomerization of the vinyl bond of the chromophore of PYP is not required for photoactivation of this photoreceptor.
CONCLUSION

The results presented in this study unequivocally demonstrate that trans/cis isomerization of the vinyl bond of the chromophore of PYP is dispensable for activation of the PYP photoreceptor and suggest that rotation of its carbonyl group may be of higher importance. Furthermore, the results obtained suggest that in hybrid I intramolecular proton transfer still takes place and that the high desorption rate of the chromophore is strongly retarded.

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Note: The x-ray structure of a pR-like intermediate of holoPYP was acknowledged (unpublished results), and a subpopulation of PYP was thought to be of great interest to resolve this relation between (variations in) NMR and pR.

4.3 Time-resolved X-ray crystallography at the European Synchrotron Radiation Facility: a discussion on the structural events in the photocycle of a xanthopsin.

**Time-resolved X-ray crystallography.** Today, the three-dimensional structures of thousands of proteins have been elucidated, but surprisingly only few molecular mechanisms of action, based on these structures, have been resolved. To understand the way proteins, or more specifically enzymes, do their job, not only a detailed picture of one state is essential, but rather of all the intermediates present during catalysis, including the rates of their interconversion. In order to obtain structural information about a protein, long X-ray exposures of hundreds of minutes are necessary, excluding the possibility of studying transient intermediates in catalysis, which typically have lifetimes on very short time scales, varying from nanoseconds to several seconds. Several ways have been found to overcome the incompatibility of long exposure times and the relatively short life times of reaction intermediates. First, short-lived intermediates can be mimicked by stable complexes like enzyme-substrate or inhibitor complexes, but these may lack key structural interactions. Second, cryocrystallography, where diffusion of a substrate into a cooled crystal prolongs the lifetimes of the intermediates, has proven to be very useful, but cooling may affect the protein structure and trapped intermediates may not be representative for those present at physiological temperatures. Third, the use of a highly intense X-ray source will strongly decrease the required X-ray exposure time. With the use of intense monochromatic X-rays however, only the second to minute time-scale is accessible, since rotation of the crystal during exposures is necessary to obtain a complete data set. Therefore, the use of polychromatic X-rays, in the so-called Laue diffraction, is the ultimate approach, since it eliminates the need for crystal rotation and reduces exposure times even further. Interestingly, the very first diffraction patterns of crystals, obtained by Knipping and von Laue in 1912, also were obtained after exposure to polychromatic X-rays. Nowadays, with the availability of extremely intense polychromatic X-rays, generated by so-called synchrotron radiation sources, the study of short-lived intermediates at physiological temperatures is feasible (Moffat, 1989).

**The European Synchrotron Radiation Facility.** Synchrotrons are extremely large facilities for circulating electrically charged particles, like electrons, at nearly the speed of light in a storage ring. Initially, they were designed for use in high-energy physics for studies on the collision of charged particles. But today, also chemists and biologists make use of synchrotrons, because such devices emit highly intense electromagnetic radiation, which has proven to be a powerful tool in a broad range of studies, to the extent that radiation-dedicated synchrotrons were constructed. Today, one of the most advanced synchrotron radiation sources in the world, also referred to as a third generation source, is the European Synchrotron Radiation Facility (ESRF) in Grenoble with a storage ring circumference of 844 m (figure 1). In the so-called synchrotron booster (a smaller ring inside the main ring), electrons are accelerated and subsequently injected into the storage
An electron in orbit emits electromagnetic radiation and this energy loss results in a drift towards the center of the ring. In addition, bending magnets guide the electrons through their orbit. The ring is not uniformly filled with electrons, but with bunches of electrons with a length of 18-60 mm, corresponding to an X-ray pulse of 60-200 picoseconds duration. Usually, the storage ring at the ESRF operates in a mode, where 1/3 of the ring (332 bunches) is filled. This a relatively stable mode of operation of the ring, with lifetime of about 52 h, so a refill is required every second day. Only nine days a year the ring will operate in the less stable single bunch mode, with a lifetime of about 11 h. This mode however, is extremely useful for time-resolved experiments of reversible phenomena on a very short time scale, provided that samples can be excited over and over again, to allow the collection of sufficient intensity by an X-ray detector. It takes a single bunch of electrons 2.82 microseconds to travel through the entire ring. This is sufficiently long to isolate a single pulse of X-rays by use of a mechanical chopper. To pump the sample with a laser flash and probe it simultaneously with X-rays at the appropriate time chosen by the user, three asynchronous clocks must be brought together in phase: (i) the X-rays generated by the synchrotron, (ii) the chopper rotation frequency and (iii) the laser pulse. In the experimental set-up, constructed at the ESRF in Grenoble, the timing sequence is triggered by software and can be synchronized with crystal rotation, such that a whole data set can be collected automatically (Bourgeois et al., 1996). Timing is controlled with a large area avalanche diode. This device can be used to detect X-rays as well as photons from the laser beam. The output from this device functions as input for the controlling software. Delays can be set with an accuracy of approximately 1 nanosecond (Bourgeois et al., 1996).

Feasibility of the experiment. New methods have only recently been developed to deal with the complications inherent in the processing of Laue diffraction data, which is much more complicated than the processing of monochromatic X-ray diffraction data. A unified solution to the problems in integration of both streaky and spatially overlapping spots and data scaling has been incorporated into a Laue diffraction data-reduction software package, LaueView (Ren & Moffat, 1995). Essential for time-resolved X-ray crystallography is the initiation of the reaction, which has to be uniform, rapid and without crystal damage. The reaction of interest can be initiated chemically, by a concentration jump, or physically, by a temperature or pressure jump or by a laser pulse. Photoactivation of a stable substrate analogue has proven successful in case of myoglobin and hemoglobin, in which a brief laser pulse ruptures the heme-iron CO bond, followed by recombination in the dark. Also, the photoactivation of caged substrates like ATP and photodestruction of inhibitors are useful alternatives (Moffat, 1989). Nevertheless, a stable, water-soluble protein with intrinsic photoactivity inducing physiologically relevant conformational changes for signal transduction would be an excellent model system for the type of experiment discussed here. Photoactive yellow protein satisfies these conditions. In addition, crystalline PYP has been shown to be photoactive (McRee et al., 1986), with photocycle kinetics similar to those of PYP in solution (Ng, Getzoff & Moffat, 1995). Probing of PYP crystals with polychromatic X-rays yield high resolution Laue diffraction patterns for different time delays (for example figure 2), which have been processed to electron density difference maps. These maps reveal the underlying conformational changes in photoactive yellow protein upon photoactivation, from 1 nanosecond to several milliseconds. The experiments described here are carried out at the edge of the physical limits of experiments with biological materials: to work with an optically sufficiently transparent crystal, one has to use a wavelength of excitation far from the absorbance maximum. To compensate for this, very high-intensity laser-pulses have to be used, which in turn cause shock- and heat-waves in the crystal. In spite of all this, the experiments described below provide extremely detailed insight into the signal...
transduction events in photoactive yellow protein.

The early intermediate pR. The PYP structure was probed at 1 nanosecond by an X-ray pulse of 150 picoseconds (single-bunch mode) after initiation of the photocycle by the delivery of a 7-ns laserpulse at 495 nm to small (80 μm by 80 μm by 150 μm) PYP crystals at 287 K (Perman et al., 1998). This resulted in a high-resolution Laue diffraction data set (see for example figure 2). After data processing, the resulting electron density difference map shows structural features of the early, red-shifted photocycle intermediate pR (chapter 1 3). As expected, the features on this map are limited to the local environment of the chromophore (figure 3). The map clearly shows that there is a lack of features on its aromatic moiety. This indicates that this moiety remains buried and does not undergo significant translational motion in the transition to the pR state. A model could be successfully refined against the Laue data, which accounted for the changes of the atoms of the chromophore and its local environment. During the process of refining this model one should consider that standard protein parameters for unstrained, stable structures may not apply to short-lived structures. The model shows that trans to cis isomerization of the chromophore vinyl double bond is completed within 1 ns, including the following structural changes: (i) torsion of the single

Figure 2. Laue diffraction pattern of a photoactive yellow protein crystal. Structure was probed 1 nanosecond after excitation at 495 nm. The synchrotron is running in the single-bunch mode. Image was taken at the ID09 beamline station, experiment LS-340 at the ESRF, Grenoble, France. 27-11-1996.
bond between the γ-sulfur of Cys69 and the chromophore carbonyl C-atom, (ii) torsion of the Tyr42 and Glu46 side chains, and (iii) minor changes in the chromophore binding pocket near Cys69 (figure 4). Although the center of the aromatic moiety of the chromophore does not move, the ring has rotated within its plane. The hydrogen-bond between Glu46 and the phenolate oxygen has been disrupted in pR, while the chromophore retains its hydrogen-bond to Tyr42. The side chain of Glu46 has moved away from Tyr42 to the protein core. The rotation of the aromatic moiety has moved the phenolate oxygen towards Arg52.

In the photocycle intermediate pB, accumulated by continuous illumination, the side chain of Arg52 has moved away from the chromophore, opening this binding pocket gateway, thus allowing solvent exposure of the chromophore (Genick et al., 1997a). The hydrogen-bond between the carbonyl oxygen of the chromophore tail and the backbone nitrogen atom of Cys69 has been broken in pR, as the carbonyl has reoriented and has formed a new hydrogen-bond with the backbone nitrogen atom of Tyr98 (figure 4). This carbonyl oxygen cannot lie in the plane of the chromophore ring in the cis isomer without severe steric hindrance between the oxygen and the ring hydrogens. In the long-lived photocycle intermediate pB, the carbonyl oxygen reforms its hydrogen-bond with the amide group of Cys69 (Genick et al., 1997a). The backbone regions that move upon pR formation largely correspond to regions of a strained secondary structure in pG. These include an underwound α-helix and the backbone adjacent to Cys69, both present in the chromophore binding pocket, in the distal and proximal region, respectively. These are

Figure 3. Electron density difference map of pR-pG. Blue and red contours indicate regions of positive and negative difference electron density, respectively. The most prominent positive feature (A) appears to result from repositioning of the vinyl carbon atoms of the chromophore. It is associated with negative features on the chromophore carbonyl oxygen (B) and the γ-sulfur of Cys69 (C) that together indicate motion of these atoms. Another prominent negative feature below the α-carbon atom of Cys69 (D) suggests that the backbone in this proximal region shifts toward the chromophore (trans to cis isomerization causes a contraction of 0.5 Å). A further set of features at the distal region of the chromophore-binding pocket, near the phenolate moiety (E,F), are associated with the disruption of the hydrogen bond between Glu46 and the phenolic oxygen. Figure is kindly provided by K. Moffat and appeared in adapted form in Perman et al. (1998).

Figure 4. Comparison of the pG and pR state structures: the chromophore and its environment are viewed from the proximal end of the chromophore binding pocket. Figure is kindly provided by K. Moffat and appeared in adapted form in Perman et al. (1998).
the only sites of protein backbone motion between the pG and pR states (Perman et al., 1998).

The cold intermediate PYP<sub>BL</sub>. It is interesting to compare the structure of the 1-
ns photocycle intermediate at 14°C (Perman et al., 1998) with that of the recently published
cryotrapped, light-activated intermediate at temperatures below -100°C (Genick et al.,
1998) and that of the photostationary state (Genick et al., 1997a). Low-temperature
spectroscopy showed that below -100°C, intermediates could be trapped that precede
PYP<sub>L</sub> (λ<sub>max</sub> = 456 nm), which most probably corresponds to the room temperature
photocycle intermediate pR<sub>s</sub> (Imamoto,
Kataoka & Tokunaga, 1996). Among these
intermediates is PYP<sub>BL</sub> (λ<sub>max</sub> = 400 nm), which is probably the intermediate that has been
studied by low-temperature X-ray crystallo-
graphy, since microspectrophotometry indi-
cated the formation of a 400-nm shoulder in
absorption spectra of light-activated PYP
crystals (Genick et al., 1998). Caution should
be taken however, with the assumption that
PYP<sub>BL</sub> represents a room temperature
photocycle intermediate, formed within 1
nanosecond after light-excitation. Especially,
since recent picosecond transient absorption
spectroscopy has demonstrated that two
intermediates are formed within the first
nanosecond, which are both red-shifted to 510
nm (Uji et al., 1998). Even though the PYP<sub>BL</sub>-
like intermediate cannot be observed at room
temperature, it seems plausible that the key-
structural events, observed in PYP<sub>BL</sub>, are
relevant and precede those in pR.

The extremely high-resolution of the
structure of the light-activated, cryotrapped
intermediate of PYP (0.85 Å) also showed
(like the pR structure) that the structural
changes, compared to pG, were confined to
the chromophore and its immediate
environment. Interestingly, this structure
shows that the flipping of the thiol ester
carbonyl group is virtually complete, at a time
when the chromophore double bond hardly
has passed the trans-to-cis transition point
(Essen & Oesterhelt, 1998; Genick et al.,
1998). Thus surprisingly, the chromophore in
PYP<sub>BL</sub> has a strongly distorted transition-
state-like conformation and the flipping of the
carbonyl group precedes the full isomerization
of the vinyl double bond. The extraordinary
finding in PYP<sub>BL</sub> that a double bond
isomerization has been trapped halfway may
explain the strong blue-shift in this
intermediate to 400 nm, because of the
interruption of the conjugated system in the
chromophore of this intermediate. Genick et al.
(1998) proposed that the flipping of the
carbonyl group drives the chromophore from
the transition state into the cis conformation,
causing a strained conformation in the protein
that drives the rest of the cycle. In chapter 4.2
we demonstrated that, after substitution of the
chromophore double bond by a triple bond,
photoactive yellow protein still displays an
authentic photocycle, be it with altered
kinetics.

Trans to cis isomerization should result in a
contraction of the chromophore. In the
transition state in PYP<sub>BL</sub>, this effect of the
partial isomerization is compensated for by
slight changes in the positions of Tyr42 and
Glu46, maintaining the hydrogen-bonds
between these residues and the phenolic
oxygen (Genick et al., 1998). In pR however,
the vinyl double isomerization has been
completed, resulting in a disruption of the
hydrogen bond with Glu46 (Perman et al.,
1998). The structural data for PYP<sub>BL</sub> are in
agreement with those obtained by low-
temperature FTIR difference spectra, which
led to the first proposal for the two-bond
isomerization process, as described in more
detail in chapter 1.3 (Xie et al., 1996). The
assumption in the FTIR-experiments however,
that the trapped intermediate at low
temperatures represents pR, is arguable. In
agreement with the X-ray crystallographic
data described above, indicating structural
differences between pR and PYP<sub>BL</sub>, a striking
difference is observed in the quantum yield
(Φ) of phototransformation of pR back to pG
between 77 K and room temperature (Gensch
et al., 1998).

A second interesting proposal, also initially
based on FTIR-experiments and now
supported by pH measurements, is that Glu46
donates a proton to the phenolic oxygen
during the transition from pR to pB (Hendriks et al., 1998; Imamoto et al., 1997; Xie et al., 1996). However, the shift of Glu46 away from the deprotonated chromophore, which remains hydrogen bonded to Tyr42, as observed by Perman et al. (1998), may indicate an alternative mechanism, in which the chromophore receives a proton from and Glu46 donates a proton to the solvent.

The long-lived intermediate pB. Structural data of the photocycle intermediate pB were obtained, during light exposure and 2 ms and 12 ms after illumination was switched off, which had previously led to the establishment of a photostationary state of intermediates (Genick et al., 1997a). As expected, in this structure of pB, isomerization had been completed. The steric hindrance between the thiol ester carbonyl group and the nearest aromatic ring proton causes a strained, nonplanar conformation of the cis isomer of the chromophore by -60°.

The aromatic ring of the chromophore has moved toward the protein surface at the position of the guanidinium group of Arg52 in pG. Arg52 has moved to a new position and consequently, the chromophore becomes exposed to the solvent and protonated. Neighboring residues of the trans chromophore have moved inward to partially fill the cavity left behind by the chromophore. A new hydrogen-bond is formed between Arg52 and the phenolic oxygen of the chromophore (Genick et al., 1997a). The exposure of two side-chain hydrogen donors of Arg52 and the chromophore to the solvent leads to a more positive electrostatic potential of the protein surface, which may trigger the signal transduction pathway to the flagellar motor (Genick et al., 1997a).

The most prominent changes in the structure of PYP are present in the blue-shifted, long-lived intermediate pB and can be described as the formation of a new hydrogen-bonding network in the chromophore-binding pocket, by replacement of hydrogen-bonds, due to rotational and translational movements of the chromophore and the surrounding amino acids. Surprisingly, one of these hydrogen-bonds in pB, which is formed and broken as a result of the rotation of the chromophore C-S bond, is already reformed to that present in pG. One of the aspects in the photocycle, which deserves further study, is the role of Glu46, as already mentioned above. In pG, the phenolic oxygen of the chromophore is deprotonated and the buried Glu46 is in the protonated, uncharged state (Borgstahl, Williams & Getzoff, 1995). In solution, these groups have a pKa of ~9 and ~4, respectively. Thus, solvent exposure will result in the protonation of the chromophore anion and a concomitant blue-shift (as observed in pB), while it will result in the deprotonation of Glu46. FTIR-studies on PYP films suggest that Glu46 is indeed deprotonated in pB (chapter 1.3), but crystallographic data indicate a shift of this residue toward the protein core (Genick et al., 1997a; Perman et al., 1998). The latter finding argues against deprotonation of Glu46, because in the protein core one would expect an uncharged, protonated Glu46. A second argument against the role of Glu46 as the proton donor of the chromorphic phenolate anion in PYP is that the E46Q mutant still displays a photocycle (Genick et al., 1997b). However, it cannot be excluded that in wild-type PYP the proton donor is Glu46, while the E46Q mutant protein receives a proton from the solvent or from an alternative amino acid residue. Furthermore, differences may be observed with respect to structural aspects of the photocycle of PYP in (concentrated) solutions and in crystals, as further discussed below.

The photocycle. One of the remaining questions concerning the photocycle of PYP deals with the order of events upon reformation to pG. The two key events that take place in the pB to pG transition are deprotonation of the phenolic oxygen and cis to trans re-isomerization. So far, no structural data on the time scale, associated with these structural events, are available. Spectroscopic data of PYP and its M100A mutant indicate that there is a light-dependent branch reaction in the photocycle of PYP, which makes pG recovery from pB much faster compared to the rate in the dark (Miller et al., 1993,
The photocycle of a xanthopsin

Devanathan et al., 1998). The reformation of pG in the light-dependent reaction takes less than 1 millisecond, while the reaction in the dark takes approximately 1 second (Hendriks & Hellingwerf, unpublished observations). This may indicate that re-isomerization of the chromophore to the trans isomer is the rate-limiting step in the recovery of pB. Probably, re-isomerization of the chromophore brings the phenolic 4-hydroxy group close to Glu46, allowing deprotonation.

The time-resolved X-ray crystallographic data obtained indicate that the structural changes associated with the photocycle of PYP are largely confined to the chromophore and its adjacent amino acids. There is however, an accumulating amount of evidence that larger conformational changes are associated with the photocycle of PYP in solution. First, a thermodynamic model, applied to the photocycle of PYP, accounting for heat capacity changes due to the exposure of hydrophobic contact surface (see also chapter 1.3), suggests that at least 40 of the 125 amino acids of PYP are involved in the pR to pB transition (Van Brederode et al., 1996). Second, FTIR-studies, which can probe light-induced structural changes in the backbone of a protein, indicate that large conformational changes occur during the pG to pB transition (Hoff et al., 1998). Third, studies on light-induced hydrogen-deuterium (H/D) exchange show that the number of buried sites, resisting H/D exchange, is reduced from 69 in pG to 53 in pB (Hoff et al., 1998). Fourth, NMR-studies indicate that pB exists as a collection of disordered, mutually interconverting conformers (Rubinstiten et al., 1998). This is in contrast to pG, which in solution, is structurally well-defined (Dux et al., 1998). In addition, NMR-studies show a differential rate of refolding of different parts of pB, starting with the central β-sheet and parts of the helical structures, followed by the region around the chromophore (Rubinsten et al., 1998). The differences, observed in the extent of the structural changes during the lifetime of pB between crystallographic data and spectroscopic studies on PYP in solution, may be explained by the relatively tight packing of the protein in a crystal lattice. However, the crystallographic and spectroscopic experiments described above provide complementary, rather than contradictory data.

Taking all these structural data into consideration, what is now the mechanism of a photocycle? What is essential in the design of a protein in order to be capable to show a self-contained light cycle? One could picture the protein as a spring, which winds up after the absorption of a photon and relaxes in the dark. Essential is to formulate a mechanism for winding up the spring: for photoactive yellow protein this is photoisomerization, in a two-bond isomerization process. In addition, a mechanism should be present to allow the subsequent relaxation to the ground state. After isomerization, the protein holds the chromophore in an iron grip, facilitating the thermal reisomerization process. The contraction of the chromophore upon isomerization, induces proton transfer and a charge redistribution in the protein, causing conformational changes that probably contribute to the lowering of the energy barrier for the eventual reisomerization. It is interesting to note that nature invented a similar strategy for the archaeal rhodopsin photosensors, where also proton transfer, induced by photoisomerization, plays an essential role in the photocycle. Reat et al. (1998) reported that bacteriorhodopsin can be considered as a hard core in a soft shell. This may also be true for photoactive yellow protein, where relatively small changes in the chromophore binding pocket, have a large impact on the more flexible structure around it, which may allow a mechanism to transfer a signal, informing the cell that there is blue-light.

References


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