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 ^{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 strong spectroscopic similarities between photoactive yellow protein and the sensory rhodopsins. This is the first demonstration of light-induced isomerization of a chromophore double bond as the photochemical basis for photosensing in the domain of Bacteria.

Key words: Capillary electrophoresis; ^{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 Eukarya rhodopsins 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 *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 Å resolution and shows that the protein has an α/β 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, $\lambda_{max} = 446$ nm) enters a photocycle in which a red-shifted intermediate, pR ($\lambda_{max} = 465$ nm), and a blue-shifted intermediate, pB ($\lambda_{max} = 355$ 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 *trans* configuration (referring to the vinyl protons) and in the deprotonated state (the *p*-hydroxy group), as was indicated by ^{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 *trans/cis* photo-isomerization of *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 (α/β fold vs. seven transmembrane α-helices) as well as in the chromophore (*trans* *p*-coumaric acid vs. all-*trans*-retinal).

2. Materials and methods

PYP from *E. halophila* was isolated as previously described [15] with minor modifications [7]. A sample of 1 ml, containing 17 μM 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-190 SA 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 PYP in the dark, however, the pH was not decreased below pH 4. Sodium dodecyl sulfate (SDS), 2% (w/v) 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 *p*-coumaric acid by ambient light. The pH of the sample was adjusted to 14 and the sample was incubated for 15 min at room temperature to

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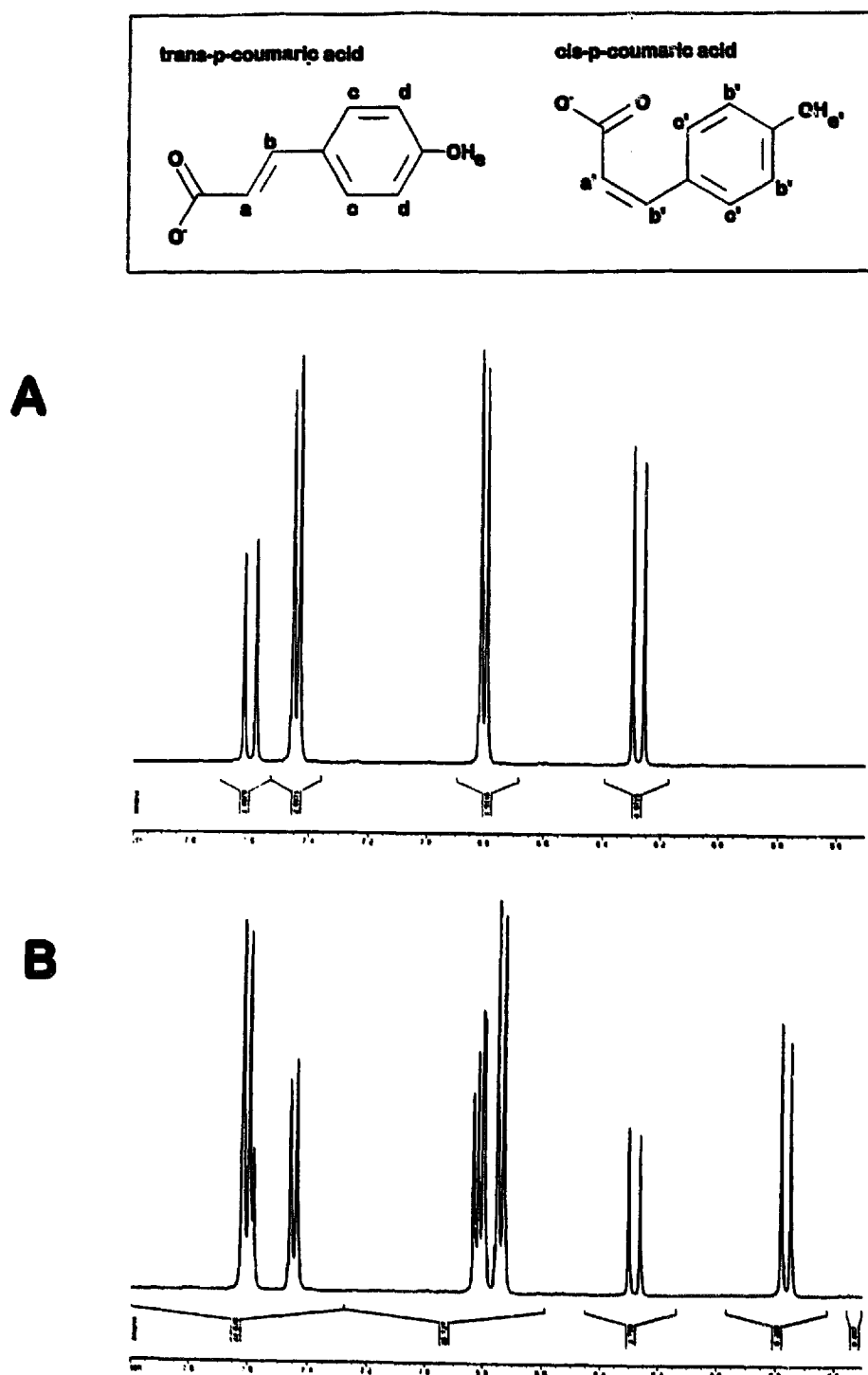
Fig. 1. (A) ¹H NMR spectrum of 60 mM *trans-p*-coumaric acid in CD₃OD. Assignment of protons (ppm): a 6.28, b 7.60, c 7.46, d 6.81, e not visible due to exchange of Hₑ with deuterium present in the solvent. Vertical numbers indicate the peak areas. (B) ¹H NMR spectrum after 3 h of UV irradiation, showing the resonances of the *trans* as 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 99.8
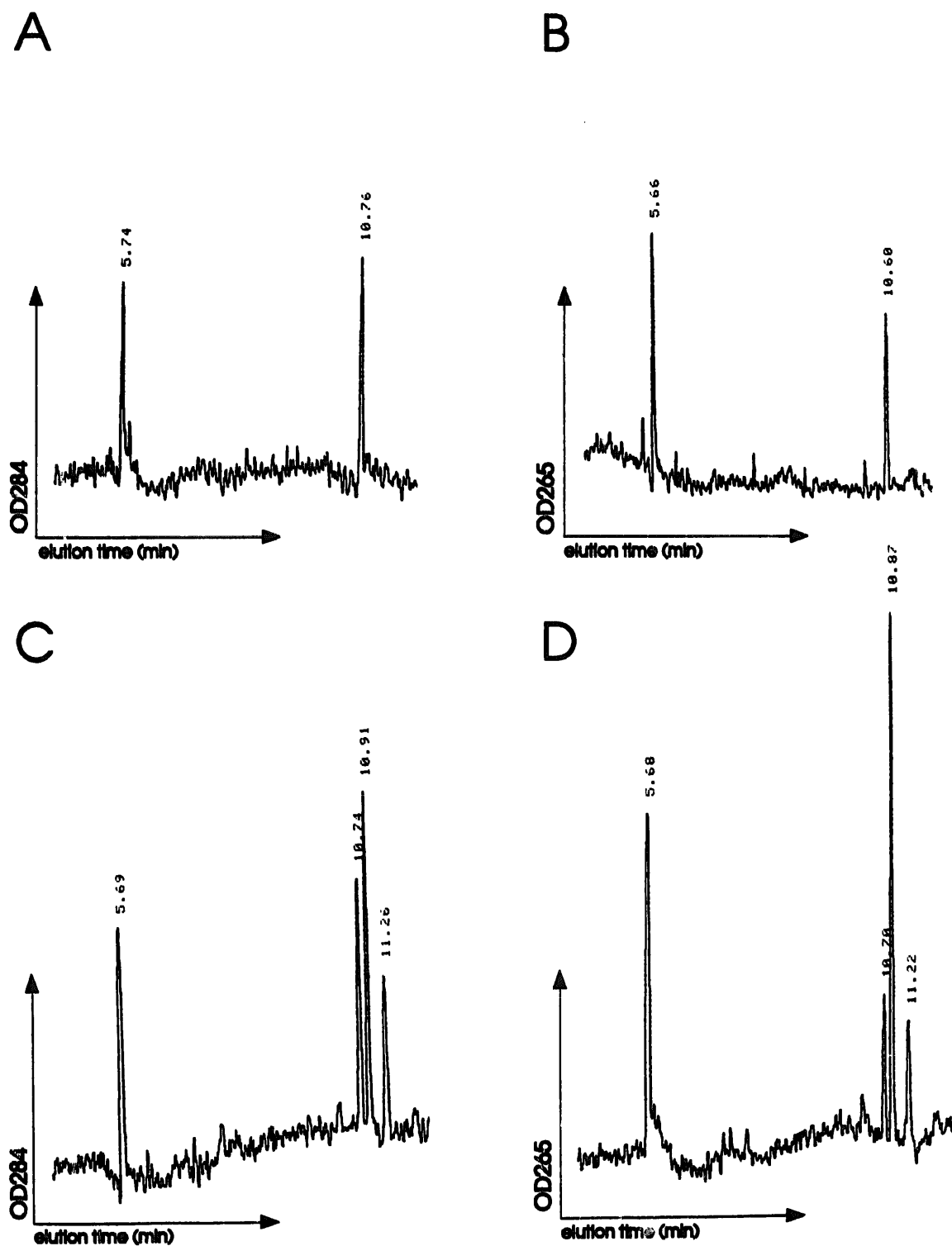
A

B

C

D

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% CD₃OD (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 (^{1}H NMR) were determined using a Bruker ARX 400 (400 MHz) spectro-

meter. 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

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

^{1}H 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 ^{1}H 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 ^{1}H 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 24 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 H$_2$O after ^{1}H 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 directly 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-
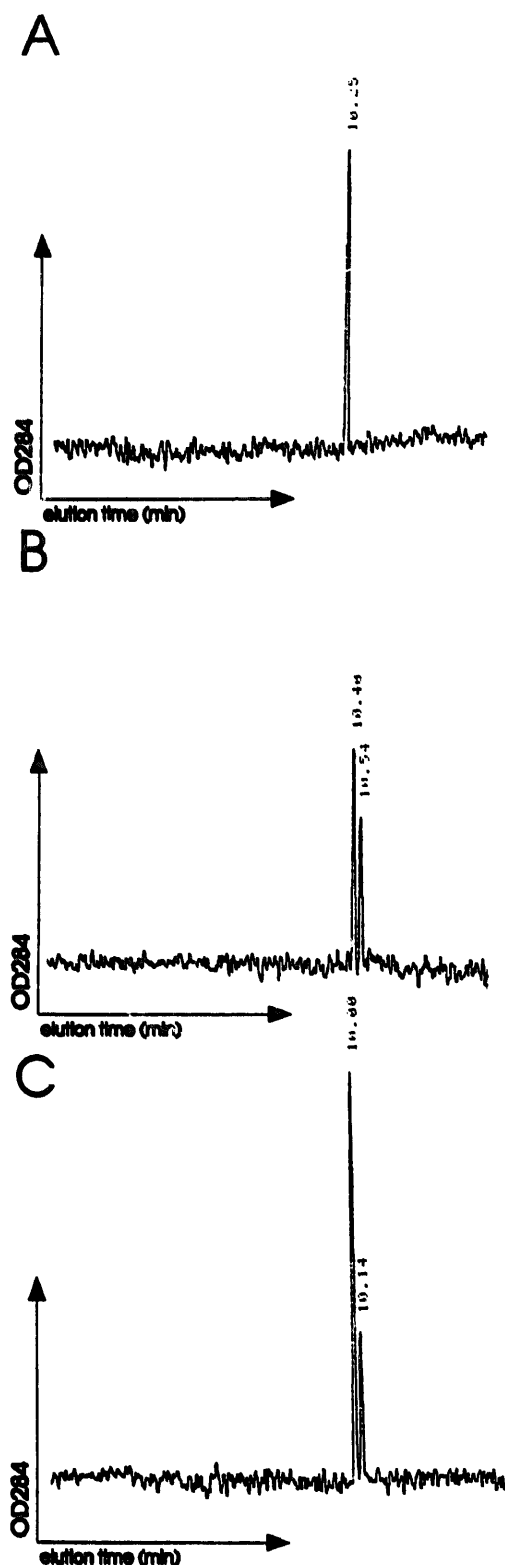

Fig. 3. Determination of the isomeric state of p-coumaric acid in photocycle intermediates of PYP. Upper part: electropherograms of p-coumaric acid extract from pG, recorded at λ_{max} of the trans isomer, 284 nm (A) and the cis isomer, 265 nm (B); the injection time is 0.1 min. Lower part: electropherograms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

ment shown in Fig. 2B, is calculated to equal 1:1.7 (trans:cis), identical to the ratio calculated from the NMR spectra. Furthermore, the effect of the solvent on this ratio, as checked by ^{1}H NMR spectra obtained from the same isomer mixture redissolved in D_2O, appeared to be negligible (data not shown). Thus, we show here an example of application of high-voltage zone electrophoresis for separation of femtomole amounts of nearly identical compounds, as demonstrated previously for other stereo-isomers [12,21]. However, our study is the first report on the electrophoretic separation of p-coumaric acid isomers, previously separated by more conventional chromatographic techniques, like paper, thin-layer and gas/liquid chromatography [18,22,23].

3.2. 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 ([1]; see also Fig. 3A,B), which was subsequently confirmed by ^{1}H 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 chromophore 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]):

$$k_1 \cdot [pG] - k_2 \cdot [pB] = 0 \qquad (1)$$

The rate constant k_1 refers to the rate of light-induced pG excitation. It equals $2.303 \cdot \Phi_{446}\varepsilon_{446}I_{446}$, with Φ_{446} and ε_{446} representing the quantum yield and molar extinction coefficient of pG and I_{446} the intensity of illumination at 446 nm. The factor 2.303 results from the conversion of the naperian to the decadic absorption coefficient. Φ_{446} equals 0.35 [25], ε_{446} is 4550 m^2 mol^{-1} [26] and I_{446}, as determined with a quantum sensor, is $\sim 3.0 \times 10^{-4}$ 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] is ~ 22, which implies that PYP is present for approx. 96% in the form of pB. As described above, only 74% of cis-p-coumaric acid (Fig. 3C,D) was observed to be present in the extracted chromophore, which can be explained by remaining catalytic activity of pB in 2% SDS, resulting in partial recovery of pG.

Extraction of the chromophore from pG, before and after exposure to light, followed by relaxation in the dark, only showed the presence of the trans isomer (Fig. 3A,B at 10.7 min and data not shown), indicating the reversibility of the photo-isomerization in the photocycle of PYP. In addition, free trans-p-coumaric acid was not converted during illumination with light of wavelengths above 430 nm, nor during the extraction procedure (data not shown). Co-elution of both compounds eluting at 10.7 and 10.9 min, present in the extract of the pB intermediate (Fig. 3C,D), was observed with the isomer mixture, which was obtained by UV irradiation of trans p-coumaric acid (see Fig. 2B for the electropherogram) and analyzed by ^{1}H NMR spectroscopy (Fig. 1B). This proves the correct assignment of the peaks at 10.7 and 10.9 min to trans-and cis p-coumaric acid, respectively. The peak at 5.7 min, present in all electropherograms of Fig. 3, indicates the presence of neutral compounds in the extract, which migrate with the same rate as the electro-osmotic flow in the capillary. Furthermore, an additional, small peak was observed at 11.2 min in the electropherograms shown in Fig. 3C,D, which was sometimes also present in electropherograms of control experiments, run in parallel. The significance of these latter findings remains unclear. Thus, we have shown that apoPYP contributes to the conversion of p-coumaric acid from the trans to the cis isomeric state during the photocycle, as a result of illumination with visible light, due to the tuning of the chromophore.

These results allow one to draw a more complete picture of the photocycle of PYP (Fig. 4). In pG the maximal absorbance of the chromophore is strongly shifted to the red (from 284 nm to 446 nm). This spectral tuning of the chromophore can be explained by contributions of (i) the thiol-ester linkage, causing a shift to 335 nm, (ii) the deprotonation of the p-hydroxy group of the chromophore, shifting the absorbance maximum to 410 nm, and (iii) unidentified aspects of the protein environment, resulting in the observed absorption maximum at 446 nm [2,4,27]. After excitation with blue light, pG is converted into the short-lived intermediate pR [6,7], in which the chromophore is probably in the cis configuration (low ε_{max}) and still deprotonated (long λ_{max}). In the dark, pR is subsequently converted to the long-lived intermediate pB (low ε_{max}, short λ_{max}), in which the chromophore is in the cis configuration, as shown here, and protonated. Finally, pG is recovered in the last step of the photocycle, at a rate
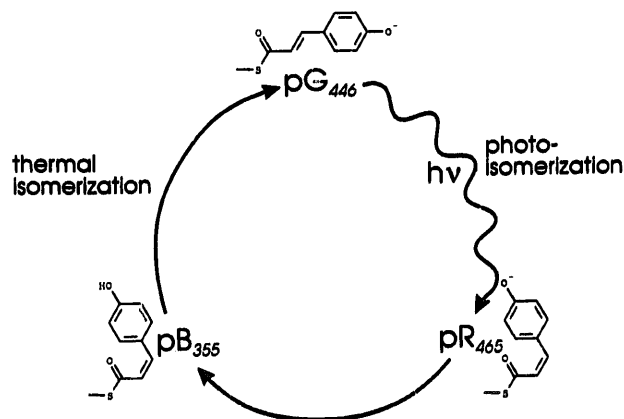

Fig. 4. Schematic representation of the photochemistry relevant for the photocycle of PYP. See text for further details.

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, trans/cis isomerization of cell wall bound 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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