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Glu46 Donates a Proton to the 4-Hydroxycinnamate Anion Chromophore During the Photocycle of Photoactive Yellow Protein†

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ABSTRACT: Photoactive yellow protein (PYP) is a photoreceptor containing a unique 4-hydroxycinnamic acid (pCA) chromophore. The *trans* to *cis* photoisomerization of this chromophore activates a photocycle involving first a short-lived red-shifted intermediate (pR), then a long-lived blue-shifted intermediate (pB), and finally recovery of the original receptor state (pG). The pCA chromophore is deprotonated in pG and protonated in pB, but the proton donor for this process has not yet been identified. Here we report the first FTIR spectroscopic data on pG, pR, and pB. The IR difference signals in the carbonyl stretching region of COOH groups (1700−1800 cm^{-1}) reveal that a buried carboxylic group close to the chromophore (i) is protonated in pG, (ii) develops a stronger hydrogen bonding in pR, and (iii) becomes deprotonated in pB. These signals are unambiguously assigned to Glu46, on the basis of the IR data and the 1.4 Å X-ray structure of PYP [Borgstahl et al. (1995) *Biochemistry 34*, 6278−6287]. Our data demonstrate that in pR Glu46 remains in hydrogen bonding contact with the negatively charged phenolic oxygen of pCA after chromophore photoisomerization. This strongly implies that the chromophore is isomerized to the 7-*cis* 9-s-*trans* conformation in pR, resulting from co-isomerization of both the C_7═C_8 and C_9−C_{10} bonds. In the pR to pB transition, Glu46 becomes deprotonated, concomitant with chromophore protonation. Therefore, we conclude that Glu46 functions as the proton donor for the protonation of pCA during the PYP photocycle. We propose a molecular mechanism in which intramolecular proton transfer in PYP leads to global protein conformational changes involved in signal transduction.

Proteins are able to perform an enormous variety of functions, while using only a limited number of underlying processes. One of these is proton transfer, found in a range of receptors and enzymes. Here we report on the light-induced proton movements during the photocycle of photoactive yellow protein (PYP).[1] PYP is the putative photoreceptor for the negative phototaxis response to blue light displayed by the photosynthetic eubacterium *Ectothiorhodospira halophila* (Sprenger et al., 1993). We propose that light-driven intramolecular proton transfer in PYP triggers large conformational changes. Such global conformational

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[1] Abbreviations: PYP, photoactive yellow protein; pR, red-shifted photocycle intermediate; pB, blue-shifted photocycle intermediate; pCA, *p*-coumaric acid (4-hydroxycinnamic acid); IR, infrared; FTIR, Fourier transform infrared; CaF_2, calcium fluoride.

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changes are a general theme in signal transduction, as they are thought to convert receptors into their signaling state.

PYP is a water soluble photosensor for blue light. The protein in the receptor state is characterized by a strong absorption band centered at 446 nm, resulting in its yellow color (Meyer, 1985). Absorption of a blue photon drives PYP into a cyclic chain of thermal processes (Meyer et al., 1987). The first observed photointermediate is a red-shifted species pR with λ_{max} at 465 nm. On a sub-millisecond time scale pR is converted into a blue-shifted species pB with λ_{max} at 355 nm. The initial state pG is recovered from pB on a sub-second time scale, completing the photocycle (Hoff et al., 1994b). This photocycle strongly resembles the one found in the archaeal sensory rhodopsins (Spudich et al., 1995). However, the chromophore of PYP is not a Schiff base-linked retinal molecule, but a novel cofactor: a 4-hydroxycinnamic acid molecule (Hoff et al., 1994a; Baca et al., 1994) covalently bound to Cys69 (Van Beeumen et al., 1993) via a thiol ester linkage (Hoff et al., 1996; see Figure 2A). The standard nomenclature for this chromophore is 4-hydroxyphenylpropenic acid and two commonly used names are 4-hydroxycinnamic acid and *p*-coumaric acid (pCA); the abbreviation pCA will be used below.

The structure of PYP in the receptor state has been reported at 1.4 Å resolution (Borgstahl et al., 1995). PYP has an α/β fold, consisting of a central antiparallel β-sheet with six strands, flanked on both sides by loops and helices. Interestingly, this structure resembles the one of a number of eukaryotic proteins involved in signal transduction (Borgstahl et al., 1995). The pCA chromophore is present as a phenolate anion in pG, as demonstrated by absorption (Baca et al., 1994; Hoff et al., 1996), X-ray (Baca et al., 1994), and resonance Raman (Kim et al., 1995) data. Key residues at the active site of PYP include Glu46 and Tyr42, whose OH groups hydrogen bond to the phenolic oxygen of pCA, and Arg52, whose positively charged guanidinium group has been proposed to stabilize and delocalized the buried negative charge on pCA (Borgstahl et al., 1995), contributing to a decrease in the apparent p*K* of pCA from 9.0 to 2.8 (Hoff et al., manuscript submitted).

The photosensing process of PYP starts with absorption of a blue photon by its buried pCA chromophore. In analogy with the retinal-containing phototransducers, it has been proposed that the photochemical reaction of the pCA chromophore involves a *trans* to *cis* isomerization (Hoff et al., 1994a). Indeed, in pG pCA is present in the *trans* form (Hoff et al., 1994a; Baca et al., 1994), and after illumination the *cis* form of pCA can be extracted from the protein (Kort et al., 1996b). A direct structural characterization of the pCA chromophore in pR has not been reported. Infrared pR − pG difference data support the hypothesis of chromophore photoisomerization (our unpublished results). Approximately half of the photon energy, 120 kJ/mol, is stored in pR (Van Brederode et al., 1995) and is subsequently utilized to drive thermal reactions leading to signaling state formation of PYP.

The pCA chromophore undergoes protonation and then deprotonation during the PYP photocycle. The protonation state of pCA is important for the folding pattern of PYP. In pG pCA is deprotonated (see above). The absorption maximum of pR is 465 nm, well above 400 nm of deprotonated pCA thiol ester model compound, indicating that its pCA remains deprotonated. The absorption maximum of pCA in pB is strongly blue-shifted to 355 nm, suggesting that pCA becomes protonated (Baca et al., 1994). The change in protonation state of pCA has been implied to trigger large protein conformational changes. However, so far the proton donor for chromophore protonation has not been identified. Clarification of this point is essential for understanding the structural basis of receptor activation of PYP.

Here we present the first Fourier transform infrared (FTIR) spectroscopic study on the pG, pR, and pB states of PYP. The results unambiguously demonstrate that a buried carboxylic group close to the chromophore is protonated in pG, structurally perturbed in pR, and becomes deprotonated in pB. These results together with examination of the X-ray structure of PYP allow the conclusion that Glu46 acts as the proton donor for the chromophore protonation in the pR to pB photocycle transition.

MATERIALS AND METHODS

Sample Preparation. The PYP samples used in this study were obtained by heterologous overexpression of a polyhistidine tagged derivative of apoPYP from *E. halophila* in *Escherichia coli*, followed by its *in vitro* reconstitution, as described in (Kort et al., 1996a; Kroon et al., in press). HoloPYP was then purified by Ni-column chromatography and stored at −70 °C after lyophilization. The purity of the resulting sample was confirmed by its purity index (the ratio between the absorbance at 280 and 446 nm) of 0.48, electrospray ionization mass spectrometry, and SDS−polyacrylamide gel electrophoresis. The holoPYP obtained in this way displays UV/vis absorption signals of the photocycle indistinguishable from those observed for the PYP isolated from *E. halophila* (our unpublished results).

PYP films for FTIR studies were prepared at pH 3.5 and 7.0. About 3 mg of freeze-dried PYP powder was dissolved in 200 μL of distilled deionized water. The buffer of the sample was changed from Tris to 20 mM citric acid at pH 3.5 (or to 10 mM potassium phosphate buffer at pH 7.0) by three washes using Microcon filters at 16000*g*. The final PYP concentration in the sample was approximately 4 mM. A homogeneous PYP film was made by spreading 10 μL of this PYP solution diluted with 20 μL of distilled deionized water over an area of 16 mm diameter on a calcium fluoride (CaF_2) plate (22 mm diameter, 2 mm thickness), immediately followed by vacuum drying in about 100 s. Prior to measurements, the PYP film was either rehydrated by adding 1 μL of water or deuterated by adding 1 μL of D_2O (99.9% pure from Sigma) sealed between two CaF_2 plates using an O-ring. The absorption of the PYP film was typically 0.6 at 446 nm.

FTIR Spectroscopy. Single-beam spectra of pG, pR, and pB were collected at 2 cm^{-1} resolution from 800 to 3800 cm^{-1} using a Mattson FTIR spectrometer (Galaxy 5000) with a KBr beamsplitter and a liquid nitrogen cooled MCT detector. The system was purged with dried nitrogen gas, prior to and during data collection, to reduce infrared absorption of water vapor. Each single-beam spectrum was averaged over 256 scans at a mirror velocity of 2.5 cm^{-1} per second. The noise level in the data reported in this paper is less than 0.1 milli-OD.

The pR − pG difference absorbance spectra of PYP films at pH 3.5 and 7.0 were measured at 80 K. At this temperature pR is thermally stable (Hoff et al., 1992). The

hydrated PYP film sandwiched between two CaF_2 plates was loaded to a copper sample holder and then mounted onto the cold finger of a liquid helium transfer optical dewar (Janis ST-100). The sample was cooled from room temperature to 80 K in the dark. The sample chamber of the cryostat was evacuated to a pressure below 1 milliTorr at 230 K. The sample temperature was monitored and regulated using a Lake Shore cryogenic temperature controller (DRC-82). Two CaF_2 windows (50 mm diameter, 3 mm thickness) on the cryostat allowed the infrared probe beam and the actinic blue light to pass through. Single beam spectra of pG were first collected at 80 K after temperature is stabilized for at least 10 min. The pR state was generated by illuminating the sample with blue light (a 150 Watt halogen tungsten light source (Cuda I-150), filtered with a water bath heat filter and a 410 nm interference filter with 10 nm bandwidth) using a light guide. A photo-equilibrated state between pR and pG was reached in about 1 min of illumination, and single beam spectra were collected immediately after illumination. The pR − pG difference spectra were calculated from the spectra recorded immediately before and after illumination.

The pB − pG difference spectra of PYP films at pH 3.5 and 7.0 and pD 3.5 were collected at 295 K. The sample pH of 3.5 was chosen to increase the accumulation of pB under continuous illumination, since the lifetime of pB is strongly prolonged at pH values below 5 (Hoff et al., manuscript submitted). The pH 7.0 sample allowed examination of the solvent accessibility of carboxylic groups. The pB state was generated by continuous blue light illumination using the halogen tungsten lamp described above but with a broadband filter. The pB − pG difference spectra were computed from the single-beam spectra recorded immediately before and during illumination.

Data Analysis. The pR − pG IR signals between 1760 and 1715 cm^{-1} were fit to two Gaussian bands using Spectra Calc. (Galactic Ind.). The 1.4 Å X-ray structure of PYP (2Phy.pdb from the Brookhaven Protein Data Bank) was examined using Insight II (Biosym) on a Silicon Graphics Indigo 2 workstation.

RESULTS AND DISCUSSION

IR difference spectroscopy is a sensitive technique to probe proton transfer processes involving protonated carboxylic groups (Rothschild, 1992). The C═O stretching mode of COOH groups exhibits characteristic frequencies around 1710−1760 cm^{-1} (Nakanishi & Solomon, 1977; Venyaminov & Kalnin, 1990), well separated from those of COO^- groups (around 1400 and 1570 cm^{-1} for a symmetric and an asymmetric O−C−O stretching, respectively; Zundel, 1988), as well as those of the chromophore and other groups of the protein. The IR difference signals in the COOH stretching region have been extensively studied concerning proton transfer events in bR [reviewed in Rothschild (1992) and Maeda (1995)]. Here we report an FTIR spectroscopic study on the protonation state of carboxylic groups that are structurally active during the PYP photocycle and identification of the proton donor for the chromophore protonation in pB formation.

Structural Perturbation of Glu46 in pR. Figure 1A shows the light-induced pR − pG difference absorption spectra of PYP at pH 3.5 and 7.0 (80 K). The two spectra are very similar, exhibiting a single negative band, peaked at 1740 cm^{-1}, and a single positive band, peaked at 1732 cm^{-1}. In these difference spectra, the negative bands represent signals originating from the vibrational modes in pG and the positive bands are signals from pR. The detection of a single negative band reveals the presence of a *protonated* carboxylic group in pG, which is structurally active during pCA photoisomerization. Observation of both a positive band and a negative band designates either protonation of a COO^- group (giving rise to a positive band) accompanied with deprotonation of a COOH group (resulting in a negative band) in the pG to pR transition or the structural perturbation of a COOH group leading to a peak shift. The data strongly favor the latter option, since the positive and negative bands show features characteristic of a peak shift: similar band shapes and close proximity of their peak positions (Figure 1A). Therefore, we conclude that a single protonated carboxylic group is structurally perturbed in the pG to pR transition.

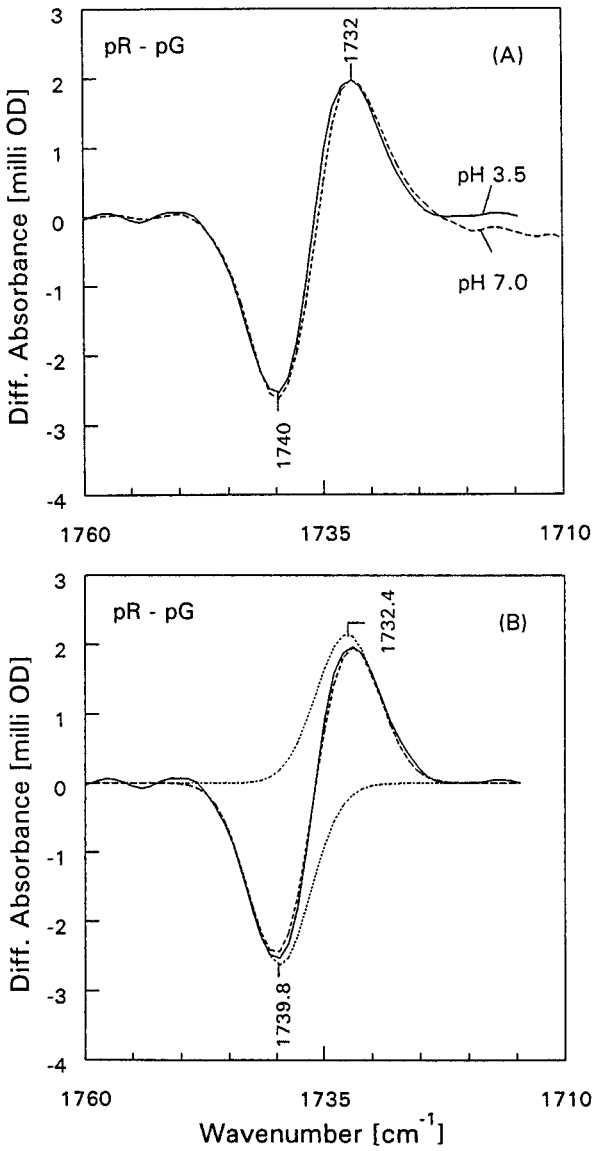

FIGURE 1: Structural perturbation of Glu46 in the pG to pR transition. (A) The pR − pG difference spectrum of PYP at 80 K at pH 3.5 (solid line) and 7.0 (dashed line). (B) A non-linear least-square fit of the pH 3.5 data to two Gaussians.

The data presented in Figure 1A allow two conclusions concerning the location of the COOH group involved. First, this carboxylic group must be buried, since the p*K* of this group is strongly shifted from the p*K* of exposed carboxylic

groups (approximately 4) to a much higher value, so that it remains protonated at pH 7. Second, this group must be in the direct vicinity of the pCA chromophore: structural perturbations in PYP caused by pCA photoisomerization at 80 K are restricted to the pCA binding site, since global conformational changes are prohibited in the frozen protein (Frauenfelder et al., 1990; Iben et al. 1989).

To assign the observed C═O stretching signals to a specific carboxylic group in PYP, we have examined its 1.4 Å resolution structure (Borgstahl et al., 1995). PYP contains a total of 20 carboxylic groups from the side chains of seven glutamic and 12 aspartic residues (E2, E9, E12, E46, E74, E81, E93, D10, D19, D20, D24, D34, D36, D48, D53, D65, D71, D97, D116) plus the C-terminus (Van Beeumen et al., 1993). To identify buried carboxylic groups, the solvent-accessible surface of each carboxylic group in pG has been examined using a molecular probe of 1.4 Å radius with Insight II. A buried carboxylic group is defined as having zero solvent-exposed surface. We found that (i) the carboxylic group of Glu46 is buried and (ii) all other 19 carboxylic groups are exposed to solvent. Since exposed carboxylic groups cannot be protonated at pH 7, the buried Glu46 is, therefore, the unique candidate contributing to the detected IR signals of a COOH group in pG and pR at neutral pH. In addition, the carboxylic group of Glu46 is located at the pCA binding site in hydrogen bonding contact with the phenolic oxygen of pCA, and therefore is likely sensitive to structural perturbation by pCA photoisomerization. Therefore, we conclude that Glu46 is protonated in pG and pR, and is structurally perturbed in the pG to pR transition.

Glu46 Remains Hydrogen Bonded to the Phenolic Oxygen of pCA in pR. The changes in hydrogen bonding interaction between the hydroxyl group of Glu46 and the phenolic oxygen of pCA in the pG to pR transition have important consequences for the mechanism of pCA photoisomerization and subsequent protonation in the PYP photocycle. In pG the OH group of Glu46 is hydrogen bonded to the negatively charged phenolic oxygen of pCA (Borgstahl et al., 1995). However, this hydrogen bond may be disrupted in pR due to pCA photoisomerization. As depicted in Figure 2, photoisomerization of pCA may result in either (i) a 7-*cis* 9-s-*cis* configuration (Figure 2B) by isomerization of the C_7═C_8 bond or (ii) a 7-*cis* 9-s-*trans* structure (Figure 2C) by co-isomerization of the C_7═C_8 and C_9—C_{10} bonds. Formation of the 7-*cis* 9-s-*cis* isomer of pCA would result in the loss of the hydrogen bonding contact between Glu46 and the phenolic oxygen of pCA, while formation of the 7-*cis* 9-s-*trans* pCA isomer would leave this hydrogen bonding intact. These two mechanisms of pCA photoisomerization can be distinguished based on the FTIR data, as discussed below.

For a COOH group, the vibrational frequency of C═O stretching mode is sensitive to the hydrogen bonding interactions of its carbonyl oxygen with a hydrogen bond donor(s) and/or hydroxyl group with a hydrogen bond acceptor(s). Without hydrogen bonding, the C═O stretching frequency is high, around 1760 cm^{-1} (Nakanishi & Solomon, 1977). Upon hydrogen bonding the C═O stretching frequency is down-shifted, to as low as 1715 cm^{-1} (Zundel, 1988). Therefore, the C═O stretching frequency of Glu46 can be employed as a sensitive spectral probe of the hydrogen bonding interaction between Glu46 and pCA in pR, broken or intact, after pCA photoisomerization (Figures 2B and 2C).

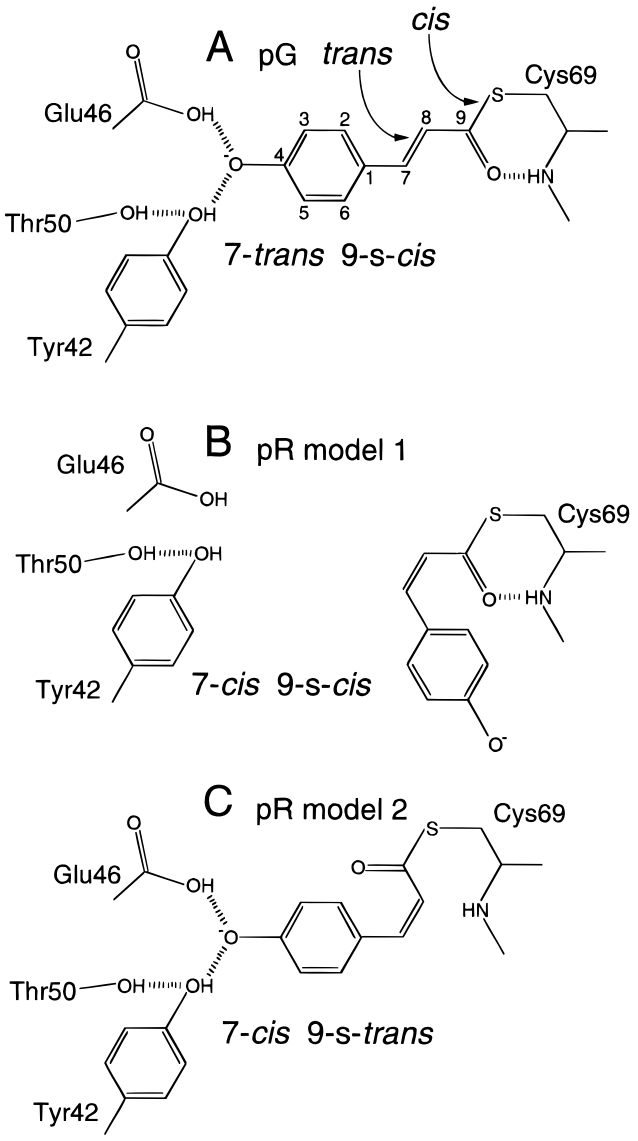

FIGURE 2: Mechanism of pCA photoisomerization. (A) In pG the pCA in 7-*trans* 9-s-*cis* form interacts with Glu46, Tyr42, and Arg52. During the pG to pR transition, the pCA is photoisomerized into either (B) a 7-*cis* 9-s-*cis* isomer or (C) a 7-*cis* 9-s-*trans* configuration.

The peak frequencies of the Glu46 C═O stretching mode in pG and pR have been determined by a nonlinear least-square fit of the IR data at pH 3.5 to two Gaussian bands: 1739.8 cm^{-1} in pG, and 1732.4 cm^{-1} in pR (Figure 1B). The peak frequency at 1740 cm^{-1} of Glu46 in pG is significantly lower than the value of 1760 cm^{-1} for non-hydrogen bonded carboxylic groups. This result shows that Glu46 is hydrogen bonded in pG, consistent with the X-ray structure of PYP. In pR the C═O stretching frequency of Glu46 is further down-shifted to 1732.4 cm^{-1}, revealing that the COOH group of Glu46 remains hydrogen bonded after pCA photoisomerization. This strongly argues against the formation of a 7-*cis* 9-s-*cis* pCA isomer. The possibility that in pR the COOH group of Glu46 forms a new hydrogen bond with a group other than the phenolic oxygen of pCA is ruled out based on the facts that (i) no alternative hydrogen bonding acceptor nor hydrogen bonding donor for the COOH of Glu46 is present within 4.0 Å center-to-center distance in pG, and the closest alternative groups for hydrogen bonding are its own backbone nitrogen and oxygen at 4.10 and 4.17 Å, respectively, but sterically not allowed, and

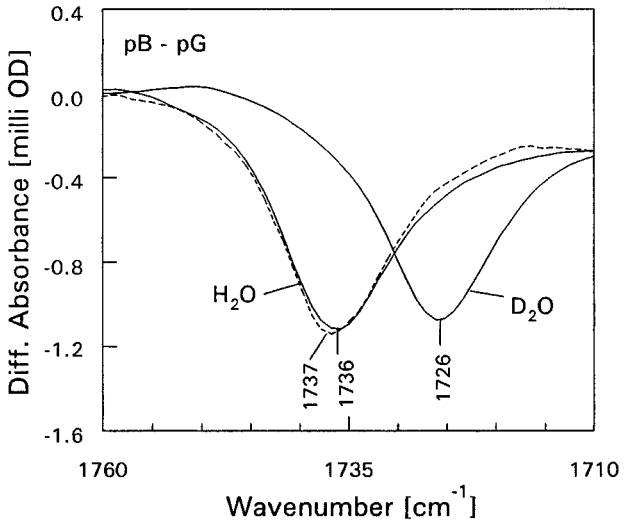

FIGURE 3: Deprotonation of Glu46 in pB. The pB − pG difference spectra of PYP at pH 3.5 (solid line) and 7.0 (dashed line) and pD 3.5 at 295 K, each shows a single negative band indicating the deprotonation of Glu46. In pG the C═O stretching of Glu46 is at 1736.0 cm^{-1} at 295 K and is shifted to 1739.8 cm^{-1} at 80 K (Figure 1), possibly due to a small temperature induced change in hydrogen bonding interaction between Glu46 and pCA.

various other backbone atoms at distances of 4.3−4.9 Å; (ii) the hydrogen bonding group for Glu46 in pR is expected to be in close to 3.0 Å distance (Sippl, 1996), ideal for forming a strong hydrogen bonding as indicated from its large down-shift in the C═O stretching frequency; and (iii) rearrangements of the protein backbones to establish a new hydrogen bonding of Glu46 are prohibited at 80 K. In addition, photoisomerization of pCA from the 7-*trans* 9-s-*cis* to the 7-*cis* 9-s-*cis* conformation would lead to a large displacement of the phenolic oxygen and would force Arg52 and Val66 completely out of their positions. Such large structural rearrangements require global conformational changes in PYP, which are frozen at 80 K (Frauenfelder et al., 1990; Iben et al. 1989). This line of reasoning strongly disfavors the formation of the 7-*cis* 9-s-*cis* pCA conformation.

The consequences of pCA photoisomerization to the 7-*cis* 9-s-*trans* structure (Figure 2C) are more favorable. With this mechanism, the positions of both the phenolic ring of pCA and the backbone of Cys69 are relatively fixed (Figure 2C), so that the resulting atomic displacements of pCA are small and can proceed without global conformational changes. This closely resembles the mechanism of retinal photoisomerization in rhodopsin (Warshel, 1976) and in bacteriorhodopsin (Smith et al., 1985; Rothschild, 1992) where the β-ionone ring of the retinal and the backbone of the Lys, which covalently bonds the retinal via a Schiff base linkage, are relatively fixed. In this mechanism, the changes in the structural environment of Glu46 during the pG to pR transition are expected to be small. This is consistent with the small peak-shift of the Glu46 C═O stretching. Therefore, we conclude that the hydroxyl group of Glu46 remains hydrogen bonded to the phenolic oxygen of pCA in pR, which implies that the pCA chromophore is photoisomerized from 7-*trans* 9-s-*cis* to 7-*cis* 9-s-*trans* conformation in the pG to pR transition.

Deprotonation of Glu46 in pB. The pB − pG difference absorbance spectra of hydrated and deuterated PYP films are shown in Figure 3. For hydrated PYP, a single negative band is observed peaked at 1737 cm^{-1} at pH 7.0, which is slightly shifted to 1736 cm^{-1} at pH 3.5. Upon deuteration of the pH 3.5 PYP sample, this band is down-shifted by 10 cm^{-1} to 1726 cm^{-1}, in line with the expected deuteration shift for the C═O stretching of COOH groups (Dollinger et al., 1986). The sensitivity of the C═O stretching to deuteration arises from its vibrational coupling with the C−OH stretching. This observed sensitivity of the negative band to deuteration excludes a possible contribution from the carbonyl stretching in the thiol ester linkage to the observed signal. As for the band assignment of the C═O stretching signal in pG and pR, the negative signal in the pB − pG difference spectrum can be unambiguously assigned to Glu46.

A noticeable result is that the pB − pG difference spectrum shows a single negative band, but no positive bands in the C═O stretching region. This lack of positive signals demonstrates that the C═O stretching mode of Glu46 in pB is shifted out of the 1700−1800 cm^{-1} frequency region. Such a large shift cannot be interpreted in terms of a structural perturbation of the protonated Glu46, but is expected if Glu46 would deprotonate (a symmetric and an asymmetric COO^- stretching are at much lower frequencies around 1400 and 1570 cm^{-1}, respectively; Zundel, 1988). These large down-shifts in frequency (by 150−300 cm^{-1}) are due to a reduction in the bond order from 2 for the C═O stretching in COOH to 1.5 for the O−C−O stretching in COO^-, and thereby the significantly decreased force constants. The signals for COO^- stretching in the regions around 1570 and around 1400 cm^{-1} are complicated by contributions from other groups in the chromophore and protein and are generally not analyzed in studies of proton transfer processes involving protonated carboxylic groups. This problem does not occur in the region of 1700−1800 cm^{-1}, where the signals can be unambiguously assigned to protonated carboxylic groups. Therefore, the presence of a negative band and the absence of a positive band in the pB − pG difference IR data conclusively demonstrate that Glu46 is deprotonated in pB.

Glu46 Donates a Proton to the Phenolic Oxygen of pCA in pB. The FTIR data reveal that the Glu46 signal shows a clear structural progression during the PYP photocycle (Figure 4). In pG Glu46 is protonated and hydrogen bonded to the phenolic oxygen of pCA. In pG to pR transition, pCA photoisomerization leads to an enhanced hydrogen bonding between Glu46 and pCA, as evidenced from a 7.4 cm^{-1} further down-shift in the Glu46 C═O stretching frequency. A stronger hydrogen bonding lowers the energy barrier for proton transfer from Glu46 to pCA (Figure 4). In the pR to pB transition, Glu46 becomes deprotonated, concomitant with the protonation of pCA (Baca et al., 1994; Hoff et al., 1996). This fact, together with the strong hydrogen bonding between Glu46 and pCA in pR, strongly argues that a direct proton transfer occurs from the hydroxyl group of Glu46 to the phenolic oxygen of pCA. Therefore, we conclude that *Glu46 is the proton donor for the protonation of the negatively charged phenolic oxygen of pCA in the pR to pB photocycle transition.*

A Molecular Mechanism for Receptor Activation of PYP. Signal transduction in general involves large changes in receptor conformation during the stimulus-induced transition from the receptor state to the signaling state. Recently formulated considerations concerning electrostatic interactions between buried charged groups involved in protein

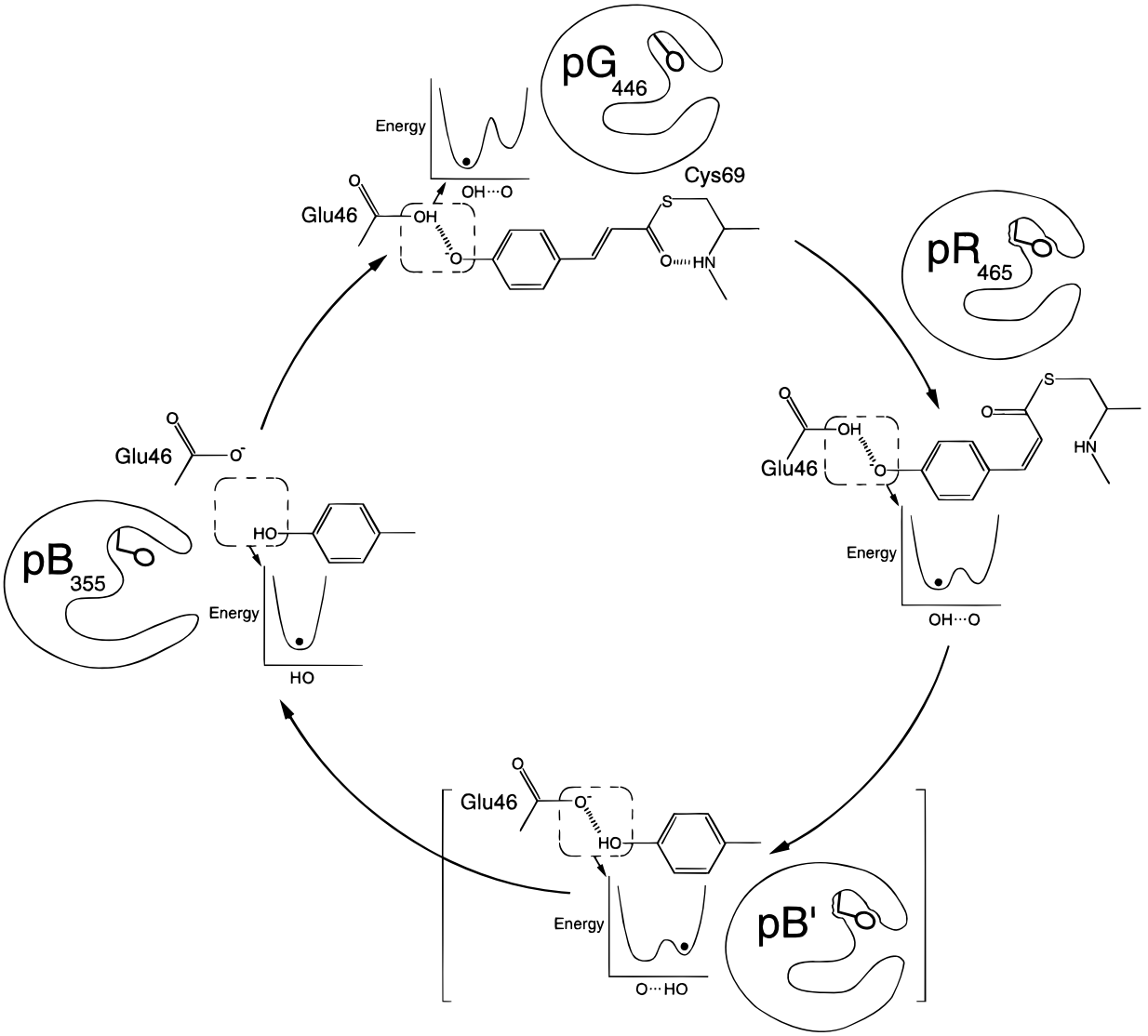

FIGURE 4: Schematic representation of the proposed molecular mechanism for receptor activation during the PYP photocycle: (i) in the pG to pR photoreaction, pCA is photoisomerized from a 7-*trans* 9-s-*cis* to a 7-*cis* 9-s-*trans* configuration, resulting in a stronger hydrogen bonding between Glu46 and pCA, while the global conformation of PYP remains unchanged; (ii) in the pR to pB′ transition, a direct proton transfer occurs from Glu46 to pCA, while the global conformation is still unchanged; (iii) the altered distribution of buried charges in pB′ triggers a large conformational change, leading to the formation of pB, the likely signaling state; (iv) recovery of pG from pB involves thermal pCA reisomerization, deprotonation of pCA, reprotonation of Glu46, and a conformational resetting of PYP. Energy potential curves are proposed to depict the hydrogen bonding interaction between Glu46 and the phenolic oxygen of the pCA chromophore and to illustrate the physical basis for this intramolecular proton transfer process during the PYP photocycle.

folding [see Honig and Yang (1995) and Dill (1990)] lead to an interpretation of how intramolecular proton transfer can result in signal transduction. Buried charges in general have a strong destabilizing effect on a folded protein, and can only be stabilized by interactions with oppositely charged and polar groups in specific conformations of the protein. In PYP, such an interaction occurs between the positively charged guanidinium group of Arg52 and the negatively charged phenolic ring of pCA in pG (Borgstahl et al., 1995; Hoff et al., manuscript submitted). Here we propose a molecular mechanism in which light-induced intramolecular proton transfer from Glu46 to the pCA chromophore triggers receptor activation during the PYP photocycle as depicted in Figure 4.

In pG, the pCA chromophore is deprotonated and in the 7-*trans* 9-s-*cis* conformation. This buried negative charge on pCA is delocalized over the phenolic oxygen and ring, and is stabilized by specific electrostatic interaction with the positively charged guanidinium group of Arg52, as well as by hydrogen bonding interactions with the OH groups of Glu46 and Tyr42 in the pCA binding pocket (Figure 2A). Upon absorption of a blue photon, the pCA chromophore is photoisomerized into a 7-*cis* 9-s-*trans* configuration, leading to the formation of pR. As a result, the interactions between the pCA chromophore and the active site residues are perturbed, while the global conformation of PYP remains unchanged. In particular, the hydrogen bonding interaction between the OH of Glu46 and the phenolic oxygen of pCA is retained and strengthened in pR, lowering the barrier for direct proton transfer between these groups. This allows Glu46 to donate a proton to the phenolic oxygen of pCA, forming a putative unstable pB′ state. This local proton transfer event neutralizes the pCA, generates a new buried unstable charge (the COO^- of Glu46), and abolishes the $pCA^-/Arg52^+$ interaction. These dramatic changes in intramolecular electrostatic interactions strongly destabilize the global conformation favorable for pG and pR and trigger a large conformational change (Meyer et al., 1989; Van

Brederode et al., 1996; our unpublished FTIR results) that leads to the formation of pB, a likely signaling state (Figure 4), and thereby receptor activation. The receptor state (pG) is recovered from pB at a much longer time scale (250 ms) than the formation of the signaling like pB state (250 μs), through a complex process, consisting of thermal reisomerization of pCA to the 7-*trans* 9-s-*cis* conformer, deprotonation of pCA, reprotonation of Glu46, and resetting of the global conformation of PYP.

In this proposed mechanism of receptor activation in PYP, Glu46 plays a central role in triggering signaling state formation. The key event in this triggering process is the proton transfer from the hydroxyl group of Glu46 to the negatively charged pCA during pB formation. As a proton donor, Glu46 changes its protonation state during the light-activated transition from pG to pB. This is accomplished by shifting its p*K* during the photocycle, through altered interactions with its neighbouring groups. Our FTIR data show that (i) Glu46 is protonated in pG, displaying an anomalously shifted p*K* to above 7, significantly above the normal p*K* of exposed Glu residues, and (ii) Glu46 is deprotonated in pB, with a p*K* shifted in the opposite direction to below 3.5. The functional importance of Glu46 as the proton donor to pCA is further supported by its evolutionary conservation among the four members of the PYP family with known sequences, all found in Proteobacteria (Koh et al., 1995; Kort et al., 1996a).

The data reported here show that the protonation of pCA in the pR to pB transition occurs through an *intramolecular* proton transfer process with Glu46 as the proton donor. On a time scale similar to the one of the pR to pB transition, proton uptake from the solvent was observed in a study employing a pH indicator dye (Meyer et al., 1993). The subsequent proton release to the solvent takes place during the pB to pG transition. The residue(s) involved in proton uptake from and release to the bulk has not been identified. One possibility is that the proton taken up from the solvent provides a counterion to stabilize the new buried negative charge on Glu46, which is created during pB formation.

Perspectives. Light-induced intramolecular proton transfer between the chromophore and its proton donor (or acceptor) at the chromophore binding pocket has also been proposed to play an important role in receptor activation of rod rhodopsin from animal eyes (Rao and Oprian, 1996) and of the sensory rhodopsins from *Halobacterium salinarium* (Spudich et al., 1995). In both rhodopsin and sensory rhodopsins, the retinal chromophore is covalently bound to the ϵ-nitrogen of a Lys via a protonated Schiff-base linkage. Absorption of a photon by the chromophore leads to *cis*−*trans* retinal photoisomerization, subsequent deprotonation of the Schiff base, and the formation of the signaling states. In rhodopsin, the negatively charged Glu113 acts as the proton acceptor, which forms a salt bridge with the positively charged Schiff base in the receptor state. Disruption of this salt bridge is important for receptor activation in rod rhodopsin. In the archaeal sensory rhodopsin II, the protonated Schiff base counterion and proton acceptor has been recently identified as Asp73, and disruption of their salt bridge is also important for receptor activation (personal communication, J. L. Spudich).

The first FTIR data reported here on the photocycle of PYP, a novel type photoreceptor different from the extensively studied photoreceptors in the rhodopsin family, suggest that light-induced chromophore photoisomerization followed by proton transfer between the chromophore and protein residues forms a general theme in receptor activation. Furthermore, this opens the way to future FTIR studies of PYP over a broad spectral region (800−3800 cm^{-1}) using isotopic labeling, chromophore analogs, and site-specific mutations. We expect that this, together with the high-resolution crystal structure of pG, will yield rich information on chromophore photoisomerization, chromophore−protein interactions involved in color regulation and energy storage, as well as large protein conformational changes for receptor activation, and will lead to a deep understanding of the mechanism of signal transduction at the molecular level.

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