Water Structural Changes Involved in the Activation Process of Photoactive Yellow Protein†

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ABSTRACT: Fourier transform infrared (FTIR) spectroscopy was applied to the blue-light photoreceptor photoactive yellow protein (PYP) to investigate water structural changes possibly involved in the photocycle of PYP. Photointermediates were stabilized at low temperature, and difference IR spectra were obtained between intermediate states and the original state of PYP (pG). Water structural changes were never observed in the >3570 cm^{-1} region for the intermediates stabilized at 77−250 K, such as the red-shifted pR and blue-shifted pB intermediates. In contrast, a negative band was observed at 3658 cm^{-1} in the pB minus pG spectrum at 295 K, which shifts to 3648 cm^{-1} upon hydration with $H_2{}^{18}O$. The high frequency of the O−H stretch of water indicates that the water O−H group does not form hydrogen bonds in pG, and newly forms these upon pB formation at 295 K, but not at 250 K. Among 92 water molecules in the crystal structure of PYP, only 1 water molecule, water-200, is present in a hydrophobic core inside the protein. The amide N−H of Gly-7 and the imidazole nitrogen atom of His-108 are its possible hydrogen-bonding partners, indicating that one O−H group of water-200 is free to form an additional hydrogen bond. The water band at 3658 cm^{-1} was indeed diminished in the H108F protein, which strongly suggests that the water band originates from water-200. Structural changes of amide bands in pB were much greater in the wild-type protein at 295 K than at 250 K or in the H108F protein at 295 K. The position of water-200 is >15 Å remote from the chromophore. Virtually no structural changes were reported for regions larger than a few angstroms away from the chromophore, in the time-resolved X-ray crystallography experiments on pB. On the basis of the present results, as well as other spectroscopic observations, we conclude that water-200 (buried in a hydrophobic core in pG) is exposed to the aqueous phase upon formation of pB in solution. In neither crystalline PYP nor at low temperature is this structural transition observed, presumably because of the restrictions on global structural changes in the protein under these conditions.

Photoactive yellow protein (PYP)[1] is the 14 kDa cytosolic photoreceptor functioning in negative phototaxis in the phototrophic bacterium *Ectothiorhodospira halophila* (*1*−*4*). Its negatively charged 4-hydroxycinnamyl chromophore is covalently attached to Cys-69 through a thioester linkage (*5*−*10*). After photon absorption (absorption maximum at 446 nm), ground-state PYP (pG) converts to a red-shifted intermediate (pR). Then, blue-shifted pB appears in 0.1 ms and returns to pG in 500 ms (*11, 12*). pB is the presumed signaling state, considering its lifetime, though a cognate transducer has not been identified yet.

PYP is a soluble protein, and its crystallographic structure has been reported (*13*). Moreover, time-resolved X-ray diffraction has been applied to PYP, which resolved the structures of both pR (*14*) and pB (*15*). These results visualize the structure of the protein at work. The snapshots of the intermediates lead to better understanding of the molecular mechanism of light-induced changes in protein structure, required for signal transduction in PYP. Furthermore, the structural homology of PYP to the PAS domains (conserved sequence motifs for functioning in sensing and signal transduction) has been revealed (*16*−*19*). Therefore, PYP is a powerful model system for studies in signal transduction.

Determination of the X-ray crystallographic structure of pB (*15*) provided important information on the structural change in the PYP protein required for signaling. That is, the crystallographic structure of pB revealed that the structural changes are limited to a small region in the vicinity of the chromophore (*15*). The mechanism of the signal transduction in PYP was generalized for the proteins pos-

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[1] Abbreviations: PYP, photoactive yellow protein; FTIR, Fourier transform infrared.

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sessing the PAS domain (*16*). This fact indicates that the molecular recognition surface of PYP with the downstream transducer protein must be near the chromophore, because there is little change in other regions. However, various spectroscopic studies of PYP in solution, in which global changes were observed (*20*−*25*), have reported opposite results. For instance, structural analysis of pB by multinuclear, multidimensional NMR showed that the protein structural changes extend over the entire protein (*20*, *21*). Thermodynamic analysis showed that buried hydrophobic sites are exposed during pB formation, leading to an increase in heat capacity (*22*, *23*). Fourier transform infrared (FTIR) spectroscopy showed considerable changes in amide bands, that reflect backbone changes (*24*). The measurement of light-dependent pH changes suggests that the side chain of His-108, which is remote from the chromophore, changes from a buried to an exposed position upon pB formation (*25*). These data, measured on PYP in solution, provide significantly different results for the structure of pB, as obtained in the time-resolved Laue diffraction experiments on crystalline PYP (*15*). Therefore, the question arises: To what extent does PYP change its structure under physiological conditions? This question is important, because global structural changes in PYP may be required for transducer activation, in a region other than the chromophore binding site. Thus, the detailed analysis of the structure of intermediates of PYP in solution becomes important even when their atomic structure in a crystalline environment has already been reported.

Previously, we have studied structural changes of functional intermediates of retinal proteins by means of Fourier transform infrared (FTIR) spectroscopy. In particular, experimental observation of water structural changes has provided useful information in the structure−function studies of proton-pumping bacteriorhodopsin (*26*−*39*), chloride-pumping halorhodopsin (*40*), and visual rhodopsin (*41*−*47*). In these retinal proteins, water molecules are present in the active center (i.e., near the chromophore), where they play a crucial functional role. A role of internal water molecules has been also shown in enzymes (*48*).

Unlike retinal proteins, no water molecules are found inside PYP, except in areas near the protein surface (*13*). In particular, water molecules are absent around the chromophore, suggesting that they do not have an important role in the primary reaction of the chromophore [i.e., photoisomerization (*8*)]. Therefore, the following question comes up: Are water structural changes not involved in the activation process of PYP?

In the present study, we applied FTIR spectroscopy to hydrated film samples of PYP to investigate the possible role of water structural changes in the photocycle of PYP. Photointermediates were trapped at low temperature, and alterations in hydrogen bonding of water molecules was examined for their O−H stretching vibrations in the >3570 cm^{-1} region. No such changes were observed for pR and pB at 77−250 K. In contrast, however, a water band was indeed observed at 3658 cm^{-1} for pB at 295 K, indicating the presence of the two pB forms, of which only one exhibits structural change of a buried water. Further mutation analysis suggested that the water molecule involved is most likely water-200 (*13*), which is far remote from the chromophore. On the basis of the present results, as well as additional spectroscopic observations, we conclude that water-200, buried in a hydrophobic core, is exposed to the aqueous phase upon formation of pB in solution. However, this happens neither in crystalline PYP nor at low temperature, presumably because of the limited flexibility of the protein under the latter conditions.

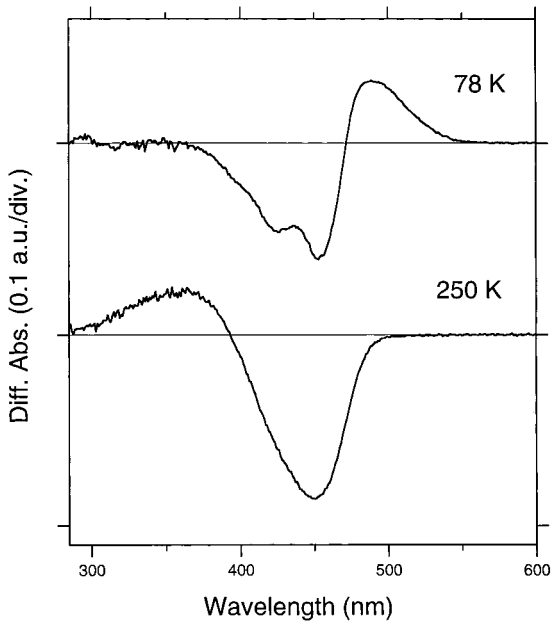

FIGURE 1: UV/Vis difference spectra of the hydrated film of PYP (pH 3.5), measured at 78 and 250 K. The pR minus pG (78 K) and pB minus pG (250 K) spectra were obtained by illumination with 440 and >400 nm light, respectively.

MATERIALS AND METHODS

Recombinant apoPYP and the PYP variant H108F (*25*), obtained through site-directed mutagenesis, were produced heterologously in *E. coli*, as described previously (*49*). The purity index of the samples (i.e., the ratio of the absorbance at 280 nm over 446 nm) was <0.8. PYP films were prepared by drying a concentrated PYP solution in 2 mM succinate buffer (pH 3.5) on a BaF_2 window. The films were rehydrated by adding ~1 μL of H_2O or $H_2{}^{18}O$. The typical absorbance of these films was 0.93, 0.74, and 0.57 at 3330, 1640, and 1550 cm^{-1}, respectively, at 295 K. Linear dichroism experiments revealed a random orientation of the PYP molecules in the film. The temperature of the sample was controlled with a cryostat.

FTIR spectroscopy was done as described previously (*32*, *33*, *35*, *44*, *50*). Spectra were obtained by calculating the difference 'after-minus-before illumination' at 78 and 250 K, or 'during-minus-before illumination' (*40*) at 295 K. In each measurement, 128 interferograms are accumulated, and the spectra in the figures are averages of 10−15 independent measurements.

RESULTS AND DISCUSSION

Water Structural Changes Are Observed Only in One Form of pB. Figure 1 shows difference spectra in the visible spectral region of a hydrated film of PYP at 78 and 250 K, upon illumination with 440 nm and with >400 nm light, respectively. At 78 K, a negative band with two maxima (at 425 and 453 nm) and a positive band at 488 nm were

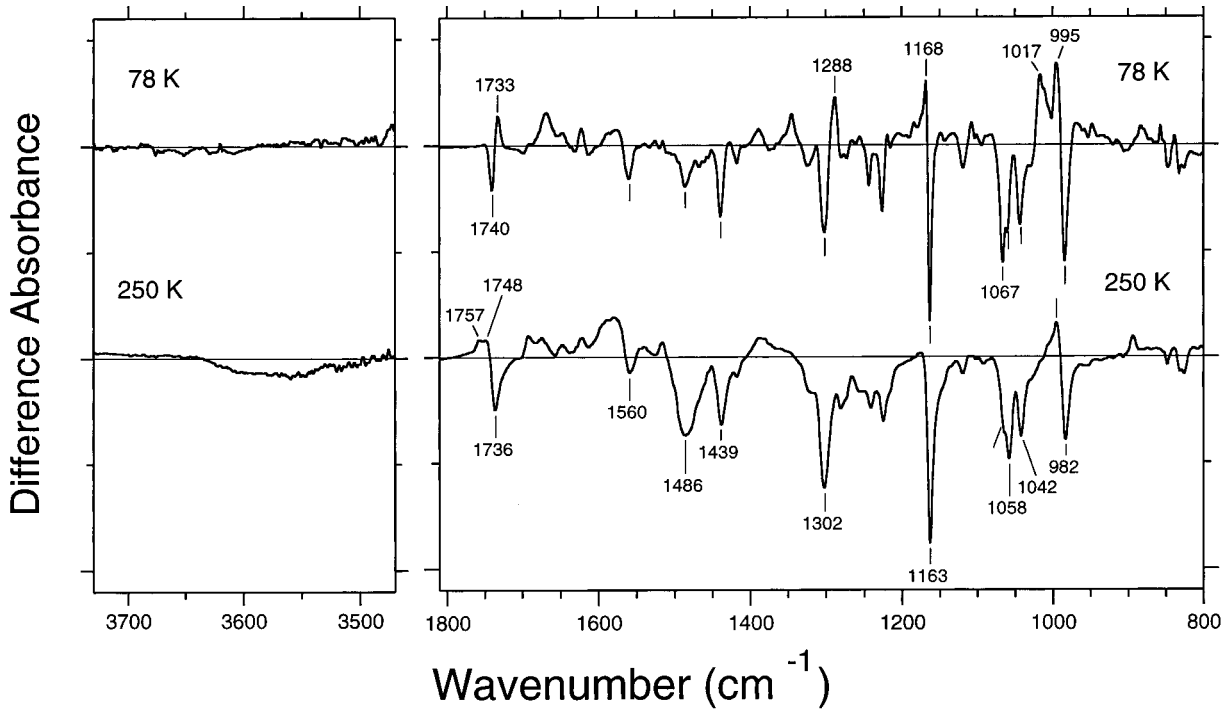

FIGURE 2: Infrared spectral changes of the hydrated film of PYP (pH 3.5) measured at 78 and 250 K. The pR minus pG (78 K) and pB minus pG (250 K) spectra were obtained by illumination with 440 and >400 nm light, respectively. The spectra were normalized by the negative band at 1163 cm^{-1}. One division of the *Y*-axis corresponds to 0.002 and 0.01 absorbance units in the 3730−3470 and 1800−800 cm^{-1} region, respectively.

observed, indicative for ground-state depletion and formation of a red-shifted photoproduct, respectively. In contrast, at 250 K, ground-state depletion causes a broad negative band at 450 nm, while simultaneously a positive band centered at about 360 nm indicates the formation of a blue-shifted photoproduct. These absorption spectra therefore confirm that pR and pB are formed from the ground state of PYP (i.e., pG) at 78 and 250 K, respectively, in agreement with previous results (*51*).

Figure 2 (right-hand panel) shows the corresponding infrared difference spectra of pR minus pG (78 K) and pB minus pG (250 K). The spectra were similar at neutral pH (7.0). Various spectral changes occur in the 1800−800 cm^{-1} region, among which are negative bands at 1560, 1486, 1439, 1302, 1163, 1067, 1058, and 1042 cm^{-1}, at both temperatures, presumably originating from the chromophore, because similar bands are observed in the resonance Raman spectra (*7*). The negative 982 cm^{-1} band is likely to be the hydrogen out-of-plane (HOOP) vibration of the chromophore. The positive bands at 1288, 1168, 1017, and 995 cm^{-1} in the spectrum of pR may be due to isomerization of the chromophore (*7*). The absence of these positive bands in pB may be caused by the neutralization of the negative charge of the phenolate moiety of the chromophore, due to its protonation. The negative bands at 1740 cm^{-1} (78 K) and 1736 cm^{-1} (250 K) are due to the C═O stretch of protonated Glu-46 (*52, 53*).

In contrast to the 1800−800 cm^{-1} region, no sharp peaks are observable in the 3730−3470 cm^{-1} region (Figure 2, left-hand panel), a region in which water O−H stretches appear in difference spectra of rhodopsin and bacteriorhodopsin (*50, 54*).[2] No further changes are observed in this spectral window, when the temperature is increased from 78 to 250 K (data not shown). From this we conclude that no water structural changes are involved in this frequency region during the initial stages of the photocycle of PYP.

Figure 2, in addition, shows two positive bands, at 1757 and 1748 cm^{-1}, respectively, in the carboxylic C═O stretch region at 250 K. This result is different from that obtained in a previous FTIR study of pB, generated with continuous illumination at 295 K (*52*). In the latter study, no positive bands were detected. Therefore, we repeated the measurement at 295 K. Formation of pB at this temperature was confirmed by recording visible absorption spectra (data not shown). Figure 3 compares the pB minus pG infrared difference spectra at 295 K (solid line) and 250 K (dotted line). The experiment at 295 K essentially reproduced the spectrum reported by Hoff et al. (*24*). The absence of positive chromophore-derived bands in this spectrum (corresponding to the negative bands marked by a circle) is in agreement with the presumed simultaneous protonation of the chromophore. However, the difference spectrum recorded at 295 K shows far greater changes in the amide-A (3500−3200 cm^{-1}) and the amide-I (1700−1600 cm^{-1}), as well as in the amide-II region (1600−1500 cm^{-1}), than the one recorded at 250 K.[3] This indicates that the structural transition in PYP, upon formation of pB, is much larger when this transition

[2] The pB minus pG spectrum at 250 K (Figure 2) shows a negative broad continuum in the 3630−3500 cm^{-1} region, which may originate from water molecules. It is however noted that it was not assigned to water molecules because of a highly broadened spectrum, and we can assign vibrational bands only when they exhibit isotope shifts.

[3] The obtained spectral changes were not due to a local heating effect, because temperature was controlled throughout the measurement. In addition, even if local heating occurs, infrared spectra tend to exhibit higher frequency shifts in such a case, which is opposite to those observed in the amide-A band (3500−3200 cm^{-1}). Therefore, great spectral changes originate from structural changes of the protein.

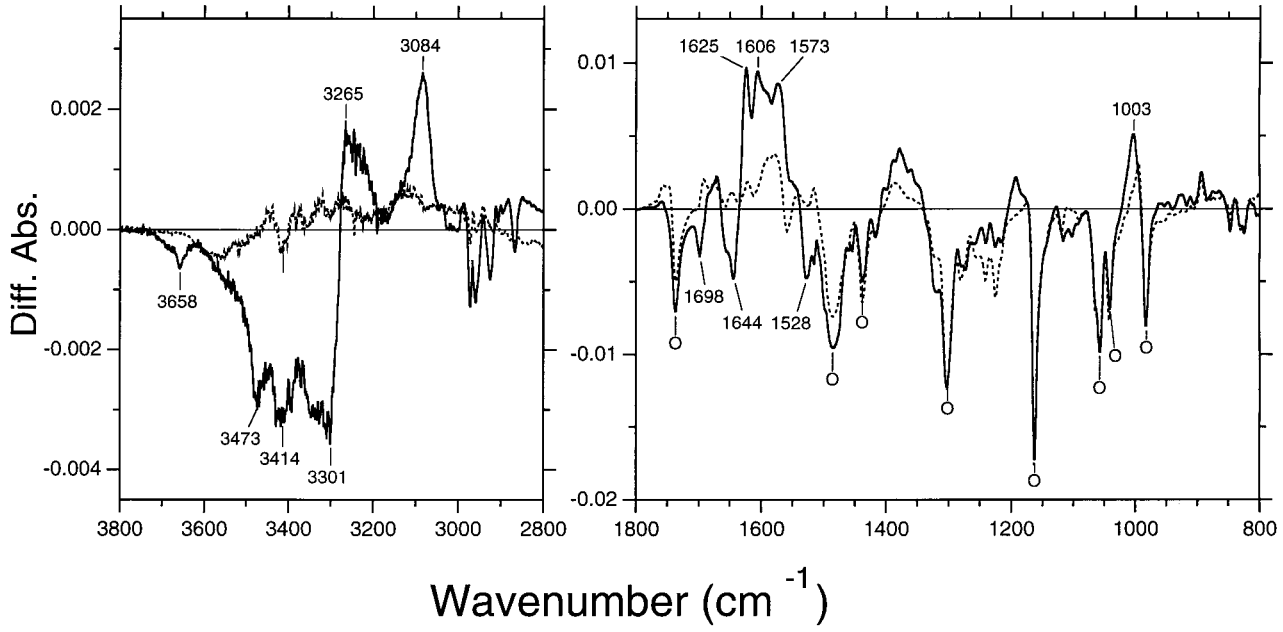

FIGURE 3: Infrared spectral changes of the hydrated film of PYP at 250 K (broken line) and 295 K (solid line). The spectra were normalized by the negative band at 1163 cm^{-1}. The common negative peaks attributable to the chromophore are marked by circles.

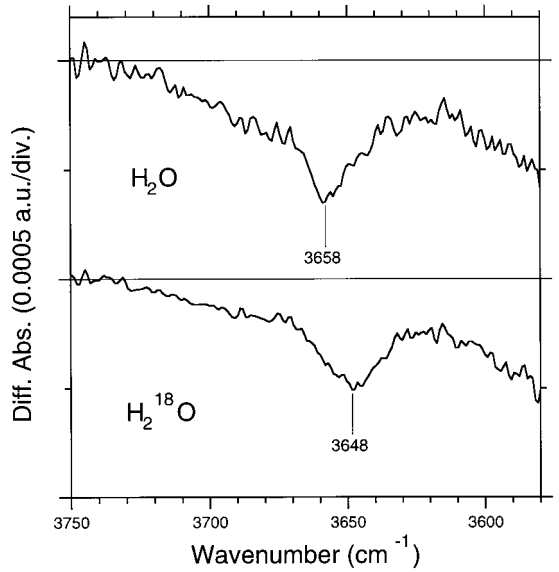

FIGURE 4: Infrared spectral changes in the 3750−3580 cm^{-1} spectral region of the PYP film hydrated with H_2O (upper panel) or $H_2{}^{18}O$ (lower panel) at 295 K.

occurs at the higher temperature. The FTIR difference spectrum at 295 K (Figure 3) furthermore shows a negative band at 3658 cm^{-1}, characteristic of the stretch vibration of water O−H (*55*). We therefore measured the difference spectrum after hydration with $H_2{}^{18}O$. Figure 4 clearly shows that the 3658 cm^{-1} band shifts to 3648 cm^{-1}, as expected for these bands when they are due to water O−H vibrations. No spectral changes were observed in other frequency regions when the spectra in H_2O and $H_2{}^{18}O$ were compared (data not shown). Thus, the present results show that, although PYP at both temperatures optically is in the pB state, the protein can be present in two different conformations: one showing large changes in the structure of the backbone of the protein (at 295 K), and a second (which is formed at 250 K) with a much more restricted difference in conformation with respect to the ground state. These observations are consistent with previous reports (e.g., *22*). Remarkably, however, of the two forms of pB, only one form exhibits a detectable change in the IR spectral region of the structural water O−H stretching vibrations.

Characteristics of the O−H Stretching Mode of the Water Band of pB. The O−H stretching vibrations of a water molecule may range from 3700 to 2700 cm^{-1}. Its frequency decreases as the strength of the hydrogen bond increases (*55*−*57*). The observed frequency of the water O−H band at 3658 cm^{-1} thus indicates that the particular water molecule is present under very weak, or even absent, hydrogen-bonding conditions. Corresponding positive peaks were not observed in the >3580 cm^{-1} region and are probably hidden by other O−H or N−H stretching bands in the <3580 cm^{-1} region (see Figure 3), implying that the water O−H forms a stronger hydrogen bond upon pB formation.

The magnitude of ∼0.0008 absorbance unit of the 3658 cm^{-1} band corresponds to an extinction of ∼120 on the basis of the molar extinction coefficient of the visible absorption band of PYP [i.e., 45 500; (*22*)]. Since the molar extinction coefficient of water has been estimated to be 100−200 (*55*), it is likely that one water O−H is responsible for the observed band in the difference spectra. Its spectral half-width (∼27 cm^{-1}; Figure 4) is comparable with those of the 3643 cm^{-1} band of bacteriorhodopsin (*36*) and the 3538 cm^{-1} band of rhodopsin (*45*). The latter two are both due to a water molecule which is fixed near the chromophore of these proteins. It is thus concluded that one water O−H group of the pG state of PYP is not involved in hydrogen bonding, whereas it does form a hydrogen bond upon formation of the pB state at 295 K, although its hydrogen-bonding condition does not give rise to a detectable change in the IR spectrum when a blue-shifted intermediate is formed at 250 K.

pB formation is accompanied by structural changes of the protein surface, which is necessary for the signal transduction between PYP and its signal transduction partner. It must therefore cause structural changes of water molecules hydrat-

ing the protein at the surface. In the present work, no water structural changes are observed of strongly hydrogen-bonded water molecules, whose O−H stretch appears in the <3580 cm^{-1} region. It is difficult to detect the isotope shift of 10 cm^{-1} (between ^{16}O and ^{18}O) for the strongly hydrogen-bonded waters, because such O−H stretches appear as broad bands in the low-frequency region, where other O−H and N−H stretches mask the change. Thus, in general, FTIR spectroscopy is less advantageous in detecting the bridged O−H stretches of water molecules through hydrogen bonds (*57*).

Location of the Water Molecule That Forms the Weak Hydrogen Bond. It is of key interest to locate the water molecule responsible for the 3658 cm^{-1} band. The location of 92 water molecules has been already reported with the crystal of PYP (*13*). The location of water molecules may be different between PYP present in solution and in a crystalline lattice, and there can be water molecules that are not observed by X-ray crystallography. The presence of such positionally disordered water molecules has been reported, using NMR methodology (*58*). However, positionally disordered water molecules may be expected to have a broader half-width than ~27 cm^{-1}. We therefore tested each of the 92 well-defined water molecules as the candidate for the water molecule that gave rise to the observed water band. In this survey, the very weak hydrogen-bonding character of this water molecule, as is evident from its frequency (at 3658 cm^{-1}), is an important criterion. A water molecule exposed to the solvent can probably form all its hydrogen bonds with solvent waters, and its O−H stretching frequency must therefore appear at a frequency lower than 3658 cm^{-1}. This restriction largely reduces the number of candidate water molecules. A water molecule that is deeply embedded in the protein, however, is a good candidate.

According to the crystallographic structure of PYP in the pG state, however, all 92 water molecules are present near the protein surface. The results of the time-resolved X-ray crystallography suggest that one of the possible candidates is a water molecule near Arg-52, because its side chain moves upon conversion from pG to pB (*15*). There are two water molecules near Arg-52: water-243 and water-244 (Figure 5a). Nevertheless, both are likely to be fully hydrogen-bonded (*13*).

There is one more water molecule which fits the current criteria. Like the other water molecules, this water is located near the PYP surface, but it is present in a restricted environment. This water molecule, water-200, is within hydrogen-bonding distance with the imidazole nitrogen of His-108 and the backbone nitrogen of Gly-7, but additional hydrogen-bonding partners are more than 4.5 Å away [Figure 5b; (*13*)]. Two phenylalanine residues, Phe-6 and Phe-121, form a binding pocket for this water molecule. Since the backbone nitrogen of Gly-7 donates a hydrogen atom, water-200 has at least one O−H group without hydrogen bonding. Interestingly, the location of water-200 is remote from the chromophore (Figure 5c), and virtually no structural changes were reported around water-200 by the time-resolved crystallographic experiments (*15*). The FTIR spectra in Figure 3 show that the water that gives rise to the 3658 cm^{-1} band forms a stronger hydrogen bond upon formation of pB. This is probably achieved by the structural change of the protein, which is restricted to 295 K. In other words, the protein

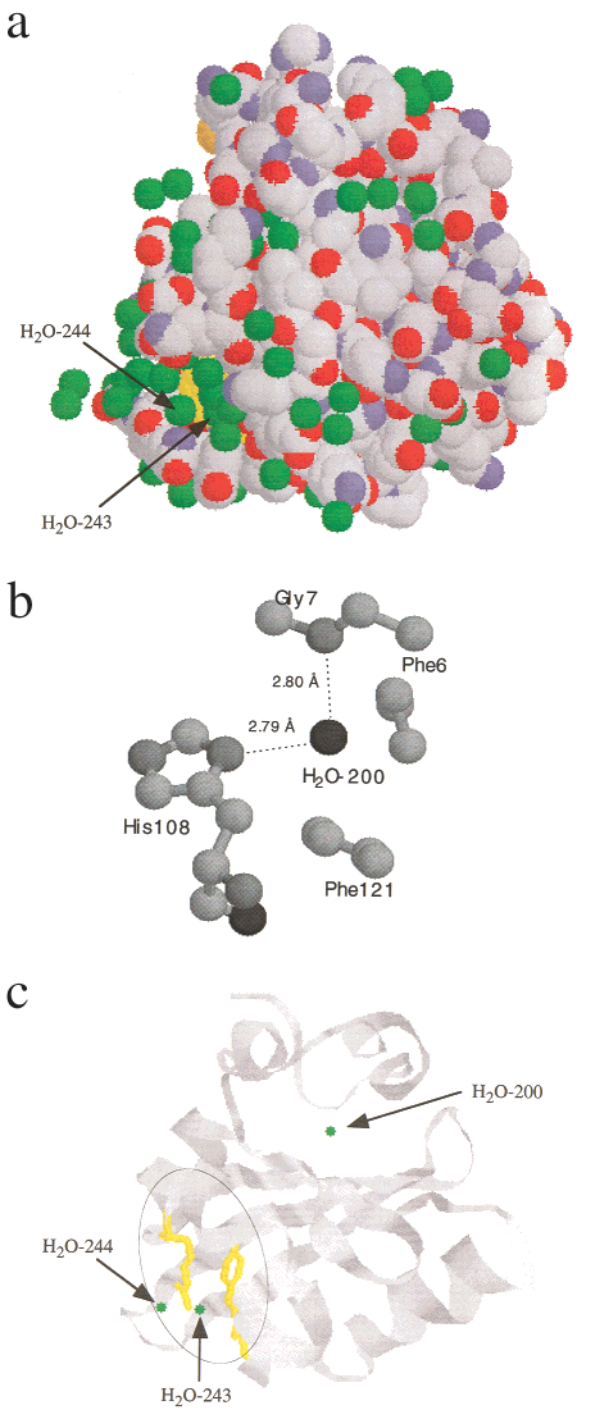

FIGURE 5: (a) Protein structure of photoactive yellow protein, as shown by a space-filling model. The chromophore is colored light yellow. Sulfur, carbon, nitrogen, oxygen atoms of water, and others are colored dark yellow, gray, blue, green, and red, respectively. Two water molecules, water-243 and water-244, are shown by arrows. (b) Structure surrounding water-200. His-108 and atoms within 4.0 Å are shown by a ball-and-stick drawing. Two nitrogen atoms are present within hydrogen-bonding distance. (c) Protein structure of photoactive yellow protein in the form of a ribbon drawing, which has identical size and orientation as those in panel a. The chromophore and Arg-52 are colored light yellow. Three water molecules, water-200, -243, and -244, are shown as green points. The oval represents the approximate area of discernible structural changes by time-resolved X-ray crystallography. The location of water-200 is remote from that.

structural change in pB at 295 K, but not at 250 K, allows the water-200 to form a stronger hydrogen bond. Thus, if we compare the 92 waters on the basis of only their

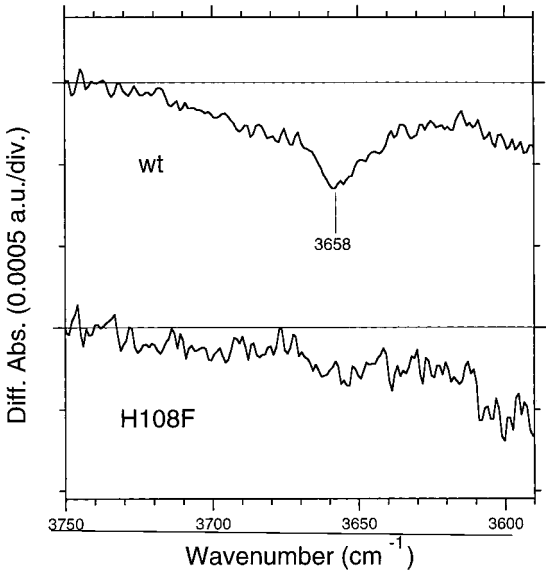

FIGURE 6: Infrared spectral changes of the PYP film of the wild-type (upper panel) and the H108F protein (lower panel), measured at 295 K.

hydrogen-bonding conditions, the most likely candidate for the 3658 cm^{-1} band is water-200.

Water molecules cannot be specifically labeled. However, our FTIR studies on bacteriorhodopsin (*27*−*35*, *37*, *39*) and visual rhodopsin (*45*, *46*) have clearly shown that mutant proteins are useful in determining the location of a water molecule. In the present study, we replaced His-108 by phenylalanine, and examined how the water band is affected. In the H108F protein, the kinetics of the photocycle are slightly affected: The formation of pB is ∼10-fold accelerated, and its decay is slightly retarded (J. Hendriks, unpublished experiments). Since the hydrogen-bonding structure of water-200 is altered in the H108F protein, we expect altered FTIR difference spectra in this particular mutant.

Assignment of the Water Molecule Near His-108. Figure 6 shows FTIR difference spectra of the water stretching vibrational region. The negative water band at 3658 cm^{-1} in the wild-type protein is strongly diminished in the H108F variant. Disappearance of the water band by mutation has been also observed for bacteriorhodopsin (*27*) and rhodopsin (*45*), upon replacement of the negatively charged carboxylates Asp-85 and Glu-113, respectively. Consequently, it was concluded that the latter two water molecules are located near these carboxylates (*25*, *26*, *34*, *43*, *50*). Accordingly, the most straightforward interpretation of the present observation is that the water band at 3658 cm^{-1} in the pB minus pG FTIR difference spectrum of PYP at 295 K originates from water-200 near His-108 (Figure 5b). This band may disappear from the FTIR difference spectra because of three different reasons: First, the water molecule indeed disappears because of the mutation. Second, the water molecule is positionally preserved, but its frequency is shifted by the mutation. Third, the position and frequency of the water molecule are preserved, but the alteration in hydrogen bonding upon formation of intermediates has disappeared by the mutation. Disruption of the hydrogen bond between His-108 and water-200 in the mutated protein results in a different environment of water-200 in the H108F protein, as compared to its wild-type counterpart.

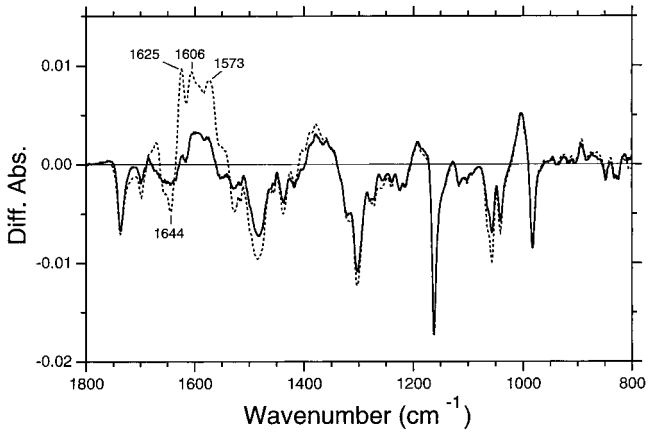

FIGURE 7: Infrared spectral changes of the PYP film of the wild-type (broken line) and the H108F protein (solid line), measured at 295 K. The spectra were normalized by the negative band at 1163 cm^{-1}.

Figure 7 compares pB minus pG spectra at 295 K between the wild-type and the H108F protein in the 1800−800 cm^{-1} region. Although vibrational bands of the chromophore and Glu-46 (Figures 2 and 3) are highly preserved, significant changes are observed in the amide-I and -II regions (1700−1500 cm^{-1}). The spectrum of H108F, measured at 295 K, exhibits much smaller amide changes than the wild-type protein, indicating that in the H108F protein the structural changes are smaller than in the wild-type protein. In fact, the spectrum of H108F at 295 K looks more similar to that of the wild type at 250 K.[4]

Based on the present observations, we assign the 3658 cm^{-1} band to the non-hydrogen-bonded O−H stretch of water-200. As mentioned, water-200 is present in a hydrophobic pocket at >15 Å from the chromophore, and probably forms a hydrogen bond with the peptide amide of Gly-7 and the imidazole nitrogen atom of His-108 (Figure 5b). The hydrogen-bonding partner of the O−H group of water-200 in PYP in the pB state at 295 K is unknown at present. We infer that in pB water-200 becomes accessible for the aqueous phase, so that it can form a hydrogen bond with surrounding water molecules or amino acid residues on the protein surface. Since the distance between the chromophore and water-200 is >15 Å, structural changes have to be transmitted through the protein from the chromophore to water-200.

Protein Structural Change: Concerted or Decoupled. Above, we have described the existence of two different blue-shifted states in the analysis of the photocycle of PYP, as a function of temperature. The intermediate state trapped at 250 K exhibits the visible absorption typical of pB (Figure 1), whereas the protein structure is significantly different from the one of the pB state formed at 295 K (Figure 3). We assume that the intermediate trapped at 295 K is closest to pB formed in the photocycle at physiological temperatures and that at 250 K a specific low-temperature pB state is trapped. At 250 K, the protein structure around the chromophore is changed as compared to the pG state, but global changes in the PYP structure do not yet occur at this temperature. Surprisingly, the pB state trapped at 250 K

[4] The structural similarity of H108F at 295 K to the wild type at 250 K indicates the important role of His-108 in the global structural changes in PYP. The hydrogen bonds of His-108 that involve water-200 could specifically work for the trigger of such changes.

presumably has a protonated Glu-46, as indicated by the positive peaks in the carboxylic C=O stretch region (Figure 2). Therefore, further structural analyses of the pB states are important particularly in relation to those by X-ray (*15*) and NMR (*20*). By FTIR, assignment of the positive bands has to be primarily done in the future.

The interconversion between the two pB states becomes measurable at 250−260 K (data not shown). In addition, we found that at reduced hydration, this transition temperature shifts to a higher value, implying that sufficient hydration is required for the global protein change to take place. Particularly the latter results suggest that the structural changes in PYP do not necessarily occur in a concerted manner (see further below).

Local vs Global Structural Change: Structural Changes in Crystal vs Solution. The extent of the conformational transition, that follows the change in configuration of the chromophore upon photoisomerization, apparently depends on the physical conditions in the molecular environment of the protein (like, e.g., the extent of hydration or the temperature). A similar conclusion can also be drawn by comparing the results of time-dependent Laue diffraction experiments (*15*) and heteronuclear spin-quantum correlation spectra of the pG and pB states of PYP (*20*). The time-resolved X-ray crystallographic studies showed that the conformational changes that accompany pB formation are essentially confined to the chromophore binding site of PYP: No global changes in protein structure were resolved (*15*). However, various spectroscopic and NMR analyses (*20*−*25*) have provided data that favor the interpretation that global structural changes do occur while PYP goes through its photocycle. The present results are consistent with such spectroscopic observations in solution, by demonstrating that the global structural changes reach the sites of His-108 and water-200. In this connection, the question comes up whether the photocycle characteristics (i.e., spectra of intermediates and rates of the relevant transitions) are affected by the molecular environment of PYP. So far, such effects have not been detected (*59*), but more detailed measurements may be warranted.

Possible structural changes beyond the chromophore domain also raise questions on the interaction surface with the—so far unknown—signal transducer protein of PYP. The crystal structure of PYP revealed the presence of a unique, large (687 Å^2), negatively charged surface patch, including a Gd^{2+} binding site (Glu-9, Asp-10, Asp-19, Asp-20), which was originally suggested as the recognition face for signal transduction (*13*). According to the crystal structure of pB, the negative patch is unlikely to activate the transducer because no change in structure is evident in this patch in the Laue diffraction experiments (*15*). However, the present study also shows that this structural change in solution can be more global, and consequently a recognition function of this patch cannot yet be excluded. We clearly need more effort in the structure−function studies of the PYP-transducer system in the future.

These observations imply that it is necessary to consider the concept of 'slip' also in signal transduction studies [compare (*60*) for the corresponding situation in bioenergetics]. Because the molecular environment of PYP dictates the extent of the conformational transition that follows light absorption, the efficiency of transmission of this conformational signal to a downstream signal transduction partner may likewise vary as a function of the physical, molecular environment of these signal transduction components. An important point further to consider is that signal transduction components in biology often function in large molecular clusters.

CONCLUSION

Water structural changes detected by means of light-induced difference FTIR spectroscopy have so far been studied mainly in retinal proteins, which are membrane proteins (*26*−*47*). The present study extends this approach to a soluble protein, PYP, in which we observed a structural change of a water molecule in the activation process of this photoreceptor protein. The water structural change does not occur in the early stage of the photocycle, but does occur upon pB formation at ambient temperatures. The O−H group of the water molecule is present under very weak hydrogen-bonding conditions in pG, while forming a stronger hydrogen bond in pB, possibly by being exposed to the aqueous solvent. Analysis of the properties of a mutant PYP protein (H108F) suggests that the water molecule responsible for the IR band is likely to be water-200, which is present in a hydrophobic pocket inside the protein. Since water-200 is >15 Å away from the chromophore, structural changes that are initiated in the vicinity of the chromophore have to be transmitted to region of the protein containing water-200.

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