Articles

Photoreaction Cycle of Photoactive Yellow Protein from *Ectothiorhodospira halophila* Studied by Low-Temperature Spectroscopy†

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ABSTRACT: The photocycle of photoactive yellow protein (PYP) from *Ectothiorhodospira halophila* was studied by low-temperature spectroscopy. Irradiation of PYP at −190 °C produced a photo-steady-state mixture composed of bathochromic and hypsochromic photoproducts (PYP_B and PYP_H). Upon warming, PYP_H was thermally converted to a slightly blue-shifted intermediate (PYP_{HL}) above −150 °C and then to a red-shifted one (PYP_L) above −80 °C. PYP_B was thermally converted to the blue-shifted intermediate (PYP_{BL}) above −180 °C and then to PYP_L above −90 °C. PYP_L thermally reverted to PYP above −50 °C, completing the photocycle. The spectral properties of PYP_L formed at low temperature suggest that it corresponds to the red-shifted photoproduct detected in the nano- to microsecond time scale at room temperature (A_{465}). The absolute absorption spectra of PYP_H, PYP_B, and PYP_L were estimated, and their absorption maxima were determined to be 442 and 489 nm at −190 °C and 456 nm at −80 °C, respectively. Although a near-UV intermediate (A_{355}) is observed in the recovery process of PYP from A_{465} at room temperature, it was not detected at low temperatures, probably due to the effects of temperature and the presence of glycerol. A scheme of the photocycle of PYP is presented.

Photoactive yellow protein (PYP)[1] is a small soluble protein (Meyer, 1985) which functions as the photoreceptor protein for the negative phototaxis of *Ectothiorhodospira halophila* (Sprenger et al., 1993). PYP maximally absorbs 446-nm light, resulting in its bright yellow color. The protein moiety of PYP has α/β structure (Borgstahl et al., 1995) composed of 125 amino acids (Van Beeumen et al., 1993). The chromophore of PYP is a *p*-coumaric acid (4-hydroxycinnamic acid) bound to the unique cysteine residue at position 69 via a thioester bond (Hoff et al., 1994a; Baca et al., 1994; Imamoto et al., 1995). PYP has a photoreaction cycle involving several intermediates, as investigated by low-temperature spectroscopy (Hoff et al., 1992) and flash photolysis at room temperature (Meyer et al., 1987; Hoff et al., 1994b) and is similar to that of the retinal proteins of halobacteria (Spudich & Bogomolni, 1988).

Among the photoreceptor proteins that function as photosensors, PYP is the first and only example whose tertiary structure has been determined to high resolution (Borgstahl et al., 1995). Therefore, PYP is eminently suitable for detailed investigations of the light capture and intramolecular signal transduction mechanisms in photoreceptor proteins. For this purpose, the identification of the intermediates appearing in the photocycle is essential because each intermediate closely correlates with the protein structural changes. However, the photocycle of PYP reported previously appears to be still unclear.

By flash photolysis at room temperature, transient red-shifted (A_{465}) and near-UV intermediates (A_{355}) are detected (Meyer et al., 1987; Hoff et al., 1994b), the lifetimes of which are, respectively, submillisecond and subsecond. The precursor of A_{465} has not been discovered even by laser photolysis with a time resolution of 5 ns. On the other hand, low-temperature spectroscopy has shown that three steady-state photoproducts are formed by irradiation of PYP at liquid nitrogen temperature (Hoff et al., 1992). They are red-shifted (A_{490}), slightly blue-shifted (A_{440}), and strongly fluorescing (F_{430}) photoproducts. However, their thermal reactions have not been reported in detail.

While the red-shifted intermediates are found at room and low temperatures (A_{465} and A_{490}), not only their absorption maxima but also their spectral shapes are quite different. Therefore, it is necessary to elucidate whether they are different species or if the spectral difference originates from the effect of temperature and/or glycerol. On the other hand, A_{440} has not been detected at room temperature, and A_{355} did not appear in the thermal reaction of A_{440} (Hoff et al., 1992).

These accumulated observations suggest that the flash photolysis at room temperature and low-temperature spectroscopy produced different intermediates. The relations of intermediates formed at room temperature and low temperature should be clarified to understand the photocycle of PYP. In the present study, the photoreaction of PYP at liquid nitrogen temperature and the subsequent thermal reactions were studied in detail to determine the photocycle of PYP. They were compared with the transient intermediates formed

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[1] Abbreviations: PYP, photoactive yellow protein from *Ectothiorhodospira halophila*; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet.

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at room temperature reported so far (Meyer et al., 1987; Hoff et al., 1994b) to understand the photoreaction process of PYP.

MATERIALS AND METHODS

Preparation of PYP. *Ectothiorhodospira halophila* strain BN 9626 was provided by Dr. Keizo Shimada of Tokyo Metropolitan University. Cells were cultured (Meyer et al., 1985) in 5-L bottles under sunlight for 7−10 days in summer and harvested by centrifugation (9000*g*, 20 min). The pellet was stored at −80 °C until use.

The cells were suspended in Tris buffer (10 mM Tris-HCl, pH 7.4) containing 10 μg/mL DNase I, using a Teflon homogenizer, and dialyzed against Tris buffer on ice overnight to disrupt the cells by osmotic shock. The supernatant collected by centrifugation (48000*g*, 1 h) from the lysate was applied to a DEAE-Sepharose CL6B column (Pharmacia) equilibrated with Tris buffer. After the column was washed, PYP was eluted with a linear gradient of NaCl (100−200 mM) in Tris buffer. The yellow fraction was collected and supplemented with ammonium sulfate powder to 60% saturation. After being stirred for 30 min on ice, the mixture was centrifuged (48000*g*, 20 min). The supernatant was desalted by dialysis against Tris buffer, and DEAE-Sepharose column chromatography was repeated. The fraction containing PYP was concentrated with an ultrafiltration membrane (Amicon Centricon-10) at 4 °C and diluted with Tris buffer containing 100 mM NaCl, followed by further concentration. The dilution and concentration were repeated several times to remove small fragments of DNA. Two volumes of glycerol were added to the sample for low-temperature spectroscopy.

Low-Temperature Spectroscopy. Absorption spectra were recorded with a Hitachi 320 spectrophotometer equipped with a glass optical cryostat and copper cell holder (light path length 5 mm) to keep the sample at low temperature (Kataoka et al., 1992; Yoshizawa & Shichida, 1982). Liquid nitrogen was used as a coolant and the temperature of the sample was monitored by a copper−constantan thermocouple attached to the sample cell. Opal glasses were placed in the sample and reference beams of the spectrophotometer to compensate for light scattering from cracks in the frozen sample. The sample was irradiated with light from a 1-kW slide projector (Master HILUX HR). A 5-cm water layer was placed in front of the projector lamp to remove heat radiation. The wavelength of the irradiation light was selected with optical glass filters (long-pass filters and/or interference filters, Toshiba).

RESULTS

Photoreaction of PYP at −190 °C. Purified PYP sample was supplemented with 66% glycerol and subjected to low-temperature spectroscopy. The absorption maximum of PYP was 446 nm in Tris buffer but shifted to 449 nm on addition of glycerol (Hoff et al., 1992). It was further shifted to 448 nm by cooling to −80 and −190 °C. The spectral shape was sharpened and the spectral shoulder appeared in the lower wavelength region due to the vibrational fine structure (Figure 1a, curves 2 and 3). The sample was then irradiated with 429-nm light at −190 °C (Figure 1b). Upon irradiation, absorbance at about 490 nm gradually increased with a concomitant decrease at about 450 nm (curves 4−9), indicating the formation of bathochromic photoproduct

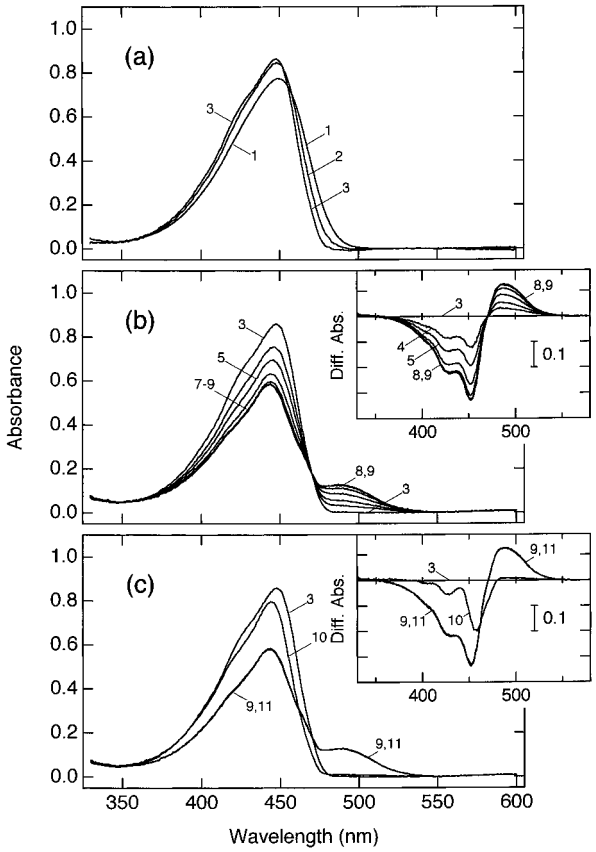

FIGURE 1: Photoreaction of PYP at −190 °C. (a) The absorption spectra of PYP/66% glycerol sample were recorded at 0, −80, and −190 °C (curves 1−3, respectively). (b) The sample at −190 °C (curve 3) was irradiated with 429-nm light for a total of 5, 10, 20, 40, 80, and 160 s (curves 4−9, respectively). (Inset) The difference spectra before and after the irradiation were calculated by subtracting curve 3 from curves 4−9. (c) The sample was then irradiated with >450-nm light for 240 s (curve 10), followed by irradiation with 429-nm light for 160 s (curve 11). (Inset) The difference spectra before and after the irradiation were calculated by subtracting curve 3 from curves 9−11.

(called PYP_B hereafter). The photo-steady-state mixture clearly contained PYP_B (curve 9). However, it should be noted that the λ_{max} of the sample was shifted from 448 (curve 3) to 444 nm (curve 9), suggesting the formation of hypsochromic photoproduct (PYP_H) simultaneously with the formation of PYP_B.

To confirm the formation of PYP_H, the photo-steady-state mixture was successively irradiated with >450-nm light at −190 °C (Figure 1C). PYP_B disappeared but the absorption maximum of the sample remained blue-shifted from the original PYP (curve 10), indicating the formation of PYP_H. The sample was then irradiated with 429-nm light, and the absorption spectrum was red-shifted again (curve 11). The spectrum was identical to that of the photo-steady-state mixture containing mainly PYP_B (curve 9), indicating that PYP, PYP_B, and PYP_H are reversibly interconvertible by light at −190 °C.

The difference spectra before and after irradiation are shown in the insets of Figure 1. The characteristic fine structures in the negative bands are due to the vibrational fine structure of the absorption spectrum of PYP (Figure 1a, curve 3). Such apparent fine structures in the difference spectra are also observed in retinal proteins when the original pigment and/or the intermediate have a spectral fine structure at low temperature like such as halorhodopsin or phobo-

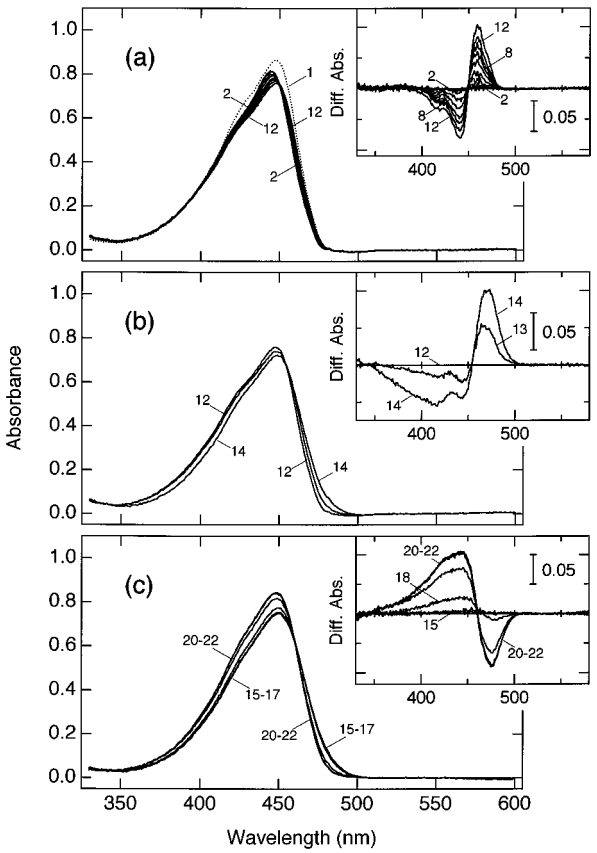

FIGURE 2: Thermal reaction of PYP_H. (a) The PYP/66% glycerol sample was cooled to −190 °C (curve 1, dotted line) and irradiated with >450-nm light for 240 s and with >500-nm light for 120 s (curve 2). The sample was warmed stepwise to −90 °C (intervals of 10 °C) and cooled to −190 °C immediately after each warming for recording the spectra (curves 3−12). (Inset) The difference spectra before and after warming were calculated by subtracting curve 2 from curves 3−12. (b) The sample was further warmed to −70 °C (intervals of 10 °C) and the spectra were recorded at −190 °C (curves 13 and 14). (Inset) The difference spectra before and after warming were calculated by subtracting curve 12 from curves 13 and 14. (c) The absorption spectrum of the sample was recorded at −80 °C (curve 15). Then it was warmed stepwise to −10 °C (intervals of 10 °C) and the spectra were recorded at −80 °C (curves 16−22). (Inset) The difference spectra before and after warming were calculated by subtracting curve 15 from curves 16−22.

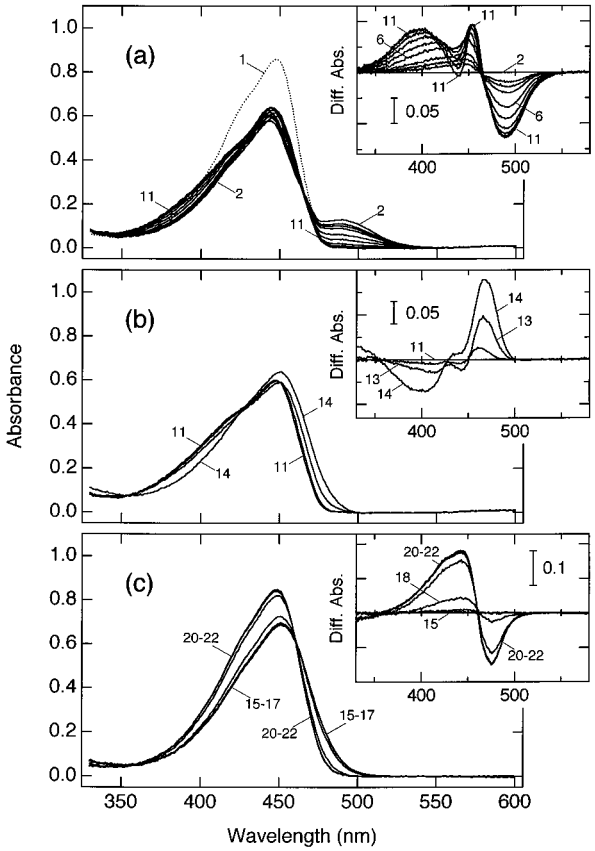

FIGURE 3: Thermal reaction of PYP_B. (a) The PYP/66% glycerol sample was cooled to −190 °C (curve 1, dotted line) and irradiated with 429-nm light for 160 s (curve 2). The sample was warmed stepwise to −100 °C (intervals of 10 °C) and cooled to −190 °C immediately after each warming for recording the spectra (curves 3−11). (Inset) The difference spectra before and after warming were calculated by subtracting curve 2 from curves 3−11. (b) The sample was further warmed to −70 °C (intervals of 10 °C) and the spectra were recorded at −190 °C (curves 12−14). (Inset) The difference spectra before and after warming were calculated by subtracting curve 11 from curves 12−14. (c) The absorption spectrum of the sample was recorded at −80 °C (curve 15). Then it was warmed stepwise to −10 °C (intervals of 10 °C) and the spectra were recorded at −80 °C (curves 16−22). (Inset) The difference spectra before and after warming were calculated by subtracting curve 15 from curves 16−22.

rhodopsin (sensory rhodopsin II) of halobacteria (Zimányi et al., 1989; Imamoto et al., 1991; Hirayama et al., 1992).

Thermal Reaction of PYP_H. The thermal reactions of PYP_B and PYP_H formed at −190 °C were studied by warming the sample in which they were contained. Because the photo-steady-state mixture mainly containing PYP_B also contained PYP_H (Figure 1b) and the interpretation of the data would be difficult, the mixture mainly containing PYP_H was first warmed to study the thermal reaction of PYP_H (Figure 2).

The sample was irradiated with >450-nm light at −190 °C for formation of PYP_H. Since this mixture contained a small amount of PYP_B, as shown by the small absorbance increase at 490 nm in the inset of Figure 1c, it was further irradiated with >500-nm light (Figure 2a, curve 2). Then the sample was warmed stepwise at intervals of 10 °C. In this experiment, the absorption spectra were recorded at −190 °C (Figure 2a,b) or at −80 °C (Figure 2c) by recooling the sample immediately after each warming to exclude the temperature effects on the absorption spectra. No spectral change was observed below −160 °C (Figure 2a, curve 5). However, two distinct spectral red shifts were observed on reaching at −70 °C: one was observed between −160 and −90 °C (Figure 2a) and the other between −90 and −70 °C (Figure 2b). They are similar to each other, but the isosbestic points (449 and 454 nm) and the spectral tails in the >490-nm and <400-nm regions are clearly different. This indicates the formation of two thermal products from PYP_H. Because the latter was stable at −70 °C, it may correspond to the L intermediate of bacteriorhodopsin, and it will be called PYP_L hereafter. The product between PYP_H and PYP_L will be called PYP_{HL}. By further warming, the spectrum was blue-shifted with the isosbestic point at 460 nm above −50 °C (Figure 2c), and the final spectrum was identical to that of PYP at −80 °C before the irradiation. Therefore, the thermal reaction of PYP_H is described as $PYP_H \rightarrow PYP_{HL} \rightarrow PYP_L \rightarrow PYP$.

Thermal Reaction of PYP_B. The thermal reaction of PYP_B was studied by warming the photo-steady-state mixture mainly containing PYP_B (Figure 3). The PYP sample was irradiated with 429-nm light at −190 °C for formation of

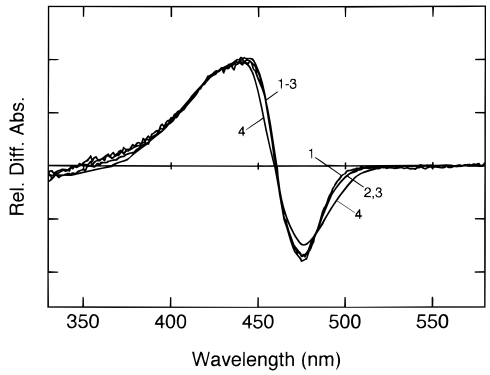

FIGURE 4: Comparison of the later stages of thermal reaction of PYP_B and PYP_H. All spectra were normalized by least-squares fitting. Curves 22 in the insets of Figure 2c and 3c are reproduced (curves 1 and 2). Difference spectrum before and after the irradiation of PYP at −80 °C with 429-nm light for 360 s (curve 3), and the transient difference spectrum 24 ns after the flash excitation at room temperature [curve 4, reproduced from Hoff et al. (1994)] are superimposed. Curves 3 and 4 are inverted for comparison.

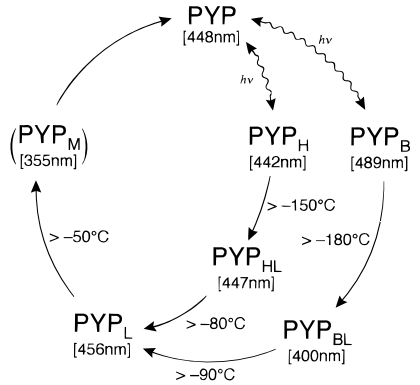

FIGURE 5: Photoreaction cycle of PYP at low temperature. The wavy and solid lines show the photochemical and thermal reactions, respectively. The (difference) absorption maxima of the intermediates are indicated in brackets. The transition temperature of each conversion is shown beside the arrows. The near-UV intermediate (PYP_M) is bracketed because it was not detected in our experimental conditions.

PYP_B (Figure 3a, curve 2), and warmed stepwise in the same way as for Figure 2. However, because the initial photo-steady-state mixture contained not only PYP_B but also PYP_H as the thermolabile species, two parallel decay processes took place and the spectral change was complex (Figure 3a). In the inset of Figure 3a, broad positive (400-nm) and negative (490-nm) bands were observed, on which sharp positive (460-nm) and negative (440-nm) peaks overlapped. Because the sharp bands were not observed below the transition temperature of PYP_H (−160 °C, see Figure 2a), the sharp peaks could be attributed to the conversion of PYP_H to PYP_{HL} (Figure 2a), and the broad positive band at 400 nm was due to the formation of the thermal product of PYP_B. It was then converted to the red-shifted intermediate above −90 °C (Figure 3b). The shape of the negative band in the difference spectrum in the inset of Figure 3b was different from that in Figure 2b, indicating that the thermal product of PYP_B (called PYP_{BL}) was not PYP_{HL}. Above −50 °C, the spectrum was blue-shifted and the final spectrum was identical to that of PYP at −80 °C before the irradiation. The spectral changes (Figure 3c) were identical to that of PYP_L formed thermally from PYP_{HL} (Figure 2c), suggesting that PYP_{BL} was thermally converted to PYP_L (see below). Therefore, the thermal reaction of PYP_B is described as $PYP_B \rightarrow PYP_{BL} \rightarrow PYP_L \rightarrow PYP$.

Comparison of the Later Thermal Reaction of PYP_B and PYP_H. In the above experiments, the thermal reactions of PYP_L formed from PYP_H and PYP_B seemed to be identical (Figures 2c and 3c). In Figure 4, it was further confirmed to characterize PYP_L. Curves 22 in the insets of Figures 2c and 3c are shown after normalization (curves 1 and 2 in Figure 4). In addition, the difference spectrum before and after the irradiation of PYP at −80 °C (curve 3) and the transient difference spectrum 24-ns after the flash excitation at room temperature [curve 4, reproduced from Hoff et al. (1994)] are superimposed. The latter is the difference spectrum between PYP and the red-shifted photoproduct at room temperature (A_{465}). Curves 1−3 were essentially identical. Curve 4 was somewhat different from curves 1−3, but it would be due to the temperature effect on the absorption spectrum of PYP_L, because the difference could be explained by the broadening of the spectrum. This similarity among the difference spectra strongly implies that all of them are the difference between PYP and the same intermediate. We conclude the photoreaction of PYP to be as follows: two independent photoproducts (PYP_H and PYP_B) are formed upon light absorption by PYP; both PYP_H and PYP_B are converted to the same intermediate (PYP_L), which is the unique intermediate formed at −80 °C; PYP_L is the same species as A_{465} found at room temperature. On the basis of the present observations, we propose here the most plausible and simple model of the photocycle of PYP in Figure 5.

Absolute Absorption Spectra of PYP Intermediates. For calculation of the absorption spectrum of an intermediate from that of a mixture of the intermediate and the original pigment, the fraction of the intermediate is required. This is easily estimated from the difference spectrum before and after the reaction, if the pigment has a remarkably blue-shifted intermediate whose absorption spectrum has little overlap with that of the original pigment (like M of bacteriorhodopsin and meta II intermediates of visual pigments). However, the near-UV intermediate (A_{355}) observed at room temperature (Hoff et al., 1994b) was not formed at low temperature, as shown in the present experiments. The absorption spectra of the intermediates identified in the present experiment (Figures 1−3) would overlap with each other and with that of the original pigment, so the fraction of the intermediate was estimated by the modified method reported previously (Imamoto et al., 1991; Fischer, 1967). The absorption spectrum of PYP_L at −80 °C was first estimated because the irradiation of PYP at −190 °C produced both PYP_H and PYP_B but PYP_L was present as the unique intermediate at −80 °C (Figure 4).

The PYP sample was irradiated with monochromatic lights at 413, 424, 429, 452, 456, 459, and 472 nm (obtained by the interference filters) until the photo-steady-state mixtures were formed at −80 °C (Figure 6a). The difference spectra before and after the irradiations were calculated and compared with each other (Figure 6b). They had the same shape, but their amplitudes were different, indicating that the fraction of PYP_L in each mixture was different (Figure 6b). In the photo-steady-state mixture under the irradiation with λ-nm light, the rate of photoreaction from PYP to PYP_L is equal to that of the reverse photoreaction. The rate depends

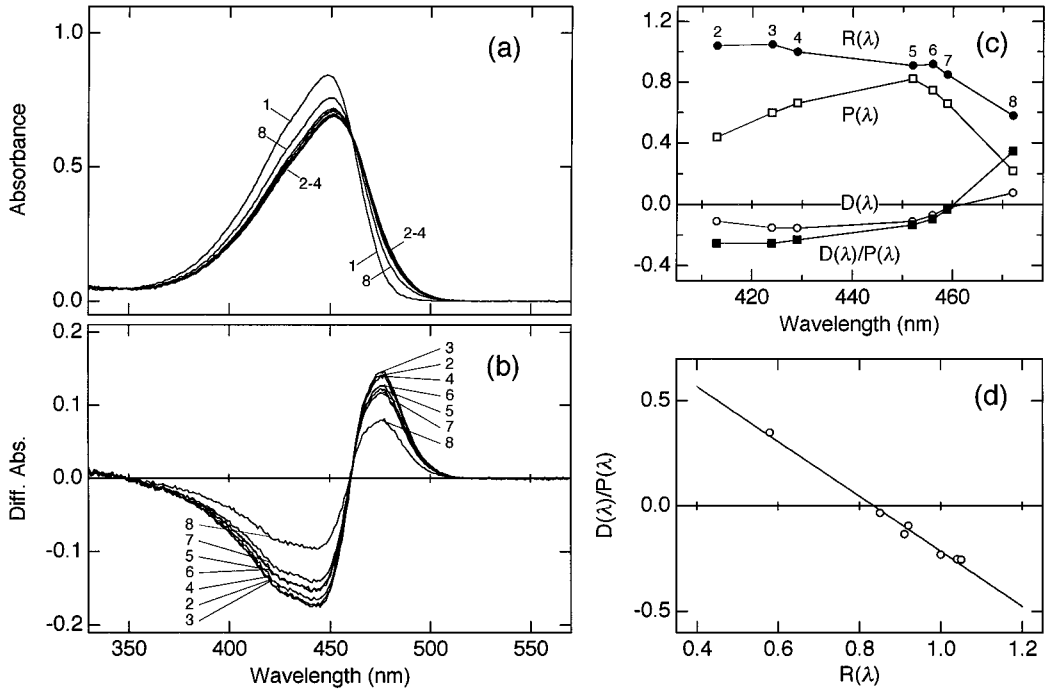

FIGURE 6: Calculation of the absorption spectrum of PYP_L. (a) The absorption spectrum of PYP sample was recorded at −80 °C (curve 1). It was irradiated with 413-, 424-, 429-, 452-, 456-, 459-, and 472-nm light until the photo-steady-state mixtures were formed (curves 2−8, respectively). (b) The difference spectra between the resulting mixtures and PYP in panel a were calculated (curves 2−8, respectively). (c) The amplitudes of curves 2, 3, and 5−8 relative to curve 4 (429-nm light irradiation) in panel b were estimated by linear least-squares fitting [$R(\lambda)$] and plotted against the wavelength of the irradiation light (closed circles). The absorbance of the PYP sample (curve 1 in panel a) at the wavelength of irradiating light [$P(\lambda)$, open squares], difference absorbance at the wavelength of irradiation lights in the respective difference spectra [$D(\lambda)$, open circles], and $D(\lambda)/P(\lambda)$ (closed squares) were plotted against the wavelength of irradiating light. (d) $D(\lambda)/P(\lambda)$ was plotted against $R(\lambda)$ (circles) and fitted with a straight line. For details, see text.

on the amount of pigment, the absorbance at λ nm, and the quantum yield of the respective pigment. On the assumption that the wavelength dependence of the quantum yield is negligible, as supported by the fact that the action spectrum of *E. halophila* is in agreement with the absorption spectrum of PYP (Sprenger et al., 1993), the following equation is derived:

$$[1 - x(\lambda)]P(\lambda) = x(\lambda)\phi L(\lambda)$$

where $x(\lambda)$ is the fraction of PYP_L in the photo-steady-state mixture under λ-nm light irradiation, $P(\lambda)$ and $L(\lambda)$ are the absorbance of PYP and PYP_L at λ nm, and ϕ is the quantum yield of PYP_L relative to PYP. In the difference absorption spectrum before and after λ-nm light irradiation, the following equation is derived:

$$D(\lambda) = x(\lambda)[L(\lambda) - P(\lambda)]$$

where $D(\lambda)$ is the difference absorbance at λ nm. On the other hand, the fraction of PYP_L is proportional to the amplitude of the difference spectrum before and after the irradiation. In the present work, the fraction relative to that with 429-nm light irradiation is used as follows:

$$x(\lambda) = x_0 R(\lambda)$$

where x_0 is the fraction of PYP_L with 429-nm light irradiation and $R(\lambda)$ is the amplitude of the difference spectrum before and after λ-nm light irradiation relative to that before and after 429-nm light irradiation. From these equations, the following equation is derived:

$$\frac{D(\lambda)}{P(\lambda)} = \frac{1}{\phi} - \left(\frac{1}{\phi} + 1\right)x_0 R(\lambda)$$

Therefore, when $D(\lambda)/P(\lambda)$ is plotted against $R(\lambda)$ and fit with a straight line, ϕ and x_0 can be estimated.

$P(\lambda)$ and $D(\lambda)$ were obtained from the absorption spectrum of PYP at −80 °C (curve 1 in Figure 6a) and the difference spectra before and after irradiation with the respective lights (i.e., Abs_{413} in curve 2, Abs_{424} in curve 3, Abs_{429} in curve 4, etc., in Figure 6b). $R(\lambda)$ was estimated by the linear least-squares fitting of curves 2, 3, and 5−8 to curve 4 in the 350−550-nm region. They are plotted in Figure 6d, and ϕ and x_0 were estimated to be 0.92 and 0.62, respectively; i.e., the photo-steady-state mixture under 429-nm light irradiation contained 38% PYP and 62% PYP_L. The absorption spectrum of PYP_L was calculated using curves 1 and 4 in Figure 6a, as follows:

$$[PYP_L] = ([\text{curve 4}] - 0.38[\text{curve 1}])/0.62$$

The calculated spectrum of PYP_L is shown with the absorption spectrum of PYP at −80 °C (Figure 7a). The absorption maximum and the relative extinction coefficient to PYP were estimated to be 456 nm and 0.72, respectively. The absorption spectrum of PYP_H was estimated on the basis of this result. The amplitude of curve 22 in the inset of Figure 2c was estimated to be 62% of curve 4 in Figure 6b, indicating that 38% (0.62 × 0.62) of PYP was converted to PYP_L in Figure 2c. According to our sequential scheme, the fraction of PYP_H at −190 °C is equal to that of PYP_L at

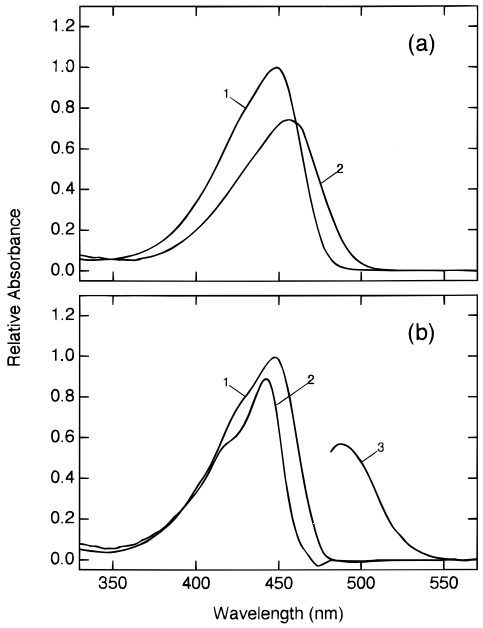

FIGURE 7: Absorption spectra of the intermediates of PYP. (a) Absorption spectra of PYP (curve 1) and PYP_L (curve 2) at −80 °C. Their absorption maxima were located at 448 and 456 nm, respectively. The extinction coefficient of PYP_L was 0.74 times that of PYP at −80 °C. (b) Absorption spectra of PYP (curve 1), PYP_H (curve 2), and PYP_B (curve 3) at −190 °C. Their absorption maxima were located at 448, 442, and 489 nm, respectively. The extinction coefficient of PYP_H was 0.89 times that of PYP at −190 °C. Note that the ordinate for curve 3 is arbitrary.

−80 °C. Therefore, the absorption spectrum of PYP_H was calculated using curves 1 and 2 in Figure 2a, as follows:

$$[PYP_H] = ([\text{curve 2}] - 0.62[\text{curve 1}])/0.38$$

The calculated spectrum of PYP_H is shown in Figure 7b. The spectral fine structure is pronounced and the bandwidth is very narrow. The absorption maximum and the relative extinction coefficient of PYP_H are estimated to be 442 nm and 0.89, respectively.

The absorption spectrum of PYP_{HL} appears to be calculated using curve 12 in Figure 2a in the similar manner. However, curve 12 would be the mixture of PYP, PYP_H, and PYP_{HL} because the conversion steps of $PYP_H \rightarrow PYP_{HL}$ and $PYP_{HL} \rightarrow PYP_L$ were not clearly separated in our experimental condition.

On the other hand, the amplitude of curve 22 in the inset of Figure 3c was estimated to be 106% of curve 4 in Figure 6b. This means that the sum of PYP_B and PYP_H was 66%, and their respective fractions remained unknown. Therefore, the absorption spectrum of PYP_B and PYP_{BL} could not be calculated accurately. However, curve 8 in the inset of Figure 1b should reflect the absorption spectrum of PYP_B in the wavelength region longer than 480 nm because the absorbance of PYP and PYP_H in this region was relatively small (Figure 1c). This is shown in Figure 7b (in the arbitrary ordinate): the absorption maximum of PYP_B was estimated to be 489 nm.

DISCUSSION

The present experiment has shown that the photocycle of PYP is apparently best represented by the scheme in Figure 5. The irradiation of PYP at −190 °C yielded PYP_B and PYP_H. They were thermally converted to PYP_L through PYP_{BL} and PYP_{HL}, respectively. The two pathways beginning with PYP_B and PYP_H joined at PYP_L and then reverted to PYP. In this model, the equilibrium between intermediates and the branched reaction are not considered.

In spite of the striking differences in the chromophores, protein moieties, and linkages, retinal proteins and PYP have some common features. The chromophores of both systems are charged (positive for retinal proteins and negative for PYP) in the dark states (Baca et al., 1994; Kim et al., 1995). The cis/trans isomerization of the chromophore takes place on photon absorption (Kort et al., 1996). The chromophore of PYP has only one double bond for isomerization but several intermediates appear, indicating that the absorption spectra of intermediates depend not only on chromophore configuration but also on the chromophore/protein interactions which alter during the photocycle. Therefore, comparison of the photocycle of PYP with those of retinal proteins may provide some suggestion for understanding the light-capturing mechanism of PYP.

The relationship between the photoproducts identified in this study and those at room temperature should be discussed first. Among the intermediates detected in the present study, the PYP_B and PYP_H formed at liquid nitrogen temperature were previously reported as A_{490} and A_{440} (Hoff et al., 1992). On the other hand, at room temperature, red-shifted (A_{465}) and near-UV (A_{355}) products have been found (Hoff et al., 1994b; Meyer et al., 1989). The candidates which correspond to A_{465} at room temperature would be PYP_B and PYP_L. In the present study, it was concluded that PYP_L corresponds to A_{465}, based upon the good agreement of their difference spectra (Figure 4). This is further supported by their stabilities. In comparison with the intermediates of bacteriorhodopsin, the thermal property of PYP_L is similar to L. The transition temperatures of PYP_L and L at low temperatures are −50 and −70 °C (Iwasa et al., 1980), while the transition temperature of PYP_B is extremely low (−180 °C). The lifetime of L is 55 μs at room temperature (Shichida et al., 1983), and that of A_{465} is ∼100 μs (Hoff et al., 1994b), indicating that PYP_L and A_{465} correspond to L of bacteriorhodopsin. While PYP_B, PYP_{BL}, PYP_H, and PYP_{HL} were clearly detected at low temperature, they have not been detected at room temperatures. Because the lifetime of K intermediate of bacteriorhodopsin is ∼10 ns, it might be possible to detect the precursors of PYP_L in the picosecond time scale.

Ultrafast spectroscopy has revealed that the first intermediates of the retinal proteins are "photo" or J intermediates. However, they are not detected at low temperatures even if liquid helium is used as a coolant. At low temperature, the first detectable products of retinal proteins are batho- or K intermediates, which commonly have red-shifted absorption spectra. The irradiation of PYP at −190 °C yielded PYP_B, the red-shifted intermediate, which would correspond to batho- or K intermediate. The blue-shifted intermediates (called iso- and hypso- pigments) are also found at liquid helium or liquid nitrogen temperatures in rhodopsins. However, they are thought to be byproducts formed from the intermediates by photon absorption (Yoshizawa et al., 1984; Sasaki et al., 1983), and the thermal reactions are different from PYP_H; e.g., hypso- pigment thermally converts to the batho- intermediate (Sasaki et al., 1992). The blue-shifted pigments are formed in halorhodopsin (Zimányi et al., 1989) and phoborhodopsin (sensory rhodopsin II) (Imamoto et al.,

1991) of *Halobacterium salinarium*, and they thermally revert to the original pigments. However, PYP_B and PYP_H were concurrently formed by irradiation with a clear isosbestic point at −190 °C (Figure 1b), and pathways from them were in parallel and joined at PYP_L (Figure 5). Such a thermal behavior of blue-shifted intermediate formed at liquid nitrogen temperature has not been reported in retinal proteins.

By low-temperature spectroscopy, PYP_L reverted directly to PYP, and the product corresponding to A_{355} (PYP_M) was not detected. This is probably because the decay of PYP_M was comparable to or faster than that of PYP_L in our experimental conditions, probably due to the effect of temperature and the presence of glycerol. It has been reported that the activation energy of the decay of A_{465} is larger than that of A_{355} by measuring the temperature dependency of their decay rate constants (Meyer et al., 1989). That is, the decay of A_{465} is much faster than that of A_{355} at room temperature, but their decay time constants converged when the temperature was reduced. Similarly, glycerol suppresses the decay of A_{465} but accelerates the decay of A_{355} (Meyer et al., 1989). Therefore, it is possible that PYP_M is not observable under our experimental conditions, in 66% glycerol buffer at low temperature: PYP_L decays to PYP_M and it immediately reverts to PYP.

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