Primary Photoreaction of Photoactive Yellow Protein Studied by Subpicosecond−Nanosecond Spectroscopy†

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ABSTRACT: The primary photochemical event of photoactive yellow protein (PYP) was studied by laser flash photolysis experiments on a subpicosecond−nanosecond time scale. PYP was excited by a 390-nm pulse, and the transient difference absorption spectra were recorded by a multichannel spectrometer for a more reliable spectral analysis than previously possible. Just after excitation, an absorbance decrease due to the stimulated emission at 500 nm and photoconversion of PYP at 450 nm were observed. The stimulated emission gradually shifted to 520 nm and was retained up to 4 ps. Then, the formation of a red-shifted intermediate with a broad absorption spectrum was observed from 20 ps to 1 ns. Another red-shifted intermediate with a narrow absorption spectrum was formed after 2 ns and was stable for at least 5 ns. The latter is therefore believed to correspond to I1 (PYP_L), which has been detected on a nanosecond time scale or trapped at −80 °C. Singular value decomposition analysis demonstrated that the spectral shifts observed from 0.5 ps to 5 ns could be explained by two-component decay of excited state(s) and conversion from PYP_B to PYP_L. The amount of PYP_L at 5 ns was less than that of photoconverted PYP, suggesting the formation of another intermediate, PYP_H. In addition, the absorption spectra of these intermediates were calculated based on the proposed reaction scheme. Together, these results indicate that the photocycle of PYP at room temperature has a branched pathway in the early stage and is essentially similar to that observed under low-temperature spectroscopy.

Photoactive yellow protein (PYP)[1] is a bright-yellow protein found in the purple phototrophic bacterium *Ectothiorhodospira halophila* (*1*). It is proposed to be a photoreceptor protein for the negative phototaxis of the bacterium (*2*), and now similar proteins have been identified in various other organisms (*3*−*6*). PYP has a *p*-coumaric acid binding to the cysteine residue at position 69 by a thioester bond as its chromophore (*7*−*9*). The absorption maximum is located at 446 nm. The chromophore is isomerized from the trans to the cis form on photon absorption (*10*−*13*), and PYP undergoes a photocycle (*14*−*22*) in which protein conformational change, proton transfer from Glu-46 to chromophore, and thermal re-isomerization of the chromophore take place. PYP is a small water-soluble protein of 14 kDa, and the tertiary structures of its intermediates (*11*−*13*) as well as the ground state have been analyzed (*23*). Due to these advantages, PYP is thought to be the most suitable target to understand the light-capturing mechanism of a photoreceptor protein. In particular, isomerization of the chromophore is the initial event upon photon absorption and is thought to be the key reaction to realize the highly effective utilization of photon energy for the photoreceptor proteins. To understand this primary process, the identification of the photocycle intermediates using ultrafast spectroscopy is considered essential. The photochemistry of PYP has been extensively studied by flash photolysis at room temperature (*14*−*19*) and low-temperature spectroscopy (*20*, *21*). As a result, several intermediates appearing in the photocycle have been identified thus far. The spectroscopic and kinetic properties of these intermediates are similar to those of the halobacterial retinal proteins, and the similarity in the mechanism of photoreactions between PYP and retinal protein has been demonstrated. Ultrafast spectroscopy has previously revealed that the stimulated emission of PYP on excitation with a laser pulse is composed of two components (τ = 0.7, 3.6 ps) (*17*). Our femtosecond up-conversion experiment also revealed that the fluorescence decay curve is multiexponential and the decay time constant depends on the wavelength, suggesting the existence of multiple excited states (*18*). As a result of relaxation of the excited state, the first photoproduct called I0 (λ_{max} = 500 nm) is formed (*19*). I0 decays to I0‡ (λ_{max} = 500 nm), which is spectrally similar to but kinetically distinct from I0 (τ = 220 ps). I0‡ decays to I1 (also called pR or PYP_L) (λ_{max} = 460 nm, τ = 3 ns), and subsequently decays to I2 (also called pB or PYP_M) (λ_{max} = 350 nm, τ = 500 μs). PYP_M has the protonated chromophore unlike ground-state PYP, and the protein conformation is largely altered. Finally, PYP is recovered from PYP_M (τ = 500 ms).

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[1] Abbreviations: PYP, photoactive yellow protein; λ_{max}, absorption maximum in the visible region; Tris, tris(hydroxymethyl)aminomethane; SVD, singular value decomposition; $\tau_{1/e}$, decay time constant.

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The photocycle of PYP revealed by low-temperature spectroscopy is more complicated. Our previous low-temperature spectroscopy trapped two intermediates, PYP_B (λ_{max} = 489 nm) and PYP_H (λ_{max} = 442 nm), by the irradiation of PYP at −190 °C (*21*). The two intermediates were thermally converted to PYP_L (λ_{max} = 456 nm), via PYP_{BL} (λ_{max} = 400 nm) and PYP_{HL} (λ_{max} = 447 nm), respectively. PYP_L corresponds to I1 (pR) (*21*) based on the absorption spectrum. Because PYP_B and PYP_H are formed at very low temperature, they are the most plausible candidates for the primary intermediate of PYP. However, the possibility that they are an artifact seen at low temperature cannot be excluded, and the formation of them should be confirmed directly by ultrafast spectroscopy at room temperature. In the present study, transient difference absorption spectra after excitation were recorded by a multichannel spectrometer. This technique permitted more precise spectral analysis than previously possible. Using this advantage, the transient difference spectra were analyzed by singular value decomposition (SVD) methods. The relationships between kinetic intermediates at room temperature and trapped intermediates at low temperature are discussed.

MATERIALS AND METHODS

Preparation of PYP. Apo-PYP was overexpressed by *Escherichia coli* (*24*). It was solubilized by 8 M urea in Tris buffer (10 mM Tris-HCl, pH 7.4) and reconstituted to holo-PYP by adding *p*-coumaric anhydride in 4 M urea, as reported previously (*24*). PYP was purified by DEAE-Sepharose column chromatography and suspended in Tris buffer containing 1 mM phenylmethanesulfonyl fluoride. The absorbance at 446 nm (λ_{max} of PYP) was adjusted to 0.6 in 1-mm light path length.

Spectroscopy. The experimental setup for transient difference absorption spectra was essentially the same as reported elsewhere (*25, 26*). Briefly, the laser system consisted of a cw self-mode-locked Ti:sapphire laser (Mira 900 Basic, Coherent), pumped by an Ar^+ laser (Innova 310, Coherent) and a Ti:sapphire regenerative amplifier system (TR70, Continuum) with a Q-switched Nd:YAG laser (Surelight I, Continuum). The fundamental output from the regenerative amplifier (780 nm, 3−4 mJ/pulse, 170 fs fwhm, 10 Hz) was frequency-doubled (390 nm) and used for excitation. The residual part of the fundamental output was focused in a 1-cm H_2O cell to generate a white continuum as a probe pulse. A computer-controlled translation stage was used to change the delay time between the pump and probe pulses. The transmitted light of the probe pulse through the sample was detected by a multichannel spectrometer (HH4-0913, Otsuka Electronics) as a function of probe delay time. The transient absorption spectral data were averaged over 300 measurements, and corrected for time dispersion of the white continuum. The time correlation profile between the white continuum and the excitation light as a function of the probe wavelength was measured by the optical Kerr effect cross-correlation method using CCl_4 as a Kerr medium.

The PYP sample was flown in an optical flow cell (1-mm light path length) using a peristaltic pump at 22 °C. The excitation energy was ∼3 mJ/cm^2, and the frequency was 10 Hz. Transient difference absorption spectra were analyzed by IGOR Pro ver. 3.01 for Macintosh (Wave Metrics Inc.).

RESULTS

The transient difference absorption spectra of PYP after femtosecond pulse were recorded in a flow cell so that the intermediates formed by the former flash were not excited. For the same purpose, the interval of the pulse was reduced to 10 Hz. Ujj et al. (*19*) reported that the linear range of the excitation energy for the photoreaction was 1.25−7.5 nJ/pulse in their experimental setup (beam waist = 20 μm) and selected 3 nJ pulse (1 mJ/cm^2) at 452 nm. The excitation energy in the present experiment was 3 mJ/cm^2 at 390 nm. Since the absorbance at 390 nm is one-fourth of that at 452 nm, the excitation efficiency is considered comparable. The transient difference absorption spectra after excitation were recorded for −1 ps to 5 ns.

Typical data are shown in Figure 1. Through the spectral analysis using a multichannel spectrometer, successive spectral changes after excitation are clearly demonstrated. Immediately after the excitation (0 ps), two negative bands were observed. The narrow band at about 450 nm was due to the decrease of PYP by excitation. The amplitude of this band was reduced as the time elapsed (4 ps). The broad band at about 500 nm was a stimulated emission signal (*19*) because PYP does not have absorbance in the wavelength region longer than 500 nm. The peak of the stimulated emission shifted to 520 nm in 1 ps. In addition, the shape of the stimulated emission was altered. The spectral waist at 480 nm and the tail longer than 550 nm became smaller (0−4 ps), resulting in the narrow bandwidth. This is in good agreement with our previous report (*18*). The stimulated emission signal was retained up to 4 ps, and then absorbance increase at 480−550 nm took place (20 ps). At 8 ps, only the decrease of PYP was observed, probably because the trace of stimulated emission was canceled by the absorbance increase. The shape of this broad positive band (λ_{max} = 500 nm) was not altered up to 1 ns. This intermediate is hereafter called PYP_B (see below). On the nanosecond time scale, the shape of the positive band was clearly different from those at 20 ps−1 ns. The difference absorption maximum was shifted to 475 nm, and the bandwidth was much narrower. This intermediate is hereafter called PYP_L (see below).

The absorbance changes at the typical wavelengths were plotted against the delay time (Figure 2). The top trace (520 nm) demonstrates the decay of stimulated emission (0.5−10 ps), formation (10−100 ps), and decay (100−5000 ps) of PYP_B. The middle trace (485 nm) demonstrates the decay of stimulated emission (0.5−10 ps), formation (10−100 ps) of PYP_B, and formation (100−5000 ps) of PYP_L. The bottom trace (440 nm) is the recovery of PYP from the excited state (0.5−10 ps) and the absorbance increase due to the formation of PYP_L (100−5000 ps). These data are suggestive for the better understanding of PYP photoreaction but are very complicated because several conversion processes are superimposed.

To understand the spectral changes in detail, singular value decomposition (SVD) analysis (*27, 28*) was applied to the data set of 24 spectra in the time region from 0.5 ps to 5 ns. Each spectum had 298 data points in the wavelength region from 700 to 406 nm. The matrix of spectral data set **A** (298 × 24) was decomposed into matrices **U** (298 × 24 column-orthonormal matrix), **S** (24 × 24 diagonal matrix), and **V** (24 × 24 orthonormal matrix) as follows:

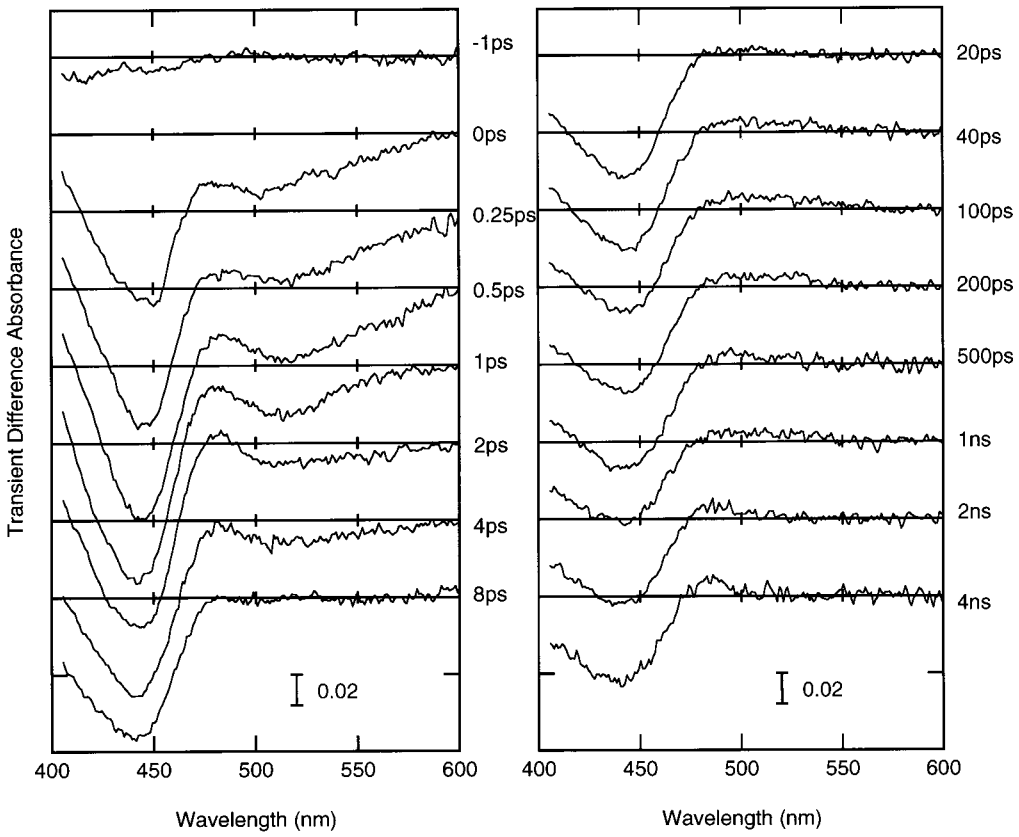

FIGURE 1: Transient difference absorption spectra of PYP after excitation by femtosecond pulse at 390 nm.

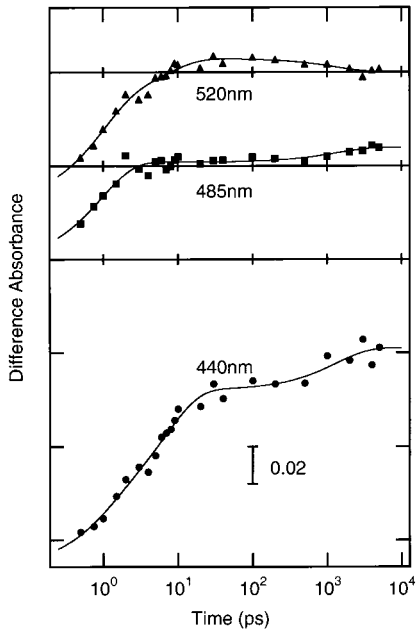

FIGURE 2: Transient absorbance changes at 520, 485, and 440 nm were plotted against delay time. These were then fitted by the sum of three exponential curves (τ = 950 fs, 7.1 ps, 1.3 ns) derived from SVD analysis.

$$\mathbf{A} = \mathbf{USV}^{\mathrm{T}}$$

Columns of **U** and **V** are called U-spectra (U*i*) and V-spectra (V*i*), respectively, and the elements of **S** are the singular values (S*i*). Results are shown in Figure 3, in which significant four sets of U- and V-spectra are demonstrated. Four V-spectra could be fitted by the sum of three exponential curves having common time constants ($\tau_{1/e}$ = 0.954, 7.08, and 1320 ps) as follows:

$$\mathrm{V1} = 0.105 + 0.0744\mathrm{e}^{-0.954t} + 0.132\mathrm{e}^{-7.08t} + 0.0512\mathrm{e}^{-1320t}$$

$$\mathrm{V2} = 0.136 - 0.988\mathrm{e}^{-0.954t} - 0.150\mathrm{e}^{-7.08t} + 0.0507\mathrm{e}^{-1320t}$$

$$\mathrm{V3} = -0.253 + 1.40\mathrm{e}^{-0.954t} - 0.979\mathrm{e}^{-7.08t} + 0.582\mathrm{e}^{-1320t}$$

$$\mathrm{V4} = 0.153 + 0.853\mathrm{e}^{-0.954t} - 0.373\mathrm{e}^{-7.08t} - 0.104\mathrm{e}^{-1320t}$$

where *t* is time after excitation (ps). These curves are also shown in Figure 3b, which shows that V1−V4 were fitted properly. Using these factors and singular values (S1, S2, S3, and S4 were 2.82, 0.497, 0.140, and 0.0854, respectively), B-spectra were calculated as follows (Figure 4):

$$\mathrm{B0} = 2.82 \times 0.105 \times \mathrm{U1} + 0.497 \times 0.136 \times \mathrm{U2} - 0.140 \times 0.253 \times \mathrm{U3} + 0.0854 \times 0.153 \times \mathrm{U4}$$

$$\mathrm{B1} = 2.82 \times 0.0744 \times \mathrm{U1} - 0.497 \times 0.988 \times \mathrm{U2} + 0.140 \times 1.40 \times \mathrm{U3} + 0.0854 \times 0.853 \times \mathrm{U4}$$

$$\mathrm{B2} = 2.82 \times 0.132 \times \mathrm{U1} - 0.497 \times 0.150 \times \mathrm{U2} - 0.140 \times 0.979 \times \mathrm{U3} - 0.0854 \times 0.373 \times \mathrm{U4}$$

$$\mathrm{B3} = 2.82 \times 0.0512 \times \mathrm{U1} + 0.497 \times 0.0507 \times \mathrm{U2} + 0.140 \times 0.582 \times \mathrm{U3} - 0.0854 \times 0.104 \times \mathrm{U4}$$

B1, B2, and B3 are the spectral changes which took place with the time constants of 950 fs, 7.1 ps, and 1.3 ns, respectively. B0 is the difference spectrum at infinite time,

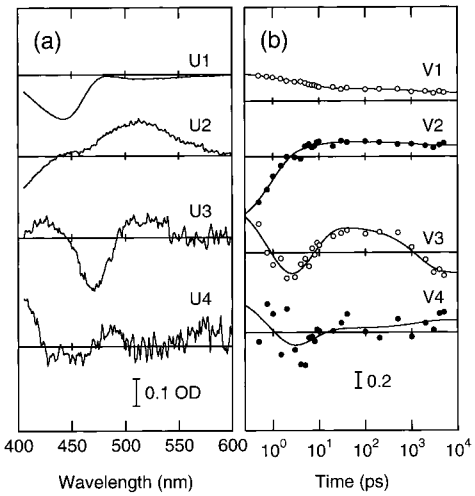

FIGURE 3: Significant four sets of U-spectra (a) and V-spectra (b) obtained by SVD. V-spectra were fitted by the sum of three exponential curves ($\tau_{1/e}$ = 0.954, 7.08, and 1320 ps).

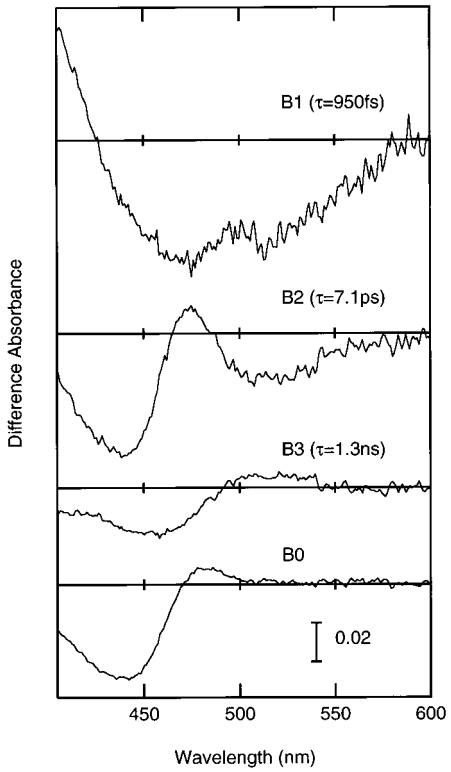

FIGURE 4: B-spectra calculated from SVD analysis. The lifetimes of B1, B2, and B3 were estimated to be 950 fs, 7.1 ps, and 1.3 ns, respectively. B0 is a constant which is the difference absorption spectrum at $t = \infty$. Note that the species in the positive side are converted to the species in the negative side in B1−B3.

extrapolated from the data set within 5 ns. Identification of these spectra is discussed under Discussion.

DISCUSSION

From the transient difference absorption spectra, we roughly see the decays of stimulated emission at 500 and 520 nm, the recovery of PYP, and formations of PYP_B and PYP_L. However, as the spectral change is complicated, SVD analysis was used to demonstrate the kinetics quantitatively. As a result, four significant sets of U- and V-spectra were obtained, and the time constants, 950 fs, 7.1 ps, and 1.3 ns, were estimated. Using these values and U-spectra, four B-spectra were calculated. To understand the primary photochemical event of PYP, the identification of B-spectra is important.

In the present experiment, two distinct spectra for excited state(s) were obtained by the combination of multichannel spectrometer and SVD analysis (B1 and B2). They show the quench of stimulated emission with the decay time constants of 950 fs and 7.1 ps. We previously reported that two fluorescence components whose lifetimes were 500−900 fs and 4−10 ps were observed in an up-conversion experiment (*18*). The spectral shape as well as decay time constant were different, suggesting the presence of two distinct excited states as previously proposed (*19*, *29*). However, the possibility that the shift in stimulated emission reflects the time-dependent Stokes shift of a single excited state cannot be excluded. Structural heterogeneity may cause the complexity of the kinetics, especially at early times. The continuous shift of stimulated emission may be apparently reproduced by the double exponential kinetics.

The fast quench of the stimulated emission shown in B1 has two peaks at 470 and 510 nm. The absorbance of the product of the first step, which should be observed in the negative region of B1, is hidden by the stimulated emission. On the other hand, B2 shows that the species which has absorbance at 470 nm and emission at 490−550 nm decays to a product having a λ_{max} at 440 nm with a time constant of 7.1 ps. Based on the spectral shape of the negative band, the product in B2 would be the ground-state PYP. B1 would be the relaxation of the excited state to primary intermediate, and the decay shown by B2 would take place parallel to the first decay. Ujj et al. (*19*) also detected two-exponential decay of stimulated emission and speculated that the decay of the 16-ps process represents the repopulation of the ground state and that the 1.5-ps process is the formation of I0.

B3 shows the decay of the species having absorbance at 500−550 nm to that at wavelength shorter than 500 nm. The broad spectral shape of the former is in good agreement with that of I0 reported previously (*19*). It is also similar to the red-shifted intermediate trapped at −190 °C (PYP_B), which has a broad absorption spectrum with a λ_{max} at 490 nm. The peak of the product (negative region) was located at 455 nm, which is longer than PYP. B0 is the final spectrum in the present time scale (5 ns), and the difference spectrum before and after the excitation of PYP. Therefore, the product is the final intermediate in this time scale, i.e., the product in B3. Because PYP_L is formed within 10 ns and its decay time constant is ∼100 μs (*16*), this intermediate is considered to be PYP_L. Therefore, B3 represents the conversion from PYP_B to PYP_L, and B0 is the difference spectrum between PYP and PYP_L.

Ujj et al. (*19*) observed I0‡ (λ_{max} = 500 nm) between I0 and I1. I0‡ has a spectrum similar to I0, but it is kinetically distinct (τ = 3 ns). In their data, the absorbance at 481 nm increased from 0 to 100 ps, then decreased slightly from 100 to 800 ps, and increased again. This decrease is due to the conversion from I0 to I0‡, which has a lower absorbance than I0. However, in our study, the absorbance decrease in ∼100 ps was not clear (Figure 2). Because the absorption spectrum of I0‡ is similar to that of I0(PYP_B), it did not contribute to the U-spectra, and the spectral changes observed in the present experiment can be explained without I0‡. It might arise from the structural heterogeneity of the protein

or from the contribution of PYP_H (see blow) to the kinetic features.

It should be noted that the ratio of the absorbance increase at 475 nm to the absorbance decrease at 446 nm in B0 is smaller than previous reports (*16*, *19*, *21*) (Figure 5). This trend is also observable in the previous reports (*19*, *30*). This suggests that the amount of photoconverted PYP is larger than that of produced PYP_L, and that the other intermediate whose absorption bands are located in the wavelength region shorter than 400 nm, or whose absorption band is overlapped but whose extinction coefficient is smaller than PYP, is formed. Among the intermediates of PYP detected at low temperature, PYP_H (λ_{max} = 442 nm), PYP_{HL} (λ_{max} = 447 nm), and PYP_{BL} (λ_{max} = 400 nm) meet these criteria (*21*). PYP_H is the photoproduct trapped at −190 °C, and the extinction coefficient is smaller than PYP. PYP_{HL} and PYP_{BL} are the thermal products of PYP_H and PYP_B, respectively, and decay to PYP_L. PYP_{BL} is the blue-shifted intermediate, but it is not the likely candidate because B3 shows the direct conversion from PYP_B to PYP_L. Therefore, PYP_H (or PYP_{HL}) is the most likely candidate. As it has no contribution to the kinetic features of the present data set, it is considered stable at least for 5 ns.

To substantiate the primary process of PYP, the absorption spectra of PYP_B, PYP_L, and PYP_H were calculated from B-spectra. The experimental evidences discussed above strongly suggest that the photocycle of PYP at room temperature is essentially similar to that at low temperature. Therefore, the absorption spectra at room temperature were calculated according to the reaction model derived by low-temperature spectroscopy.

Large negative absorbance of B0 strongly suggests that B0 is not the pure PYP_L minus PYP spectrum; the additional intermediate, presumably PYP_H, contributes to B0. The PYP_L minus PYP spectrum at room temperature has been previously reported by Hoff et al. (*16*). In Figure 5, B0 and the PYP_L minus PYP spectrum (*16*) were reproduced. The shapes of their positive bands in the long-wavelength sides are identical. The amplitude of the PYP_L minus PYP spectrum was normalized so that the positive band of B0 was canceled, and the PYP_L minus PYP spectrum was subtracted from B0. This calculation decomposed B0 into the PYP_L minus PYP spectrum and the putative PYP_H minus PYP spectrum.

We previously calculated the absorption spectrum of PYP_L at −80 °C (*21*). Because the shape of the PYP_L minus PYP spectrum at −80 °C was almost identical to that at room temperature, the amount of PYP converted to PYP_L can be estimated from the amplitude of the PYP_L minus PYP spectrum using the parameters obtained by low-temperature experiments. As the negative band of the PYP_L minus PYP spectrum is temperature-independent, we used the amplitude of the negative band for calculation. In the present set of experiments, the PYP converted to PYP_L was estimated to be 0.033 absorbance (5.5%). The absorption spectrum of PYP_L was calculated by adding the spectrum of PYP whose maximal absorbance was normalized to 0.033 to the PYP_L minus PYP spectrum in Figure 5. Because the time constant of B3 (1.3 ns) is enough separated from those of B1 (950 fs) and B2 (7.1 ps), the contribution of the components other than PYP_B and PYP_L to B3 was negligible (<1%). The absorption spectrum of PYP_B was therefore calculated by adding the absorption spectrum of PYP_L to B3.

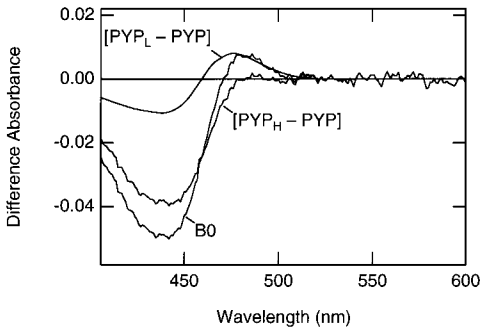

FIGURE 5: Comparison of B0 with the PYP_L minus PYP spectrum [reproduced from (*16*)]. The latter was normalized so that the positive band of B0 was canceled. B0 was then decomposed into the PYP_L minus PYP spectrum and the putative PYP_H minus PYP spectrum for calculation of the absorption spectrum of PYP_H.

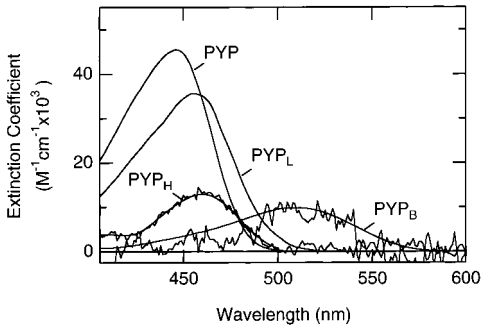

FIGURE 6: Calculated absorption spectra of PYP_B, PYP_H, and PYP_L.

With the assumption that no PYP recovers directly from PYP_B, PYP_H, or PYP_L, the total amount of PYP converted to PYP_B and PYP_H is calculated by the excitation energy (3 mJ/cm^2 at 390 nm), the optical density of the sample (0.148 at 390 nm), the molar extinction coefficient of PYP [45 500 M^{-1} cm^{-1} at 446 nm (*15*)], and the quantum yield for formation of intermediates [0.64 (*15*)]. We used the value of 0.64 for the quantum yield, which was estimated from the amount of bleached state (PYP_M). It is considered to be the most reliable because PYP_M is formed after two parallel pathways join and the amount of PYP_M is equal to the sum of PYP_B and PYP_H. The percentages of excited PYP and PYP converted to the intermediates were calculated to be 21.4% (0.128 absorbance) and 13.7% (0.082 absorbance), respectively, and thus PYP converted to PYP_H was 0.049 absorbance (8.2%), which was added to the PYP_H minus PYP spectrum. In this calculation, the amount of PYP recovered directly from the excited state was estimated to be 0.046 absorbance (7.7%), which is in good agreement with the negative absorbance of B2.

The calculated absorption spectra are shown in Figure 6. The λ_{max} of PYP_B, PYP_H, and PYP_L were located at 509, 458, and 456 nm, respectively. The extinction coefficients of PYP_B and PYP_H were remarkably smaller than that of PYP, which would cause no observation of PYP_B and PYP_H in B1 or B2. We tentatively assigned the new intermediate to PYP_H, but it may correspond to PYP_{HL}, because its λ_{max} and extinction coefficient relative to those of PYP_H are red-shifted and reduced (*21*).

The photocycle of PYP is summarized in Figure 7. Low-temperature spectroscopy revealed the existence of six intermediates: PYP_B, PYP_{BL}, PYP_H, PYP_{HL}, PYP_L, and PYP_M. The present experiments suggest the existence of two excited

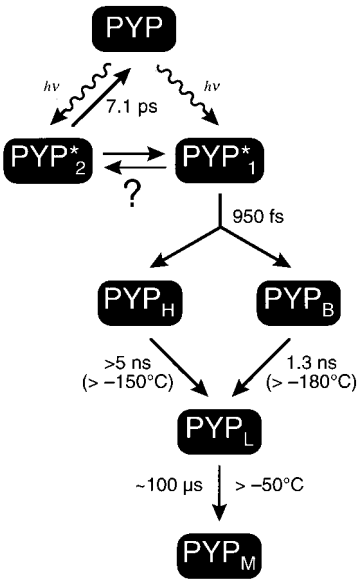

FIGURE 7: Photocycle of PYP at room temperature. The decay time constants ($\tau_{1/e}$) are shown beside the arrow. The transient temperatures estimated by the low-temperature spectroscopy are also indicated.

states, PYP^*_1 and PYP^*_2. Two red-shifted intermediates, PYP_B and PYP_L, were observed clearly. In addition, the existence of another intermediate, presumably PYP_H, was shown by the transient spectrum at 5 ns, and its absorption spectrum was calculated. Because the calculated amount of PYP recovered from the excited state agreed with the negative absorbance of B2, PYP^*_2 would decay mainly to PYP. While the thermal product of PYP_B at low temperature is PYP_{BL}, at room temperature PYP_B decays to PYP_L with a time constant of 1.3 ns as shown by B3. It may be explained by the difference in the temperature effect on the decay of PYP_B and PYP_{BL}: at low temperature, the decay of PYP_B is faster than that of PYP_{BL}. Because these are thermal reactions, they are much faster at room temperature. If the temperature effect on the decay of PYP_{BL} is larger than that of PYP_B, the former is faster than the latter, resulting in no observation of PYP_{BL}. The conversion from PYP_H to PYP_L could not be observed within 5 ns. However, it is reasonable to speculate that PYP_H decays to PYP_L because the PYP_L minus PYP spectrum was obtained at 24 ns after excitation (*16*). Therefore, the PYP photocycle at room temperature has a parallel pathway like that at low temperature.

PYP_B likely corresponds to the J intermediate of bacteriorhodopsin, considering the spectral and kinetic properties. J has never been trapped at low temperature whereas the present experiments showed that PYP_B is formed at physiological temperature as well as at low temperature. The combination of the ultrafast technique and the low-temperature experiment have the potential to reveal the primary intermediate of PYP and to clarify the light-capturing mechanism of PYP.

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