Effects of Modification of Protein Nanospace Structure and Change of Temperature on the Femtosecond to Picosecond Fluorescence Dynamics of Photoactive Yellow Protein

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The effect of the protein environment surrounding a chromophore [protein nanospace (PNS)] on the photoinduced ultrafast reaction of the chromophore (deprotonated p-coumaric acid covalently bonded to the protein via a thioester linkage) has been investigated by comparing the femtosecond to picosecond fluorescence dynamics of wild-type (w-t) photoactive yellow protein (PYP) with those of the chromophore in aqueous solution as well as in various PNSs modified by site-directed mutagenesis. The fluorescence decay dynamics of the chromophore are considerably accelerated in PNSs compared with those in solution. The ultrafast nonexponential fluorescence decay dynamics of the w-t PYP in the 100 fs to 10 ps time regime, which are probably due to the formation of a twisted state by the flipping of the thioester linkage of the chromophore, have been slowed in the mutants, which seem to have a looser PNS structure because of the weakening of the chromophore−amino acid residue hydrogen-bonding interaction and the partial destruction of the H-bonding network surrounding the chromophore in PNS by mutation. Specifically, the more restricted structure of the PNS in w-t PYP seems to be favorable for twisting by the flipping mechanism. In addition, we have examined temperature effects on the fluorescence dynamics of the w-t PYP and several mutants to elucidate the mechanisms underlying the highly nonexponential decay of the w-t PYP as well as the remarkable influences on the fluorescence dynamics of modifications in the PNS structure by site-directed mutations. We have revealed by these investigations that the fastest decay in the femtosecond to 1 ps regime in w-t PYP is temperature-independent and that the twisting reaction responsible for it could be of a coherent or barrierless origin, whereas thermal activation is necessary for the reaction in the few picosecond to 10 ps regime. For the mutants, however, no activationless process could be observed. Therefore, rapid relaxation into traps seems to occur, competing with the barrierless reaction in the case of the w-t PYP and becoming overwhelming in the mutants. After such trappings, slow reactions may take place by thermal activation.

Introduction

In photosensory or photoactive proteins with chromophores absorbing in the visible region, such as rhodopsin (Rh) for vision,[1] bacteriorhodopsin (bR)[1] and other photoactive proteins of various photoresponsive bacteria, and even several other enzymes[2] that are not necessarily involved in the photobiological reactions, light absorption can lead to ultrafast and highly efficient reactions. Among those proteins, photoactive yellow protein (PYP), which functions as a blue light photoreceptor for the negative phototaxis of the purple sulfur bacterium *Ectothiorhodospira halophila*, seems to have the simplest structure[3] and to show a relatively simple photoreaction cycle.[4−10] The chromophore, p-coumaric acid (deprotonated) covalently bonded to the side chain of Cys69 via a thioester bond, which has an absorption peak at $\lambda_{max} = 446$ nm (ground state, G), undergoes trans → cis photoisomerization, leading to a red-shifted intermediate state, I_1, which has an absorption peak near $\lambda_{max} \approx 460$ nm and which probably can be attributed to the cis form. Then, I_1 undergoes one-proton uptake, leading to a blue-shifted intermediate, I_2, with an absorption peak at $\lambda_{max} = 355$ nm, which then re-forms G by deprotonation[2−10] (see Figure 1).

We examined the dynamics of PYP in the early stages of relaxation immediately after photoexcitation by means of femtosecond fluorescence up-conversion measurements for the first time.[11] According to our results, fluorescence decay curves of wild-type (w-t) PYP were nonexponential and approximately reproduced by the superposition of three exponentials of the several hundred femtosecond, few picosecond, and 10 ps time regimes. These fluorescence decay dynamics can be ascribed to the primary reaction process of forming the twisted state (Tw^-) from the Franck−Condon (FC) excited state or fluorescence state (Fl) in the course of the trans → cis isomerization, about which we provide more or less detailed discussions in the following part of this paper. In a sub-picosecond pump−probe absorption spectral study of the w-t PYP[12] reported at the same time as the above work,[11] spectral changes in the several hundred femtosecond to few picosecond time regime were also indicated. Other sub-picosecond fluorescence up-conversion measurements on the w-t PYP were also reported recently,[13] results of which were in good agreement with those of our previous measurements.[11] We have assumed in our

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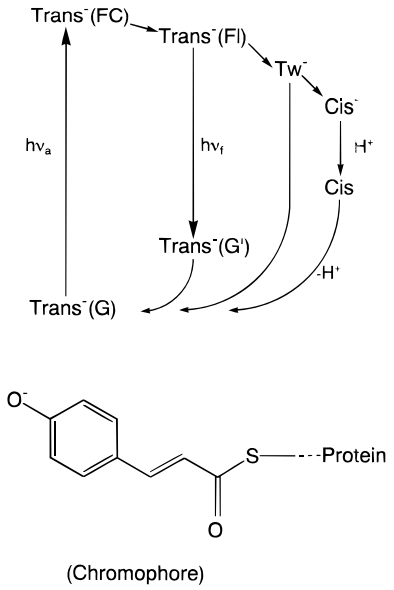

Figure 1. Photoreaction cycle of the w-t PYP. Chromophore: deprotonated p-coumaric acid thioester bound to Cys69 in PNS.

previous papers[11] as well as in this work that the fluorescence quenching dynamics of the w-t PYP are due to the formation of the twisted state (Tw^-), which is nonfluorescent in the wavelength region of the fluorescence band of w-t PYP (450−600 nm). This assumption is supported by various investigations, as follows.

Usually, the energy gap between the excited- and the ground-state potential surfaces at the twisted state in the course of the trans → cis photoisomerization is small because of the (avoided) crossing between the excited reactant and ground-state product potential surfaces, and radiationless deactivation to the original ground state or to the product state takes place rapidly. Therefore, fluorescence from the twisted state can barely be observed in some typical examples.[19] Moreover, in the case of w-t PYP, there are several experimental results that indicate the important role of Tw^- state formation in the photoisomerization and fluorescence quenching processes, as discussed below.

With respect to the structures of the chromophore and the surrounding protein environment [protein nanospace (PNS)] of w-t PYP in the course of the photocycle, remarkable advances have been achieved by millisecond time-resolved X-ray crystallography studies of w-t PYP crystals,[14] as well as nanosecond time-resolved measurements[15] at room temperature. On the basis of these studies, structures of the red-shifted intermediate I_1, with the deprotonated chromophore in its cis form, and the blue-shifted intermediate I_2, with the protonated chromophore in its cis form, including the structures of amino acid residues surrounding the chromophore in the PNS, have been elucidated. Moreover, detailed X-ray crystallography on w-t PYP crystals in which the photoreaction intermediates are cryotrapped below −100 °C has revealed that the early transient intermediate state with the twisted structure of the chromophore (Tw^-) seems to be formed by flipping its thioester linkage with the protein, avoiding collisions resulting from the large-scale movement of its aromatic ring during the initial photoreaction.[16] Detailed analysis of the nanosecond X-ray crystallographic measurements also suggests the existence of the initial structural change of the chromophore primarily in its tail part.[15] This initial Tw^- state seems to be identified with the intermediate PYP_B, which has an absorption peak at $\lambda_{max} = 489$ nm, previously found by difference FTIR static spectroscopy at −190 °C.[17,18] A recent picosecond transient absorption spectral study on the w-t PYP at room temperature has revealed a new transient absorption band (with a maximum near 500 nm) due to an intermediate (I_o) that appeared ≤3 ps after excitation with the picosecond laser pulse, and the intermediate I_o has been demonstrated to be converted to I_1.[10] In light of the rather close values of their absorption maxima (489 nm for PYP_B at −190 °C[17,18] and 500 nm for I_o at room temperature), I_o might be identified with the highly distorted intermediate Tw^-. In addition, despite a strong similarity in the chromophore structure of w-t PYP to that of GFP (green fluorescent protein) from *Aequorea victoria*, the tail of the GFP chromophore is covalently locked in its conformation and evidently lacks the flexibility of the tail part of the PYP chromophore. This results in the highly efficient green fluorescence emission of GFP, in contrast to the low fluorescence yield ($\sim 10^{-3}$) of w-t PYP.[15] This fact also seems to support a fluorescence quenching mechanism attributable to formation of the Tw^- state by the flipping of the tail part of the w-t PYP chromophore in the course of the photoisomerization.

In the highly distorted geometry of the chromophore in the early transient state ($Tw^- \approx PYP_B$), much of the initial photon energy may still be stored, and all subsequent steps of I_1 (deprotonated cis) and I_2 (protonated cis) state formation, accompanied by changes in the surrounding PNS structures, and completion of the cycle by cis → trans isomerization accompanied by deprotonation may be driven mainly by the stored energy. Therefore, for the elucidation of the reaction mechanisms, detailed femtosecond to picosecond spectroscopic studies are of crucial importance, along with the above structural studies by X-ray crystallography. Specifically, the way in which the PNS of w-t PYP affects the ultrafast relaxation processes in the excited state leading to the formation of the highly distorted Tw^- state should be studied in detail. In this respect, we have compared the fluorescence dynamics of the chromophore (in deprotonated form) in aqueous solution with those of the chromophore in the PNS of w-t PYP, and we also have examined the effects of modifications of the amino acid residues surrounding the chromophore on the dynamics of Tw^- state formation by using the site-directed mutagenesis method.

We reported in the previous short communication[11] our preliminary results on the fluorescence dynamics of the PYP chromophore in solution, in the w-t PYP, and in several site-directed mutants and provided a brief discussion of their mutual relations and the mechanisms of their photoinduced primary processes. In the present report, we will give more detailed experimental results of the femtosecond to picosecond fluorescence dynamics studies of the problems indicated above. Moreover, we will show results of our investigations concerning temperature effects on the fluorescence dynamics of w-t PYP and several mutants. These studies on temperature effects may be of crucial importance for disclosing the nature of the nonexponential fluorescence dynamics and for the elucidating the fundamental mechanisms of the ultrafast reaction dynamics in the initial Tw^- state formation, as discussed later in this paper. On the basis of these studies, we will discuss the fundamental mechanisms underlying these experimental results.

Experimental Section

Measurements of fluorescence dynamics were made by a fluorescence up-conversion apparatus similar to that described elsewhere.[11] The fwhm of the over-all instrumental response was 210 fs. The sample solutions for the measurements were made to flow through a 1-mm cell at room temperature. For

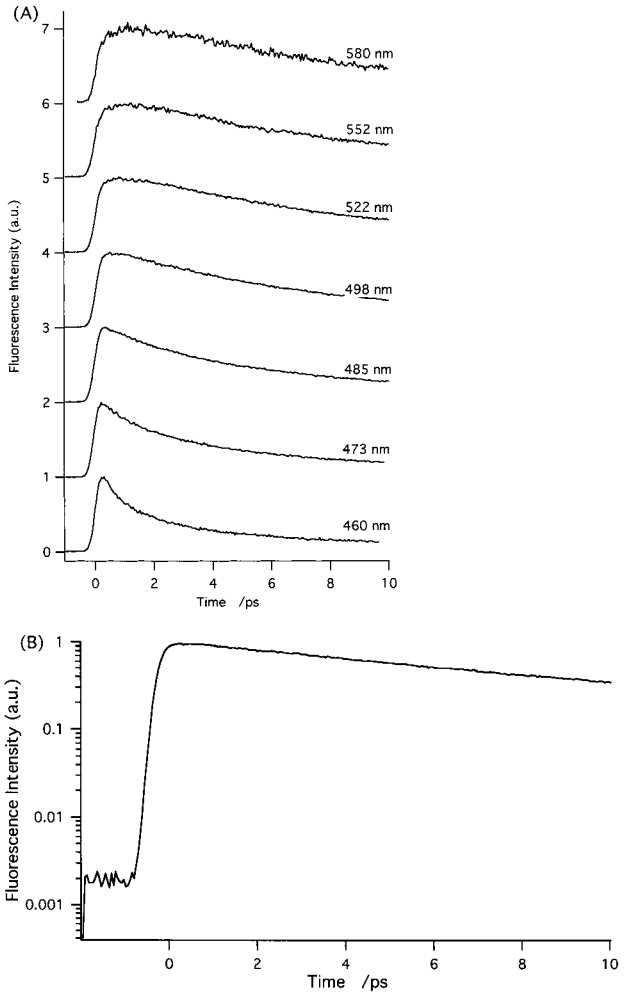

Figure 2. (A) Fluorescence dynamics of the p-coumaric acid (deprotonated) chromophore linked to the unfolded protein of w-t PYP (denatured through the addition of guanidinium HCl to a solution of pH 9.5) excited at 410 nm and observed at various wavelengths. (B) Single-exponential decay of the chromophore fluorescence of the denatured solution in A near the peak position of the stationary fluorescence spectrum (498 nm).

measurements of the temperature dependencies of the fluorescence decay curves, we used the apparatus equipped with a homemade Peltier device and monitored fluorescence decay curves at temperatures between 1 and 35 °C.

The preparations of w-t PYP and its site-directed mutants were described elsewhere.[20,21] We examined the E46Q, T50A, T50V, R52Q, and E46Q/T50V mutants, in which the amino acid residues placed contiguous to the chromophore and rather strongly interacting with it in the PNS are replaced as follows: Glu46 → Gln for E46Q, Thr50 → Ala for T50A, Thr50 → Val for T50V, Arg52 → Gln for R52Q, and Glu46 → Gln/Thr50 → Val for E46Q/T50V.

Results and Discussion

Comparison of the Fluorescence Dynamics of the PYP Chromophore in Aqueous Alkaline Solution and in PNS. Fluorescence dynamics of the deprotonated chromophore linked to the unfolded protein of w-t PYP (denatured through the addition of guanidinium HCl to a solution of pH 9.5), excited at 410 nm with a femtosecond pulse and observed at various wavelengths, are indicated in Figure 2A. The observed fluorescence dynamics show typical features of the dynamic Stokes shift due to solvation, namely, a rapid initial decay in the short wavelength region and a corresponding fluorescence rise curve in the long wavelength region, in accordance with the large Stokes shift ($\Delta\nu_s \sim 4840$ cm^{-1}) of the fluorescence spectrum. The fluorescence decay observed at wavelengths near the peak position of the stationary fluorescence spectrum, where the effect of the dynamic Stokes shift is negligible, fits a single exponential with a decay time of 11 ps, as shown in Figure 2B. Thus, the exponential fluorescence decay dynamics of the PYP chromophore in aqueous solution with an 11-ps decay time are quite different from the highly nonexponential and ultrafast decay dynamics of w-t PYP in the 100 fs to few picosecond regimes, indicating also a large enhancement of the initial photoinduced reaction rate in the PNS, as discussed in detail later (see Figure 4A).

Although detailed studies of the photoisomerization of the PYP chromophore itself in aqueous solutions do not seem to be available, some 4-substituted styrene derivatives are known to undergo trans → cis photoisomerization. For example, 4-methoxy and 4-*N*,*N*-dimethylamino-β-(1-pyrenyl) styrenes in solution undergo trans → cis photo-isomerization exclusively from the S_1 state,[22] where the isomerization occurs by a twisting about the vinyl bond. The reaction yield is enhanced with an increase in the electron-donating ability of the 4 substituent.[22] In light of the strong electron-donating ability of the O^- substituent of the present chromophore and the relatively short fluorescence lifetime of 11 ps, it is probable that the chromophore itself undergoes fairly rapid isomerization by twisting in the S_1 state, although it is not very clear whether the twisting takes place at the vinyl bond or at the thioester bond in the tail part.

At any rate, in relation to the problem that the twisting of the chromophore in the excited state seems to cause the fluorescence quenching, we emphasize again its importance in the fluorescence quenching of the PYP chromophore in light of the following fact. Specifically, upon replacement of the p-coumaric acid chromophore in the PNS of w-t PYP by 7-hydroxycoumarin-3-carboxylic acid, where a twisting at the vinyl bond is not possible and that at the tail part also seems to be not easy, the fluorescence decay time becomes much longer (>60 ps)[13] compared with the several hundred femtosecond to few picosecond decay time of the w-t PYP. Together with the highly efficient green fluorescence of GFP from *A. victoria* in which the tail part of the chromophore similar to that of PYP is covalently locked[15] (as discussed already in the Introduction), this result for the 7-hydroxycoumarin-3-carboxylic acid chromophore strongly indicates the importance of the fluorescence quenching mechanism due to Tw^- state formation in the PYP.

Effects of Modifications of the Protein Nanospace on the Primary Photoreaction Process of PYP. In Figure 3A and B, absorption and fluorescence spectra, respectively, of the w-t PYP and its mutants are shown. In general, spectra of the mutants are red-shifted compared with those of the w-t PYP, and the extent of the red shift is largest in the case of the double mutation, E46Q/T50V.

On the other hand, the fluorescence Stokes shift $\Delta\nu_S$, i.e., the reorganization energy associated with the photoexcitation and fluorescence emission, is generally smaller for mutants compared with those of the wild type. Specifically, $\Delta\nu_S$ (cm^{-1}) is 1340 (E46Q/T50V), 1780 (E46Q), 1760 (T50V), 1865 (T50A), and 2180 (w-t PYP), and the $\Delta\nu_S$ value of R52Q (2220 cm^{-1}) is rather close to and slightly larger than that of the wild type. The phenyl$-O^-$ group of the chromophore [p-coumaric acid (deprotonated) covalently bonded to the side chain of Cys69 via a thioester linkage] is surrounded by proton-donating amino

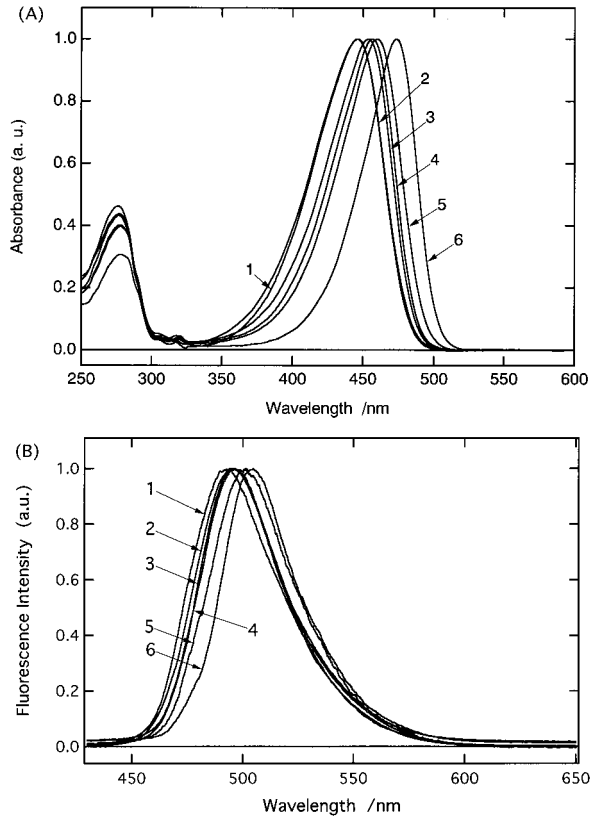

Figure 3. (A) Absorption and (B) fluorescence spectra of wild-type and mutant PYPs. (1) Wild-type, (2) R52Q, (3) T50A, (4) T50V, (5) E46Q, and (6) E46Q/T50V.

acid residues, which make a hydrogen-bonding network and a short-range H-bonding interaction with the phenyl$-O^-$ group. In the mutants we examined, except for R52Q, H-bonding interactions are weakened and the H-bonding network is partly destroyed by replacing the amino acid residues with only weakly H-bonding or non-H-bonding moieties. Such modifications of the amino acid residues in the PNS make the direct interactions between the chromophore and the H-bonding amino acid residues weaker, leading to smaller reorganization energies for the excitation by light absorption and the fluorescence emission. Moreover, such modifications of the H-bonding network and H-bonding interactions presumably cause a small change of the PNS, leading to a looser structure.

In the case of R52Q, although the H-bonding network and interactions may be kept almost invariant, the arginine residue with its positive charge is replaced by a neutral amino acid residue, leading to the loss of the Coulomb attraction with the negative charge of the phenyl$-O^-$ group of the chromophore. Because this interaction is not as short-range and direct as the H-bonding interaction, the influence on the reorganization energy accompanied by the light absorption and emission transitions may be small. Nevertheless, the loss of the chromophore−amino acid residue Coulomb attraction in the PNS will also contribute to the modification of the PNS to a looser structure.

In Figure 4, fluorescence dynamics of the w-t PYP and several mutants observed at various wavelengths are presented. As discussed in the previous section, fluorescence dynamics of the chromophore (deprotonated) linked to the unfolded protein of PYP (denatured by the addition of guanidinium HCl to an alkaline solution) showed typical features of the dynamic Stokes shift due to solvation. Because the chromophore is contained within the PNS for the w-t PYP and also for the mutants, solvent reorganization surrounding the protein in the course of the relaxation process may not be important for the fluorescence dynamics. Nevertheless, the excited PYP should undergo ultrafast relaxation from the FC excited state to the Fl state corresponding to the $\Delta\nu_S$ value given above. Because we could not observe the dynamic Stokes shift of the fluorescence, the ultrafast conversion from the FC to the Fl state presumably takes place within the time resolution of our measurement. Such ultrafast conversion might be realized by coherent couplings with intrachromophore high-frequency vibrations and with reorganizations of the excited chromophore−amino acid residue interactions, as proposed for rhodopsin and bacteriorhodopsin.[23] Thus, both the w-t PYP and mutant PYPs seem to undergo ultrafast FC−Fl conversion, presumably by coherent coupling, and the fluorescence dynamics may be determined predominantly by formation of the Tw^- state from an FC−Fl coherently coupled state or a relaxed Fl state, as discussed in detail later. We have already discussed in detail the very important role of Tw^- state formation for the fluorescence quenching dynamics of w-t PYP in the Introduction and in the previous section of the Results and Discussion. We assume that fundamentally the same mechanism of the fluorescence quenching dynamics also occurs in the case of mutant PYPs, although the details of the Tw^- state formation process might be modified because of changes in the PNS structure caused by site-directed mutagenesis, as described later.

The fluorescence decay curves indicated in Figure 4 are nonexponential, especially in the case of the w-t PYP, while the nonexponentiality is more moderate in the case of the mutants. In particular, the fastest decay component in the early stage is very conspicuous in the w-t PYP, while it is not so remarkable in the mutants and is especially inconspicuous in the case of the double mutant E46Q/T50V. In relation to the above-described problems of ultrafast conversion from the FC excited state to the Fl state, we should note that we can recognize in Figure 4 a slightly faster decay of the fluorescence intensity on both the blue and the red sides of the spectrum in the early stage. This seems to mean a slight narrowing of the fluorescence band shape at the early stage of the decay as the displacements of the high-frequency modes decrease along the twisting coordinate. In this respect, we have constructed a time-dependent fluorescence spectrum of the w-t PYP from the observed decay curves at various wavelengths. Although it was rather difficult to obtain accurate time-dependent fluorescence spectra, we could recognize a slight time-dependent narrowing in the blue and red bottom part of the fluorescence spectrum. We give a more detailed discussions of this problem later in this article in connection with our studies on the temperature dependence of the fluorescence dynamics.

The nonexponential fluorescence decay curves in Figure 4 can be reproduced approximately by the superposition of exponential decay functions. The superposition of three exponential functions is sufficient to reproduce satisfactorily the observed decay curves. In the case of mutants, for which nonexponentiality is considerably more moderate than that for the wild type, especially at the peak wavelength of the fluorescence spectrum, the decay curve can be reproduced equally well by the superposition of two exponential decay functions.

To compare the fluorescence decay dynamics of the w-t PYP and several mutants, we show in Figure 5 their fluorescence decay curves observed at the peak wavelength of the fluorescence spectra. Because the decay dynamics in the subpicosecond to several picosecond time regime, which should

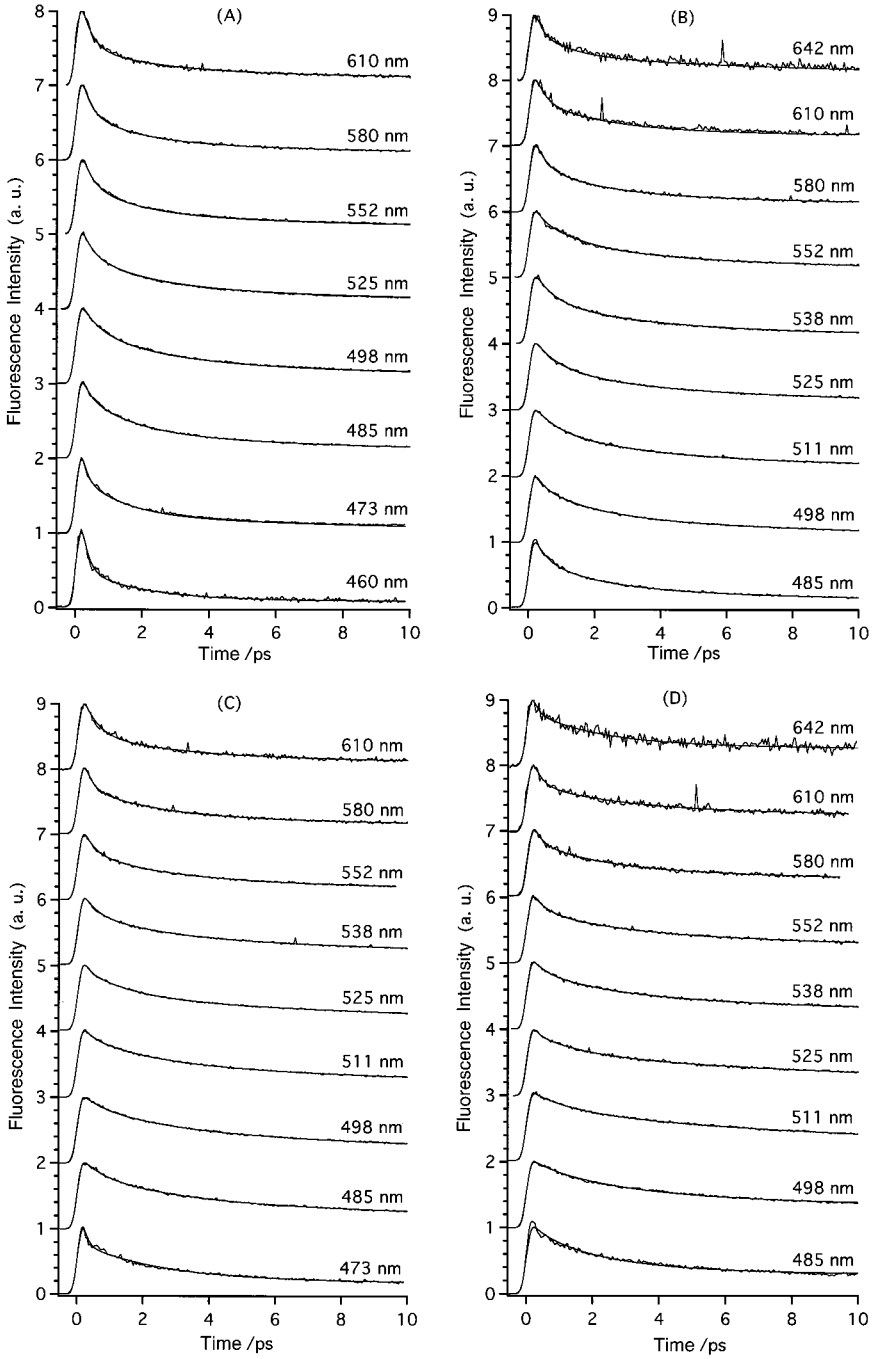

Figure 4. Fluorescence dynamics of w-t PYP and several mutants excited at 410 nm and observed at various wavelengths. (A) Wild-type, (B) E46Q, (C) T50V, and (D) E46Q/T50V. In addition to the observed decay curves, smooth curves simulated by the superposition of exponential decay functions are also indicated.

be related to the initial process of Tw^- state formation, are not in line with the value of the fluorescence Stokes shift $\Delta\nu_S$ given in the beginning of this section, the short-range interactions between the chromophore and the H-bonding amino acid residues in the PNS do not seem to be directly responsible for determining the rate of Tw^- state formation. This finding is in accord with the flipping mechanism of the thioester bond in the tail part of the chromophore for Tw^- state formation without the large-scale movement of the aromatic ring part (phenyl$-O^-$), which seems to keep short-range interactions (including H-bonding) with amino acid residues approximately unchanged during initial photoreaction. Therefore, for a determination of the dynamics and mechanisms of ultrafast Tw^- state formation, the structures and functions of the PNS as a whole in the excited state may be of crucial importance.

As described already to some extent at the beginning of this section, in the mutants E46Q, T50V, T50A, and E46Q/T50V, the weakening of the chromophore−amino acid residue H-bonding interaction, the partial destruction of the H-bonding network surrounding the chromophore, and also the loss of the Coulomb attraction between the positively charged amino acid residue and the negative charge in the chromophore will result in a modification of the PNS to a slightly looser structure, which seems to reduce the rate of Tw^- state formation compared with the rate for the w-t PYP. This effect of modifications in the PNS by site-directed mutagenesis is largest in the doubly mutated system, as indicated in Figure 5.

Thus, our investigations of the fluorescence dynamics of several site-directed mutants of PYP suggest that the photochemical reaction rate of the initial Tw^- state formation by the

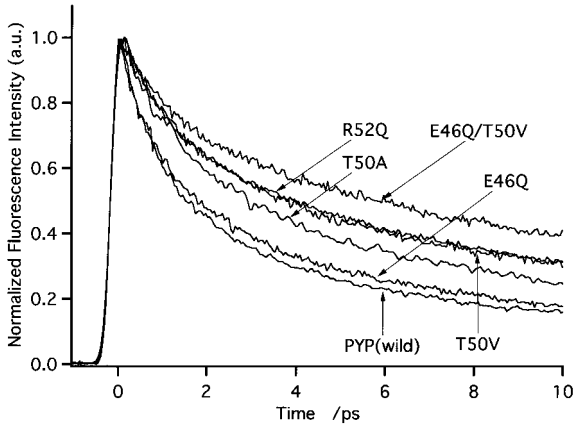

Figure 5. Fluorescence dynamics of w-t PYP and its site-directed mutants excited at 410 nm and observed at the wavelength of the maximum of the steady-state fluorescence spectrum. The fluorescence decay curves are normalized at the peak intensity.

mechanism of the flipping of the thioester linkage seems to be enhanced in a more restricted PNS environment. Specifically, among the native PYP and site-directed mutants examined here, the native one seems to be best-engineered for fast Tw^- state formation. In the case of the E46Q/T50V mutant, a rather large modification by double mutation of the PNS to a considerably looser structure may take place, leading to much slower twisting dynamics compared with those of the w-t PYP. To elucidate further the detailed mechanisms of Tw^- state formation from the excited state of the w-t PYP and site-directed mutants, we have investigated temperature effects on the fluorescence dynamics of these chromophores, results of which are described and discussed in the following.

Temperature Effects on the Fluorescence Dynamics of Wild-Type and Mutant PYPs. Fluorescence decay curves of the w-t PYP observed at the peak position (500 nm) and the blue side (470 nm) as well as the red side (570 nm) of the fluorescence spectrum undergo the changes indicated in Figure 6A, B, and C, respectively, when the temperature increases from 1 to 35 °C. These results show clearly that the nonexponential decay curve of the PYP fluorescence depends considerably on the temperature change, and the decay becomes slower at lower temperatures, especially in the few picosecond to 10 ps time regime.

The fluorescence decay dynamics shown here reflect the primary photoreaction process of forming the Tw^- state from the FC excited state in the course of the trans → cis isomerization. Therefore, the results in Figure 6 suggest that, for the Tw^- state formation reaction in the few picosecond to 10 ps time regime, thermal activation is necessary. However, the fastest decay component in the few hundred femtosecond to 1 ps time regime of the w-t PYP seems to be independent of temperature.

The fastest decay component in the decay curve is more conspicuous at the blue or red side of the spectrum, probably because of a slight narrowing in the blue and red bottom parts of the fluorescence spectrum in the early stage of the decay, as discussed in the preceding section. Nevertheless, the essential features of the decay curves on the blue and red sides of the spectrum with respect to temperature dependence are practically the same as that of the peak position. Specifically, the component in the few hundred femtosecond to 1 ps region is temperature-independent, while decays of the components in the few picosecond to 10 ps regime become slower at lower temperatures. For an analysis of the temperature dependence

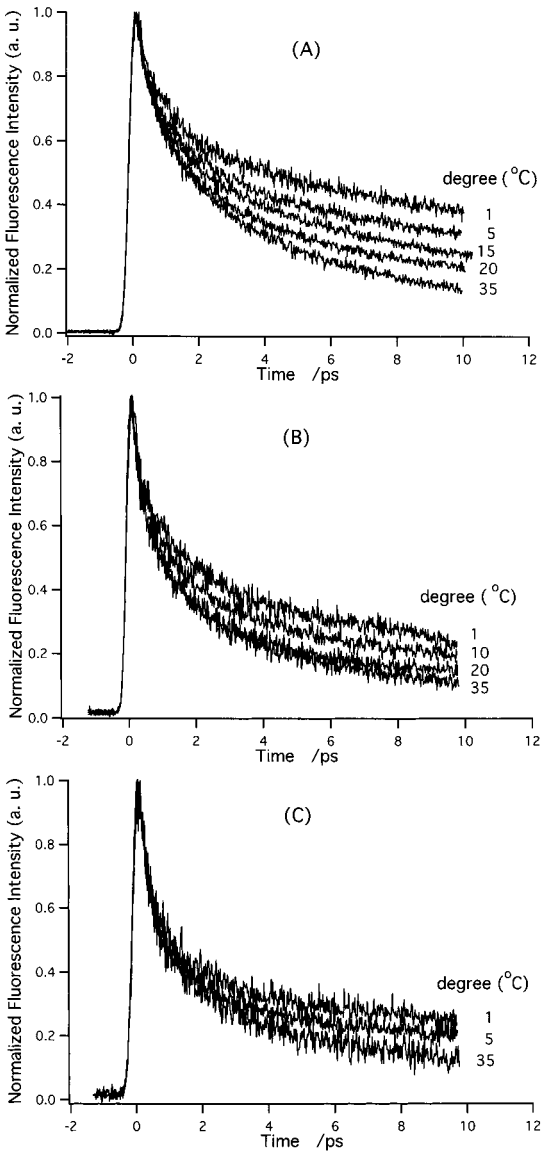

Figure 6. Temperature effects on the fluorescence decay curves of the w-t PYP observed at (A) 500, (B) 470, and (C) 570 nm. Sample solutions for A and C were excited at 410 nm, whereas that for B was excited at 430 nm to avoid the effect of the Raman scattering of water.

of the fluorescence decay curves, however, we have used only the result for the peak position, because the results at the blue and red bottom of the spectrum are not accurate because of low fluorescence intensity.

The above results for the temperature dependencies of the fluorescence decay curves indicate that different modes of the Tw^- state formation process coexist in the w-t PYP. One is an ultrafast, temperature-independent process probably of a barrierless and coherent origin, and the other is a slower process that needs thermal activation. In general, the relaxation process of the coherent wave packet from the FC−Fl coupled state or Fl state along the reaction coordinate competing with fluorescence seems to combine not only with the twisting motion but also with other vibrational modes, including high-frequency vibrations of the chromophore, and also with vibrations of environmental proteins.[23,25] In the almost relaxed (or critically damped) state, the coherent crossing of the excited state to the Tw^- state seems to become difficult, and the chromophore falls into shallow traps from which the slow Tw^- state formation process, in competition with fluorescence, takes place by thermal

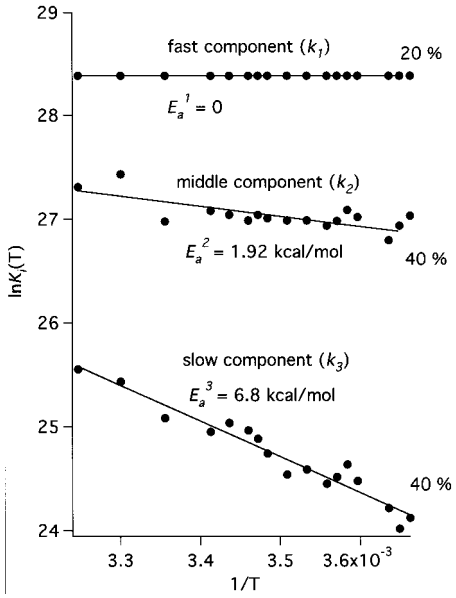

Figure 7. Temperature dependence of the rate constants $k_i(T)$ and activation energies E_a^i for the w-t PYP.

activation. On the basis of such a reaction scheme for Tw^- state formation, we have reproduced the fluorescence decay curves observed at the peak wavelength of the fluorescence band of the w-t PYP at various temperatures by superposing exponential decay functions as follows:

$$I(t) = \sum_i a_i \exp[-k_i(T)t] \quad (1)$$

$$1/\tau_i(T) = k_i(T) = k_{io} \exp(-E_a^i/RT) \quad (2)$$

where a_i is assumed to be temperature-independent and E_a^i is the activation energy for *i*th decay component. In the case of w-t PYP, decay curves can be reproduced by superposing three exponentials, such that $a_1 \approx 20\%$ for the fast component with $\tau_1 \approx 430$ fs, $a_2 \approx 40\%$ for middle component with $\tau_2 \sim 1$ ps (308 K) to 3 ps (274 K), and $a_3 \approx 40\%$ for slow component with $\tau_3 \sim 10$ ps (308 K) to 30 ps (274 K). From the linear plot, $\ln k_i(T) = \text{const} - (E_a^i/R)(1/T)$, shown in Figure 7, activation energies have been evaluated as follows: $E_a^1 = 0$, $E_a^2 = 1.92$, and $E_a^3 = 6.8$ kcal/mol. These results indicate that the highly nonexponential fluorescence decay dynamics of the w-t PYP in the 100 fs to 10 ps time regime can be ascribed to the existence of (real) different pathways undergoing Tw^- state formation, one of which is the ultrafast coherent process.

In the comparative studies of the fluorescence dynamics of the w-t PYP and site-directed mutants described in the previous section, we demonstrated that the fluorescence decay dynamics, which are determined by the reaction rate of Tw^- state formation, are slowed in the site-directed mutants, probably because of modifications in the PNS by mutations to a somewhat looser structure compared with that of the w-t PYP, and for the mechanism of Tw^- state formation by flipping of the thioester linkage, the tighter PNS of the w-t PYP seems to be favorable. In light of the above results of the temperature effects on the fluorescence dynamics of the w-t PYP, we examined the temperature dependencies of the fluorescence dynamics of a few mutants to gain more insight into the mechanisms of Tw^- state formation in the PNS of PYP.

As examples, we show temperature effects on the fluorescence decay curves of E46Q/T50V and E46Q in Figure 8. Although the observed decay curves are rather noisy because

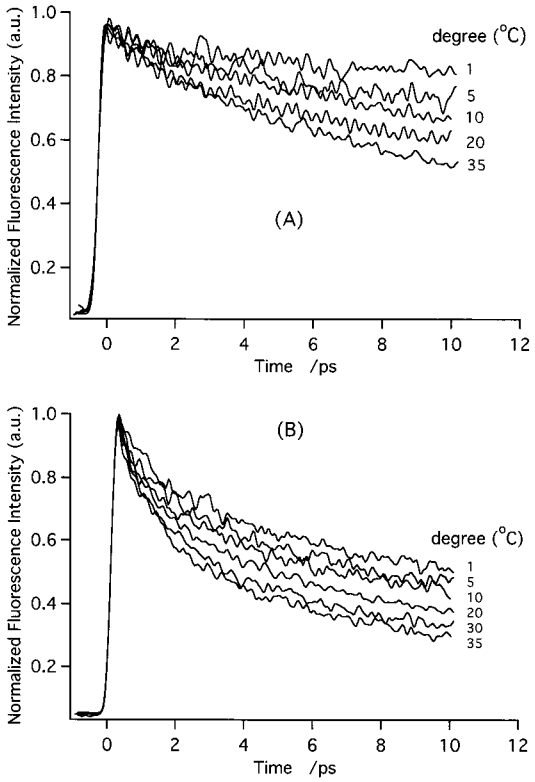

Figure 8. Temperature effects on the fluorescence decay curves of the site-directed mutants. (A) E46Q/T50V excited at 410 nm and observed at 510 nm. (B) E46Q excited at 410 nm and observed at 500 nm.

of the scarcity of the mutant samples, we can recognize that not only the decay curves in the few picosecond to 10 ps time regimes but also those in the shortest decay time regime show an increase in decay times at lower temperatures. The decay time of E46Q/T50V is considerably long already at room temperature, while that of E46Q in the early time regime is pretty short, as we can see from Figure 5. Nevertheless, we cannot recognize in Figure 8 a temperature-independent decay component in the early time regime, as observed in the case of the w-t PYP (Figure 6). We observed similar results also for R52Q and T50V. These results indicate that the barrierless process of Tw^- state formation observed for the w-t PYP becomes negligible in the mutants.

Our analysis of the temperature effects on the fluorescence decay curves of E46Q/T50V and E46Q according to eqs 1 and 2 are shown in Figure 9. As discussed above, the temperature-independent ultrafast components are absent in these mutants. Activation energies for the middle (E_a^2) and slow components (E_a^3) were obtained as follows: $E_a^2 = 3.73$ kcal/mol and $E_a^3 = 7.0$ kcal/mol for E46Q/T50V and $E_a^2 = 2.4$ kcal/mol and $E_a^3 = 4.9$ kcal/mol for E46Q. Furthermore, by the same procedure, E_a^2 and E_a^3 values for T50A and R52Q were obtained as follows: $E_a^2 = 0.86$ kcal/mol and $E_a^3 = 4.93$ kcal/mol for T50A and $E_a^2 = 3.07$ kcal/mol and $E_a^3 = 3.8$ kcal/mol for R52Q. These activation energies may depend delicately on the chromophore−protein interactions in the PNS, and it is not easy to give any systematic interpretation concerning the effect of the site-directed mutagenesis on these barrier heights at the present stage of the investigations. For a full understanding of the mechanisms underlying the above-described results concerning the wild-type and mutant PYPs, more detailed optical spectroscopic investigations, including measurements with better time resolutions and more extensive investigations of various

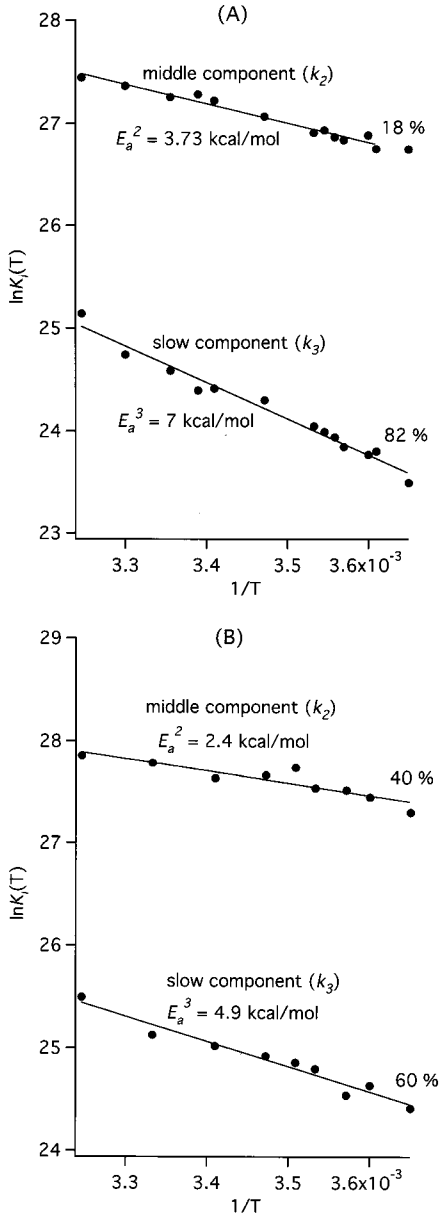

Figure 9. Temperature dependencies of the rate constants $k_i(T)$ and activation energies E_a^i for the mutants (A) E46Q/T50V and (B) E46Q.

mutants, may be necessary, and efforts in such directions are proceeding in our laboratory.

On the other hand, it should be noted here that, in the case of the E46Q/T50V mutant, the PNS may be modified to a considerably looser structure in which Tw^- state formation might take place not only by the mechanism of a slighty slowed flipping of the thioester linkage but also with some contributions from a large-scale rotation about the vinyl bond. Specifically, the mechanism of Tw^- state formation in a PNS with a rather loose structure might approach, to a small extent, that of the chromophore in solution. In this respect, a comparison of the temperature dependence of the fluorescence decay curve of the chromophore in solution with that of the E46Q/T50V mutant might give some useful information. However, because various solute−solvent interactions depend on solvent viscosity, dielectric constant, friction coefficient in the rotational motions, etc., which are functions of temperature, any accurate measurement of the rate constant and its temperature dependence for comparison with the above-described measurements of the fluorescence dynamics in the PNS seems to be rather difficult.

Similarities between PYP and Visual Rhodopsin. Before concluding the present paper, we should note here that the work described above seems to suggest similarities between the present results for PYP and those of ultrafast laser spectroscopic studies of visual Rh, especially with respect to the ultrafast barrierless or coherent reaction processes from the excited FC and/or Fl state. Although the chromophore molecule of PYP is quite different from that of Rh, both undergo isomerization in the primary process of the photoinduced reaction in the PNS. It should be noted here that many previous studies[1,24,25] on wild-type and mutant bRs seem to indicate similarities in their reaction mechanisms to those of Rh and also to those of PYP. Nevertheless, according to recent ultrafast laser spectroscopic studies on bR systems, including systems with an artificially "locked" chromophore, the nature of the twisted intermediate in the primary process of photoisomerization does not seem to be very clear.[26] Accordingly, we briefly compare the reaction mechanisms of PYP and Rh here.

According to previous femtosecond transient absorption spectral measurements on bovine Rh,[27,28] its ultrafast barrierless photoisomerization takes place from the FC excited state to the primary product by the coherent process within 200 fs, and an investigation of the Rh fluorescence spectrum by a stationary method indicates that Rh fluorescence is emitted from the vibrationally unrelaxed state in the course of relaxation from the FC excited state.[29] Very efficient and ultrafast primary product formation by the coherent process from the FC excited state has been emphasized by these studies. However, our study on the dynamics of bovine Rh fluorescence by femtosecond up-conversion has demonstrated that the fluorescence decay is nonexponential and can be reproduced satisfactorily by the superposition of three exponential functions in which the fastest components, with time constants in the hundred femtosecond time regime, are overwhelming.[30] An important result obtained by this study is the dependence of the decay curve on the monitoring wavelength.[30] Specifically, the time constants of the predominant (fastest) components have been determined to be 146 fs at a short wavelength (578 nm) and 330 fs at a longer wavelength (635 nm).[30] The decay time of 146 fs at the shorter wavelength agrees with the primary product formation time (<200 fs) by the coherent process, as determined by pump−probe measurements,[27,28] but the value at the longer wavelength is a little longer than this measurement. These results suggest the similar dependence of the nonexponential fluorescence decay curves on the monitoring wavelength, as observed for the w-t PYP (Figure 4A).

From such a viewpoint, we recently made a more detailed measurement of the fluorescence dynamics of bovine Rh over a wider wavelength range. We observed not only a faster initial decay at short wavelength but also a slightly faster initial decay at sufficiently longer wavelengths (730−780 nm), as in the case of w-t PYP.[31] On the basis of the dependence of the fluorescence decay curve of Rh on the monitoring wavelength[30,31] and the stationary fluorescence measurements,[29] which indicate that the Rh fluorescence is emitted from the vibrationally nonrelaxed state, we can conclude that a coherent ultrafast conversion from the FC state to the Fl state may take place (no dynamic Stokes shift of fluorescence could be observed) through a coupling with the high-frequency modes of the chromophore also for Rh.

Coupling with the high-frequency modes will bring a slight narrowing of the fluorescence band shape at the early stage of the decay as the displacements of the high-frequency modes decrease along the twisting coordinate, resulting in the slightly faster initial decay of the fluorescence intensity at the blue and

red sides of the fluorescence spectrum. It should be noted here that a similar conclusion about the participation of the high-frequency modes in the twisting reaction was derived also for Rh from the recent femtosecond pump−probe measurement.[32]

Thus, on the basis of the experimental results concerning the femtosecond to picosecond fluorescence dynamics of PYP given in the present article and their relation to the results of ultrafast laser spectroscopic studies of Rh, we can conclude that, although the chromophore molecule of PYP is quite different from that of Rh, their behaviors in the photoinduced primary processes in the PNS in the course of the relaxation and twisting, including coherent processes, seem to be similar. This is an interesting and very important issue concerning various photoactive proteins, and further elucidation of the mechanisms underlying the reaction dynamics and PNS structures of these molecules is needed.

Concluding Remarks

By examining the effects of modifications of the PNS (protein nanospace) by site-directed mutagenesis and changes in temperature on the femtosecond to picosecond fluorescence dynamics of PYP, we have revealed the following results.

(a) The ultrafast nonexponential decay of fluorescence in the 100 fs to 10 ps time regime of the w-t PYP because of Tw^- state formation is slowed considerably in the mutants.

(b) The mutants seem to have looser PNS structures, and the more restricted PNS structure of the w-t PYP is the most favorable for the rapid formation of the Tw^- state by the mechanism of flipping the thioester bond.

(c) The fastest fluorescence decay of the w-t PYP in the 100 fs to 1 ps time regime is temperature-independent, and the Tw^- formation process responsible for it could be a barrierless or coherent reaction from the vibrationally nonrelaxed Fl state, whereas thermal activation is necessary for the reaction in the few picosecond to 10 ps regime.

(d) No activationless process for Tw^- state formation can be observed for the mutants; specifically, the ultrafast coherent process from the vibrationally nonrelaxed Fl state to the Tw^- state becomes difficult in the loose PNS structure of the mutants.

(e) Although the chromophore of PYP is quite different from that of Rh, their behaviors in the photoinduced primary processes in the PNS in the course of converting from the FC state to the Fl state and twisting, including coherent processes, are very similar.

For an elucidation of the mechanisms underlying the above results of the ultrafast laser spectroscopic studies, detailed studies of the photocycle and X-ray structures of PYP mutants and theoretical as well as computer simulation studies on the structure and dynamics of the wild-type and mutant PYPs may be of crucial importance, even though they are rather difficult tasks.

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References and Notes

(1) See, for example: Kochendoerfer, G. G.; Mathies, R. A. *Isr. J. Chem.* **1995**, *35*, 211.

(2) Flavoproteins, for example: Mataga, N.; Chosrowjan, H.; Shibata, Y.; Tanaka, F. *J. Phys. Chem. B* **1998**, *102*, 7081.

(3) (a) Baca, M.; Borgstahl, G. E. O.; Boissinot, M.; Burke, P. M.; Williams, D. R.; Slater, K. A.; Getzoff, E. D. *Biochemistry* **1994**, *33*, 14369. (b) Borgstahl, G. E. O.; Williams, D. R.; Getzoff, E. D. *Biochemistry* **1995**, *34*, 6278.

(4) Meyer, T. E.; Yakali, E.; Cusanovich, M. A.; Tollin, G. *Biochemistry* **1987**, *26*, 418.

(5) Hoff, W. D.; van Stockkum, I. H. M.; van Ramesdonk, H. J.; van Brederode, M. E.; Brouwer, A. M.; Fitch, J. C.; Meyer, T. E.; van Grondelle, R.; Hellingwerf, K. J. *Biophys. J.* **1994**, *67*, 1691.

(6) Meyer, T. E.; Cusanovich, M. A.; Tollin, G. *Arch. Biochem. Biophys*. **1993**, *306*, 515.

(7) Hoff, W. D.; Dux, P.; Hard, K.; Devreese, B.; Nugteren-Roodzant, I. M.; Crielaard, W.; Boelens, R.; Kaptein, R.; van Beeumen, J.; Hellingwerf, K. J. *Biochemistry* **1994**, *33*, 13959.

(8) Kim, M.; Mathies, R. A.; Hoff, W. D.; Hellingwerf, K. J. *Biochemistry* **1995**, *34*, 12669.

(9) Kort, R.; Vonk, H.; Xu, X.; Hoff, W. D.; Crielaard, W.; Hellingwerf, K. J. *FEBS Lett*. **1996**, *382*, 73.

(10) Ujj, L.; Devanathan, S.; Meyer, T. E.; Gusanovich, M. A.; Tollin, G.; Atkinson, G. H. *Biophys. J.* **1998**, *75*, 406.

(11) (a) Chosrowjan, H.; Mataga, N.; Nakashima, N.; Imamoto, Y.; Tokunaga, F. *Chem. Phys. Lett*. **1997**, *270*, 267. (b) Chosrowjan, H.; Mataga, N.; Shibata, Y.; Imamoto, Y.; Tokunaga, F. *J. Phys. Chem. B* **1998**, *102*, 7695.

(12) Baltuska, A.; van Stokkum, I. H. M.; Kroon, A.; Monshouwer, R.; Hellingwerf, K. J.; van Grondelle, R. *Chem. Phys. Lett*. **1997**, *270*, 263.

(13) Changenet, P.; Zhang, H.; van der Meer, M. J.; Hellingwerf, K. J.; Glasbeek, M. *Chem. Phys. Lett*. **1998**, *282*, 276.

(14) Genick, U. K.; Borgstahl, G. E. O.; Ng, K.; Ren, Z.; Praderwand, C.; Burke, P. M.; Srajer, V.; Teng, T.-Y.; Schildkamp, W.; Mcree, D. E.; Moffat, K.; Getzoff, E. D. *Science* **1997**, *275*, 1471.

(15) Perman, B.; Srajer, V.; Ren, Z.; Teng, T.-Y.; Praderwand, C.; Ursby, T.; Bourgeois, D.; Schotte, F.; Wulff, M.; Kort, R.; Hellingwerf, K. J.; Moffat, K. *Science* **1998**, *279*, 1946.

(16) Genick, U. K.; Soltis, S. M.; Kuhn, P.; Canestrelli, I. L.; Getzoff, E. D. *Nature* **1998**, *392*, 206.

(17) Imamoto, Y.; Kataoka, M.; Tokunaga, F. *Biochemistry* **1996**, *35*, 14047.

(18) Imamoto, Y.; Mihara, K.; Hisatomi, O.; Kataoka, M.; Tokunaga, F.; Bojkova, N.; Yoshihara, K. *J. Biol. Chem.* **1997**, *272*, 12905.

(19) See, for example: Fleming, G. R. *Chemical Applications of Ultrafast Spectroscopy*; Oxford University Press: New York, 1986.

(20) Imamoto, Y.; Ito, T.; Kataoka, M.; Tokunaga, F. *FEBS Lett.* **1995**, *374*, 157.

(21) Mihara, K.; Hisatomi, O.; Imamoto, Y.; Kataoka, M.; Tokunaga, F. *J. Biochem.* **1997**, *121*, 876.

(22) Maeda, Y.; Okada, T.; Mataga, N. *J. Phys. Chem.* **1984**, *88*, 2714.

(23) (a) Warshel, A.; Chu, Z. T.; Hwang, J.-K. *Chem. Phys.* **1991**, *158*, 303. (b) Kakitani, T.; Hatano, Y.; Shichida, Y.; Imamoto, Y.; Tokunaga, F.; Kakitani, H. *Photochem. Photobiol.* **1992**, *56*, 987.

(24) Song, L.; El-Sayed, M. A.; Lanyi, J. K. *Science* **1993**, *261*, 891.

(25) Du, M.; Fleming, G. R. *Biophys. Chem.* **1993**, *48*, 101.

(26) Zhong, Q.; Ruhman, S.; Ottolenghi, M.; Sheves, M.; Friedman, N.; Atkinson, G. P.; Delaney, J. K. *J. Am. Chem. Soc.* **1996**, *118*, 12828 and references therein.

(27) (a) Schoenlein, R. W.; Peteanu, L. A.; Mathies, R. A.; Shank, C. V. *Science* **1991**, *254*, 412. (b) Peteanu, L. A.; Schoenlein, R. W.; Wang, Q.; Mathies, R.; Shank, C. V. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11762.

(28) Wang, Q.; Schoenlein, R. W.; Peteanu, L. A.; Mathies, R. A.; Shank, C. V. *Science* **1994**, *266*, 422.

(29) Kochendoerfer, G. G.; Mathies, R. A. *J. Phys. Chem*. **1996**, *100*, 14526.

(30) Chosrowjan, H.; Mataga, N.; Shibata, Y.; Tachibanaki, S.; Kandori, H.; Shichida, Y.; Okada, T.; Kouyama, T. *J. Am. Chem. Soc.* **1998**, *120*, 9706.

(31) Kandori, H.; Furutani, Y.; Nishimura, S.; Shichida, Y.; Chosrowjan, H.; Shibata, Y.; Mataga, N. Manuscript in preparation.

(32) Haran, G.; Morlino, E. A.; Matthes, J.; Callender, R. H.; Hochstrasser, R. M. *J. Phys. Chem. A* **1999**, *103*, 2202.