Environmental Effects on the Femtosecond−Picosecond Fluorescence Dynamics of Photoactive Yellow Protein: Chromophores in Aqueous Solutions and in Protein Nanospaces Modified by Site-Directed Mutagenesis

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The effect of the protein environment surrounding chromophores (protein nanospaces) on the photoinduced ultrafast twisting of chromophores in photoactive yellow protein (PYP) was investigated by comparing the femtosecond−picosecond fluorescence dynamics of wild type PYP with those of the chromophore in aqueous solution as well as in protein nanospace modified by site-directed mutagenesis. The rate of the twisted state formation of the chromophore (the primary step of the trans → cis photoisomerization) was demonstrated to be considerably enhanced in protein compared with the aqueous solution. Moreover, results of the measurements of fluorescence dynamics of the chromophore in the protein nanospace modified by site-directed mutagenesis implied that the twisted state formation by flipping the thioester linkage of the chromophore was slowed by modifying the nanospace structure to a slightly looser one. Namely, the more restricted structure of the protein nanospace in the wild-type PYP seems to be best engineered for the twisting by the flipping mechanism.

Introduction

In photosensory or photoactive proteins with chromophores absorbing in the visible region such as rhodopsin (Rh) for vision and photoactive proteins of various photoresponsive bacteria, light absorption usually leads to ultrafast and highly efficient reactions. Among those proteins, photoactive yellow protein (PYP), which functions as a blue light photoreceptor for a negative phototaxis of the purple sulfur bacterium Ectothiorhodospira halophila, seems to have the simplest structure[1] and to show a simple photoreaction cycle.[2−7] The chromophore, *p*-coumaric acid (deprotonated) covalently bound to the side chain of Cys69 via thioester linkage, with an absorption peak at $\lambda_{max} = 446$ nm (ground state, G), undergoes trans → cis photoisomerization leading to a red-shifted intermediate state I_1 (with absorption peak at $\lambda_{max} = 465$ nm) in less than 10 ns, and then I_1 decays into a blue-shifted intermediate I_2 (with $\lambda_{max} = 355$ nm) coupled with one proton uptake on the submicrosecond time scale followed by the reformation of the G state coupled with deprotonation on a subsecond time scale.[2−7]

On the other hand, we have examined dynamics of PYP in the early stages of relaxations immediately after photoexcitation by means of femtosecond fluorescence up-conversion measurements for the first time.[8] According to our results, fluorescence decay curves of wild type PYP were nonexponential and approximately reproduced by superposing three exponentials, where main contributions were from several hundreds of femtoseconds to a few picoseconds time regimes and only minor contributions from several tens of picoseconds regimes. The main components in the hundreds of femtoseconds to a few picoseconds regimes were ascribed to the reaction to form the twisted state (Tw) from the fluorescent state (Fl).[8] Recent subpicosecond pump−probe absorption spectral studies on the wild-type PYP also indicated spectral changes in the several hundreds of femtoseconds to a few picoseconds time regions.[9] It should be noted here that other subpicosecond fluorescence up-conversion measurements on the wild-type PYP were reported very recently,[10] results which were in good agreement with our previous measurements.[8]

Concerning structures of the chromophore as well as the surrounding protein environment in the course of the photocycle, remarkable advances have been achieved by X-ray crystallography on PYP crystal in millisecond time-resolved[11] as well as nanosecond time-resolved[12] measurements at room temperature. By these studies, detailed structures of the red-shifted intermediate state I_1 (with deprotonated chromophore in cis form) and the blue-shifted intermediate I_2 (with protonated chromophore in cis form) including the structures of amino acid residues surrounding the chromophores have been elucidated. Moreover, detailed X-ray crystallography on the PYP crystal where the reaction intermediates are cryotrapped below −100 °C has revealed that the early transient state of the chromophore seems to be formed by flipping its thioester linkage with the protein avoiding collisions resulting from large-scale movement of its aromatic ring during the initial photoreaction.[13] This initial Tw state may be identified with the intermediate PYP_{BL} previously found by static spectroscopy at −190 °C.[14] It should be noted here that detailed analysis of the nanosecond X-ray crystallographic measurements also suggests the existence of the initial structural changes of the chromophore mainly in its tail part.[12] In addition, despite a strong similarity in the chromophore structure of the PYP and GFP (green fluorescent protein) from Aequorea victoria, the tail of the GFP chro-

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mophore is covalently locked in its conformation and evidently lacks the flexibility of the tail part of the PYP chromophore, resulting in its highly efficient green fluorescence emission contrary to the low fluorescence yield ($\sim 10^{-3}$) of PYP.[12,15] This fact also seems to support the mechanism of the flipping in the tail part of the PYP chromophore for the initial Tw state formation in the photoisomerization.

In this early transient state, much of the initial photon energy may still be stored in its highly distorted geometry of the chromophore, and all subsequent steps of I_1 state formation, I_2 state formation accompanied by changes of surrounding protein structures, protonation and deprotonation of the chromophore, and completion of the cycle by G state formation seems to be driven by this stored energy.[13] We should note here that this initial Tw state is formed in the course of the ultrafast relaxations from the FC excited state. Accordingly, for the elucidation of the reaction mechanisms, detailed femtosecond spectroscopic studies on PYP are of crucial importance along with the above structural studies by X-ray crystallography. Namely, in what way the protein nanospace affects the ultrafast relaxation processes in the excited state leading to the highly distorted Tw state formation should be studied in detail. In this respect, we have compared the fluorescence dynamics of the chromophore (in deprotonated form) in aqueous solution with that in protein nanospace and also we have examined effects of the modifications of amino acid residues surrounding the chromophore on the dynamics of the Tw state formation using the site-directed mutagenesis method. We discuss our recent results in the following.

Experimental Section

The fluorescence dynamics measurements were made by an fluorescence up-conversion apparatus similar to that described elsewhere.[8] The fwhm of the instrumental response was 210 fs. The sample solutions for the measurements were made to flow through a 1 mm cell. The preparations of wild-type PYP and its site-directed mutants were described elsewhere.[16,17] We have examined R52Q, T50V, E46Q, and E46Q/T50V mutants, where the amino acid residues Arg52, Thr50, and Glu46 contiguously placed to the chromophore and rather strongly interacting with it are replaced with Gln, Val, and Gln, respectively. For the measurements of the fluorescence dynamics of the chromophore in solution, PYP was denatured by addition of guanidinium hydrochloride at pH 9.5, where the protein was unfolded and the deprotonated chromophore was exposed to water environment.

Results and Discussion

The stationary absorption and fluorescence spectral band peaks of the deprotonated chromophore linked by thioester bond to the unfolded protein and exposed to water were observed at $\lambda_a \sim 400$ nm and $\lambda_f \sim 496$ nm, respectively, with a fluorescence Stokes shift of $\Delta\nu_S \sim 4840$ cm^{-1}. In contrast to this, the corresponding quantities of wild-type PYP were obtained to be $\lambda_a \sim 446$ nm, $\lambda_f \sim 494$ nm and $\Delta\nu_S \sim 2200$ cm^{-1}, respectively. This difference between the $\Delta\nu_S$ values of the same chromophore in aqueous solution and in protein nanospace indicates the large effect of the solvation dynamics in aqueous solution. In Figure 1 the wavelength dependence of the fluorescence rise and decay curves of the deprotonated chromophore of the denatured PYP is shown. One can recognize clearly the rapid initial decay in the short wavelength region and fluorescence rise curve in the long wavelength region owing to the dynamic Stokes shift due to solvation. The fluorescence decay observed

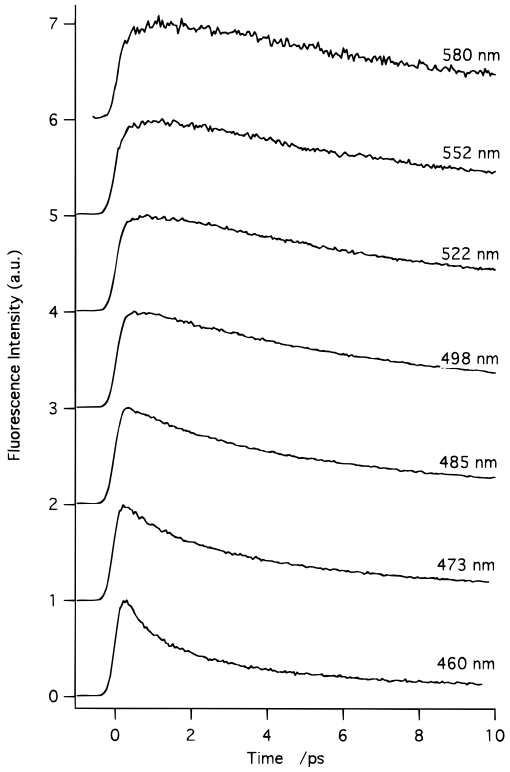

Figure 1. Fluorescence dynamics of *p*-coumaric acid (deprotonated) chromophore linked to unfolded protein of PYP denatured with addition of guanidinium HCl to solution of pH 9.5, excited at 410 nm and observed at various wavelengths.

at the wavelengths near the band peak of the stationary fluorescence spectra, where the effect of the dynamic Stokes shift is negligible, was actually single exponential with 11 ps decay time.

Although detailed studies on the photoisomerization of this 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 solutions undergo trans → cis photoisomerization exclusively from the S_1 state,[18] where the isomerization occurs by twisting around the vinyl bond. The reaction yield is enhanced with increase of the electron-donating ability of the 4-substituent.[18] In view of the strong electron-donating ability of the $-O^-$ substituent of the present chromophore and relatively short fluorescence decay time of 11 ps, it is highly probable that the chromophore itself undergoes fairly rapid isomerization by twisting in the S_1 state.

On the other hand, the fluorescence dynamics of the wild-type PYP observed at various wavelengths are indicated in Figure 2, where, contrary to the case of the chromophore in aqueous solution, no indication of the fluorescence rise curve in the long wavelength region due to the dynamic Stokes shift was recognized. Because the chromophore is contained within the protein nanospace for the PYP, the 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 the relaxation processes from the excited FC state to the Fl state coupled with intrachromophore vibrational modes and with reorganizations of the excited chromophore−amino acid residue interactions. These relaxation processes corresponding to the fluorescence Stokes shift $\Delta\nu_S$ of the wild type PYP are presumably too fast

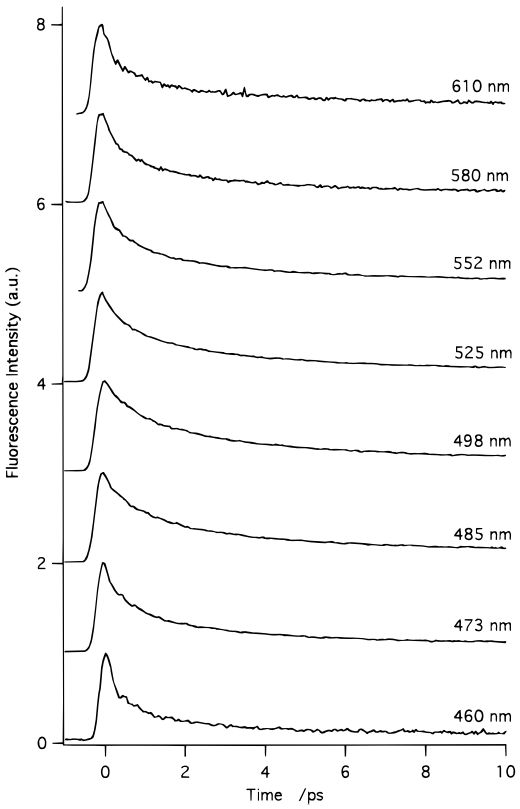

Figure 2. Fluorescence dynamics of wild-type PYP excited at 410 nm and observed at various wavelengths.

to time-resolve with the present measurements by using the 100 fs laser pulses.

In addition to the above problem, we can recognize in Figure 2 slightly faster time-dependent decrease of fluorescence intensity on both blue and red sides of the spectrum, i.e., a slight narrowing of the fluorescence band in the early stage of the decay. This result might be related also to the mechanisms of the ultrafast relaxation processes from the FC to Fl state coupled with many vibrational modes of chromophore and protein environments including coherent processes.[19]

In any case, the rate of Tw state formation by photoexcitation is much more enhanced in protein nanospace compared with the aqueous solution of the chromophore. Possible mechanisms for this result might be as follows: (a) In both cases, the twisting takes place by flipping thioester linkage, and this process is accelerated in protein nanospace. (b) In aqueous solution, where the chromophore can move more freely, a large-scale rotational motion around the vinyl bond of the chromophore takes place, while the more rapid twisting by flipping thioester linkage occurs in the protein nanospace for the PYP. In this respect, effects of the modification of the protein nanospace by site-directed mutagenesis on the fluorescence spectra and dynamics of PYP may be very interesting and important.

There are two main features to compare among the wild-type PYP and various mutants. One is the fluorescence Stokes shift $\Delta\nu_S$ (cm^{-1}): 2178 (wild type PYP), 2219 (R52Q), 1761 (T50V), 1779 (E46Q), and 1339 (E46Q/T50V), and the other is the fluorescence decay dynamics. For the latter comparison, we show in Figure 3 fluorescence decay curves observed at the peak wavelength of the fluorescence spectrum for native PYP and various mutants. We should note that slightly faster time-dependent decrease of fluorescence intensity on both blue and red sides of the spectrum in the early stage of the decay was observed also in these mutants.

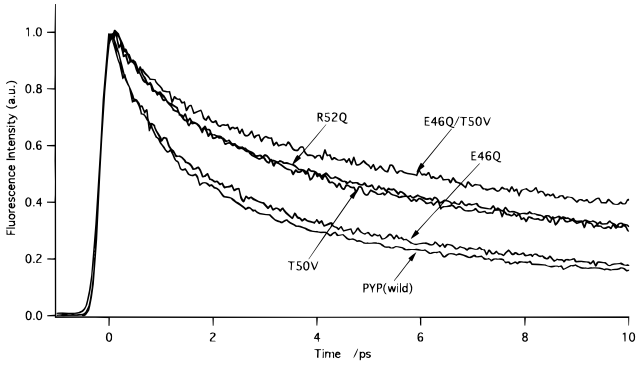

Figure 3. Fluorescence dynamics of wild-type PYP and its site-directed mutants excited at 410 nm and observed at the wavelength of the maximum of the steady-state fluorescence spectrum. The fluorescence decay curves are normalized at the peak intensity.

In the wild-type PYP, the $-O^-$ substituent of the chromophore is surrounded by proton-donating amino acid residues Glu46, Tyr42, and Thr50, which make a hydrogen (H)-bonding network and undergo short-range H-bonding interaction with the $-O^-$ group.[1,11−13] In the mutants E46Q, T50V, and E46Q/T50V, H-bonding interaction with the $-O^-$ group may be weakened and the H-bonding network may be partly destroyed because Glu46 and Thr50 are replaced, respectively, by Gln and Val with much weaker or no function as proton donor in H-bonding. On the other hand, Arg52 is replaced by Gln, leading to the loss of positive charge and accordingly the loss of Coulomb interaction between the positive charge and negative charge of the chromophore in R52Q, although the H − bonding network seems to remain approximately unchanged.

Presumably, the above-described changes of the amino acid residues by mutations will affect not only the short-range H − bonding interactions but also the structures and functions of the protein nanospace containing the chromophore as a whole, which in turn will affect seriously the ultrafast relaxation processes from the excited FC to Fl and Tw state. The relatively large $\Delta\nu_S$ values of wild-type PYP and R52Q given above may be ascribed to the reorganization energies that arise from the short-range H − bonding interactions in the photoexcitation and fluorescence transitions. In the case of the other mutants, the short-range H − bonding interactions and the H − bonding network are more or less weakened leading to the smaller reorganization energies associated with the transitions, and evidently, such decrease of the reorganization energies is especially large in the doubly mutated system.

Because the decay dynamics in subpicosecond to several picosecond time regions, which should be related to the initial process of the Tw state formation, is not parallel with the $\Delta\nu_S$ value, the structures and functions of protein nanospace as a whole in the excited state may be of crucial importance for the rapid twisting dynamics as described above. The short-range H − bonding interactions with the chromophore should become very weak in the fluorescence state owing to the decrease of charge density on the substituent oxygen $-O^-$ due to the charge transfer to other parts of the chromophore, leading to the poor correlation between $\Delta\nu_S$ and fluorescence decay dynamics determined by Tw state formation process.

Presumably, the partial destruction of the H − bonding network surrounding the chromophore by replacing Thr50 with Val in the T50V will result in the modification of the protein nanospace to a slightly looser one, which seems to reduce slightly the twisting rate. In the case of R52Q, loss of electrostatic attraction between the positive charge on Arg52

and the negative charge on the chromophore might result also in a modification of the protein nanospace to a looser one and/or some modification of the electronic structure of the excited chromophore leading to the slowing down of the twisting process, as observed in the case of the photoisomerization of bacteriorhodopsin.[20] The largest effect of the mutation in slowing down the fluorescence decay dynamics due to the retardation of the twisting reaction among the mutants examined here was observed in E46Q/T50V. Rather large modification by double mutation of the protein nanospace to a much looser one may take place, leading to a slower twisting dynamics. In this case, it might be possible that the Tw state formation takes place not only by the mechanism of a slightly slowed flipping of the thioester linkage but also with some contributions from a large-scale rotation around the vinyl bond.

Thus, our recent results of investigations on the fluorescence dynamics of several site-directed mutants of PYP suggest that the photochemical reaction rate of the initial Tw state formation by the mechanism of flipping thioester linkage seems to be enhanced in a more restricted environment of the protein nanospace and the reaction is retarded in a modified protein nanospace probably with a slightly looser structure. Namely, among the native PYP and site-directed mutants examined here, the native one seems to be best engineered for the fast twisting reaction. It should be noted here also that we cannot recognize the indication of the fluorescence rise curve due to Stokes shift originating from the excited FC → Fl relaxation process with the measurements by means of the 100 fs laser pulses.

To establish the above suggested reaction mechanisms and to elucidate the details of the FC → Fl relaxation processes leading to the initial Tw state formation, systematic investigations employing different mutants and including measurements with higher time resolutions are now going on in our laboratory, results of which will be reported in the near future.

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