Early Photocycle Kinetic Behavior of the E46A and Y42F Mutants of Photoactive Yellow Protein: Femtosecond Spectroscopy

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ABSTRACT In the photoactive yellow protein, PYP, both Glu46 and Tyr42 form hydrogen bonds to the phenolic OH group of the *p*-hydroxycinnamoyl chromophore. Previous work on replacement of the carboxyl group of Glu46 by an amide group (Glu46Gln) has shown that changing the nature of this hydrogen bond has a minimal effect on the rate constant for the formation of the first intermediate (I_0) and on the excited state lifetime, whereas the rate constants for the formation of the second ($I_0^{\neq}$) and third (I_1) intermediates were increased by factors of ~30 and 5, respectively. In the present experiments, two additional mutants (Glu46Ala and Tyr42Phe) have been studied. These two mutants are shown to behave kinetically very similarly to one another. In both cases, the rate constant for I_0 formation is decreased by a factor of ~2, with little or no effect on the photochemical yield as a consequence of a compensating increase in the excited state lifetime. Although we are unable to resolve the rate constant for the formation of the second intermediate from that of the first intermediate, the rate constant for the formation of the third intermediate is increased by a factor of ~100. The structural implications of these results are discussed.

INTRODUCTION

Photoactive yellow protein (PYP) from *Ectothiorhodospira halophila* (Meyer, 1985; Meyer et al., 1987, 1991) is a cytosolic 14-kD protein that has a covalently attached *p*-hydroxycinnamoyl cofactor (Baca et al., 1994; Hoff et al., 1994) as the light absorbing entity (λ_{max} = 446 nm). It undergoes a self-contained photocycle upon light activation involving several intermediate steps (Meyer et al., 1987, 1989, 1991; Ujj et al., 1998; Devanathan et al., 1999b, 2000), which include protein conformational changes (Genick et al., 1997a, 1998; Düx et al., 1998), *trans-cis* isomerization of the *p*-hydroxycinnamoyl chromophore side chain double bond (Kort et al., 1996; Genick et al., 1997a; Unno et al., 2000), and protonation/deprotonation of the chromophore phenolic hydroxyl group (Meyer et al., 1993; Genick et al., 1997b). The behavior of this protein and its mutants have been well studied by biophysical techniques (Genick et al., 1997a, 1998; Ujj et al., 1998; Devanathan et al., 1998, 1999b, 2000; Masciangioli et al., 2000; Xie et al., 2001; Brudler et al., 2001; Imamoto et al., 2001), although its biology remains obscure. In *Rhodospirillum centenum*, a PYP-phytochrome hybrid is involved in the regulation of chalcone synthase expression (Jiang et al., 1999). Understanding the structural implications of the interaction of light energy with photoactive proteins is crucial to the elucidation of the signal transduction mechanism for photosensory receptors. Transient photochemical intermediates are key features of these mechanisms, and their characterization for PYP is the primary goal of this study.

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The crystal structure for ground state (unphotolyzed) PYP at 1.4 Å resolution (Borgstahl et al., 1995) shows its chromophore buried in a hydrophobic pocket with the anionic phenolate oxygen hydrogen-bonding with the protonated carboxyl group of Glu46 and the hydroxyl group of Tyr42. This H-bond network is extended by the side-chain of Thr50, which forms a H-bond with the carbonyl oxygen of Glu46 and with the hydroxyl of Tyr42. The main chain oxygen of Thr50 also forms a H-bond with the side chain of Arg52, which encloses the chromophore within the pocket and shields it from the solvent. This active site hydrogen-bonded network to the anionic phenol oxygen is critical for the overall stabilization of the protein, the maintenance of the chromophore's negative charge and thus its spectral properties, the photocycle kinetics, and likely for signal transduction as well. Glu46 is dominant in these roles as evidenced by previous studies of site-specific mutants such as Glu46Gln (Genick et al., 1997b; Devanathan et al., 2000), Glu46Asp, and Glu46Ala (Devanathan et al., 1999a). Thus, the red-shift in the PYP absorption maximum is largest for the Glu46Ala mutant (shift of 23 nm), and is likely the result of an increase in the anionic character of the phenolate oxygen because of complete removal of the Glu46 H-bond to the chromophore. The H-bonding interaction between the protein and the chromophore is also weakened upon mutation of Tyr42 to Phe (Brudler et al., 2001). In this case as well, the electron density on the chromophore oxygen is increased resulting in a red shift of 12 nm in the absorption maximum (Mihara et al., 1997; Brudler et al., 2000).

The Glu46Ala mutant shows a ground state pH-dependent spectral transition between two species: a basic (deprotonated; λ_{max} = 469 nm), and an acidic (protonated; λ_{max} = 365 nm) form. Both of these species are photoactive and have been characterized in detail (Devanathan et al., 1999a).

Photobleaching kinetics ($I_1 \rightarrow I_2$ transition) for the 469-nm species is accelerated ($\tau < 1$ μs; at least 50 times faster than the Glu46Gln mutant and 200 times faster than wild-type (WT)) (Devanathan et al., 1999a). Interestingly, Tyr42Phe shows both a predominant wavelength maximum at 458 nm and a second PYP population absorbing at shorter wavelengths, giving rise to a shoulder at 391 nm. The latter species presumably has an altered chromophore conformation and has a decreased protein stability (Brudler et al., 2000). This indicates that this H-bond between the Tyr42 hydroxyl and the chromophore is also crucial in stabilization of the chromophore and protein conformations in the ground state. However, in the Tyr42Phe mutant, these two conformations can be interconverted by kosmotropic (stabilizing the 458-nm protein form) and chaotropic reagents (stabilizing the 391-nm altered form) (Brudler et al., 2000). Further, the $I_1 \rightarrow I_2$ transition for the Tyr42Phe mutant is also accelerated compared with WT ($\tau = 120$ μs) for the 458-nm form when it is excited near its wavelength maximum (Brudler et al., 2000).

Recently, we characterized the early part of the photocycle using femtosecond and picosecond spectroscopy for both WT-PYP and the Glu46Gln mutant (Devanathan et al., 1999b, 2000; Zhou et al., 2001). The photocycle kinetics for the mutant were significantly altered compared with WT. Thus, in the mutant, the time constants for formation of the second intermediate $I_0^{\neq}$ (8 ps vs. 220 ps for WT), and the third intermediate I_1 (700 ps vs. 3 ns), were appreciably faster, although the formation constant for the first intermediate I_0 and the excited state lifetime were essentially unaffected (Devanathan et al., 2000). The recent paper by Zhou et al. (2001) confirms this result, although the time constants obtained were approximately 2-fold larger (14 and 1700 ps, respectively); this was attributed to better signal-to-noise characteristics. The subsequent stages of the photocycle also showed decreased time constants in the mutant, with the I_1 to I_2 transition being ~4 times faster and the recovery step I_2 to P being ~3 times faster (Devanathan et al., 2000; Genick et al., 1997b). To further understand the role of the H-bonding network in influencing the early times of the photocycle, we have undertaken the ultrafast spectroscopic characterization of two additional mutants (Glu46Ala and Tyr42Phe), each of which involves the removal of one H-bond to the chromophore, leaving the other one intact. The structural implications of the results for the photochemical pathways of these mutants are discussed.

MATERIALS AND METHODS

Sample preparation

The Glu46Ala mutant was prepared as previously reported (Devanathan et al., 1999a) and the Tyr42Phe mutant (Brudler et al., 2000) was a generous gift from Dr. E. Getzoff (Scripps Research Institute, La Jolla, CA). Four milliliters of Glu46Ala-PYP (1.5 OD/ml) in 20 mM CHES buffer (pH = 9.5) was used in the sample wheel to obtain predominantly the 469-nm basic form. Similarly, 4 ml of the Tyr42Phe mutant (1.5 OD/ml) in 20 mM Tris-Cl buffer at pH 7.5 was used. Data were collected at ambient temperature. The sample wheel (radius = 10 cm) had an optical pathlength of 2 mm and was rotated at 5 Hz. The excitation wavelength was at 470 nm for both mutants.

Instrumental setup

Transient absorbance change spectra were recorded on a femtosecond spectrometer described previously (Devanathan et al., 1999b, 2000). The laser pulses were provided by a Ti:S regenerative amplifier (Model CPA-1000, Clark-MXR, Dexter, MI) pumped by a diode-pumped solid state laser (Model Millenia V, Spectra Physics, Mountain View, CA). The output of the CPA-1000 was at 790 nm, with 1 kHz repetition rate. Most of the CPA output (>80%) was used to pump an optical parametric amplifier (Model IR-OPA, Clark-MXR) to generate excitation at 470 nm. The rest of the laser output was focused into a 1-cm flowing water cell to generate a white light continuum, which was further split into two identical parts used as probe and reference beams. The probe and reference signals were recorded using a dual diode array detector (Model DPDA-1024, Princeton Instruments, Trenton, NJ). Excitation energy at the sample was adjusted to ~1 μJ per pulse using neutral density filters. The beam size at the sample was 0.5 mm in diameter. The polarization of the excitation beam was set at the magic angle (54.7°) relative to the probe and reference beams. Detailed data correction and analysis procedures were the same as described in the previous paper (Devanathan et al., 2000).

Data fitting

Levenberg-Marquardt algorithms were incorporated into the nonlinear least-squares fitting routine of the Microcal Origin software package (Microcal Origin, Northampton, MA) for analysis of the dispersion-corrected kinetic data. The data were analyzed for two different time regions: −1 and 7.0 ps and −1 and 500 ps, using selected wavelengths in the spectral range from 420 to 550 nm. A Gaussian cross-correlation function was used to represent the instrumental response time of the pump-probe laser pulse, as described previously (Ujj et al., 1998; Devanathan et al., 1999b, 2000); this was found to be 0.5 ps full width half-maximal height over the entire measured spectral range. The decay kinetics were fit with a single exponential term for the early time ranges. Kinetic fits in the −1 to 500 ps time range required one exponential for the early times and an additional time constant for the later times. Global analysis fits for selected transients were also performed. As noted previously (Ujj et al., 1998; Devanathan et al., 1999b, 2000) ground-state bleaching and recovery kinetics are observed in the 420–480 nm region and the 490–550 nm wavelength region reflects stimulated emission and photochemical intermediate formation and decay processes.

RESULTS

Transient absorption difference spectra obtained at selected times after excitation are presented in Figs. 1 and 2 for the Glu46Ala and Tyr42Phe mutants, respectively. These clearly show ground-state bleaching at shorter wavelengths and stimulated emission at longer wavelengths in the earlier time intervals, and positive absorbance changes at long wavelengths because of photochemical intermediate formation at the later times. This is consistent with our previous work (Ujj et al., 1998; Devanathan et al., 1999b, 2000). Both the $I_0/I_0^{\neq}$ intermediates at earlier times (~50 ps) and the I_1 intermediate at later times (~330 ps) can be seen. As

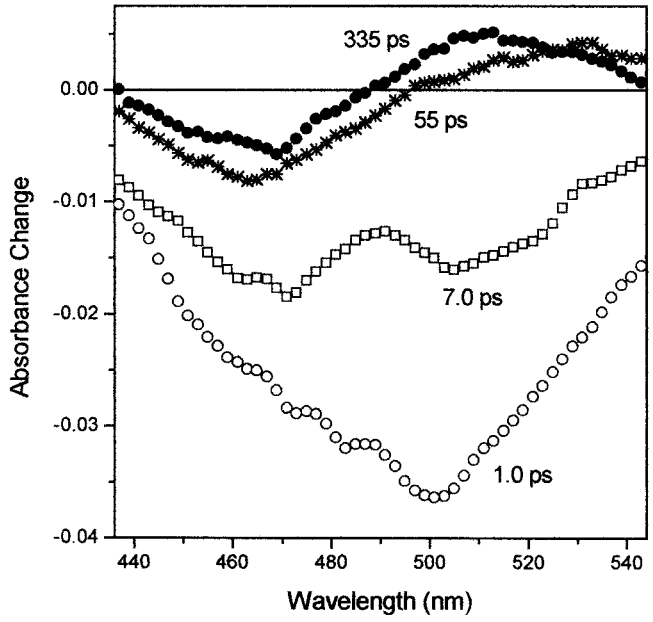

FIGURE 1 Transient absorption difference spectra obtained at selected times after excitation of Glu46Ala-PYP mutant at 470 nm.

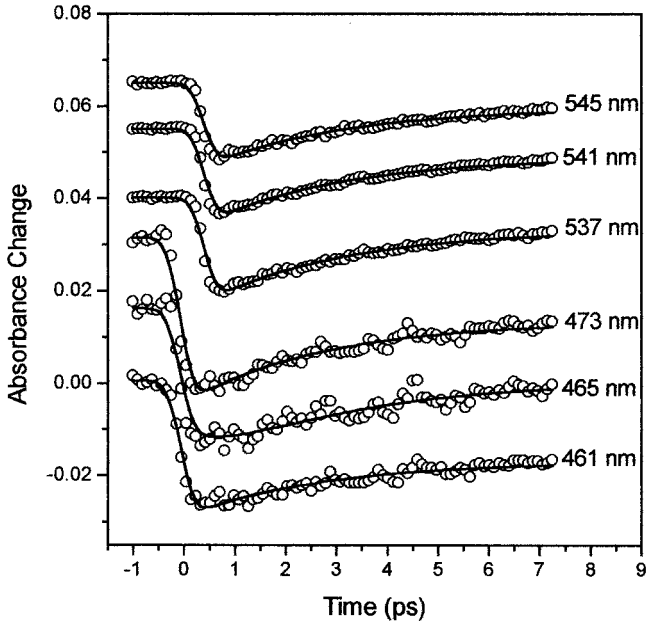

FIGURE 3 Kinetic transients obtained from −1 ps to 7 ps for the Glu46Ala-PYP mutant at selected wavelengths. *Solid lines* are global fits to the data. Baselines have been shifted for clarity.

in the earlier work, the partial recovery of ground state bleaching is attributable to the decay of the excited state to regenerate the ground state and to form the photochemical intermediates.

Figs. 3 and 4 show transient decay curves obtained from −1 ps to 7 ps over a wavelength range from 430–550 nm for the Glu46Ala and Tyr42Phe mutants, respectively. Fig. 5 shows decay data obtained in the time range from −1 ps to 250 ps for the Tyr42Phe mutant between 500 nm and 530 nm (similar results were obtained with the Glu46Ala mutant; data not shown). Global nonlinear least-squares fit to these data are shown by the solid lines in the figures, and the

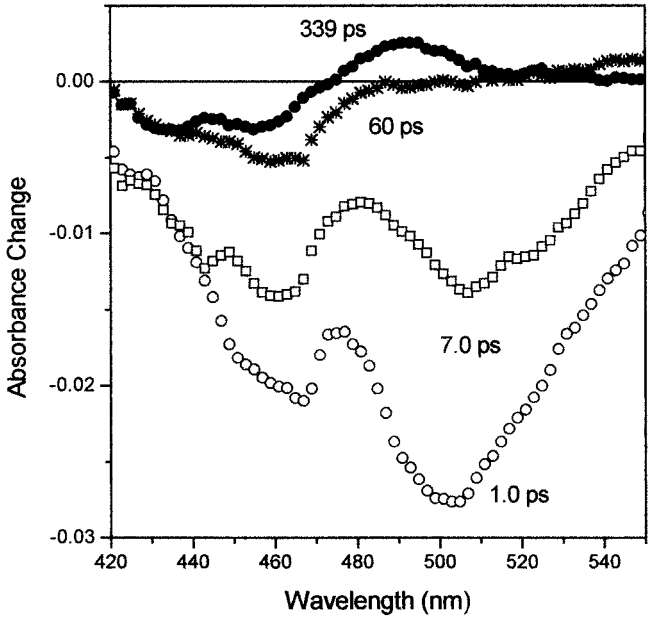

FIGURE 2 Transient absorption difference spectra obtained at selected times after excitation of Tyr42Phe-PYP mutant at 470 nm.

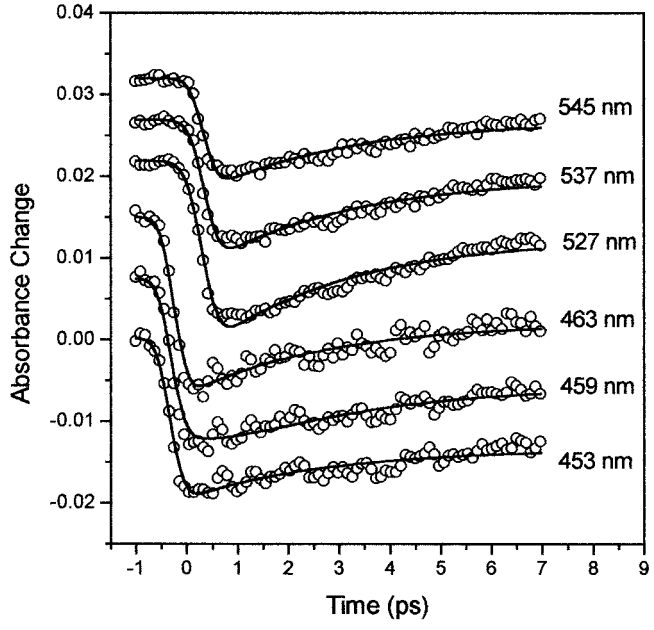

FIGURE 4 Kinetic transients obtained from −1 ps to 7 ps for the Tyr42Phe-PYP mutant at selected wavelengths. *Solid lines* are global fits to the data. Baselines have been shifted for clarity.

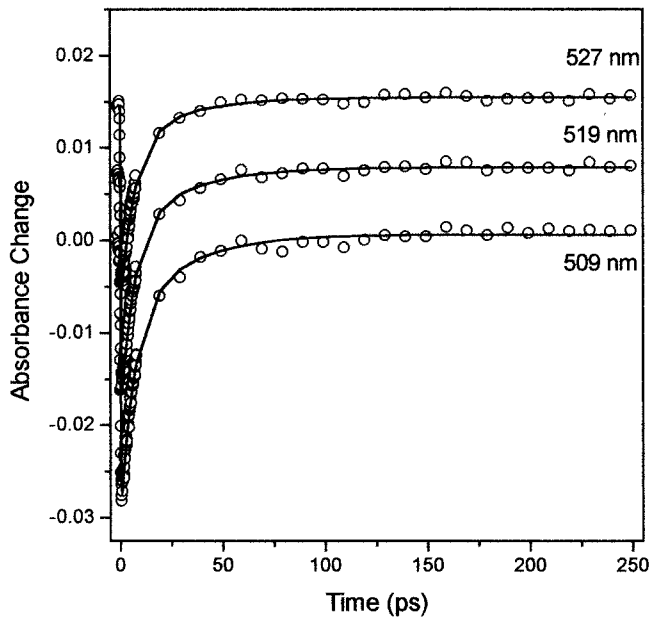

FIGURE 5 Kinetic transients obtained from −1 ps to 250 ps for the Tyr42Phe-PYP mutant. *Solid lines* are global fits to the data. Baselines have been shifted for clarity.

time constants obtained from these are given in a kinetic scheme shown in Fig. 6, along with the previous results obtained for WT PYP and the Glu46Gln mutant for comparison purposes. The decay portions of the curves in Figs. 3 and 4 can be fit by a single exponential using the same rate constant at all wavelengths [Glu46Ala: $\tau = 2.70 \pm 0.03$ ps; $k_{obs} = 0.37 \times 10^{12}$ s^{-1}; Tyr42Phe: $\tau = 2.81 \pm 0.02$ ps; $k_{obs} = 0.36 \times 10^{12}$ s^{-1}]. The fits in Fig. 5 were obtained using this rate constant and one additional rate constant [Tyr42Phe: $\tau = 30.04 \pm 0.15$ ps; $k_{obs} = 33.29 \times 10^{9}$ s^{-1}]. The corresponding value for the second time constant for Glu46Ala was $\tau = 40.10 \pm 0.18$ ps; $k_{obs} = 24.94 \times 10^{9}$ s^{-1} (data not shown).

As was done previously, the rate constants in the early times were deconvoluted using a simple kinetic model that involves two competing processes: repopulation of the ground state from the excited state (k_d) and formation of the first photochemical intermediate I_0 (k_p), where $k_{obs} = k_d + k_p$. A photochemical quantum yield (equal to $k_p/(k_p + k_d)$) was determined from the decay data in the 440–480 nm region ($\Phi = 0.54$) for both mutants. As shown in Fig. 6, both mutants have excited state lifetimes that are approximately 2-fold longer than obtained previously for WT PYP and Glu46Gln (Devanathan et al., 2000). Conversion of this excited state to the first photochemical intermediate occurs with a time constant of 5.3 ps for both mutants, in comparison to 2.3 ps for WT and 3.0 ps for Glu46Gln. The intrinsic decay rate of excited state (P^*) to the ground state has slowed by about the same amount as the rate of formation of the first photochemical intermediate state from P^*. This is the reason that the same photochemical quantum yields are observed for both mutants as previously determined for WT PYP and the Glu46Gln mutant.

As noted above, subsequent to the decay of the stimulated emission, a positive absorption at longer wavelengths is observed corresponding to the formation of $I_0/I_0^{\neq}$ (Figs. 1 and 2). We are unable to resolve the formation kinetics of

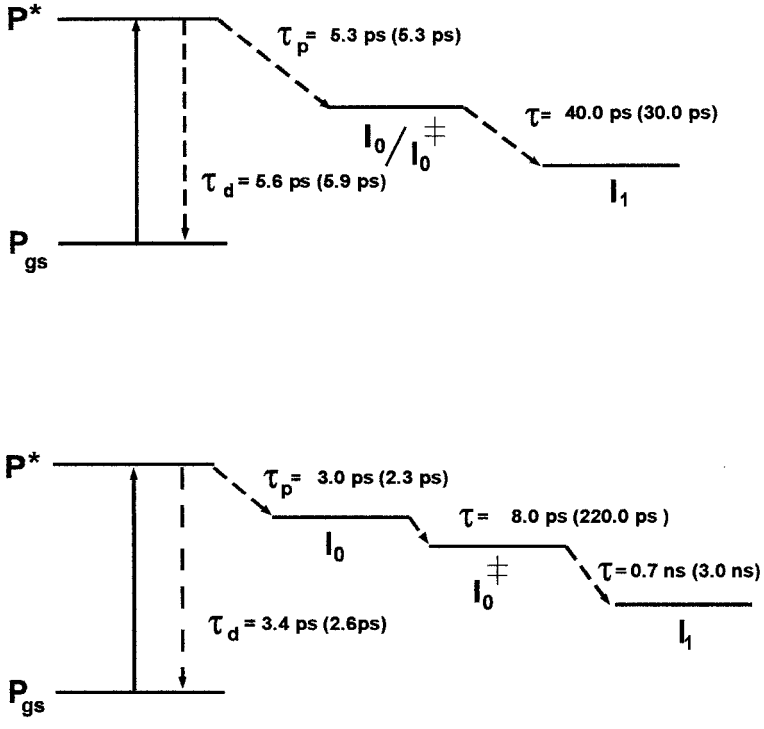

FIGURE 6 Kinetic scheme showing the proposed mechanism of photoproduct formation for WT and mutant PYPs. (*A*) Time constant values are given for Glu46Ala-PYP and for Tyr42Phe-PYP (values in parentheses). (*B*) Time constant values are given for Glu46Gln-PYP and for WT PYP (values in parentheses).

these two intermediate species for these mutants, probably because of the slower kinetics of I_0 formation and excited state decay. In our previous work (Devanathan et al., 2000), we were barely able to resolve these two kinetic processes in the Glu46Gln mutant, whereas they were easily resolved in the WT protein (Devanathan et al., 1999b). Thus, as was the case for the Glu46Gln mutant, $I_0^{\neq}$ formation is greatly accelerated for these mutants relative to WT PYP. We associate the time constants of 30–40 ps observed for these mutants with the conversion of $I_0/I_0^{\neq}$ to I_1, consistent with the fact that the species formed by this process does not decay during the 500-ps time window used in these experiments. It is interesting that this conversion is also accelerated, by a factor of ~100 for these mutants compared with WT protein, and by a factor of 25 compared with the Glu46Gln mutant. We attribute this large effect to the presence of only one H-bond attachment of the chromophore phenolic hydroxyl, compared with two in both WT PYP and Glu46Gln.

DISCUSSION

Direct kinetic measurements with sufficient time resolution to observe dynamic processes such as formation and decay of the excited state and of primary and subsequent photointermediates are essential to understanding the mechanism of conversion of light energy to a photobiological signaling state upon absorption of light by a chromophore. Femtosecond spectroscopy provides such a sensitive probe of the early time dynamics of the chromophore-protein environment. This primary process of light absorption initiates a cascade of events, beginning with excited state formation and its subsequent decay to both the ground state and to photochemical intermediates. These processes have been well characterized for WT PYP and the Glu46Gln mutant (Ujj et al., 1998; Devanathan et al., 1999b, 2000; Zhou et al., 2001).

In the present study, subpicosecond kinetics of mutants in two key active site residues, Glu46Ala and Tyr42Phe, show the formation of transient excited states (observed as ground-state bleaching at shorter wavelengths and stimulated emission at longer wavelengths) having time constants for internal conversion to the ground state that are ~2-fold longer in comparison to WT and Glu46Gln. Presumably, this is a consequence of the removal of one H-bond to the phenolic oxygen of the chromophore. It would seem that these H-bonds provide a pathway for increasing the vibrational coupling of the chromophore excited state to the surrounding protein.

We also find that the conversion of the excited state to the initial photochemical intermediate (I_0) occurs more slowly for these mutants than for either WT PYP or the Glu46Gln mutant (again by a factor of ~2). It has been previously suggested from the crystal structure of a cryotrapped photocycle intermediate (Xie et al., 1996; Genick et al., 1998) that this process involves a rotation of the C-S single bond of the thioester linkage with little or no movement of the phenolic ring, which is tethered to the protein by its two H-bonds. This introduces torsional strain that is subsequently relieved by allowing the phenolic ring to rotate. In this early intermediate, the largest movements are found for the chromophore carbonyl oxygen (3.4 Å) and sulfur (1.4 Å) atoms, whereas the chromophore aromatic ring undergoes only a small lengthwise contraction (0.4 Å). Further, the H-bond between the chromophore and Glu46 is approximately unchanged in the early photocycle intermediate and may actually become stronger in the I_1 intermediate. Additional corroboration for this hypothesis comes from recent time-resolved Fourier transform infrared studies (Brudler et al., 2001; Xie et al., 2001). Apparently, when this phenolic ring tethering is diminished by removal of one of these H-bonds in the present mutants, the thioester single bond rotation process occurs more slowly. This may be a consequence of allowing rotational motions of the phenolic ring that decrease the coupling between the vibronic states of the thioester bond and those of the π-electron system of the aromatic portion of the chromophore.

Although altering the strength of one H-bond in the Glu46Gln mutant did not significantly affect the primary photochemistry (I_0 formation), large differences (3 to 30-fold) were found in the time constants for the subsequent steps (Devanathan et. al, 2000). In this study, we have shown that totally removing one H-bond in both the Glu46Ala and Tyr42Phe mutants causes an even more dramatic acceleration of these subsequent kinetic processes. Thus, the formation of I_1 becomes 100 times more rapid than in WT PYP, and this rate is increased by a factor of 25 compared with the Glu46Gln mutant. Apparently, the time constants for the later photocycle processes are appreciably slowed by the tethering of the phenolic hydroxyl to the protein in WT PYP. This is not unexpected inasmuch as the completion of the *trans-cis* isomerization requires the phenolic ring to move from the chromophore binding pocket in the I_2 intermediate. Thus, the further increase in the rate of the I_1 formation process observed here upon removal of one of the H-bonds is consistent with a more facile movement of this region of the chromophore.

CONCLUSION

It has previously been shown that the H-bonding interactions between the phenolic oxygen, Glu46 and Tyr42, which form part of the chromophore-protein environment in PYP, tether the chromophore within the active site and are major factors in the spectral tuning responsible for the yellow color of the protein (Genick et al., 1997b; Devanathan et al., 1999a; Brudler et al., 2000; Imamoto et al., 2001). The present study demonstrates that abridgment of this H-bonding network also has a striking influence on the dynamics of the photocycle intermediary states. The torsional strain induced in the chro-

mophore by thioester bond rotation has to relax within the cofactor binding pocket of the protein by inducing a series of conformational changes of the surrounding amino acid residues. The demonstration that removal of one H-bond to the chromophore leaving the other intact allows this to happen much more rapidly indicates that it is the movement of the phenolic ring that causes the protein conformation surrounding the chromophore to open sufficiently to produce the I_2 intermediate, which is presumed to be the signaling state of PYP. It is interesting that although these two H-bonds make unequal contributions to spectral shifts, their effects on photocycle kinetics are closely similar. This suggests that these H-bonds differ in the level of mechanical coupling versus electronic coupling to the chromophore. These observations shed new light on the internal dynamics underlying the mechanistic functioning of the protein.

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