New Insights into the Photocycle of *Ectothiorhodospira halophila* Photoactive Yellow Protein: Photorecovery of the Long-Lived Photobleached Intermediate in the Met100Ala Mutant†

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ABSTRACT: There are previously two known intermediates (I_1 and I_2) in the room-temperature photocycle of the photoactive yellow protein (PYP) from *Ectothiorhodospira halophila*. The three-dimensional structures of ground-state PYP and of I_2 have shown that light-induced conformational changes are localized to the active site. Previous site-specific mutagenesis studies of PYP in our laboratories have characterized two active site mutants (Glu46Gln and Arg52Ala). We now report the construction and characterization of a mutant at a third active site position (Met100Ala) in order to establish the role of this residue in the photocycle. Met100Ala PYP has an absorption spectrum which is very similar to wild-type (WT) PYP, but exhibits very different kinetic properties. At pH 7.0, the light-induced bleaching reaction (I_2 formation) has a half-life <1 μs and the recovery in the dark has a half-life of 5.5 min, as compared with half-lives of 100 μs and 140 ms for the same reactions in WT PYP. The slow rate of recovery from I_2 for Met100Ala results in the accumulation of the bleached intermediate even under room light illumination. These results are qualitatively similar to what has been observed with the Arg52Ala mutant of PYP, and with WT PYP in the presence of alcohols or urea, and suggest that Met100 acts to stabilize the ground state of the protein. The midpoint for guanidine denaturation confirms this. The slow recovery of I_2 in the Met100Ala mutant has allowed us to obtain direct evidence that this intermediate species is also photoactive and can be returned to the ground state by a 365 nm laser flash, with kinetics (half-life $= 160$ μs; $k = 6300$ s^{-1}) which are 6 orders of magnitude faster than dark recovery. This implies that chromophore reisomerization limits the rate of conversion of I_2 to the ground state in PYP. Met100 is in van der Waals contact with the chromophore in the I_2 state, and we suggest that the sulfur atom catalyzes *cis-trans* isomerization in WT PYP.

The photoactive yellow proteins (PYPs)[1] of phototrophic bacteria constitute a class of signal transduction proteins which change conformation as a result of trans-cis photoisomerization of their anionic *p*-hydroxycinnamyl cysteine thioester chromophore. To date, PYPs have been found only in the halophilic purple phototrophs (*1*, *2*), where they presumably mediate a photophobic response to blue light (*3*). PYPs are small (14 kDa) water-soluble, cytoplasmic proteins which have maximal absorption at 446 nm ($\epsilon = 45$ mM^{-1} cm^{-1}) and a relatively simple photocycle. Recent characterization of the photocycle of WT PYP at picosecond time resolution (*21*) has revealed two new red-shifted intermediates, I_0 and $I_0^{\ddagger}$, which precede the previously established I_1 and I_2 intermediates. The less red-shifted I_1 species is produced from $I_0^{\ddagger}$ in 3 ns, a blue-shifted form (I_2) appears on a millisecond time scale, and I_2 returns to the ground state in several hundred milliseconds (*4*−*6*).

Several amino acid and nucleotide sequences of PYP are known (*7*−*9*), and the three-dimensional structure of the protein from *Ectothiorhodospira halophila* has been determined to 1.4 Å resolution (*10*). The structure of I_2 has also been solved to 1.9 Å by millisecond time-resolved Laue crystallography (*11*). These latter results show that light-induced conformational changes are confined to the region of the chromophore, and involve *trans-cis* photoisomerization of the *p*-hydroxycinnamyl double bond (*12*, *13*) and movement of a small number of nearby amino acid residues. Among the latter is Arg52, and mutation of this residue to Ala has been shown (*6*) to have significant effects on the photocycle; i.e., it increases the rate of formation of the I_2 intermediate by approximately 4-fold and slows its return to the ground state by about 5-fold at pH 7.0. The slightly red-shifted spectrum of this mutant indicates that the positive charge of Arg52 is not required to stabilize the anionic form

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[1] Abbreviations: PYP, photoactive yellow protein; WT, wild type; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactopyranoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CD, circular dichroism.

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FIGURE 1: α-Carbon backbone structure of PYP showing the *p*-hydroxycinnamyl group attached to Cys69, and the side chains of Arg52 and Met100.

of the chromophore (*6*, *14*). Instead, the kinetic results correlate with a decreased overall stability of the protein.

Met100 is part of the peptide loop that connects strands 4 and 5 of PYP's central β sheet (*10*), as shown in Figure 1. This loop forms the back of the active site pocket which shields the ground-state chromophore from solvent. The distance (3.3 Å) between one of the terminal nitrogens of Arg52 and the Met100 sulfur atom is well below the maximum value for N−H−S hydrogen bonding (*15*) and exhibits reasonable bonding angles. It is therefore likely that this interaction, along with the hydrogen bonds described earlier (*10*), helps stabilize the ground-state position of Arg52; Met100 may therefore be involved in influencing the movement of Arg52 during the process of I_2 formation. In the present work, we have constructed and characterized the Met100Ala mutant in order to establish the role of this residue in the photocycle and on protein stability. As will be demonstrated below, the kinetic consequences of the mutation are to greatly increase the rate of I_2 formation and to dramatically slow the dark return of I_2 to the ground state. As a consequence of these effects, the Met100Ala mutant is partially bleached on exposure to room light. This has allowed us to directly demonstrate that photoexcitation of I_2 greatly accelerates the ground-state re-formation process, thereby implying that the rate-limiting process in the recovery of the ground-state conformation is chromophore isomerization.

MATERIALS AND METHODS

Site-Directed Mutagenesis. For the generation of the site-directed mutation, we used a PCR-based approach as implemented in the QuickChange kit (Stratagene). The template for the PCR was the pPYP vector (*6*), and the sequences of the primers were:

5′CCTTCGATTACCAAGCGACGCCCACGAAGG3′

5′CCTTCGTGGGCGTCGCTTGGTAATCGAAGG3′

The generation of the correct mutation was confirmed by sequencing with an Applied Biosystems automated sequencer.

Protein Production and Purification. Protein expression and chromophore attachment were performed as described for previously prepared mutants (*6*). During the purification, the concentration of ammonium sulfate was reduced to 60% to prevent excessive precipitation of PYP holoprotein. The rest of the purification was performed as previously described (*6*). As judged by SDS−PAGE and isoelectric focusing, the mutant protein was >90% pure.

Protein Stability and Circular Dichroism. Protein stability was assessed by denaturation of 1 μM solutions of Met100Ala PYP in 20 mM Tris−chloride buffer (pH 7.5) plus 40 mM NaCl with increasing concentrations of guanidine (USB, ultrapure), following a procedure developed by Pace (*4*, *16*) and by monitoring changes in the secondary structure at 222 nm with circular dichroism. These changes as well as visible circular dichroism spectra were obtained using an Aviv-modified Cary 60 spectropolarimeter with 20 μM protein in 20 mM HEPES buffer, pH 7.0.

Time-Resolved Optical Spectroscopy. The laser flash photolysis, time-resolved spectroscopy, and data analysis procedures have been described previously (*4*). The laser flash and steady-state photobleaching and recovery experiments were done either in 10 mM HEPES buffer, pH 7.0, or in universal buffer (10 mM MES/MOPS/Bicine; pH 5.0−9.5) for studies of the pH dependence of the dark recovery and photorecovery kinetics. In the latter experiments, the protein was first bleached by a few seconds exposure to a tungsten lamp, and subsequent recovery of absorbance was measured at 446 nm either in the dark or after a 365 nm laser flash. A modified Cary-15 spectrophotometer (OLIS Corp., Bogart, GA) was used to follow the dark recovery kinetics. Absorbance changes were determined over a 30−60 min time period after photobleaching the sample, and the data were fit with a single-exponential function using OLIS software.

RESULTS AND DISCUSSION

Steady-State Properties. The Met100Ala mutant of *E. halophila* PYP has an absorption spectrum similar to WT protein, except for the presence of a ∼350 nm shoulder on the 446 nm peak; this is shown in Figure 2. The relative intensities of the 350 and 446 nm peaks were found to be dependent on the length and intensity of exposure to room light. When a solution of this mutant PYP was placed in the dark at 4 °C overnight to obtain maximal intensity at 446 nm, the protein still retained significant 350 nm absorbance. Nevertheless, the mutant protein still had only half the absorptivity of the WT protein at 446 nm, when the spectra were normalized at 280 nm. A short exposure to weak room light caused the 446 nm peak to partially bleach and the 350 nm peak to increase in magnitude, with an isosbestic point at approximately 380 nm at pH 7.0. When it is fully bleached by steady-state illumination, the difference spectrum for the Met100Ala mutant has a shape similar to the time-resolved difference spectrum of protein bleached by laser flash photolysis (*4*). Thus, the I_2 photocycle intermediate is kinetically, and perhaps also thermodynamically, stabilized in this mutant, and even weak light can lead to a further accumulation of this species. In contrast, WT protein is unaffected by room light and requires a high-intensity laser flash to give an equivalent amount of bleaching. The visible CD spectrum for Met100Ala is qualitatively the same as that of WT PYP (data not shown),

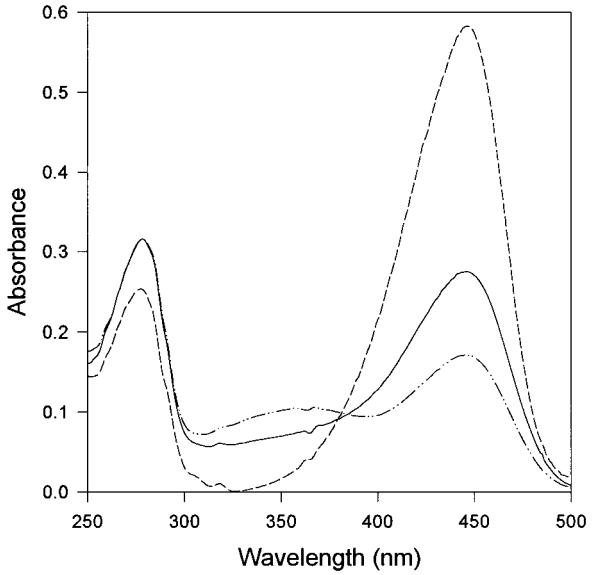

FIGURE 2: UV−vis absorption spectra of dark-adapted Met100Ala (solid line), Met100Ala partially bleached by irradiation with a 40 W tungsten lamp for 15 s (dash−dotted line), and WT PYP (dashed line), in HEPES buffer at pH 7.0.

suggesting that the mutation does not result in significant changes in the overall protein structure and chromophore environment.

The behavior of the Met100Ala mutant toward room light is similar to that observed with the *Rhodospirillum salexigens* PYP in pure water (*2*), and to native *E. halophila* PYP in urea (*4*). The latter result suggests that the mutant protein is relatively unstable in the ground-state conformation. Guanidine denaturation was used to test this possibility. The energy of unfolding was obtained by measuring changes in the UV circular dichroism at 222 nm of the dark-adapted Met100Ala protein as a function of the concentration of the denaturant guanidine hydrochloride. As expected, the ΔG_{unfold} (6.3 kcal/mol) for the Met100Ala mutant is lower than that of the WT PYP (7.2 kcal/mol), and the midpoint of denaturation is 2.4 M compared to the WT value of 2.75 M (data not shown). A similar decrease in the energy of unfolding has been observed for the Arg52Ala mutant (6.2 kcal/mol) (*6*). However, in this mutant the effects on the photocycle kinetics were much less pronounced than those reported here for Met100Ala. In Arg52Ala, the flash-induced formation of I_2 is accelerated 3−4-fold and the return from I_2 to the ground state is slowed 6−8-fold, compared with a more than one 100-fold acceleration and several 1000-fold deceleration of the respective reactions in Met100Ala (see below). This indicates that destabilization of the ground state alone cannot account for the observed kinetic properties, and that Met100Ala must have an additional effect that leads to the exceptionally large kinetic changes, especially the decrease in the rate of the I_2 to ground-state reaction. One possibility is that Met100 plays a central role in the, as yet undetermined, mechanism that drives the reisomerization of the cis chromophore to initiate the return to the ground state.

Laser Flash Photolysis. Laser flash-induced formation of I_2 in dark-adapted Met100Ala is too fast to measure with the present apparatus (half-life $< 1\ \mu$s; $k > 10^6\ s^{-1}$), as shown in Figure 3a. Furthermore, dark recovery of the photobleached protein is too slow to measure with our flash

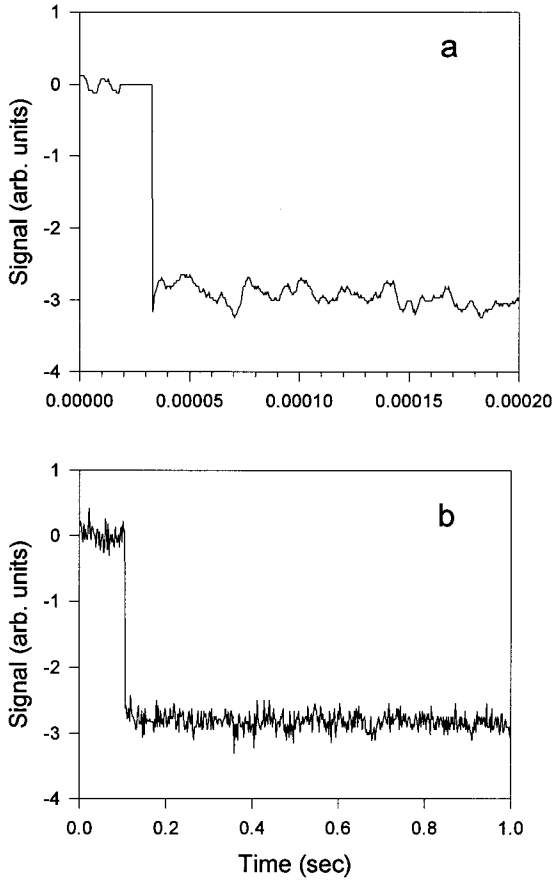

FIGURE 3: Transient absorbance changes observed at 446 nm upon laser flash photolysis of dark-adapted Met100Ala PYP in 10 mM HEPES at pH 7.0. Excitation was at 446 nm; measurements were made on 200 μs (panel a) and 1 s (panel b) time scales.

photolysis system (Figure 3b), which is limited to a time range of several seconds due to diffusion processes occurring at longer times. Thus, the kinetics of dark recovery were measured on an ordinary spectrophotometer, following steady-state bleaching by a tungsten lamp, as shown in Figure 4. As with WT PYP, the return of I_2 to the ground state is a single-exponential process (see inset to Figure 4). However, the half-life at pH 7.0 is more than 3 orders of magnitude (half-life = 5.5 min; $k = 21 \times 10^{-4}\ s^{-1}$) larger than that obtained with WT PYP. These large differences in photocycle kinetics between WT PYP and the Met100Ala mutant account for the ability of room light to effectively bleach the mutant protein. Removal of the supposed hydrogen bond between Met100 and Arg52 by the mutation, as well as the destabilization of the ground state, which occur in both the Arg52Ala and the Met100Ala mutants (*6*), may account in part for the observed increases in the rate of I_2 formation. The loss of the hydrogen bond could also account in part for the slowing of the dark recovery process, if this interaction is an important thermodynamic barrier which keeps the chromophore "locked" into the active site cavity in the dark-adapted state. Arg52 H-bonds to the chromophore hydroxyl in the I_2 form of PYP (*11*), an interaction which is presumably still present in Met100Ala but which is lost in Arg52Ala. This may play a role in the differences in the kinetics for the two mutants.

pH Effects on Dark Recovery. The dark recovery of the Met100Ala mutant is faster by an order of magnitude at pH 9.5 (half-life = 3.3 min) compared with pH 5.0 (half-life =

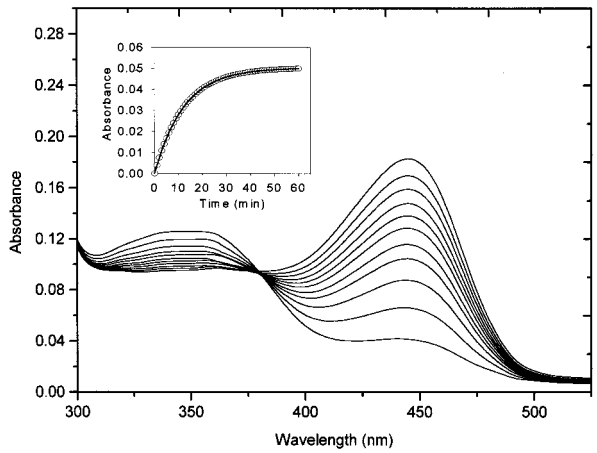

FIGURE 4: Dark recovery kinetics of Met100Ala PYP in universal buffer (10 mM MES/MOPS/Bicine, pH 7.0) following steady-state bleaching with a 40 W tungsten lamp. Spectra were taken at intervals of approximately 2 min. Note the isosbestic point at 380 nm. The inset shows data (open circles) obtained at 446 nm at pH 6.5 over a time range of 60 min, and a single-exponential fit (solid line) with $k = 1.35 \times 10^{-3}$ s^{-1}.

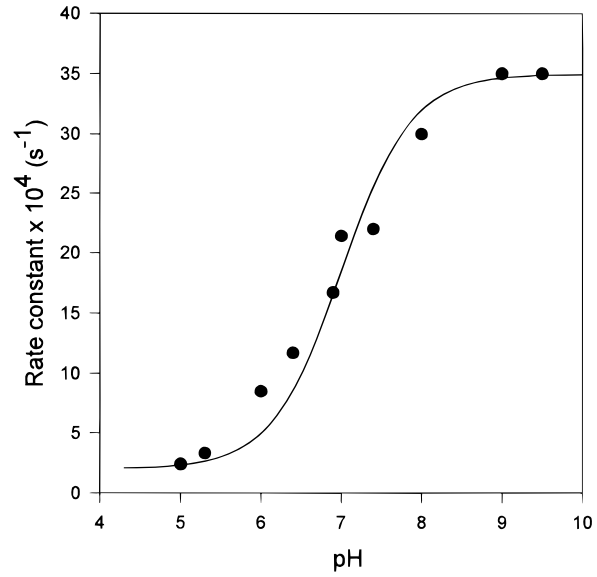

FIGURE 5: pH dependence of dark recovery kinetics of Met100Ala PYP. Conditions as in Figure 4. The solid line corresponds to a theoretical one-proton ionization curve with a pK_a value of 7.0, obtained using a nonlinear least-squares fitting procedure.

60 min) (Figure 5; the kinetics were not reproducible above pH 9.5); a sigmoidal curve was obtained over this pH range governed by a pK_a of about 7.0. In contrast, WT PYP has a biphasic pH dependence for dark recovery of I_2, with pK_a values of 6.4 and 9.4 (the half-life is 0.1 s at the pH 8.0 maximum and 1 s at pH 5.0; *6*). Thus, the pK_a for Met100Ala is similar to one of those of WT PYP, and both proteins show a 10-fold effect of pH on kinetics. The difference is that the rate of dark recovery of the mutant is more than 3 orders of magnitude slower than the WT and has a monophasic pH dependence. The ionizable groups responsible for these effects are uncertain at present.

Photorecovery of the Ground State from the I_2 Intermediate. The long lifetime of I_2 in the Met100Ala mutant has allowed us to directly demonstrate that this intermediate is itself photoactive. Previous steady-state experiments with white light illuminated WT PYP suggested that one of the

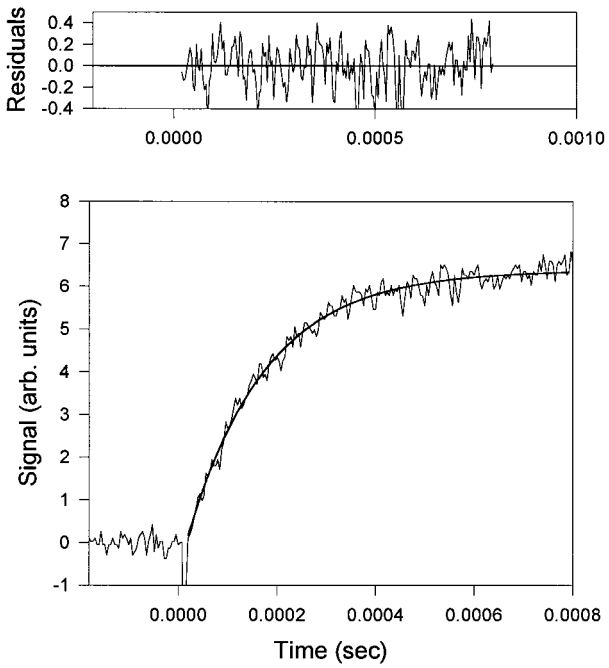

FIGURE 6: Transient absorbance change obtained at 446 nm upon laser flash excitation at 365 nm of steady-state bleached Met100Ala PYP. The solid line through the data corresponds to a single-exponential fit with a rate constant of 6300 s^{-1}; residuals are shown in the upper panel. Conditions are as in Figure 3.

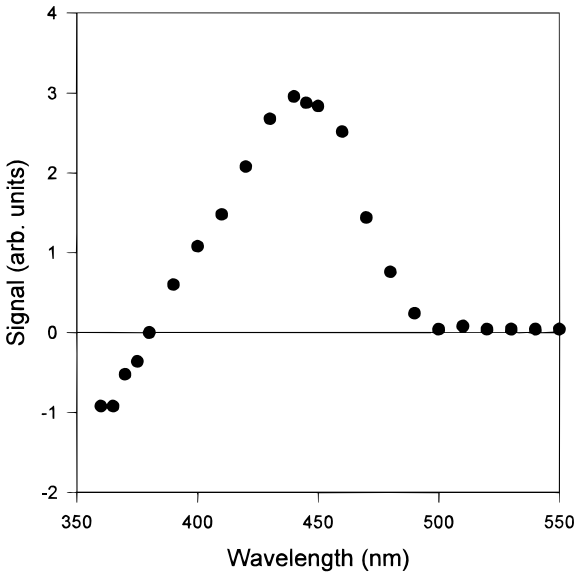

FIGURE 7: Laser flash-induced difference spectrum for recovery of bleached Met100Ala PYP; points correspond to signal amplitudes at 1 ms following the laser flash. Conditions are as in Figure 3; compare with the spectra in Figure 4.

intermediates might be photoconverted back to the ground state (*17*), but it was not established which intermediate was involved nor was the effect quantified. When laser flash photolysis was carried out on white light-bleached Met100Ala protein, with excitation at 365 nm and observation at 446 nm, a single-exponential absorbance increase was observed which occurred more than 6 orders of magnitude faster than spontaneous dark recovery (half-life = 160 μs; $k = 6300$ s^{-1}); this is shown in Figure 6. The time-resolved difference spectrum shown in Figure 7 indicates that the increase at 446 nm occurs concomitantly with the bleaching at 350 nm. No evidence for the existence of any kinetic complexities

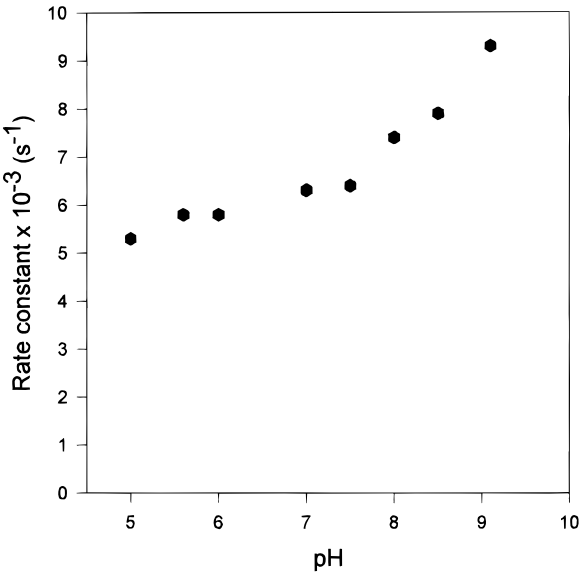

FIGURE 8: pH dependence of kinetics of laser-induced recovery of bleached Met100Ala PYP. Buffer conditions are as in Figure 4; other conditions are as in Figure 6.

was obtained from these data. Thus, the protein returns directly to the ground state without any apparent intermediates. Presumably, this process involves an initial cis-trans photoisomerization of the chromophore in the I_2 form, which probably occurs on a picosecond time scale (cf. *18*), followed by a rate-limiting protein relaxation to complete the return to the ground state. This observation suggests that the rate-limiting step in the dark recovery of I_2 in WT PYP (half-life = 140 ms) is the dark reisomerization of the chromophore.

We had previously shown that alcohols increase the rate of formation of I_2 and decrease the rate of dark return to the ground state in WT PYP (*19*). Thus, it is conceivable that such treatment could also affect the kinetic properties of the photorecovery process in the Met100Ala mutant. However, 30% methanol had no effect on these kinetics. We also tested the possibility that in 30% methanol the mutant protein, which is mostly bleached in the dark, might show spontaneous dark bleaching following photorecovery induced by a 365 nm laser flash. However, the magnitude of the photorecovery signal was smaller under these conditions, and there was no measurable dark bleaching on a 2 s time scale. This indicates that the dark bleaching reaction is kinetically quite slow, even when the thermodynamic equilibrium is shifted toward unfolded protein by methanol addition.

pH Effects on Photorecovery. The effect of pH on photoinduced recovery in Met100Ala is shown in Figure 8. The reaction is about twice as fast at pH 9.0 as at pH 5.0 and appears to be controlled by a p*K* of 8 or greater (it was not possible to go to higher pH values because dark recovery became too rapid and thus photorecovery could not be measured reproducibly). The approximate p*K* observed in this experiment suggests that it is due to the chromophore itself (p*K* = 9) (*8*). Thus, the anionic form of the trans chromophore may return to the ground-state conformation more rapidly than the protonated form. It is known that the chromophore is negatively charged in the ground state, so that releasing a proton while the chromophore is still exposed to solvent could speed the return of the chromophore to its position in the active site pocket (*8, 20*). Perhaps hydrogen bond formation between the anionic chromophore and the carboxyl group of Glu46 is part of the rate limitation.

The above results clearly establish that I_2 is photoactive. The large flash-induced signal observed in these experiments indicates a high quantum yield for the photorecovery of ground state from I_2 in Met100Ala (we estimate that it is comparable to the photoinduced formation of I_2 from ground-state PYP), which is consistent with previous estimates for photorecovery in the steady-state experiment (*17*).

We have also examined the effect of ionic strength on I_2 photorecovery and found that the kinetics in 0.1 M NaCl were essentially the same as in 0.2 mM HEPES, pH 7.0., and that the reaction slowed approximately 2-fold in 0.8 M NaCl. The normal photocycle kinetics of WT protein are also relatively insensitive to ionic strength (*4*).

CONCLUSIONS

The present studies have shown that the I_2 intermediate in PYP is photoactive, that isomerization of the chromophore is the rate-limiting step in the I_2 to ground-state reaction, and that the protein adapts to the configuration of the isomerized chromophore very quickly (rate constant of 6300 s^{-1} for the light-driven reaction). Mutation of Met100 [as well as of Arg52 (*6*)] does not produce a large shift in the ground-state chromophore absorption spectrum compared to WT PYP. This demonstrates that these residues make negligible contributions to charge stabilization of the negatively charged hydroxylate of the chromophore. The results also indicate that both Met100 and Arg52 play a role in controlling photocycle kinetics and in stabilizing the ground state of PYP. This is not surprising since they are adjacent to one another in the 3-D structure and appear to interact via a hydrogen bond. Both mutants show a faster bleach and a slower recovery in the photocycle kinetics, thereby indicating that these residues influence the rates of the processes involved in these photocycle steps in a qualitatively similar manner. The kinetic effects appear to be due both to decreases in ground-state PYP stability (i.e., a global property change) and to specific influences of these residues on the conformational transitions occurring during the bleaching and recovery reactions. We therefore suggest that Met100 catalyzes the rate-limiting *cis-trans* isomerization in the I_2 to ground-state conversion in WT PYP.

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