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The rate-determining step in the dark state recovery process in the photocycle of PYP

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The last step in the photocycle of photoactive yellow protein (PYP) is a spontaneous recovery of the dark state from the active state in which the *p*-coumaric acid chromophore is thermally isomerized, concomitantly with the deprotonation of the chromophore and the refolding of the protein moiety. For the purpose of understanding the mechanism of the thermal back-isomerization, we have investigated the rate-determining step by analyzing mutant PYPs of Met100, which was previously shown to play a major role in facilitating the reaction (1). The mutation to Lys, Leu, Ala, or Glu decelerated the dark state recovery by 1 to 3 three orders of magnitude. By evaluating temperature-dependence and pH-dependence of the kinetics of the dark state recovery, it was found that the retardation by mutations resulted from elevation of the activation enthalpy ($\Delta H^{\ddagger}$) and that the pKa of the chromophore, which was affected by the mutation, is in a linier correlation with the amplitude of the rate constants. It was, therefore, deduced from the correlation that the free energy for crossing the activated state in the dark recovery process is proportional to the free energy for the deprotonation of the chromophore, identifying the rate-determining step as the deprotonation of the chromophore.
(1) Devanathan, S. Genick, U. K. Canestrelli, I. L. Meyer, T. E. Cusanovich, M. A. Getzoff, E. D. Tollin, G., *Biochemistry* 1998, 37, 11563 - 11568

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INTRODUCTION

Photoactive Yellow Protein (PYP) in *Ectothiorhodospira halophila* is a small water-soluble protein (14 kDa) (1) and has been demonstrated to serve as a photoreceptor for the repellent phototactic response to the blue light (2). The chromophore for the light-reception is carried out by a *p*-coumaric acid (*p*CA) molucule bound via a thiol-ester linkage to Cys69 and via hydrogen bonds to Tyr42, Glu 46, Thr50 and Arg52 with the anionic phenolic oxygen and to the peptide amide of Cys69 with the carbonyl group.

Upon light absorption, the chromophore undergoes isomerization from *trans* to *cis* by flipping the carbonyl group over to the other side, as was shown by X-ray crystallography and resonance Raman spectroscopy in so-called PYP_B and PYP_L intermediate states whose absorption spectra are red-shifted with respect to that of the dark state (PYP_{dark}). Subsequent protonation of the phenolic oxygen by Glu46 (as can be seen by the blue-shift of the absorption maximum to 350 nm) causes disruption of the hydrogen-bonding network around it, triggering partial unfolding of the protein moiety to form a signaling state intermediate PYP_M. It finally decays within 0.5 sec to PYP_{dark}, completing the photocycle. In the last step of the photocycle, all the events that occurred in the course of the formation of PYP_M, namely the chromophore isomerization/protonation and the protein conformational cha-nges, are spontaneously reversed simultaneously. Here we are interested in the catalytic mechanisms of the protein moiety to facilitate the thermal isomerization of the *p*CA thiol-ester with the protonated phenolic oxygen, which is highly unlikely to occur in solvents.

Earlier it was shown that a mutant protein M100A exhibited an enormously stable PYP_M state whose decay rate constant was smaller by three orders of magnitude, suggesting a crucial role of Met100 in facilitating PYP_M decay (3). However, the recovery of PYP_{dark} in M100A was shown to be completed within 1 ms when the chromophore in the PYP_M was photo-isomerized with near-UV light, suggesting that the rate-determining step in

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PYP_M-to-PYP_{dark} is the isomerization of the chromophore and that Met100 plays the role of a catalyst for facilitating the thermal isomerization of the chromophore (3).

However, it is possible that the photoreaction of the chromophore in PYP_M elicits another different reaction pathway from the authentic one. Furthermore, the fact that the thermal isomerization of the chromophore still occurs in M100A PYP suggests the existence of a fundamental mechanism for inducing thermal isomerization besides Met100.

In the present paper, we reinvestigated the mechanism of the isomerization of the chromophore and the role of Met100, by measuring thermodynamic parameters in the PYP_M-to-PYP_{dark} process in Met-100 mutants of PYP.

RESULTS & DISCUSSION

PYP_M-to-PYP_{dark} process comprises three events, the thermal isomerization and the deprotonation of the chromophore, and refolding of the protein moiety. Since these events appear to take place simultaneously, the occurrence of one of them must be prerequisite for the other two to take place.

PYP_M decay is accompanied by proton release presumably from the chromophore or a protonatable group that relays the release from the chromophore. Because the process is dependent on the bulk pH, we first investigated the possibility that the deprotonation of the chromophore is rate-determining. In this case, we assume that, in the activated state ($PYP_M^{\ddagger}$) in going from PYP_M to PYP_{dark}, the phenolic oxygen of the chromophore is deprotonated. The equilibrium constant ($K^{\ddagger}$) between PYP_M and $PYP_M^{\ddagger}$ is propoctional to the dissociation constant of the pCA chromophore,

$$K^{\ddagger} \propto K_d = \frac{[\mathrm{pCA}^-][\mathrm{H}^+]}{[\mathrm{pCA\text{-}H}]} \qquad (1)$$

, where pCA^- and pCA-H represent the unprotonated and protonated form of the pCA chromophores, respectively. Because the rate constant of PYP_M decay (k) is linearly proportional to $K^{\ddagger}$,

$$k \propto K^{\ddagger} \qquad (2)$$

it follows that the logarithm of k is negatively proportional to the acidity of the chromophore in PYP_M (pK_a), the negative customary logarithm of K_d.

$$\ln k \propto -pK_a \qquad (3)$$

, where $pKa = -\log_{10} K_d$. It is, therefore, supposed that the deceleration of PYP_M decay in M100A is brought about by an elevation of pKa of the chromophore. Thus, the goal for proving that the rate-determining step is the chromophore deprotonation is to demonstrate the correlation between the pKa of a proton release group in PYP_M state and the rate constant.

For this purpose, we constructed mutant genes of PYP, in which Met100 is replaced either with Ala, Leu, Glu, or Lys, and over-produced them in an *E.coli* strain BL21. These mutant proteins showed absorption maxima identical to that of wild-type PYP (WT), although the spectra were partially contributed to by a blue-shifted species as well in which the chromophores were presumably protonated, suggesting elevation of the pKa in the dark state in these mutants (not shown).

The decay rates of PYP_M for the Ala, Leu and Lys mutants were extraordinarily slowed and the full recovery of the PYP_{dark} state took > 1 hr. The Glu mutant also showed a slowed PYP_{dark} recovery rate as compared to WT, but in contrast to the other mutants, the photocycle was accomplished in $\sim$ 2 min (not shown).

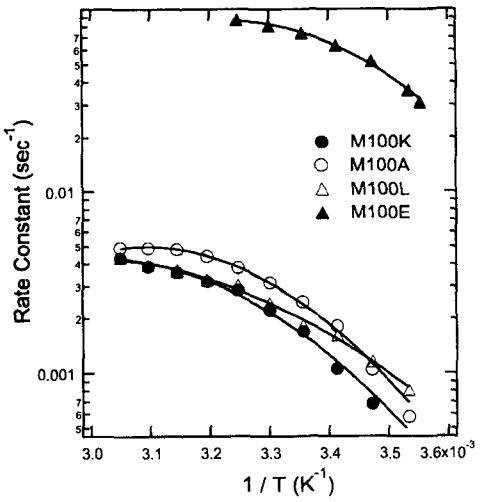

Figure 1: Rate constants of the PYP_M-to- PYP_{dark} process plotted in logarithmic scale against reciprocal of the temperature for the mutant PYPs.

Temperature dependence of the rate constants. The rate constants for these mutants were obtained by fitting the recovery kinetics with a single exponential term. The same measurements were carried out at several temperatures and the rate constants were plotted as a function of the reciprocal temperatures as shown

in Fig. 1. The curvatures in the plots in the case of WT has been ascribed to a heat capacity (Cp) decrease in going from PYP_M to $PYP^{\ddagger}_M$, suggesting more folded protein structure in $PYP^{\ddagger}_M$ than in PYP_M. Because these mutants show similar curvatures as WT does, it is suggested that the PYP_M state of these mutants are similarly unfolded and become refolded in the activated state ($PYP^{\ddagger}_M$) as in the case of WT (4).

	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta Cp^{\ddagger}$
WT	5.5	0.21	-2.81
M100K	43.6	0.152	-1.42
M100A	42.1	0.154	-1.76
M100L	30.9	0.193	-0.86
M100E	17.6	0.207	-1.78

Table 1: Activation thermodynamic parameters obtained after approximation of the temperature dependence of the rate constants with the eq (4).

By taking account of the activation heat capacity changes ($\Delta Cp^{\ddagger}$), the temperature dependences of the rate constants were approximated with the Arrhenius equation,

$$\ln k + \ln \frac{h}{k_B T} = -\frac{\Delta G^{\ddagger}}{RT}$$

$$= \frac{\Delta S^{\ddagger}}{R} - \frac{\Delta H^{\ddagger}}{RT} - \frac{\Delta Cp^{\ddagger}}{R}(1 - \frac{T_0}{T} + \ln \frac{T_0}{T}) \quad (4)$$

, where h, k_B, R and T_0 are the Planck constant, Boltzmann constant, gas constant, and a reference temperature, respectively. From the fit, the activation thermodynamic parameters were obtained as presented in Table 1. Remarkably, the activation enthalpy was elevated upon mutation at the 100th position, in contrast to the other parameters, for which the effects of 100th mutations were relatively small. Since the process in going from PYP_M to $PYP^{\ddagger}_M$ is exothermic ($\Delta H^{\ddagger} > 0$), it is likely to involve disruption of bondages in the molecule. The function of the methionine is therefore to facilitate the disruption of the bondages presumably with the negatively polar sulfur atom in view of the fact that Glu, which also provides for a negatively polar environment, renders the activation enthalpy smaller than the other substitutions.

pH-dependence of the rate constants. Measurements of rate constants of PYP_M decay in the mutant proteins were carried out at varying pHs at a constant temperature (25 ℃). Figure 2 shows the rate constants plotted as a function of the pH for M100E (a) and M100A (b). The titration profile of the rate constants is rationalized by assuming presence of a titratable group whose protonated form prevent PYP_M from decaying. Since one proton is released to the bulk concomitantly with PYP_M decay, the prevention indicates that the proton release from the group is prerequisite for PYP_M decay.

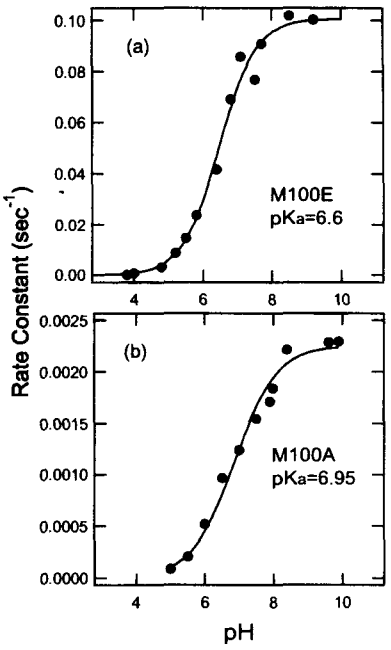

Figure 2: Rate constants of the PYP_M-to- PYP_{dark} process at various pHs for M100E (a) and M100A (b).

The pKa of the titratable group (X) for these mutants were obtained by fitting the titration profiles of the rate constants with the Henderson-Hasselbalch equation.

$$k = k_{max} \cdot \frac{10^{(pH-pK_a)}}{1 + 10^{(pH-pK_a)}} \quad (5)$$

The maximal rate constants (k_{max}) also obtained from the fit for each mutant are plotted as a function of the respective pKa as shown in Fig. 3a. Since $\Delta H^{\ddagger}$ is the sole contributor to deceleration of the rates in the mutants, it is also in a linier correlation with the pKa as shown in Fig. 3b.

The pKa, which is a negative of the customary logarithm of K_d, is correlated with the standard free energy (ΔG^0) for the dissociation of the proton from X,

$K_d = \exp(\frac{\Delta G^0}{RT})$, and, taking eq. (4) into consideration, the eq. (3) can be further simplified as

$$\Delta G^{\ddagger} \propto \Delta G^0 \tag{6}$$

The fact that the free energy required for crossing the activation free energy is proportional to the free energy for the deprotonation of X means that, in the active state($PYP^{\ddagger}_{M}$), X is deprotonated.

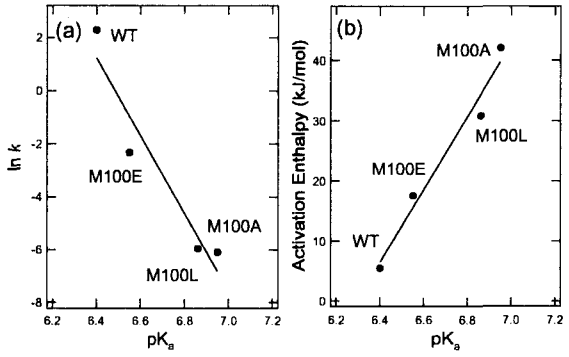

Figure 3: Correlation between the maximum rate constants and the pKa (a) and $\Delta H^{\ddagger}$ and pKa

CONCLUSIONS

In the activated state ($PYP^{\ddagger}_{M}$) in the PYP_M-to-PYP_{dark} process, it was shown that a titratable group (X) becomes deprotonated. The simplest explanation is that X is the *p*-coumaric acid chromophore, which could reasonably explain the facilitated isomerization (rotation) around the double bond that follows, because the anionized phenolic oxygen brings about a more conjugated π-electronic system in the chromophore. Another possibility is that X is a group that relays the proton transfer from the chromophore to the bulk. In this case, the candidate for X is Arg52, because it is the only residue that is located closely enough to be affected by Met100.

In any case, the role of Met100 is to stabilize the unprotonated form of X by lowering the pKa as the result of reducing the enthalpy difference between the protonated and unprotonated forms with the electrically negative nature of the sulfur atom.

The fact that in $PYP^{\ddagger}_{M}$ the Cp is smaller as compared to that in PYP_M indicates more folded protein conformation in $PYP^{\ddagger}_{M}$ than in PYP_M. Therefore, the refolding of the protein moiety is also prerequisite for the PYP_M decay process. However, because the free energy barrier is mostly contributed to by the energy required for the deprotonation of X, the rate-determining step must be the latter.

Finally, it should be noted that the pKas of the chromophore in PYP_{dark} are also elevated in the Met100 mutants in the same way as in PYP_M (our unpublished results), although the absolute values were shifted to the lower values. This means that, in the *trans* chromophore, the activation barrier for the deprotonation is lower than in the *cis* form according to eq. (3), explaining well the fast recovery of PYP_{dark} in M100A once the chromophore in the PYP_M is photo-isomerized to *trans*.

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