Light Induces Destabilization of Photoactive Yellow Protein[†]

Shin'ichi Ohishi, Nobutaka Shimizu, Ken'ichi Mihara, Yasushi Imamoto, and Mikio Kataoka*

Department of Materials Science, Nara Institute of Science and Technology, Ikoma, Nara 630-0101, Japan

Received August 7, 2000; Revised Manuscript Received December 22, 2000

ABSTRACT: To understand the effect of visible light on the stability of photoactive yellow protein (PYP), urea denaturation experiments were performed with PYP in the dark and with PYP_M under continuous illumination. The urea concentrations at the midpoint of denaturation were 5.26 ± 0.29 and 3.77 ± 0.19 M for PYP and PYP_M, respectively, in 100 mM acetate buffer, and 5.26 ± 0.24 and 4.11 ± 0.12 M for PYP and PYP_M, respectively, in 100 mM citrate buffer. The free energy change upon denaturation ($\Delta G_D^{H_2O}$), obtained from the denaturation curve, was 11.0 ± 0.4 and 7.6 ± 0.2 kcal/mol for PYP and PYP_M, respectively, in acetate buffer, and 11.5 ± 0.3 and 7.8 ± 0.1 kcal/mol for PYP and PYP_M, respectively, in citrate buffer. Even though the $\Delta G_D^{H_2O}$ value for PYP_M is almost identical in the two buffer systems, the urea concentration at the midpoint of denaturation is lower in acetate buffer than in citrate buffer. Although their CD spectra indicate that the protein conformations of the denatured states of PYP and PYP_M are indistinguishable, the configurations of the chromophores in their denatured structures are not necessarily identical. Both denatured states are interconvertible through PYP and PYP_M. Therefore, the free energy difference between PYP and PYP_M is 3.4−3.7 kcal/mol for the protein moiety, plus the additional contribution from the difference in configuration of the chromophore.

The first event after light absorption by a photoreceptor protein such as rhodopsin, bacteriorhodopsin, and photoactive yellow protein (PYP)[1] is light-induced isomerization of a chromophore, which triggers the reactions that follow. A global protein conformational change is probable in certain photocycle intermediates, and this would be a key reaction for the conversion of light energy into signal or chemical energy. In fact, conformational changes of photocycle intermediates have been revealed and characterized for bacteriorhodopsin (*1*−*7*). To understand the photoreactions of photoreceptor proteins, it is useful to describe the reaction in terms of free energy. The wavelength of maximal absorption (λ_{max}) gives the electronic transition energy. If the energy difference between the Franck−Condon state of each photointermediate and the minimum energy level of the excited state is estimated, the difference in energy level between the photointermediate and the ground state can be obtained. Kakitani et al. (*8*) described the potential energy diagrams of rhodopsin through spectral analyses of the photointermediates.

In addition to studies of the absorption spectrum, one effective method to estimate the free energy of a protein is to study its denaturation (*9*, *10*). If a denaturation experiment is performed with the ground state and with a specific photointermediate of a light-absorbing protein, the free energy difference between the ground state and the intermediate can be estimated. For example, thermal denaturation of bacteriorhodopsin has been performed in the presence and absence of light (*11*, *12*). However, this procedure induces denaturation from several photointermediates, and it is impossible to estimate the free energy change upon denaturation for a specific photointermediate. Instead, the activation free energy was estimated through kinetic measurements.

We applied thermodynamic analysis of denaturation to the photointermediate of PYP. PYP was isolated from *Ectothiorhodospira halophila* (*13*) and is proposed to be a photoreceptor protein for negative phototaxis of this bacterium (*14*). Many photoreceptor proteins are membrane proteins, but in contrast PYP is a small water-soluble protein (MW = 14 kDa). According to its high-resolution structure, PYP has an α/β fold structure composed of 125 amino acids with a chromophore, 4-hydroxycinnamic acid, bound to the unique cysteine residue at position 69 via a thioester bond (*15*−*17*). Thus, PYP belongs to a novel group of photoreceptor proteins (*18*, *19*). The photoreaction of PYP has been intensively studied and the photocycle has been established (*20*−*22*). Despite the novel structure of PYP, photochemical properties are similar to those of the retinal proteins in *Halobacterium sarinarum* (*20*). Among several photointermediates, PYP_M (or pB) is considered to be a functional signaling state.

The stability of PYP in the dark has been investigated by denaturation experiments: PYP is very stable at high temperature or low pH (*20*, *23*, *24*). Therefore, PYP is a suitable protein to examine the effect of light on denaturation. Under dark conditions, denaturation of PYP can be expressed as the following reaction equilibrium:

$$\mathrm{PYP} \rightleftarrows \mathrm{D} \qquad \text{(I)}$$

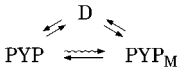
In the presence of continuous illumination, the photoreaction of PYP alters the denaturation equilibrium as follows:

[†] This work was partly supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan to M.K.

* To whom all correspondence should be addressed. Phone: +81-743-72-6100. Fax: +81-743-72-6109. E-mail: kataoka@ms.aist-nara.ac.jp.

[1] Abbreviations: PYP, photoactive yellow protein; ΔG_D, free energy change upon denaturation; D_{PYP}, denatured state of PYP; D_M, denatured state of PYP_M.

10.1021/bi001846i CCC: $20.00
Published on Web 02/10/2001



(II)

Denaturation from the other photointermediates should be also included. To apply thermodynamic analysis to PYP_M, it is necessary to attain the following denaturation condition:

$$PYP_M \rightleftarrows D \qquad \text{(III)}$$

If we can measure reactions I and III separately, the free energy change upon denaturation (ΔG_D) can be estimated for both PYP and PYP_M. Since the protein conformation of the denatured state is considered to be identical for both PYP and PYP_M, the difference in ΔG_D corresponds to the difference in free energy of PYP and PYP_M. Meyer et al. (*20*) reported urea denaturation of PYP in the dark and in the light. They estimated ΔG_D for PYP as 10.8 kcal/mol at pH 7.0. They also estimated ΔG_D for PYP_M by recoloring kinetics with flash photolysis and with steady-state measurements. However, if reaction II shown above is attained, the recoloring kinetics is irrelevant to estimate ΔG_D, because both the conversion from D to PYP and the conversion from PYP_M to PYP affect the recoloring. In fact, our present results show the irrelevance of the recoloring kinetics.

Van Brederode et al. (*23*) applied a thermodynamic analysis to the photoreaction of PYP. They evaluated the activation free energy between photointermediates through the temperature dependence of the kinetics of the photoreaction. They also estimated the free energy difference between PYP and PYP_M by the acid-induced formation of the PYP_M-like state. Although the acid-induced spectral change is quite similar to the light-induced spectral change, detailed characterization and comparison of the protein conformations have not been performed. The pH-induced spectral change was assumed to be acid denaturation by Hoff et al. (*24*). However, the spectrum of PYP under low pH is different from that of the denatured state of PYP. We have performed urea denaturation of PYP in the presence and absence of continuous illumination to estimate the free energy difference between PYP and PYP_M. We accumulated PYP_M exclusively without any ground-state fraction of PYP at pH 5.0. Under these conditions, we can apply reaction III to estimate the ΔG_D for PYP_M. The present results show that PYP_M is less stable against urea than is PYP and that $\Delta\Delta G$ is about 3.5 kcal/mol.

MATERIALS AND METHODS

Sample Preparation. PYP was overproduced in *Escherichia coli* and isolated by a urea extraction method (*25*, *26*). Anhydrous 4-hydroxycinnamic acid was added to the urea extract, and the reconstituted PYP was purified with a DEAE Sepharose CL6B (Pharmacia) column (*25*, *26*). The purified PYP was dissolved into either 100 mM acetate buffer (pH 5.0) or 100 mM citrate buffer (pH 5.0). The purity index of the samples (the ratio of the absorbance at 275 nm over 446 nm) used in this study was 0.44.

Denaturation Experiment. Urea denaturation experiments were performed at 10 °C under illumination as well as in the dark. Two procedures were used for denaturation in the presence of light: (i) urea was added to the illuminated PYP solution; (ii) a PYP solution with the desired concentration of urea was illuminated. Both methods gave the same results. The PYP solution was illuminated by a cold light source (LA-60Me, Hayashi Watch Engineering) with a yellow glass filter (Y43, Asahi Techno Glass; $\lambda \geq 415$ nm).

Spectroscopy. Denaturation was monitored by a Shimadzu spectrophotometer (UV2400). Under illumination, the detector window of the spectrophotometer was covered with a UV band-pass glass filter (UV-D36A, Asahi Techno Glass; 300 nm $\leq \lambda \leq$ 390 nm) to avoid monitoring of the excitation light. CD spectra were measured with a JASCO spectropolarimeter (J-725), using a 1-mm light path. All of the measurements were performed at 10 °C, which was maintained with a Peltier device.

Thermodynamic Analysis. The fraction of denatured state, F_D, can be described by the observed absorbance at an appropriate wavelength, A, as:

$$F_D = (A - A_N)/(A_D - A_N) \qquad (1)$$

where A_N and A_D are the absorbance values for native and denatured protein, respectively. The wavelength used to estimate ΔG_D was 446 nm for the denaturation curve of PYP and 375 nm for that of PYP_M. Free energy difference between native and unfolded state, ΔG_D, is related to the equilibrium constant for the unfolding, K_D, as:

$$\Delta G_D = -RT \ln K_D \qquad (2)$$

$$K_D = F_D/(1 - F_D)$$

where R is the gas constant. ΔG_D is a function of the urea concentration, as indicated below:

$$\Delta G_D = \Delta G_D{}^{H_2O} + m[\text{urea}] \qquad (3)$$

where $\Delta G_D{}^{H_2O}$ is ΔG_D in the absence of urea and m is a proportional constant.

RESULTS

PYP is known to be a very stable protein. At neutral pH, the concentration of urea at the midpoint of denaturation is 7.4 M (*20*). We selected pH 5.0 for the denaturation experiment because we need to obtain the urea denaturation curve of PYP_M. At pH 5.0, the lifetime of PYP_M is prolonged, as shown in Figure 1. The absorption maximum was shifted from 446 to 360 nm upon formation of PYP_M (Figure 2). This is identical to the previous observations (*20*, *22*). Figure 1 shows the time course of decay of PYP_M as measured by the decrease of absorbance at 360 nm and the urea concentration dependence of the rate constant. The rate constant is affected by urea denaturation if the urea concentration is over 2.4 M (see Figure 2a). Therefore, the apparent extrapolation with linear fit of Figure 1b is meaningless. The recovery from PYP_M to PYP in 5 M urea requires more than 1 day. Because the decay rate becomes very slow, we can accumulate sufficient amount of PYP_M (almost 100%) confirmed by the whole spectrum shown in Figure 1c, which is corresponding to the initial state of Figure 1a.

Figure 2 shows the urea-dependent spectral change for PYP (a) and PYP_M (b) in 100 mM acetate buffer (pH 5.0). For PYP, the spectrum is unchanged between 0 and 4 M urea. The clear isosbestic point at 376 nm indicates that the urea denaturation of PYP is a two-state transition. The denatured state of PYP (D_{PYP}) has an absorption maximum

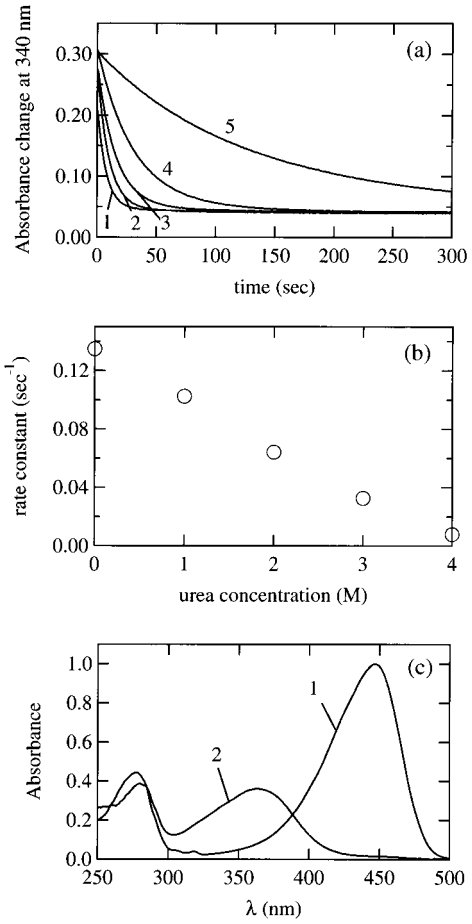


FIGURE 1: Properties of PYP_M examined in the present study: (a) decay kinetics of PYP_M in urea solutions of various concentrations, (b) urea concentration dependence of its time constant, and (c) spectrum of PYP (curve 1) and PYP_M (curve 2) in 100 mM acetate buffer (pH 5.0). The urea concentrations in panel a were 0 (curve 1), 1.0 M (curve 2), 2.0 M (curve 3), 3.0 M (curve 4), and 4.0 M (curve 5), respectively.

at 340 nm, 20 nm shorter than that of PYP_M. In the case of PYP_M, the spectrum is unchanged between 0 and 2.6 M urea. The spectrum of PYP_M was measured under continuous illumination. Since the detector window was covered with a UV band-pass filter (λ_{max} = 345 nm, fwhm = 50 nm) to protect the photomultiplier of the spectrophotometer, the spectrum of PYP_M is limited to the wavelength region between 300 and 385 nm. The photo-steady-state under continuous illumination at pH 5.0 contained only PYP_M, which is confirmed by the full spectrum. Thus, the addition of urea brought PYP_M into its denatured state (D_M). The isosbestic point at 336 nm indicates that the urea denaturation of PYP_M is a two-state transition between PYP_M and its denatured state. The existence of the isosbestic point also confirms that PYP is fully converted to PYP_M in the light, because the spectrum of each denatured state is different (Figure 4). The absorption maximum of the denatured state of PYP_M is around 340 nm, which is similar to that of D_{PYP}. Since the peak of the difference spectrum of Figure 2b is located at 375 nm, the denaturation curve for PYP_M was obtained from the absorption change at 375 nm, whereas the denaturation curve for PYP was obtained from the absorption change at 446 nm.

The existence of the isosbestic point indicates that the denaturation of PYP and PYP_M by urea is a two-state

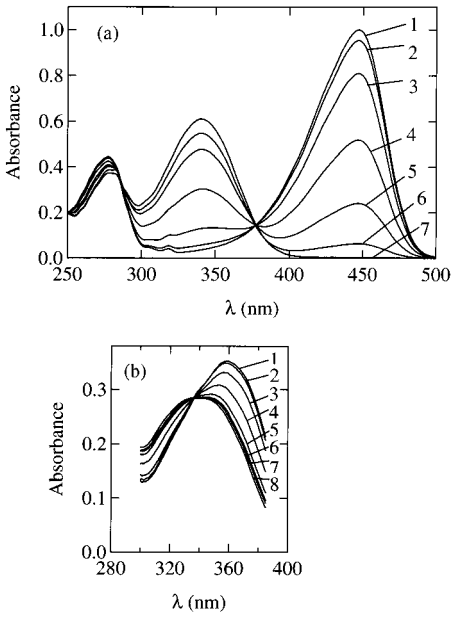


FIGURE 2: Urea denaturation of PYP (a) and PYP_M (b) monitored by absorption spectrum. PYP was dissolved in 100 mM acetate buffer (pH 5.0). The urea concentration was 4.0 M (curve 1), 4.4 M (curve 2), 4.8 M (curve 3), 5.2 M (curve 4), 5.6 M (curve 5), 6.0 M (curve 6), and 7.0 M (curve 7) for PYP, and 2.6 M (curve 1), 3.0 M (curve 2), 3.4 M (curve 3), 3.8 M (curve 4), 4.2 M (curve 5), 4.6 M (curve 6), 5.0 M (curve 7), and 6.0 M (curve 8) for PYP_M. The spectrum of PYP with no urea was identical to the spectrum in 4 M urea (curve 1, panel a), and the spectrum of PYP_M with no urea was identical to the spectrum in 2.6 M urea (curve 1, panel b).

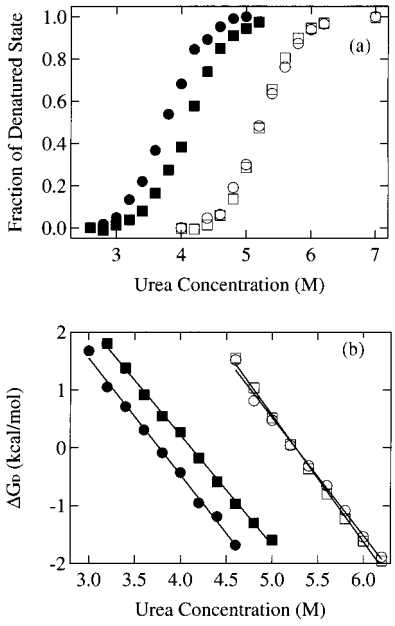


FIGURE 3: Urea concentration dependence of the denatured state fraction (a) and the urea concentration dependence of ΔG_D (b). Open symbols (○, □) represent PYP, and filled symbols (●, ■) denote PYP_M. Circles (○, ●) denote the data for PYP in acetate buffer, and squares (□, ■) denote the data for PYP in citrate buffer.

transition. Thus, we can obtain the denaturation curve for PYP and PYP_M by applying eq 1. Figure 3 shows the denaturation curve for PYP and PYP_M. As shown in the figure, the midconcentration of denaturation is higher for PYP than for PYP_M, indicating that PYP_M is less stable against urea than PYP. Figure 3 also shows that the stability

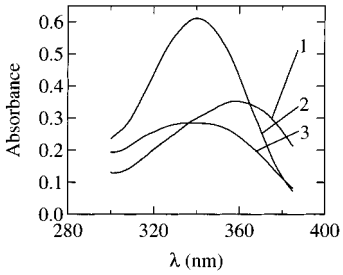


FIGURE 4: Comparison of the absorption spectra of PYP_M (curve 1), D_{PYP} (curve 2), and D_M (curve 3). Protein was dissolved in 100 mM acetate buffer.

Table 1: Thermodynamic Parameters Obtained by the Urea Denaturation Experiments in Equilibrium

experimental conditions	$[urea]_{1/2}$[a] (M)	$\Delta G_D^{H_2O}$ (kcal/mol)	m (kcal/mol/M)
PYP in acetate buffer	5.26 ± 0.29	11.0 ± 0.4	2.09 ± 0.06
PYP in citrate buffer	5.26 ± 0.24	11.5 ± 0.3	2.18 ± 0.05
PYP_M in acetate buffer	3.77 ± 0.19	7.6 ± 0.2	2.02 ± 0.05
PYP_M in citrate buffer	4.11 ± 0.12	7.8 ± 0.1	1.90 ± 0.03

[a] The urea concentration at the midpoint of denaturation.

of PYP_M is dependent on the buffer. We used two different buffer systems: 100 mM Na-acetate buffer (pH 5.0) and 100 mM Na-citrate buffer (pH 5.0). The denaturation curve for PYP is almost identical for these two buffer systems (open symbols in Figure 3); however, the denaturation curve for PYP_M in acetate buffer is clearly different from that in citrate buffer. The midconcentration of denaturation is lower in acetate buffer than in citrate buffer, suggesting that PYP_M is more stable in the latter buffer.

The free energy change upon unfolding was obtained by the equations given in Materials and Methods, as shown in Figure 3b. ΔG_D is linear with urea concentration, as would be expected from eq 3. The thermodynamic parameters obtained are summarized in Table 1. $\Delta G_D^{H_2O}$ for PYP is 11.0 kcal/mol in acetate buffer and 11.5 kcal/mol in citrate buffer, which are typical values for globular proteins (*27*). These values are also comparable to the reported $\Delta G_D^{H_2O}$, 10.8 kcal/mol, for the urea denaturation of PYP in 5 mM phosphate buffer (pH 7.0) (*20*). The $\Delta G_D^{H_2O}$ value for PYP_M is 7.6 kcal/mol in acetate buffer and 7.8 kcal/mol in citrate buffer. We can conclude that, within experimental error, the thermodynamic parameters for PYP are independent of the buffer systems. On the other hand, although $\Delta G_D^{H_2O}$ for PYP_M seems to be independent of buffer system, the midconcentration of denaturation differs in the two buffer systems as a result of the difference in the m value (eq 3). The m value for PYP_M in citrate buffer is smaller than that in acetate buffer, indicating that the urea denaturation of PYP_M is less cooperative in citrate buffer than in acetate buffer.

$\Delta G_D^{H_2O}$ for PYP_M is smaller than that for PYP by 3.4 kcal/mol in acetate buffer and by 3.7 kcal/mol in citrate buffer. If the structure of D_M is assumed to be identical to that of D_{PYP}, these values correspond to the free energy difference between PYP and PYP_M. The value is larger than the reported value, 1 kcal/mol, obtained for the equilibrium between PYP and acid dark PYP_M (*22*). As discussed later, this quantitative interpretation is not straightforward because the structure of D_M is not necessarily identical to that of D_{PYP}.

To understand the properties of D_{PYP} and D_M, we compared the spectra of these states. Figure 4 shows absorption spectra

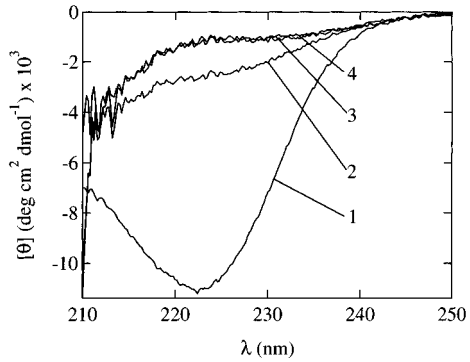


FIGURE 5: Comparison of the far-UV CD spectra of PYP (curve 1), D_{PYP} (curve 3), and D_M (curve 4). D_{PYP} and D_M were measured in the presence of 6 M urea. Since PYP is in equilibrium between native PYP and D_{PYP} in the presence of 6 M urea, the CD spectrum for D_{PYP} was estimated from the CD spectrum with 6 M urea (curve 2) and the CD spectrum of native PYP (curve 1) as: [curve 3] = ([curve 2] − 0.14[curve 1])/0.86.

for PYP_M, D_{PYP}, and D_M. The absorption maximum for both denatured states is located at 340 nm, but the spectral shape and absorption coefficient are different from each other. CD spectra for D_{PYP} and D_M in 6 M urea are shown in Figure 5. It should be noted that the CD spectrum for PYP in 6 M urea results from 14% native PYP and 86% D_{PYP} (see Figure 3). The CD spectrum of D_M (curve 4 in Figure 5) is completely identical to that of D_{PYP} (curve 3 in Figure 5), strongly demonstrating that there are no significant differences in protein conformation for both denatured states. This is consistent with the general concept that globular proteins are completely unfolded in a high concentration of urea. Therefore, the observed spectral properties suggest that the difference in structure between D_{PYP} and D_M occurs in the chromophore itself.

A solution of PYP in 5.6 M urea and 100 mM acetate buffer (pH 5.0) was irradiated by light from a 60-W cold source with a glass filter, Y-43, to examine the interconvertibility between D_{PYP} and D_M. The excitation light contains only wavelengths longer than 410 nm, which are not absorbed by D_{PYP} or D_M. The absorbance change at 340 nm was monitored. An increase in the absorbance at 340 nm is expected for the reactions PYP → D_{PYP}, PYP → PYP_M, D_M → PYP_M, and D_M → D_{PYP}, whereas a decrease is expected for the reactions D_{PYP} → PYP, PYP_M → PYP, PYP_M → D_M, and D_{PYP} → D_M (Figures 1 and 4). Upon illumination, the absorbance increases at first and then subsequently decreases. In 5.6 M urea, the fraction of PYP is about 20% (Figure 3). This PYP is converted to PYP_M upon illumination, and then the resultant PYP_M is converted to D_M. At the same time, D_{PYP} is converted to PYP, according to the equilibrium in 5.6 M urea. When the illumination is shut off, the absorbance gradually decreases and reaches a constant value. The second illumination at 300 s causes a slight increase in absorbance. Therefore, some portion of PYP_M is converted to PYP after shutting off the light, and this portion of PYP is redistributed to PYP and D_{PYP} in the dark according to the equilibrium in 5.6 M urea. Finally, at 2000 s, no absorbance changes were observed despite irradiation. This suggests that all the PYP and D_{PYP} fractions have been converted to D_M and that no direct conversion from D_M to D_{PYP} occurs. At urea concentrations lower than 5.6 M, the D_M fraction is converted to PYP and D_{PYP} through PYP_M during an overnight incubation

in the dark. This is because the reaction rate of the recovery from PYP_M to PYP becomes very slow in the presence of urea (Figure 1). From these observations, the denaturation scheme of PYP and PYP_M can be summarized as:

$$D_{PYP} \rightleftharpoons PYP \rightleftharpoons PYP_M \rightleftharpoons D_M \qquad (IV)$$

Visible light changes the equilibrium between PYP and D_{PYP} at certain urea concentrations into the equilibrium between PYP_M and D_M at the same urea concentration. This indicates that the urea denaturation curve of PYP_M can be obtained by urea denaturation experiments in the presence of visible light.

DISCUSSION

The three major findings of the present study are summarized as follows: (1) The free energy change upon denaturation, $\Delta G_D^{H_2O}$, for PYP is 11.0 kcal/mol in acetate buffer and 11.5 kcal/mol in citrate buffer, whereas that for PYP_M is 7.6 and 7.8 kcal/mol, respectively, indicating that PYP_M is less stable against urea than PYP. (2) The stability of PYP_M is affected by the nature of the buffer. (3) The protein conformation of the denatured structure of PYP_M (D_M) is identical to that of PYP (D_{PYP}), whereas the configuration of the chromophore is different. Direct conversion between D_M and D_{PYP} does not occur, but they can be interconverted through PYP and PYP_M.

In the present study, we have shown that the urea-induced unfolding of PYP and PYP_M is approximated closely by a two-state transition. Thus, the free energy change upon unfolding can be obtained by equilibrium denaturation measurements. This is the first thermodynamic analysis of unfolding of a photointermediate of a light-absorbing protein in equilibrium. Meyer et al. (*20*) reported urea denaturation of PYP in the dark and in the light. In their study, recoloring kinetics were determined by flash photolysis and steady-state measurements to obtain $\Delta G_D^{H_2O}$. Since PYP_M and its denatured state coexist under their measurement conditions, as is shown in reaction IV, the recoloring kinetics would not give a relevant $\Delta G_D^{H_2O}$ value. The light-induced denaturation of bacteriorhodopsin (*11*, *12*) has been reported. In this case, a kinetic analysis was carried out to estimate activation free energy upon denaturation. These investigators assumed that several photointermediates were responsible for the light-induced denaturation. It is impossible to determine the free energy difference between the dark-adapted bacteriorhodopsin and a specific photointermediate. Van Brederode et al. (*23*) applied thermodynamic analysis to the photocycle of PYP and could estimate the activation free energy between certain photointermediates, a procedure that was quite effective to understand the property of the photocycle. However, their described relative free energy between each photointermediate and the original state of PYP should be reexamined, because the photointermediate of interest and its thermally denatured state could coexist under their measurement conditions.

The stability of PYP_M is affected by the buffer system, whereas the stability of PYP itself seems to be independent of the buffer system, as shown in Figure 3. PYP_M in citrate buffer is more stable against urea than PYP_M in acetate buffer. The difference is mainly due to the difference in the *m* value, because $\Delta G_D^{H_2O}$ for PYP_M is identical in the two buffer systems. The *m* value for PYP_M is larger in acetate buffer than in citrate buffer. The *m* value is interpreted to represent the difference in solvent-accessible surface area between the native and unfolded states (*30*). The protein conformation of D_M is considered to be similar to that of D_{PYP} (Figure 5). If we assume that the solvent-accessible surface area is almost identical for the denatured states, the difference in the *m* value indicates the difference in the surface area of the native state. Thus, the surface area of PYP_M appears to be slightly larger than that of PYP, and the surface area of PYP_M in citrate buffer is larger than that in acetate buffer. We observed that the radius of gyration (R_g) of PYP_M, obtained by solution X-ray scattering, is larger than that of PYP and that the R_g in acetate buffer is smaller than that in citrate buffer (unpublished results), supporting the interpretation of the *m* value. PYP_M corresponds to a partially unfolded state (*23*), which leads to an increase of the surface area. The mechanism of the buffer effect is unsolved at the moment. The buffer solution we used contained only Na^+ as a metal ion. Na^+ itself does not show any effects on the spectrum of PYP_M (*20*). Thus the metal-ion-chelating activity of citrate is not the origin of the buffer effect.

The CD spectrum of the denatured state (Figure 5) suggests strongly that the protein conformation of D_M is identical to that of D_{PYP}. However, their absorption spectra are different. Therefore, the difference in the structures of D_{PYP} and D_M is considered to be the isomeric state of the chromophore. In the ground state, the phenolic hydroxyl group of the chromophore is deprotonated and the chromophore is present in the *trans* configuration (*16*, *28*, *29*). In contrast, the blue-shifted intermediate PYP_M contains the chromophore in a protonated state and in the *cis* configuration (*28*, *29*). Upon protein unfolding by urea, the chromophore would be exposed to the aqueous milieu, leading to deprotonation of the chromophore 4-hydroxyl group. D_{PYP} and D_M are formed from PYP and PYP_M, respectively. Visible light is required for the conversion of D_{PYP} into D_M. Therefore, D_{PYP} and D_M should maintain the original isomeric state of the chromophore. The activation energy of the *cis*−*trans* isomerization of 4-hydroxycinnamic acid is typically 40 kcal/mol at room temperature (*32*), which is considerably larger than the thermal energy at room temperature. Therefore, under the present experimental conditions, 10 °C, thermal isomerization would hardly occur, as suggested by Figure 6.

The free energy change upon denaturation, $\Delta G_D^{H_2O}$, can be estimated for PYP and PYP_M by thermodynamic analysis. The value of $\Delta G_D^{H_2O}$ obtained is 11.0−11.5 kcal/mol for PYP and 7.6−7.8 kcal/mol for PYP_M. If the denatured structure was identical for PYP and PYP_M, $\Delta\Delta G_D^{H_2O}$ would correspond to the free energy difference between PYP and PYP_M. This free energy increase is a result of light absorption. However, as mentioned above, the structure of D_M differs from that of D_{PYP} in the chromophore configuration. Therefore, the free energy difference between PYP and PYP_M is the sum of $\Delta\Delta G_D^{H_2O}$ and the free energy difference between the *cis* and *trans* forms of 4-hydroxycinnamic acid. The free energy difference between the *trans* and *cis* isomers of retinal in solution is reported to be almost 0 (*31*). In the case of 4-hydroxycinnamic acid, however, it is expected that the *cis* form is higher in free energy than the *trans* form. The free energy difference between the *trans* and *cis* forms

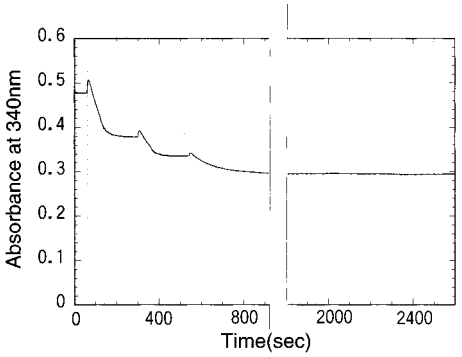


FIGURE 6: Light-induced conversion of D_{PYP} into D_M. The absorption change at 340 nm was monitored to evaluate the formation of D_M. The sample was kept in the dark during the shaded region of the time axis, and continuous illumination was applied during the unshaded region.

can be estimated to be 3−8 kcal/mol by simple quantum chemical calculations (unpublished results). The free energy difference between PYP and PYP_M is, therefore, expected to be 6.5−11.5 kcal/mol.

Van Brederode et al. (*23*) suggested that the formation of PYP_M is similar to an unfolding reaction and that the decay of PYP_M is close to a refolding reaction. We also have several lines of evidence from mutation studies that PYP_M is in a partially unfolded state (unpublished results). The $\Delta\Delta G_D^{H_2O}$ value, 3.4−3.7 kcal/mol, would thus be related to the difference in stability between the folded and partially unfolded states of PYP. In the case of apomyoglobin, the $\Delta\Delta G_D^{H_2O}$ value between the native and molten globule states is between 0.7 and 4.5 kcal/mol (*33*), which is a similar value to that found in the present study, 3.4−3.7 kcal/mol. These $\Delta\Delta G_D^{H_2O}$ values support the idea that PYP_M is in a partially unfolded state. The crystal structure of PYP_M has been obtained by time-resolved X-ray diffraction (*34*). In this structure, however, the difference between the dark state and PYP_M was very small and confined to the chromophore region. This observation seems inconsistent with the NMR study (*35*), H−D exchange study (*36*), water structural changes (*37*), and our observed increase in R_g. Constraints forced upon the protein by the crystal lattice could suppress a relatively large conformational change of PYP upon light absorption. The solution structure analysis of PYP_M is now underway in our laboratory.

ACKNOWLEDGMENT

The authors express their sincere thanks to Dr. Hironari Kamikubo (Institute of Materials Structure Science) for fruitful discussions and to Dr. Remco Kort (Nara Institute of Science and Technology) for critical reading of the manuscript.

REFERENCES

1. Dencher, N. A., Dresselhaus, D., Zaccai, G., and Büldt, G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7876−7879.
2. Nakasako, M., Kataoka, M., Amemiya, Y., and Tokunaga, F. (1991) *FEBS Lett. 292*, 73−75.
3. Subramaniam, S., Gerstein, M., Oesterhelt, D., and Henderson, R. (1993) *EMBO J. 12*, 1−8.
4. Kamikubo, H., Kataoka, M., Váró, G., Oka, T., Tokunaga, F., Needleman, R., and Lanyi, J. K. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1386−1390.
5. Luecke, H., Schobert, B., Richiter, H. T., Cartailler, J. P., and Lanyi, J. K. (1999) *Science 286*, 255−261.
6. Edman, K., Nollert, P., Royant, A., Belrhali, H., Pebay-Peyroula, E., Hajdu, J., Neutze, R., and Landau, E. M. (1999) *Nature 401*, 822−826.
7. Kataoka, M., and Kamikubo, H. (2000) *Biochim. Biophys. Acta 1460*, 166−176.
8. Kakitani, T., Hatano, Y., Schichida, Y., Imamoto, Y., Tokunaga, F., and Kakitani, H. (1992) *Photochem. Photobiol. 56*, 977−987.
9. Brandts, J. F. (1964) *J. Am. Chem. Soc. 86*, 4291−4301.
10. Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167−241.
11. Etoh, A., Itoh, H., and Mitaku, S. (1997) *J. Phys. Soc. Jpn. 66*, 975−978.
12. Mukai, Y., Kamo, N., and Mitaku, S. (1999) *Protein Eng. 12*, 755−759.
13. Meyer, T. E. (1985) *Biochim. Biophys. Acta 806*, 175−183.
14. Sprenger, W. W., Hoff, W. D., Armitage, J. P., and Hellingwerf, K. J. (1993) *J. Bacteriol. 175*, 3096−3104.
15. Baca, M., Borgstahl, G. E. O., Boissinot, M., Burke, P. M., Williams, W. R., Slater, K. A., and Getzoff, E. D. (1994) *Biochemistry 33*, 14369−14377.
16. Borgstahl, G. E. O., Williams, D. R., and Getzoff, E. D. (1995) *Biochemistry 34*, 6278−6287.
17. Hoff, W. D., Düx, P., Hard, K., Devreese, B., Nugteren-Roodzant, I. M., Crielaard, W., Boelens, R., Kaptein, R., Van Beeumen, J., and Hellingwerf, K. J. (1994) *Biochemistry 33*, 13959−13962.
18. Koh, M., Van Driessche, G., Samyn, B., Hoff, W. D., Meyer, T. E., Cusanovich, M. A., and Van Beeumen, J. J. (1996) *Biochemistry 35*, 2526−2534.
19. Kort, R., Hoff, W. D., Van West, M., Kroon, A. R., Hoffer, S. M., Vlieg, K. H., Crielaard, W., Van Beeumen, J. J., and Hellingwerf, K. J. (1996) *EMBO J. 15*, 3209−3218.
20. Meyer, T. E., Yakali, E., Cusanovich, M. A., and Tollin, G. (1987) *Biochemistry 26*, 418−423.
21. Hoff, W. D., Kwa, S. L. S., Van Grondelle, R., and Hellingwerf, K. J. (1992) *Photochem. Photobiol. 56*, 529−539.
22. Imamoto, Y., Kataoka, M., and Tokunaga, F. (1996) *Biochemistry 35*, 14047−14053.
23. Van Brederode, M. E., Hoff, W. D., Van Stokkum, I. H. M., Groot, M.-L., and Hellingwerf, K. (1996) *Biophys. J. 71*, 365−380.
24. Hoff, W. D., van Stokkum, I. H. M., Gural, J., and Hellingwerf, K. (1997) *Biochim. Biophys. Acta 1322*, 151−162.
25. Mihara, K., Hisatomi, O., Imamoto, Y., Kataoka, M., and Tokunaga, F. (1997) *J. Biochem. 121*, 876−880.
26. Imamoto, Y., Ito, T., Kataoka, M., and Tokunaga, F. (1995) *FEBS Lett. 374*, 157−160.
27. Pace, C. N. (1975) *CRC Crit. Rev. Biochem. 3*, 1−43.
28. Kort, R., Vonk, H., Xu, X., Hoff, W. D., Crielaard, W., and Hellingwerf, K. J. (1996) *FEBS Lett. 382*, 73−78.
29. Imamoto, Y., Mihara, K., Hisatomi, O., Kataoka, M., Tokunaga, F., Bojkova, N., and Yoshihara, K. (1997) *J. Biol. Chem. 272*, 12905−12908.
30. Shellman, J. A. (1978) *Biopolymers 17*, 1305−1322.
31. Ann, H., and Krzysztof, K. (1998) *Biochemistry 37*, 2843−2853.
32. Ross, D. L., and Blanck, J. (1971) in *Photochromism* (Brown, G. H., Ed.) pp 471−556, Wiley-Interscience, New York.
33. Loh, S. N., Kay, M. S., and Baldwin, R. L. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 5446−5450.
34. Gernick, U. K., Borgstahl, G. E. O., Ng, K., Ren, Z., Pradervand, C., Burke, P. M., Šrajer, V., Teng, T. Y., Schildkamp, W., McRee, D. E., and Getzoff, E. D. (1997) *Science 275*, 1471−1475.
35. Rubinstenn, G., Vuister, G. W., Mulder, F. A. A., Düx, P. E., Boelens, R., Hellingwerf, K. J., and Kaptein, R. (1998) *Nat. Struct. Biol. 5*, 568−570.
36. Hoff, W. D., Xie, A., Van Stokkum, I. H., Tang, X. J., Gural, J., Kroon, A. R., and Hellingwerf, K. J. (1999) *Biochemistry 38*, 1009−1017.
37. Kandori, H., Iwata, T., Hendriks, J., Maeda, A., and Hellingwerf, K. J. (2000) *Biochemistry 39*, 7902−7909.

BI001846I