Temperature-Dependent Volume Change of the Initial Step of the Photoreaction of Photoactive Yellow Protein (PYP) Studied by Transient Grating

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Abstract: The energetics and protein dynamics of photoactive yellow protein (PYP) were studied by transient grating (TG) and photoacoustic (PA) spectroscopies. The enthalpy difference (ΔH) between the ground state (pG) and the first intermediate (pR) at 20 °C was determined by the TG method as 160 kJ/mol, which is much larger than ΔH of the photoisomerized chromophore (*p*-coumaric acid) in water (50 kJ/mol). By simultaneous measurements of the TG and PA signals, the volume change (ΔV) for the pG → pR process is determined to be −7 cm^3/mol (contraction) at 20 °C. Interestingly, the volume contraction increases with decreasing temperature. At 0 °C the volume change becomes ca. −15 cm^3/mol. This temperature-dependent volume change may indicate the structural fluctuation of PYP protein in the solution phase.

1. Introduction

In any chemical reaction, the energy levels (or enthalpy difference, ΔH) and the volume change (ΔV) of intermediates are fundamental and important quantities. For reversible reactions, ΔH and ΔV data have been extensively accumulated so far by the temperature or pressure dependence of the equilibrium constants.[1−4] On the other hand, for intermediate species of irreversible reactions, knowledge of ΔH and ΔV is very limited. A technique for determining ΔH and ΔV of such irreversible reactions developed so far is the photoacoustic (PA) method.[5−11] Since the pressure wave after the pulsed photoexcitation is created by thermal expansion and molecular volume change, the signal intensity reflects the energetics and the volume change of the reaction. However, the observed PA signal consists of the thermal as well as the volume contributions simultaneously, and both contributions have to be separated. For aqueous solution, the temperature-variation method always has been used since the first study on these properties by Callis et al.[5] The PA intensities due to thermal energy are determined by changing the thermal expansion coefficients of the solution, which is accomplished by changing the temperature. This method is based on the assumption that ΔH and ΔV are temperature independent. However, the adequacy of this assumption has never been tested rigorously. The linear relationship of the PA intensity against α_{th} has sometimes been used as evidence for the temperature independence of ΔH and ΔV. However, unless the temperature change is very large, it may be difficult to distinguish whether the plot is actually linear or slightly nonlinear, in other words, whether this assumption is valid or not. In this paper, we report nonnegligibly temperature-dependent volume change in the initial step of the photocycle reaction of photoactive yellow protein (PYP) using the transient grating (TG) and PA hybrid methods. As far as we know, this is the first report of the temperature-dependent ΔV for any intermediate species of irreversible reactions. This temperature dependence suggests the importance of the structural fluctuation of this biological protein.

PYP is a relatively newly isolated protein from the purple sulfur bacterium *Ectothiorhodospira halophila*, in 1985.[12] The interest in the photophysical and photochemical processes of PYP has rapidly increased in the last several years.[12−24] The

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chromophore of PYP is *p*-coumaric acid (4-hydroxycinnamic acid) covalently bound to the side chain of Cys 69 via a thioester linkage.[15,20,21] Upon excitation of the chromophore, the ground-state PYP (pG, $\lambda_{max} = 446$ nm) is converted into the red-shifted intermediate (pR, $\lambda_{max} = 465$ nm) in less than 2 ns.[23] Subsequently pR decays in the submillisecond time scale into a blue-shifted intermediate (pB, $\lambda_{max} = 355$ nm), which returns to pG in a subsecond time scale.[22,23] This cyclic reaction is triggered by the trans → cis isomerization of the chromophore. As early as 1994, Van Brederode et al. quantitatively measured the photoacoustic (PA) signal intensity to determine ΔH and ΔV associated with the early transformation (pG → pR) of PYP using the PA method.[24] However, the quantity strictly depends on the assumption stated above. We investigated this reaction by the time-resolved TG and PA methods without any assumption and found a remarkably large temperature dependence of ΔV.

2. Experimental Section

The details of the TG and PA setup have been described previously.[25−28] In this study, the third harmonic of a Nd:YAG laser (Spectra-Physics-CGR-170-10)-pumped dye laser (Lumonics, Hyper-Dye 300; $\lambda = 465$ nm) was used for the photoexcitation. The excitation wavelength was 465 nm and the pulse length was 10 ns. The laser light was split by a beam splitter and crossed inside a quartz sample cell (2 mm path length). The created interference pattern (transient grating) was probed by a He−Ne laser (633 nm) as a Bragg diffracted signal (TG signal). The TG signal was detected by a photomultiplier (Hamamatsu R928), averaged by a digital oscilloscope, and transferred to a computer for averaging and analysis.

For the PA measurements, the sample was excited by the dye laser light and the pressure wave was detected by a piezoelectric transducer as described previously.[27] The signal was directly recorded by the digital oscilloscope and averaged about 100−300 times.

The transient absorption signal was detected after the photoexcitation with the dye laser pulse. A probe light (476 nm line of an Ar^+ laser) was made nearly collinear to the pump beam in the sample. The intensity change of the probe light was detected by the photomultiplier and averaged by the computer system.

The temperature of the sample was controlled by flowing methanol from a thermostatic bath around the sample holder. The temperature of the sample was measured with a thermocouple and a voltometer. The laser repetition rate was about 0.5−3 Hz depending on the experiment. The repetition rate was set as low as possible for low-temperature measurements.

PYP was prepared as reported previously.[20,21] PYP was dissolved in 10 mM Tris-HCl (pH 8.0) with 1 mM PMSF (phenylmethylsulfonyl fluoride). BCP (bromocresol purple) in water was used as a calorimetric reference. The thermal grating intensity of BCP in water and that in the buffer solution were the same within our experimental accuracy. Concentration of the sample and reference was adjusted so that the absorbance in the cell was the same at the excitation wavelength. Absorbance was about 0.7−1.0 in each experiment.

3. Analysis

The procedure for determination of ΔV from the TG and PA signals was reported previously.[25−29] If the absorptive contribution is negligible, the TG signal intensity is proportional to the square of the refractive index change.

The refractive index change consists of the following three components: (1) contribution of released heat (δn_{th}, thermal grating), (2) the molecular refractive index difference between the reactant and products due to the change of absorption spectrum (δn_{pop}, population grating), and (3) the density change of the solvent caused by the reaction volume change (δn_{vol}, volume grating).

We can separate thermal contribution (1) from the other two components (2, 3) by the time-resolved method.[25−27] The key point of the separation is based on the fact that the thermal grating signal decays with a rate constant given by the thermal diffusivity but the time development of the other signal is determined by the kinetics of the reaction and the molecular diffusion. Since the thermal diffusivity is usually 1 or 2 orders of magnitude larger than the molecular diffusion constant in solution, the thermal component can be easily separated from the species grating signal. Therefore, we often represent the sum of components 2 and 3 as the species grating (δn_{spe}). The square root of the TG signal ($I_{TG}^{1/2}$) is given by

$$I_{TG}^{1/2}(t) = \alpha|\delta n_{th}(t) + \delta n_{spe}(t)| \quad (1)$$

$$\delta n_{th}(t) = \delta n_{th} \exp(-q^2 D_{th} t)$$

$$\delta n_{spe}(t) = \sum_i \delta n_{spe}^i \exp(-q^2 D_i t)$$

where α is a constant representing the sensitivity of the system, D_{th} is the thermal diffusivity, D_i is the diffusion coefficient of species i, and q is the grating wavenumber. δn_{th} and δn_{spe}^i are the magnitude of the refractive index changes caused by the released heat from the excited state and creation (or extinction) of species i, respectively.

The magnitude of the thermal grating is given by

$$\delta n_{th} = \frac{dn}{dT} \frac{h\nu\phi W}{\rho C_p} \Delta N \quad (2)$$

where

$$\phi \equiv 1 - \frac{\Phi \Delta H}{h\nu}$$

W is the molecular weight, C_p is the heat capacity, ρ is the density, $h\nu$ is the photon energy of excitation light, ΔN is the number of the reacting molecules per unit volume, and Φ is the quantum yield of the reaction. We determine $\Phi\Delta H$ by comparison with the signal intensity of a reference sample, which releases the photon energy as heat with unity quantum yield. This method is applicable to the measurement of ΔH at any temperature. The ratio of the refractive index change for the sample (δn(sample)) to that for reference (δn(ref)) is given by

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$$\frac{\delta n_{\text{th}}(\text{sample})}{\delta n_{\text{th}}(\text{ref})} = 1 - \frac{\Phi \Delta H}{h\nu} \tag{3}$$

The magnitude of the volume grating is given by

$$\delta n_{\text{vol}} = \frac{({n_0}^2 + 2)^2 \alpha_{\text{solvent}}}{18 n_0 \epsilon_0 V_{\text{solvent}}} \Delta N V \tag{4}$$

where V is the partial molar volume of the solute, V_{solvent} is the partial molar volume of the solvent, α_{solvent} is the molecular polarizability of the solvent, n_0 is the unperturbed refractive index of solution, and ϵ_0 is the vacuum permittivity.

The intensity of the PA signal is given by

$$I_{\text{PA}} = \alpha'' \Delta N \left| \frac{h\nu\phi W}{\rho C_{\text{p}}} \alpha_{\text{th}} + \Phi \Delta V \right| \tag{5}$$

where α'' is a proportional constant which includes the sensitivity of the apparatus and α_{th} is the thermal expansion coefficient. If we know the value of ϕ from the TG signal analysis, we can determine $\Phi\Delta V$ from the PA intensity of the sample (I_{PA}(sample)) and of the reference (I_{PA}(ref)) by using the following relation at any temperature.

$$\frac{I_{\text{PA}}(\text{sample})}{I_{\text{PA}}(\text{ref})} = \phi + \Phi \Delta V \left(\frac{\rho C_{\text{p}}}{h\nu W \alpha_{\text{th}}} \right) \tag{6}$$

4. Results and Discussion

Figure 1a depicts the TG signal of the PYP solution after excitation at 465 nm at various temperatures. The signal rises within the excitation pulse and decays with a rate constant of $D_{\text{th}}q^2$. Therefore, the decaying component is easily identified as thermal grating. The intensity of this component represents the thermal energy released by the first photocycle step pG* → pR. The very fast rise (<20 ns) of the thermal grating signal is consistent with the previous transient absorption measurements[23] and, furthermore, it indicates that there is no slower structural relaxation, which may not be detected by the optical detection method. Since there is no optical absorption from the original species (pG) or any intermediates of PYP at the probe wavelength (633 nm), the observed TG signal must be due to the refractive index change after photoexcitation. The background signal beneath the thermal grating is due to the contributions from the population (δn_{pop}) and volume gratings (δn_{vol}). Hence, this signal intensity represents the absorption spectrum change as well as the volume change during this process.

First we measure ΔH and ΔV at 20 °C. The thermal contribution of the signal can be isolated by fitting the signal with eq 1. The preexponential factor with a rate constant of $D_{\text{th}}q^2$ gives the magnitude of the thermal grating intensity. We observed that the TG signal intensity saturated at a strong laser power (>2 μJ/pulse). This is consistent with the observations by Van Brederode et al. for the PA signal intensity.[24] However, as long as the laser power was kept within <1−2 μJ/pulse, we found that the TG intensity was proportional to the excitation power and we generally measured the signal within this weak power range. By comparing the intensity of the thermal grating with that of the reference sample, we determine ϕ in eq 1 and, from this value, we obtain $\Phi\Delta H = (57 \pm 7)$ kJ/mol. The quantum yield of the reaction was measured as $\Phi = 0.35$,[24] and hence we determine ΔH to be (160 ± 20) kJ/mol. The enthalpy difference between trans and cis isomer of *p*-coumaric acid was determined to be about 50 kJ/mol by our group.[29] ΔH of PYP is much larger than this value. This large difference indicates that pR has a strain structure (especially around the chromophore) and stores large energy in the protein part.

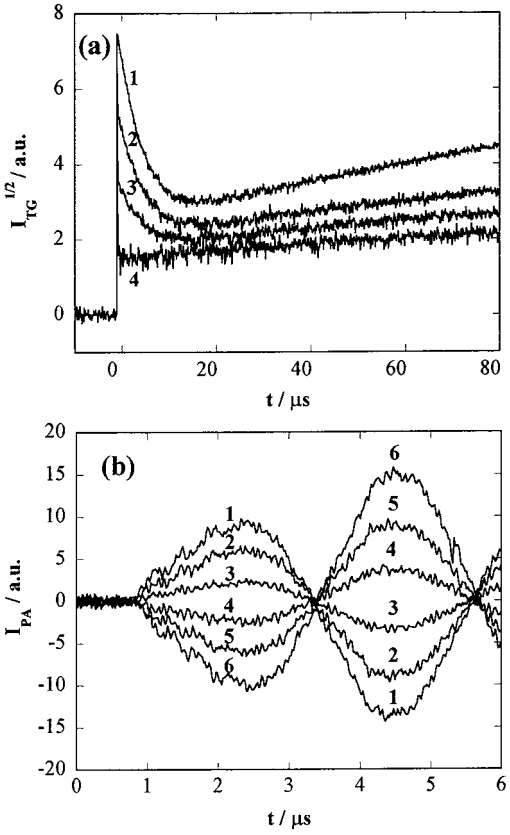

Figure 1. Time profiles of (a) the TG signals and (b) the PA signals observed after photoexcitation of PYP in buffer solution at various temperatures. Traces 1−4 in part a are for 20.5, 12.4, 8.4, and 4.5 °C, respectively. Traces 1−6 in part b are for 20.1, 15.4, 11.0, 8.2, 5.7, and 2.7 °C, respectively.

The observed PA signal of the PYP solution is shown in Figure 1b. The PA signal consists of two components: the thermal expansion of solvent and the net volume change of the molecule. With ΔH determined from the TG measurement, we calculate $\Phi\Delta V$ from the PA intensities of the sample and the reference. Using $\Phi = 0.35$, the volume contraction of $\Delta V = (-7 \pm 2)$ cm^3/mol is obtained at 20 °C. Van Brederode et al. estimated ΔH and ΔV between pG and pR as 120 kJ/mol and −14 cm^3/mol using the PA method under the assumption of temperature independence.[24] Although the observed molecular volume contraction in the initial stage of the PYP photocyclic reaction is consistent with this, these values are quantitatively different from our result by the TG method. We should note that, in the previous PA study, ΔH is determined by the temperature-dependent part of the PA intensity and ΔV from the extrapolation to $\alpha_{\text{th}} = 0$. If ΔH and ΔV depend on temperature, the analysis is not appropriate. The difference may be rationalized by the temperature dependence of ΔH and ΔV as will be described next.

Before investigating the temperature dependence of the TG and PA signals, we examined the temperature dependence of the absorption spectrum and Φ. Essentially, the absorption spectrum is temperature independent except for the slight sharpening of the red edge of the spectrum with decreasing temperature. The slight change of the absorbance at the excitation wavelength can be easily corrected. The temperature dependence of Φ is examined by the transient absorption

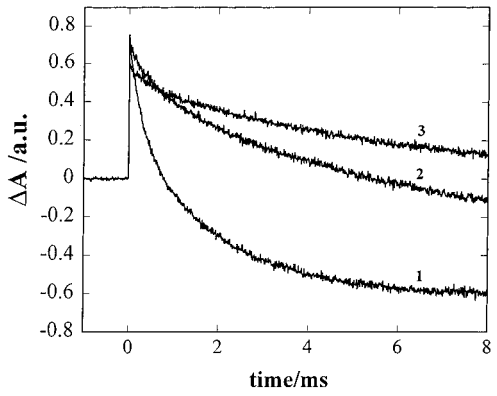

Figure 2. Temperature dependence of the transient absorption (ΔA) probed at 476 nm of PYP. Traces 1−3 are for 16.4, 5.8, and 0.8 °C, respectively.

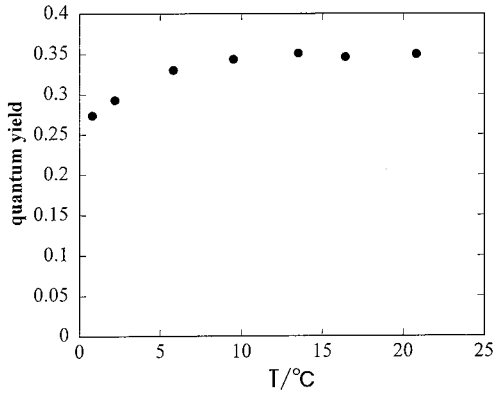

Figure 3. Temperature dependence of the reaction quantum yield of PYP.

method. The temporal profile of the transient absorption, which was probed at 476 nm, is depicted in Figure 2. Initially, the probe light intensity decreases (enhanced absorption) quickly after the excitation within the 10 ns pulse width, then it gradually changes to the bleach signal. This feature is consistent with the previous transient absorption studies of the PYP system.[22,23] The initial enhanced absorption and the subsequent bleach signals are attributed to pR and pB, respectively. The temporal profile associated with the pR → pB transformation can be fitted by a biexponential function with lifetimes of 170 μs and 1.0 ms. These lifetimes agree well with those reported before from the global analysis of the absorption change.[23] The initial rise of the signal represents the pG → pR process and the second change from the enhanced absorption to the bleach signal represents the pR → pB process. Clearly the lifetime of the pR → pB process becomes longer with decreasing temperature. We measure the relative quantum yield of the reaction by monitoring the amplitude of the initial rise at various temperatures. We found that the quantum yield does not change in a temperature range of 20−10 °C. However, when temperature becomes lower than 10 °C, Φ becomes gradually smaller (Figure 3). Around 0 °C, Φ is about 80% of that at 20 °C. We took into account this temperature-dependent Φ in the analysis of the TG and PA signals.

When the temperature of the sample decreases, the thermal grating intensity decreases. This change is expected because the temperature dependence of the refractive index (dn/dT) decreases with temperature. More importantly, the background signal intensity, which is a sum of δn_{pop} and δn_{vol}, also decreases with temperature at the same time. Since the change

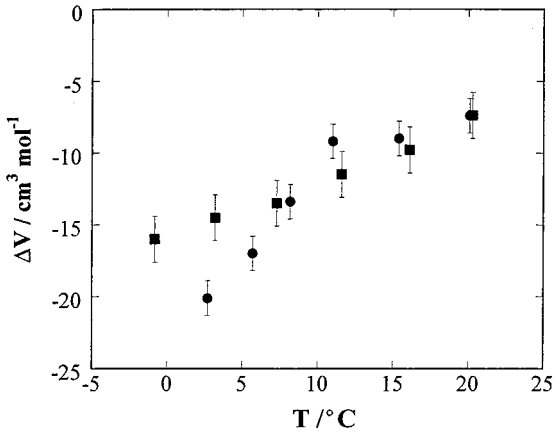

Figure 4. Plot of the volume change (cm^3/mol) determined from the intensity of the volume grating (squares) and PA signal (circles) against the temperature.

of the absorption spectrum with temperature is negligibly small in this temperature range, the temperature dependence of δn_{spe} should come from that of the volume change. Taking ΔV determined at 20 °C as the reference volume change, we calculated ΔV at various temperatures using eq 4 and plotted the results in Figure 3.

The temperature-dependent volume change is also confirmed from the PA intensity. By subtracting the thermal contribution from the PA signal, ΔV at various temperatures can be calculated from the PA intensity. The obtained ΔV from TG or PA are shown in Figure 4. The value of ΔV from PA and TG are slightly different especially at low temperature, but the difference is almost within experimental error. It is surprising that volume contraction becomes larger as the temperature goes down. The volume contraction around 0 °C becomes about twice as large as that at room temperature.

Previously, the volume contraction was interpreted in terms of the protein reorganization around a phototransformed chromophore induced by the dipole moment change.[24] We also consider that the volume change detected in the present study comes from the change of the protein part of PYP based on the following two observations. First, ΔV associated with the trans−cis photoisomerization of *p*-coumaric acid in aqueous solution is found to be negligibly small.[29] Hence, even if the dipole moment of the chromophore is changed by the isomerization, the volume contraction of the water part induced by the electrostatic interaction cannot be large. Second, a large temperature dependence of ΔV is not expected from such contraction of the water part by the electrostatic interaction. Since there is no new bond formation or dissociation during the reaction, the volume change should be attributed to the void volume change of the protein part or change of the hydophobic (or hydrophilic) part of the protein associated with the photoisomerization of the chromophore and our finding indicates that this protein structural change should depend on the temperature. Many biological systems such as PYP are not rigid reaction systems, but there are many local free energy minima (substates) along the reaction coordinate. This thermal fluctuation of the protein structure is essential for the biological function. The observed temperature-dependent volume change may reflect the structural fluctuation of PYP. We are currently investigating the structural change of PYP in detail by using a site mutation technique.

In summary, we applied the PA-TG hybrid method to the study of PYP photocyclic dynamics. Using the quantity of released heat estimated by TG, we determine the ΔH and ΔV

in the pG → pR process. ΔH indicates that the first intermediate pR stores the large energy. More importantly, we found a temperature-dependent volume change for the first time in the photocyclic reaction of PYP. The complete analysis of the TG signal in the nanoseconds to tens of milliseconds range and also discussions of the temperature-dependent ΔH and ΔV will be presented in the near future.

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