Time-Resolved Thermodynamics: Heat Capacity Change of Transient Species during Photoreaction of PYP

Javaid Shahbaz Khan,[†] Yasushi Imamoto,[‡] Mikio Kataoka,[‡] Fumio Tokunaga,[§] and Masahide Terazima*,[†]

Contribution from the Department of Chemistry, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan, Graduate School of Materials Science, Nara Institute of Science and Technology (NAIST), Nara 630-0101, Japan, and Department of Earth and Space Science, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan

Received August 16, 2005; E-mail: mterazima@kuchem.kyoto-u.ac.jp

Abstract: Heat capacity changes of short-lived transient species in different time ranges were measured for the first time by using the thermal component of the transient grating and transient lens signals at various temperatures. This method was applied to the transient intermediates of Photoactive Yellow Protein (PYP). The temperature dependence of the enthalpy change shows that the heat capacity of the short-lived intermediate pR_2 (also called I_1 or PYP_L) species is the same as that of the ground state (pG) species within our experimental accuracy, whereas that of the long-lived intermediate pB (I_2 or PYP_M) is much larger (2.7 ± 0.4 kJ/mol K) than that of pG. The larger heat capacity is interpreted in terms of the conformational change of the pB species such as melted conformation and/or exposure of the nonpolar residues to the aqueous phase. This technique can be used for photochemical reactions in general to investigate the conformational change and the hydrophobic interaction in a time domain.

1. Introduction

In chemistry, thermodynamics has been playing a central role in characterizing the nature of states. Measurements of the thermodynamical properties are essential to understand the conformational states of macromolecules and/or mechanisms of chemical reactions. For instance, in protein researches, the nature of the native and denatured states under the equilibrium condition have been studied from a viewpoint of the free energy, entropy, enthalpy, or the heat capacity differences.[1-10] The heat capacity (C_p) is in particular a fundamental thermodynamical quantity, from which other quantities may be derived. The heat capacity has served for a long time in characterizing the state of noncovalent interactions of macromolecules including proteins.[5-10] For example, a positive change in C_p has been regarded as a characteristic signature of protein unfolding. The nature of unfolded proteins has been investigated from a viewpoint of C_p.[5-10]

A variety of thermodynamical data has been accumulated for a number of stable compounds for a long time.[1-11] On the basis of the accumulated knowledge, a next step is to study the thermodynamical properties of short-lived intermediate species for understanding chemical reactions. However, experimentally, it has been very difficult to measure these properties for short-lived species. To overcome this difficulty, the transient lens (TrL) and photoacoustic (PA) techniques combined with the temperature dependence method or solvent variation method have been developed to determine the enthalpy change (ΔH) and the volume change (ΔV) of irreversible reactions so far.[12-18] Despite the importance of these methods, it is unavoidable to use an assumption that these thermodynamical properties do not depend on the temperature or solvent. On the other hand, we have demonstrated the usefulness of the pulsed laser induced transient grating (TG) method for quantitative measurements of the partial molar volume change and the enthalpy change during photochemical reactions in a time domain at one temperature under one pressure in one solvent without any assumption.[19-28] This method allows a kinetic description of

† Kyoto University.
‡ Nara Institute of Science and Technology (NAIST).
§ Osaka University.

(1) Murphy, K. P.; Freire, E. *Adv. Protein Chem.* **1992**, *43*, 313.
(2) Cooper, A. *Biophys. Chem.* **2000**, *85*, 25.
(3) Kitakuni, E.; Kuroda, Y.; Oobatake, M.; Tanaka, T.; Nakamura, H. *Protein Sci.* **1994**, *3*, 831.
(4) Makhatadze, G. I.; Privalov, P. L. *J. Mol. Biol.* **1993**, *232*, 639.
(5) Privalov, P. L. *Annu. Rev. Biophys. Chem.* **1989**, *18*, 47.
(6) Sturtevant, J. M. *Proc. Nat. Acad. Sci., U.S.A.* **1977**, *74*, 2236.
(7) Pace, C. N.; Grimsley, G. R.; Thomas, S. T.; Makhatadze, G. I. *Protein Sci.* **1999**, *8*, 1500. Loladze, V. V.; Ermolenko, D. N.; Makhatadze, G. I. *Protein Sci.* **2001**, *10*, 1343.
(8) Privalov, P. L.; Gill, S. J. *Adv. Protein Chem.* **1989**, *39*, 191. Privalov, P. L. *Adv. Protein Chem.* **1979**, *33*, 245. Privalov, P. L.; Makhatadze, G. I. *J. Mol. Biol.* **1992**, *224*, 715.
(9) Myers, J. K.; Pace, C. N.; Scholtz, J. M. *Protein Sci.* **1995**, *4*, 2148.
(10) Cooper, A. *Biophys. Chem.* **2005**, *115*, 89.

(11) Lazaridis, T.; Archontis, G.; Karplus, M. *Adv. Protein Chem.* **1995**, *47*, 231.
(12) Callis, J. B.; Parson, W. W.; Gouterman, M. *Biochim. Biophys. Acta* **1972**, *267*, 348.
(13) Herman, M. S.; Goodman, J. L. *J. Am. Chem. Soc.* **1989**, *111*, 1849.
(14) Braslavsky, S. E.; Heibel, G. E. *Chem. Rev.* **1992**, *92*, 1381.
(15) Westrick, J. A.; Goodman, J. L.; Peters, K. S. *Biochemistry* **1987**, *26*, 8313.
(16) Strassburger, J. M.; Gartner, W.; Braslavsky, S. E. *Biophys. J.* **1997**, *72*, 2294.
(17) Gensch, T.; Viappiani, C.; Braslavsky, S. E. *J. Am. Chem. Soc.* **1999**, *121*, 10573.
(18) Terazima, M.; Hara, T.; Hirota, N. *J. Phys. Chem.* **1993**, *97*, 13668.
(19) Terazima, M.; Hara, T.; Hirota, N. *Chem. Phys. Lett.* **1995**, *246*, 577.
(20) Hara, T.; Terazima, M.; Hirota, N. *J. Phys. Chem.* **1996**, *100*, 10194.

both the energetics and the molecular volume during chemical and biological transformations. Furthermore, this technique has an important and unique advantage; i.e., not only enthalpy change or volume change but other thermodynamical properties can be extracted by changing external parameters, such as the temperature or pressure. For example, the volume change measurements of carboxymyoglobin[21] and Photoactive Yellow Protein (PYP)[28] clearly showed that ΔV depends on temperature. From this temperature dependence, we determined, for the first time, the thermal expansion coefficient change ($\Delta \alpha_{th}$) of transient intermediate species, which lives only 700 ns in the Mb case and 200 μs in the PYP case. Hence, we have so far succeeded in measuring ΔH, ΔV, and $\Delta \alpha_{th}$ of transient species. In this paper, we report the first measurement of the heat capacity change (ΔC_p) of transient species by improving an experimental accuracy with a new type of sample cell holder. We applied this technique to the study of the conformational change of two intermediate species of PYP photoreaction.

PYP is first isolated from the purple sulfur bacterium *Ectothiorhodospira halophila*[29−31] and is considered to possess a function of a blue light photoreceptor for a negative phototactic response.[32] It is a relatively small (14 kDa) water soluble protein.[33] The chromophore of PYP is *p*-coumaric acid (4-hydroxycinnamic acid) covalently bound to the side chain of Cys 69 via a thioester linkage.[34,35] After the photoexcitation, PYP shows a complete photocycle triggered by the photoisomerization of this chromophore.[36,37] Upon flash excitation of the chromophore, the ground state (pG) is converted into red-shifted intermediate pR_1 in less than 2 ns.[37] (For describing the intermediate species, different nomenclatures are used depending on the research groups.[30] The pR_1 is sometimes called I_1 or PYP_L.) Subsequently, pR_1 is converted into pR_2 without the spectrum change.[38,39] The pR_2 species decays on the submillisecond time scale into blue-shifted intermediate pB′ (or

I_1'), which is converted to pB (or I_2 or PYP_M) without a spectrum change.[30,40] This pB is transformed to pB^{deprot} (or I_2') and finally returns to pG in a seconds time scale.[30,31,40]

The reaction as well as the conformational change associated with this reaction has been a very interesting and stimulating topic in the protein science.[29−51] From the kinetic measurements, it was found that the reaction rates of $pR_2 \rightarrow pB$ and $pB \rightarrow pG$ cannot be expressed with the Arrhenius equation with a single activation energy and the deviation was explained in terms of the activation heat capacity changes ($\Delta C_p^{\ddagger}$) for the $pR \rightarrow pB$ and $pB \rightarrow pG$ processes.[41−43] The $\Delta C_p^{\ddagger}$ values were determined to be 0.35 and −2.35 kJ/mol K, respectively.[42,43] Since there is no time-resolved capability in the traditional thermodynamical techniques, ΔC_p of any intermediate has not been measured. Instead, using the reversible denaturation of PYP by decreasing the pH below 3.25, van Brederode et al. have reported ΔC_p of the acid-denatured PYP at low pH.[42] Since the absorption spectrum of this denatured species is similar to that of the photocreated PYP (pB), this species is called pB_{dark} and the character of this species is considered to resemble that of pB. From the temperature dependence of the equilibrium between pG and pB_{dark} with a two-state transition assumption, ΔC_p of pB_{dark} was determined to be 1.59 kJ/mol K.[42] This positive heat capacity change is interpreted in terms of the exposure of the hydrophobic group in this state. However, it is not certain if the pH-denatured conformation is really similar to the transient pB species, because the absorption spectrum can ensure a structural resemblance only around the chromophore, but it does not reflect the protein conformation far from it. In other words, although the pH sensitive parts around the chromophore of pB and pB_{dark} resemble each other, the conformation of the other part could be totally different. Naturally, it should be essentially important to characterize the heat capacity change of the photocreated pB species in a time domain. Here, we show the ΔC_p measurement of pR and pB in a time domain by TG and TrL methods.

This is the first measurement of the transient change of the heat capacity during any chemical reaction, and this technique can be used for other chemical reactions in principle.

2. Principle

In this study, we used two experimental methods to monitor the heat capacity changes in different time regions; the transient grating (TG) and the transient lens (TrL) methods. The principle

(21) Sakakura, M.; Yamaguchi, S.; Hirota, N.; Terazima, M. *J. Am. Chem. Soc* **2001**, *123*, 4286.
(22) Nishioku, Y.; Nakagawa, M.; Tsuda, M.; Terazima, M. *Biophy. J.* **2001**, *80*, 2922.
(23) Sakakura, M.; Morishima, I.; Terazima, M. *J. Phys. Chem. B* **2001**, *105*, 10424.
(24) Sakakura, M.; Morishima, I.; Terazima, M. *Biochemistry* **2002**, *41*, 4837. Nishioku, Y.; Nakagawa, M.; Tsuda, M.; Terazima, M. *Biophy. J.* **2002**, *83*, 1136. Nada, T.; Terazima, M. *Biophys. J.* **2003**, *85*, 1876. Nishida, S.; Nada, T.; Terazima, M. *Biophys. J.* **2004**, *87*, 2663.
(25) Nishihara, Y.; Sakakura, M.; Kimura, Y.; Terazima, M. *J. Am. Chem. Soc.* **2004**, *126*, 11877.
(26) Inoue, K.; Sasaki, J.; Morisaki, M.; Tokunaga, F.; Terazima, M. *Biophys. J.* **2004**, *87*, 2587.
(27) Terazima, M. *Bull. Chem. Soc. Jpn.* **2004**, *77*, 23.
(28) Takeshita, K.; Hirota, N.; Imamoto, Y.; Kataoka, M.; Tokunaga, F.; Terazima, M. *J. Am. Chem. Soc.* **2000**, *122*, 8524.
(29) Meyer, T. E. *Biochim. Biophys. Acta* **1985**, *806*, 175.
(30) (a) Hellingwerf, K. J.; Hendriks, J.; Gensch, T. *J. Phys. Chem. A* **2003**, *107*, 1082. (b) Cusanovich, M. A.; Meyer, T. E. *Biochemistry* **2003**, *42*, 4759.
(31) Losi, A.; Braslavsky, S. E. *Phys. Chem. Chem. Phys.* **2003**, *5*, 2739.
(32) Sprenger, W. W.; Hoff, W. D.; Armitage, J. P.; Hellingwerf, K. J. *J. Bacteriol.* **1993**, *175*, 3096.
(33) Borgstohl, G. E. O.; Williams, D. R.; Getzoff, E. D. *Biochemistry* **1995**, *34*, 6278.
(34) Baca, M.; Borgstahl, G. E. O.; Boissinot, M.; P. M. Burke; Williams, D. R.; Slater, K. A.; Getzoff, E. D. *Biochemistry* **1994**, *33*, 14369.
(35) Hoff, W. D.; Düx, P.; Hård, K.; Devreese, B.; Nugteren-Roodzant, I. M.; Crielaard, W.; Boelens, R.; Kaptein, R.; Van Beeumen, J.; Hellingwerf, K. J. *Biochemistry* **1994**, *33*, 13959.
(36) Meyer T. E.; Tollin, G.; Hazzard, J. H.; Cusanovich, M. A. *Biophys. J.* **1989**, *56*, 559.
(37) Hoff, W. D.; van Stokkum, I. H. M.; van Ramesdonk, H. J.; van Brederode, M. E.; Brouwer, A. M.; Fitch, J. C.; Meyer, T. E.; van Grondelle, R.; Hellingwerf, K. J. *Biophys. J.* **1994**, *67*, 1691.
(38) Takeshita, K.; Hirota, N.; Imamoto, Y.; Kataoka, M.; Tokunaga, F.; Terazima, M. *Biochemistry* **2002**, *41*, 3037.
(39) Takeshita, K.; Hirota, N.; Imamoto, Y.; Kataoka, M.; Tokunaga, F.; Terazima, M. *Biophys. J.* **2002**, *83*, 1567.
(40) Hendriks, J.; van Stokkum, I. H. M.; Hellingwerf, K. J. *Biophys. J.* **2003**, *84*, 1180.
(41) Hoff, W. D.; van Stokkum, I. H. M.; van Ramesdonk, H. J.; van Brederode, M. E.; Brouwer, A. M.; Fitch, J. C.; Meyer, T. E.; van Grondelle R.; Hellingwerf, K. J. *Biophys. J.* **1994**, *67*, 1691.
(42) (a) van Brederode, M. E.; Hoff, W. D.; van Stokkum, I. H. M.; Groot, M.-L.; Hellingwerf, K. J. *Biophys. J.* **1996**, *71*, 365.
(43) Hoff, W. D.; Xie, A.; van Stokkum, I. H. M.; Tang, X.; Gural, J.; Kroon, A. R.; Hellingwerf, K. J. *Biochemistry* **1999**, *38*, 1009.
(44) Hendriks, J.; Gensch, T.; Hviid, L.; van der Horst, M. A.; Hellingwerf, K. J.; van Thor, J. J. *Biophys. J.* **2002**, *82*, 1632.
(45) van Brederode, M. E.; Gensch, T.; Hoff, W. D.; Hellingwerf, K. J.; Braslavsky, S. E. *Biophys. J.* **1995**, *68*, 1101.
(46) Meyer T. E.; Tollin, G.; Hazzard, J. H.; Cusanovich, M. A. *Biophys. J.* **1989**, *56*, 559.
(47) Imamoto, Y.; Kataoka M.; Tokunaga, F. *Biochemistry* **1996**, *35*, 14047.
(48) Harigai, M.; Imamoto, Y.; Kamikubo, H.; Yamazaki, Y.; Kataoka, M. *Biochemistry* **2003**, *42*, 13893.
(49) Imamoto, Y.; Kamikubo, H.; Harigai, M.; Shimizu, N.; Kataoka, M. *Biochemistry* **2002**, *41*, 13595.
(50) Sasaki, J.; Kumauchi, M.; Hamada, N.; Oka, T.; Tokunaga, F. *Biochemistry* **2002**, *41*, 1915.
(51) Ohishi, S.; Shimizu, N.; Mihara, K.; Imamoto, Y.; Kataoka, M. *Biochemistry* **2001**, *40*, 2854.

for the quantitative extraction of the thermal component from the TG and TrL signals is based on the characteristic decay rate due to the thermal diffusion as explained below.

(i) Transient Grating (TG) Method. When the absorption change at the probe wavelength is negligible, the TG signal intensity ($I_{TG}(t)$) is proportional to the square of the refractive index change $\delta n(t)$ under a weak diffraction condition:[52,53]

$$I_{TG}(t) = \alpha \delta n(t)^2 \qquad (1)$$

where α is a constant. There are two main contributions to the refractive index change; the thermal effect and a change of chemical species by the reaction. We represent the former as $\delta n_{th}(t)$ (thermal grating; hereafter abbreviated as ThG), the latter as $\delta n_{spe}(t)$ (species grating).[54−56]

$$I_{TG}(t) = \alpha\{\delta n_{th}(t) + \delta n_{spe}(t)\}^2 \qquad (2)$$

The refractive index changes due to the absorption change as well as the partial molar volume change are included in the $\delta n_{spe}(t)$ term.

The temporal profile of $\delta n_{th}(t)$ is determined by the convolution between the thermal diffusion decay and intrinsic temporal evolution of the temperature change ($\Delta T(t)$).

$$\delta n_{th}(t) = (dn/dT)\,\Delta T(t)^* \exp(-D_{th}q^2 t) \qquad (3)$$

where $*$ represents the convolution integral, dn/dT is the refractive index change by the temperature variation of the solution, D_{th} is the thermal diffusivity, and q is the grating wavenumber given by $q = \pi \sin(\theta/2)/\lambda_{ex}$ (λ_{ex} is the wavelength of the excitation light). We can vary q by varying the crossing angle (θ) between two excitation beams. The function of $\Delta T(t)$ represents the heating process due to the nonradiative transition from the excited state and the enthalpy change (ΔH) of the chemical reaction. The temperature rise (ΔT) is related to the released thermal energy, Q (J/mol), by the next equation.

$$\Delta T = \frac{QW}{\rho C_p}\Delta N \qquad (4)$$

where W is the molecular weight (g/mol), ρ is the density (g/cm^3) of the solvent, and ΔN is the molar density of the excited molecule (mol/cm^3). Since the quantum yield of the fluorescence of PYP is almost negligible, the enthalpy change is calculated from the equation

$$Q = h\nu - \Delta H\Phi \qquad (5)$$

where $h\nu$ is the photon energy, and Φ is the quantum yield of the reaction. Here, ΔH is defined by the enthalpy difference from the ground state of PYP.

Since the value of $D_{th}q^2$ is known under the experimental conditions from the ThG signal of a calorimetric reference sample, which converts all the absorbed photon energy to the thermal energy within a time response of our system, the ThG

component of a sample can be extracted from the observed TG signal by the curve fitting with a function of $\exp(-D_{th}q^2 t)$, and the amplitude of ThG (δn_{th}) can be accurately determined from the pre-exponential factor. By comparison with the amplitude from the calorimetric reference sample, the absolute value of Q, hence ΔH, is obtained.

The decay lifetime of the ThG signal ($(D_{th}q^2)^{-1}$) can be varied in a range of 100 ns−100 μs depending on the q^2 value. If the lifetime of a transient species is longer than this, the ThG signal associated with this decay process becomes very weak and we cannot accurately determine Q from the signal. Hence, ΔH measurement by this method can be applied to a species that possesses a lifetime shorter than $(D_{th}q^2)^{-1}$. For measuring ΔH of a relatively long-lived species, we used the TrL method.

(ii) Transient Lens (TrL) Method. In the TrL experiment, a spatially Gaussian shaped laser beam is used for the excitation.[14,57−61] The photoinduced refractive index distribution with the Gaussian shape is monitored by the light intensity change at the central part of another probe beam. The signal intensity ($I_{TrL}(t)$) is proportional to the photoinduced refractive index change, $\delta n(t)$,

$$I_{TrL}(t) = \alpha'\delta n(t) \qquad (6)$$

where α' is a constant including the sensitivity of the system. The origins of the refractive index change are the same as those in the TG case: the thermal effect (thermal lens; ThL) and a change of chemical species by the reaction (species lens), which is composed of the volume lens and the population lens contributions.[61]

$$I_{TrL}(t) = \alpha'\{\delta n_{th}(t) + \delta n_{spe}(t)\} \qquad (7)$$

Similar to the TG case, the temporal profile of the ThL signal is determined by the thermal diffusion process and the heat releasing process. Assuming the impulsive response of the ThL signal as $f(t)$, one may find that the temporal profile of the ThL signal is given by

$$I_{ThL}(t) = \alpha'(dn/dT)\,\Delta T(t)^* f(t) \qquad (8)$$

If the excitation beam shape is the Gaussian shape with a radius of w, $f(t)$ is proportional to $1/(1 + 2t/\tau_c)^2$, where τ_c is given by[57,58]

$$\tau_c = w^2/4D_{th} \qquad (9)$$

Similarly to the ThG case, the released thermal energy and hence the enthalpy change (ΔH) can be calculated from the amplitude of the ThL signal by comparison with that of the calorimetric reference sample. The decay rate of the ThL signal is approximately given by $w^2/4D_{th}$, which is about 100 ms under our experimental conditions. This TrL method can be used for quantitative measurement of ΔH for a species, which lives shorter than this time.

(52) Eichler, H. J.; Gunter, P.; Pohl, D. W. *Laser induced dynamic gratings*; Springer-Verlag: Berlin, 1986.
(53) Kogelnik, H. *Bell Syst. Tech. J.* **1969**, *48*, 2909.
(54) Terazima, M. In *Advances in multiphoton processes and spectroscopy*; Lin, S. H., Villaeys, A. A., Fujimura, Y., Eds.; World Scientific: Singapore, 1996.
(55) Terazima, M. *Adv. Photochem.* **1998**, *24*, 255.
(56) Terazima, M. *J. Photochem. Photobiol.* **2002**, *24*, 1.

(57) Bialkowski, S. E. *Photothermal spectroscopy methods for chemical analysis*; John Wiley: 1996.
(58) Fang, H. L.; Swofford, R. L. In *Ultrasensitive Laser Spectroscopy*; Kliger, D. S., Ed.; Academic Press: New York, 1983.
(59) Gordon, J. P.; Leite, R. C. C.; Moore, R. S.; Porto, S. P. S.; Whinnery, J. R. *Bull. Am. Phys. Soc.* **1964**, *9*, 501. Leite, R. C. C.; Moore, R. S.; Whinnery, J. R. *Appl. Phys. Lett.* **1964**, *5*, 141.
(60) Twarowski, A. J.; Kliger, D. S. *Chem. Phys.* **1977**, *20*, 253.
(61) Terazima, M. *Isr. J. Chem.* **1998**, *38*, 143.

(iii) Heat Capacity Change. The heat capacity change is calculated from the definition

$$\partial \Delta H / \partial T = \Delta C_p \qquad (10)$$

Assuming that ΔC_p does not depend on temperature, at least in the temperature range we investigated, integration of eq 10 leads to

$$\Delta H(T) = \Delta H(T_0) - (T_0 - T)\Delta C_p \qquad (11)$$

where T_0 is any reference temperature. Therefore, from the slope of ΔH vs T plot, ΔC_p is calculated.

3. Experimental Section

The experimental setup for the TG[19−26] and TrL[62,63] experiments was similar to that reported in previous papers. A XeCl excimer laser-pumped dye laser (Lamda Physik Compex 102xc, Lumonics Hyper Dye 300; $\lambda = 465$ nm) was split into two by a beam splitter and crossed inside a quartz sample cell (optical path length = 2 mm). The laser power of the excitation was < 5 μJ/pulse. The created refractive index modulation (transient grating) in the sample was probed by a He−Ne laser (633 nm) or a diode laser (840 nm) as a Bragg diffracted signal (TG signal). The TG signal was detected by a photomultiplier tube (Hamamatsu R-928) and fed into a digital oscilloscope (Tetronix 2430A). The TG signal was averaged by a microcomputer to improve the signal-to-noise (S/N) ratio.

In the TrL experiment, the same laser systems as the TG measurement were used for the excitation and the probe beams. The excitation beam was slightly focused by a lens with a focal length of 20 cm. The probe beam was brought into the sample collinearly with the pump beam. The probe beam exiting the sample was expanded by a concave lens, and the TrL signal was detected as a light intensity change at the beam center by using a pinhole. The excitation light was removed by an optical filter. The repetition rate of the excitation beam is less than 0.2 Hz for the TG and TrL experiments to avoid multiexcitation of the photoproduct of PYP. In particular, the repetition rate was set as low as possible for low-temperature measurements. We confirmed that reducing the repetition rate further did not change the signal intensity.

For the ΔC_p measurement, it is important to measure the TG and TrL signal intensities quantitatively and accurately at various temperatures. For a quantitative measurement of the thermal energy, we have to compare the signal intensity of the sample with that of a calorimetric reference sample to eliminate all factors that depend on the experimental conditions. Conventionally, the solution exchange method has been frequently used; i.e., the sample solution in a cell is exchanged by the calorimetric reference sample solution without moving the sample cell and the other optical alignments. This technique is essential, because the signal intensity is very sensitive to the cell position against the crossing region of the three beams for TG and the monitor position of the probe beam for TrL. This has been a standard technique normally used for the TG and TrL experiments as well as the PA experiment.[12−28] However, we found that this method is not suitable in this case, because of the following reasons. First, in this research, it required relatively long times for a series of experiments at various temperatures. Particularly, the repetition rate in this PYP experiments cannot be high to avoid multiexcitation of the product. Therefore, it might be possible that, during the long time measurement at various temperatures, the laser beam position could be slightly changed due to changes of any laser condition or fluctuations of mirrors. Since the signal intensity is very sensitive to the optical alignment of the three (TG) and two (TrL) beams, any minor variations will change the signal intensity. Second, the reproducibility of temperature should be also very strict, because
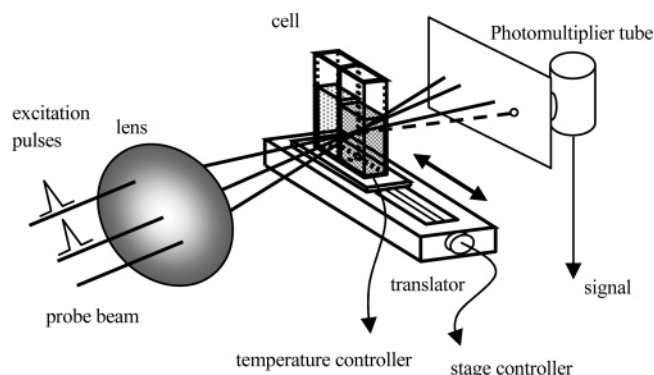
(62) Terazima, M.; Azumi, T. *Chem. Phys. Lett.* **1987**, *141*, 237.
(63) Terazima, M.; Azumi, T. *J. Am. Chem. Soc.* **1989**, *111*, 3824.

Figure 1. Schematic illustration of the experimental setup for quantitative measurements of the TG signal intensities. For the TrL experiments, the same setup was used.

the thermal component intensity (ThG and ThL) is sensitive to the solution temperature due to the dn/dT term in eq 3.[14] To improve the reproducibility and decrease the influence of fluctuations, the intensities of the signals for sample and reference were compared at each temperature. However, if we measure the signal of the PYP solution and the calorimetric reference solution at every temperature, the solutions should be changed many times, which will cause contamination of the sample or loss of a part of the sample solution. To avoid sample exchange as well as to obtain a precise comparison of two signal intensities at the same temperature, we made a quartz cell with a partition to separate it into two compartments (Figure 1). This cell was fixed on a mechanical translator by a metal cell holder, of which temperature can be controlled. One of the compartments was filled with the sample solution, and the other one, with the calorimetric reference sample. By moving the translator stage, we can exchange the sample any time. The translator was controlled by a computer (Figure 1). This way, we can compare the signal intensities of the sample with that of the reference many times at exactly the same temperature to examine the accuracy of the measurement. We confirmed that the signal intensities from the same sample in different compartments were the same within experimental accuracy ($\pm 2\%$)

PYP was prepared as reported previously.[28,38] PYP (ca. 100 μM) was dissolved in 10 mM Tris-HCl (pH = 8.0). BCP (bromocresol purple) was used as a calorimetric reference for the TG and TrL experiments.[14] Concentrations of the sample and reference were adjusted so that the absorbance in the cell was the same at the excitation wavelength. The absorbance was about 0.7−1.0 in each experiment.

3. Results

3.1. Heat Capacity Change of pR. Figure 2a shows the temporal profiles of the TG signal after photoexcitation of PYP in the buffer solution. The signal is composed of several components; the qualitative features and the assignment of the signal components in the TG signal have already been presented previously.[28,38] Briefly, the features are summarized as follows. The signal rises quickly after photoexcitation with the instrumental response of our system and then shows a weak slow rising component (~ 1 μs at 20 °C). This component has been attributed to the protein conformational change apart from the chromophore representing the conversion from pR$_1$ to pR$_2$. After this, the signal decays to a certain intensity with a time constant $D_{th}q^2$ and shows growth−decay curves twice during the observation time. The component that decays with $D_{th}q^2$ should be the thermal grating (ThG) component, and the latest growth−decay curve was attributed to the protein diffusion process of pG and pB.

The signal in a time range of the ThG component at 302 K is depicted in Figure 2b. Since the decay lifetime of the ThG
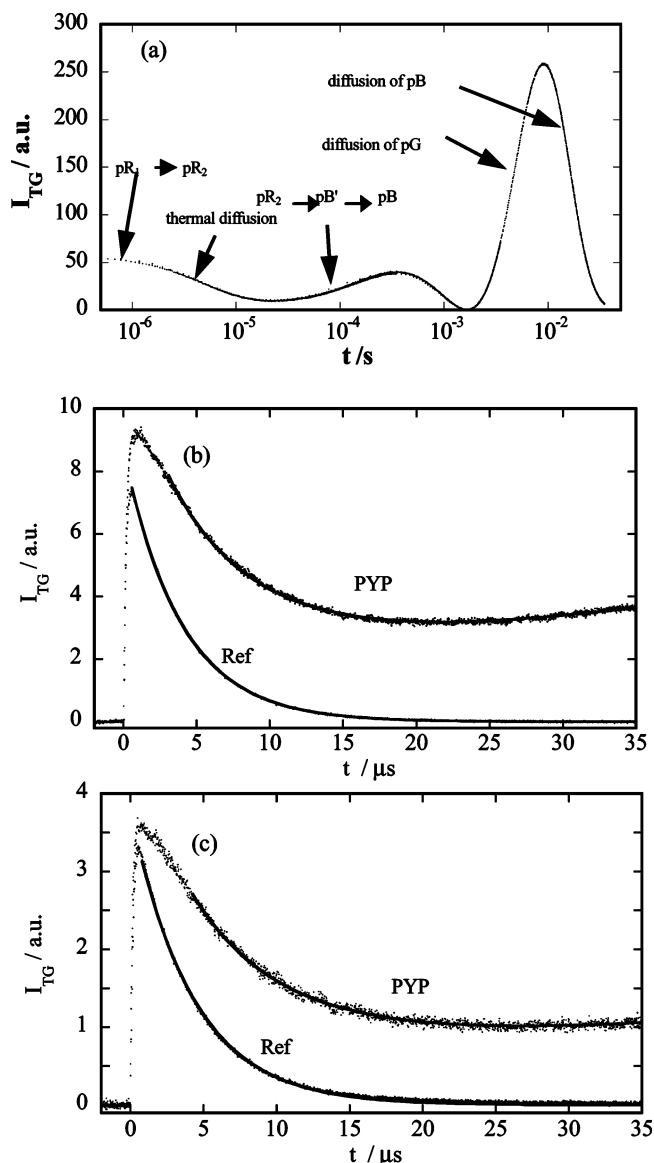
Figure 2. (a) A typical TG signal of PYP in the phosphate buffer in a wide time range. The assignments of the kinetic components are labeled in the figure. Some examples of the TG signals in a time range of the thermal grating decay of PYP (PYP) and the calorimetric reference sample (Ref) at various temperatures; (b) 302 K and (c) 287 K. The observed signals and the best fitted curves are shown by the dotted lines and the solid lines.

Figure 3. ΔH of pR_2 (■) against temperature calculated from the ThG signal intensities.

Table 1. Enthalpy Change (ΔH/kJ/mol) at 302 K and the Heat Capacity Change (ΔC_p/kJ/mol K) of the Intermediates and the Activation States of PYP from the Ground State (pG) Values

	pR_2	$pR_2{}^{\ddagger}$	pB	pB‡
ΔH	173 ± 10	230^a	75 ± 12	84^a
ΔC_p	0 ± 0.5	0.35^b	2.7 ± 0.4	0.4^c

^{a} Calculated from ΔH of the present study and $\Delta H^{\ddagger}$ in ref 41. ^{b} Calculated from ΔC_p of the present study and $\Delta C_p{}^{\ddagger}$ in ref 41. ^{c} Calculated from ΔC_p of the present study and $\Delta C_p{}^{\ddagger}$ in ref 42.

assume that D_{th} of the PYP solution is the same as that of the reference solution. This assumption is reasonable, because of the low concentrations of PYP (ca. 100 μM) and the buffer solution. Furthermore, this assumption was confirmed by measuring the thermal diffusion decay rate of the buffer solution with a calorimetric reference sample. However, it should be noted that this is not an essential assumption for the measurement. Even if D_{th} of the sample solution is different from that of solvent, we can still measure D_{th} independently by other means, e.g., traditional thermodynamical methods.) The second and third terms of the right-hand side represent the species grating terms. The constant term, a_2, represents a long-lived component. ΔH of pR_2 (ΔH_{pR}) is calculated from the amplitude of a_{th} in eq 12 and the quantum yield of the reaction $\Phi = 0.35$ [45] to be $\Delta H_{pR} = 173 \pm 10$ kJ/mol at 302 K.

When we decrease the solution temperature, the ThG signal intensity decreases. This decrease is mostly due to the temperature dependence of the dn/dT term in eqs 3 and 8. Comparing the signal intensity of PYP sample with that of the calorimetric reference sample at the same temperature (Figure 2c), we calculate ΔH_{pR} at this temperature. We made sure that the ThG signal intensity is strong enough in this experimental temperature range to determine ΔH accurately. Thus determined ΔH_{pR} values are plotted in Figure 3. This value does not depend on the temperature within our experimental accuracy. According to eq 11, this result means that the heat capacity of pR_2 is the same as that of pG within our experimental accuracy (± 0.5 kJ/mol K) (Table 1).

3.2. Heat Capacity Change of pB. Using the same method as that in the previous section, the heat capacity change of the pB species could be determined. However, since the decay of the thermal grating signal is generally much faster than the kinetics of $pR_2 \rightarrow pB$, the TG signal due to the thermal energy

signal is 7.3 μs under this condition, the signal intensity reflects the thermal energy that comes out by the formation of pR_2. For reliable measurement of the ThG signal intensity, the signal should be fitted accurately with a small number of adjustable parameters to avoid ambiguity. For this, we used the following procedure. First, we fitted the ThG signal of the calorimetric reference sample by a single-exponential function to determine the amplitude of the ThG signal and $D_{th}q^2$ under this condition. Then, the TG signal of PYP after the slow rising component, which represents the $pR_1 \rightarrow pR_2$ process, is fitted by the function

$$I_{TG}(t) = (a_{th}\exp(-D_{th}q^2 t) + a_1\exp(-k_1 t) + a_2)^2 \quad (12)$$

By neglecting the slow rising dynamics from the fitting region, the number of the adjustable parameters can be reduced. Furthermore, the $D_{th}q^2$ value was fixed to that obtained from the calorimetric reference sample during the fitting. (Here, we
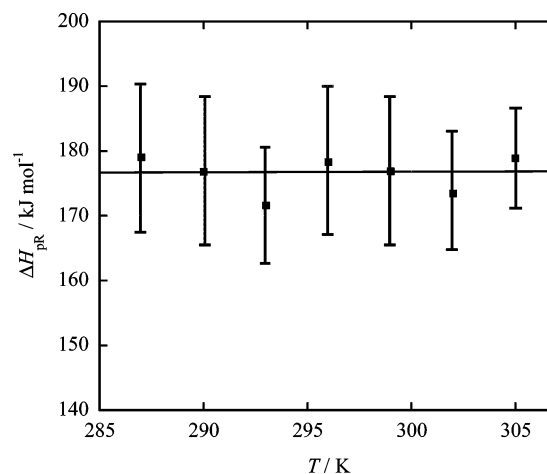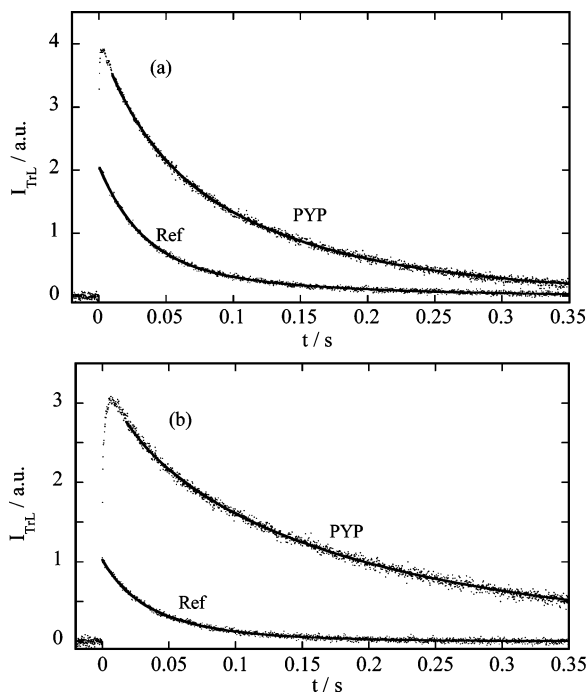
Figure 4. Some typical TrL signals in a time range of the thermal lens decay of PYP (PYP) and the calorimetric reference sample (Ref) at various temperatures; (a) 301 K and (b) 287 K. The observed signals and the best fitted curves are shown by the dotted lines and the solid lines.

released by this process is too small to be determined. To study the enthalpy of the pB state (ΔH_{pB}), we used the TrL method. The decay of the TrL signal is in an order of tens of milliseconds, and the thermal energy released from the photo-excited state to the pB formation can be detected as a decaying component of the TrL signal ($Q = h\nu - \Phi\Delta H_{pB}$).

Figure 4 shows the TrL signals of PYP and the reference sample. The TrL signal of PYP rises fast (with the response time of the thermal lens signal) and then slowly. The first rise corresponds to the transformation pG→pR. The second slower rise should be attributed to the transformation pR$_2$→pB. The TrL signal intensity of PYP is larger than that of the reference sample, because δn_{spe} is constructively overlapped with δn_{th} of PYP (both δn_{spe} and δn_{th} possess the negative sign). The sign of δn_{spe} is consistent with the result from the TG measurement. For measuring the intensity of the thermal component, we decomposed the TrL signal into ThL and species lens components in the following way.

First, the temporal profile of the ThL signal from the calorimetric sample is fitted by a biexponential function. Although this is not a theoretically predicted decay function of the thermal lens signal, this function reproduces the observed decay profile quite well (Figure 4). Hence, we used this function as the impulsive response function f(*t*) for the ThL signal. The observed TrL signal of PYP was fitted with this function and a species lens component,

$$I_{TrL}(t) = b_1 f(t) + b_2 \exp(-kt) + b_3 \qquad (13)$$

Similar to the TG case, data after the initial slow rising component representing pR$_2$ → pB process was used for the fitting to reduce the adjustable parameters. The second and the third terms represent the slow dynamics of the pB → pB^{deprot} process. With this fitting method, relative thermal energy

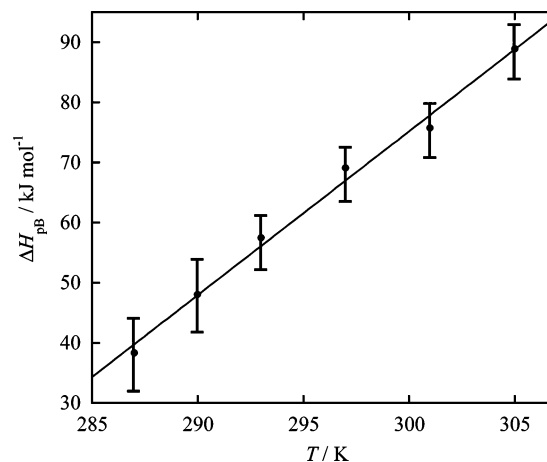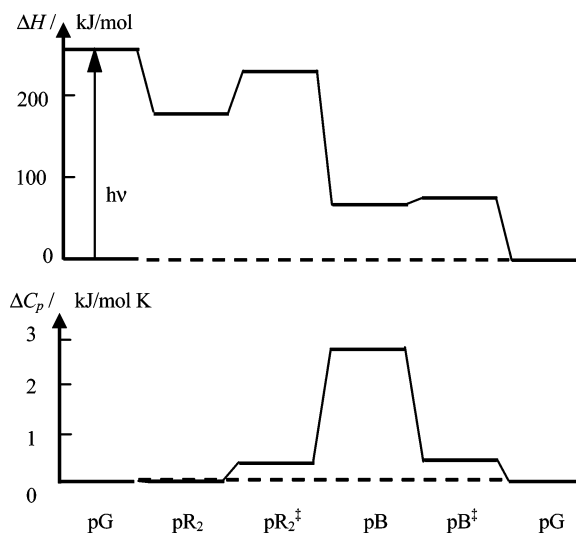**Figure 5.** ΔH of pB (●) against temperature calculated from the ThL signal intensities.

Figure 6. Schematic summary of the enthalpy changes (ΔH) and heat capacity change (ΔC_p) during the PYP photocycle (Table 1).

compared with the calorimetric reference sample can be determined from the amplitude of b_1; ΔH of pB$_1$ (ΔH_{pB}) is determined to be (75 ± 12) kJ/mol at 301 K.

Previously, we used the temperature variation method to separate the thermal intensity from the other contributions; the species grating signal measured at the temperature of dn/dT = 0 is subtracted from the observed signal.[38] In this method, we assumed that ΔH and species grating terms are temperature independent. On the other hand, in this present research, we determined ΔH_{pB} without using this assumption for the first time.

Figure 5 shows the temperature dependence of ΔH_{pB}. The ΔH_{pB} value decreases with decreasing the temperature. According to eq 11, this temperature dependence indicates nonzero ΔC_p of pB species. The temperature dependence of C_p may be neglected in this small temperature range we used. From the linear fitting in the observation temperature range, $\Delta C_p = (2.7 \pm 0.4)$ kJ/mol K is obtained (Table 1). The determined ΔH and ΔC_p including the activation parameters along the PYP photoreaction coordinate is schematically shown in Figure 6.

4. Discussion

The molecular origin of ΔC_p has been investigated for a long time.[5-10] Transfer of small molecules from nonaqueous to

aqueous liquids is generally accompanied by a positive ΔC_p, which has been interpreted in terms of the construction of hydrogen bonding structure of water molecules around the nonpolar group. Generally, the large changes in C_p have been taken as evidence for the involvement of hydrophobic interaction. For supporting this, good correlations between ΔC_p and changes in solvent accessible surface area (SASA) have been reported in several systems.[5-10] However, hydrophobic interaction may not be the only source of the positive ΔC_p. The unfolding of protein can be regarded as the melting of a well-ordered cluster. An increase of heat capacity is a fundamental property of most order−disorder transitions in a condensed phase, because of the increase in accessible degrees of freedom of molecules in the disordered state.[10] If the intermolecular interaction of substance is thermally disrupted, such a substance should show an increase in C_p, when going from ordered to disordered states.

On the basis of these investigations, one may conclude that the similar C_p values between pG and pR_2 indicate a similar SASA and similar hydrophobic interaction of these species, hence, that the protein conformation including the solvation structure of pR_2 does not change so much from the pG state. Previously, we found that the thermal expansion coefficient (α_{th}) of pR_2 is larger than that of pG.[28] Since α_{th} is proportional to the cross-correlation of the volume (V) and entropy (S) fluctuations,[64,65]

$$\langle SV - \langle S \rangle \langle V \rangle \rangle = k_B T V \alpha_{th} \qquad (14)$$

where $\langle \ \rangle$ indicates the ensemble average and k_B is the Boltzmann constant, we concluded that the protein structure is loosened even in this initial intermediate species pR_2. These two facts can be consistently interpreted, if we consider that the pR_2 conformation is fluctuating around the equilibrium structure of pR_2, which is similar to that of pG. It is interesting to note that ΔH_{pR} is quite large ($\sim$170 kJ/mol). We consider that the high energy of the pR species is the cause of the large fluctuation of the conformation and it could be an important driving force for the subsequent thermal reaction of the $pR_2 \rightarrow pB$ transformation.

Upon the transformation of $pR_2 \rightarrow pB$, the energy of the protein drastically relaxed and C_p increases (Figure 6). The increase of C_p for pB species implies that the protein conformation is drastically changed. We may suggest that this large conformational change is the cause of the energetic stabilization of pB. Based on the effect of hydrophobicity on the photocycle kinetics of PYP, Meyer et al. have proposed that pB exposes more hydrophobic surface area than pG or pR.[46] This transient exposure of the hydrophobic group is consistent with the other observations such as the fluorescence study of Nile Red, which is a fluorescent hydrophobic probe.[44] From the fluorescence detection of another hydrophobic probe, ANS, it was suggested that the pB state resemble a molten globule state. The large conformational change in the pB state has been also suggested from the diffusion measurement,[28] IR and CD detection of mutants,[48,50] and small-angle X-ray scattering.[49]

Interestingly, the observed ΔC_p of pB (2.7 kJ/mol K) is close to that of pB_{dark} (2.11 kJ/mol K), which is the acid-denatured species.[42,43] This similarity might indicate a possibility that the photocreated conformation in pB may be similar to the denatured conformation. However, on the other hand, this similarity could be accidental, because pB, which may be a biological signaling state, should not have a random unfolded structure. More interestingly, the activation heat capacity change ($\Delta C_p^{\ddagger}$) of $pB^{\ddagger}$ is almost negligible (Table 1). This fact suggests that the hydrophobic part is already buried in the activation state; in other words, the burying of the hydrophobic part could be the triggering step for the pB to pG process.

It may be instructive to roughly estimate how many residues are exposed to water in the pB state. Several empirical equations for the calculation of ΔC_p upon protein unfolding have been proposed based on the changes in ASAS. For example, Myers et al. have obtained the following equation by fitting experimental data for a number of proteins;

$$\Delta C_p \ (\text{J/mol K}) = 1.17 \Delta \text{SASA}_{\text{npol}} - 0.38 \Delta \text{SASA}_{\text{pol}}$$

where $\Delta \text{SASA}_{\text{npol}}$ and $\Delta \text{SASA}_{\text{pol}}$ represent the change of SASA of nonpolar and polar residues, respectively. If we apply this equation to pB by assuming SASA change of only nonpolar residues, ΔC_p =2.7 kJ/mol K corresponds to the exposure of nonpolar residues with 2300 Å^2. If we assume the mean surface area of nonpolar residues is 93 Å^2,[66] the observed ΔC_p can be explained by the exposure of ca. 25 nonpolar residues. Although this value should be considered as just a rough estimation because of a number of assumptions we used, it may be interesting to note that the number of amino acid residues at the N terminal region, which is suggested to be important for the conformational change in pB,[67] is about 25 residues. In fact, it was found that $\Delta C_p^{\ddagger}$ for the pB→pG processes becomes almost negligible by the N-terminal truncation up to the 25th residue.[67] We need to consider the role of the N-terminal domain by using this time-resolved ΔC_p measurement in future.

Conclusion

The heat capacity changes of chemical intermediate species during chemical reaction are measured for the first time. This technique is applied to the photochemical reaction of PYP. We found that C_p of pR_2 is almost similar to that of pG, whereas C_p of pB is larger than that of pG. Combining the present data with the previous data on the enthalpy change (ΔH) as well as the thermal expansion coefficient ($\Delta \alpha_{th}$), it is concluded that the conformational fluctuation is enhanced in the pR_2 state around the equilibrium conformation, which is similar to that of pG. Considering the accumulated thermodynamical data of proteins showing the C_p increase upon protein unfolding, we conclude that the conformation of the pB species is drastically changed and several nonpolar amino acid residues are exposed to water to increase the hydrophobic interaction in pB. This technique will be a powerful tool to elucidate protein reactions.

(64) Heremans, K.; Smeller, L. *Biochim. Biophys. Acta* **1998**, *1386*, 353.
(65) Landau, L.; Lifsitz, E. *Statistical Physics, Theoretical Physics*; Pergamon Press: Oxford, 1969; Vol. 5.

(66) Miller, S.; Janin, J.; Lesk, A. M.; Chothia, C. *J. Mol. Biol.* **1987**, *196*, 641.
(67) van der Horst, M. A.; van Stokkum, I. H.; Crielaard, W.; Hellingwerf, K. J. *FEBS Lett.* **2001**, *497*, 26.