

Headline Articles

Transient Thermal Expansion of a Protein in Solution after Photo-Excitation of the Chromophore: Deoxymyoglobin

Ryoji Miyata and Masahide Terazima*

Department of Chemistry, Graduate School of Science, Kyoto University, Kyoto 606-8502

Received March 25, 2003; E-mail: mterazima@kuchem.kyoto-u.ac.jp

The energy releasing process from a photo-excited chromophore embedded in a protein structure to the surrounding protein matrix and to the solvent is studied for deoxymyoglobin by the acoustic peak delay method with the transient grating technique. The acoustic peak delay time is usually a good indicator of the solvent heating time by the nonradiative transition after the photoexcitation and should be a positive value. However, the peak delay after the photoexcitation of deoxymyoglobin was found to be −15 ps. The observed negative peak delay was interpreted in terms of interference between two acoustic waves: an acoustic wave due to the thermal expansion of the protein and a wave of the medium. A theoretical calculation suggests that most of the energy of the heme is first transferred to the protein part and subsequently dissipates into the solvent. During this energy-releasing process, the protein experiences the transient thermal expansion and it creates an acoustic wave.

There are many biological proteins that use light energy to create chemical energy or for signal transducing function. Such proteins generally have chromophores inside the proteins to absorb the light from the sun. The energy that is not stored as chemical energy or not used for the biological function should be eventually damped into the surrounding medium. If the chromophore is completely surrounded by the protein structure, the energy of the chromophore should be first passed to the protein part and the protein energy is subsequently transferred to the medium. However, if the chromophore is partially exposed to the solvent, how is the energy dissipated into the solvent; through the protein indirectly or directly into the solvent? How can we observe the route? Energy transfer from a photo-excited chromophore embedded in a protein matrix is important to understand the molecular mechanism of the damping of the light energy that is used for triggering protein dynamics. Such study has been attracting many scientists. In this paper, we report the first investigation on the energy dissipation route of deoxymyoglobin in buffer from the photo-excited chromophore (heme) to the solvent by the time-resolved transient grating (TG) method.

Myoglobin (Mb) is a small globular heme protein that consists of a single polypeptide chain of 157 amino acids and a single iron–porphyrin (heme).[1] Although Mb does not have a light driven biological function, it has a chromophore, heme, inside the protein, and a ligand of the heme is dissociated upon the photoexcitation. This photo-induced ligand dissociation is one of the most extensively studied chemical reactions of biological molecules.[1,2] At the same time, deoxyMb has been a typical model system to study the photo-physical processes among many biological proteins. When the heme of deoxyMb is photo-excited, the electronically excited heme relaxes to the ground electronic state within 3 ps nonradiatively.[3] The excess energy should be dissipated into the matrix of either protein or solvent. If the energy of the photon would be distributed among the vibrational modes of the heme, it would cause a temperature jump of the protein structure. Li and Champion modeled the thermal response of an instantaneously heated chromophore embedded in a protein as one boundary thermal transport problem.[4] They found that the numerical solutions to the model were well described by two-exponential functions. The cooling process of the heme has been studied by the hot band detection of the band Ⅲ region.[5,6] This research revealed that the temperature of the heme is cooled by time constants of 3.4 ps and 6.2 ps. A time-resolved Raman scattering experiment of carboxymyoglobin showed that the cooling of the heme is expressed by a bi-exponential function, 3.0 ps decay with an amplitude of 93% and 25 ps with 7% amplitude.[7] On the other hand, the heating process of water was investigated by Lian et al. by monitoring the IR band of water.[8] They found that heat transfer to the water is biphasic: 60% of the temperature rise occurs with a time constant of 7.5 ps and 40% occurs with a time constant of approximately 20 ps. They suggested that the faster heating process could be due to a collective energy transfer or the direct energy transfer from the heme to water, and the slower one could be due to the diffusive energy dissipation through the protein. Miller and co-workers detected the pressure wave induced by the ligand photodissociation of carboxymyoglobin and obtained energy and structural dynamics after the reaction.[9] Recently a MD simulation of

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deoxyMb was carried out to show that a part of the heme energy could be directly transferred to the medium through the carboxyl group of the heme.[10,11] However, there is no experimental information on the energy transfer route.

In this paper, we report the time-resolved acoustic measurement of deoxyMb to study the energy transport process from the photo-excited heme to the solvent. As shown in a series of our previous papers,[12–14] the heat releasing time after the photoexcitation can be measured by the peak delay time from the arrival time of instantaneously created acoustic wave and it should be a positive value. On the contrary, the peak delay time of the deoxyMb in the present study was found to be −15 ps; i.e., the acoustic peak delay of the signal was anomalously negative after the photoexcitation of deoxyMb. This negative value is explained by an interference effect of two acoustic waves created from the medium as well as from the protein thermal expansion. We believe that this is the first experimental fact that suggests the temporal heating of the protein matrix after the heme excitation and the transient thermal expansion of the protein structure due to the excess energy.

1. Experimental

The experimental setup for the picosecond time-resolved transient grating experiment is similar to that reported previously.[12–14] A pulse from a fiber laser regeneratively amplified by a YAG laser (Clark CPA2001, wavelength = 775 nm) was frequency doubled by a BBO crystal and split into two. These pulses were used as excitation beams. The laser power was ~1 μJ/pulse. These beams were slightly focused by lenses ($f = 25$ cm) and crossed at about 30° inside the quartz sample cell in order to generate an optical interference pattern. The fundamental light after the SHG crystal was used as a probe beam. The probe beam that had passed through an optical delay line was slightly focused by a lens ($f = 20$ cm) and brought into the sample cell at an angle that satisfied the Bragg condition. The diffracted TG signal was separated from the other beams with a pinhole and a glass filter (Toshiba R-60), and detected by a photomultiplier. The detected TG signal was averaged with a boxcar-integrator (EG&G Model 4400 Series) and with a personal computer. The pulse shape was monitored by the optical Kerr signal from neat carbon tetrachloride. In order to reduce the peak power of the laser pulse, the pulse width was intentionally stretched to ~2 ps. The repetition rate was 100 Hz.

Horse heart Mb dissolved in 10 mM Tris–HCl buffer (pH = 7) was put in a 1 mm path length quartz cell after removing small particles or dust in solution by filtering through a membrane filter. The sample cell was sealed with a rubber septum and the sample was deoxygenated by passing N_2 for 30 min through the sample cell. Mb was then reduced anaerobically by adding a slight excess of sodium dithionite. The stage of ligation of the protein was checked by measuring absorption spectrum with an UV–visible spectrometer (Shimadzu UV-2500 PC). The sample concentration was about 100 μM.

2. Principle

In the TG experiment, two excitation beams with a parallel polarization are crossed to create the interference pattern of the light intensity. After the photo-excitation by this light, the concentration of the photo-excited Mb was spatially modulated. When the thermal energy is released from the photo-excited molecules, the thermal expansion of the medium takes place and the pressure wave is launched. If the releasing of the thermal energy is instantaneous, the acoustic wave starts to be launched instantaneously and the arrival time of the acoustic wave is determined by the sound speed and the fringe length. On the other hand, if the thermal energy is released with a finite rate, the creation of the pressure wave is temporally delayed. The heating lifetime as well as the amount of the thermal energy from that process can be extracted by curve fitting based on a theoretical equation.

However, if the thermalization rate is very fast, the difference in the acoustic shape for different relaxation rate is very subtle and it is almost impossible to determine these quantities by fitting the TG signal on a wide time scale. Through this difficulty, we have noticed that the arrival time of the acoustic peak is also delayed and that this delay can be a good indicator of the heating time.[12] Experimental determination of the peak arrival time is much easier and more accurate than the curve fitting of the whole grating signal. The TG signal around the peak position was recorded with a rather high time resolution and the time profiles were fitted by a function of $A(t - t_i)^2 + B$, where t_i gives the peak time. The accuracy of the measurement of the peak delay time depends on several factors. First, we can find the time origin ($t = 0$) accurately enough (within 0.1 ps accuracy) by monitoring the population grating or the electronic response of the optical Kerr signal of the sample. Second, the accuracy of the peak arrival time is determined by several factors, such as the time interval (e.g., step length of the optical delay line) of the data points, the number of the data points around the peak, the shot-to-shot fluctuation of the laser power (noise of the signal), and the long-term drift of the power. We examined the accuracy of the fitting using a calculated signal of $\sin^2(t)$ with a random noise. Using similar S/N and a number of the data points as the present experimentally observed signal, we found that the peak position can be determined within an accuracy of 10% of the time interval of the data point. Hence, if we used 3 ps interval for the data collection, the accuracy of the peak arrival time is 0.3 ps, which is good enough for the discussion on the thermalization time. A larger error may be expected for a slow drift of the laser power, but it can be eliminated by a careful adjustment of the laser. We measured the TG curves at the peak part, and about 10 data sets of the curves were averaged to determine the peak position. We repeated similar measurements more than 10 times and also on different days and found that the peak delay time can be determined within an accuracy of ±2 ps.

When the acoustic damping is neglected, we have shown that the acoustic peak delay time (τ_{pd}) is almost the same as the thermalization time (τ_{temp}) under a short τ_{temp} condition. The peak delay time is almost linear in a fast heating region (~40 ps under our experimental conditions). The time delay also depends on the acoustic attenuation rate. If the attenuation constant is increased, the peak delay decreases. This effect can be easily corrected.[12] If there are several thermalization processes, τ_{pd} provides the energy weighted averaged thermalization time.

3. Results and Discussion

3.1 Acoustic Peak Delay. Figure 1 show the TG signal after the photoexcitation of deoxyMb in the buffer solution at 23 °C on two different time scales. First a TG signal appears around $t \sim 0$ and decays to the baseline within 3 ps. This signal is due to the population grating, which represents the creation and decay of the excited state of the heme. After the complete decay of the population grating signal, a temporally oscillating grating signal appears. The signal comes from the pressure wave propagation along the grating vector. To find the acoustic peak times of the first and the second peaks (t_1 and t_2), the signals around the peaks were recorded in detail (Figs. 1(b), (c)). The time profiles were fitted by a function of $A(t - t_i)^2 + B$, where A and B are constants and $i = 1$ and 2 for the first and second peaks, respectively. The acoustic frequency (ω) was calculated from these t_1 and t_2 values and a relation of $\omega = 2\pi/(t_2 - t_1)$ under a condition of a fast rate region.[12] The solid curve in Fig. 1(a) represents the calculated signal with $\omega = 8.6 \times 10^9$ s^{-1}, which reproduces the experimental signal well. The acoustic peak delay time was calculated using a relation of $\tau_{pd} = (3t_1 - t_2)/2$.[12] Measuring the peak position carefully, we determined the arrival time of the first and the second peaks within 2 ps accuracy. From these values, we found the acoustic peak delay time after the photoexcitation of deoxyMb to be -15 ± 2 ps.

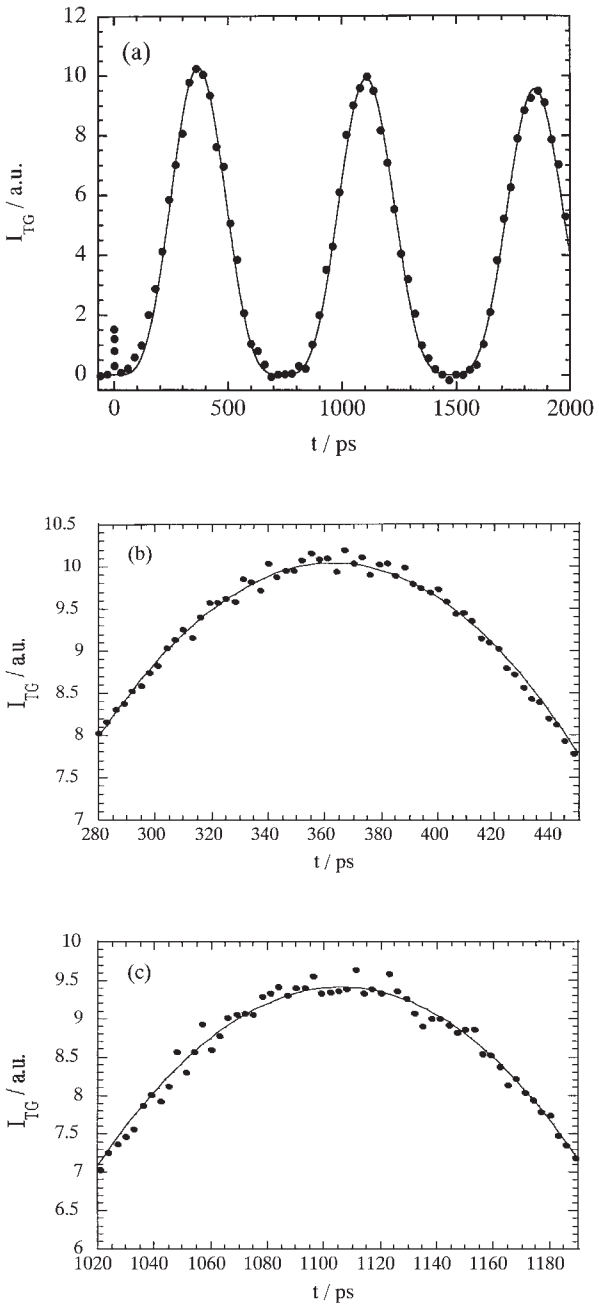

Fig. 1. (a) The observed acoustic signal after photoexcitation of deoxymyoglobin at 23 °C. The peak parts are amplified in (b) and (c). The arrival time of the peaks are determined by the fitting with a function of $A(t - t_i)^2 + B$.

In order to confirm that this negative delay time is not caused by any experimental artifact such as misalignment of the excitation beams or the probe beam, we measured τ_{pd} under the same condition just by changing the sample to betaine-30/ethanol. The observed τ_{pd} is +10 ps, which agrees well with that reported before.[13] Hence, the negative delay time is not due to any experimental artifact.

3.2 Transient Thermal Expansion of the Protein. It is rather surprising to find that the peak delay is negative, because τ_{pd} generally indicates the averaged thermalization time and it should be positive.[12] We first explain this negative value qualitatively. Since the acoustic wave cannot be launched *before* the photo-excitation, we have to consider that the negative τ_{pd} may be somehow related with the creation mechanism of the pressure wave. Here we suggest that the interference between two different sources of the acoustic waves is the cause of the observed shift of the wave phase as follow. The created electronic photo-excited state is relaxed within 3 ps to the ground state. The excess energy is damped to the surrounding protein and/or the water molecules. If the energy that is transferred to the protein part heats up the structure, the protein should experience the thermal expansion of the structure. The thermal expansion should launch the pressure wave, which creates the acoustic TG signal due to the protein expansion. At the same time, the energy that is transferred to the solvent heats up the matrix and the thermal expansion of the medium takes place. This expansion also creates the pressure wave. These two waves should interfere each other and it could be possible to create the observed anomalous negative τ_{pd}.

Next, this interpretation is quantitatively examined. The pressure wave created under a similar condition has been treated theoretically by Chen and Diebold.[15] They derived an analytical equation for describing the temporal profile of the acoustic wave under a condition that pressure waves are created by the thermal expansion of the medium and the molecular volume change by a chemical reaction. When the excited species evolves instantaneously and decays exponentially with a time constant of τ, the medium density change is given by:[15]

$$\delta(\hat{x},\hat{t}) = u(\hat{t})\cos\hat{x}\,\frac{E\alpha}{C_p}\left\{\frac{1}{1-\hat{l}_h\hat{\tau}}\right.$$
$$\times\left[\frac{-\exp(\hat{l}_h\hat{t})+\exp(-\hat{\gamma}\hat{t})[\cos\hat{t}+(\hat{\gamma}-\hat{l}_h)\sin\hat{t}]}{1+(\hat{\gamma}-\hat{l}_h)^2}\right]$$
$$+\left[-\frac{1}{1-\hat{l}_h\hat{\tau}}+\frac{C_p\beta_c}{\tilde{h}\alpha}\right]$$
$$\left.\times\left[\frac{-\exp(-\hat{t}/\hat{\tau})+\exp(-\hat{\gamma}\hat{t})[\cos\hat{t}+(\hat{\gamma}-1/\hat{\tau})\sin\hat{t}]}{1+(\hat{\gamma}-1/\hat{\tau})^2}\right]\right\} \quad (1)$$

where $\hat{t}=ckt$, $\hat{\tau}=ck\tau$, $\hat{x}=kx$, $\hat{l}_h=l_hk=D_{th}k/c$, $\hat{\gamma}=[\hat{l}_v'+(\gamma-1)\hat{l}_h]/2$, $\gamma=C_p/C_v$, $\hat{l}_v'=(\eta+3\mu/4)/\rho c$, α is the thermal expansion coefficient of the solvent, C_p is the heat capacity of the solvent at a constant pressure, $u(t)$ is the Heaviside function, E is the laser energy absorbed per unit volume, c is the sound velocity, k is the grating vector, D_{th} is the thermal diffusion constant, η is the bulk viscosity, μ is the shear viscosity, $\gamma=C_p/C_v$, and ρ is the density.

For the Mb case, the previous studies showed that the surrounded water is heated up by a bi-exponential function.[7,8] Here we assume that the thermal energy of the water matrix is expressed by

$$Q_{solv}(t) = Q_{tot}\{1-b_1\exp(-t/\tau_{b1})-b_2\exp(-t/\tau_{b2})\} \quad (2)$$

where Q_{tot} is the total energy for the heating, which should be equal to the photon energy of the excitation. Here b_i ($i=1,2$) denote the relative amplitudes for the processes with lifetimes of τ_{bi}. We further modeled that the energy transfer from the heme takes place in two routes independently: from the heme to protein (amount of the thermal energy: Q_p) and from the heme to the solvent ($Q_{tot}-Q_p$). Since the thermalization process is frequently expressed by a bi-exponential function,[4–8] the thermal energy of the heme (Q_{heme}) can be written as

$$Q_{heme}(t) = Q_p\{a_1\exp(-t/\tau_{a1})+a_2\exp(-t/\tau_{a2})\}$$
$$+(Q_{tot}-Q_p)\{b_1\exp(-t/\tau_{b1})+b_2\exp(-t/\tau_{b2})\} \quad (3)$$

If a part of the energy of the heme does not go through the protein, but goes directly into the buffer, Q_p/Q_{tot} should be less than 1.

By this heat releasing process, the acoustic wave may be calculated from Eq. 1 as

$$\delta(\hat{t}) \propto \left[\frac{-\exp(-\hat{l}_h\hat{t})+\exp(-\hat{\gamma}\hat{t})[\cos\hat{t}+(\hat{\gamma}-\hat{l}_h)\sin\hat{t}]}{1+(\hat{\gamma}-\hat{l}_h)^2}\right]$$
$$-\frac{Q_p}{Q_{tot}}a_1\left[\frac{\alpha'}{C_p'}\Big/\frac{\alpha}{C_p}\right]$$
$$\times\left[\frac{-\exp(-\hat{t}/\hat{\tau}_{a1})+\exp(-\hat{\gamma}\hat{t})[\cos\hat{t}+(\hat{\gamma}-1/\hat{\tau}_{a1})\sin\hat{t}]}{1+(\hat{\gamma}-1/\hat{\tau}_{a1})^2}\right]$$
$$-\frac{Q_p}{Q_{tot}}a_2\left[\frac{\alpha'}{C_p'}\Big/\frac{\alpha}{C_p}\right]$$
$$\times\left[\frac{-\exp(-\hat{t}/\hat{\tau}_{a2})+\exp(-\hat{\gamma}\hat{t})[\cos\hat{t}+(\hat{\gamma}-1/\hat{\tau}_{a2})\sin\hat{t}]}{1+(\hat{\gamma}-1/\hat{\tau}_{a2})^2}\right]$$
$$+b_1\left[-\frac{1}{1-\hat{l}_h\hat{\tau}_{b1}}+\frac{Q_p}{Q_{tot}}\frac{\alpha'}{C_p'}\Big/\frac{\alpha}{C_p}\right]$$
$$\times\left[\frac{-\exp(-\hat{t}/\hat{\tau}_{b1})+\exp(-\hat{\gamma}\hat{t})[\cos\hat{t}+(\hat{\gamma}-1/\hat{\tau}_{b1})\sin\hat{t}]}{1+(\hat{\gamma}-1/\hat{\tau}_{b1})^2}\right]$$
$$+b_2\left[-\frac{1}{1-\hat{l}_h\hat{\tau}_{b2}}+\frac{Q_p}{Q_{tot}}\frac{\alpha'}{C_p'}\Big/\frac{\alpha}{C_p}\right]$$
$$\times\left[\frac{-\exp(-\hat{t}/\hat{\tau}_{b2})+\exp(-\hat{\gamma}\hat{t})[\cos\hat{t}+(\hat{\gamma}-1/\hat{\tau}_{b2})\sin\hat{t}]}{1+(\hat{\gamma}-1/\hat{\tau}_{b2})^2}\right] \quad (4)$$

where $\hat{\tau}_i=ck\tau_i$ ($i=a_1, a_2, b_1, b2$), α' is the thermal expansion coefficient of Mb, and C_p' is the heat capacity of Mb.

First, we consider one of the extreme cases that all of the electronic energy is transferred to the thermal mode of the medium directly, not through the protein. In this case, the acoustic wave should be the same as in the case that the electronic energy of a relatively small organic molecule exposed in medium is transferred to the solvent. In this case, Eq. 4 is simplified and the acoustic wave is expressed by

$$\delta(\hat{t}) \propto \left[\frac{-\exp(-\hat{l}_h\hat{t})+\exp(-\hat{\gamma}\hat{t})[\cos\hat{t}+(\hat{\gamma}-\hat{l}_h)\sin\hat{t}]}{1+(\hat{\gamma}-\hat{l}_h)^2}\right]$$
$$+b_1\left[-\frac{1}{1-\hat{l}_h\hat{\tau}_{b1}}\right]$$
$$\times\left[\frac{-\exp(-\hat{t}/\hat{\tau}_{b1})+\exp(-\hat{\gamma}\hat{t})[\cos\hat{t}+(\hat{\gamma}-1/\hat{\tau}_{b1})\sin\hat{t}]}{1+(\hat{\gamma}-1/\hat{\tau}_{b1})^2}\right]$$
$$+b_2\left[-\frac{1}{1-\hat{l}_h\hat{\tau}_{b2}}\right]$$
$$\times\left[\frac{-\exp(-\hat{t}/\hat{\tau}_{b2})+\exp(-\hat{\gamma}\hat{t})[\cos\hat{t}+(\hat{\gamma}-1/\hat{\tau}_{b2})\sin\hat{t}]}{1+(\hat{\gamma}-1/\hat{\tau}_{b2})^2}\right] \quad (5)$$

In this case, the peak delay time should be positive. When we use $b_1=0.6$, $\tau_{b1}=7.5$ ps, $b_2=0.4$, and $\tau_{b2}=20$ ps,[8] τ_{pd} is calculated to be 12 ps. In fact, we have experimentally shown that the peak delay time is in a range of 5–20 ps for most of the organic molecules in an organic solvent.[12–14] Hence this energy transfer process cannot explain the observed result as long as only the heating of solvent is considered.

Second, we calculate τ_{pd} for the other extreme case: all of the excited energy is transferred from the heme to the protein ($Q_p/Q_{tot}=1$). The acoustic wave created by the protein part is depicted in Fig. 2. The acoustic signal initially shows the expansion wave and then the compressed wave. When the acoustic wave from the medium interferes with this wave, the arrival time of the peak shifts to an earlier time. For a quantitative calculation, since we have no knowledge of a_i or τ_{ai} ($i=1,2$), we assumed that the time profile for the energy transfer from the heme is given by the time profile of the heme cooling monitored for carboxymyoglobin ($a_1=0.93$, $\tau_{a1}=3$ ps, $a_2=0.07$, $\tau_{a2}=25$ ps).[7] Figure 3 depicts the τ_{pd} as a function of the parameter of $R=$

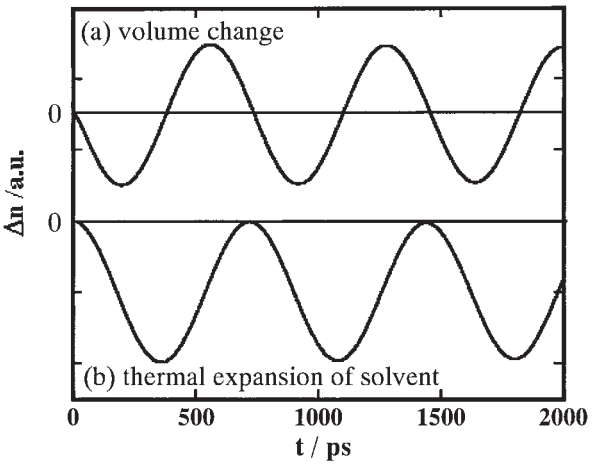

Fig. 2. Calculated acoustic signal due to the thermal expansion of the protein (a) and the medium (b).

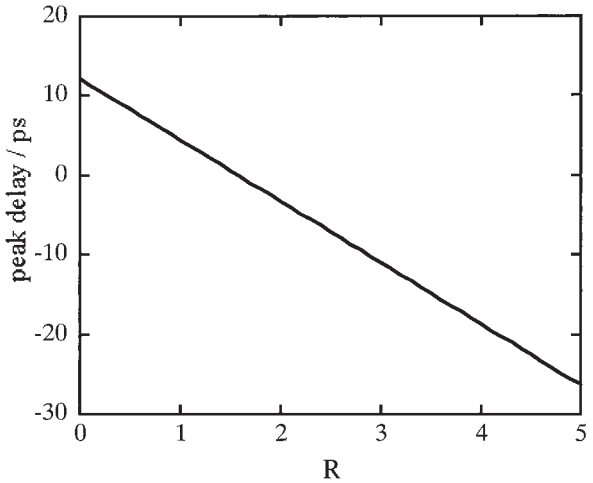

Fig. 3. Peak delay time (τ_{pd}) as a function of $R = (\alpha'/C_p')/(\alpha/C_p)$ under a condition that all of the excited energy of the heme is transferred to the protein.

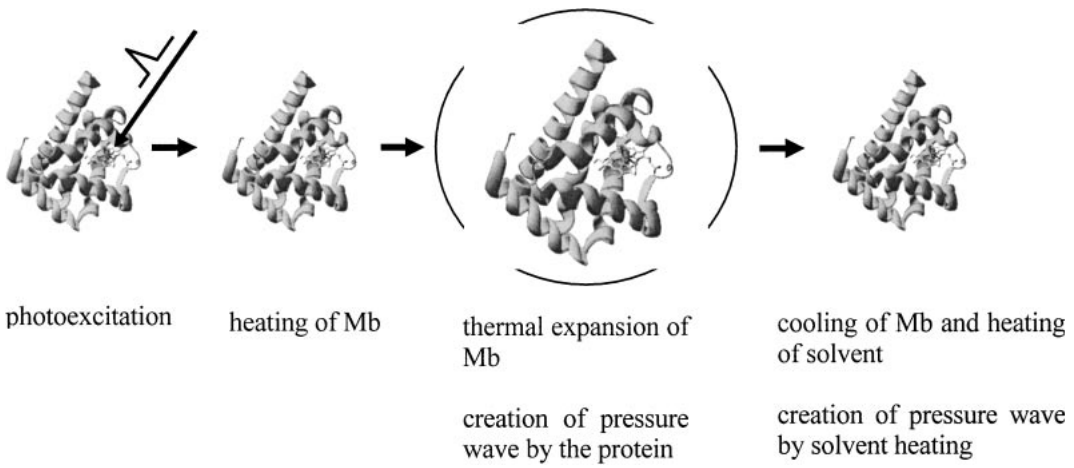

Fig. 4. Schematic illustration of the energy releasing process from the photoexcited heme to the solvent. The energy of the heme is first transferred mainly to the protein part and then flows into the solvent. During the transient temperature rise by this energy transfer, the protein structure is expanded, which creates the pressure wave.

$(\alpha'/C_p')/(\alpha/C_p)$ with these parameters. Using the thermal expansion coefficient (345×10^{-6} K^{-1})[16] and heat capacity (1.36 $J\,K^{-1}\,g^{-1}$)[17] of Mb measured under the steady state condition; $R = 4.1$, we found that τ_{pd} is largely negative (-19.4 ps), which is rather close to the experimentally observed value (-15 ps).

If a part of the energy is transferred to the aqueous solution and the protein matrix simultaneously, the expected peak delay time should be the average of the positive value (former contribution, 12 ps) and the negative value (latter contribution; -19.4 ps). In order to reproduce the observed τ_{pd} by changing Q_p/Q_{tot}, we found that about 17% of the energy should be transferred to the water first. This result strongly supports a mechanism that most of the photon energy absorbed by the heme is first transferred to the protein matrix and then the energy is dissipated to the aqueous solution (Fig. 4).

In summary, we measured the acoustic peak delay time after the photoexcitation of deoxymyoglobin by the transient grating technique, and found that τ_{pd} was -15 ps. Such a negative delay has not been observed before. The anomalous negative peak delay was explained in terms of the interference of two acoustic waves created by the thermal expansion of the protein and the medium. We calculated the expected peak delay time using a theory by Chen and Diebold.[15] Assuming that all of the photon energy is first transferred to the protein matrix, we found that the observed peak delay is almost reproduced by the calculated one with the reported thermal expansion coefficient (345×10^{-6} K^{-1}) and heat capacity (1.36 $J\,K^{-1}\,g^{-1}$) of Mb measured under the steady state condition. Although there have been several reports about the protein structural changes after photochemical reactions, this is the first experimental fact that suggests the transient thermal expansion of any protein by the energy released from the photo-excited chromophore without any photochemical reaction. This technique can be used for other proteins and we will study the energy transfer process from the chromophore to the protein matrix from a point of view of the protein volume expansion.

References

1 E. Antonini and M. Brunori, in "Hemoglobin and Myoglobin in Their Reaction with Ligands," North Holland, Amsterdam (1971).

2 a) J. S. Olson and G. N. Phillips, Jr., *J. Biol. Chem.*, **271**, 17593 (1996). b) E. R. Henry, W. A. Eaton, and R. H. Hochstrasser, *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 8982 (1986).

3 J. W. Petrich, C. Poyart, and J. L. Martin, *Biochemistry*, **27**, 4049 (1988).

4 P. Li and P. M. Champion, *Biophys. J.*, **66**, 430 (1994).

5 M. Lim, T. A. Jackson, and P. A. Anfinrud, *J. Phys. Chem.*, **100**, 12043 (1996).

6 T. A. Jackson, M. Lim, and P. A. Anfinrud, *Chem. Phys.*, **180**, 131 (1994).

7 Y. Mizutani and T. Kitagawa, *Science*, **278**, 443 (1997).

8 Y. Lian, B. Locke, Y. Kholodenko, and R. M. Hochstrasser, *J. Phys. Chem.*, **98**, 11648 (1994).

9 J. Deák, H.-L. Chiu, C. M. Lewis, and R. J. D. Miller, *J. Phys. Chem. B*, **102**, 6621 (1998); L. Richard, L. Genberg, J. Deak, H.-L. Chiu, and R. J. D. Miller, *Biochemistry*, **31**, 10703 (1992).

10 D. E. Sagnella and J. E. Straub, *J. Phys. Chem. B*, **105**, 7057 (2001).

11 I. Okazaki, Y. Hara, and M. Nagaoka, *Chem. Phys. Lett.*, **337**, 151 (2001).

12 a) M. Terazima, M. Takezaki, S. Yamaguchi, and N. Hirota, *J. Chem. Phys.*, **109**, 603 (1998). b) T. Okazaki, N. Hirota, and M. Terazima, *J. Chem. Phys.*, **110**, 11399 (1999). c) N. Saga, Y. Kimura, N. Hirota, and M. Terazima, *Chem. Phys. Lett.*, **332**, 496 (2000).

13 M. Terazima, *Chem. Phys. Lett.*, **305**, 189 (1999).

14 M. Terazima, *Bull. Chem. Soc. Jpn.*, **74**, 595 (2001).

15 H. Chen and G. J. Diebold, *J. Chem. Phys.*, **104**, 6730 (1996).

16 H. Frauenfelder, H. Hartmann, M. Karplus, I. D. Kuntz, Jr., J. Kuriyan, F. Parak, G. A. Petsko, D. Ringe, R. F. Tilton, Jr., M. L. Connolly, and N. Max, *Biochemistry*, **26**, 254 (1987).

17 P. L. Privalov, E. I. Tiktopulo, S. Yu. Venyaminov, Yu. V. Griko, G. I. Makhatadze, and N. N. Khechinashvili, *J. Mol. Biol.*, **205**, 737 (1989).