

Real-Time Observation of Conformational Fluctuations in Zn-Substituted Myoglobin by Time-Resolved Transient Hole-Burning Spectroscopy

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ABSTRACT Equilibrium fluctuations of the protein conformation have been studied in myoglobin by a novel method of time-resolved transient hole-burning spectroscopy over a temperature range of 180–300 K and a time range of 10 ns to 10 ms. The temporal shift of the hole spectrum has been observed in a wide temperature region of 200–300 K. It has been found that the time behavior of the peak position of the hole is highly nonexponential and can be expressed by a stretched exponential function with a β value of 0.22. As compared with the results for a dye solution sample, the time scale of the fluctuation of the protein conformation is much more weakly dependent on temperature. The time scale of the observed conformational dynamics shows a temperature dependence similar to that associated with the ligand escape process of myoglobin.

INTRODUCTION

The time-domain observation of conformational dynamics of protein molecules is important for the understanding of their biological functioning. The conformational dynamics has been taken into account in the analysis of the ligand rebinding kinetics of heme proteins (Austin et al., 1975; Steinbach et al., 1991). In the case of myoglobin (Mb), it has been revealed that that protein's conformational dynamics affects its reaction kinetics in various time scales. In the so-called geminate phase of the ligand rebinding process, the conformational relaxations of Mb affect the rebinding kinetics by perturbing the barrier height of the rebinding process (Agmon and Hopfield, 1983; Steinbach et al., 1991). In a longer time scale, on the other hand, the ligand molecule escapes into the solvent, which requires the conformational fluctuations of Mb (Case and Karplus, 1979; Tian et al., 1993).

Two types of conformational dynamics have been proposed in Mb (Ansari et al., 1985; Steinbach et al., 1991). One is the functionally important motion, which is a non-equilibrium conformational motion occurring in the course of the protein reaction, leading from one state to another. The other is the equilibrium fluctuation, which occurs in resting proteins and corresponds to the interconversion among many metastable conformations. Both of these dynamics have been considered to affect the ligand rebinding kinetics of Mb (Steinbach et al., 1991). So far, nonequilibrium conformational motions of heme proteins triggered by cutting the ligand-heme bond have been studied extensively by several experimental approaches (Lambright et al., 1991; Ansari et al., 1992; Jackson et al., 1994; Huang et al., 1997).

In contrast, there are only a few experimental reports on the direct time-domain observation of equilibrium conformational fluctuations of proteins, such as those observations made by means of time-resolved fluorescence (TRF) (Pierce and Boxer, 1992; Murakami and Kushida, 1996) and three-pulse photon echo techniques (Thorn Leeson and Wiersma, 1995). These experiments do not cover the whole time range and temperature range of interest, because the time range of the fluorescence method is limited by the excited-state lifetime, whereas the temperature range of the photon echo experiment is limited by the shortening of the dephasing time with increasing temperature. Because the protein dynamics are known to extend over many orders of magnitude of time, and because the reaction kinetics of proteins shows complicated temperature dependence in a wide temperature region (Austin et al., 1975; Steinbach et al., 1991), observations over both a wide time range and a wide temperature range are necessary.

In this paper we report the first real-time observation of the equilibrium conformational fluctuations in a chromoprotein over a wide time range of 10 ns to 10 ms and a temperature range of 180 to 300 K, using time-resolved transient hole-burning (TRTHB) spectroscopy (Shibata et al., 1996). Some of the results have already been reported briefly (Shibata et al., 1997). In a chromoprotein, the optical transition energy of the chromophore is dependent on the conformation of the protein. Hence, by the irradiation of monochromatic light, proteins with a certain conformation are selectively excited, and a hole is formed in the absorption spectrum. The hole spectrum changes with time because of the conformational fluctuation of the unexcited molecules. Therefore, if we measure the hole spectrum as a function of time after the excitation, we can obtain information on the conformational fluctuations of the protein in the electronic ground state. In the Zn-protoporphyrin-substituted myoglobin (ZnPP-Mb) that we employed, the intersystem crossing from the singlet S_1 state to the triplet T_1 state has a high efficiency and the lifetime of the triplet state

Received for publication 5 June 1997 and in final form 20 March 1998.

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0006-3495/98/07/521/07 \$2.00

is rather long (15–10 ms at 200–300 K). Therefore, the hole remains for a long time, and the conformational dynamics over a very wide time range from a few nanoseconds up to several tens of milliseconds can be studied by this technique. Furthermore, because the hole-burning efficiency is high even at room temperature, this technique can be used over a wide temperature range from cryogenic temperatures to room temperature, or even to higher temperatures.

PRINCIPLE OF MEASUREMENT

Here we explain the principle of the present TRTHB method on the basis of the model depicted in Fig. 1, where Q is the configuration coordinate denoting the conformation of the protein. It is known that the conformational energy surfaces of a protein molecule have a large number of local energy minima, i.e., the so-called conformational substates. We assume that these minima are on the curves $U_g(Q)$ and $U_e(Q)$ for the electronic ground and excited states of the chromophore, respectively. At very low temperatures, the atoms constituting the molecule vibrate around the equilibrium positions corresponding to the local energy minima, and a protein molecule behaves like a glass. The coupling of these vibrations of various modes to the electrons of the chromophore, as well as the finite lifetime of the excited state, causes the homogeneous broadening of the absorption spectrum due to the optical transition from the ground state to the excited state of the chromophore. At high temperatures, on the other hand, thermally induced conformational transitions among these local energy minima are not negligible, and a protein molecule behaves like a liquid. This

type of conformational change corresponds to the transition between the two points on $U_g(Q)$ along the Q axis.

Under the irradiation of a monochromatic light pulse, the molecules with a certain value of Q are selectively excited, although the selectivity is reduced by the homogeneous broadening of the absorption spectrum. When some of the excited molecules are transferred to the T_1 state, a hole is burned in the ground-state population distribution until the molecules accumulated in the T_1 state relax to the ground state. This hole can be observed as the difference between the absorption spectra before and after the monochromatic light excitation. As a result of the conformational fluctuation of the unexcited molecules, the hole profile varies as time passes, and its peak position is expected to approach that of the absorption spectrum before the burning, because the thermal equilibrium distribution is restored with time. Thus, by detecting the time evolution of the hole spectrum, one can obtain information concerning the conformational fluctuation dynamics of molecules in the ground state.

It should be noted that the dynamics observed in this TRTHB method is essentially that due to the equilibrium fluctuation process in the ground state. This is because one observes the conformational dynamics of the unexcited molecules, which do not undergo perturbation by the burning pulse. It is also possible to consider the observed process as corresponding to the time evolution of the nonequilibrium population distribution produced by the burning. However, this time evolution is strictly dominated by the equilibrium fluctuation process among the conformational substates. This is one of the remarkable characteristics of this method as compared with the flash photolysis measurement in which the relaxation from the ligated to the unligated state is observed.

The difference between the absorption spectra without and with the burning pulse obtained in the present experiment also contains the triplet-triplet (T-T) absorption component, which reflects the conformational dynamics in the triplet state. As mentioned later, however, the T-T absorption peak in the sample we employed was found to be located far from the spectral region of our interest. Therefore, the effect of the T-T absorption can be neglected in our experiment. This is a conspicuous advantage of our nanosecond to millisecond TRTHB method as compared with the femtosecond to picosecond TRTHB technique, in which the stimulated emission component generally appears in the same spectral region as the hole.

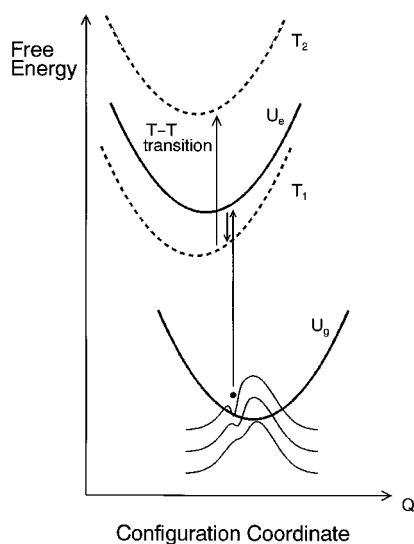


FIGURE 1 The schematic description of the TRTHB measurement. Q is the configuration coordinate denoting the conformation of the protein. U_g and U_e (—) are the envelopes of the local energy minima of the conformational potential surfaces for the electronic ground and the excited state of the chromophore, respectively. T_1 and T_2 (---) are the lowest and a higher electronic triplet state, respectively.

EXPERIMENTAL PROCEDURES

The preparation of ZnPP-Mb was described in a previous paper (Kurita et al., 1990). ZnPP-Mb was dissolved in a water-glycerol mixture with a volume ratio of 1:3 with a concentration of ~ 0.3 mM (ZnPP-Mb-W:3G). In Fig. 2, we compare the far-UV circular dichroism (CD) spectrum of ZnPP-Mb with that of the native met-Mb. The two samples show almost the same spectra, which suggests that the secondary structure of Mb is not altered substantially by the Zn substitution. The sample solution was sealed in a glass cell with an optical path length of 2 mm, and its temperature was controlled within ± 1 K accuracy in a N_2 gas flow-type cryostat. The hole

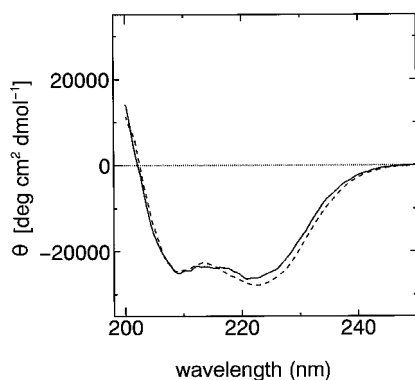


FIGURE 2 The circular dichroism spectrum of ZnPP-Mb in pH 6.0 buffered water (—) and that of native Mb-solution (---) at room temperature.

was burned in the low-energy tail of the $Q(0,0)$ absorption band around 590 nm with a dye laser (Scanmate using rhodamine B dye with a pulse duration of ~ 7 ns; Lambda Physik) pumped by a frequency-doubled and Q -switched Nd:YAG laser (Spectra Physics GCR-130). In our previous work (Shibata et al., 1996) we also tried to observe the temporal change in the hole spectra. In that case, however, no temporal change was observed over the 1–100 μ s time range, probably because the burning was too close to the peak of the absorption spectrum. In the present study, the burning wavelength was carefully chosen so that the optical density was 20–30% of the absorption maximum at the burning wavelength. Because the peak energy of the absorption spectrum is temperature-dependent, we adjusted the burning wavelength at each temperature. We burned a hole also in the high-energy tail of the Q -band. In this case, however, the observed hole shift was much smaller than that observed in the low-energy excitation case because the site selectivity is very small, owing to the presence of a sideband in the high-energy side (see Results and Analysis). In the following, therefore, we discuss only the experimental results in the low-energy excitation case.

For the probe light, we used spontaneous emission from a dye solution (rhodamine 6G in ethanol with a concentration of ~ 0.3 g/liter) pumped by the frequency-doubled light of a Q -switched Nd:YLF laser (Spectra Physics, TFR) with a pulse duration of ~ 7 ns. The temporal resolution of our measurement of ~ 10 ns was limited by the pulse durations of the burning and the probe light. The spectrum of the light transmitted through the sample was measured by a triple polychromator (Spex 1877 Triplemate) and a CCD camera system cooled by liquid nitrogen (Photometrics AT200). The transient hole-burning spectrum at time t_d after the burning was obtained as

$$H(\nu, t_d) \equiv A'(\nu, t_d) - A(\nu) \quad (1)$$

$$= \log_{10} \left[\frac{I_{\text{THB}}(\nu, t_d) - I_{\text{laser}}(\nu)}{I_{\text{abs}}(\nu)} \right].$$

Here, $A(\nu)$ is the ordinary absorption spectrum before the burning, and $A'(\nu, t_d)$ is the absorption spectrum at t_d after the burning. Furthermore, I_{THB} , I_{laser} , and I_{abs} are the intensity of light transmitted through the sample under the irradiation of both burning and probing pulses, that under the burning pulse, and that under the probe pulse, respectively. We obtained the hole spectrum free from rotational relaxations of the chromophore by correcting the data as

$$H(\nu, t_d) = \{H_{\parallel}(\nu, t_d) + 2H_{\perp}(\nu, t_d)\}/3, \quad (2)$$

where H_{\parallel} and H_{\perp} are the hole spectra measured using probe pulses polarized in the parallel and perpendicular directions with respect to the polarization of the burning light, respectively. It is possible to extract the Q_x and Q_y components of the absorption band from the analysis of the H_{\parallel} and H_{\perp} hole spectra. The details have been reported in a separate paper

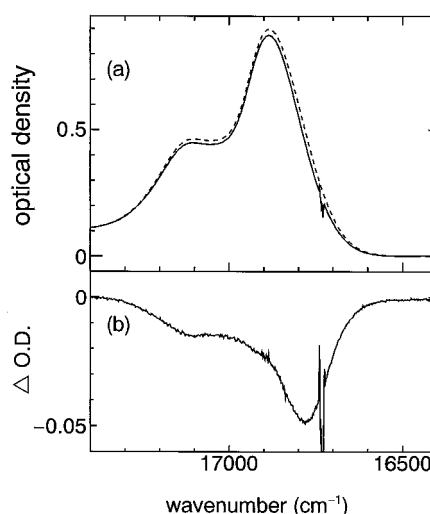


FIGURE 3 (a) The ordinary absorption spectrum before the burning, A (---), and the absorption spectrum under the burning, A' (—) at 200 K. The latter spectrum is corrected by subtracting I_{laser} . (b) The difference spectrum between the two spectra in a.

(Shibata and Kushida, 1998). Fig. 3 shows a typical hole spectrum together with $A(\nu)$ and $A'(\nu)$ at 200 K. Because the burning light pulse is rather intense, the hole spectrum is considered to be distorted around the burning wavenumber despite the subtraction of $I_{\text{laser}}(\nu)$ because of the fluctuation in the scattered light intensity. Although the difference between A and A' is very small, it is obvious that the hole is shifted from the position of the ordinary absorption spectrum to the low-energy side, where the burning was made.

We also obtained the T-T absorption spectrum of ZnPP-Mb by the same procedure as that in the literature (Tsvirko et al., 1973). The obtained spectrum was found to be similar to those reported for various metal porphyrins by Tsvirko et al., and is peaked around 470 nm. Because only a small low-energy tail of the T-T absorption band overlaps the hole spectrum shown in Fig. 3, we can ascribe the temporal variation of the observed hole spectrum to the conformational dynamics in the ground state.

The time interval t_d between the burning and probing pulses was varied logarithmically from 10 ns to 10 ms. We took precautions that the hole depth would not exceed 5% of the absorption at the burning wavenumber to avoid a saturation effect, and the typical burning energy density employed was ~ 1 mJ/cm² per pulse. Here we discuss the heating effect of the sample by the burning light. The heat diffusion process from the excited chromophore into the surrounding matrix is inferred to be completed within a few hundred picoseconds from the heat conductivity of the sample. This is much faster than the time scale of our measurement. Furthermore, the temperature rise created by a burning laser pulse is estimated to be very small. We consider the case in which a single burning pulse with an energy density of 1 mJ/cm² irradiates a sample area of ~ 0.05 cm². If the energy of the burning pulse is completely transformed into heat energy, a volume of 0.01 cm³ in the sample obtains a heat energy of 0.05 mJ from a single burning pulse. Using the specific heat of the sample, ~ 1 J \cdot K⁻¹ \cdot g⁻¹, the rise in the sample temperature caused by a burning pulse is estimated to be only 5 mK. Thus the influence of the heating effect is considered to be negligible in our experiment.

RESULTS AND ANALYSIS

Protein solution sample

The solid lines in Fig. 4 show a temporal variation of the TRTHB spectrum of ZnPP-Mb-W:3G at 240 K. The hole

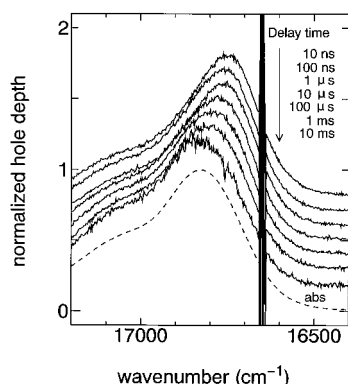


FIGURE 4 Time-resolved transient hole-burning spectra of ZnPP-Mb in a water-glycerol mixture at 240 K. The hole depths are normalized to unity, and the spectra are offset vertically to avoid overlap. The excitation wavenumber is $16,653 \text{ cm}^{-1}$. The dashed line denotes the ordinary absorption spectrum whose height is also normalized to unity.

spectrum has a broad width even just after the burning. This hole width is induced by the fluctuation process, occurring in a time scale faster than the temporal resolution of 10 ns. As reported in our previous paper (Shibata et al., 1996), the hole spectra at 1 ms after burning are rather broad even below 180 K; this profile can be reproduced fairly well by considering the interaction between the chromophore and the low-frequency vibrational modes of Mb. At temperatures higher than 200 K, on the other hand, the hole spectrum at $t_d = 1 \text{ ms}$ is shifted to the low-energy side and has a larger width compared with that calculated under the assumption of no diffusive conformational fluctuation. This deviation was ascribed to the presence of diffusive conformational fluctuations at these temperatures. In Fig. 4, despite the large hole width, it is clear that the main peak of the TRTHB spectrum approaches that of the ordinary absorption spectrum with increasing t_d , and a shift in the hole spectrum, amounting to $\sim 60 \text{ cm}^{-1}$, is observed over the time range of 10 ns to 10 ms. A time-dependent hole broadening also occurs, although it is not clear in Fig. 4. A sideband is seen in the hole spectrum around $17,100 \text{ cm}^{-1}$, which is assigned to the absorption from the ground state into a higher-energy excited state. This sideband shows little time-dependent shift at all temperatures examined, probably because the homogeneous width of this band is much broader than that of the main peak.

To characterize the conformational fluctuation observed in our experiment, we introduce a function $C(t_d)$, which is the difference between the first moment of the ordinary absorption spectrum and that of the time-resolved hole spectrum. This function is expected to tend to zero with time as the deformed distribution of the conformation due to the burning approaches the thermal equilibrium distribution, as discussed above under Principle of Measurement. From the temporal evolution of $C(t_d)$, one can deduce the time scale of the conformational fluctuation of the protein in the electronic ground state (Kinoshita, 1989). To reduce the influence of the drift in the tail region of the spectrum, we

calculated the first moments of the TRTHB and absorption spectra over a limited wavenumber range. We selected the spectral positions where the height of the spectrum is 20% and 80% of its maximum as the low- and high-energy limits of the calculation, respectively. Thus we excluded the sideband around 17100 cm^{-1} from the calculation. Furthermore, we eliminated the distortions due to the laser scattering in the observed TRTHB spectra by approximating the spectra around the laser wavelength with appropriate quadratic functions.

Fig. 5 *a* shows the temporal evolution of $C(t_d)$ for ZnPP-Mb-W:3G at various temperatures. At 180 K, $C(t_d)$ is almost time-independent, except for the time region of $t_d \geq 1 \text{ ms}$. In this time region, the population accumulated in the T_1 state begins to relax to the ground state, and this hole-filling process affects the hole spectrum through the fast conformational relaxation in the excited state. Therefore, it is difficult to determine whether the temporal change observed in $C(t_d)$ for $t_d \geq 1 \text{ ms}$ is due to the conformational fluctuations in the excited state or in the ground state. If the former is the case, our result is consistent with the interpretation that the glass-like transition of ZnPP-Mb in the ground state occurs around 180 K (Ahn et al., 1995).

Above 200 K, $C(t_d)$ shows highly nonexponential t_d dependence over a time range of 10 ns to 10 ms, and the time scale of the change in $C(t_d)$ becomes faster with increasing temperature. Nonexponential time evolution has been observed in a great variety of glasses and proteins, and is often reproduced well by some empirical expressions, such as a stretched exponential function, $\exp[-(t_d/\tau_r)^\beta]$, where $0 < \beta < 1$ (Steinbach et al., 1991; Jäckle, 1986; Iben et al., 1988). We fitted our data to a stretched exponential form,

$$C(t_d) = A + B \exp[-(t_d/\tau_r)^\beta], \quad (3)$$

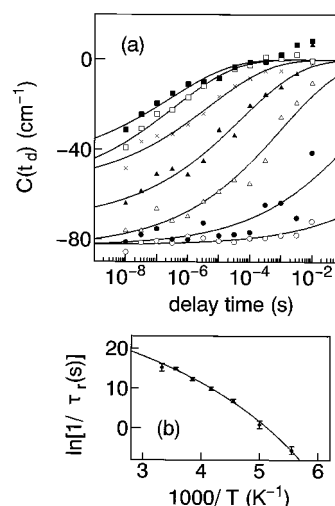


FIGURE 5 (a) The time evolution of $C(t_d)$ for ZnPP-Mb in a water-glycerol mixture at 180 K (○), 200 K (●), 220 K (△), 240 K (▲), 260 K (×), 280 K (□), and 300 K (■). The solid lines are the fitting curves to Eq. 3 obtained by the least-squares method. (b) The temperature dependence of τ_r (points with error bars) and its fitting curve to Eq. 4 (—).

where A and B are the parameters adjusting the value at $t_d \gg \tau_r$ and the amount of the time-dependent shift of $C(t_d)$, respectively. Here we call τ_r the correlation time of the conformational fluctuation of Mb. The solid lines in Fig. 5 *a* are the fitting curves of the data to Eq. 3 for $\beta = 0.22$ and $A = 0$. They reproduce the observed data well. In the fitting, the data points of $C(t_d)$ for $t_d = 10$ ms were neglected, because the shape of $C(t_d)$ in this time region is probably affected by the hole filling. The time dependence of the width of the hole spectrum has also been reproduced well by using the same correlation function with the same values of β and τ_r . This will be discussed in detail in a separate paper.

We plot $(1/\tau_r)$ versus $1000/T$ in Fig. 5 *b*, where it is clear that the temperature dependence of τ_r is no longer expressed by a simple Arrhenius law. In glassy materials, the temperature dependence of τ_r is known often to obey the relation $\tau_r = \tau_0 \exp[(T_0/T)^2]$, or the Vogel-Fulcher law,

$$\tau_r = \tau_0 \exp[E/(T - T_0)], \quad (4)$$

rather than a simple Arrhenius law (Steinbach et al., 1991; Jäckle, 1986; Iben et al., 1988). We fitted the temperature dependence of the determined τ_r to these relations. The best fit was obtained for the Vogel-Fulcher expression (the *solid line* in Fig. 5 *b*), with $\tau_0 = 1.14 \times 10^{-13}$ s, $E = 2670$ K, and $T_0 = 105$ K.

Dye solution sample

We also made a control experiment using a dye solution. A rhodamine-like dye, merbromin, was dissolved in the same solvent of a water-glycerol mixture as the protein solution sample (Mm-W:3G), and the hole was burned with the frequency-doubled light (532 nm, pulse duration ~ 7 ns) of a Q -switched Nd:YAG laser. In this measurement, we used a Xe flashlamp (Hamamatsu photonics L4633-01) with a pulse duration of ~ 1 μ s for the probing light source, because the absorption band of merbromin is so broad that it cannot be covered by spontaneous emission from a dye solution. Hence the temporal resolution of this measurement is ~ 1 μ s. The temporal change in $C(t_d)$ was found to be observed in a narrower temperature range with stronger temperature dependence of the correlation time compared with the case of ZnPP-Mb-W:3G. That is, as shown in Fig. 6 *a*, the temporal change in $C(t_d)$ in the case of Mm-W:3G was observed only in a limited temperature region of 200–250 K (only the data at every 20 K are shown in Fig. 6 *a*). In this temperature region, the observed data were well reproduced by a stretched exponential function with $\beta = 0.56$. This value for β is roughly the same as that determined from a dielectric dispersion measurement in a water-glycerol mixture (Abe et al., 1990). Therefore, $C(t_d)$ for this sample is considered to be determined by the configurational fluctuations of the solvent around the dye molecule.

We fitted the temperature dependence of τ_r for Mm-W:3G in the 200–250 K temperature range to the Vogel-Fulcher law. Although it has been suggested theoretically

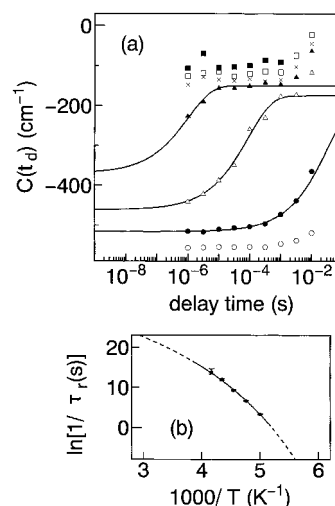


FIGURE 6 (a) The time evolution of $C(t_d)$ for merbromin in a water-glycerol mixture. The meanings of the symbols and the solid lines are the same as in Fig. 5. The dashed lines are the fitting curves to $C(t_d)$ obtained using τ_r estimated through Eq. 4. (b) Arrhenius plot of τ_r over the temperature range of 200–250 K. The solid line is the fitting curve to Eq. 4, and the dashed line is its extension to the temperature region in which the temporal change in $C(t_d)$ was not observed.

that the correlation time obtained by TRTHB or TRF measurements differs slightly from τ_r determined by the dielectric dispersion measurements (Bagchi et al., 1984), we assume here a similar temperature dependence for the time constants obtained by both measurements. Thus, in this fitting, we employed the value of 120 K for T_0 , which has been used to reproduce the results of the dielectric measurement (Abe et al., 1990). The solid line in Fig. 6 *b* is the fitting curve, which reproduces well the experimental data. τ_0 and E were obtained as 1.48×10^{-15} s and 2466 K, respectively.

DISCUSSION

Comparison among various time scales

Comparison of the time evolution of $C(t_d)$ of ZnPP-Mb-W:3G with that of Mm-W:3G leads to some interesting observations. That is, the conformational fluctuations of Mb occur on a much wider time scale than those of water-glycerol mixture. Around room temperature, the time scale of the conformational fluctuations of solvent seems to be much shorter than that of Mb molecules. Assuming that the Vogel-Fulcher law is still valid outside of the temperature range of 200–250 K for Mm-W:3G, we estimate that the time of the configurational fluctuation of the solvent at 280 K lies in the nanosecond scale. In ZnPP-Mb-W:3G, on the other hand, a slow fluctuation process on the microsecond time scale exists even at 280 K. Thus, around room temperature, the time scales of the structural fluctuations of Mb and the solvent are inferred to be separated from each other. When the temperature is decreased, this separation of the time scale becomes vague. Below ~ 200 K, these two time

scales become almost the same, which suggests that the diffusive structural motions of Mb and the solvent occur as one body. Furthermore, these structural fluctuations of both samples become much slower than the experimental time scale of 10 ms around almost the same temperature of 180 K.

This fact supports the idea that the glass-like transition of Mb in water-glycerol mixture around 180 K is caused by the freezing of the solvent molecules around a Mb molecule. The solvent effect on the freezing of the conformational dynamics of proteins has been intensively discussed so far. Flash photolysis studies on MbCO for a wide variety of solvent viscosity and in a solid matrix have clarified that the solvent friction, rather than the protein's internal friction, plays an important role in the suppression of the conformational dynamics of proteins (Ansari et al., 1992; Hagen et al., 1995). Similar results have also been derived from the temperature dependence of the relative spectral area of three CO stretching bands of MbCO (Ansari et al., 1987). Therefore, the glass-like transition of Mb in solution around 180 K is probably attributed to the freezing of the solvent. The present study gives the first direct comparison of the equilibrium structural fluctuations of proteins and the surrounding solvent molecules, which supports the idea of the solvent-induced glass-like transition of Mb.

It is also interesting to compare the conformational dynamics observed in the present study with the nonequilibrium conformational dynamics observed so far. Steinbach et al. (1991) introduced the conformational relaxation function $\Phi^*(t)$ to reproduce the temperature dependence of the geminate phase of the ligand rebinding kinetics. They employed the expression $\tau_r = A \exp[(E/T)^2]$ with $A \approx 10^{-18}$ s and $E \approx 1200$ K for the temperature dependence of the relaxation time of $\Phi^*(t)$. Jackson et al. (1994) made direct observation of the temporal shift of the charge transfer band near 760 nm (band III) of MbCO triggered by cutting the ligand-heme bond at 301 K. For both cases, the conformational relaxation time at room temperature was estimated at $\sim 10^{-11}$ s, which is four orders of magnitude shorter than the correlation time estimated in the present study. A much slower time constant of the dynamics studied in the present work may suggest that the process observed here corresponds to a conformational motion of a much larger scale. In the case of band III measurement, the observed conformational dynamics has been interpreted to correspond to the iron motion relative to the plane of the porphyrin (Jackson et al., 1994). Very recently, the temporal variation in the band III after photolysis has been investigated in more detail, and the existence of a slower process has been disclosed (Huang et al., 1997). Although these investigations have mainly been made for a hemoglobin sample, a precise comparison between this slow process and the process observed here is considered to be important and is now in progress.

It has been also pointed out that there exists a slower conformational relaxation process that is associated with the escape of the ligand molecule from the heme pocket of Mb.

The double-pulse flash photolysis experiments (Tian et al., 1996) and the transient IR measurements (Young et al., 1991) have given the time scale of this conformational process in a temperature region of 185–293 K. Although the time scale estimated in these measurements is about one order of magnitude slower than the present ones, the temperature dependence of the time constant obtained is very similar to ours. This correspondence is very interesting and might be evidence of the relation between these two processes, although we must take care in comparing the dynamical properties between the artificial ZnPP-Mb and intact Mb. The above-mentioned difference in the time constant may not be serious, because the process observed here occurs in a very wide temporal region beyond four orders of magnitude, and the time constant of a stretched exponential function is no more than one of the ways to characterize such a process. In any event, we may be able to say that the above processes correspond to rather global conformational motions, as compared with the localized process observed by the band III measurement of Jackson et al.

In the case of intact Mb, it is widely accepted that there are three conformers in the highest tier of the hierarchy of the potential landscape (Ansari et al., 1985, 1987; Iben et al., 1988). Tian et al. claimed that the conformational dynamics observed by them corresponds to the interconversion process among these conformers. If this is also the case in our experiment, $C(t_d)$ is expected to be composed of a few different curves. In the present study, on the other hand, the experimental results for $C(t_d)$ were found to be roughly reproduced by a single stretched exponential function. This suggests that our model, assuming the conformational potential surface with one global energy minimum, is sufficient as a first approximation to describe the overall features of our experimental results. However, if we look more closely at Fig. 5 *a*, $C(t_d)$ for ZnPP-Mb-W:3G at 240 K and 260 K appears to be expressed by a sum of two stretched exponential functions rather than a single one, which suggests the existence of two distinct correlation times. Perhaps this tendency is related to the hierarchical structure of the conformational potential surface of a Mb molecule. A more detailed study of the shape of $C(t_d)$ is one of the important future problems. Furthermore, we must investigate carefully the effect of the metal substitution on the conformational dynamics of Mb, although it is supposed that the global motion treated here is not substantially affected by the substitution. Such studies will give more valuable information about the conformational energy landscape of Mb.

CONCLUSION

We have observed the TRTHB spectra of ZnPP-Mb-W:3G and Mm-W:3G, and determined the function $C(t_d)$ for the conformational and the configurational fluctuations of these samples. For both protein and dye-solution samples, $C(t_d)$ has been found to be well represented by stretched expo-

nential functions. The estimated parameter β is much smaller for ZnPP-Mb than for the merbromin solution, suggesting much more nonexponential behavior for the former. The fairly good agreement between the observed $C(t_d)$ and the curve calculated using a stretched exponential form suggests that a simple model in Fig. 1 gives a rather good description of the real system. The validity of this picture must be examined in the future by more detailed studies of the shape of $C(t_d)$.

The comparison of the TRTHB results for ZnPP-Mb-W:3G and Mm-W:3G has shown that the structural fluctuations of Mb and the surrounding solvent molecules are suppressed around almost the same temperature, 180 K. This is consistent with the idea of the solvent-induced glass-like transition of Mb. Furthermore, it has been found that the time scale of the conformational dynamics of ZnPP-Mb observed in the present study shows a temperature dependence similar to that of the conformational dynamics associated with the ligand escape process from the heme pocket. At present, we cannot clarify the meaning of this almost accidental correspondence. More precise studies may shed light on this problem.

The authors express their gratitude to Prof. Y. Goto, Dr. D. Hamada, and Prof. K. Yutani of Osaka University for their help in the measurements of the CD spectra.

This work was supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

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