Thermodynamic Study of Protein Dynamic Structure in the Oxygen Binding Reaction of Myoglobin*

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We examined the flash photolysis of oxy complexes of sperm whale myoglobin (Mb) on the nanosecond time scale at ambient temperatures. In this time range, we can observe the geminate reaction of Mb with the O₂ ligand existing in the protein matrix after the photodissociation from the heme iron. We found that the fraction of the geminate component to the total O₂ photodissociation exhibited temperature dependences. The geminate fraction decreased with rising temperature, indicating that the protein fluctuation is enhanced at high temperature because of thermal agitation. However, the temperature-dependent behavior showed a break at 20 °C. Concerning the geminate O₂ escaping reaction from the protein matrix to the solvent region, the activation energy above 20 °C (0.4 ± 0.4 kcal/mol) is significantly lower than that below 20 °C (6.1 ± 0.4 kcal/mol). Thermodynamic analysis on the basis of the transition state theory indicated that the O₂ escaping reaction above 20 °C is entropy dominated whereas that below 20 °C is enthalpy dominated. The results were qualitatively compatible with the theoretical prediction by J. Kottalam and D. A. Case (1988 J. Am. Chem. Soc. 110, 7690-7697). Comparing the kinetic and thermodynamic process of the O₂ geminate reaction among several Mbs, we concluded that the geminate O₂ reaction with Mb is governed by the dynamic motion of the protein which is sensitively controlled by the static interaction of the heme moiety with the surroundings.

The mechanism involved in binding of gaseous ligands to hemoproteins has been investigated intensively in recent years (1-12). Kinetic intermediates in the ligand binding process were originally identified in low temperature studies (13-17) and have been characterized recently at room temperature in solution containing agents such as glycerol or ethylene glycol. In those studies, they carried out the thermodynamic analysis of the ligand binding reaction to hemoproteins and extrapolated the results to the biologically significant situation at room temperature in water. On the other hand, Case and his co-workers (21, 22) recently carried out theoretical studies of the O₂ binding to Mb. In particular, they dealt with the ns geminate reaction of the O₂ recombining to Mb and predicted the possibility that temperature-dependent behavior of the reaction rate at room temperature might be different from that implied by Frauenfelder et al. (19-20). Their results have allowed us to infer the characteristic mechanism of the O₂ geminate binding to Mb, which is associated with the protein dynamic structure.

In the present study, we have applied flash photolysis to oxy complexes of several Mbs and followed the several hundred ns (10⁻⁷ s) geminate recombination kinetics of the photodissociated O₂ in the temperature range of 5-40 °C, where the reaction of the ns geminate pair C (see Equation 1) is observable. On the basis of the thermodynamic analysis of the kinetic data obtained, we discuss the details of the dynamics between O₂ and the protein moiety in Mb.

**MATERIALS AND METHODS**

Sperm whale Mb was obtained from Sigma. Oxy complexes of Mb (MbO₂) and Mb reconstituted with cobalt-protoporphyrin (CoMbO₂)
were prepared and purified with both Sephadex G-25 (Pharmacia LKB Biotechnology Inc.) and ion-exchange CM52 (Whatman) chromatographies as described previously (23, 24). Oxy complex of Aplysia Mb (AplysiaMbOz) was isolated directly from the radular muscle of Aplysia kurodai and was purified further as previously reported (23, 25). Deutero-, meso-, and 2,4-diacetyldeutero-hemins were prepared according to the literature methods (26-28). The reconstituted Mbs with the modified hemes were prepared and purified according to the standard methods (29-31). The sample solutions for the flash photolysis measurements were kept at 25 μM in 0.1 M potassium phosphate buffer at pH 7.0.

O2 concentration in the sample solution was regulated by gas dividers (Etec model SGD-XC-0.5L) and was monitored by an oxygen electrode (Diamond Electro-Tech Inc.) inserted into the solution.

The ns photolysis experiments were performed by using the second harmonic (532 nm) of a Quantar-Ray DCR-1 Nd:YAG laser, producing an excitation flash with a pulse width of 6 ns (6 mJ). The sample solutions in the quartz rectangular cells with 2-mm path length were exposed to the laser pulse with a spot size of 0.8 x 0.8 mm. In order to avoid much exposure of the sample solutions to the laser, the samples were changed at each temperature. The laser pulse was not exposed to one sample more than 100 shots. Before and after the laser exposure, the sample was checked by the visible absorption spectrum (Hitachi U 2910). Details of the measurement system were reported previously (23, 32).

RESULTS

Structural Dependence of the Nanosecond Geminate Components—In Fig. 1 are illustrated the transient absorbance changes of MbOs at 437 nm corresponding to the Soret band region of deoxyMb on the ns time range (~800 ns) after a laser irradiation. These kinetic traces show that the iron-bound O2 is photodissociated from Mb and subsequently rebinds to the heme iron in two, rapid and slow, processes. The slow phase after 500 ns, which is nearly constant in this time range, represents a bimolecular reaction between Mb and O2, as has been observed in the μs flash photolysis (23, 33). By contrast, the rapid process was the ns geminate recombination of O2 with Mb (16, 17, 34, 35). When we estimated the apparent rate constant of the rapid process (k∞) by the single exponential curve fitting under various O2 concentrations (inset in Fig. 1), the k∞ value was independent of the O2 concentration (k∞ = 0.91 ± 0.03 x 107 s⁻¹ at 22 °C).

We performed the ns geminate kinetics after flash irradiation for several Mbs having different protein structures, MbOz, CoMbOz, AplysiaMbOz, and CO complex of Mb (MbCO). As illustrated in Fig. 2, the ns geminate recombination is detectable for MbOz and CoMbOz but not for AplysiaMbOz and MbCO. It is also found that the fraction of the geminate process of CoMbOz is much larger than that of MbOz. In order to quantify the geminate component, we introduce the following parameter

\[
\Gamma = \frac{\Delta A_\infty - \Delta A_0}{\Delta A_\infty}
\]

where ΔA0 and ΔA∞ represent the initial change in absorbance and the value of absorbance at plateau, respectively, which were determined on the basis of the single exponential fitting curve (see Fig. 1). Γ is defined as the fraction of the geminate pair relative to the total photodissociated species. According to this definition, Γ values in MbOz and CoMbOz at 22 °C are 0.37 and 0.75, respectively, whereas those in AplysiaMbOz and MbCO are practically zero. Γ value implies the ratio of the ligand trapped in the protein, which subsequently recombines

![Fig. 1. Nanosecond transient absorption kinetics of MbOz. The absorbance change monitored at 437 nm corresponding to the Soret band region of deoxyMb. The employed measurement condition were [MbOz] = 25 μM, [O2] = 272 μM, in 0.1 M potassium phosphate buffer at pH 7.0, 22 °C. In this trace, the fast process is observable between 0 and 500 ns; the slow bimolecular process, which appeared after 500 ns, is constant in the trace, relaxes firmly in μs time region (data not shown). The apparent rebinding rate constant k∞ of the rapid process was obtained by the semilogarithmic plot, ln(ΔA0 - ΔA∞) vs. t, and the fitting curve was shown along the trace (dashed line). k∞ values are plotted against O2 concentration in the inset, in which the wave-lengths at 437 and 416 nm are corresponding to the Soret regions of deoxy- and oxyMb, respectively. Independence of k∞ to the O2 concentration shows that the rapid process is a geminate reaction between Mb and O2. The average value of k∞ at 22 °C was estimated to be 0.91 ± 0.03 x 10⁷ s⁻¹.

![Fig. 2. Nanosecond transient absorption kinetics of CoMbOz, MbOz, MbCO, and AplysiaMbOz. The absorbance changes of CoMbOz, MbOz, MbCO, and AplysiaMbOz monitored at 427, 437, 440, and 438 nm, respectively, are shown. Although the trace of CoMbOz at 427 nm (top) showed the bleaching curve it was illustrated as the absorption curve for comparison. Other experimental conditions were the same as in Fig. 1.]}
Nanosecond Geminate Reaction of O₂ to Myoglobin

Correlation between pKₐ and the geminate kinetic constants (T, k₁, k₋₁) of O₂ to Mbs

All results are at pH 7.0, 22 °C. Uncertainty indicates the standard deviation from the mean of the five measurements.

<table>
<thead>
<tr>
<th>MbO₂</th>
<th>pKₐ</th>
<th>Γ</th>
<th>k₁</th>
<th>k₋₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diacetyldeutero</td>
<td>3.4</td>
<td>0.417</td>
<td>1.20 ± 0.02 x 10⁷</td>
<td>0.70 ± 0.03 x 10⁷</td>
</tr>
<tr>
<td>Proto (native)</td>
<td>4.8</td>
<td>0.370</td>
<td>0.91 ± 0.03 x 10⁷</td>
<td>0.58 ± 0.03 x 10⁷</td>
</tr>
<tr>
<td>Deutero</td>
<td>5.5</td>
<td>0.411</td>
<td>1.18 ± 0.04 x 10⁷</td>
<td>0.66 ± 0.05 x 10⁷</td>
</tr>
<tr>
<td>Meso</td>
<td>5.8</td>
<td>0.311</td>
<td>0.94 ± 0.01 x 10⁷</td>
<td>0.65 ± 0.01 x 10⁷</td>
</tr>
<tr>
<td>CoMb</td>
<td>0.747</td>
<td>0.747</td>
<td>1 ± 0.8 x 10⁸</td>
<td>0.2 ± 0.1 x 10⁸</td>
</tr>
</tbody>
</table>

Fig. 3. Nanosecond transient absorption kinetics of native (proto-) (A) and deutero-MbO₂ (B) from 10 to 40 °C. The absorbance changes of native and deutero-MbO₂ were monitored at 437 and 423 nm, respectively. The maximal absorbance change immediately after the O₂ photodissociation was almost constant, but the extrapolation absorbance level of the slow phase becomes lower with decreasing the temperature. However, the traces of native MbO₂ (A) above 20 °C are almost piled up on each other and hardly change with increasing the temperature. Other experimental conditions were the same as in Fig. 1.

with the iron or escapes to the solvent region in the geminate process.

We applied the ns flash photolysis to the MbO₂ derivatives containing hemes modified at 2,4-side chains, such as deutero-, diacetyldeuter-, and meso-hemes. Their Γ values at 22 °C are compiled in Table I. It becomes smaller in the order of deutero-, diacetyldeutero-, proto-(native), and meso-MbO₈. This order was not correlated with the pKₐ value of the porphyrin, which has been used as a measure of the electron density on the heme iron. Further, in the temperature-dependent profiles of Γ values for all Mbs, it was not so simple to correlate the Γ value with either the electronic or steric parameter of porphyrin itself, as described below (see Fig. 4).

Temperature Dependence of the Nanosecond Geminate State—Fig. 3, A and B, shows the temperature dependences of the ns kinetic traces in native (proto-) and deutero-MbO₂ from 10 to 40 °C, respectively. In both cases, the initial changes in absorbance (ΔA₀) were essentially constant between 10 and 40 °C. However, the temperature dependence of the absorbance value at plateau (ΔAₜ) is different between native and deutero-MbO₂. With rising temperature, ΔAₜ of native MbO₂ hardly changes above 20 °C whereas that of deutero-MbO₂ clearly increases. Those features were substantiated in Fig. 4, in which the Γ values are plotted against the temperature for proto-, deutero-, diacetyldeutero-, meso-MbO₈, and CoMbO₈. All of the Γ values tend to decrease with rising temperature, showing that the fraction of the geminate recombination is smaller at higher temperature. Here, we must note that all of the five lines show breaks at 20 °C and that the temperature-dependent profile is drastically different between native and other artificial Mbs. The Γ value of native Mb decreases with increasing temperature from 5 to 20 °C but is almost constant above 20 °C. By contrast, in the case of artificial Mbs, the slope is steeper above 20 °C than below 20 °C. CoMbO₂ is the extreme case, in which the Γ value is almost constant below 20 °C.

From the kinetic traces, we obtained the apparent rate constants (kₐ) of the O₂ geminate recombination at each temperature. The kₐ values calculated were compiled at each condition in Table II.² In the O₂ binding scheme of Mb with

² In the case of CoMbO₂, we cannot obtain the accurate value of the kₐ because the half-width in the ns geminate kinetic trace (10 ns) is comparable to the pulse width of the excitation laser (6 ns). Therefore, we did not represent the Arrhenius plot for CoMbO₂.
In the present study, we obtained the I values and their temperature dependences of the ns O₂ geminate reaction for several Mbs (Fig. 4). From these data, we can calculate the value of kₚₐₜ at various temperatures, which represents the O₂ determined in this work because the temperature dependence Therefore we have referred to kₚₐₜ as "kₚₐₜ." By contrast, the kₜCB value and its temperature dependence were not well escaping rate from the protein matrix to the solvent region. types of Mbs. Each plot has a break at 20 °C, resulting in two different activation energy (EₜCB) and preexponential factors (AₜCB) for the ns O₂ escaping reaction below and above 20 °C (Equation 4).

To get further insight into the nature of the potential barrier on the ns O₂ escaping reaction, the entropy of activation ΔSₚₐₜ of this reaction was estimated by the following equations on the basis of the transition state theory under the assumption that the transmission coefficient κ = 1.

$$k_{\text{out}} = A_{\text{out}} \cdot e^{-\Delta G_{\text{out}}/kT} = \frac{k_B}{h} e^{-\Delta H_{\text{out}}/kT}$$

$$\Delta G_{\text{out}} = \Delta H_{\text{out}} - T \Delta S_{\text{out}} = E_{\text{out}} - RT - T \Delta S_{\text{out}}$$

$$\Delta S_{\text{out}} = R (\ln A_{\text{out}} - h e^{-\Delta H_{\text{out}}/kT} - 1)$$

where kₘ is Boltzmann and Planck constants, respectively. ΔGₚₐₜ and ΔHₚₐₜ represent the free energy and enthalpy of activation, respectively. On this analysis, we obtained two ΔGₚₐₜ values at 20 °C, each of which is ascribed to the ns O₂ escaping reaction below or above 20 °C, respectively. The obtained values of four Mbs are compiled in Table III together with other parameters, EₜCB, AₜCB and ΔSₚₐₜ. On the basis of these results, we will consider the thermodynamic process of the geminate O₂ escaping reaction under "Discussion."
the photodissociated $O_2$ escapes from the protein moiety to the solvent more readily at higher temperatures. This may be general and reasonable for the geminate $O_2$ process in Mbs employed because the $\Gamma$ is most probably related to the fluctuation of the protein structure due to thermal agitation enhanced at higher temperatures.

Here, it is worthy to note that the temperature dependences of the $\Gamma$ have a break at 20 °C to all of the native (proto-) and artificial Mbs. These novel findings might be explained in term of the change in the static structures of Mbs around 20 °C. However, the visible spectra of both MbO2 and deoxyMb were insensitive to the temperature variation between 5 and 40 °C. Moreover, the temperature dependence of the $^1H$ NMR signal position of valine E11 methyl protons in MbO2, which has served as a probe for the change in the fine static structure of the heme vicinity (36), did not show any break at 20 °C (data not shown). Thus, the break of the temperature dependence of $\Gamma$ at 20 °C may be attributable to the change in the thermal fluctuation of the local protein structure along the pathway of $O_2$ rather than to the drastic change in the whole (static) structure of protein; namely, the ns geminate process may reflect the dynamic motion of the protein in the reaction of the $O_2$ binding to Mb. Then, in order to explore quantitatively the nature of the protein dynamic structure, we tried to make thermodynamic analysis of the ns $O_2$ geminate reaction as described in the following section.

**Thermodynamic Analysis of the Nanosecond Geminate State**—As the most significant example, we focused our attention on the $O_2$ geminate escaping process from native (proto) Mb ($C(Mb \cdots O_2)\kappa_{\text{out}} D(Mb + O_2)$) because the rate constant $k_{\text{out}}$ for this process is more quantitatively estimated from the parameters $\Gamma$ and $k_{\text{out}}$, which are the same as $k_{\text{out}}$, $k_{\text{out}}$, and $k_{\text{CT}}$. The Arrhenius plot of $k_{\text{out}}$ also has a break at 20 °C (Fig. 5), resulting in the different values of the activation energy for the reaction ($E_{\text{out}}$) above and below 20 °C (Table III). This result indicates that the break in the Arrhenius plot comes from a change in mechanism of the $O_2$ geminate escaping reaction. Furthermore, we also note that the $E_{\text{out}}$ value above 20 °C (0.4 ± 0.4 kcal/mol) is substantially smaller than that below 20 °C (5.1 ± 0.4 kcal/mol).

According to the thermodynamic model of Case and Karplus (37), the $O_2$ ligand in the geminate state must cross an energy barrier $\Delta G_{\text{out}}$ to pass through a narrow path between histidine E7 and valine E11 which opens up enough to just let $O_2$ escape from the protein moiety to the solvent. In our experiments, the $\Delta G_{\text{out}}$ value (8.1 kcal/mol) for the $O_2$ escape is almost constant in the temperature range examined (5–40 °C), showing that the barrier height that the $O_2$ must cross does not change in this temperature range. As shown in Equation 7, $\Delta G_{\text{out}}$ is represented in the form of a sum of the enthalpic term ($E_{\text{out}} - RT$) and the entropic term ($-T \Delta S_{\text{out}}$).

Therefore, in the case of the reaction with large $E_{\text{out}}$, the contribution of the enthalpic term to the total energy barrier is predominant. In other words, the escaping rate of the $O_2$ ligand should be predominantly determined by the height of the enthalpic barrier, an enthalpy-dominated reaction. This is the case for the $O_2$ geminate reaction below 20 °C.

By contrast, above 20 °C, the enthalpic term ($-0$ kcal/mol) is too small to contribute to the total energy barrier. In this case, the entropic term ($-T \Delta S_{\text{out}}$) is much more important. Indeed, the entropic contribution to $\Delta G_{\text{out}}$ above 20 °C is about 8 kcal/mol, which is a main fraction of the total barrier of 8.1 kcal/mol. The value 28 e.u. of $\Delta S_{\text{out}}$ is large enough for the $O_2$ ligand to rotate freely in the protein matrix (state C) since even a free $O_2$ molecule has 36 e.u. and 13 e.u. for the translational and rotational entropies, respectively. Much of the rotational freedom of $O_2$ in the protein will be lost in ascending the barrier to escape from the protein.

Our experimental results obtained here are in good agreement with the theoretical prediction by Kottalam and Case (22), who recently calculated the free energy of activation on the ns geminate $O_2$ escaping process of Mb on the basis of the transition state theory. They simulated the potential of mean force along the $O_2$ exit path in the “active site” of Mb defined by them and estimated the rate constant for the $O_2$ escape ($k_{\text{out}}$; our $k_{\text{out}}$). Their simulation predicted that the Arrhenius plot of $k_{\text{out}}$ has a break around 270 K and that the activation energy of this process is much smaller above 270 K than below 270 K. Together with their theoretical study, our experimental one suggests that above 20 °C, the protein moiety is “soft” or “fluid” enough for the $O_2$ rotation and that the $O_2$ motion in the protein matrix happens to find the pathway to escape from the heme pocket to the solvent water. In this situation, the rate constant of the $O_2$ geminate escaping reaction is limited by the rate of occurrence or frequency with which $O_2$ finds the proper pathway, the entropy-dominated reaction.

<table>
<thead>
<tr>
<th>Mbo2</th>
<th>Above 20 °C</th>
<th>Below 20 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E_{\text{out}}$</td>
<td>$\Delta G_{\text{out}}$</td>
</tr>
<tr>
<td></td>
<td>kcal/mol</td>
<td>kcal/mol</td>
</tr>
<tr>
<td>Diacetyldeutero</td>
<td>6.9</td>
<td>9.6 x 10^{-11}</td>
</tr>
<tr>
<td>Proto (native)</td>
<td>0.4</td>
<td>1.2 x 10^{-9}</td>
</tr>
<tr>
<td>Deutero</td>
<td>5.6</td>
<td>9.5 x 10^{-9}</td>
</tr>
<tr>
<td>Meso</td>
<td>5.3</td>
<td>2.5 x 10^{-11}</td>
</tr>
</tbody>
</table>

a Uncertainty is ±0.4 kcal/mol.

b Uncertainty is ±1.5 e.u.

\* Uncertainty is ±1 in the form of ln $A_{\text{out}}$.

At 20 °C. \* Uncertainty is ±0.1 kcal/mol.

Here, it is worthy to note that the temperature dependences of the $\Gamma$ have a break at 20 °C to all of the native (proto-) and artificial Mbs. These novel findings might be explained in term of the change in the static structures of Mbs around 20 °C. However, the visible spectra of both MbO2 and deoxyMb were insensitive to the temperature variation between 5 and 40 °C. Moreover, the temperature dependence of the $^1H$ NMR signal position of valine E11 methyl protons in MbO2, which has served as a probe for the change in the fine static structure of the heme vicinity (36), did not show any break at 20 °C (data not shown). Thus, the break of the temperature dependence of $\Gamma$ at 20 °C may be attributable to the change in the thermal fluctuation of the local protein structure along the pathway of $O_2$ rather than to the drastic change in the whole (static) structure of protein; namely, the ns geminate process may reflect the dynamic motion of the protein in the reaction of the $O_2$ binding to Mb. Then, in order to explore quantitatively the nature of the protein dynamic structure, we tried to make thermodynamic analysis of the ns $O_2$ geminate reaction as described in the following section.

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Structural Control of the Protein Dynamic Structure—Our present results and analysis indicate that the ns geminate process in the O_2 escaping reaction from Mb is controlled by the dynamic motion of the protein. In addition to these observations, both $\Gamma$ and $k_{\text{on}}$ of artificial Mbs exhibited entirely different temperature dependence from those of native Mb (Figs. 4 and 5), showing that the modification of the heme side chains may result in changing the dynamic structure of Mb for the O_2 binding. According to our thermodynamic analysis, the O_2 escaping process for artificial Mb containing the modified heme is the enthalpy dominated even above 20 °C (see Figs. 4 and 5 and Table III). In artificial Mbs, this low entropic barrier (less than 3 kcal/mol) restricts rotation of the O_2 ligand in the protein matrix, contrasted with entropy-dominated barrier in native one (8 kcal/mol). Moreover, the $k_{\text{on}}$ value of artificial MbO_2 is larger than the native one by 3 order of magnitude (Table III). Thereby, the O_2 motion in protein matrix of artificial Mb seems randomly collided rather than ballistic in the native one. In addition, we must note that the behavior of $k_{\text{on}}$ of the artificial Mbs is entirely similar among them but different from that of native Mb. Therefore, it is considered that even the dynamic motion of the protein is sensitively controlled by some structural factors in the static state, e.g., the interaction of the heme with surroundings. In artificial Mbs, the mode of the interaction may delicately alter because of some reason such as the change in bulkiness of 2,4-side chains (38).

On the other hand, it seems interesting that MbCO and AplysiaMbO_2 did not exhibit the ns geminate process whereas MbO_2 and CoMbO_2 did. When the structure is compared among these Mbs, the most remarkable difference is the presence of a hydrogen bonding of the iron-coordinated ligand with the distal histidyl (histidine E7) imidazole in MbO_2 (39) and CoMbO_2 (40, 41) but not in the others (42, 43). Recently, Gibson (44) reported the ns flash photolysis of methyl isonitrile for several mutants of Mbs in which histidine E7 is replaced by valine, glycine, or phenylalanine. In the ns kinetic traces, the $\Gamma$ value varies among these mutants. These results suggest that interaction of histidine E7 with the bound ligand (23), e.g., hydrogen bonding or steric one, is at least a factor to determine whether the $\Gamma$ value is zero or not. This suggestion may be plausible because the distal histidyl imidazole has been thought to serve as a "gate" in controlling the ligand exit from or entry to the heme pocket (18–20, 37).

During the submission this manuscript, an independent investigation reporting results of the O_2 geminate escaping kinetics was published (45). Their results indicated that temperature dependence of $\Gamma$ (eq 1 − $\Gamma_0$) and the Archenius plot of $k_{\text{on}}$ (their $k_s$) has no break between 6.3 and 34.9 °C, which were different from our present data. Here, we note that the both $\Gamma$ and $k_{\text{on}}$ behaviors are sensitive to the solution condition. For example, we found recently that the temperature dependence of $\Gamma$ changes into the normal type (45) upon addition of glycerol to the sample solution. Details will be reported elsewhere.

This discrepancy has not been simply explained yet except for solution, sample, and instrumental conditions.

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