Effects of Magnesium Sulfate on an Unfolding Step of Human Cyanomet Myoglobin

Takashi Konno and Isao Morishima
Division of Molecular Engineering, Graduate School of Engineering, Kyoto University, Kyoto 606-01, Japan

ABSTRACT The effects of magnesium sulfate ($MgSO_4$) on an unfolding step of human cyanomet myoglobin (Mb) were examined for wild-type and three L→A mutant Mbs. The unfolding was induced at acidic pH (3.6–4.5) with various concentrations of $MgSO_4$ (0–2 M). The monophasic process was monitored by visible absorption spectroscopy. We observed quite nonlinear $\Delta G^{\ddagger}$-[$MgSO_4$] relations for all the Mbs. $\Delta G^{\ddagger}$-[$MgCl_2$] relations were also determined for a comparative study. Thermodynamic evaluation of the results indicated that an upward reflection of $\Delta G^{\ddagger}$-[$MgSO_4$] relations in high [$MgSO_4$] is caused by the strong Hofmeister effect of the salt. Results obtained for three mutants (L29A, L72A, and L104A) at pH 4.0 and 4.5 were consistent with our previous observation that the structure of the transition state is determined by the stability of Mb cores in the balance with the pH conditions of unfolding (T. Konno and I. Morishima. 1993. *Biochim. Biophys. Acta.* 1162:93–98).

INTRODUCTION

Recently, many works on protein folding-unfolding events have been reported (1–3), but we still encounter much difficulty in studying structures of proteins in unstable states, including transition states of dynamic processes (3). Although there are methods such as x-ray diffraction or nuclear magnetic resonance spectroscopy for determining the structures of native proteins, they cannot be applied directly to the transition state. We therefore need to study the unstable structure kinetically.

Protein-solute interactions have long been convenient and powerful probes for macroscopic structures of proteins (4–6). They are applicable also to the transition state structure in the folding-unfolding processes (1, 4, 7). Following this approach, our previous study on an urea-induced unfolding step of human myoglobin (Mb) revealed that the structure of the transition state of the process is dynamically determined by pH conditions and the stability of the hydrophobic cores of Mb (1). The study was possible because protein-urea interactions could probe the exposed surface area of the protein (4, 5). In the present report, we have studied the effects of $MgSO_4$ on an unfolding rate of the same process. Compared to urea, $MgSO_4$ is interesting because it interacts with protein via not only a direct binding of the salt but also its strong Hofmeister effect, which is known to be important in determining the stability of proteins (8). Mg^{2+} and SO_4^{2-} will bind to charged sites of proteins, and SO_4^{2-} facilitates the hydration of proteins (9–11). We performed our experiments over a wide concentration range of the salt. Our present purpose is to demonstrate the complex properties of $MgSO_4$ effects and to see how perturbations in protein sequences and external conditions affect them. For the latter purpose, we used three mutant Mbs (L29A, L72A, and L104A), which we prepared in previous studies (1, 12). To gain insight into the dynamic events underlying our results, we also presented a kinetic scheme for the salt effects that allows for dynamic alterations in the structure of the transition state.

MATERIALS AND METHODS

Human Mb expression vector pMb3 (pLcIIFXMb) was a gift from Prof. S. G. Boxer and Dr. R. Varadarajan (Stanford University) (13). The details of mutagenesis and protein preparation and purification procedures were described previously (12). Mbs are notated as follows: the original Mb coded by pMb3 is indicated as WT (wild type), a leucine→alanine mutant at position 104 of WT as L104A, etc. We used L29A, L72A, and L104A, in each of which a leucine→alanine mutation is introduced in one of three hydrophobic cores of Mb (1). We previously reported that the three mutations drastically reduced the stability against acid or urea denaturation (1). Some spectroscopic studies on the mutants were also done, which indicated that the native structure of the mutants is largely similar to that of WT (12).

Magnesium sulfate and magnesium chloride were of reagent grade. Salt-containing solutions were freshly prepared for each experiment with a volumetric adjustment of concentration. Unfolding solutions contained 10 mM sodium acetate, 0.5 mM sodium cyanide, and various concentrations of the salts (pH adjusted with acetic acid to 3.6, 4.0, or 4.5). An aliquot of concentrated protein solutions (10 mM sodium acetate, pH 5.5, and 0.5 mM sodium cyanide) were added manually into the 2-ml unfolding solution in a stirring cuvette (final Mb concentration, ~2–10 μM), and changes in absorbance at 422 nm were monitored with a Hitachi U-3210 spectrometer. Mixing dead times were about 2 s. All of the experiments were performed at 23°C. Reaction traces could be fitted well to

$$A(t) = A_0 + A_1\exp(-t/\tau), \quad (1)$$

where $A(t)$ is the absorbance at 422 nm at time t. A_0, A_1, and τ are fitting free parameters. t was measured in minutes. The activation free energy of the process ($\Delta G^{\ddagger}$) was calculated using

$$\Delta G^{\ddagger} = -RT[\ln(1/\tau) - \ln(1/b)], \quad (2)$$

where b is a vibrational constant. Since we are interested only in relative changes in $\Delta G^{\ddagger}$, we arbitrarily assumed $RT \ln(1/b) = 0$.

RESULTS

Nonlinear $\Delta G^{\ddagger}$-[$MgSO_4$] relations

Time-dependent changes in absorbance at 422 nm in our unfolding conditions were fitted well to monophasic

Received for publication 23 November 1992 and in final form 10 March 1993.

Address reprint requests to Dr. Takashi Konno

0006-3495/93/08/907/05 $2.00

functions. A typical trace is shown in Fig. 1*A*. The process is a part of Mb unfolding events (1, 14). In Fig. 1*B*, $\Delta G^{\ddagger}$-[$MgSO_4$] relations are shown for WT at pH 3.6 and 4.0 and for L104A at pH 4.0. Their common noticeable properties are: (*a*) the plots show upward curvatures; (*b*) in low [$MgSO_4$], $\Delta G^{\ddagger}$ decreases sharply when [$MgSO_4$] increases; (*c*) in high [$MgSO_4$], $\partial(\Delta G^{\ddagger})/\partial([MgSO_4])$ even becomes positive. The nonlinear $\Delta G^{\ddagger}$-[$MgSO_4$] relations indicate that the effect of $MgSO_4$ is quite different from that of urea, since we previously observed linear $\Delta G^{\ddagger}$-[urea] relations at the same pH range (1). Complex mechanisms of interactions of SO_4^{2-} (and Mg^{2+}) with protein must be the origin of the nonlinear behavior.

$\Delta G^{\ddagger}$-[$MgCl_2$] relation

To see anionic contributions of the $MgSO_4$ effects, we performed unfolding experiments in the presence of $MgCl_2$ instead of $MgSO_4$. It is known that Cl^- has the much weaker Hofmeister effect as compared to SO_4^{2-} (11). We used L104A at pH 4.0 because of its typical V-shaped $\Delta G^{\ddagger}$-[$MgSO_4$] relations (Fig. 1*B*). The result is shown in Fig. 2. In low salt concentrations, the negative slope is larger and $\Delta G^{\ddagger}$ values are smaller for $MgSO_4$ than for $MgCl_2$. By increasing the salt concentration, the slope changes to positive values for both the salts but much more strongly for $MgSO_4$, resulting in a larger $\Delta G^{\ddagger}$ for $MgSO_4$ in more than 0.5 M. In short, the V shape is more prominent in $MgSO_4$ than in $MgCl_2$. We also determined the $\Delta G^{\ddagger}$-[$MgCl_2$] relation for WT at pH 3.6 and

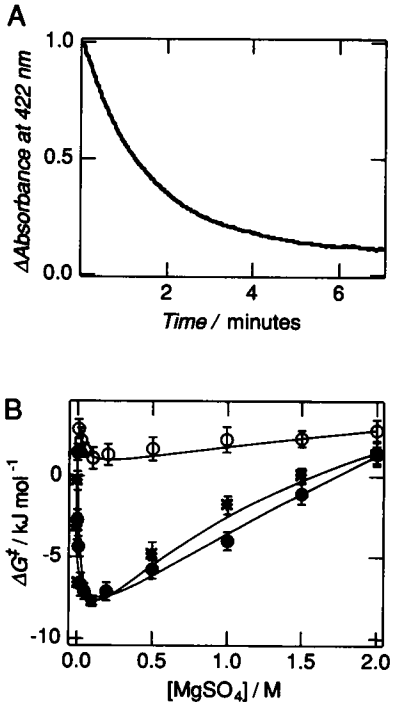


FIGURE 1 (*A*) A time course of changes in absorbance at 422 nm for WT at pH 4.0 and [$MgSO_4$] = 0.1 M. (*B*) $\Delta G^{\ddagger}$-[$MgSO_4$] relations for WT at pH 3.6 (*) and 4.0 (○) and for L104A at pH 4.0 (●). Continuous lines have no theoretical meaning.

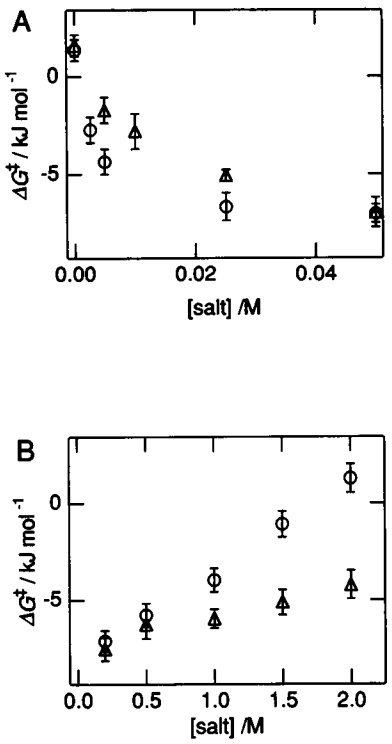


FIGURE 2 Relations between $\Delta G^{\ddagger}$ and [$MgSO_4$] (○) or [$MgCl_2$] (△) at pH 4.0 for L104A, in the low (*A*) and the high (*B*) concentration ranges of the salts.

obtained essentially the same results as in Fig. 2 (data not shown). Possibly, anionic properties of the salts cause the difference in the slope of the $\Delta G^{\ddagger}$-[salt] relations.

Mutational and pH effects on $\Delta G^{\ddagger}$-[$MgSO_4$] relations

In our previous study, probed by interactions of urea with Mb, we found that L→A mutations in cores of Mb or pH reduction of unfolding solutions affect the slope of $\Delta G^{\ddagger}$-[urea] relations for the unfolding process. We investigated similar aspects of $MgSO_4$ effects. Some of the results are shown in Fig. 1*B*. Fig. 3*A* additionally shows $\Delta G^{\ddagger}$-[$MgSO_4$] relations for L29A, L72A, and L104A at pH 4.0 and 4.5 in salt concentrations of more than 0.2 M. At pH 4.0, as compared to WT in the same condition, the mutation L104A not only decreases $\Delta G^{\ddagger}$ values in any salt concentrations but also increases the positive slope of $\Delta G^{\ddagger}$-[$MgSO_4$] relations in high salt concentrations (Fig. 1*B*). The results are almost the same for the other mutations, L29A and L72A (Fig. 3*A*). Fig. 1*B* also shows some pH effects. We notice that the $\Delta G^{\ddagger}$-[$MgSO_4$] relation for WT at pH 3.6 becomes quite similar to that for L104A at pH 4.0 (Fig. 1*B*). On the other hand, the relations for all three mutants at pH 4.5 are similar to that of WT at pH 4.0 (Fig. 3*A*). The mutational effects seem to be compensated by increasing the pH value of the unfolding solution. As compared to our previous results (1), it may be expected that these mutational and pH effects on the $\Delta G^{\ddagger}$-[$MgSO_4$] relations are related to structural changes in the transition state of the unfolding process (see Discussion).

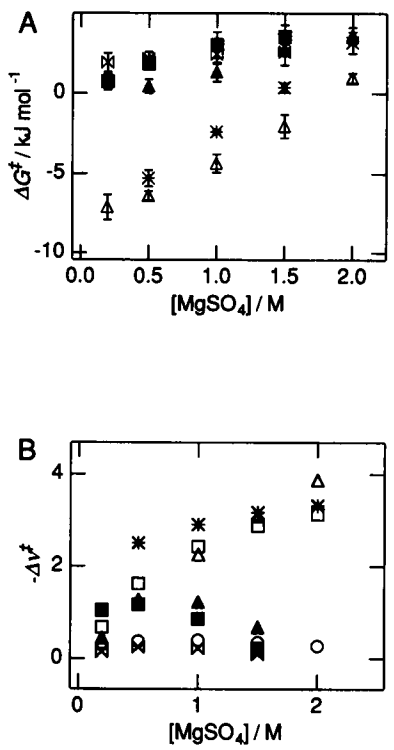


FIGURE 3 (*A*) $\Delta G^{\ddagger}$-[$MgSO_4$] relations for L29A and L72A at pH 4.0 and L29A, L72A and L104A at pH 4.5. (*B*) $-\Delta\nu^{\ddagger}$ in the $MgSO_4$ solutions, estimated using Eq. 3, is plotted against [$MgSO_4$]. Only in *B*, WT (○), L104A (□) at pH 4.0; in both *A* and *B*, L29A (*) and L72A (△) at pH 4.0, and L29A (▷◁), L72A (▲), and L104A (■) at pH 4.5.

DISCUSSION

A simple view of the thermodynamics of $MgSO_4$ effects

We have shown that $\Delta G^{\ddagger}$-[$MgSO_4$] relations for WT and mutants are quite nonlinear, which is possibly caused by both the preferential interaction and the preferential hydration effects of $MgSO_4$ (8–10). The Wynman linkage relation is convenient for considering the effects (15):

$$-\partial(\Delta G^{\ddagger}/RT)/\partial(\ln a_3)_{T,P,m_2} = \Delta\nu_3^{\ddagger} - (m_3/55.5)\Delta\nu_1^{\ddagger} = \Delta\nu^{\ddagger}, \quad (3)$$

where

$$\Delta\nu^{\ddagger} = \nu_T^{\ddagger} - \nu_N^{\ddagger}, \qquad \Delta\nu_3^{\ddagger} = \nu_{3,T}^{\ddagger} - \nu_{3,N}^{\ddagger},$$

$$\Delta\nu_1^{\ddagger} = \nu_{1,T}^{\ddagger} - \nu_{1,N}^{\ddagger} \qquad \nu_N^{\ddagger} = \nu_{3,N}^{\ddagger} - (m_3/55.5)\nu_{1,N}^{\ddagger},$$

$$\nu_T^{\ddagger} = \nu_{3,T}^{\ddagger} - (m_3/55.5)\nu_{1,T}^{\ddagger}$$

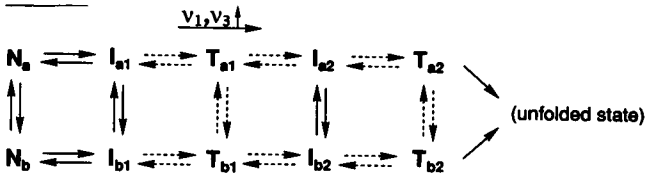
a_3 and m_3 are the activity and the molal concentration of the third component (or $MgSO_4$ in this case). $\nu_{3,T}^{\ddagger}$ and $\nu_{3,N}^{\ddagger}$ (or $\nu_{1,T}^{\ddagger}$ and $\nu_{1,N}^{\ddagger}$) are the total numbers of bound molecules of the salt (or water) in the transition and the native states, respectively (in moles/mole of protein). The first and second terms of Eq. 3 represent contributions of solute binding and hydration, respectively. We suggest that the impressive upward curvatures of $\Delta G^{\ddagger}$-[$MgSO_4$] relations in high $MgSO_4$ concentrations (Figs. 1 and 3) are explained mainly by the second term, based on the following three reasons: (*a*) It can be expected from Eq. 3 that the hydration term $(m_3/55.5)\Delta\nu_1^{\ddagger}$ will be large in high m_3, whereas the interaction term $\Delta\nu_3^{\ddagger}$ is important in lower salt concentrations (9). (*b*) Because of the ionic character of the salt in solutions and its high affinity for charged sites, $\Delta\nu_3^{\ddagger}$ is probably saturated in low salt concentrations (9). (*c*) Since the transition state must have a larger exposed surface than the native state, $\Delta\nu_3^{\ddagger}$ will be positive, and this term in Eq. 3 cannot explain any positive slopes of $\Delta G^{\ddagger}$-[$MgSO_4$] relations.

The suggestion also seems consistent with the results in Fig. 2. Because of a smaller activity coefficient for $MgSO_4$ than for $MgCl_2$ (16), $-\Delta\nu^{\ddagger}$ in high salt concentrations is larger for $MgSO_4$ than for $MgCl_2$. On the other hand, in low salt concentrations, the negative slope for $MgSO_4$ is slightly larger than for $MgCl_2$ (Fig. 2*A*). Thus, along the salt concentration axis, $MgSO_4$ must increase $-\Delta\nu^{\ddagger}$, probably via the hydration term, more strongly than $MgCl_2$, which agrees with the well-known larger Hofmeister effect of SO_4^{2-} versus Cl^- (11).

Salt effects and dynamic structures of the transition state

The simple interpretation above has assumed only one native state (*N*) and one transition state (*T*). However, both *N* and *T* are ensembles of various substates or molecular conformations, among which mutations or alterations of external conditions may affect distributions. Our previous study, for example, indicated that the macroscopic structure of the transition state of the unfolding step is drastically altered by pH conditions or mutations (1). Alternatively, we should also consider the possibility that the pathway of the unfolding transition is altered by changing external conditions such as salt concentrations or pH. In addition, it is widely believed that the native structure is composed of many conformational substates (17, 18). Of course, our experiments cannot directly detect such a detailed composition for each state. This section is presented only for examining possible events underlying our results.

We use Scheme 1, which is general enough to consider the dynamic view of the unfolding pathway and the complex composition of each state. The number of each state is limited for simplification. Extension to more general cases is not difficult.



SCHEME 1. The pathway of unfolding.

N, I, and T are native, intermediate, and transition states, respectively. According to the transition state theory (19), T's are in quasi-equilibrium with other states (dotted arrows). Differences among conformers of each state can be characterized by the binding parameters ν_1 and ν_3. It may be natural to suppose that, given a salt concentration, states on the left side of the scheme have smaller binding parameters than those on the right.

Since our experiments exhibited monophasic kinetics of the unfolding reaction, the following two conditions will be fulfilled: (*a*) there exists one dominant rate-limiting step and (*b*) the system is under a steady-state condition. In case (*a*), we arbitrarily suppose that T_{a1} and T_{b1} are the transition states of the rate-limiting step. N's and I's on the left side of the step must be in fast equilibrium. The apparent $\Delta G^{\ddagger}$ and $\Delta\nu^{\ddagger}$ will be given by

$$\Delta G^{\ddagger}_{app} = -RT(\ln Z_T - \ln Z_N) \tag{4}$$

$$\Delta\nu^{\ddagger}_{app} = \nu_T - \nu_N, \tag{5}$$

where

$$\nu_{T1} = \frac{\nu_{Ta1}\cdot\exp(-G_{Ta1}/RT) + \nu_{Tb1}\cdot\exp(-G_{Tb1}/RT)}{Z_T} \tag{6}$$

$$\nu_N = [\nu_{Na}\cdot\exp(-G_{Na}/RT) + \nu_{Nb}\cdot\exp(-G_{Nb}/RT) + \nu_{Ia1}\exp(-G_{Ia1}/RT) + \nu_{Ib1}\cdot\exp(-G_{Ib1}/RT)]/Z_N \tag{7}$$

$$Z_{T1} = \exp(-G_{Ta1}/RT) + \exp(-G_{Tb1}/RT) \tag{8}$$

$$Z_N = \exp(-G_{Na}/RT) + \exp(-G_{Nb}/RT) + \exp(-G_{Ia}/RT) + \exp(-G_{Ib}/RT) \tag{9}$$

G_X and ν_X are the free energy and the binding parameter of a state X. In case (*b*), ν_N and Z_N are given by Eqs. 7 and 9, respectively, and by

$$Z_T = Z_{T1}\cdot Z_{T2}/[Z_{T1} + Z_{T2}] \tag{10}$$

$$\nu_T = \nu_{T1} + \nu_{T2} - \frac{\nu_{T1}\cdot Z_{T1} + \nu_{T2}\cdot Z_{T2}}{Z_{T1} + Z_{T2}} \tag{11}$$

Equations 10 and 11 are derived from the well-known formula for the steady-state sequential reaction (19).

According to Eqs. 4–11, the apparent $\Delta\nu^{\ddagger}$ value is determined not only by ν for each state but also by relative stabilities among them. The latter depend on the salt concentration, and thus relative contributions of various conformers of the transition (intermediate or native) state are changed in different salt concentrations. In case (*a*), the rate-limiting step may be shifted from one transition step to the other. For a complete analysis of the $MgSO_4$ effects, we have to know the structures and relative stabilities of all of the conformers in the native as well as the transition states, which is left for the future. Note, however, that the positive slope of $\Delta G^{\ddagger}$-[$MgSO_4$] relations in high salt concentrations cannot be explained by the positive $\Delta\nu_3^{\ddagger}$ terms in these cases and can reasonably be attributed to hydration effects.

Interpretations in pH and mutational effects

Using the experimental results, $\Delta\nu^{\ddagger}$ values in concentrations of $MgSO_4$ greater than 0.2 M were estimated for WT and the mutants at pH 4.0 and 4.5. The calculation was performed using activity coefficients of $MgSO_4$ (16) and differentiating 4th-order polynominal functions that fit well to the data of Figs. 1*B* and 3*A*. The estimated values are plotted against [$MgSO_4$] in Fig. 3*B*. The L→A mutations or the pH reduction drastically increases the apparent $|\Delta\nu^{\ddagger}|$ values in a high concentration range at around pH 4.0. Since the mutational and pH increases in $-\Delta\nu^{\ddagger}$ become stronger by increasing the salt concentration where salt binding to Mb is probably saturated, contributions of the hydration term are probably dominant in the pH and mutational effects. Note, however, that our experiments do not give estimates for $\Delta\nu_3^{\ddagger}$ and $\Delta\nu_1^{\ddagger}$ separately, and further works may be necessary to confirm this point.

Scheme 1 and Eqs. 4–11 are useful for interpreting the results, according to which there are three possible origins of increase in the apparent $\Delta\nu^{\ddagger}$: (*a*) a decrease in ν_N caused by alterations in relative stabilities among N's and I's (Eq. 7); (*b*) an increase in ν_T caused by alterations in a relative stability between T_a and T_b (Eqs. 6 and 11); (*c*) a shift in the rate-limiting step between (T_{a1}, T_{b1}) and (T_{a2}, T_{b2}). In the present context, the structure of each substate itself is supposed to be conserved by perturbations, although mutations introduced in the protein will surely affect the structure slightly.

In fact, our present results alone cannot determine which of the three possibilities is the real origin of our observations. However, our previous study showed that the macroscopic structure of the transition state is determined by the stability of Mb hydrophobic cores in the balance with pH conditions of unfolding (1). The extent of the expansion caused by the L→A mutations, estimated from m_{urea}, was 15 to 30% of the surface area of the random coil configuration (1). Referring to such dynamics of the transition state, our present results may be reasonably explained by case b or c. The large amplitude of alteration in the tertiary structure of the transition state is surprising since the structure of the transition state of major unfolding or folding processes is believed to be compact and very similar to the native structure (3, 7). Possibly some native-like local structures are essential for a large free energy of the transition state, and the other parts are in equilibrium between compact and expanded conformations. This view may agree with mutational mappings on barnase, which showed that tertiary interactions in the transition state of unfolding are not uniformly formed (20).

Limitation on the usage of $MgSO_4$

Considering the quite nonlinear $\Delta G^{\ddagger}$-[$MgSO_4$] relations, $MgSO_4$ can be an interesting probe for unfolding steps of protein. However, there exist some limitations to be overcome in the future. First, the estimated $\Delta\nu^{\ddagger}$ values contain contributions of both the strong hydration and the binding phenomena. In addition, by the $\Delta\nu^{\ddagger}$ value, we can know only

the difference between the native and the transition states but not the structure of the transition state itself. Some of the limitations will be overcome by structural studies on the native structure and probing unfolding steps by more than one spectroscopic method. Another related limitation is that $MgSO_4$ in high concentrations will affect the structure of the transition state or the transition pathway, as we discussed in some detail above. The unfolding pathway under our conditions is possibly different from that in physiological conditions. What we have concluded about the unfolding step in particular cannot be extended to the corresponding folding step. However, the limitation is applicable to almost all other unfolding experiments. On the other hand, by altering solution conditions drastically, we can study the dynamic responses of the unstable structure (1).

CONCLUSIONS

In this study, we have shown that $MgSO_4$ affects $\Delta G^{\ddagger}$ of the Mb unfolding step in a quite nonlinear manner and that the $MgSO_4$ effect is potentially valuable for surveying the dynamic structure of the transition state. This work considers the phenomena on a qualitative level. Future works should elucidate the molecular mechanisms of $MgSO_4$ effects and the transition pathways.

We are grateful to Prof. S. G. Boxer and Dr. R. Varadarajan (Stanford University) for their gift of expression vector of the human Mb gene and Dr. Masashi Unno for his interest and constructive criticism of the manuscript.

REFERENCES

1. Konno, T., and I. Morishima. 1993. Transition state of an unfolding step in human cyanomet myoglobin. *Biochim. Biophys. Acta.* 1162:93–98.
2. Ghelis, C., and J. Yon. 1982. Protein Folding. Academic Press, New York and London.
3. Kim, P. S., and R. L. Baldwin. 1990. Intermediates in the folding reactions of small proteins. *Annu. Rev. Biochem.* 59:631–660.
4. Tanford, C. 1970. Protein denaturation Part C. *Adv. Protein Chem.* 24: 1–95.
5. Pace, C. N. 1986. Denaturation and analysis of urea and guanidine hydrochloride denaturation curves. *Methods Enzymol.* 131:266–280.
6. Arakawa, T., and S. N. Timasheff. 1982. Preferential interactions of proteins with salts in concentrated solutions. *Biochemistry.* 21:6545–6552.
7. Chen, B., W. A. Baase, and J. A. Schellman. 1989. Low-temperature unfolding of a mutant of phage T4 lysozyme. 2. Kinetic investigations. *Biochemistry.* 28:691–699.
8. Arakawa, T., R. Bhat, and S. N. Timasheff. 1990. Why preferential hydration does not always stabilize the native structure of globular proteins. *Biochemistry.* 23:1924–1931.
9. Arakawa, T., and S. N. Timasheff. 1984. Mechanism of protein salting in and salting out by divalent cation salts: balance between hydration and salt binding. *Biochemistry.* 23:5912–5923.
10. Arakawa, T., and S. N. Timasheff. 1984. Protein stabilization and destabilization by guanidinium salts. *Biochemistry.* 23:5924–5929.
11. Collins, K. D., and M. W. Washabaugh. 1985. The Hofmeister effect and the behaviour of water at interfaces. *Q. Rev. Biophys.* 18:323–422.
12. Adachi, S., N. Sunohara, S. Ishimori, and I. Morishima. 1992. Structure and ligand binding properties of leucine 29 (B10) mutants of human myoglobin. *J. Biol. Chem.* 267:12614–12621.
13. Varadarajan, R., A. Szabo, and S. G. Boxer. 1985. Cloning, expression in *Escherichia coli*, and reconstitution of human myoglobin. *Proc. Natl. Acad. Sci. USA.* 82:5681–5684.
14. Shen, L. L., and J. Hermans. 1972. Kinetics of conformation change of sperm-whale myoglobin. I. Folding and unfolding of metmyoglobin following pH jump. *Biochemistry.* 11:1836–1841.
15. Wyman, J. 1964. Linked functions and reciprocal effects in hemoglobin: a second look. *Adv. Protein. Chem.* 19:223–286.
16. Robinson, R. A., and R. H. Stokes. 1959. Electrolyte Solutions. Butterworths, London. 491–502.
17. Austin, R. H., K. W. Beeson, E. H. Frauenfelder, and I. C. Gunsalus. 1975. Dynamics of ligand binding to myoglobin. *Biochemistry.* 14: 5355–5373.
18. Frauenfelder, H., F. Parak, and R. D. Young. 1988. Conformational substates in proteins. *Annu. Rev. Biophys. Biophys. Chem.* 17:451–479.
19. Steinfeld, J. I., J. S. Francisco, and W. L. Hase. 1989. Chemical Kinetics and Dynamics. Prentice-Hall, Englewood Cliffs, NJ.
20. Kellis, J. T., K. Nyberg, D. Sali, and A. R. Fersht 1988. Mapping the transition state and pathway of protein folding by protein engineering. *Nature (Lond.).* 333:784–786.