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Salt-induced formation of the molten globule state of apomyoglobin studied by isothermal titration calorimetry ☆,1

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Abstract

Whereas apomyoglobin is unfolded at pH 2 by HCl in the absence of salt, the addition of anions either from salts or acids stabilizes the molten globule state, i.e. a compact denatured state with a significant native-like secondary structure but with largely flexible side-chains. To clarify the thermodynamic mechanism responsible for the conformational stability of the molten globule state of apomyoglobin, we studied the salt-induced formation of the molten globule state of horse apomyoglobin at pH 2 by isothermal titration calorimetry (ITC). By titrating the acid-unfolded apomyoglobin with $NaClO_4$ or Na_2SO_4, an exothermic reaction was observed. The titration curve obtained from the heat was cooperative and consistent with the conformational transition curve measured by circular dichroism at 222 nm. This suggested that the salt-induced conformation change can be approximated by a two-state transition between the acid-unfolded and molten globule states. However, the heat for formation of the molten globule state estimated by ITC was slightly larger in magnitude than the enthalpy change for unfolding of the salt-stabilized molten globule state at pH 2, suggesting a relatively small contribution of heat other than the conformational change. These results support a view that the conformational transition of apomyoglobin at pH 2 can be represented, as a first approximation, by a two-state transition between the molten globule state and the fully unfolded state.

Keywords: Apomyoglobin; Calorimetry; Hydrophobic interaction; Molten globule; Protein folding

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☆ Dedicated to Hiroshi Suga on the Occasion of his 65th Birthday.

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1. Introduction

Stable conformational states located between the native and unfolded states have been found for many proteins [1–3]. For example, whereas several proteins including apomyoglobin and cytochrome–C are unfolded significantly at pH 2 in the absence of salt, the addition of anions either from salt or acid stabilizes the intermediate conformational state (Fig. 1) [4–8]. Many of these states share common properties, i.e. the molecule is compact and contains a significant amount of secondary structure but with largely flexible side-chains, and are collectively called the molten globule state [1–4]. Application of high-resolution NMR techniques in conjunction with amide proton/deuterium exchange methods has revealed the location of secondary structures and their dynamics in the molten globule state of several proteins including apomyoglobin [9]. Kinetic studies have indicated that the molten globule state is, indeed, a major kinetic intermediate of protein folding [10, 11]. At the same time, it is becoming clear that various molten globule states exist, ranging from a significantly disordered but collapsed molten globule state, to a molten globule state with several native characteristics [12–15].

However, the thermodynamic mechanism responsible for the conformational stability of the molten globule state is unclear and controversial [12, 15]. There are two types of molten globule state with respect to the thermodynamics of unfolding [15]. One type shows cooperative thermal unfolding. A typical example is the acidic molten globule state of cytochrome–C [16–18]. Thermal unfolding of the acidic molten globule of cytochrome–C is a cooperative process with enthalpy change (ΔH_U) and heat capacity change ($\Delta C_{p,U}$) of unfolding, although their amplitudes are much smaller than those of the native state. This suggests that the molten globule state is a third conformational state separated by a first-order phase transition from the native state and the more significantly unfolded state [3, 19]. A very different situation has been reported for the other type of molten globule state. A typical example of this is the molten globule of α-lactalbumin [1, 20]. The thermal unfolding of the molten globule state of α-lactalbumin has been regarded as a gradual process which cannot be represented by a cooperative two-state transition. The apparent change in structure has been considered to represent the gradual redistribution of a population of microscopic states within the same macroscopic state, i.e. the denatured state. In a recent paper [21], the thermal unfolding of the molten globule state of α-lactalbumin was explained by a statistical thermodynamic formalism in which the distribution of substates with varying degrees of residual structure changes with temperature. Although the variation in structure of the molten globule states can be related to the variation in thermodynamic characteristics, little is known about the details of this relationship.

So far, thermodynamic studies of the intermediate conformational state have depended mostly on DSC measurements [20–22]. DSC curves were analyzed in order to determine the nature of the conformational transition and the thermodynamic parameters, such as ΔH_U and $\Delta C_{p,U}$, of unfolding. However, since $\Delta C_{p,U}$ of the intermediate conformational state is generally small, it has often been difficult to obtain accurate thermodynamic data. To provide a more general view of the thermodynamic stability of the molten globule state, it is important to explore a novel approach, which is complementary to DSC.

Recently, we reported that isothermal titration calorimetry (ITC) is a promising calorimetric method for following the salt-dependent folding transition of cytochrome-C [18]. By titrating acid-unfolded cytochrome–C with salts such as $NaClO_4$, we obtained the enthalpy change of folding (ΔH_F) at various temperatures, which is consistent with ΔH_U obtained by DSC. Because the sensitivity of ITC is high, it is capable, under suitable conditions, of detecting a reaction with a small enthalpy change undetectable by DSC. To explore further the usefulness of ITC and the thermodynamic properties of the molten globule state, we studied the salt-induced formation of the molten globule state of apomyoglobin. The results indicated that the reaction can be represented, as a first approximation, by a two-state transition mechanism.

2. Materials and methods

2.1. Materials

Horse myoglobin (Type I) was purchased from Sigma. Apomyoglobin was prepared from myoglobin by 2-butanone extraction of the heme [23] and then filtered using Sephadex G-50 equilibrated with 50 mM sodium acetate at pH 5.5 to remove the aggregate [24]. The content of the holoprotein remaining was less than 1%, judging from the ratio of absorption at 280 and 410 nm.

2.2. Methods

Most experiments in this study were done in the presence of 10 mM HCl at pH 2. The pH was measured using a Radiometer PHM83 at 20°C. Stock protein solution (2–10 mg ml^{-1}) was prepared by dialysis against 10 mM HCl.

Stepwise titration calorimetry was performed using an isothermal titration calorimeter, OMEGA, from MicroCal, Northampton, MA, USA, as described before [18]. Briefly, the titration was performed with injections of 1–3 μl each of a 1–3 M salt solution in 10 mM HCl with a 100-μl syringe at temperatures ranging from 20 to 40°C. The recorded time course of the change in heat from the baseline corresponds to the heat effect due to the change in salt concentration before and after each injection.

Circular dichroism (CD) spectra were measured with a Jasco spectropolarimeter, model J-500 A, equipped with an interface and a personal computer. The instrument was calibrated with ammonium *d*-10-camphorsulfonate. The results are expressed as the mean residue ellipticity $[\theta]$, which is defined as $[\theta] = 100 \times \theta_{obs}(lc)^{-1}$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue mol l^{-1}, and l is the length of the light path in cm.

Thermal unfolding transitions of the molten globule state stabilized by various concentrations of Na_2SO_4 in 10 mM HCl (pH 2) were measured using the ellipticity at 222 nm at a protein concentration of 0.2 mg ml^{-1} with a 1-mm cell. Temperatures were increased from 5 to 97°C at a rate of 1.0 K min^{-1} for the heat-denaturation measurements and decreased from 20°C at 0.5 K min^{-1} for the cold-denaturation measurements. The temperature changes were monitored with a thermocouple (Sensorteck

BAT-12 and a flexible probe) inserted directly into the cell and were stored simultaneously with the ellipticities in the computer. The reversibility of the unfolding transitions was checked by examining the ellipticities after returning the samples to the starting temperatures: unfolding was found to be quantitatively reversible.

Salt-induced transitions were measured by the ellipticity at 222 nm in 10 mM HCl (pH 2). Apomyoglobin solutions in 10 mM HCl containing various concentrations of salts were prepared and the CD measurements were carried out a few minutes after the sample preparation. There was no time-dependent change in signal.

The protein concentrations were determined spectrophotometrically. The extinction coefficient used to calculate the concentration of native apomyoglobin at 280 nm was $1.43 \times 10^4\ M^{-1}\,cm^{-1}$ [25].

3. Results

3.1. ITC of the salt-induced folding transition

The molten globule state is stabilized at pH 2 in the presence of high salt (Fig. 1) [5–8]. Although various salts stabilize the molten globule state, the potential of the salt

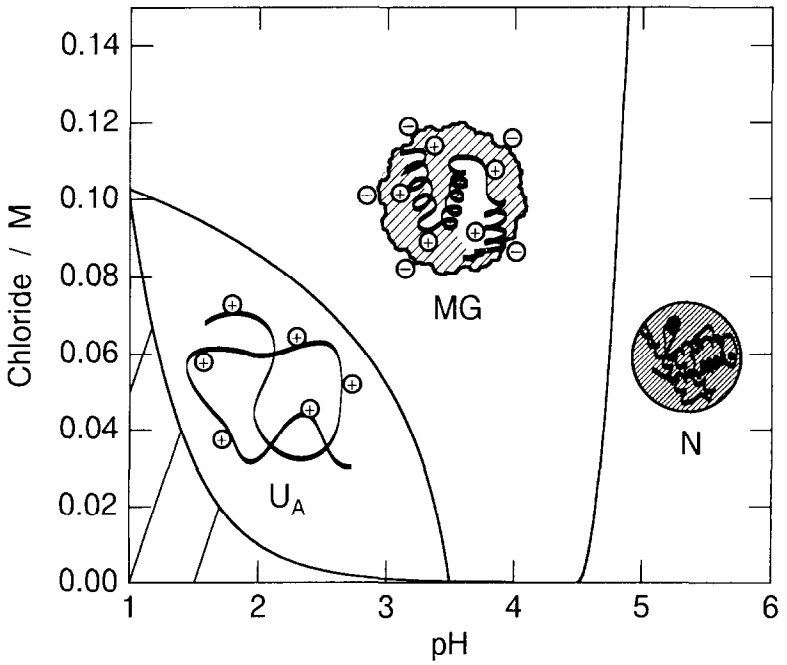

Fig. 1. Schematic representation of the pH and salt-dependent phase diagram for the native (N), acid-unfolded (U), and molten globule (MG) states of apomyoglobin. The lines show the boundaries between the conformational states. The concentration of salt required to stabilize the molten globule state changes significantly depending on anion species. The hatched area is prohibited owing to the increase in the minimal chloride concentration with decrease in pH ($[Cl^-] = 10^{-pH}$).

varies substantially depending on the anion species. As described previously [18], the choice of salt species is important because a large volume of protein solution (1.3 ml) is titrated with a small volume of salt solution (100 µl at most). We first used $NaClO_4$, which is an effective salt having a C_m value of 13 mM at 20°C [6,8].

First, we carried out the ITC measurement at 40°C, a relatively high temperature, because ΔH_F might be undetectable at lower temperatures due to its temperature dependence (see Fig. 6, below) [24]. Figs. 2A and B show the raw data for the titration

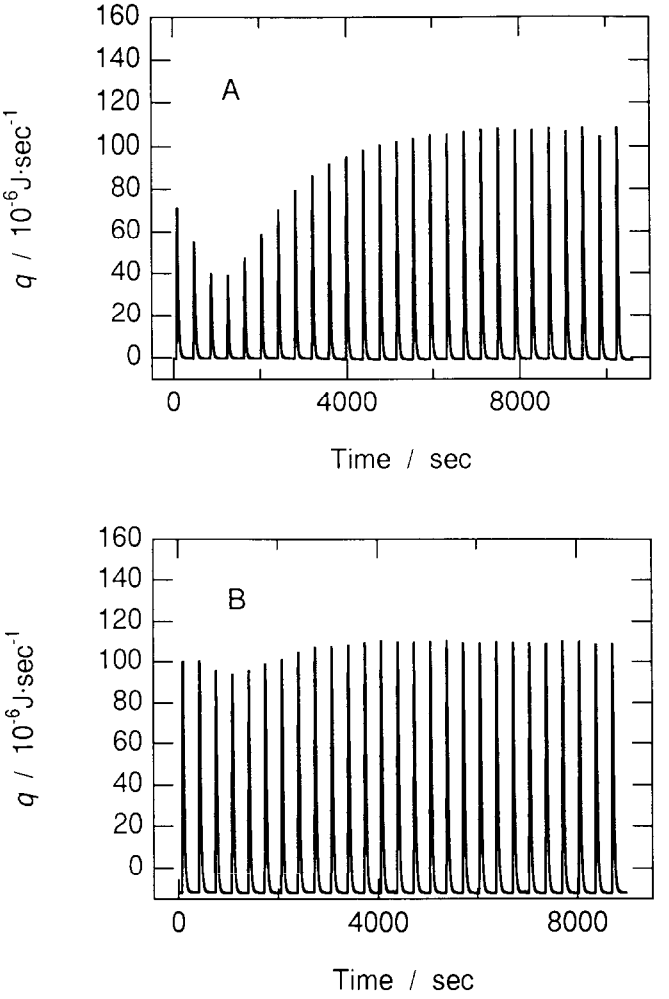

Fig. 2. Isothermal titration calorimetry of the $NaClO_4$-induced conformational transition of apomyoglobin at 40°C in 10 mM HCl (pH 2). A and B are typical calorimetric titrations of the acid-unfolded apomyoglobin with $NaClO_4$; A, protein concentrations 10 mg ml^{-1}; B, 2 mg ml^{-1}; 1 µl of 2 M $NaClO_4$ was added for each injection; C, Control titration in the absence of protein; D, Net heat effect involved in each injection, which was obtained after subtracting the effect of salt dilution. Protein concentrations: ○, 10 mg ml^{-1}; □, 2 mg ml^{-1}.

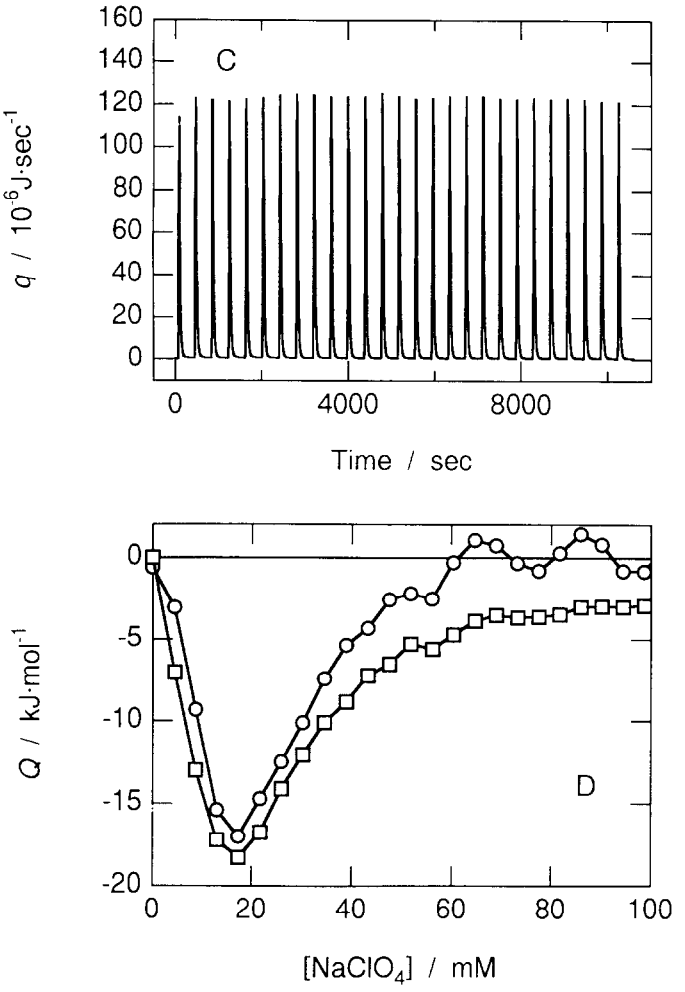

Fig. 2 *(continued)*

of apomyoglobin at 10 and 2 mg ml^{-1}, respectively, with $NaClO_4$ in 10 mM HCl (pH 2) at 40°C, and Fig. 2C shows the control titration carried out in the absence of protein. Each peak in Figs. 2A–C corresponds to the shift of heat q arising from the change in $NaClO_4$ concentration before and after each injection. As shown in Fig. 2C, in the absence of protein, a large positive heat of dilution was observed, indicating that the dilution of $NaClO_4$ solution is an endothermic reaction. In the presence of apomyoglobin, the titration pattern indicated a negative heat effect in addition to the heat of dilution of $NaClO_4$. The amplitude of the negative heat effect was dependent on protein concentration: it was several times larger at 10 mg ml^{-1} than at 2 mg ml^{-1}.

Fig. 2D shows the net heat effect Q observed at each injection of $NaClO_4$, which was obtained by subtracting the heat of salt dilution. As can be seen, the molar negative heat effect was independent of protein concentration and had a minimum value, leveling off with the progress of titration. This suggested that the net exothermic heat change arose from the formation of the molten globule state, and that it largely represents ΔH_F. Whereas the negative heat effect was zero above 60 mM $NaClO_4$ at 2 mg ml^{-1}, it continued to be slightly negative at 10 mg ml^{-1} (Fig. 2D). Because the conformational transition measured by CD is complete at around 60 mM $NaClO_4$ (see below), we consider that the negative heat effect observed at 10 mg ml^{-1} above 60 mM $NaClO_4$ represents a contribution not directly related to the folding transition, and possibly caused by intermolecular interactions at high protein concentrations, i.e. aggregation of the molten globule state.

The net heat effect observed at each injection of $NaClO_4$ at a protein concentration of 2 mg ml^{-1} was cumulated to obtain the total heat of reaction Q_{total}. Fig. 3A shows the plot of Q_{total} against $NaClO_4$ concentration at 40°C, which represents a saturation curve with a sigmoidal characteristic. To confirm that the transition measured by heat represents the folding transition stabilizing the molten globule state, we followed the $NaClO_4$-induced transition at 40°C by CD (Fig. 3B). Whereas the ellipticity in the absence of $NaClO_4$ is small, reflecting the unfolded conformation, it increased cooperatively with increase in the $NaClO_4$ concentration, showing the formation of α-helical structures [6, 7].

We normalized the transition curves assuming a two-state transition mechanism between the acid-unfolded state (U) and the molten globule state (M) [6, 7]

$$U \rightleftharpoons M \tag{1}$$

The fraction of the molten globule state (f_{MG}) is defined by

$$f_{MG} = [M]/([U] + [M]) = K_M/(1 + K_M) \tag{2}$$

where K_M is the equilibrium constant for the formation of the molten globule state: $K_M = [M]/[U]$. Fig. 3C compares the normalized transition curves detected by heat and CD. It is clear that the transition monitored by heat agreed well with that monitored by CD. These results suggest that the salt-induced conformational transition can be represented by a cooperative two-state mechanism and that the heat of titration arises substantially from the conformational change, i.e. ΔH_F. This situation is very similar to that observed for the salt-titration of acid-unfolded horse cytochrome-C at pH 2 [18]. By extrapolating the linear portion after the cooperative transition to zero salt concentration, we estimated the maximal Q_{total} value in the absence of salt ($Q_{total,0}$) to be -105 kJ mol^{-1} at 40°C.

3.2. Temperature dependence of ITC measurements

Nishii et al. [24] reported that the unfolding of the molten globule state of apomyoglobin is accompanied by distinct ΔH_U and $\Delta C_{p,U}$ values, as is the case of the native state. However, since their amplitudes are much smaller than those of the native state, it

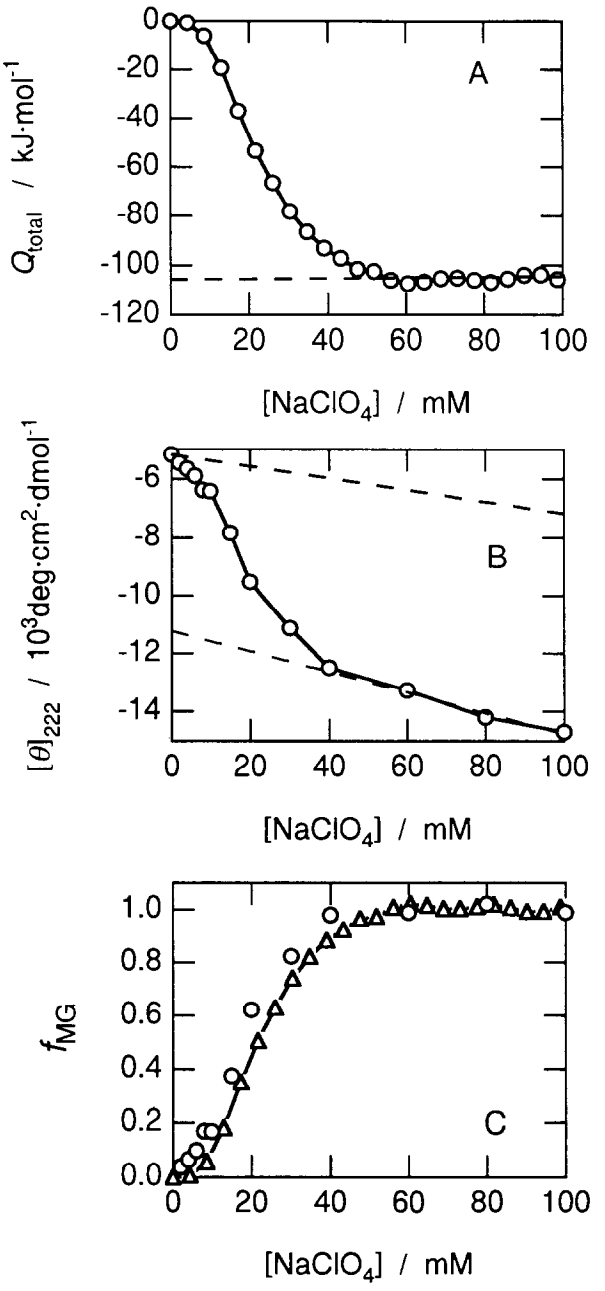

Fig. 3. $NaClO_4$-induced transition of apomyoglobin in 10 mM HCl (pH 2) at 40°C: A, transition monitored by ITC; B, transition monitored by the ellipticity at 222 nm; C, normalized transition curves measured by: △, ITC; and ○, CD.

is difficult to estimate precisely these values from the DSC measurements [26]. Provided that the heat of titration largely represents ΔH_F, it should become larger with an increase in the temperature, and its temperature dependence provides $\Delta C_{p,U}$ [27,28].

To examine the temperature dependences of the ITC measurements, we used Na_2SO_4 instead of $NaClO_4$, because the former is more effective than the latter in stabilizing the molten globule state, i.e. C_m for Na_2SO_4 is one fifth of that for $NaClO_4$ [6,8]. Thus, the contribution of the heat from salt dilution was slightly smaller than that of $NaClO_4$, even though it was still significant (data not shown).

Fig. 4A shows the Na_2SO_4-induced transitions of apomyoglobin at 2 mg ml^{-1} monitored by ITC at various temperatures. It is evident that the heat of titration depends significantly on temperature: whereas it was close to zero at 10°C, it was more than 100 kJ mol^{-1} above 30°C. The major cooperative transition was followed by a gradual change in heat, the direction of which varied depending on the temperature. Fig. 4B shows the Na_2SO_4-induced conformational transitions monitored by the ellipticity at 222 nm. As can be seen, the transition measured by CD showed a saturating curve without the later gradual change in signal. With increase in temperature, whereas the final CD intensity decreased, the C_m value increased slightly (2.5 mM at 0°C and 6 mM at 40°C). The range of salt concentration for the major cooperative transition measured by ITC is consistent with the cooperative transition measured by CD. We estimated the $Q_{total,0}$ values by extrapolating the linear portion after cooperative transition to zero salt concentration (Table 1) and plotted them against the temperature of measurement (Fig. 6).

3.3. *Unfolding of the molten globule state measured by CD*

To obtain the thermodynamic parameters which are complementary to those obtained from ITC measurements, we measured by CD the thermal unfolding of the

Table 1
Heat of titration for the $NaClO_4$ or Na_2SO_4-induced refolding transition of horse apomyoglobin at pH 2 determined by ITC

Temperature/°C	$Q_{total,0}$/kJ mol^{-1}
$NaClO_4$	
30	−98.4
40	−105.8
Na_2SO_4	
10	−10.3
20	−26.8
25	−53.3
30	−66.6
35	−85.2

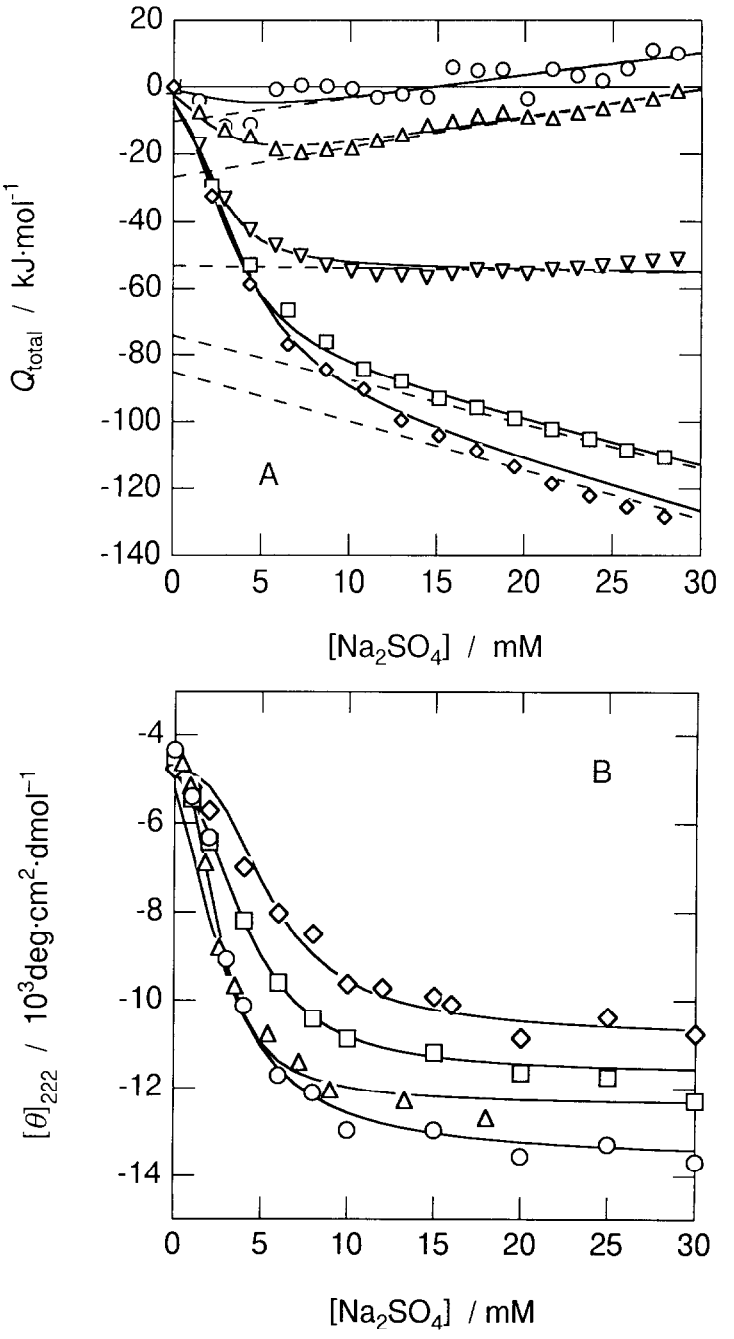

Fig. 4. Na_2SO_4-induced conformational transition of apomyoglobin in 10 mM HCl (pH 2) at various temperatures measured by heat (A) and the ellipticity at 222 nm (B): A. temperatures are 10 (○), 20 (△), 25 (▽), 30 (□) and 35 (◇)°C. B. Temperatures are 10 (○), 20 (△), 30 (□), and 40 (◇)°C.

molten globule state at pH 2, which was stabilized by various concentrations of Na_2SO_4 (Fig. 5). At around 10°C, whereas the protein was maximally unfolded in the absence of salt, the molten globule state was stabilized as the concentration of Na_2SO_4 increased. As was reported for the trichloroacetate- or chloride-stabilized molten globule state [24, 26], it showed a tendency for unfolding upon cooling below 10°C, i.e. cold denaturation, in addition to the unfolding at higher temperatures, i.e. heat denaturation, demonstrating that the unfolding is accompanied by a significant increase in heat capacity.

The thermal unfolding processes at various salt concentrations were analyzed on the basis of the two-state mechanism to obtain a common ΔH_U function with a constant $\Delta C_{p,U}$ value [24]. As shown in Fig. 5, a common ΔH_U function and the baselines for the molten globule and unfolded states satisfactorily reproduced the observed CD transition curves. The ΔH_U function was consistent with that obtained previously by analyzing the unfolding of the trichloroacetate- or chloride-stabilized molten globule states measured by CD (Fig. 6) [24].

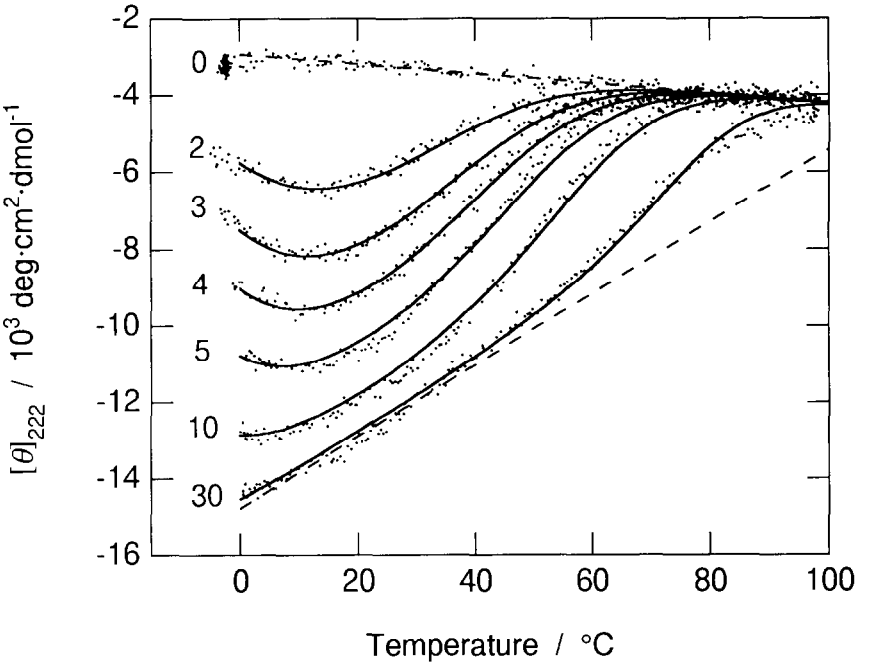

Fig. 5. The thermal unfolding transitions of the molten globule state of apomyoglobin in the presence of various concentrations of Na_2SO_4 at pH 2 (10 mM HCl) measured by the ellipticity at 222 nm. The numbers refer to the salt concentration in millimolar units. Dots are the observed signals and the solid lines are theoretical lines calculated with the baselines shown by the dashed lines and Eqs. (3)–(6) of Ref. [24]. The parameters used for the theoretical lines were $T_0 = 20$°C, $\Delta H_U(T_0) = 7.7$ kJ mol^{-1}, $\Delta C_{p,U} = 2.1$ kJ mol^{-1} K^{-1}, and $\Delta S_U(T_0) = 32.7$, 26.9, 22.5, 17.4, 9.3, 2.7, and -9.0 J mol^{-1} K^{-1} with increasing concentration of Na_2SO_4.

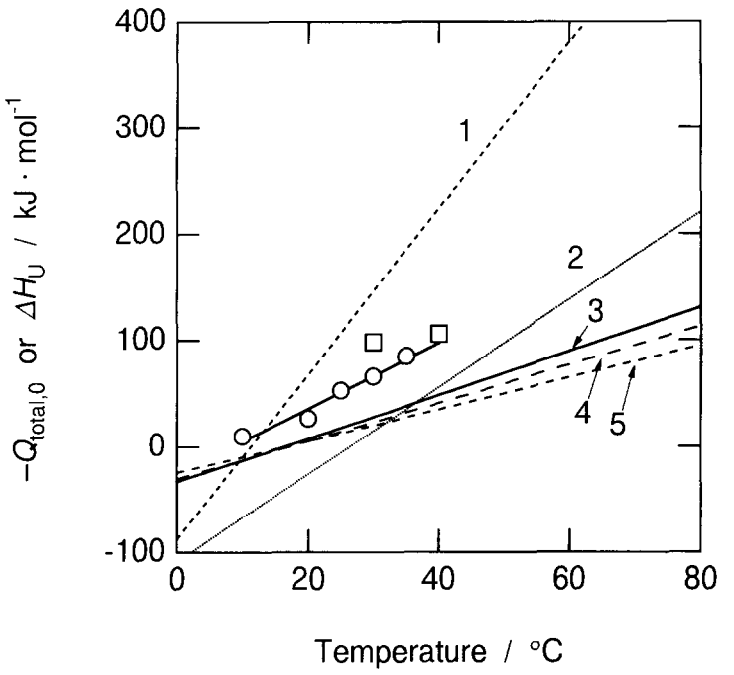

Fig. 6. Dependence on temperature of $Q_{total,0}$ of the acid-unfolded apomyoglobin titrated with Na_2SO_4 (○) and $NaClO_4$ (□). For comparison, the ΔH_U functions of the various conformational states are shown: 1, the native state of holomyoglobin [31]; 2, the native state of apomyoglobin [24]; 3, the molten globule state stabilized by Na_2SO_4; 4, the molten globule state stabilized by NaCl [24]; 5, the molten globule state stabilized by Na trichloroacetate [24]. Line 3 was drawn on the basis of the parameters determined in this study (see caption of Fig. 5) and other lines were taken from the literature.

4. Discussion

4.1. Heat of titration

Protein folding is a process in which an extended polypeptide chain aquires a maximally compact structure through formation of specific secondary and tertiary architectures [29, 30]. A large amount of hydrophobic residues are buried inside the molecule upon protein folding, resulting in a decrease in heat capacity, although a significant and opposite contribution of the burial of polar groups is also evident [27, 28]. Therefore, in addition to the structural characterization of the intermediate state, thermodynamic characterization is of critical importance for understanding the mechanism of protein folding. In the present study, we showed that ITC is useful for characterizing the salt-induced stabilization of the molten globule state of horse apomyoglobin, as we reported previously for horse cytochrome-C [18].

Whereas several proteins including apomyoglobin are substantially unfolded in the absence of salt at pH 2, the addition of anions either from salt or acid stabilizes the

molten globule state [5–8]. Goto et al. [6] proposed that, since anions can interact with positive charges of both the unfolded and molten globule states, the salt-induced formation of the molten globule state should be explained in terms of preferential binding of anions to the compactly packed molten globule state compared to the expanded unfolded state. At pH 2, most of the titratable groups are protonated and hence the net charge of the molten globule state is essentially the same as that of fully unfolded state at the same pH. However, since the anion binding arises from an electrostatic interaction, the compact molten globule state with higher charge density binds anions more tightly than the unfolded state. This results in stabilization of the molten globule state with an increase in salt concentration.

On the basis of the preferential binding model, Q_{total} observed by ITC consists of the heat of conformational change (Q_{conf}), including the heat of interaction with water, and those of anion binding to the molten globule (Q^{M}_{bind}) and the unfolded states (Q^{U}_{bind}) [24]

$$Q_{total} = Q_{conf} + Q^{M}_{bind} + Q^{U}_{bind} \quad (3)$$

To obtain Q_{conf}, it is necessary to know the contribution of the heat of anion binding, i.e. Q^{M}_{bind} and Q^{U}_{bind}. Although direct measurement of the heat of anion binding is difficult, several results obtained for cytochrome–C have suggested that the heat of anion binding (Q^{M}_{bind} and Q^{U}_{bind}) is small compared with the heat of conformational change (Q_{conf}), and that Q_{total} is approximated by $Q_{conf}(= \Delta H_F f_{MG})$ [18].

To decide if Q_{total} represents Q_{conf}, $Q_{total,0}$ was obtained by extrapolating the linear portion after the cooperative transition to zero salt concentration. Fig. 6 shows the temperature dependence of $-Q_{total,0}$. Fig. 6 also shows the ΔH_U functions of the molten globule states, which were determined from the thermal unfolding experiments using CD. For comparison, we also plotted the ΔH_U functions of the native states of apomyoglobin [24] and holomyoglobin [31]. The temperature dependence of $-Q_{total,0}$ was similar to that of ΔH_U for the molten globule states, although the $-Q_{total,0}$ values were slightly larger than the ΔH_U value. Assuming that ΔH_U makes a large contribution to $-Q_{total,0}$, we estimated $\Delta C_{p,U}$ to be 3.1 kJ mol^{-1} K^{-1} from the temperature dependence of $-Q_{total,0}$. Nishii et al. [24] reported $\Delta C_{p,U}$ values of 1.8 and 1.5 kJ mol^{-1} K^{-1} for the molten globule states stabilized by Na trichloroacetate and NaCl, respectively. The value estimated from ITC is comparable to that obtained from the thermal unfolding of the molten globule state and smaller than that of the native state of apomyoglobin (4.0 kJ mol^{-1} K^{-1}) or holomyoglobin (8.7 kJ mol^{-1} K^{-1}) [24, 31].

However, it is clear that $Q_{total,0}$ obtained from ITC is slightly larger in magnitude than ΔH_U obtained from CD. Although we do not know the exact reason for this at present, two explanations can be suggested. Since the salt concentration required for stabilizing the molten globule state of apomyoglobin is about twice that of cytochrome-C, the contribution of anion binding to the heat of titration might be higher than that of cytochrome–C. Therefore, it is possible that the heat of anion binding is not negligible in the case of apomyoglobin and contributes to the apparent heat of titration.

It should be noted that the net heat effect arising from apomyoglobin, in comparison with the heat of salt dilution, is significantly small at 2 mg ml^{-1} (Fig. 2B). Therefore, we cannot exclude the possibility that the high basal heat may produce some errors when

estimating the heat of titration. Alternatively, the apparent difference might be caused by a slight deviation of the mechanism of folding from the exact two-state mechanism, and will be described below.

4.2. *Mechanism of the conformational transition*

The molten globule state of apomyoglobin is one of the most extensively studied intermediate conformational state of proteins. The native structure of holomyoglobin consists of eight α-helices (A–H) [9, 11]. In addition to the salt-stabilized molten globule state, a similar molten globule state is stable at pH 4 under conditions of low salt [9, 32]. The structural characterization of the molten globule state at pH 4 showed that it retains the A, G, and H helices. A solution X-ray scattering study has suggested that the trichloroacetate-stabilized molten globule state at pH 2 is composed of a helical core, probably consisting of the A, G, and H helices, and flaring tails [13].

A series of studies carried out by Goto and coworkers indicated that the salt-induced stabilization of the molten globule state of apomyoglobin can be interpreted in terms of a two-state transition between the unfolded and molten globule states [6–8]. However, Nishii et al. [26] studied the thermal unfolding of the molten globule state of apomyoglobin and suggested that, although the transition might be approximated by a two-state transition, there are several observations which are apparently inconsistent with a strict two-state mechanism. Taken together, they concluded that a combined mechanism of the two-state transition and a gradual structural change, as suggested from statistical mechanical theories [33, 34], is a more general mechanism describing the unfolding transition of the molten globule state of proteins [26]. Therefore, it is possible that the slight deviation in the $-Q_{\text{total},0}$ value from the ΔH_{U} value might be caused by the two-state approximation of the thermal unfolding, which underestimates the ΔH_{U} value.

Griko and Privalov [35] reported the thermal unfolding of the molten globule state of sperm whale apomyoglobin at pH 4 measured by DSC. They showed that the process is not accompanied by excess heat absorption, as expected from the rather cooperative unfolding transition measured by far-UV CD, and concluded that the unfolding process cannot be represented by a first-order phase transition, i.e. two-state transition. They suggested that the transition might be explainable in terms of a second-order phase transition, although a quantitative analysis was not proposed. Their results for the molten globule state of apomyoglobin at pH 4 are apparently inconsistent with ours for the salt-stabilized molten globule state at pH 2. Although a combined mechanism involving gradual disordering of the molten globule state and a two-state transition may accommodate both our results and theirs [26], at present we cannot offer a clear explanation for the difference.

5. Conclusion

We have shown that ITC is useful for studying the salt-induced formation of the molten globule state of apomyoglobin. The transition measured by heat was consistent

with the conformational transition measured by far-UV CD. However, $Q_{total,0}$ was slightly higher in magnitude than ΔH_F estimated from the thermal unfolding of the molten globule state. These results suggest that, although ΔH_F makes a major contribution to $Q_{total,0}$, other contributions have to be considered. Our results support a view that the salt-induced formation of the molten globule state of apomyoglobin can be approximated by a two-state transition between the unfolded and molten globule states.

To understand the mechanism of protein folding, it is critically important to elucidate the thermodynamic mechanism responsible for the stability of the intermediate conformational states in comparison with that of the native state. The results presented here and those obtained with cytochrome–C indicate that ITC provides a unique and important approach for this purpose.

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