Thermal unfolding of tetrameric melittin: Comparison with the molten globule state of cytochrome *c*

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Abstract

Whereas melittin at micromolar concentrations is unfolded under conditions of low salt at neutral pH, it transforms to a tetrameric α -helical structure under several conditions, such as high peptide concentration, high anion concentration, or alkaline pH. The anion- and pH-dependent stabilization of the tetrameric structure is similar to that of the molten globule state of several acid-denatured proteins, suggesting that tetrameric melittin might be a state similar to the molten globule state. To test this possibility, we studied the thermal unfolding of tetrameric melittin using far-UV CD and differential scanning calorimetry. The latter technique revealed a broad but distinct heat absorption peak. The heat absorption curves were consistent with the unfolding transition observed by CD and were explainable by a 2-state transition mechanism between the tetrameric α -helical state and the monomeric unfolded state. From the peptide or salt-concentration dependence of unfolding, the heat capacity change upon unfolding was estimated to be 5 kJ (mol of tetramer)⁻¹ K⁻¹ at 30 °C and decreased with increasing temperature. The observed change in heat capacity was much smaller than that predicted from the crystallographic structure (9.2 kJ (mol of tetramer)⁻¹ K⁻¹), suggesting that the hydrophobic residues of tetrameric melittin in solution are exposed in comparison with the crystallographic structure. However, the results also indicate that the structure is more ordered than that of a typical molten globule state. We consider that the conformation is intermediate between the molten globule state and the native state of globular proteins.

Keywords: circular dichroism; differential scanning calorimetry; melittin; molten globule state; thermal unfolding

Melittin is a major component of honeybee venom. It consists of 26 amino acid residues, 5 of which are basic, but none of which are acidic (Habermann, 1972): NH₃⁺-G-I-G-A-V-L-K⁺-V-L-T-T-G-L-P-A-L-I-S-W-I-K⁺-R⁺-K⁺-R⁺-Q-Q-NH₂. The amino group is free and the carboxyl group is amidated; as a consequence, it has no negative charge. The conformation and stability of melittin have been studied extensively (Brown et al., 1980; Bello et al., 1982; Quay & Condie, 1983; Ramalingam et al., 1991; Wesson & Eisenberg, 1992; Wilcox & Eisenberg, 1992; Ramalingam & Bello, 1993). It has been shown that, whereas melittin at micromolar concentrations is unfolded under conditions of low salt at neutral pH, it transforms to an α -helical tetramer under high-salt conditions or at alkaline pH.

Because it is a monomer-to-tetramer reaction, tetrameric melittin is also stabilized with an increase in peptide concentration.

Whereas several acid-denatured proteins including cytochrome *c* and apomyoglobin are unfolded extensively under conditions of low salt at pH 2, they transform to a molten globule state upon addition of salt or acid (Goto & Fink, 1989; Goto et al., 1990a, 1990b). The molten globule state is a compact denatured state with a significant amount of native-like secondary structure, but a largely disordered tertiary structure, and has been proposed to be a major intermediate of protein folding (Kuwaitjima, 1989; Ptitsyn, 1992; Baldwin, 1993; Jennings & Wright, 1993). A series of studies carried out by Goto and co-workers (Goto et al., 1990a, 1990b; Goto & Nishikiori, 1991) has shown that the conformation of acid-denatured proteins is determined by a balance of various forces stabilizing or destabilizing the molten globule state: whereas the proteins are unfolded significantly in the absence of salt by charge repulsion between the protein positive groups, an increase in anion concentration either from salt or acid stabilizes the molten globule state because the anions bind to the positive charges and shield the repulsive forces.

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Abbreviations: ANS, 1-anilino-8-naphthalene sulfonate; ASA, accessible surface area; $\Delta C_{p,U}$, heat capacity increase upon unfolding; DSC, differential scanning calorimetry; Mes, 2-(*N*-morpholino)ethanesulfonic acid; R_g, radius of gyration; SAXS, small angle X-ray scattering; T, absolute temperature.

As a model of the anion-dependent conformational transition of the molten globule state, Goto and Aimoto (1991) prepared an amphiphilic model peptide of 51 amino acid residues consisting of tandem repeats of a Lys-Lys-Leu-Leu sequence and containing a turn sequence at the center of the molecule. Whereas the model peptide showed no significant conformation under conditions of low salt at neutral pH, addition of anion, either from salt or from acid, induced the folding transition to a monomeric α -helical state, in which 2 amphiphilic α -helices interacted with each other intramolecularly through hydrophobic Leu residues. These results showed that the anion-dependent conformational transition is not limited to acid-denatured proteins but may occur with peptides and proteins even at neutral pH.

At the same time, we noticed that the conformational transition of melittin is very similar to those of acid-denatured proteins or the synthetic model peptide (Goto & Hagihara, 1992; Hagihara et al., 1992). We showed that the effectiveness of different salts at stabilizing the α -helical state of melittin depends on the anion species and follows the electroselectivity series, confirming that the anion binding is responsible for the transition. We showed that strong acids also stabilize the α -helical state with a potential similar to that of the corresponding salt. In addition, we demonstrated that the pH- and anion-dependent conformational change can be explained by a minimal 2-state mechanism in which the anion binding and protonation of the titratable groups are coupled to the conformational transition (Goto & Hagihara, 1992). This simple mechanism of conformational stability is similar to that of the acidic molten globule of proteins but differs from that of the native proteins and raises a question: is the tetrameric structure of melittin more similar to the structure of the molten globule state than to the native structure of globular proteins?

There are 2 additional results supporting this suggestion (Table 1):

1. *Contribution of the charge repulsion.* Hagihara et al. (1992) estimated the contribution of charge repulsion in destabilizing the α -helical state of melittin to be 2.5 kJ (mol of tetramer)⁻¹ (mol of charge)⁻¹ at 20 °C. By chance, the acidic molten globule of horse cytochrome *c*, consisting of the same number (104) of amino acid residues as tetrameric melittin (26 × 4), has the same number of positive charges (24 at pH 2) as (6 × 4) and a similar size ($R_g = 16.1$ Å) to melittin (17.5 Å).

They concluded that the contribution of electrostatic repulsion to the free energy of unfolding (ΔG_U) of the molten globule of cytochrome *c* (1.9 kJ mol⁻¹ (mol of charge)⁻¹) is similar to that of melittin.

2. *Radius of gyration.* The R_g value of the native state of horse ferricytochrome *c* is estimated to be 13.8 Å from the SAXS measurement and 12.7 Å from the crystallographic structure (Hagihara et al., 1992; Kataoka et al., 1993). Therefore, R_g (16.1 Å) of the molten globule state is larger by 27% than that of the crystallographic structure. In the case of melittin, because the R_g value calculated from the crystallographic structure was 14.0 Å (Hagihara et al., 1992), the measured value (17.5 Å) was larger by 25% than that of the crystallographic structure. This indicates that tetrameric melittin in solution is expanded, compared with the crystallographic structure, to an extent similar to the molten globule of cytochrome *c*.

Conformational transition of melittin has been assumed to be an excellent model for understanding the mechanism of protein folding. In the study of protein folding, much attention has been paid recently to the conformation and stability of the molten globule state and the elucidation of a novel state, which might be intermediate between the molten globule and native states or the molten globule and more extensively unfolded states (Baldwin, 1993; Goto et al., 1993; Redfield et al., 1994). Therefore, we consider the detailed characterization of tetrameric melittin in comparison with the molten globule state of proteins to be of great importance.

In the present study, to characterize the conformation and stability of tetrameric melittin in solution, we measured the thermal unfolding of melittin using CD and differential scanning calorimetry. Although several groups have examined the thermal unfolding of melittin, no successful DSC result has been reported (Wilcox & Eisenberg, 1992). We showed for the first time a distinct heat absorption during the unfolding of tetrameric melittin, demonstrating a contribution of hydrophobic interactions in stabilizing the tetrameric α -helical state. However, the heat capacity increase upon unfolding is evidently smaller than that predicted from the X-ray crystallographic structure using a method based on accessible surface area (Ooi & Oobatake, 1991; Oobatake & Ooi, 1993). This strongly suggests that the hydrophobic residues of the tetramer in solution are more exposed than expected from the crystallographic structure. We consider

Table 1. Comparison of the conformational states of melittin and horse cytochrome *c*^a

	Net charge	R_g^b (Å)	Electrostatic repulsion ^c (kJ mol ⁻¹ charge ⁻¹)	$\Delta C_{p,U}$ (kJ mol ⁻¹ K ⁻¹)	ΔH_U (110 °C) (kJ mol ⁻¹)
Melittin tetramer	24	17.45 (13.96)	2.5	4.9 ^d (9.2)	280–350
Cytochrome <i>c</i>					
Native	—	13.75 (12.65)	—	5.3 ^e , 7.0 ^f (8.5)	574 ^e , 660 ^f
Molten globule at pH 2	24	16.11	1.9	1.6 ^e	227 ^e

^a The values in parentheses were estimated for the crystallographic structure.

^b Taken from Hagihara et al. (1992) for melittin and from Kataoka et al. (1993) for horse cytochrome *c*.

^c Taken from Hagihara et al. (1992).

^d The value was estimated at 30 °C.

^e Taken from Hagihara et al. (1994) for horse cytochrome *c*.

^f Taken from Privalov and Gill (1988) for bovine cytochrome *c*.

that the tetrameric structure of melittin in solution is intermediate between the typical molten globule state and the native state of globular proteins.

Results

Thermal unfolding measured by CD

Figure 1 shows the far-UV CD spectra of 138 μM melittin in 10 mM sodium acetate buffer at pH 4.5 in the presence of 0.2 M NaClO_4 at various temperatures. Whereas the tetrameric α -helical structure was substantially stabilized at 30 $^\circ\text{C}$, it unfolded with increasing temperature. At 95 $^\circ\text{C}$, melittin was unfolded largely to a structure close to a random coil. The spectra at various temperatures showed an isodichroic point at 203 nm, consistent with a 2-state transition mechanism.

Figure 2A shows the thermal transition curve of 27.6 μM melittin at pH 4.5 in the presence of various concentrations of NaClO_4 , measured by the ellipticity at 222 nm. As reported previously (Goto & Hagihara, 1992), the NaClO_4 -induced folding transition is a cooperative process with a midpoint concentration of 0.1 M at 27.6 μM melittin at 20 $^\circ\text{C}$ and the α -helical structure is stable in the presence of 1 M NaClO_4 at 20 $^\circ\text{C}$. The α -helical structure is cooperatively unfolded upon heating. With increase in the salt concentration, the apparent midpoint temperature ($T_{m,\text{app}}$) of the unfolding transition was increased. It should be noted that the unfolding transition was much broader than those observed for the native state of typical globular proteins.

We then measured the thermal unfolding curves of melittin at various peptide concentrations at pH 4.5 in the presence of 0.2 M NaClO_4 . Because measurement of the unfolding transitions using the ellipticity at 222 nm was difficult for melittin solutions above 150 μM due to high absorption, we used 238.5 nm, in addition to 222 nm, to follow the thermal unfolding (Fig. 2B). A broad but cooperative thermal transition was observed, and the transition was shifted to a higher-temperature region as the peptide concentration increased. The pattern of unfolding tran-

Fig. 1. Far-UV CD spectra of 138 μM melittin in 0.2 M NaClO_4 and 10 mM sodium acetate buffer (pH 4.5) at various temperatures. Figures indicate the temperature in $^\circ\text{C}$.

Fig. 2. Dependence on NaClO_4 concentration and peptide concentration of the thermal unfolding transition of melittin measured by the ellipticity at 222 nm and 238.5 nm, respectively. **A:** 27.6 μM melittin at various NaClO_4 concentrations in 10 mM sodium acetate buffer at pH 4.5. Figures indicate the molar NaClO_4 concentrations with increasing thermal stability. Clear circles indicate the transition in the absence of NaClO_4 . **B:** Various concentrations of melittin in 0.2 M NaClO_4 and 10 mM sodium acetate buffer at pH 4.5. Figures indicate the micromolar melittin concentrations with increasing stability. The dots show the observed data points and the solid lines show the theoretical curves calculated with Equation 12 and the parameters in Table 2. Broken straight lines show the baselines for the helical state and unfolded state.

sitions observed at various melittin concentrations is apparently similar to that at various salt concentrations (Fig. 2A). Figure 3 shows the dependence of $T_{m,\text{app}}$ upon melittin concentration at pH 4.5 and 0.2 M NaClO_4 . $T_{m,\text{app}}$ increased with increasing melittin concentration and exhibited a tendency for saturation at high melittin concentration.

Thermal unfolding measured by calorimetry

Provided that the unfolding transition of melittin is a cooperative process, it should accompany the change in enthalpy. Because the thermal unfolding measured by CD indicates that the transition is broad, extending over a temperature range of more than 50 $^\circ\text{C}$, we carefully carried out DSC measurements between 10 $^\circ\text{C}$ and 120 $^\circ\text{C}$.

Fig. 3. Dependence of $T_{m,app}$ on melittin concentration in 10 mM sodium acetate buffer at pH 4.5 and 0.2 M NaClO_4 obtained by CD (clear circles) and DSC (solid circles). Solid and broken lines are the theoretical curves calculated using Equation 16 and the parameters in Table 2. $\alpha_0 = 0.08$ mM and $T_{m,app,0} = 51.8$ °C for the CD data; $\alpha_0 = 0.7745$ mM and $T_{m,app,0} = 80.2$ °C for the DSC data.

Figure 4A shows the DSC curve of 2.68 mM melittin in 10 mM sodium acetate buffer at pH 4.5 in the presence of 0.1 M NaClO_4 . It is clear that the thermal unfolding of melittin shows a distinct heat absorption peak. In accordance with the unfolding transition measured by CD, the DSC curve was much broader than those observed for the thermal unfolding of globular proteins (see, for example, Privalov, 1979). The temperature of the DSC peak is consistent with the $T_{m,app}$ estimated from the CD measurement (data not shown). The DSC scan shown in Figure 4A was repeated 4 times, keeping the same melittin solution in the cell, up to 100 °C for the first 2 scans and to 120 °C for the latter 2 scans. As can be seen from the superimposition of the DSC curves, the reversibility of the unfolding transition was remarkably high.

Figure 4B shows the dependence on NaClO_4 concentration of the DSC curve of 2.68 mM melittin at pH 4.5. Whereas no significant heat absorption was observed in the absence of salt, a clear heat absorption peak was observed at NaClO_4 concentrations higher than 50 mM. The DSC curve at 20 mM NaClO_4 showed a tendency for cold denaturation. The peak temperature increased with increasing salt concentration, similar to that observed by CD.

Figure 4C shows the DSC curves at 0.42, 1.54, 3.10, and 6.42 mM melittin concentrations at pH 4.5 in 0.2 M NaClO_4 . With an increase in melittin concentration, the peak temperature was increased, consistent with the results observed by CD (Fig. 2B).

Mechanism of conformational transition

The conformational transitions of melittin under various conditions were well approximated by a minimal 2-state mechanism between the folded tetrameric state (F_4) and the monomeric unfolded state (U): $F_4 \rightleftharpoons 4U$ (Brown et al., 1980; Quay & Condie, 1983; Goto & Hagihara, 1992; Hagihara et al., 1992; Wilcox & Eisenberg, 1992). Therefore, we analyzed the thermal transitions measured by CD and DSC on the basis of the 2-state assumption.

Fig. 4. DSC curves of the unfolding transition of tetrameric melittin. **A:** Repeated DSC scans of 2.68 mM melittin in 10 mM sodium acetate buffer at pH 4.5 and 0.1 M NaClO_4 . The first and second scans were run up to 100 °C and the third and fourth scan up to 120 °C. **B:** Dependence on NaClO_4 concentration at 2.68 mM melittin in 10 mM sodium acetate buffer at pH 4.5. Figures represent the concentration of NaClO_4 in mM. The dotted and broken line represents the baseline for the transition in 100 mM NaClO_4 . **C:** Dependence on melittin concentration in 10 mM sodium acetate buffer at pH 4.5 and 200 mM NaClO_4 . Figures represent the millimolar melittin concentration. The broken line represents the baseline for the transition at 1.54 mM melittin. In B and C, the symbols show the observed data and the solid lines show the theoretical curves.

First, we tried to analyze the CD and DSC curves assuming that $\Delta C_{p,U}(T)$ is constant, an approach often employed for analysis of the thermal unfolding transition of proteins. However, the theoretical transition curves obtained using this assumption did not give a good fit to the observed CD and DSC curves for melittin. In fact, it is evident from the DSC curves in Figure 4 that the C_p for the tetrameric state increases substantially with increase in temperature, approaching the value for the unfolded state at around 100 °C. In other words, $\Delta C_{p,U}(T)$ decreases with increase in temperature. Similar temperature dependence of $\Delta C_{p,U}(T)$, although less pronounced than that for melittin, has been reported for globular proteins (Makhataadze & Privalov, 1990). Because the temperature range of the unfolding transition for melittin is so wide, the temperature dependence of $\Delta C_{p,U}(T)$ cannot be neglected. Therefore, we assumed that the amplitude of $\Delta C_{p,U}(T)$ decreases with increase in temperature and can be represented by $\Delta C_{p,U}(T) = \Delta C_{p,U}(T_R) + a(T - T_R) + b(T - T_R)^2$, where T_R is a reference temperature in Kelvin. Introduction of the T_R is necessary in order to obtain the thermodynamic parameters accurately near the transition temperatures (see Materials and methods). We also assumed that the heat capacity of the unfolded monomer, $C_{p,U}(T)$, is independent of temperature, being a reasonable assumption if we neglect the slight temperature dependency of $C_{p,U}$ observed in the absence of salt (Fig. 4B). As a consequence, the heat capacity for the tetrameric helical state, $C_{p,F}(T)$, is calculated as $C_{p,F}(T) = C_{p,U}(T) - \Delta C_{p,U}(T)$.

First, each CD transition curve (Fig. 2) was analyzed using the thermodynamic equations described in the Materials and methods. In the analysis, because the baselines for the folded tetramer and unfolded monomer were not explicit, they were also included as the fitting parameters, both being assumed to be linear against temperature. With a nonlinear least-squares fitting program, the calculated CD transition curves can be fitted to the observed curves so that a set of linear baselines, $\Delta H_U(T)$ and $\Delta C_{p,U}(T)$ functions, and the $T_{m,app}$ value are determined. T_R was set at 353.15 K (80 °C). However, because the transition curves were broad, the fitting errors were significant and, in particular, the uncertainty of the baselines was serious. Therefore, to minimize these errors, the transition curves in the presence of various concentrations of salt were fitted simultaneously. Simultaneous curve-fitting analysis was also carried out for the transitions observed at various peptide concentrations. The theoretical curves in Figure 2A and B were drawn on the basis of the parameters in Table 2 and Equation 12, described in the Materials and methods. As can be seen from Figure 2, the calculated transition curves agreed well with the observed curves.

We then analyzed the heat absorption curves. Because each heat absorption curve was so broad, fitting again produced significant errors. Therefore, as was the case in CD analysis, we analyzed a series of heat absorption curves obtained at various salt concentrations or peptide concentrations simultaneously to estimate a set of parameters, i.e., the baselines for the helical tetramer and unfolded monomer, $\Delta H_U(T)$ function and the respective $T_{m,app}$ values. It will be noted that the unfolding transition at 20 mM NaClO₄ in Figure 4B was not included in the analysis because its inclusion significantly worsened the fitting. Probably the heat absorption peak was too small to be fitted correctly, compared with the baseline fluctuation. Table 2 shows the parameters obtained with $T_R = 353.15$ K (80 °C) and the solid lines in Figure 4B and C show the theoretical curves drawn using the parameters in Table 2 and Equation 14. As can be seen, fittings to the observed curves were fairly good.

Figure 5A and B shows the $\Delta H_U(T)$ and $\Delta C_{p,U}(T)$ functions, respectively, obtained from the CD and DSC analyses. Although the $\Delta H_U(T_{m,app})$ values obtained from calorimetry measurements are slightly smaller than those obtained by the CD measurements, they are relatively consistent with each other if we consider the broadness of the transitions and the uncertainty of the baselines. ΔH_U increased with increase in $T_{m,app}$. However, it showed saturation around 90 °C because $\Delta C_{p,U}$ decreased with increase in temperature, becoming zero at around 90 °C (Fig. 5B). From the CD measurements, at 30 °C, ΔH_U and $\Delta C_{p,U}$ were estimated to be about 120 kJ (mol of tetramer)⁻¹ and 4.9 kJ (mol of tetramer)⁻¹ K⁻¹, respectively. At 50 °C, these values were 210 kJ (mol of tetramer)⁻¹ and 4.5 kJ (mol of tetramer)⁻¹ K⁻¹, respectively. Table 2 shows the ΔH_U and $\Delta C_{p,U}$ values at the reference temperature, 80 °C. The maximum value of ΔH_U estimated from the DSC data was about 280 kJ (mol of tetramer)⁻¹. ΔH_U obtained from the CD data suggests that the maximum value is slightly higher (i.e., about 350 kJ (mol of tetramer)⁻¹).

As shown in Figure 3, $T_{m,app}$ increased with the increase in melittin concentration but exhibited a tendency for saturation at high concentration. Figure 3 also includes the plots of $T_{m,app}$ against melittin concentration detected by DSC, which were consistent with those obtained by the CD measurements. Because $T_{m,app}$ is related to melittin concentration (see Materials and methods), we can examine the validity of the parameters obtained from analysis of the CD or heat absorption curves at various melittin concentrations. On the basis of Equation 16 described in the Materials and methods and the thermodynamic parameters obtained above (Table 2), we calculated the relation between melittin concentration and $T_{m,app}$. The lines in Figure 3

Table 2. Parameters for the $\Delta C_{p,U}(T)$ function of melittin

Method	Conditions changed	$\Delta C_{p,U}(T_R)^a$ (kJ mol ⁻¹ K ⁻¹)	a	b	$\Delta H_U(T_R)^a$ (kJ mol ⁻¹)
CD	Salt concentration	3.15	-6.44×10^{-2}	-5.98×10^{-4}	332
	Peptide concentration	3.14	-6.45×10^{-2}	-6.00×10^{-4}	311
DSC	Salt concentration	1.73	-0.13	-7.24×10^{-4}	266
	Peptide concentration	1.63	-0.15	-9.25×10^{-4}	263

^a $T_R = 353.15$ K (80 °C).

Fig. 5. Dependence of ΔH_U (A) and $\Delta C_{p,U}$ (B) on $T_{m,app}$ of the unfolding transition of tetrameric melittin. Circles and squares show the results obtained from the CD and DSC experiments, respectively. Clear and solid symbols are the results obtained for the peptide concentration dependence and NaClO₄ concentration dependence, respectively, of the unfolding transition. Solid lines are the theoretical ones drawn on the basis of parameters of Table 2.

show the theoretical lines, which reproduce well the observed dependence, showing the validity of the 2-state approximation and the estimated thermodynamic parameters.

Aromatic CD spectrum and ANS binding

The aromatic CD spectrum has been useful for examining the molten globule hypothesis (Goto & Fink, 1989; Kuwajima, 1989; Ptitsyn, 1992). Whereas the presence of intense and sharp CD peaks suggests specific side-chain interactions, which are representative of the native tertiary structure, a weak and monotonous spectrum suggests a disordered or fluctuating tertiary structure. Figure 6 shows the near-UV CD spectra of melittin under various conditions. The spectrum at pH 7.5 in the absence of salt is monotonous with weak intensity, showing that the peptide is significantly unfolded. The near-UV CD spectrum of the helical states showed sharp peaks, indicating the presence of unique side-chain structures, at least around the tryptophan residue. Interestingly, the spectra for the various tetrameric states are different from each other, although the far-UV CD spectra are essentially the same (Goto & Hagihara, 1992). Whereas the CD intensity at around 280 nm was negative for the anion-stabilized helical state, it was positive for the helical state sta-

Fig. 6. Aromatic CD of melittin under various conditions at 20 °C. 1, Helical state at 20 mM melittin in 10 mM Tris-HCl at pH 7.5; 2, helical state in 0.5 M NaCl and 10 mM NaOH at pH 11; 3, unfolded state in 10 mM Tris-HCl at pH 7.5; 4, helical state in 1.8 M NaClO₄ and 10 mM Tris-HCl at pH 7.5. Spectra 2, 3, and 4 were measured at 27.6 μ M melittin with a 4-cm cell and spectrum 1 was measured with a 0.1-mm cell.

bilized at high melittin concentration. The CD intensity of the helical state induced by alkaline pH was close to zero, although the spectrum showed fine peaks. These results suggest that the distinct side-chain structures are produced in accordance with the conditions stabilizing the tetrameric α -helical state.

ANS binding has been considered to be evidence of the molten globule state having water-accessible hydrophobic surfaces (Goto & Fink, 1989; Ptitsyn, 1992). Figure 7 shows the fluorescence spectra of ANS in the presence of melittin in different conformational states. An increase in ANS fluorescence, accompanying a blue shift of the maximum emission wavelength, was observed in the presence of the tetrameric state stabilized at alkaline pH or the tetrameric state stabilized by high salt at pH 6. However, only a slight increase in ANS fluorescence was observed with the unfolded monomeric melittin.

Discussion

Thermal unfolding of tetrameric melittin

We have reported here for the first time a clear heat absorption peak for the unfolding of tetrameric melittin. Significant $\Delta C_{p,U}$ and ΔH_U values for the unfolding of tetrameric melittin have been suggested by several groups on the basis of CD measurements (Quay & Condie, 1983; Wilcox & Eisenberg, 1992). Wilcox and Eisenberg (1992) reported the $\Delta C_{p,U}$ of unfolding of the melittin tetramer to be 4.1–4.9 kJ K⁻¹ (mol of tetramer)⁻¹, which is similar to our value at the lower temperature (Fig. 5B). Bello and coworkers (Ramalingam et al., 1991; Ramalingam & Bello, 1993) examined systematically the conformation and stability of melittin and its derivatives and demonstrated cold denaturation in addition to thermal unfolding. Cold denaturation

Fig. 7. Fluorescence spectra of 50 μ M ANS in the presence of various conformational states of 27.6 μ M melittin at 20 $^{\circ}$ C. 1, Helical state in 20 mM NaOH at pH 12; 2, helical state in 10 mM Mes buffer at pH 6.0 and 0.5 M NaClO₄; 3, unfolded state in 10 mM Mes buffer at pH 6.0. Broken line (4) shows the fluorescence spectrum of 50 μ M ANS in the absence of melittin. Excitation was 350 nm.

is evidence that $\Delta C_{p,U}$ is positive and significant (Oobatake & Ooi, 1993; Privalov & Makhatadze, 1993). Nevertheless, no heat absorption peak for the unfolding of melittin has been reported so far. Wilcox and Eisenberg (1992) claimed that they were unable to detect the heat absorption peak by DSC within the temperature range 10–60 $^{\circ}$ C. The present results showed that a much wider temperature range is necessary in order to observe the unfolding of tetrameric melittin by DSC.

Because the transition, measured by either CD or DSC, was so broad, precise analysis was hampered, mainly due to the uncertainty of the baselines. However, the results of DSC and CD were consistent with each other and explained satisfactorily on the basis of the 2-state transition mechanism. Therefore, we consider that the 2-state mechanism is a reasonable approximation, even though our analysis was not capable of detecting slight deviations from the model.

The CD and DSC curves, measured over a wide temperature range, indicated that $\Delta C_{p,U}$ decreases significantly with increasing temperature. Similar temperature dependence of $\Delta C_{p,U}$, although less pronounced than that of melittin, has also been reported for globular proteins (Makhatadze & Privalov, 1990). As a result, the ΔH_U value became saturated at a temperature (i.e., around 90 $^{\circ}$ C) lower than that of a typical globular protein (i.e., 100–110 $^{\circ}$ C). Although the exact mechanism responsible for the saturation of ΔH_U at the lower temperature is unknown, it may be related to the suggestion that tetrameric melittin in solution is molten globule-like, showing weaker hydrophobic interactions and internal packing than those of the native state (see below).

Molten globule state of proteins

One of the most controversial aspects of the molten globule state is the role of hydrophobic interactions in its stability (Xie et al.,

1991, 1993; Yutani et al., 1992; Griko & Privalov, 1994; Griko et al., 1994). By hydrophobic interaction, we mean the total interactions accompanying the transfer of a hydrophobic substance from a hydrophobic environment to water (see Dill, 1990; Privalov & Makhatadze, 1993). Thus, it involves both the disruption of the van der Waals interactions between the nonpolar substances and the process of their hydration. It has been proposed that the hydration of nonpolar groups contributes much less significantly to the stability of the native structure than previously imagined (Privalov & Makhatadze, 1993).

The thermal unfolding of the molten globule state has been assumed to be noncooperative, and hydrophobic interactions are suggested to be of minor importance in its stabilization (Griko et al., 1988; Kuwajima, 1989; Yutani et al., 1992). In other words, the molten globule state is enthalpically equivalent to the fully unfolded state, or $\Delta C_{p,U}$ and ΔH_U are zero. However, it is now becoming clear that this is not the case. Significant $\Delta C_{p,U}$ and ΔH_U values, although smaller than those of the native state, are well established for the molten globule state of cytochrome *c* (Potekhin & Pfeil, 1989; Hagihara et al., 1994). Nishii et al. (1994) reported a small but significant $\Delta C_{p,U}$ upon unfolding of the molten globule state of apomyoglobin. Xie et al. (1991, 1993) proposed significant $\Delta C_{p,U}$ and ΔH_U values for the unfolding of the molten globule state of α -lactalbumin, although Yutani et al. (1992) reported contradictory results. Haynie and Freire (1993), on the basis of theoretical considerations, proposed that the $\Delta C_{p,U}$ value of the molten globule state is significant, although smaller than that of the native state. Kitakuni et al. (1994) reported large $\Delta C_{p,U}$ and ΔH_U values for the unfolding of the molten globule state with a helical pentamer for an artificially designed 30-residue peptide.

Taken together, small but non-zero $\Delta C_{p,U}$ and ΔH_U values upon unfolding of the molten globule state seem to be common to many proteins (see also Griko et al., 1994). Consequently, the presence of positive $\Delta C_{p,U}$ and ΔH_U values does not necessarily prove that the conformational state is a state corresponding to the tightly packed native state of globular proteins. Different criteria are necessary to define the native-like state of proteins and peptides.

Prediction of thermodynamic parameters

The $\Delta C_{p,U}$, ΔH_U , and ΔG_U functions of the native state of small globular proteins can be predicted successfully on the basis of their crystallographic structures (Ooi & Oobatake, 1988, 1991; Murphy & Freire, 1992; Privalov & Makhatadze, 1992, 1993; Oobatake & Ooi, 1993). For the native states of proteins, the predicted values are consistent with the experimental values, indicating that the native structure in solution is similar to the crystallographic structure. Of these parameters, the positive $\Delta C_{p,U}$ value largely represents the exposure of the hydrophobic residues on unfolding, although the negative contribution of the polar groups is also involved (Murphy & Freire, 1992; Privalov & Makhatadze, 1992, 1993; Oobatake & Ooi, 1993). Thus, we expected that comparison of the experimental $\Delta C_{p,U}$ value with the predicted value on the basis of the crystallographic structure would be useful for considering whether tetrameric melittin in solution is similar to the crystallographic structure.

On the basis of the method of Oobatake and Ooi (1993), we calculated the hydration heat capacity change ($\Delta C_{p,h}$ at 25 $^{\circ}$ C) for the process of transition from the helical tetramer to the un-

folded monomer (Table 3). Exposed polar and nonpolar surfaces were calculated using the crystallographic structure reported by Terwilliger and Eisenberg (1982). For comparison, Table 3 shows the corresponding values for horse cytochrome *c*. The predicted value of $\Delta C_{p,h}$ is $9.2 \text{ kJ K}^{-1} (\text{mol of tetramer})^{-1}$ for the transition from the helical tetramer to the unfolded monomer (see also Table 1). The $\Delta C_{p,h}$ value, which represents mainly the contribution from the nonpolar amino acid residues ($\Delta C_{p,h}^{\text{nonp}}$), is about 2 times larger than that obtained from the experiments at 30°C . The difference expands with increase in temperature because, whereas the former is assumed to be independent of temperature, the latter decreases with increasing temperature: at 80°C , the predicted value is 3–4 times larger than the observed value. Because the agreements of the predicted $\Delta C_{p,h}$ and experimental $\Delta C_{p,U}$ values are excellent for globular proteins (Oobatake & Ooi, 1993), this disagreement suggests that the hydrophobic residues of the tetrameric structure in melittin are more exposed in comparison with the crystallographic tetramer.

Comparison with the molten globule state of cytochrome *c*

As a typical example of the molten globule state, we will consider the acidic molten globule state of horse cytochrome *c*, for which a cooperative unfolding transition has been well established (Potekhin & Pfeil, 1989; Goto et al., 1993; Kataoka et al., 1993; Hagihara et al., 1994). Tables 1 and 3 compare several structural and thermodynamic properties of melittin tetramer in solution and the molten globule states of cytochrome *c*.

First, it should be recalled that both tetrameric melittin and cytochrome *c* consist of 104 amino acid residues and that both have a net charge of 24 at pH 2. In addition, the relative size compared to the crystallographic structure and the role of charge repulsion show close similarity between the melittin tetramer in solution and the molten globule state of cytochrome *c*. Now, it is clear that the $\Delta C_{p,U}$ value of tetrameric melittin in solution is significantly smaller than that predicted from the crystallographic structure. The $\Delta C_{p,U}$ value for the molten globule state of cytochrome *c* was estimated to be 1.6 kJ mol^{-1} , being about 20% that of the native state (7.0 kJ mol^{-1} ; Privalov & Gil, 1988).

The ΔH_U value at around 110°C , where the contribution of hydrophobic hydration becomes small, was proposed to provide a measure of the contribution of van der Waals and hydrogen bond interactions stabilizing the folded state, although the details are considerably complex (Privalov & Makhatadze, 1993). We estimated the maximal ΔH_U value for melittin at 110°C to be $280\text{--}350 \text{ kJ mol}^{-1}$ (Fig. 5A). This value is smaller than that predicted from the crystallographic structure (647 kJ mol^{-1})

but similar to the ΔH_U value at 110°C of the molten globule state of cytochrome *c* (Table 1). These results suggest that the hydrophobic residues of tetrameric melittin in solution are exposed in comparison with the crystallographic structure, and that the contribution of the intrinsic forces stabilizing the tetrameric state (van der Waals and hydrogen bond interactions) is closer to that of the molten globule state than to that of the native state.

ANS binding to tetrameric melittin is consistent with the molten globule hypothesis. In the case of the molten globule of cytochrome *c*, however, because of the contribution of heme absorption, ANS binding has not been confirmed (Y. Goto & Y. Hagihara, unpubl. results). In addition, the anion-dependent stabilization of tetrameric melittin is similar to that of the acidic molten globule of various proteins (Goto & Hagihara, 1992). All these observations suggest that the tetrameric structure in water is significantly disordered in comparison with the crystallographic structure and may be similar to the molten globule state of globular proteins.

However, we have also obtained results inconsistent with the molten globule hypothesis. As shown in Figure 6, the presence of definite aromatic CD, which has been considered to be absent in the molten globule state (Kuwaitjima, 1989; Goto & Nishikiori, 1991; Ptitsyn, 1992), indicates that tetrameric melittin has a specific tertiary structure, at least around tryptophan 19. One-dimensional H-NMR of tetrameric melittin in aqueous solution has revealed a distinct spectrum with dispersed chemical shifts (Brown et al., 1980; Y. Hagihara & Y. Goto, unpubl. results). These results suggest that tetrameric melittin has unique side-chain structures. On the other hand, the 2-dimensional H-NMR spectra suggested that the structure is at least partly disordered and fluctuating, in comparison with the rigid native structure of proteins (Y. Hagihara & Y. Goto, unpubl. results).

A molten globule state with significant native-like characteristics

Taken together, we consider that tetrameric melittin has properties of both the native state and the typical molten globule state and therefore is intermediate between them. The structure may be considered as a molten globule state with significant native-like characteristics. The crystallographic structure of melittin indicates that the charged residues near the C-terminus, i.e., lysine 21, arginine 22, lysine 23, and arginine 24, are in close proximity to each other. Such proximity is energetically unfavorable and, in solution, these residues will repel each other. This un-

Table 3. Structural thermodynamic analysis of the crystallographic structure of melittin and horse cytochrome *c*^a

	ASA^U ($\text{\AA}^2$)	ASA^F ($\text{\AA}^2$)	ΔASA ($\text{\AA}^2$)	ΔASA^{pol} ($\text{\AA}^2$)	ΔASA^{nonp} ($\text{\AA}^2$)	$\Delta C_{p,h}^{\text{tot}}$ ($\text{kJ mol}^{-1} \text{K}^{-1}$)	$\Delta C_{p,h}^{\text{pol}}$ ($\text{kJ mol}^{-1} \text{K}^{-1}$)	$\Delta C_{p,h}^{\text{nonp}}$ ($\text{kJ mol}^{-1} \text{K}^{-1}$)	ΔH_U (110°C) (kJ mol^{-1})
Melittin tetramer	15,846	5,910	9,936	3,313	6,623	9.2	1.6	7.6	647
Cytochrome <i>c</i>	16,124	5,571	10,353	4,861	5,492	8.5	2.9	5.6	779

^a Accessible surface areas for the unfolded (ASA^U) and folded (ASA^F) states, contribution from polar amino acid residues (ΔASA^{pol}) and nonpolar amino acid residues (ΔASA^{nonp}), calculated hydration heat capacity change at 25°C ($\Delta C_{p,h}^{\text{tot}}$), contributions from polar amino acid residues ($\Delta C_{p,h}^{\text{pol}}$) and nonpolar amino acid residues ($\Delta C_{p,h}^{\text{nonp}}$), and calculated enthalpy change at 110°C (ΔH_U (110°C)).

favorable repulsion may result in a somewhat disordered and solvated tetrameric structure in solution.

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-like characteristics. For example, Redfield et al. (1994) studied the intermediate conformational state of the 4-helix-bundle protein interleukin-4 at low pH using NMR and showed that the protein retains a highly ordered hydrophobic core in which most of the secondary structure is preserved, but that extensive disorder exists in the polypeptide chain regions linking the structural elements. They suggested that interleukin-4 exists in a highly ordered molten globule state, occurring late in the protein-folding pathway. On the other hand, Feng et al. (1994) studied the structure of apocytochrome *b*₅₆₂ using NMR and showed that this protein retains a 4-helix-bundle topology similar to the holoprotein, whereas significant differences in helix-helix packing between the 2 states are evident. Although the detailed structure of tetrameric melittin in solution is unknown, we believe that it has properties similar to these highly structured molten globules.

Finally, it should be noted that Thompson et al. (1993), using DSC and CD, examined the thermal unfolding of a 56-amino acid fragment of GCN4, which contains a leucine zipper sequence. The thermal unfolding transition of the leucine zipper peptide can be approximated by a 2-state mechanism between the folded dimer and unfolded monomer. Although the oligomeric state (dimer) is different from that of melittin (tetramer), the apparent results including the peptide concentration dependence are very similar to those of melittin. On the basis of NMR data (Goodman & Kim, 1991), the folded state of GCN4 in solution is known to be a compactly packed coiled-coil structure, similar to that in the crystal and not the molten globule state. In accordance with this observation, the $\Delta C_{p,U}$ and ΔH_U values calculated using the crystallographic structure are consistent with the observed values within the limits of experimental error (Thompson et al., 1993).

Conclusions

Although the molten globule state is assumed to be a major intermediate of protein folding, much is still unknown about its details and generality. Examination of the molten globule state of various proteins indicates that the uniform melting of the tertiary structure, as originally proposed, is not an accurate picture of the molten globule state. Instead, there is a range of molten globule states from the relatively disordered to the highly ordered. The disordered molten globule may be similar to the collapsed state of polymers in a poor solvent. On the other hand, in the highly ordered state, local melting of the tertiary and secondary structures with maintenance of significant tertiary and secondary structure at other regions may occur. We demonstrated by DSC that the unfolding of tetrameric melittin in solution is accompanied by positive and significant $\Delta C_{p,U}$ and ΔH_U values. However, the $\Delta C_{p,U}$ value was evidently smaller than that predicted on the basis of the crystallographic structure, suggesting exposure of the hydrophobic residues in comparison with the crystal tetramer. Tetrameric melittin in solution may correspond to an ordered molten globule structure, i.e., a molten globule state with significant native-like characteristics.

Materials and methods

Materials

Crude melittin, purchased from Sigma, was applied to a Nacalai 5C18-AR HPLC column (20 × 250 mm), and the major sharp peak, eluted with a gradient of acetonitrile in 0.05% trifluoroacetic acid, was collected and stored after lyophilization. The melittin concentration was determined from the absorption at 280 nm using a molar absorption coefficient of 5,570 (Quay & Condie, 1983). Other chemicals were of reagent grade and used without further purification.

CD measurements

CD measurements were carried out with a Jasco spectropolarimeter, model J-500A, equipped with an interface and a personal computer. The instrument was calibrated with ammonium *d*-10-camphorsulfonic acid. The results are expressed as the mean residue ellipticity, $[\theta]$, which is defined as $[\theta] = 100\theta_{\text{obs}}/lc$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue moles per liter, and l is the length of the light path in centimeters. Far-UV CD spectra were obtained using cells with a 1-mm or 0.1-mm light path according to the melittin concentration. Near-UV CD spectra were obtained with 4-cm or 0.1-mm cells. The temperature was controlled with a water-circulating cell holder.

Thermal unfolding was monitored by the ellipticity at 222 or 238.5 nm using the 1-mm cell. The temperature was increased at a rate of 1 °C min⁻¹ and was monitored with a thermocouple (Sensortek, BAT-12 and a flexible probe) inserted directly into the cell. The dependence of the unfolding transition of melittin on salt concentration was measured at a peptide concentration of 27.6 μM in 10 mM sodium acetate buffer at pH 4.5. The dependence of the unfolding transition on the peptide concentration was measured within the range 27.6–290.4 μM melittin in 10 mM sodium acetate buffer at pH 4.5 in the presence of 200 mM NaClO₄. The pH of the sample solution was measured using a Radiometer pH meter, model PHM83, at 20 °C.

Calorimetric measurements

Calorimetric experiments were carried out with a differential scanning microcalorimeter, DASM-4, equipped with a NEC PC-9801 personal computer. The scan rate was 1.0 deg min⁻¹ for all experiments. The dependence of the unfolding transition of melittin on salt concentration was measured at 7.6 mg mL⁻¹ melittin in 10 mM sodium acetate buffer at pH 4.5. The dependence on melittin concentration was measured within the range 1.2–18.3 mg mL⁻¹ in 10 mM sodium acetate buffer at pH 4.5 containing 200 mM NaClO₄.

Fluorescence measurements

Fluorescence spectra were measured with a Hitachi-Perkin Elmer fluorescence spectrophotometer, model MPF-4. ANS fluorescence was measured by excitation at 350 nm. The measurements were carried out at 20 °C with a thermostatically controlled cell holder.

Thermodynamic analysis

CD and DSC results were analyzed on the basis of the 2-state transition mechanism between the folded tetramer (F_4) and unfolded monomer (U):

$$F_4 \rightleftharpoons 4U. \quad (1)$$

The equilibrium constant of the unfolding transition (K_U) is defined by:

$$K_U = [U]^4/[F_4] = 4\alpha^3 f_U^4 / (1 - f_U), \quad (2)$$

where $[F_4]$ and $[U]$ are the concentrations of tetrameric and monomeric melittins, respectively, α is the total concentration of monomeric melittin, and f_U is the fraction of unfolded melittin, i.e., $f_U = [U]/([U] + 4[F_4])$. It is clear that, because the reaction is a tetramer-to-monomer reaction, the midpoint of the transition where $f_U = 0.5$ does not correspond to $K_U = 1$.

In order to express the thermodynamic functions, we define an ambient reference temperature, T_R . Introduction of the T_R scale is important for obtaining the thermodynamic parameters accurately within the temperature region where many data points are available. The free energy change of unfolding at a temperature T is:

$$\Delta G_U(T) = -RT \ln K_U = \Delta H_U(T) - T \Delta S_U(T), \quad (3)$$

where $\Delta H_U(T)$ and $\Delta S_U(T)$ are the enthalpy and entropy changes of unfolding, respectively. Although $\Delta C_{p,U}$ is often approximated to be independent of temperature, we consider that $\Delta C_{p,U}(T)$ is a function dependent on temperature and can be approximated by:

$$\Delta C_{p,U}(T) = \Delta C_{p,U}(T_R) + a(T - T_R) + b(T - T_R)^2, \quad (4)$$

where a and b are constants and $\Delta C_{p,U}(T_R)$ is the heat capacity change at the reference temperature. Then the enthalpy and entropy changes of the unfolding are represented by:

$$\begin{aligned} \Delta H_U(T) &= \int \Delta C_{p,U} dT \\ &= \Delta H_U(T_R) + \int_{T_R}^T \Delta C_{p,U} dT \\ &= \Delta H_U(T_R) + \Delta H_T(T), \end{aligned}$$

where

$$\begin{aligned} \Delta H_T(T) &= \Delta C_{p,U}(T_R)(T - T_R) + (a/2)(T - T_R)^2 \\ &\quad + (b/3)(T - T_R)^3 \end{aligned} \quad (5)$$

and

$$\begin{aligned} \Delta S_U(T) &= \int \Delta C_{p,U} \cdot T^{-1} dT \\ &= \Delta S_U(T_R) + \int_{T_R}^T \Delta C_{p,U} \cdot T^{-1} dT \\ &= \Delta S_U(T_R) + \Delta S_T(T), \end{aligned}$$

where

$$\begin{aligned} \Delta S_T(T) &= (a - b \cdot T_R)(T - T_R) + (b/2)(T - T_R)^2 \\ &\quad + [\Delta C_{p,U}(T_R) - a \cdot T_R + b \cdot T_R^2] \ln(T \cdot T_R^{-1}), \end{aligned} \quad (6)$$

respectively.

In order to represent the thermodynamic functions in terms of T , T_R , and $\Delta H_U(T_R)$, $\Delta S_U(T_R)$ in Equation 6 must be rearranged. We can relate $\Delta S_U(T_R)$ and $\Delta H_U(T_R)$ introducing the apparent midpoint temperature, $T_{m,app}$, of the unfolding transition, where $f_U = 0.5$. From Equations 2 and 3, the Gibbs free energy of unfolding of melittin is:

$$\begin{aligned} \Delta G_U(T_{m,app}) &= -RT_{m,app}(3 \ln \alpha - \ln 2) \\ &= \Delta H_U(T_{m,app}) - T_{m,app} \Delta S_U(T_{m,app}). \end{aligned} \quad (7)$$

On the other hand,

$$\Delta H(T_{m,app}) = \Delta H_U(T_R) + \Delta H_T(T_{m,app}) \quad (8)$$

and

$$\Delta S(T_{m,app}) = \Delta S_U(T_R) + \Delta S_T(T_{m,app}). \quad (9)$$

By inserting Equations 8 and 9 into Equation 7, $\Delta S_U(T_R)$ is related to $\Delta H_U(T_R)$:

$$\begin{aligned} \Delta S_U(T_R) &= [\Delta H_U(T_R) + \Delta H_T(T_{m,app})] T_{m,app}^{-1} \\ &\quad - \Delta S_T(T_{m,app}) + R(3 \ln \alpha - \ln 2). \end{aligned} \quad (10)$$

Then, $\Delta G_U(T)$ at any temperature can be obtained with parameters T_R , $T_{m,app}$, and $\Delta C_{p,U}(T)$ functions:

$$\begin{aligned} \Delta G_U(T) &= \Delta H_U(T_R) + \Delta H_T(T) \\ &\quad - T\{[\Delta H_U(T_R) + \Delta H_T(T_{m,app})] T_{m,app}^{-1} \\ &\quad - \Delta S_T(T_{m,app}) + R(3 \ln \alpha - \ln 2) + \Delta S_T(T)\}. \end{aligned} \quad (11)$$

Now, the ellipticity at temperature T is represented by

$$[\theta](T) = (1 - f_U)[\theta]_F(T) + f_U[\theta]_U(T), \quad (12)$$

where $[\theta]_F(T)$ and $[\theta]_U(T)$ are the ellipticities for the folded and unfolded states, respectively.

The van't Hoff equation for the tetrameric transition is related to f_U by:

$$\frac{\Delta H_U(T)}{RT^2} = \frac{d \ln K_U}{dT} = \frac{4 - 3f_U}{f_U(1 - f_U)} \frac{df_U}{dT}. \quad (13)$$

Therefore, the heat capacity $C_p(T)$ of melittin is represented from Equation 13 by the following equation:

$$\begin{aligned}
C_p(T) &= \Delta H_U(T) \frac{df_U}{dT} + C_{p,U}(T) - \Delta C_{p,U}(T)(1 - f_U) \\
&= [\Delta H_U(T)^2 f_U(1 - f_U)] [RT^2(4 - 3f_U)]^{-1} \\
&\quad + C_{p,U}(T) - \Delta C_{p,U}(T)(1 - f_U), \quad (14)
\end{aligned}$$

where $C_{p,U}(T)$ is the heat capacity of the unfolded state.

Because the unfolding transition is a tetramer-to-monomer reaction, $T_{m,app}$ increases with increasing melittin concentration. We can relate $T_{m,app}$ with melittin concentration. From Equation 7,

$$\Delta G_U(T_{m,app}) = -RT_{m,app}(3 \ln \alpha - \ln 2). \quad (15)$$

By using Equation 11, we assume another relation at a different protein concentration, α_0 , and at a different apparent midpoint temperature, $T_{m,app,0}$:

$$\begin{aligned}
\Delta G_U(T_{m,app}) &= \Delta H_U(T_R) + \Delta H_T(T_{m,app}) - T_{m,app} \\
&\quad \times \{[\Delta H_U(T_R) + \Delta H_T(T_{m,app,0})]T_{m,app,0}^{-1} \\
&\quad - \Delta S_T(T_{m,app,0}) + R(3 \ln \alpha_0 - \ln 2) \\
&\quad + \Delta S_T(T_{m,app})\}. \quad (16)
\end{aligned}$$

By equating Equations 15 and 16,

$$\begin{aligned}
\alpha &= \alpha_0 \exp\{-[\Delta G_U(T_{m,app}) + RT_{m,app}(3 \ln \alpha_0 - \ln 2)] \\
&\quad \times (3RT_{m,app})^{-1}\}. \quad (17)
\end{aligned}$$

Equation 17 indicates that melittin concentration is a function of $T_{m,app}$ with parameters, $\Delta C_{p,U}$, $\Delta H_U(T)$, reference temperature, $T_{m,app,0}$, and the reference melittin concentration, α_0 .

Calculation of thermodynamic values

The thermodynamic values were calculated on the basis of the melittin (2MLT in Protein Data Bank) and oxidized horse heart cytochrome *c* crystal structures (Bushnell et al., 1990) using the method of accessible surface area (Ooi & Oobatake, 1991; Oobatake & Ooi, 1993). In the present calculation of *ASA* for horse cytochrome *c*, a heme group as well as the polypeptide chain were taken into account, whereas only the polypeptide chain of tuna cytochrome *c* was considered in the previous calculation (Ooi & Oobatake, 1988, 1991; Oobatake & Ooi, 1993). *ASA* for the unfolded state is evaluated by a single extended conformation using ECEPP/2 (Némethy et al., 1983). The values of ΔASA and $\Delta C_{p,h}$ are divided into the contributions of the polar amino acid residues (D, E, G, K, N, Q, R, S, and T) and nonpolar amino acid residues (A, C, F, H, I, L, M, P, V, W, and Y). Horse cytochrome *c* has 42 polar and 62 nonpolar amino acid residues, and tetrameric melittin has 52 polar and 52 nonpolar amino acid residues.

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