Charge Repulsion in the Conformational Stability of Melittin†

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ABSTRACT: Electrostatic repulsion between positively charged groups has been suggested to be critical in determining the conformation of melittin. To clarify the role of repulsive forces, we prepared a series of succinylated melittins, an acetylated melittin, and a synthetic melittin mutant, with various degrees of charge repulsion. The conformation of the melittin derivatives was examined by far-UV circular dichroism under various conditions of pH and salt at 20 °C. The stability of the tetrameric helical state was found to be dependent on the net charge of the peptides. The charge repulsive forces destabilized the helical state of intact melittin by 600 cal/(charge·mol of tetramer). This value was close to the corresponding one (450 cal/(charge·mol)) obtained for the acidic molten globule of horse cytochrome *c* [Goto, Y., & Nishikiori, S. (1991) *J. Mol. Biol. 222,* 679–686], which has a molecular weight and a net charge comparable to those of the tetrameric melittin. Small-angle X-ray scattering of the tetrameric melittin and the molten globule of cytochrome *c* showed that the two states are also comparable to each other in the radius of gyration. These results suggest that the contribution of electrostatic repulsion to the conformational stability of melittin is similar to that of the molten globule.

The conformation of proteins and peptides is determined by a balance of various forces favoring folding or unfolding, and the net stability of the folded state is marginal (Creighton, 1984; Dill, 1990). To extend our understanding of protein folding, it is important to clarify the detailed properties of the respective forces. Electrostatic repulsion is one of the classical forces assumed to disfavor protein folding. Although much attention has been paid recently to electrostatics in protein folding (Dill, 1990; Stigter et al., 1991; Alonso et al., 1991), there is a paucity of experimental data showing the importance of electrostatic repulsion, and thus it is important to obtain such data. Goto and co-workers have indicated the importance of charge repulsion in determining the conformation of acid-denatured proteins (Goto & Fink, 1990; Goto et al., 1990a,b; Goto & Nishikiori, 1991) and a basic amphiphilic helical peptide (Goto & Aimoto, 1991). They have also reported that charge repulsion determines the conformation of melittin (Goto & Hagihara, 1992).

Melittin, a bee venom toxin, consists of 26 amino acid residues, 5 of which are basic but none of which are acidic (Habermann, 1972). The conformational properties of melittin have been studied extensively (Talbot et al., 1979; Brown et al., 1980; Bello et al., 1982; Quay & Condie, 1983; Ramalingam et al., 1991). Whereas melittin at micromolar concentrations is unfolded under conditions of low ionic strength at neutral pH, it adopts a tetrameric helical structure under conditions of high ionic strength, either by salt or acid, or at alkaline pH (Goto & Hagihara, 1992). The X-ray structure of tetrameric melittin crystallized from aqueous solution has been reported (Terwilliger & Eisenberg, 1982a,b). Because the conformational transition accompanies a monomer-to-tetramer reaction, high peptide concentrations also favor the helical structure.

In the previous study (Goto & Hagihara, 1992), we examined the anion- and pH-dependent conformational transition of melittin in detail. We have shown that the conformation of melittin is determined by a balance of unfolding forces including the electrostatic repulsive forces and the folding forces and that the anion binding to the protonated groups and the deprotonation of these groups at alkaline pH modulate the balance by decreasing the repulsive forces. The conformational transitions of melittin under various conditions were interrelated satisfactorily on the basis of an equation in which the conformational transition is linked to the proton and anion bindings to the titratable groups. On the basis of the previous study, it is predicted that removal of the positive charges by chemical modification or amino acid mutation should result in stabilization of the helical state under the conditions where the intact melittin is unfolded. To confirm this prediction, we prepared a series of melittin derivatives, and by removal of the positive charges, the helical state, as expected, became stabilized.

The molten globule state, a compact denatured state with a significant amount of secondary structures but showing a largely disordered tertiary structure, has been indicated to be the major intermediate of protein folding (Ohgushi & Wada, 1983; Ptitsyn, 1987; Kuwajima, 1989; Kim & Baldwin, 1990; Christensen, & Pain, 1991; Baldwin, 1991). Goto and Nishikiori (1991) determined the energetic contribution of charge repulsion to the stability of the acidic molten globule of cytochrome *c*. We noticed that the size and the net charge of the molten globule of cytochrome *c* are comparable to those of tetrameric melittin and that the charge repulsion of tetrameric melittin is also similar in magnitude to that of the molten globule of cytochrome *c*. These results suggest a common mechanism determining the marginal conformational state of proteins and peptides.

MATERIALS AND METHODS

Materials. Crude melittin, purchased from Sigma, was purified by reverse-phase HPLC as described previously (Goto & Hagihara, 1992). Intact and 9-acetylated horse ferricy-

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Table I: Amino Acid Sequences of Intact Melittin and Its Derivatives[a]

Peptide	Sequence
	1 11 21 26
Intact	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-NH_2
	1
1-Suc	^{Suc}G----------
	1 21
2-Suc	^{Suc}G----------K^{Suc}----------
	1 21 23
3-Suc	^{Suc}G----------K^{Suc}-K^{Suc}------
	1 7 21 23
4-Suc	^{Suc}G----------K^{Suc}----------K^{Suc}-K^{Suc}------
	1 7 21 23
4-Ac	^{Ac}G----------K^{Ac}----------K^{Ac}-K^{Ac}-------
	1 10 18 25
3-Glu	----------E----------E----------E------

[a] For the melittin derivatives, only the modified residues are indicated. ^{Suc}G and ^{Ac}G indicate glycine which has the α-amino group succinylated and acetylated, respectively, and K^{Suc} and K^{Ac} indicate lysine which has the ϵ-amino group succinylated and acetylated, respectively.

tochrome *c* were obtained as described previously (Goto & Nishikiori, 1991). *Achromobacter lyticus* protease I (endoproteinase lys-C) was obtained from Boehringer Mannheim.

To prepare succinylated melittins, intact melittin (20 mg/mL in 0.4 M phosphate buffer, pH 7.5) was reacted with succinic anhydride by stepwise addition of the solid compound with stirring at room temperature. The concentration ratio of reagent to amino groups was altered to obtain various degrees of modification. The extent of modification was followed by reverse-phase HPLC. The four major peaks with increasing retention time that appeared on modification were isolated with a preparative HPLC column. The sites of modification were determined on the basis of analysis of N-terminal residues after digestion with *A. lyticus* protease I and peptide mapping of the digests. The melittins (0.2 mg/mL) were digested at an enzyme to substrate ratio of 1:100 in 10 mM sodium borate buffer (pH 9.0) for 12 h. The N-terminal amino acid residues of the digests were determined by a protein sequencer, Model 473A (Applied Biosystems), and the peptide mapping was carried out by reverse-phase HPLC with absorbance detection at 215 and 280 nm. For the intact melittin, Val, Arg, and Gly were detected as the N-terminal residues after the digestion. The peptide mapping of the digests provided four peaks corresponding to Arg(22)–Lys(23), Arg(24)–Gln(26), Gly-(1)–Lys(7), and Val(8)–Lys(21) in order of the retention time. On acetylation of the lysyl residues, the adjacent residues to the modified site disappeared from the N-terminal analysis and the mapping pattern was changed. By taking into account these results, the sites of modification were identified as shown in Table I.

To prepare acetylated melittin, melittin (20 mg/mL in 0.4 M phosphate buffer, pH 7.5) was reacted with acetic anhydride by stepwise addition with stirring. All the amino groups were modified after reaction for 1 h with a 1.5-fold molar excess of acetic anhydride. The acetylated melittin was purified by reverse-phase HPLC with a preparative column.

A melittin mutant with three Glu residues introduced at positions 10, 18, and 25 (see Table I) was synthesized on a peptide synthesizer, Model 430A (Applied Biosystems), employing a double-coupling protocol. After the peptide chain assemblage, the peptide resin was treated with anhydrous hydrogen fluoride containing 15%, 1,4-butanedithiol and 5% *p*-cresol at 0 °C for 90 min. The crude material was purified on a reverse-phase HPLC. The amino acid composition and mass spectrum were consistent with the designed sequence.

Methods. The peptide concentrations were determined from absorption at 280 nm using a molar absorption coefficient of 5570 (Quay & Condie, 1983) for all the intact, modified, and synthetic melittins.

Circular dichroism (CD)[1] 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*-camphorsulfonate. The results are expressed as the mean residue ellipticity, $[\theta]$, which is defined as $[\theta] = 100\theta_{obsd}/lc$, where θ_{obsd} is the observed ellipticity in degrees, c is the concentration in moles of residue per liter, and l is the length of the light path in centimeters. The measurements were carried out at 20 °C with thermostatically controlled cell holders.

Typically, 0.1 mL of melittin solution, dissolved in deionized water, was mixed with 0.15 mL of buffer solution. The CD spectra were measured at a peptide concentration of 27.6 μM using a 1-mm cell. The buffers used were sodium acetate (pH 3–5), MES (pH 5–7), Tris-HCl (pH 7–9), CHES (pH 9–10), and CAPS (pH 10–11). The buffer concentrations were 10 mM, and the ionic strength was controlled by NaCl. Solutions below pH 3 and above pH 11 were prepared using the appropriate concentration of HCl and NaOH, respectively.

SAXS of the intact melittin and the intact and 9-acetylated cytochrome *c* species were collected at the Photon Factory in Japan on a small-angle scattering station as described (Ueki et al., 1985). Protein concentrations were varied within the range 30–5 mg/mL, and correction for the concentration dependence of the scattered intensity was made to obtain the scattering curves at infinite dilution (Kataoka et al., 1989).

Sedimentation equilibrium measurements of the intact, 3-Suc, and 3-Glu melittins were carried out at 20 °C with a Beckman-Spinco Model E analytical ultracentrifuge monitored with the absorbance at 280 nm. The partial specific volumes of intact, 3-Suc, and 3-Glu melittins were assumed to be 0.781, 0.781, and 0.775, respectively.

The pH was measured using a Radiometer PHM83 at 20 °C.

RESULTS

Conformation of Modified Melittins. Figure 1A shows the far-UV CD spectra of intact and succinylated melittins (27.6 μM) at pH 6 and 20 °C. In the absence of salt, the CD spectrum of intact melittin indicated the absence of any significant secondary structure. With the progress of succinylation, the CD spectra showed minima at 208 and 222 nm, indicating the formation of α-helix. The spectrum of intact melittin in 2 M NaCl, where the salt-induced transition is over [see Figure 2 of Goto and Hagihara (1992)], is shown in Figure 1B. The spectrum of 4-Suc melittin is close to the spectrum in 2 M NaCl, indicating that succinylation of four amino groups stabilizes almost completely the helical conformation of melittin even in the absence of salt.

Figure 1B shows the far-UV CD spectra of 4-Ac and 3-Glu melittins. The spectrum of 3-Glu melittin indicated the presence of a small amount of α-helix. In the process of

[1] Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CD, circular dichroism; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; HPLC, high-performance liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid; Rg, radius of gyration; SAXS, small-angle X-ray scattering.

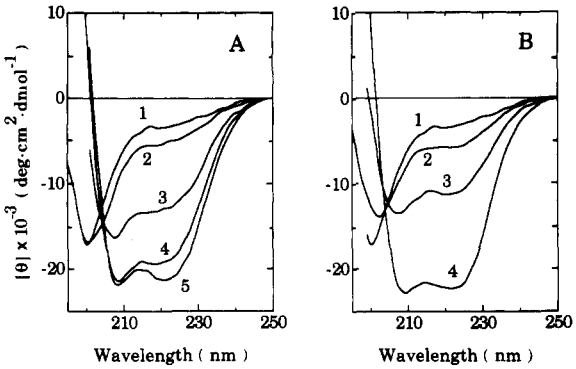

FIGURE 1: Far-UV CD spectra of various melittin derivatives (27.6 μM) in 10 mM MES buffer (pH 6) at 20 °C. Panel A: Intact (1), 1-Suc (2), 2-Suc (3), 3-Suc (4), and 4-Suc (5) melittins in the absence of salt. Panel B: Intact (1), 3-Glu (2), and 4-Ac (3) melittins in the absence of salt and intact melittin in 2 M NaCl (4).

preparation of 3-Glu melittin, we also synthesized six melittin mutants in which one or two Glu residues were introduced at position 10, 18 or 25. These mutants at a peptide concentration of 27.6 μM showed a slight tendency to form α-helix at pH 6 without salt, the ellipticity values at 222 nm being smaller than that of 3-Glu melittin (Y. Hagihara, S. Aimoto, and Y. Goto, unpublished results). These results suggested that although the stability of the helical state increases with the number of Glu residues introduced, the introduction of three Glu residues is not enough to stabilize substantially the helical state at pH 6 in the absence of salt.

The helical state of the intact melittin is tetrameric. To examine the effects of modification on the oligomeric state, we measured the sedimentation equilibrium of 3-Suc and 3-Glu melittins along with the intact melittin at a peptide concentration of 70 μM in 10 mM MES buffer at pH 6.0. Molecular weight values of the intact melittin were determined to be 3900 in the presence of 0.2 M NaCl and 12 200 in the presence of 2 M NaCl, indicating the transformation from monomer to tetramer by the increase in salt concentration. Molecular weight values of 3-Suc and 3-Glu melittins in 10 mM MES buffer at pH 6.0 and 2 M NaCl were 15 200 and 9500, respectively. These values were consistent with those expected for the tetramer.

Effects of Salts. With regard to the mechanism of helix stabilization induced by succinylation or introduction of Glu, we can assume two possibilities. One is a decrease in net charge, which results in a decrease of charge repulsion in the helical state. The other is the formation of salt bridges between the positive charges and the introduced carboxyl groups, stabilizing specifically the helical state. To distinguish these two possibilities, we examined the effects of salts, assuming that a salt bridge is destabilized by increasing the salt concentration.

Figure 2 shows the effects of sodium perchlorate (A) and sodium chloride (B) on the conformation of modified melittins. Neither sodium perchlorate nor sodium chloride destabilized the helical conformations. On the contrary, the salts further stabilized the helical conformations. These results suggest that salt bridges, even if present, are unimportant energetically. Further stabilization of helices by salts as observed for 1-Suc, 2-Suc, 4-Ac, and 3-Glu melittins is explainable by counterion binding to the positive groups (Goto & Hagihara, 1992).

pH-Dependent Conformational Transition. Participation of salt bridges in the stabilization of the helical state of the succinylated or 3-Glu melittins can be examined further by measuring the pH-dependent conformational transitions. A

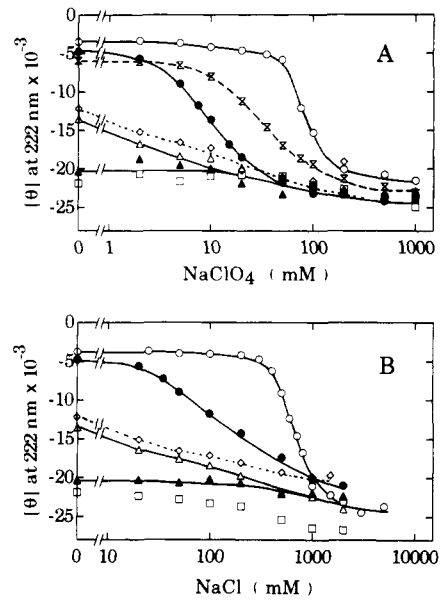

FIGURE 2: Effects of sodium perchlorate (A) and sodium chloride (B) on the ellipticity at 222 nm of various melittin derivatives in 10 mM MES buffer (pH 6) at 20 °C. Intact (○), 1-Suc (●), 2-Suc (△), 3-Suc (▲), 4-Suc (□), 4-Ac (◇), and 3-Glu (⧖) melittins were used.

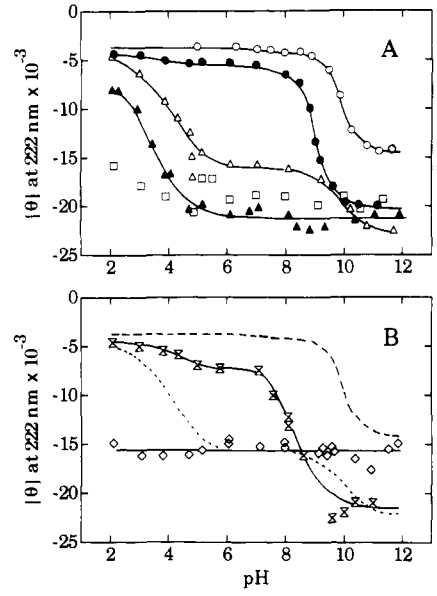

FIGURE 3: Dependence of the ellipticity of various melittin derivatives at 222 nm on pH at 20 °C. Ionic strength of the solution was 0.01. The symbols are the same as in Figure 2. The broken lines in panel B indicate the dependencies of the intact and 2-Suc melittins taken from panel A.

salt bridge formed between a carboxyl group and a basic group is typically stable at neutral pH and is unstable in the acidic and alkaline pH regions (Marqusee & Baldwin, 1987; Merutka & Stellwagen, 1991). Thus, if salt bridges are important, the helical conformation would be destabilized at both extremes of pH. On the other hand, if the net charge is important, the helical conformation should be stabilized further in the alkaline pH region because the net charge decreases with increase in pH.

Figure 3A shows the pH dependence of ellipticity at 222 nm of the intact and succinylated melittins. For all these species, the helical structure became stabilized with an increase in pH. Cooperative destabilization in the acidic pH region, having an apparent pK_a at around 4, indicates that carboxyl groups are responsible for it. Stabilization of the helical

Table II: Net Charges of Various Species of Melittin at pH 2, 6, and 11

species	net charge		
	pH 2	pH 6	pH 11
intact	+6	+6	+2
1-Suc	+5	+4	+1
2-Suc	+4	+2	0
3-Suc	+3	0	−1
4-Suc	+2	−2	−2
4-Ac	+2	+2	+2
3-Glu	+6	+3	−1

Table III: Comparison of the Conformational Properties of Tetrameric Melittin at Acidic pH and the Molten Globule of Horse Cytochrome *c* at pH 2

	molecular weight	net charge	charge repulsion at 20 °C (cal/(mol·charge))	radius of gyration (Å)
tetrameric melittin	11 400	+24	600	17.45 + 0.04[a]
molten globule of cyctochrome *c*	12 360	+24	450	16.11 + 0.10[b] 16.06 + 0.07[c]

[a] The value was obtained in 0.1 M $NaClO_4$, 10 mM sodium acetate buffer (pH 4.7). For comparison, the value calculated from the X-ray structure was 13.96 Å. [b] The values were obtained with intact cytochrome *c* in 0.5 M NaCl and 20 mM HCl (pH 2). For comparison, the Rg of the native state in 10 mM sodium phosphate buffer (pH 7.0) and that of the unfolded state in 20 mM HCl (pH 2) were determined to be 13.75 ± 0.06 Å and 22.80 ± 0.06 Å, respectively. The value calculated from the X-ray structure was 12.65 Å. [c] The value was obtained with the 9-acetylated cytochrome *c* in 20 mM HCl (pH 2).

structure in the alkaline pH region suggests the contribution of α- and ε-amino groups. We estimated the net charge values at pH 2, 6, and 11 assuming that the titratable groups are fully protonated at pH 2, carboxyl groups are deprotonated at pH 6, and all titratable groups except for two arginyl groups are deprotonated at pH 11 (Table II). Figure 3B shows the pH-dependent transitions of 4-Ac and 3-Glu melittin. Alkaline stabilization of the helical structure of 3-Glu melittin suggests that deprotonation of the α- and ε-amino groups is responsible for it, consistent with the assumption that net charge is important. In contrast, 4-Ac melittin showed no pH-dependent conformational change. This is as expected because the acetylated melittin has no titratable groups between pH 2 and 11 (Table II).

Small-Angle X-ray Scattering. We noticed structural similarities between the tetrameric melittin at acidic pH and the molten globule of cytochrome *c* at pH 2. Whereas the molecular weight and net charge of the former are 11 400 (2850 × 4) and +24 (6 × 4), respectively, those of the latter are 12 360 and +24, respectively (Table III).

To compare the two states exactly, we measured the radius of gyration (Rg) by SAXS. The SAXS of the intact melittin was measured in 10 mM acetate buffer (pH 4.7) and 0.1 M $NaClO_4$ at the peptide concentrations in the range of 5–30 mg/mL. In this peptide concentration range, the fraction of the tetrameric melittin was calculated to be 97–99% on the basis of a value of the equilibrium constant, K_F, presented in the previous report [i.e., $K_F = 1 \times 10^{15}$ M^{-3}; see Figure 4c of Goto and Hagihara (1992)]. Figure 4 shows the Guinier plots, ln $I(Q)$ vs Q^2, of the intact melittin and cytochrome *c*, where $Q = (4\pi \sin\theta)/\lambda$, 2θ and λ are scattering angle and the wavelength of X-ray, respectively. A SAXS profile, $I(Q)$, is expressed as $I(Q) = I(0) \exp[-Rg^2(Q^2/3)]$, where $I(0)$ and Rg are the intensity at 0 angle and the radius of gyration, respectively. Therefore, a Guinier plot should be approximated by a straight line, and the slope gives Rg. $I(0)$ is proportional

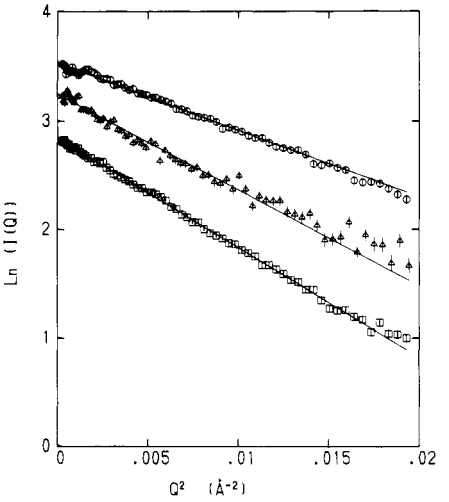

FIGURE 4: Comparison of SAXS profiles of the intact melittin and cytochrome *c* at infinite dilution in the form of Guinier plot. Symbols: (□) melittin in 0.1 M $NaClO_4$, 10 mM sodium acetate buffer (pH 4.7); (○) cytochrome *c* in 0.1 M NaCl, 10 mM sodium acetate buffer (pH 4.7); and (△) cytochrome *c* in 0.5 M NaCl, 20 mM HCl (pH 2). For clarity, the values for the melittin and the molten globule of cytochrome *c* are shifted on the ln *I* axis.

to molecular weight. The molecular weight of the intact tetrameric melittin was determined to be 11 300 from its $I(0)$ with the native cytochrome *c* as a standard. This confirms that the melittin assumes a tetrameric helical state in 0.1 M $NaClO_4$ at pH 4.7.

The Rg of the molten globule of cytochrome *c* in 0.5 M NaCl at pH 2 was determined to be 16.1 Å, being a little larger than that (13.7 Å) of the native state, whereas that of the highly unfolded state at pH 2 was 22.8 Å (Table III). These results were consistent with the previous reports that the acidic molten globule of cytochrome *c* has a compactness near to that of the native state (Goto et al., 1990; Ohgushi & Wada, 1983). It should be noted that the Guinier plot of the molten globule of cytochrome *c* is well approximated by a straight line over a rather wide range, up to $QRg \approx 2.5$, as well as that of the native cytochrome *c* (Figure 4). It was shown that the truncated staphylococcal nuclease is compact (Flanagan et al., 1992). However, a straight line region of Guinier plot extends only up to $QRg \approx 1.5$. We can again say that the molten globule of cytochrome *c* is quite compact and close to a native-like globular structure. The Rg (16.1 Å) of the molten globule induced by acetylation agreed well with that of the molten globule induced by salt. This indicates that the molecular conformation of the molten globule state induced by salt is close to the corresponding state induced by the acetylation, supporting the assumption that the acetylation of amino groups replaces the salt effects on the acid-unfolded cytochrome *c* (Goto & Nishikiori, 1991).

The Rg (12.7 Å) of the native state calculated from the X-ray crystallographic structure is smaller than the measured value by 1.0 Å. Trewhella et al. (1988) observed a similar difference with ferri- and ferrocytochrome *c* molecules. Thus, the measured Rg of the molten globule state is larger by 3.5 Å than the value calculated on the basis of the X-ray structure.

On the other hand, the Rg of tetrameric melittin was determined by SAXS to be 17.5 Å, being larger by 1.3 Å than that of the molten globule of cytochrome *c*. Because the Rg calculated from the crystal structure of tetrameric melittin is 14.0 Å, the difference (3.5 Å) between the measured and calculated values is relatively large, being comparable to that of the molten globule and the crystal structure of cytochrome *c*.

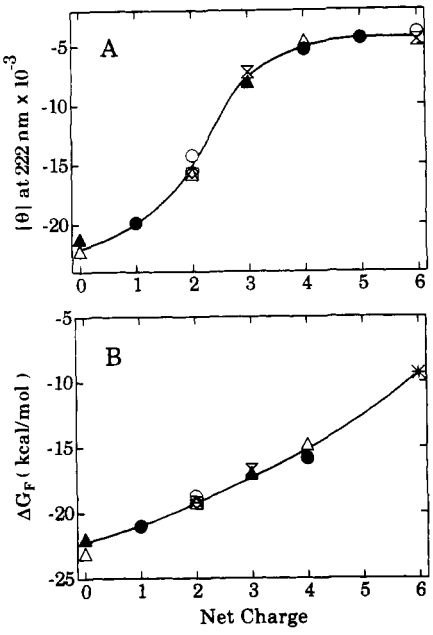

FIGURE 5: Dependence on net charge of the ellipticity at 222 nm (A) and the free energy of the folding transition (ΔG_F) (B) of various melittin derivatives at 20 °C. Net charge values of the various species under different pH conditions were assumed as shown in Table II. The symbols are the same as in Figure 2. In panel B, the ΔG_F of intact melittin obtained from the concentration-dependent transition (*), taken from Goto and Hagihara, (1992), was also shown.

DISCUSSION

Role of Charge Repulsion. We were able to increase the stability of the tetrameric helical structure of melittin to various extents by modifying the amino groups or introducing Glu residues. The formation of salt bridges was a possible mechanism explaining the stabilization of the helical state. However, both salt and alkaline stabilizations of the helical state ruled out a major contribution of salt bridges. Salt bridges have been reported to be important in the stability of the folded state of proteins and peptides (Marqusee & Baldwin, 1987; Merutka & Stellwagen, 1991). On the other hand, several cases have been reported where salt bridges on the surface of protein and peptide molecules do not contribute to stability (Bradley et al., 1990; Sali et al., 1991; Horovitz et al., 1990). The stabilization of the helical structure of melittin should be explained largely by the decrease in unfavorable charge repulsion between the positive groups by the decrease in net charge.

Because the melittins used in the present study take various ionic forms depending on pH (Table II), we can consider the effects of net charge on the conformation of melittin in detail. Figure 5A shows the dependence of ellipticity at 222 nm on the net charge of melittin. The data were obtained from the ellipticity at pH 2, 6, and 11, assuming the net charge values shown in Table II. In the present study, we did not consider the conformational states with negative net charge. The ellipticities obtained from the various melittin species formed a single cooperative transition, showing that net charge is a critical factor determining the conformation of melittin.

The conformational transition was analyzed assuming a monomer-to-tetramer mechanism, in which the monomer is unfolded (U) and the tetramer folded (F_4):

$$4U \rightleftarrows F_4 \qquad (1)$$

The validity of this model has been indicated previously (Goto & Hagihara, 1992). The equilibrium constant (K_F) for the folding transition was obtained from the ellipticity at 222 nm by

$$K_F = \frac{[F_4]}{[U]^4} = \frac{[\theta]_U - [\theta]}{4([\theta] - [\theta]_F)^4} \qquad (2)$$

where $[\theta]$ is the observed ellipticity and $[\theta]_U$ and $[\theta]_F$ are the ellipticities for the unfolded and folded states, respectively, assumed to be −4400 and −24 000, respectively. The change in free energy (ΔG_F) for the formation of the tetrameric state is calculated by the relation $\Delta G_F = -RT \ln K_F$ (Figure 5B).

In Figure 5B, we have also plotted the ΔG_F of intact melittin obtained from the concentration-dependent folding transition at pH 5 (Goto & Hagihara, 1992). It should be noted that a midpoint of the conformational transition (i.e., net charge +2) does not provide a free energy value of zero. From the slope between net charge +4 and +6, charge repulsion is estimated to destabilize the tetrameric state of intact melittin by 600 cal/(charge·mol of tetrameric melittin). With a decrease in net charge, the slope decreases, indicating the decrease in electrostatic contribution to the stability of the tetrameric state. This is as expected from the nature of electrostatic repulsion. ΔG_F at net charge zero (23 kcal/mol) indicates that intrinsic stability without electrostatic effects.

Intact melittin has no negative charges, and the unfolded state dissociates into monomers, in which the charge repulsions are small. Therefore, the electrostatics of melittin should be determined largely by the charge repulsion in the compactly folded state, and the decrease in ΔG_F upon reduction of the net charge reflects the release of charge repulsion in the folded state. However, it is probable that the reduction in net charge also affects, to a lesser extent, the other factors.

Dill, Stigter, and co-workers considered protein stability through the use of a statistical mechanical theory of polyelectrolytes (Dill, 1990; Stigter et al., 1991; Alonso, et al., 1991). The theory treats protein stability as a balance between hydrophobic interactions, conformational entropy, and electrostatics. Whereas the hydrophobic interactions favor a compact state, the conformational entropy and charge repulsions favor an unfolded state. Their results suggest that reduction of the net charge affects not only the charge repulsion in the folded state but also the repulsion in the unfolded state and, furthermore, modulates the balance of conformational entropy and hydrophobic interactions. Consideration of such effects will be necessary for a complete explanation of our results.

Comparison with the Acidic Molten Globule of Cytochrome c. Conformation and stability of the molten globule state is one of the most important topics of protein folding (Kuwajima, 1989; Kim & Baldwin, 1989; Christensen & Pain, 1991; Baldwin, 1991). While several proteins including cytochrome *c* are fully unfolded under conditions of low salt at pH 2, they assume a molten globule state in the presence of high salt (Goto et al., 1990a,b; Ohgushi & Wada, 1983). Goto and co-workers (1990a,b) explained the salt effects by the anion binding to the positive charges of the proteins. The anion bindings shield the charge repulsion, which results in the manifestation of the intrinsic forces stabilizing the molten globule state. To confirm this mechanism, Goto and Nishikiori (1991) prepared a series of acetylated cytochrome *c* species, in which acetylation of the lysyl amino groups progressively removed the positive charges. They showed that the decrease in net charge stabilized the molten globule state. They estimated the contribution of charge repulsion in destabilizing the molten globule of intact cytochrome *c* to be 450 cal/(charge·mol) at 20 °C. This contribution comes largely from

the charge repulsion in the folded state, although we have to consider the other effects described above.

We compared the structural properties of tetrameric melittin with those of the molten globule of cytochrome *c*. As shown in Table III, although the Rg of the tetrameric melittin in solution is slightly larger than that of the molten globule of cytochrome *c*, the two states are similar in molecular weight, size, and net charge. The present results clarified that the contributions of electrostatic repulsion to the folding transition are also similar to each other. It is remarkable that essentially different systems, i.e., the monomeric molten globule and tetrameric melittin, provide a comparable measure of charge repulsion. Melittin has a region of condensed charge (i.e., Lys(21)-Arg-Lys-Arg). This may explain the slightly higher contribution of charge repulsion in melittin in spite of its slightly larger size.

It has been suggested that the mechanism of conformational stability of the molten globule state is simpler than that of the native state (Goto & Nishikiori, 1991; Baldwin, 1991; Hughson et al., 1991). Although proteins have a tendency to be most stable near their isoelectric point (Pace et al., 1990), the stability of the native state is not necessarily proportional to the net charge. On the other hand, the stability of the molten globule state depends largely on the net charge (Goto & Nishikiori, 1991). The critical role of charge repulsion in the tetrameric helical state of melittin suggests that the mechanism responsible for the conformational stability of melittin is similar to that of the molten globule. The fact that the Rg of tetrameric melittin in solution is comparable to that of the molten globule of cytochrome *c* supports this assumption.

It should be mentioned, however, that we do not propose that the tetrameric state of melittin in solution is equivalent to the molten globule state as originally proposed (Ohgushi & Wada, 1983; Ptitsyn, 1987; Kuwajima, 1989). Our data are not enough to conclude this. On the contrary, the near-UV CD spectrum of the tetrameric melittin showed fine peaks, suggesting the presence of specific tertiary structure (Y. Hagihara and Y. Goto, unpublished data). In addition, thermal unfolding of the tetrameric melittin measured by the far-UV CD was cooperative, and the calorimetric measurements indicated that it accompanies a significant heat absorption (Y. Hagihara and Y. Goto, unpublished data). Although these results are apparently inconsistent with the original definition of the molten globule, we do not know at present if the definition is general or not. For example, it is known that the acidic molten globule of cytochrome *c*, the first example for which the term molten globule was used, shows cooperative thermal unfolding, which is not observed for the molten globule of other proteins (Potekhin & Pfeil, 1989; Ptitsyn, 1987; Kuroda et al., 1992). The location of secondary structure in the molten globule of cytochrome *c* was examined by two-dimensional NMR using the H–D exchange of amido protons (Jeng et al., 1990). This revealed that the major helices and their common hydrophobic domain are largely preserved, while the loop region of the native structure is flexible and partly disordered. It was suggested that while some regions of the tertiary structure are highly flexible, other regions remain intact, i.e., local increases in structural flexibility rather than a uniform melting of the tertiary structure. In addition, confusion has arisen from use of the term "molten globule" for various intermediate states with different extents of structure (Kim & Baldwin, 1990; Baldwin, 1991). Further characterization of the tetrameric state of melittin in solution may shed light on these problems.

Conclusion. We have confirmed that the electrostatic charge repulsion destabilizes the tetrameric helical structure of melittin and estimated its energetic contribution. The contribution is comparable to that in the acidic molten globule of cytochrome *c*, which has the size and net charge comparable to that of the tetrameric melittin, suggesting that the mechanism of the stability of these states are similar. The results provide exact experimental data on a measure of charge repulsion in the stability of proteins and peptides.

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REFERENCES

Alonso, D. O. V., Dill, K. A., & Stigter, D. (1991) *Biopolymers 31*, 1631–1649.
Baldwin, R. L. (1991) *Chemtracts 2*, 379–389.
Bello, J., Bello, H. R., & Granados, E. (1982) *Biochemistry 21*, 461–465.
Bradley, E. K., Thomason, J. F., Cohen, F. E., Kosen, P. A., & Kuntz, I. D. (1990) *J. Mol. Biol. 215*, 607–622.
Brown, L. R., Lauterwein, J., & Wüthrich, K. (1980) *Biochim. Biophys. Acta 622*, 231–244.
Christensen, H., & Pain, R. H. (1991) *Eur. Biophys. J. 19*, 221–229.
Creighton, T. E. (1984) *Proteins*, W. H. Freeman and Co., New York.
Dill, K. A. (1990) *Biochemistry 29*, 7133–7155.
Flanagan, J. M., Kataoka, M., Shortle, D., & Engelman, D. M. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 748–752.
Goto, Y., & Fink, A. L. (1990) *J. Mol. Biol. 214*, 803–805.
Goto, Y., & Aimoto, S. (1991) *J. Mol. Biol. 218*, 387–396.
Goto, Y., & Nishikiori, S. (1991) *J. Mol. Biol. 222*, 679–686.
Goto, Y., & Hagihara, Y. (1992) *Biochemistry 31*, 732–738.
Goto, Y., Calciano, L. J., & Fink, A. L. (1990a) *Proc. Natl. Acad. Sci. U.S.A. 87*, 573–577.
Goto, Y., Takahashi, N., & Fink, A. L. (1990b) *Biochemistry 29*, 3480–3488.
Habermann, H. (1972) *Science 177*, 314–322.
Horovitz, A., Serrano, L., Avron, B., Bycroft, M., & Fersht, A. R. (1990) *J. Mol. Biol. 216*, 1031–1044.
Hughson, F. M., Barrick, D., & Baldwin, R. L. (1991) *Biochemistry 30*, 4113–4118.
Jeng, M.-F., Englander, S. W., Elove, G. A., Wand, A. J., & Roder, H. (1990) *Biochemistry 29*, 10433–10437.
Kataoka, M., Head, J. F., Seaton, B. A., & Engelman, D. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6944–6948.
Kim, P. S., & Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631–660.
Kuroda, Y., Kidokoro, S., & Wada, A. (1992) *J. Mol. Biol. 223*, 1139–1153.
Kuwajima, K. (1989) *Proteins Struct., Funct., Genet. 6*, 87–103.
Marqusee, S., & Baldwin, R. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8898–8902.
Merutka, G., & Stellwagen, E. (1991) *Biochemistry 30*, 1591–1594.
Ohgushi, M., & Wada, A. (1983) *FEBS Lett. 164*, 21–24.
Pace, C. N., Laurents, D. V., & Thomson, J. A. (1990) *Biochemistry 29*, 2564–2572.
Potekhin, S., & Pfeil, W. (1989) *Biophys. Chem. 34*, 55–62.
Ptitsyn, O. B. (1987) *J. Protein Chem. 6*, 273–293.

Quay, S. C., & Condie, C. C. (1983) *Biochemistry 22*, 695–700.
Ramalingam, K., Bello, J., & Aimoto, S. (1991) *FEBS Lett. 295*, 200–202.
Sali, D., Bycroft, M., & Fersht, A. R. (1991) *J. Mol. Biol. 220*, 779–788.
Stigter, D., Alonso, D. O., & Dill, K. A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4176–4180.
Talbot, J. C., Dufourcq, J., deBony, J., Faucon, J. F., & Lussan, C. (1979) *FEBS Lett. 102*, 191–193.
Terwilliger, T. C., & Eisenberg, D. (1982a) *J. Biol. Chem. 257*, 6010–6015.
Terwilliger, T. C., & Eisenberg, D. (1982b) *J. Biol. Chem. 257*, 6016–6022.
Trewhella, J., Carlson, V. A. P., Curtis, E. H., & Heidorn, D. B. (1988) *Biochemistry 27*, 1121–1125.
Ueki, T., Hiragi, Y., Kataoka, M., Inoko, Y., Amemiya, Y., Izumi, Y., Tagawa, H., & Muroga, Y. (1985) *Biophys. Chem. 23*, 115–124.