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Thermodynamics of melittin tetramerization determined by circular dichroism and implications for protein folding

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Abstract

The tetramerization of melittin, a 26-amino acid peptide from *Apis mellifera* bee venom, has been studied as a model for protein folding. Melittin converts from a monomeric random coil to an α-helical tetramer as the pH is raised from 4.0 to 9.5, as ionic strength is increased, as temperature is raised or lowered from about 37 °C, or as phosphate is added. The thermodynamics of this tetramerization (termed "folding") are explored using circular dichroism. The melittin tetramer has two pK_a values of 7.5 and 8.5 corresponding to protonation of the N-terminus and Lys 23, respectively. pK_a values calculated with the program DelPhi (Gilson, M.K., Sharp, K.A., & Honig, B.H., 1987, *J. Comp. Chem. 9*, 327–335; Gilson, M.K. & Honig, B.H., 1988a, *Proteins 3*, 32–52; Gilson, M.K. & Honig, B.H., 1988b, *Proteins 4*, 7–18) agree with experimental titration data. Greater electrostatic repulsion of these protonated groups destabilizes the tetramer by 3.6 kcal/mol at pH 4.0 compared to pH 9.5. Increasing the concentration of NaCl in the solution from 0 to 0.5 M stabilizes the tetramer by 5–6 kcal/mol at pH 4.0. The effect of NaCl is modeled with a ligand-binding approach. The melittin tetramer is found to have a temperature of maximum stability ranging from 35.5 to 43 °C depending on the pH, unfolding above and below that temperature. ΔC_p^0 for folding ranges from −0.085 to −0.102 cal g^{-1} K^{-1}, comparable to that of other small globular proteins (Privalov, P.L., 1979, *Adv. Protein Chem. 33*, 167–241). ΔH^0 and ΔS^0 are found to decrease with temperature, presumably due to the hydrophobic effect (Kauzmann, W., 1959, *Adv. Protein Chem. 14*, 1–63). Phosphate is found to perturb the equilibrium substantially with a maximal effect at 150 mM, stabilizing the tetramer at pH 7.4 and 25 °C by 4.6 kcal/mol. The enthalpy change due to addition of phosphate (−7.5 kcal/mol at 25 °C) can be accounted for by simple dielectric screening. Both circular dichroism and crystallographic results suggest that phosphate may bind Lys 23 at the ends of the elongated tetramer. These detailed measurements give insight into the relative importance of various forces for the stability of melittin in the folded form and may provide an experimental standard for future tests of computational energetics on this simple protein system.

Keywords: circular dichroism; melittin; protein folding; thermodynamics

Melittin is a 26-amino acid peptide that composes 50% (Habermann, 1972) of the dry weight of venom of *Apis mellifera* honey bees. Its sequence[2] is (Habermann & Jentsch, 1967)

Gly Ile Gly Ala Val Leu Lys Val Leu Thr Thr Gly
Leu Pro Ala Leu Ile Ser Trp Ile Lys Arg Lys
Arg Gln Gin-NH_2.

In solution, under certain conditions, melittin aggregates to form an α-helical tetramer. The amount of tet-

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Abbreviations: CD, circular dichroism; far UV, 260–185 nm; near UV, 330–245 nm; A_{280}, light absorption at 280 nm; ΔC_p^0, the standard change in heat capacity from the unfolded to the folded form; $\Delta\epsilon = A_L - A_R$, where A_L and A_R are the absorbance of left and right circularly polarized light, respectively; $\Delta\epsilon_{222}$, $\Delta\epsilon$ at 222 nm; $\Delta\epsilon_{282}$, $\Delta\epsilon$ at 282 nm; T_h, a temperature at which $\Delta H^0(T) = 0$; T_s, a temperature at which $\Delta S^0(T) = 0$; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

[2] In bee venom, the N-terminus is formylated to the extent of 10 (Kreil & Kreil-Kiss, 1967) to 20% (Lauterwein et al., 1980). Formylated melittin tetramerizes at a lower concentration than unformylated melittin (Lauterwein et al., 1980). Formylated melittin was not detected in the melittin preparation used in this study and melittin is not formylated in the refined crystal structure (M. Gribskov, L. Wesson, & D. Eisenberg, in prep.).

ramer is increased by increasing ionic strength (Faucon et al., 1979; Talbot et al., 1979, 1982; Yunes, 1982), concentration (Talbot et al., 1979; Quay & Condie, 1983; Quay et al., 1985), pH (Yunes, 1982), temperature between −4 and 25 °C (Faucon et al., 1979), sulfate (Strom et al., 1980), and phosphate (Drake & Hider, 1979; Podo et al., 1982; Strom et al., 1980).

Tetrameric melittin has been crystallized in two forms with ammonium sulfate and the structure of both determined (Terwilliger & Eisenberg, 1982a,b; Terwilliger et al., 1982). The structure of form II has been refined to a residual R-factor of 0.16 at 2.0 Å resolution (M. Gribskov, L. Wesson, & D. Eisenberg, in prep.). These coordinates are the basis of the calculations here and are illustrated in the kinemages.

In many respects the melittin equilibrium

$$4 \times \text{monomer (random coil)} \underset{K_{eq}}{\rightleftharpoons} \text{tetramer } (\alpha_4) \quad (1)$$

represents a simple experimental model of protein folding. The apparently simultaneous formation of secondary, tertiary, and quaternary structure in melittin (see Discussion) is similar to the typical two-state transition (primary to tertiary structure) often found in protein folding (Go, 1983; Creighton, 1984; Kuwajima & Schmid, 1984; Jaenicke, 1987). The difference between melittin and other well-studied systems is that the units of secondary structure in melittin are not joined by peptide linkages. Consequently, a convenient feature of melittin is the ability to measure the thermodynamics of folding under a broad range of conditions without addition of chemical denaturants by simply adjusting the concentration of melittin.

The forces that stabilize the melittin tetramer relative to the unfolded monomer include hydrogen bonds, hydrophobic interactions, dipole-dipole interactions (Hol, 1985a,b), and van der Waals dispersion forces. Destabilizing influences include the loss of conformational entropy upon folding and electrostatic repulsion of the 24 positive charges (the C-terminus is amidated, and there are no acidic groups). By evaluating the equilibrium under different conditions of pH, temperature, and ionic strength, the relative contributions of some of these forces can be assessed.

CD at 222 nm is useful for monitoring the α-helical content (Greenfield & Fasman, 1969) of melittin, whereas CD at 282 nm reflects the quaternary structure through the environment of the single Trp residue (Kahn, 1979), and the ratio of CD at 282 nm to 222 nm is a sensitive indicator of changes in structure.

Results

Melittin self-association as a function of concentration

The dependence of melittin tetramerization on concentration was studied in the near and far UV. Sample near and far UV spectra of melittin in predominantly the tetrameric form are shown in Figure 1. For further analysis in the far UV, $\Delta\epsilon_{222}$ (CD at 222 nm) was chosen as a parameter to follow because of its relation to helicity (proportional to $\Delta\epsilon_{222}$). $\Delta\epsilon_{282}$ was chosen to monitor the

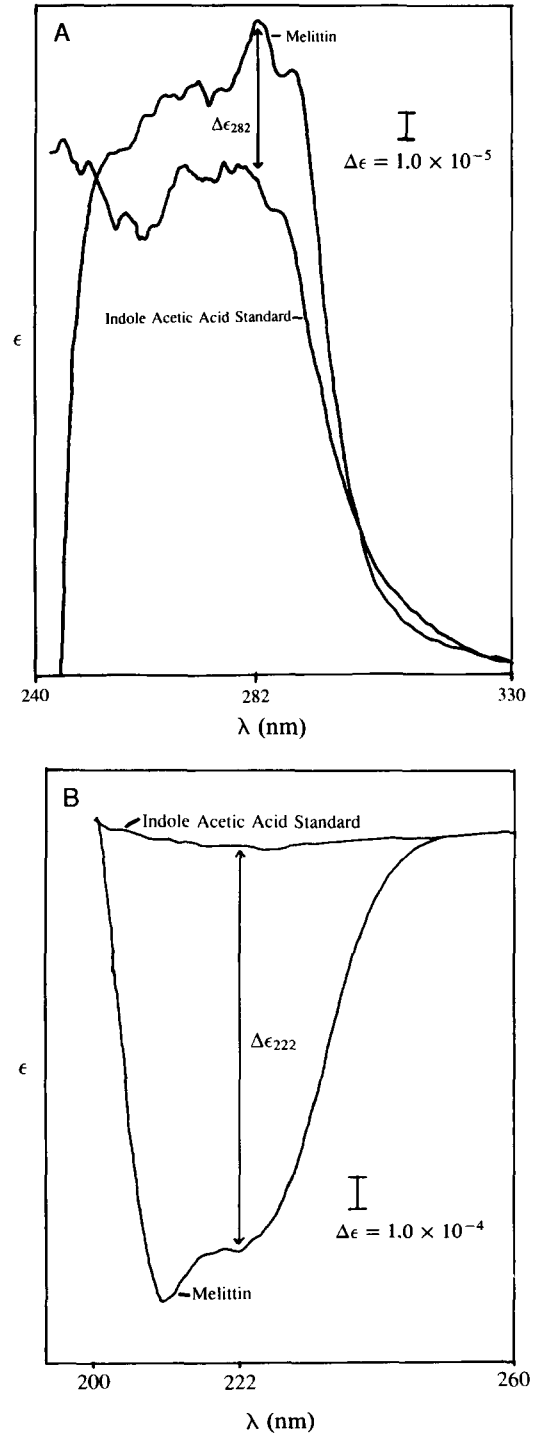

Fig. 1. Sample CD spectra of tetrameric melittin. CD spectra of a 0.535 mM solution of melittin in 150 mM NaCl, 20 mM Tris, pH 7.4 at 25 °C. **A**: $\Delta\epsilon$ in the near UV from 330 to 245 nm. **B**: $\Delta\epsilon$ in the far UV from 260 to 185 nm.

near UV because it was the largest peak in the spectra and reflects the environment of the single tryptophan (Kahn, 1979).

Melittin self-association as a function of pH

The proportion of tetramer is expected to increase with increasing pH because of decreasing electrostatic repulsions, given that melittin has only basic titratable groups, a phenomenon that has been observed by other workers (Bello et al., 1982; Podo et al., 1982; Yunes, 1982; Strom et al., 1980). Near and far UV spectra of melittin were obtained at different pH values as shown in Figure 2.

Melittin self-association as a function of [NaCl]

Increasing ionic strength might be expected to stabilize the tetramer by screening electrostatic repulsions between the basic titratable groups of melittin (Hill, 1956), although salt may have an effect on other interactions such as hydrophobic bonds (lyotropic effects [Tanford, 1968; von Hippel & Schleich, 1969]). Figure 3 shows how the helicity of melittin varies with pH as the concentration of NaCl is increased. $\Delta\epsilon$ is divided by A_{280} to adjust for small differences in concentration between different samples.

Melittin self-association as a function of [PO_4^{2-}]

The amount of melittin tetramer is known to be increased by including phosphate in the buffer (Strom et al., 1980; Drake & Hider, 1979; Podo et al., 1982), particularly at physiologic pH (Strom et al., 1990), but the concentration dependence has not been previously explored. Phosphate causes an increase in helicity from 5 mM to a maximum at 100 mM, with a greater effect at pH 7.4 than pH 4.0 or 9.5 (data not shown). Figure 4 shows how titrations are perturbed by 100 mM phosphate.

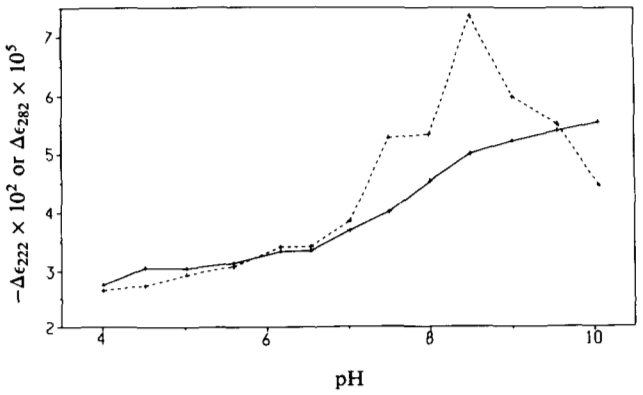

Fig. 2. Near and far UV CD of melittin as a function of pH. $-\Delta\epsilon_{222}$ (solid line) and $\Delta\epsilon_{282}$ (dashed line) versus pH for 0.334 mM melittin in 0.15 M NaCl, 20 mM Tris. Titration of the samples caused at most a 0.0008 mM change in concentration from dilution.

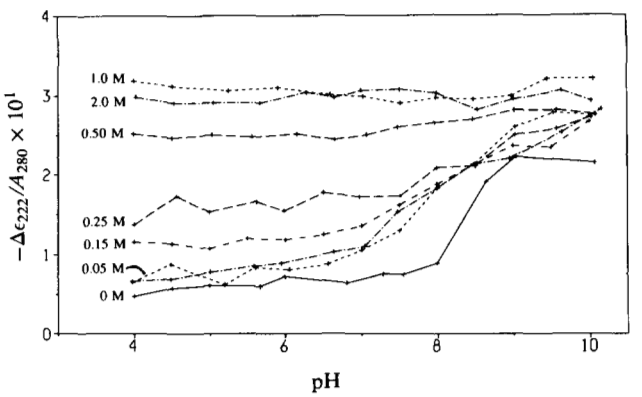

Fig. 3. Dependence of melittin helicity (proportional to $-\Delta\epsilon_{222}$) on pH. The ratio $-\Delta\epsilon_{222}/A_{280}$ versus pH varying the concentration of NaCl. The concentrations of melittin are 0.195, 0.221, 0.203, 0.212, 0.198, 0.185, 0.207, 0.203 mM, respectively, for samples of 0, 0.05, 0.10, 0.15, 0.25, 0.50, 1.0, and 2.0 M NaCl. Dilution of the samples during titration caused at most a 0.0003 mM decrease in concentration.

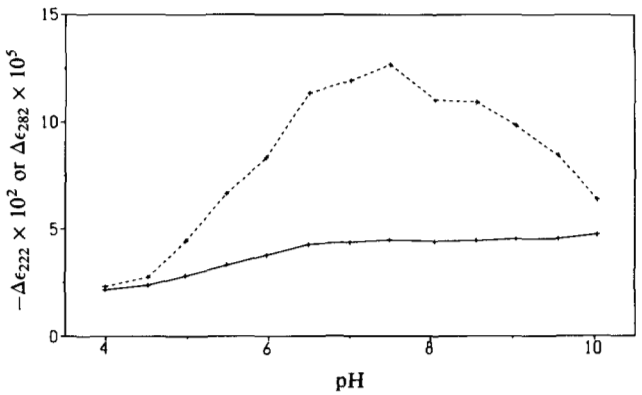

Fig. 4. The pH dependence of near and far UV CD of melittin in 100 mM phosphate, 50 mM NaCl. $-\Delta\epsilon_{222}$ (solid line) and $\Delta\epsilon_{282}$ (dashed line) versus pH. The concentration of melittin is 0.243 mM in 0.15 M NaCl, 20 mM Tris. Dilution during titration caused no more than a 0.0007 mM change in concentration.

Melittin self-association as a function of temperature

The melittin tetramer is known to be destabilized upon elevating (Quay & Condie, 1983) or lowering (Faucon et al., 1979) the temperature, but the thermodynamics of these transitions have not been explored under a variety of conditions. The dependence of the helicity of melittin on temperature for pH 4.0, pH 7.4, pH 9.5, and pH 7.4 with 150 mM phosphate is shown in Figure 5. These par-

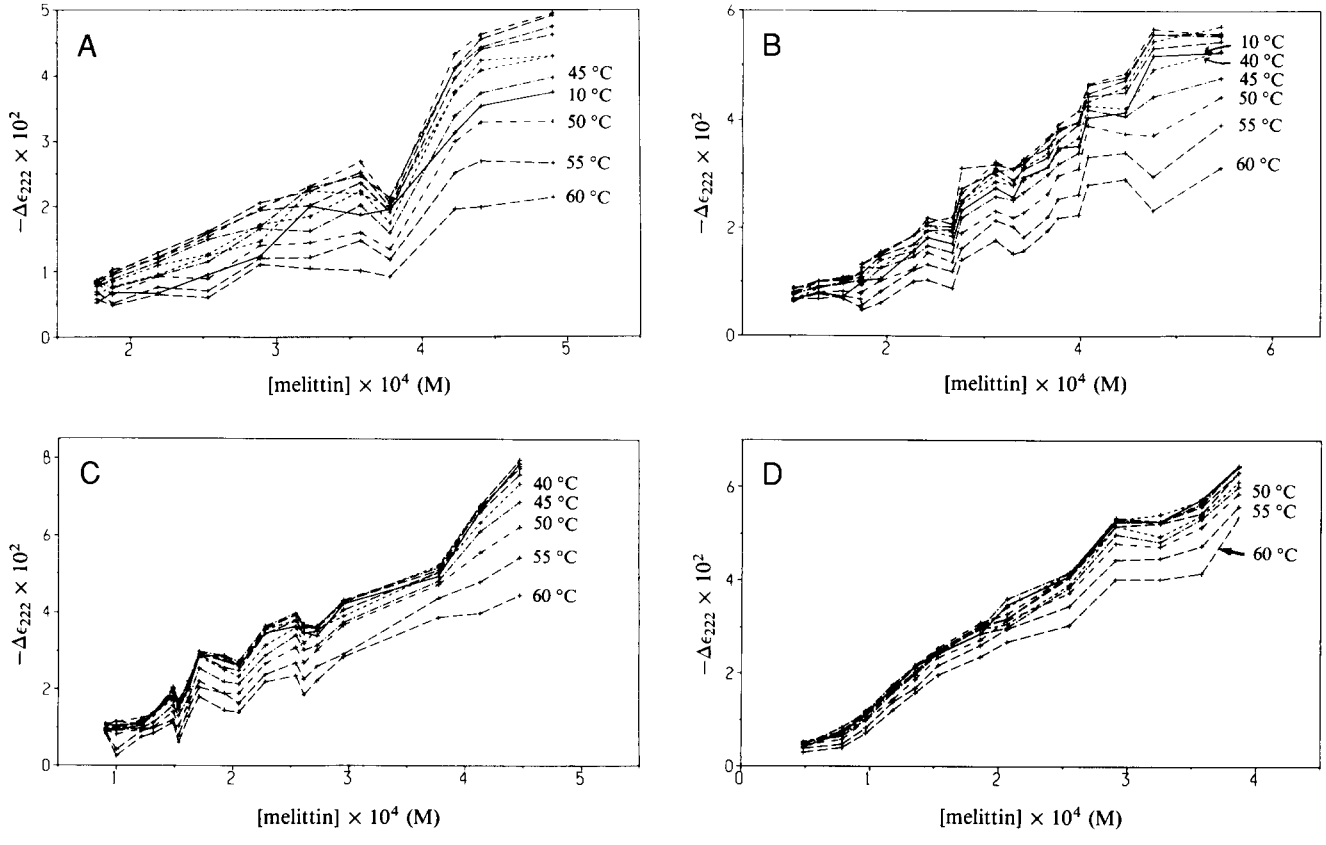

Fig. 5. Melittin helicity at different concentrations as a function of temperature used to calculate thermodynamic parameters. $-\Delta\epsilon_{222}$ versus concentration for temperatures between 10 and 60 °C in 5 °C increments (selected temperature values are labeled). **A**: pH 4.0. **B**: pH 7.4. **C**: pH 9.5. **D**: pH 7.4 with 150 mM phosphate.

ticular pH values were chosen for further study based on the titration curves in Figure 3: at pH 4.0 all ionizable groups should be protonated; at pH 9.5 the pK_a at pH 8.5 has been passed (see Discussion); pH 7.4 represents physiologic conditions; and pH 7.4 with 150 mM phosphate was chosen to observe the maximum effect of phosphate (see above).

Attempts failed to measure directly ΔC_p^0 for unfolding: differential scanning calorimetry between 10 and 60 °C on concentrated solutions of melittin revealed no discrete transitions as might have been expected from melittin's denaturation over a broad temperature range.

Discussion

The tetramerization of melittin is a two-state process

The tetramerization of melittin presents an unusual opportunity to study the thermodynamics of protein folding under a broad range of conditions without the use of chemical denaturants. This is because the units of secondary structure are unlinked in the melittin tetramer, unlike most other proteins. This allows folding of melittin to be observed under many conditions simply by changing its concentration.

In this work we assume that melittin tetramer formation is a thermodynamic two-state process (random coil monomer and α-helical tetramer) similar to many single-domain proteins that show a one-step conversion between denatured and folded forms (Go, 1983; Creighton, 1984; Kuwajima & Schmid, 1984; Jaenicke, 1987). Studies of melittin from several laboratories under a variety of conditions with a number of different techniques including NMR have failed to reveal any other form of melittin in significant concentration (Brown et al., 1980; Quay & Condie, 1983; Schwarz & Beschiaschvili, 1988). Although melittin aggregation may proceed through a dimer, the dimer seems not to be very stable in solution. Fluorescence experiments on melittin in sodium sulfate solutions reveal a dimer concentration of approximately 1% (Schwarz & Beschiaschvili, 1988). Dimer formation was not detected in this work. Presumably, in a dimer, the environment of the tryptophan would be different than in the tetramer, changing the near UV spectra (Kahn, 1979) and altering the ratio $-\Delta\epsilon_{282}/\Delta\epsilon_{222}$. However, although both $-\Delta\epsilon_{222}$ and $\Delta\epsilon_{282}$ increase nonlinearly with concentration (data not shown), the ratio is approximately constant (except

at low concentrations of melittin where experimental error is high particularly in the near UV).

The helicity of a melittin tetramer appears to be the same no matter what the conditions. This is supported by the work of Strom et al. (1980) who observed that there are small variations in the structure of the melittin tetramer at different pH values, discernable with ^{1}H NMR even though the helicity remained the same. These small spectral changes may be due to changes in side-chain positions to compensate for electrostatic repulsion. However, because the C-terminus contains four positive charges, it might be expected to unfold locally at low pH, changing the near UV CD.[3] With the exception of pH 7.4 at low temperature, the near and far UV CD parallel each other within the limits of the experiment for a given pH (data not shown). The increase in near UV signal for pH 7.4 at low temperatures is probably due to the 0.4 unit increase in the pH of the buffer that occurs when the temperature is decreased from 25 to 10 °C causing some deprotonation of the N-terminus (see below). In the following discussion, it will be assumed that there is no local unfolding, and thus the amount of tetramer is proportional to the helicity ($-\Delta\epsilon_{222}$).

Effect of pH

The conformational state of melittin is greatly influenced by pH (Bello et al., 1982; Podo et al., 1982; Yunes, 1982; Strom et al., 1980). Titration experiments (Figs. 2, 3) reveal two inflection points (pH 7.5 and 8.5) in the $-\Delta\epsilon_{222}$ versus pH curves, which we will henceforth call pK_a values, recognizing that these are not direct protometric titration experiments. Nevertheless, these observed pK_a values may represent the actual pK_a values of titratable groups in the melittin tetramer. The reason is that the amount of tetramer is expected to increase as the pH is raised above the pK_a of a titratable group, because of decreased electrostatic repulsion.

Our results compare favorably with pK_a values determined by other methods: 8.8 in 0.15 M NaCl from NMR (Strom et al., 1980); 6.8 in 120 mM NaCl for 0.63 mM melittin in phosphate buffer from CD (Bello et al., 1982); 8.8 and 7.8 for 0.49 and 0.18 mM melittin, respectively, in 5 mM TES, 5 mM piperazine, and 0.15 M NaCl by CD (Podo et al., 1982); 6.8 and 8.7 at 2.3–3.4 μM melittin, 0.05 M NaCl, whereas this same concentration in 2.02 M NaCl, 0.02 M phosphate had only one pK_a of 7.4, determined by acrylamide quenching of melittin fluorescence (Quay et al., 1985).

[3] Hydrogen exchange experiments on melittin show the C-terminal helix (residues 12–26) to be more stable than the N-terminal helix (residues 1–11) regardless of the ionization state of the positive charges (Dempsey, 1988). However, these experiments were carried out in methanol where melittin exists as a monomeric helix with less of a bend at Pro 14 than in the crystal structure (Bazzo et al., 1988), and thus these results may not be applicable to aqueous solutions.

Which of the titratable groups on melittin is responsible for the two observed pK_a values? Tentative assignments can be made with a combination of near and far UV spectra by noting that the near UV CD represents the environment of the sole tryptophan (Kahn, 1979) and that changes in the charge surrounding it affect its electronic environment and thus its rotational strength (Cantor & Schimmel, 1980). In Figure 2 the near and far UV signal increase in concert from pH 4 until pH 7. The near UV signal then increases faster than that of the far UV until pH 8.5, when it decreases. Examination of the crystal structure reveals that Arg 22 and Lys 23 of one monomer and the N-terminus of an adjacent monomer are close enough to interact with the tryptophan. Because the N-terminus has a lower intrinsic pK_a[4] (8.0), and because the change in near UV signal starts at pH 7, the pK_a at pH 7.5 probably corresponds to titration of the N-terminus. As the pH is raised, the next group to be titrated would be lysine (pK_{int} 10.4). The titration of lysines 7 and 21, both far from the tryptophans, are probably not detectable in the near UV, so the change in near UV and the pK_a at pH 8.5 can be assigned to titration of Lys 23.

Electrostatic calculations using the crystal structure can also help with assignment of pK_a values. Classical electrostatic calculations using Coulomb's law (with dielectrics of the form C, Cr, Cr^2, C$e^{0.33r}$ where C is a constant and r is the interatomic distance) and the method of Matthew et al. (1985) failed to agree with experiment. However, experimental values could be reproduced using the Poisson-Boltzman model employed in the DelPhi program (Gilson et al., 1987; Gilson & Honig, 1988a,b). Although adjustment of parameters in the program changed the absolute ΔpK_a values, the titration order remained the same. The best agreement with the experimental titration curve (Figs. 2, 3) was with an internal dielectric of 2 and a 3-Å Stern layer. Inclusion of crystallographic waters did not improve the results. Calculated ΔpK_a values yielded pK_a's of N-terminus (7.3), Lys 23 (8.8), Lys 7 (9.4), and Lys 21 (9.8).

Effect of ionic strength

The melittin tetramer is stabilized by increasing concentrations of NaCl (Faucon et al., 1979; Talbot et al., 1979, 1982; Yunes, 1982). Increasing NaCl causes increased association and increased thermal stability. The maximal effect of NaCl on increasing the amount of tetramer is at about 0.5 M (Fig. 3). The effect appears mainly for pH values less than 8.5 implying that the effect of ionic strength is due to Debye-Huckel dielectric screening (Hill, 1956) of the electrostatic repulsions between basic

[4] The intrinsic pK_a (pK_{int}) is the pK_a of a group isolated from other charges. The actual pK_a values in the melittin tetramer will be lower because of electrostatic effects from the surrounding positive charges. The pK_{int} values for the side chains in melittin are N-terminus 8.0, lysine 10.40, and arginine 12.00 (Matthew et al., 1985).

titratable groups. Assuming the CD absorption coefficients derived below, ΔG^0 for any point in Figure 3 can be calculated using $\Delta G^0 = -RT \ln K_{eq}$, where K_{eq} is defined in Equation 3 below. At low pH, increasing the ionic strength from 0 to 0.5 M stabilizes the tetramer by 5–6 kcal/mol.

The concentration dependence of ionic strength on the equilibrium may be analyzed in two different ways. Calculations of electrostatic energy on the crystal structure using Coulomb's law and the Debye-Huckel screening dielectric of Hill (1956) did not agree with the experimental data at low pH (where all ionizable groups are protonated). Whereas Debye-Huckel theory strictly applies only to electrolyte solutions of ionic strength less than 0.01 M (McQuarrie, 1976), a ligand-binding approach is useful at higher concentrations of salt. Ions can bind to charged residues in a pH-dependent manner at low ionic strength thus screening the charge. The binding is nonlinear with salt concentration (Steinhardt & Beychok, 1964; von Hippel & Schleich, 1969; Record et al., 1978). Assuming that there are n identical binding sites for a ligand L on the tetramer at a given pH and any ions bound to the monomer are not dissociated on tetramer formation, we can define a perturbed equilibrium:

$$4 \times \text{monomer} + nL \underset{K_a}{\rightleftharpoons} \text{tetramer} \cdot nL,$$

where

$$K_a = \frac{[\text{tetramer} \cdot nL]}{[\text{monomer}]^4 [L]^n},$$

which is equivalent to

$$\ln\left(\frac{[\text{tetramer} \cdot nL]}{[\text{monomer}]^4}\right) = n \ln[L] + \ln K_a. \quad (2)$$

The data of Figure 3 were analyzed with Equation 2 (assuming the CD absorption coefficients determined below). The correlation coefficients (Hoel, 1971) for this fit are rather poor at pH 9.5 and 10.0 (0.59 and 0.48), so there are probably no salt ligands for pH values greater than 8.5 (a pK_a value; see above). However, below pH 8.5, the effect of salt is well approximated by a ligand-binding model in which there are four additional Cl^- ion binding sites on tetrameric melittin compared to the monomeric form (correlation coefficients 0.73, 0.88, 0.92, 0.88, 0.92, 0.83, 0.96, 0.95, 0.96, 0.88, and 0.84 at pH 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, and 8.5, respectively).

Thermodynamics of melittin tetramerization

Calculation of ΔG^0

The equilibrium constant K_{eq} of Equation 1 is defined by

$$K_{eq} = \frac{[\text{tetramer}]}{[\text{monomer}]^4}. \quad (3)$$

The observed $\Delta\epsilon$ is the sum of the contributions from the monomeric and tetrameric forms:

$$\Delta\epsilon_{observed} = \Delta\epsilon_{tetramer}[\text{tetramer}] + \Delta\epsilon_{monomer}[\text{monomer}], \quad (4)$$

where $\Delta\epsilon_{tetramer}$ and $\Delta\epsilon_{monomer}$ are the molar CD coefficients for the tetrameric and monomeric forms, respectively. K_{eq} may be varied in Equation 3 so that the correlation between the observed $\Delta\epsilon$ and that calculated with Equation 4 is maximal. Unfortunately, the molar CD coefficients are not known and cannot be reliably calculated based on structure because CD depends not only on the conformation of a peptide, but on its length (Chen et al., 1974), polarity of secondary structures with respect to each other (Chen et al., 1974), amino acid composition (Applequist, 1979a,b), side-chain conformation (Applequist, 1982), nonplanarity of the amide bond (Woody, 1983), and the presence of disulfides and aromatics (Labhardt, 1986). Therefore, experimental measures of the $\Delta\epsilon$ coefficients were desired. A 0.0983 mM solution of melittin in 20 mM Tris, pH 4.0, 10 °C had a $\Delta\epsilon_{222}$ of 0 (the same ellipticity as the indole acetic acid baseline); therefore the monomeric CD absorption coefficient is 0. A 0.207 mM solution of melittin in 1.0 M NaCl, 20 mM Tris, pH 10.06, at 25 °C was found to have the largest molar $\Delta\epsilon_{222}$, corresponding to a tetrameric CD absorption coefficient of -178.8 (cm^{-1} mol-tetramer^{-1}). Several other concentrated samples under conditions expected to induce maximal tetramer formation yielded similar values for the tetrameric coefficient. Analysis of the pH 9.5 data using the method of Schwarz and Beschiaschvili (1988) yielded a value of -171 cm^{-1} mol-tetramer^{-1} for $\Delta\epsilon_{tetramer}$. It is assumed that the melittin in the sample used for determining the tetrameric CD absorption coefficient is 100% in the α-helical tetramer form, and that the CD absorption coefficients do not change with the varying conditions employed in this study. If, for example, at low pH the C-terminus unfolds, then the tetrameric coefficient ($\Delta\epsilon_{tetramer}$) will give an underestimate of the amount of tetramer in solution under these conditions. Similarly, it is assumed that the structure of monomeric melittin is identical under all conditions. The tetrameric absorption coefficient ($\Delta\epsilon_{tetramer}$) corresponds to a $[\theta]$ ($[\theta] = 330 \times \Delta\epsilon$: Cantor & Schimmel, 1980) of $-14{,}800$ deg cm^{-2} dmol-melittin^{-1}, somewhat smaller than the $-15{,}700$ obtained by DeGrado et al. (1981).

Using these experimentally derived coefficients, ΔG^0 ($= -RT \ln K_{eq}$) was calculated, and Figure 6 was obtained. The values for ΔG^0 are similar to those obtained by other workers. At pH 7 in 0.16 M KCl, 0.02 M phosphate, DeGrado et al. (1981) obtained, using CD, a ΔG^0

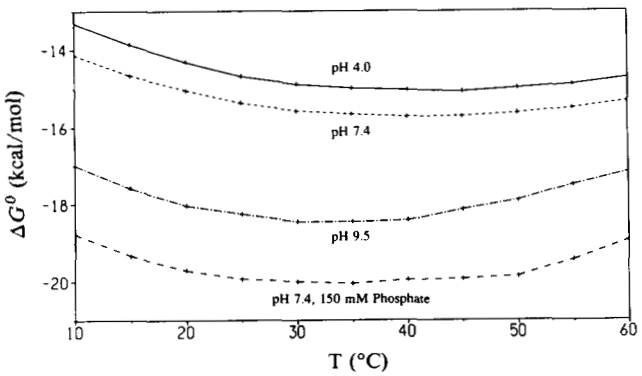

Fig. 6. Standard free energy change of melittin tetramerization (ΔG^0) versus temperature for the samples shown in Figure 5. Correlation coefficients ranged from 0.94 to 0.99 for the fit of the lines shown using Equation 8.

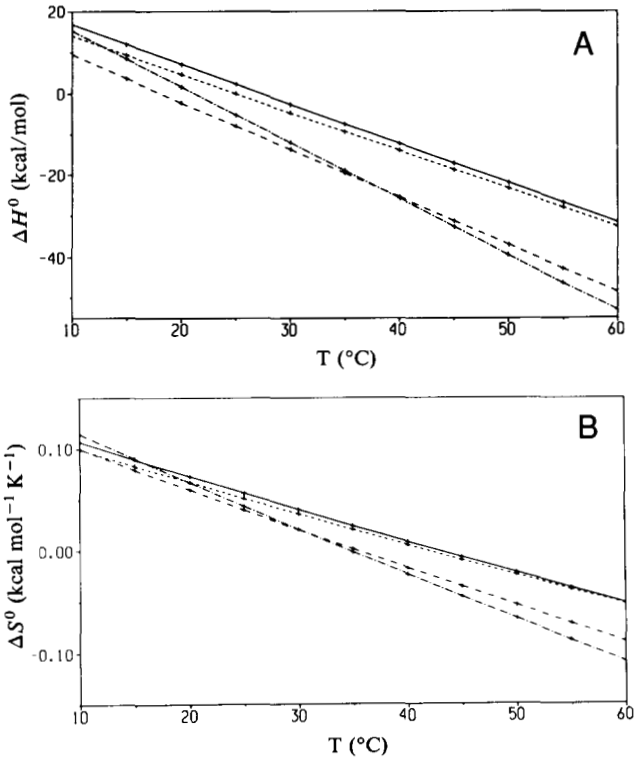

Fig. 7. Standard enthalpy and entropy changes of melittin association (assuming ΔC_p^0 is invariant with temperature). ——, pH 4.0; – – –, pH 7.4; -·-·-, pH 9.5; ———, pH 7.4 with 150 mM phosphate. **A**: ΔH^0 versus temperature calculated from Equation 5 for the samples in Figure 5. **B**: ΔS^0 versus temperature calculated from Equation 6.

of −16.1 kcal/mol for tetramer formation, compared to our value of −15.4 kcal/mol at pH 7.4, 25 °C. In the most complete study to date, Quay and Condie (1983) studied melittin association under a variety of conditions using fluorescence. At pH 7.5, 0.15 M NaCl, 0.01 M phosphate, 23 °C, they found a ΔG^0 of −17.75 kcal/mol. The ΔG^0 of folding for melittin is also comparable to the values of −11.4 (Eisenberg et al., 1986) and −18.6 kcal/mol (Ho & DeGrado, 1987) found for designed peptides that form α-helical tetramers.

The melittin tetramer is destabilized by elevating or lowering the temperature (see Fig. 6). Cold denaturation is consistent with the observations of Faucon et al. (1979) who found an increase in tetramer as the temperature was increased from −4 to 25 °C, whereas cold denaturation between 12 and 69.5 °C was not seen by Quay and Condie (1983). Cold denaturation is not unusual for proteins: it has been seen for numerous systems (for reviews see Franks, 1988; Privalov, 1990).

The melittin tetramer at pH 9.5 is stabilized over that at pH 4.0 by 3.6 kcal/mol at 25 °C, and the stabilization decreases with increasing temperature. This may be due to increased mobility of the structure at higher temperatures allowing positive charges to move away from each other and thus decrease electrostatic destabilization at low pH. Above 40 °C, some of the destabilization of melittin at pH 9.5 at due to a drop in the actual pH of the buffer (actual pH 9.0 at 40 °C) approaching the pK_a of 8.5.

Determination of ΔH^0, ΔS^0, *and* ΔC_p^0

Because ΔG^0 does not vary linearly with temperature, ΔH^0 and ΔS^0 cannot be calculated by the van't Hoft method of fitting a straight line to the curves in Figure 6. However, the curves can be approximated by straight lines between any two adjacent data points and ΔH^0 and ΔS^0 calculated in those ranges. The values of ΔH^0 and ΔS^0 obtained by this method were similar to those shown in Figure 7.

Another method for calculating thermodynamic quantities is by assuming a form for $\Delta C_p^0(T)$, the change in heat capacity between the monomeric and tetrameric states (Becktel & Schellman, 1987; Schellman, 1987). In general,

$$\Delta H^0(T) = \int_{T_h}^{T} \Delta C_p^0(T)\, dT \tag{5}$$

$$\Delta S^0(T) = \int_{T_s}^{T} \frac{\Delta C_p^0(T)}{T}\, dT \tag{6}$$

$$\Delta G^0(T) = \Delta H^0(T) - T\Delta S^0(T), \tag{7}$$

where T_h and T_s are the temperatures at which $\Delta H^0(T)$ and $\Delta S^0(T)$ are 0, respectively. Assuming that $\Delta C_p^0(T)$ for melittin tetramerization does not change in the temperature range being studied ($\Delta C_p^0[T] = \Delta C_p^0$) as is the case for most other proteins (Privalov & Potekhin, 1986),

Table 1. *Heat capacity changes, ΔC_p^0 (assumed to be invariant with temperature), for melittin tetramerization and related quantities, determined by a least-squares fit to Equation 8*[a]

Sample	ΔC_p^0		T_h, °C	T_s, °C	$T_{\Delta G=0}$, °C
	cal mol^{-1} K^{-1}	cal g^{-1} K^{-1}			
pH 4.0	−970	−0.085	27.4	43.0	−50.9, 161.9
pH 7.4	−934	−0.082	25.1	42.0	−55.3, 163.8
pH 9.5	−1,369	−0.121	21.3	34.7	−51.8, 150.9
pH 7.4, 150 mM PO_4^{-2}	−1,161	−0.102	18.2	35.5	−61.9, 159.4

[a] $T_{\Delta G=0}$, T_h, and T_s are the temperatures at which $\Delta G^0(T)$, $\Delta H^0(T)$, and $\Delta S^0(T)$ are 0, respectively.

although this is not always the case (e.g., Franks, 1988; Privalov et al., 1989), we can derive

$$\Delta G^0(T) = \Delta C_p^0(1 + \ln T_s)T - \Delta C_p^0 T \ln T - \Delta C_p^0 T_h, \quad (8)$$

from which it can be shown that $\Delta G^0(T)$ is a minimum when $T = T_s$. Equation 8 describes a U-shaped ΔG^0 versus temperature curve and may be written in the form $\Delta G^0(T) = AT + BT\ln T + C$, which is fit using least-squares methods to the experimental data in Figure 6. Table 1 shows the results along with the temperatures at which $\Delta G^0(T)$ would be 0 if the data could be extrapolated. The correlation coefficient for all fits was 0.99. The values obtained for ΔC_p^0 are similar to those found by Privalov (1979) for other small globular proteins (−0.090 to −0.155 cal g^{-1} K^{-1}). Using Equations 5 and 6, $\Delta H^0(T)$ and $\Delta S^0(T)$ can be calculated and are shown in Figure 7. Our values for $\Delta H^0(T)$ and $\Delta S^0(T)$ compare favorably with ΔH^0 of −4.85 kcal/mol and ΔS^0 of 50.4 cal K^{-1} mol^{-1} at pH 7.15, 0.05 M NaCl, 0.01 M phosphate obtained with the van't Hoft method by Quay and Condie (1983).

Interpretation of thermodynamic quantities

The variation of thermodynamic quantities with pH and temperature can be rationalized by considering the forces stabilizing and destabilizing the melittin tetramer.

Varying the pH changes both the enthalpy and entropy of folding: at 25 °C, the change in ΔG^0 between pH 9.5 and 4.0 ($\Delta[\Delta G^0]$) is −3.6 kcal/mol; $\Delta(\Delta H^0)$ is −7.5 kcal/mol, and $-T\Delta(\Delta S^0)$ is 3.8 kcal/mol. The intersection of $\Delta H^0(T)$ at two different pH values seen in Figure 7 is probably due to a combination of experimental error and pH changes in the buffer with temperature (at 15 °C the actual pH values are 7.7 and 9.7 for the pH 7.4 and 9.5 samples, respectively). Examination of this figure also reveals that $\Delta S^0(T)$ varies with pH even though electrostatic interactions are sometimes assumed to cause only enthalpic changes. An entropic change is reasonable, however, because there is altered freedom of motion in a system with electrostatic repulsions.[5] In the case of melittin, $\Delta S^0(T)$ decreases with decreasing pH implying that electrostatic repulsion inhibits the motion of the monomer more than the tetramer or there is some unraveling of the tetrameric helices at lower pH resulting in increased mobility of the backbone atoms. The entropy change apparently decreases at lower temperatures, and at 15 °C, $\Delta S^0(T)$ is approximately independent of pH (the +0.4 pH change for the pH 7.4 and 9.5 samples that occurs as temperature is decreased from 25 to 10 °C should only affect folding at pH 7.4 because of the proximity to the minor pK_a of 7.5).

How can the temperature dependence of the thermodynamic quantities be interpreted? As temperature increases, electrostatic interactions and hydrogen bonds will become weaker, yet $\Delta H^0(T)$ is found to decrease with increasing temperature (that is, greater enthalpic stability). This behavior is consistent with the hydrophobic interaction (Kauzmann, 1959) being a major force in stabilizing melittin. From data on the solubilities of hydro-

[5] *Note added in proof.* An anonymous referee suggested the following argument. The work of bringing two charges q_1 and q_2 from infinity to a distance r from one another is given by

$$\Delta A = \frac{1}{\epsilon}\frac{q_1 q_2}{r}.$$

Then

$$\Delta S = -\frac{\partial A}{\partial T} = \frac{1}{\epsilon^2}\frac{q_1 q_2}{r}\frac{\partial \epsilon}{\partial T}$$

$$\Delta E = \Delta A + T\Delta S = \Delta A\left(1 + \frac{T}{\epsilon}\frac{\partial \epsilon}{\partial T}\right).$$

ΔE is a good approximation to ΔH. Substituting the dielectric for water, $\epsilon = 233.76 + 5{,}321T^{-1} - 0.9297T + (0.1417 \times 10^{-2})T^2 - (0.8292 \times 10^{-6})T^3$ (Akerlof & Oshry, 1950) and evaluating at 25 °C, ΔH is $-0.27 \times \Delta A$, of opposite sign from ΔA. This shows that in aqueous solution electrostatic effects are entropy driven. In contrast, in apolar solvents, $\partial\epsilon/\partial T$ is small and $\Delta A \approx \Delta E$.

carbons in water, it is known that solubility increases above and below a minimum temperature ($=T_h$), which is 22 ± 5.5 °C for model aliphatic hydrocarbons (Baldwin, 1986). For aliphatic hydrocarbons, both the ΔH^0_{trans} (the unitary enthalpy of transfer from an apolar environment to water) and ΔS^0_{trans} are negative (Kauzmann, 1959). This is commonly interpreted to mean that water orders around nonpolar solutes to maximize the number of hydrogen bonds it makes causing a decrease in its entropy and, below T_h, the formation of slightly stronger hydrogen bonds (the so-called "iceberg" of Frank & Evans [1945]). As the temperature is increased the order of water is diminished (the iceberg structure is broken down), so that ΔH^0_{trans} and ΔS^0_{trans} increase. Presumably, ΔH^o_{trans} would decrease again at some temperature as water becomes more like an ideal liquid. Similarly, as the temperature is decreased and water itself becomes more ordered, ΔH^0_{trans} decreases and ΔS^0_{trans} approaches zero. From this argument, ΔH^0 for a hydrophobic interaction should decrease monotonically with temperature, just as $\Delta H^0(T)$ for melittin does. It is interesting that the T_h values calculated for melittin are in the range found for hydrocarbons (Baldwin, 1986).

The decrease of $\Delta S^0(T)$ with increasing temperature can be viewed as arising from two effects. One is the increase in conformational entropy of the monomer compared to that of the tetramer, because of its greater flexibility (Dill et al., 1989). The other is the decrease in ΔS^0 for the hydrophobic interaction with increasing temperature (T_s = 113 °C [Baldwin, 1986]).

Effect of phosphate

Phosphate has a profound stabilizing effect on melittin tetramerization and flattens out the titration curve (Fig. 4) above 7.4, suppressing the pK_a at pH 8.5 (Fig. 2). The effect of phosphate appears to be predominantly at physiologic pH, consistent with the results of Strom et al. (1980). This indicates a specific divalent effect, which is maximal between 100 to 150 mM (data not shown), because phosphate is a divalent anion only above its second pK value of 7.21 (Weast, 1983).

Phosphate is often used as a buffer in experiments without regard to its possible effects on protein stability. It is also the major physiologic divalent anion with intracellular concentrations of 75 mM and extracellular concentrations of 4 mM (Guyton, 1981). The intracellular concentration is well within the range of 5–100 mM for phosphate effects on melittin. Other examples of the effect of phosphate in proteins include the aggregation of apo-C-I, which increases with the addition of phosphate >0.5 M (Osborne et al., 1977), and the conformational change that cytochrome b_{562} (particularly in the ferrous form) undergoes when phosphate is increased from 0.01 M to 0.1 M (Myer & Bullock, 1978).

A ligand-binding analysis similar to that for NaCl above was applied to similar data on phosphate (not shown) to yield 0.14, 1.35, and 1.26 sites for the pH 4.0, 7.4, and 9.5 samples, respectively (correlation coefficients 0.78, 0.92, and 0.78). The data at pH 7.4 can almost equally well be fit with two sites (correlation coefficient 0.92 for both). The only other experimental data on phosphate binding to melittin was obtained with ^{31}P-NMR, which showed that 20–24 phosphates bind per tetramer (Podo et al., 1982).

Possible binding sites for phosphate on the melittin tetramer may be proposed by comparing the crystal structure with the near UV data in Figure 4. The refined crystal structure (Gribskov et al., in prep.) shows that eight sulfates bind to the tetramer (Kinemage 3). Four of these (two at each end of the tetramer) bridge the Arg 24 guanidinium nitrogen of one monomer to the N-terminus of the adjacent monomer. Another four sulfates bridge the nitrogen of the Trp 19 indole ring and the epsilon amino group of Lys 23 of one monomer to the guanidinium nitrogen of Arg 24 and C-terminal amide nitrogen of an adjacent monomer. Changes in the near UV CD upon binding anions must be caused by the second set of four sulfates described above, because their location is near the tryptophans. In fact, phosphate causes a change in the near UV at pH 7.4 that falls off above pH 8.5 (see Fig. 4), suggesting its site of action is at Lys 23, consistent with the structure of melittin in sulfate.

The specific effect of sulfate or phosphate in their divalent forms is probably due to bridging electrostatic interactions with two or more positively charged atoms on adjacent monomers and screening the electrostatic repulsion of those charges. A similar effect with divalent cations (and negative charges) has been noted in other protein systems (Voordouw et al., 1976; Filimonov et al., 1978; Bashford et al., 1986; Linse et al., 1988; Pantoliano et al., 1988). The effect of phosphate above its second pK_a is expected to be essentially the same as sulfate.

Screening of these positive charges can account for the stabilization of the tetramer by phosphate. Calculation of the change in the electrostatic energy (with the dielectric of Hill [1956] and an ionic strength of 0.15 M) by removing the charges on all N-termini, Lys 23, and Arg 24 residues with all other groups fully charged (the expected effect of binding eight phosphates) suggests an increased stabilization of 8.3 kcal/mol. This value correlates well with the experimental enthalpy change of −8.0 kcal/mol at 25 °C. The experimental free energy change of −4.6 kcal/mol is smaller than the enthalpy change because of a large experimental entropy change ($-T\Delta S^0$ = 3.4 kcal/mol at 25 °C) associated with the binding of phosphate (Fig. 7), probably due to immobilization of the phosphate anions and the charged side chains they bind. Similar large negative entropy and enthalpy changes have been observed upon addition of Ca^{2+} to calcium-binding proteins (Voordouw et al., 1976).

Conclusions

Our principal conclusions on the energetics of the melittin tetramer are the following:

1. Electrostatic forces: The melittin tetramer exhibits pK_a values of about 7.5 and 8.5, the former corresponding to titration of the N-terminus, and the latter to the titration of Lys 23, and probably also in part Lys 7 and Lys 21. The pK_a values calculated with the program DelPhi (Gilson et al., 1987; Gilson & Honig, 1988a,b) agree with experimentals. The melittin tetramer at pH 9.5 is stabilized by 3.6 kcal/mol (24% of the stability of the tetramer at pH 4.0) over that at pH 4.0, because of decreased electrostatic repulsions at higher pH values. Electrostatic interactions have both enthalpic and entropic contributions to the stabilization of the melittin tetramer.
2. Salt effects on stability: The melittin tetramer is stabilized at acidic pH (see Fig. 3) as the ionic strength is increased from 0 to 0.5 M by about 5–6 kcal/mol because of dielectric screening of its interchain electrostatic repulsions. This dependence of stability on ionic strength is well approximated by a ligand-binding model at pH values less than 8.5.
3. Temperature effects on stability: The melittin tetramer is destabilized by increasing or decreasing the temperature from a temperature of maximum stability ranging from 35.5 to 43 °C, depending on the pH. Cold denaturation may be viewed as a consequence of a constant ΔC_p^0 of folding, which is typical of proteins (Privalov & Potekhin, 1986). ΔC_p^0 estimated for melittin tetramerization ranges from -0.085 to -0.102 cal g^{-1} K^{-1} comparable to that of other small globular proteins (Privalov, 1979). $\Delta H^0(T)$ and $\Delta S^0(T)$ decrease with increasing temperature largely due to the properties of the hydrophobic interaction, a major stabilizing force in the melittin tetramer. The T_h for melittin is comparable to that for the transfer of hydrocarbons to water.
4. Effect of phosphate: Phosphate in physiologic concentrations greatly stabilizes the melittin tetramer by binding to positive charges on the tetramer. The stabilization is maximal at 150 mM phosphate, amounting to 4.6 kcal/mol at pH 7.4, 25 °C. Dielectric screening of positive charges on melittin by bound phosphate anions can account for the experimental enthalpy change, the entropy change being secondary to immobilization of the amino acid side chains and phosphate anions.

Materials and methods

Lyophilized bee venom (grade IV) was purchased from Sigma (approximately 50% melittin by weight). All solvents used were HPLC grade (Fischer). All chemicals used were reagent grade or better.

Purification of melittin

One gram of bee venom and 50 g of sucrose were dissolved in 3 mL of 50 mM sodium formate buffer, pH 4.0. After vortexing and allowing stingers and other insoluble matter to settle, the solution was syringe-filtered through a Millex-GV 0.22-μm Millipore filter. A small amount of buffer (~10 drops) was then used to wash out the bee venom bottle and likewise filtered through the syringe. Approximately 4 mL of amber liquid was obtained and loaded onto a tandem gel filtration column (Shepherd et al., 1974) consisting of a descending Sephadex G-50 (20–80 μm) and an ascending G-75 (40–120 μm) column each packed in 2.5 × 120-cm columns and flushed with buffer. The flow rate was 0.66 mL/min controlled by a Pharmacia FPLC; 6.5-ml fractions were collected from 22.5 to 25 h during the major 280 nm absorbance peak. These fractions were pooled to a total volume of 105 mL and raised to a volume of 155 mL with water. The absorbance at 280 nm (A_{280}) of this solution diluted 1:10 was 0.484. Ammonium sulfate fractionation (Anderson et al., 1980) was then performed by slowly adding 80 g of solid ammonium sulfate with stirring until an amber cloudy solution was obtained. This mixture was centrifuged at 27,000 × g for 30 min, and the supernatant was saturated with ammonium sulfate and allowed to stand for at least 12 h. This solution was likewise centrifuged for 30 min, and the white pellets obtained were resuspended in about 10 mL of water to yield a slightly yellow liquid. This was desalted in four fractions on a 1.7 × 69-cm Sephadex G-10 column with a water eluant at a 1.5-mL/min flow rate; 1.5-mL fractions were obtained and analyzed for conductivity and A_{280}. Fractions with the highest A_{280} and lowest conductivity were pooled to a total of 54 mL, which was then lyophilized to dryness. The white powder was redissolved in 3 mL of water to obtain a yellow solution, which was again desalted in four aliquots as above to obtain 39 mL that were again lyophilized to dryness, obtaining 260.8 mg of white powder for a yield of 52%. Reverse-phase analysis on a Pharmacia PepRPC HR 5/5 column revealed a single peak. N-terminal sequencing showed that the material is pure melittin with the N-terminus unformylated.

Concentrations of melittin were calculated using a monomeric molecular mass of 2,840 g/mol and an extinction coefficient of 5,570 cm^{-1} M^{-1} (that of the single tryptophan [Sober, 1970]). The extinction coefficient of melittin was not found to change significantly with changes in pH or ionic strength. Tris (20 mM) was used as a buffer in all melittin solutions, and the pH was adjusted with formic acid and NaOH. Titration experiments were carried out by adding small amounts of NaOH with a micropipette to a buffered solution of melittin. The con-

centration of melittin changed by no more than 0.03 mM because of dilution in these titrations. Unless otherwise specified, all melittin solutions contained 150 mM NaCl. Solutions containing $NaHPO_4$ had the amount of added NaCl reduced to maintain a total [Na^+] of 150 mM.

The variation of pH with temperature was measured for several of the buffers used in this work with a Ross pH electrode (Orion Research Inc.) attached to a Beckman pHI 45 pH meter with automatic temperature compensator. The pH was found to decrease linearly with increasing temperature from 10 to 60 °C with slopes 0.0, −0.023, and −0.024 pH units/°C for the pH 4.0, 7.4, and 9.5 (at 25 °C) buffers with 20 mM Tris, 150 mM NaCl; and −0.0032 pH units/°C for the pH 7.4 with 150 mM $NaHPO_4$, 20 mM Tris buffer. The pH values reported subsequently refer to values measured at 25 °C.

Circular dichroism

All measurements were obtained on a modified Beckman CD spectrophotometer as previously described (Horwitz et al., 1979) in the laboratory of Joseph Horwitz (Jules Stein Eye Institute, UCLA) equipped with a Beckman T_m analyzer (model 139570W), platinum temperature probe and bridge, and a water-cooled, heated cell holder. The temperature of the experimental cell was obtained by measuring the temperature of an adjacent cell containing water. The temperature was maintained ±0.5 °C for each measurement. Near UV (330–245 nm) measurements were obtained in a 1-cm quartz cell, and far UV (260–185 nm) data were obtained with a 0.2-mm cell composed of a 0.2-mm-thick teflon spacer interposed between two flat quartz plates. To prevent evaporation during temperature experiments, the 1-cm cell was covered with a teflon cap, and the edges of the far UV cell were coated with Dow #4 vacuum grease and covered with teflon spacers. This effectively stopped leakage and evaporation from the cell. That melittin does not bind to the grease is supported by unchanged A_{280} and CD spectra after heating to 60 °C and cooling again to 25 °C (data not shown). All measurements were standardized with a 0.2147-mg/mL camphor-sulfonic acid solution between 340 and 255 nm. CD spectra of indole acetic acid solutions of the same A_{280} to the melittin solutions were obtained at 25 °C to estimate the baseline. These spectra do not vary with temperature from 0 to 60 °C. Circular dichroism is reported as $\Delta\epsilon$ referred to a 1-cm cell. To improve the signal-to-noise ratio, near UV spectra were composed of an average of 16 spectra, whereas 4 were averaged for the far UV. For most experiments, far UV was employed because of a better signal-to-noise ratio.

DelPhi electrostatic calculations

Electrostatic calculations were performed on the melittin refined crystal structure (Gribskov et al., in prep.) with the DelPhi version 2.1 (Gilson et al., 1987; Gilson and Honig, 1988a,b) module of the Insight II package (Biosym Technologies) on a Silicon Graphics IRIS workstation. Atomic charges were from the Amber partial charge set, the solvent dielectric was 80 with an ionic strength of 0.15 M, the radius of the Stern (ion exclusion) layer was varied from 0 to 3 Å, the internal dielectric was varied from 2 to 4, and boundary conditions were calculated using the focusing option (the whole molecule focused to an 8-Å radius from the ionizable atom of interest). Values reported for ΔpK_a are averages of the corresponding values for the four chains of the tetramer.

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