Conformational Studies of Aqueous Melittin: Thermodynamic Parameters of the Monomer–Tetramer Self-Association Reaction†

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ABSTRACT: The self-association reaction in which four melittin molecules associate to form an aqueously soluble tetramer was studied by fluorescent spectroscopy. At 23 °C, pH 7.15, $\Gamma/2$ 0.50, the dissociation constant, K_d, is 3.20×10^{-16} M^3. At 23 °C, $\Gamma/2$ 0.60, melittin has an amino acyl group with a proton ionization constant at ca. 10^{-6} M, which must be un-ionized for tetramer formation to occur. The change in K_d with temperature indicates the forward reaction (tetramer formation) proceeds primarily by entropic changes, with $\Delta H°$ = −20.3 kJ/mol of monomer and $\Delta S°$ = 211 J/(K·mol of monomer). The observed enthalpic and entropic values for the tetramerization reaction are consistent with the expected contributions of both nascent hydrogen bonds and hydrophobic stabilization to the reaction. The ionic strength dependence of the tetramerization reaction was found to be consistent with an Edsall–Wyman treatment of activity coefficients. Specifically, the calculated charge of melittin varied from 2.5 (pH 10.53, $\Gamma/2$ <0.08) to ca. 6 (pH 7.15, $\Gamma/2$ >0.3) and showed a strong dependence on $\Gamma/2$.

Melittin is the major component of the venom of *Apis mellifera* (honey bee). The pharmacological properties of melittin, especially its neurotoxicity, have been the subject of numerous studies [see review in Habermann (1972)], which indicate that its ability to disrupt membrane function by its strong membrane binding is an important part of its action. The recent observation that melittin can produce voltage-dependent anion conductance in bilayer membranes (Tosteson & Tosteson, 1981) is intriguing and may be more specifically related to the mechanism of its neurotoxicity.

The hexacosapeptide melittin contains four clustered basic amino acids and an N-terminal sequence that appears to be an amphipathic helix (Habermann & Jeutsch, 1967; DeGrado et al., 1981). The amino acid sequence of melittin is largely

^+H_3N-Gly-Ile-Gly-Ala-Val_5-Leu-Lys-Val-Leu-Thr_{10}-
Thr-Gly-Leu-Pro-Ala_{15}-Leu-Ile-Ser-Trp-Ile_{20}-
Lys-Arg-Lys-Arg-Gln_{25}-Gln-$CONH_2$

melittin

homologous to sequences determined for leader peptides of eukaryotic proteins (Garnier et al., 1980), and the possibility that melittin evolved from such a primordial role has been suggested (Knoppel et al., 1979). Melittin is soluble as a tetramer in water to over 60 mM (Knoppel et al., 1979) and yet rapidly forms lipid–peptide complexes at extremely low melittin concentrations (<1 μM). It also undergoes a hitherto partially characterized dissociation from the tetramer structure to water-soluble monomers (Talbot et al., 1979). All of these observations suggest major conformational diversity in melittin structure: diversity that responds to environmental factors.

Our laboratory is interested in determining the conformationally important features of each of the four major structures of melittin: monomeric, water-soluble melittin; the aqueous tetramer; the membrane-bound conformation; and the voltage-dependent, anion channel state. Before beginning studies of melittin–lipid interactions, we have examined the conformation of aqueous melittin by a number of techniques. Our work is complementary to the ongoing X-ray diffraction studies of Eisenberg and colleagues on the conformation of melittin in aqueously grown crystals (Terwilliger et al., 1982). This first paper is concerned with determining the thermodynamic parameters of the self-association reaction of melittin in aqueous solution.

Experimental Procedures

Materials. Melittin and *N*-acetyl-L-tryptophanamide (NATA) were purchased from Sigma Chemical Co., St. Louis, MO. Sephadex G-25 (medium) was purchased from Pharmacia Fine Chemicals, Piscataway, NJ. All other materials were the highest grade commercially available.

Methods. Melittin was purified by dissolving it in 0.02 M sodium phosphate, pH 7.20, at ca. 10 mg/mL and passing it down a Sephadex G-25 column equilibrated with the same buffer at room temperature. The absorbance at 280 nm was monitored, and the major peak fractions, eluting at K_d = 0.25–0.35, were pooled. The brown, crude melittin was rendered a white powder upon lyophilization. The purified melittin was a single spot (R_f 0.54) by thin-layer chromatography [TLC; silica gel GHLF, 250 μm; 1-butanol–acetic acid–pyridine–water (15:3:9:12 v/v)]. The purified melittin was subjected to amino acid analysis (Spackman et al., 1958) on a Beckman amino acid analyzer. The results were as follows [observed (expected)]: Lys, 3.14 (3); Arg, 2.09 (2); Asx <0.03 (0); Thr, 2.08 (2); Ser, 1.0 (1); Glx, 0.93 (2); Pro, 1.12 (1); Gly, 3.32 (3); Ala, 1.46 (2); Val, 1.78 (2); Ile, 2.58 (3); Leu, 3.37 (3); His, Cys, Met, Tyr, Phe, <0.01 (0). In addition, a phospholipase activity (as judged by generation from egg lecithin of free fatty acid on TLC; Haverkate & Van Deenen, 1965) present in crude melittin was not detected in purified melittin. The extinction coefficient of purified melittin is 5570 M^{-1} cm^{-1} at 280 nm.

Ultraviolet Spectroscopy. Absorption spectra were recorded with a Hitachi 110 spectrometer.

Fluorescence Spectroscopy. Fluorescence measurements were made with a Perkin-Elmer Model MPF-44B fluorescence spectrometer. Temperature-dependent studies were made on a similar instrument equipped with a constant temperature bath and appropriate cell holder (generously made available

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by R. Simoni, Stanford University). Purified melittin was dissolved in an appropriate buffer and the concentration determined by measuring the absorbance at 280 nm in a 1-cm quartz fluorescence cell. The fluorescence emission spectra were recorded from 300 to 400 nm with the excitation wavelength set at 280 nm. The excitation and emission bandwidths varied from 2 to 8 nm; the minimum setting that gave a satisfactory spectrum was used. The peak emission wavelength was assigned as the average of the wavelengths at half-maximum fluorescence. The λ_{max} determined in this way was insensitive to the small changes in bandwidth. The concentration of melittin was varied by diluting the melittin solution with the appropriate buffer equilibrated at the experimental temperature or by making small additions of melittin (5–25 μL) to the cuvette. Typically, experiments were made by the dilution method from the highest $[melittin]_{total}$ to one-third of this value and by the addition technique from the lowest $[melittin]_{total}$ to 3 times this value. Thus, data from the two techniques overlapped with respect to $[melittin]_{total}$. Experiments could not be performed above 10^{-2} M melittin due to inner filter effects.

The fluorescence quantum yields (ϕ_R) of monomeric and tetrameric melittin were determined relative to NATA by using $\phi_R = [F_A(melittin)/A_{280}(melittin)][A_{280}(NATA)/F_A(NATA)]$, where F_A is the area under the fluorescence emission spectrum and A_{280} is the absorbance at the exciting wavelength. Since the spectral distribution of fluorescence did not differ significantly between melittin and the NATA reference, the emission spectra were not corrected for instrumental response. The A_{280} values were always kept below 0.05 to obviate inner filter corrections, and both melittin and NATA A_{280} values were adjusted to near equality.

Measurements of pH were made with a Beckman Century SS-1 pH meter that was standardized with phosphate NBS primary standard solutions at 23 °C (Bates, 1964). The electrode response was corrected for temperature. The observed pH was corrected for ionic strength by using $[H^+] = 10^{-pH}/a_{H^+}$, where a_{H^+} is the hydrogen ion activity coefficient at the appropriate ionic strength (Weast, 1970).

Buffers were prepared by mixing a solution of 0.01 M acidic component with a 0.01 M solution of the basic form of the buffer until the desired pH was obtained. The buffers used were as follows: $NaHPO_4$–Na_2PO_4, pH 6.00–8.09; $Na_2H_2P_2O_7$–$Na_3HP_2O_7$, pH 5.36–6.50; $Na_3HP_2O_7$–$Na_4P_2O_7$, pH 7.5–9.00; $NaHCO_3$–Na_2CO_3, pH 9.50–10.53. The ionic strength was varied with NaCl and was corrected to molality, m, by using $c = m\rho/(1 + mM)$, where c = [NaCl], ρ is solution density, and M is the molar mass of the solute (58.45 g). The densities of NaCl solutions were taken from the *Handbook of Chemistry and Physics* (Weast, 1970). Ionic strength was determined from the calculated molality and the activity coefficients for various [NaCl] (Weast, 1970). The experiments at $\Gamma/2 \leq 0.01$ were done with 0.001 M buffers ($\Gamma/2$ 0.003–0.006) and purified melittin (initially in NaCl) dialyzed exhaustively against glass-distilled water ($\Gamma/2$ for an ideal, molal solution of melittin chloride is calculated to be 10.6–4.4 from pH 7.15 to 10.53).

Results

The intrinsic fluorescence of the single tryptophan of melittin has been used to define some experimental conditions under which melittin protomers self-associate to form tetramers (Talbot et al., 1979). These authors have shown that high [NaCl] or $[melittin]_{total}$ shifted the λ_{max} for fluorescence emission from 353 to 337 nm. This shift in λ_{max} correlated with changes in the apparent molecular weight of melittin (as measured by gel filtration chromatography) from ca. 3500 to 12000. We have extended the use of this technique to obtain detailed thermodynamic parameters for this reaction. Under our experimental conditions, the λ_{max} of melittin at low [NaCl] and $[melittin]_{total}$ is 353.7 ± 0.2 nm (n = 27). At 2.25 M NaCl and at lower salt concentrations when melittin concentrations are very high, the λ_{max} is 342.5 ± 0.4 nm (n = 37). Our value for protomers agrees well with the value determined by Talbot et al. (1979) although our λ_{max} for tetramers is ca. 5.5 nm higher than theirs. The reason for this difference is not known but may be related to differences in the buffers used [Talbot et al. (1979) used Tris-HCl[1] while we generally used phosphate]. The presence of phosphate anions has been shown to change the helical content (Drake & Hider, 1979), lysine chemical reactivity (S. C. Quay and L. P. Tronson, unpublished experiments), and tryptophan exposure of melittin to solvent (unpublished data). Similar experiments (unpublished data) on the gel filtration chromatography of melittin under the different experimental conditions of [NaCl] confirm the monomer and tetramer molecular weights as determined by Talbot et al. (1979).

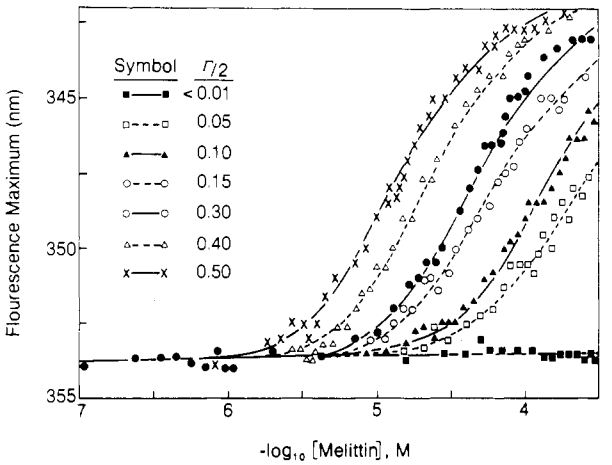


FIGURE 1: Effect of increasing $[melittin]_{total}$ on λ_{max} of fluorescence emission at 23 °C, pH 7.15. The λ_{max} of Trp-19 fluorescence emission was measured by the half-height method and plotted at the indicated $-\log_{10}[melittin]_{total}$ at constant $\Gamma/2$ from <0.01 to 0.50. The solid and dotted lines were drawn from eq 2 and 3 and the data in Table I.

At various constant ionic strengths from ≤0.01 to 0.50 the λ_{max} varied in a regular fashion with increasing $[melittin]_{total}$ (Figure 1). The data indicate that the equilibrium between monomers and tetramers is shifted toward monomers as $\Gamma/2$ decreases until, at $\Gamma/2 \leq 0.01$, melittin is totally monomeric to at least a concentration of 3×10^{-4} M.

If the self-association reaction is represented by eq 1, then

$$4melittin_{mon} \overset{K_d}{\rightleftharpoons} melittin_{tet} \quad (1)$$

$K_d = [melittin]_{mon}^4/[melittin]_{tet}$. Since $[melittin]_{total} = [melittin]_{mon} + [melittin]_{tet}$, the equilibrium constant, K_d, can be expressed as a function of $[melittin]_{mon}$ and $[melittin]_{total}$ by eq 2. If the observed fluorescence emission maximum (λ_o)

$$[melittin]_{mon} + [melittin]_{mon}^4/K_d = [melittin]_{total} \quad (2)$$

represents that expected from taking the mean of the con-

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; $melittin_{mon}$, monomeric melittin; $melittin_{tet}$, tetrameric melittin; MH_{2mon}, equilibrium concentrations of monomeric melittin species differing in ionization states; MH_{tet} and M_{tet}, equilibrium concentrations of tetrameric melittin species differing in ionization states.

Table I: Equilibrium Constants for the Reversible Melittin Monomer–Tetramer Self-Assocation in Water

$$4\text{melittin}_{mon} \underset{}{\overset{K_d}{\rightleftharpoons}} \text{melittin}_{tet}$$

pH	T (°C)	$\Gamma/2$	K_d (M^3)[a]
7.15	23	<0.01	$\geq 2.5 \times 10^{-11}$
7.15	23	0.05	2.40×10^{-12}
7.15	23	0.10	5.01×10^{-13}
7.15	23	0.15	7.96×10^{-14}
7.15	23	0.30	1.40×10^{-14}
7.15	23	0.40	1.80×10^{-15}
7.15	23	0.50	3.20×10^{-16}
7.15	23	0.60	8.91×10^{-17}
7.15	12	0.05	1.55×10^{-12}
7.15	37	0.05	3.24×10^{-12}
7.15	51	0.05	4.68×10^{-12}
7.15	69.5	0.05	1.10×10^{-11}
5.36	23	0.60	2.52×10^{-14}
5.70	23	0.60	2.00×10^{-15}
6.28	23	0.60	3.55×10^{-16}
7.15	23	0.60	7.08×10^{-17}
8.09	23	0.60	5.00×10^{-17}
9.12	23	0.60	3.98×10^{-17}
7.10	23	0.02	3.39×10^{-12}
8.09	23	0.02	4.47×10^{-15}
10.53	23	0.60	4.00×10^{-17}
10.53	23	0.02	7.94×10^{-16}
10.53	23	0.08	6.30×10^{-16}
10.53	23	0.23	2.53×10^{-16}
10.53	23	0.50	7.95×10^{-17}
10.53	23	0.85	4.13×10^{-17}

[a] $K_d = [\text{melittin}]_{mon}^4/[\text{melittin}]_{tet}$.

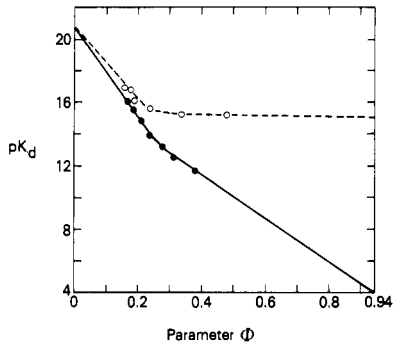


FIGURE 2: Primary salt effect for melittin tetramerization. The data are taken from Figure 1 and Table I. The parameter Φ was calculated from eq 23. The slope of the lines at pH 7.15 (●) and pH 10.53 (○) is equal to $-z_m^2$, the square of the charge of a melittin monomer. The intersection of the plotted line with the left vertical axis, where Φ equals zero, yields the dissociation constant if the protein were uncharged ($\Gamma/2 \infty$). The intersection of the right vertical axis with the plotted line [$\Phi = 0.94$ and $\kappa/(1 + \kappa a) = 0$] yields the K_d at an ionic strength of zero where none of the charge of the protein would be neutralized.

tributions of monomeric and tetrameric melittin (see Appendix I for the justification of this procedure), then eq 3 can be used

$$\lambda_o = \lambda_m \frac{[\text{melittin}]_{mon}}{[\text{melittin}]_{total}} + \lambda_t \frac{[\text{melittin}]_{mon}^4}{K_d[\text{melittin}]_{total}} \quad (3)$$

to relate λ_o to K_d and $[\text{melittin}]_{total}$. The experimental data, i.e., λ_o, λ_m, and λ_t, and $[\text{melittin}]_{total}$ were used to determine the best estimate of $[\text{melittin}]_{mon}$ and K_d by trial and error computation. The solid and dotted lines in Figure 1 are theoretical curves using the values from Table I in eq 2 and 3.

Returning to the primary salt effect of melittin tetramerization, the decrease in K_d as the ionic strength is increased is consistent with the expectedly unfavorable electrostatic interaction that results from bringing four cationic peptides together in the tetramer. To analyze the effect of ionic strength on fluorometrically determined K_d values and to calculate the experimentally determined charge of a melittin monomer (z_m), we related the measured K_d to the thermodynamic K_d, K_d^0, and z_m by eq 4, where $pK_d = -\log_{10} K_d$ (the

$$pK_d = pK_d^0 - z_m^2\Phi \quad (4)$$

derivation of Φ is given in Appendix II). A plot of pK_d vs. Φ will have a slope of $-z_m^2$ (Figure 2). At pH 7.15, the data at $\Gamma/2$ <0.15 indicate a charge of 3.9 while at $\Gamma/2$ >0.15 z_m approaches 6. At pH 10.53, z_m is 2.5 below $\Gamma/2$ 0.23 and becomes 3.7 at higher $\Gamma/2$. These values of z_m are in reasonable agreement with the expected z_m for a peptide with six ionizable basic aminoacyl residues, four of which are clustered in the carboxy-terminal portion of melittin.[2]

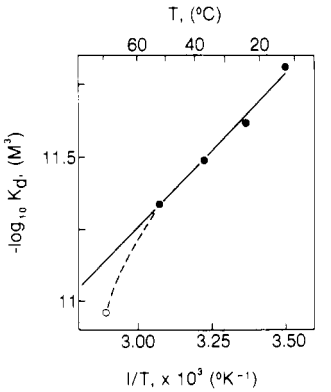


FIGURE 3: Relationship between $-\log K_d$ for tetramer formation and (thermodynamic temperature)$^{-1}$ at pH 7.15, $\Gamma/2$ 0.05. The solid line is the interpolated and extrapolated relationship from eq 5–7 with $\Delta H° = -20.3$ kJ/mol and $\Delta S° = 211$ J/(K·mol).

By varying the temperature and measuring the K_d for melittin self-association, we were able to determine the enthalpic and entropic contribution to the reaction using eq 5–7.

$$\Delta G° = -2.303RT \log_{10} K_d \quad (5)$$

$$\Delta G° = \Delta H° - T\Delta S° \quad (6)$$

$$-\log_{10} K_d = (\Delta H°/2.303R)(1/T) - \Delta S°/2.303R \quad (7)$$

Figure 3 shows a plot of $-\log_{10} K_d$ vs. $1/T$ (K), which is linear from 12 to 51 °C. The value at 69.5 °C is lower than that expected from extrapolation of the data at lower temperatures, suggesting some other process, perhaps denaturation, occurs at 69.5 °C. The data from 12 to 51 °C (pH 7.15, $\Gamma/2$ 0.05) indicate $\Delta H° = -20.3$ kJ/mol and $\Delta S° = 211$ J/(K·mol) for the forward reaction, i.e., tetramer formation.

Finally, the effect of changes in the hydrogen ion concentration on the self-association reaction was measured to determine if the ionization state of melittin changed with pH in a manner important for association and to determine the

[2] The ionizable residues of melittin that determine its charge are the N-terminal glycine, three lysine residues, and two arginine residues. Lys-21 and -23 have perturbed pK values of 6.5 and 8.6 (23 °C, $\Gamma/2$ 0.05), as determined by 2,4,6-trinitrobenzenesulfonate reactivity (Quay & Tronson, 1983). The pK values of Lys-7 and Gly-1 have not been directly determined but would be expected to also be somewhat less than their unperturbed values of 10.5 and 8.0 in this positively charged protein. Thus at pH 7.15 the charge is predicted to be somewhat less than 5.0. At pH 10.53 these same considerations suggest a charge of less than 2.5. As the ionic strength increases the perturbation in pK values would become less due to electrostatic neutralization by solute anions and thus z_m would be expected to increase.

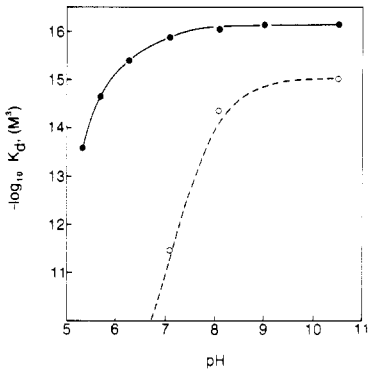


FIGURE 4: Relationship between $-\log K_d$ and pH at 23 °C, $\Gamma/2$ 0.60 (●) and 0.02 (○). The solid and dotted lines were drawn from eq 9 and $\Gamma/2$ 0.60, $K_1 = 1 \times 10^{-6}$ M, $K_3 = 8 \times 10^{-17}$ M^3, and $K_4 \geq 2 \times 10^{-19}$ M^4; at $\Gamma/2$ 0.02, $K_1 = 1 \times 10^{-8}$ M, $K_3 = 1 \times 10^{-15}$ M^3, and $K_4 \geq 1 \times 10^{-25}$ M^4.

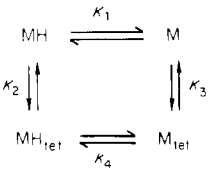
magnitude of this effect. The K_d for melittin association at 23 °C was measured at $\Gamma/2$ 0.02 and 0.60 over the pH range 5.36–10.53 (Figure 4). The data indicate that at $\Gamma/2$ 0.60, pH values below ca. 7 lead to a lower pK_d while at $\Gamma/2$ 0.02 this effect begins at a higher pH (ca. 8.5).

Discussion

In this paper, we have determined the dissociation constant for the aqueous self-association reaction of melittin under conditions of varied pH, ionic strength, and temperature. This study was performed by measuring the changes in fluorescence emission λ_{max} and relating these to changes in the concentrations of melittin monomer and tetramer. The validity of this treatment is examined and supported in Appendix I. For summarization of this examination, the experimental observation that the portions of the emission spectra between the wavelengths where emission is half-maximum for both monomeric and tetrameric melittin are well approximated by parabolic functions of emission intensity and wavelength makes the λ_{max} a simple function of $[\text{melittin}]_{mon}$ and $[\text{melittin}]_{tet}$ (specifically, eq 3).

The exact λ_{max} values for monomeric and tetrameric melittin are 353.7 and 342.5 nm, respectively. Since the λ_{max} for tryptophan emission is sensitive to environmental changes (Teale, 1960), it is interesting to consider the values for melittin in this context. Burnstein et al. (1973) have classified the emission maximum of tryptophan fluorescence and concluded that fully solvent-exposed tryptophans (class I) have an emission λ_{max} near 352 nm, "surface" tryptophans (class II) near 341 nm, and "buried" tryptophans (class III) at 331 nm. By these criteria, one would conclude that the tryptophan in monomeric melittin is fully solvent exposed (class I) while in the tetrameric form the tryptophans are less accessible to solvent but still on the tetramer surface (class II). The small size of the melittin tetramer makes this perfectly reasonable; i.e., there would be very few tetramer conformations that would permit a buried position for the four tryptophans. In related studies (S. C. Quay, C. C. Condie, and K. Minton, unpublished work), we have shown by collisonal quenching of tryptophan fluorescence that the tryptophan of monomeric melittin is fully solvent exposed while the tetramer fluorescence is quenched 0.39 times as well. Interestingly, melittin bound to lipid vesicles has a fluorescence emission spectrum consistent with class III tryptophans (Dufourcq & Faucon, 1977).

The pH dependence of the tetramerization reaction can be used to estimate approximate protonic ionization constants that are important for tetramerization. The data in Figure 4 indicate that the formation of tetramers is hindered at acid pH values and that this effect is sensitive to ionic strength. The simplest mechanism to explain these data is eq 8.[3] For eq



(8)

8, the variation of the observed K_d with pH is eq 9. The solid

$$K_d = K_3 \frac{(1 + [H^+]/K_1)^4}{1 + [H^+]^4/K_4} \quad (9)$$

line in Figure 4 represents values obtained from eq 9 with $K_1 = 1 \times 10^{-6}$ M, $K_3 = 8 \times 10^{-17}$ M^3, and $K_4 \geq 2 \times 10^{-19}$ M^4. The lower limit for K_4 was obtained by imagining that the K_d value at pH 5.36 is at the inflection point of the curve in Figure 4 [i.e., where $\partial^2(-\log K_d)/\partial(\text{pH})^2 = 0$] and from the identity, $K_1^4 K_2 = K_3 K_4$. Since at pH 5.36 $[H^+]^4 = 3.6 \times 10^{-22}$ M^4, $1 \gg [H^+]^4/K_4$ and precludes an accurate determination of K_4. At $\Gamma/2$ 0.02, the dotted line in Figure 4 is obtained from eq 9 by using $K_1 = 1 \times 10^{-8}$ M, $K_3 = 1 \times 10^{-15}$ M^3, and $K_4 \geq 1 \times 10^{-25}$ M^4. Since one would have expected K_1 to increase as $\Gamma/2$ was lowered (due to electrostatic shielding), our finding that K_1 decreased was somewhat unexpected and is not completely understood at this point. One explanation might be that *different* titratable groups affect tetramer formation at different ionic strengths. Elsewhere (Quay & Tronson, 1983), we present evidence that Lys-21 and -23 in monomeric melittin have ionization constants of $10^{-6.5}$ and $10^{-8.6}$, respectively.

The ionic strength dependence of the dissociation constant can be used to estimate the effective charge of a melittin monomer in the self-association reaction (Appendix II). The results, obtained from analysis of the slope of the data in Figure 2, show that only at the highest $\Gamma/2$ does the calculated charge approach the expected maximum value of 6, supporting the earlier suggestion that the ionization constants for melittin aminoacyl residues are perturbed downward to minimize the total charge of the protein. This is especially apparent at low $\Gamma/2$. From the data in Figure 2, the value of K_d where Φ is zero ($\Gamma/2$ ∞) could be as small as 10^{-22} M^3. On the other hand, the K_d at an ionic strength of zero, where none of the charge of the protein would be neutralized, would be 10^{-4} M^3 at pH 7.15 and 10^{-15} M^3 at pH 10.53. Thus, the concentration range of melittin in which the monomer–tetramer association reaction occurs can change by as much as 10^6 M with changes in ionic strength and by over 10^3 M with changes in the charge on melittin (brought about by pH changes). These considerations underscore the important role that electrostatic interactions play in melittin self-association.

The temperature dependence of the association reaction indicates a $\Delta H° = -20.3$ kJ/mol and a $\Delta S° = 211$ J/(K·mol) at pH 7.15, $\Gamma/2$ 0.05. These values suggest that the major energetic contribution to tetramer formation is the entropic energy change. This is consistent with previous work, which indicated hydrophobic interactions are important for stabilization of melittin in the tetramer form (Bello et al., 1982; DeGrado et al., 1981). In addition, since monomeric melittin is largely present as a random coil in solution, while tetrameric

[3] We use the symbols ⇄ for reactions occurring at measurable rates and ⇌ for reactions in which a rapidly obtained equilibrium is assumed to exist (King, 1964). Reaction schemes contain several simplifying features: omission of hydrogen ion in ionization reactions and omission of the charge on melittin species.

melittin has an α-helical conformation (Terwilliger et al., 1982), hydrogen-bond formation accompanying self-association should also contribute to the measured enthalpy and entropy of the reaction. These considerations of the thermodynamics of melittin tetramerization are similar to those of Formisano et al. (1977) for the formation of glucagon trimers. Specifically, the formation of glucagon trimers also involves both new hydrogen-bond formation and hydrophobic stabilization.

Acknowledgments

We gratefully acknowledge useful discussions with Dr. J. Shafer of the University of Michigan concerning the electrostatic interactions of melittin self-association.

Appendix

(I) Determination of Dissociation Constants from Fluorescence Emission Maximum. Assume that a portion of the fluorescence emission spectra of monomeric (M) and tetrameric (T) melittin can be expressed by a set of equations relating E (the observed emission) and λ (the wavelength of emission):

$$E_M = f_M(\lambda) \tag{10}$$

and

$$E_T = f_T(\lambda) \tag{11}$$

The λ_{max} for each relationship is the wavelength where the first derivative of eq 10 and 11, $\partial E/\partial f_x$, equals zero. If we assume that an experimentally obtained spectrum is the linear summation of the contributions of monomeric and tetrameric melittin and set $x = [\text{melittin}]_{mon}/[\text{melittin}]_{total}$ and $1 - x = [\text{melittin}]_{tet}/[\text{melittin}]_{total}$, we obtain

$$E_{obsd} = xf_M(\lambda) + (1 - x)f_T(\lambda) \tag{12}$$

In the cases of monomeric and tetrameric melittin, the portion of the spectrum between the wavelengths where $E = 0.5E_{max}$ is well approximated by a parabolic function of E and λ. Thus, eq 10 and 11 become

$$E_M = -\frac{1}{4p}(\lambda_o - \lambda_{max}{}^M)^2 + k_M \tag{13}$$

and

$$E_T = -\frac{1}{4p}(\lambda_o - \lambda_{max}{}^T)^2 + k_T \tag{14}$$

where p is the directrix of the solution of these equations, λ_{max}, k is the position of the vertex, and k is the maximum fluorescence and is related to the fluorescence quantum yield. The relative quantum yields (ϕ_R) for monomeric and tetrameric melittin are 0.82 ± 0.02 and 0.83 ± 0.02, respectively (n = 4).

The validity of assigning the fluorescence emission spectra of melittin monomers and tetramers to parabolic functions was tested by integrating (with a Hewlett-Packard 9874A digitizer) the area inscribed by actual recorded spectra and comparing this area to that obtained from calculation of the identity expression for a parabolic function, $A_{parabola} \equiv (2/3)(\text{base})(\text{height})$. For melittin monomers and tetramers, the error in the expression $A_{integration} = (2/3)(\text{base})(\text{height})$ was less than 4% (n = 8 for each melittin species).

In addition, the term p, which determines the "width" of the parabolic curve, can be determined by solving eq 13 and 14 for p at given E,λ_o values. For monomeric and tetrameric melittin, these values are equal ($p_M = p_T$) within the standard deviation of the measurement (n = 8). Using eq 13 and 14, eq 12 becomes

$$E_{obsd} = -\frac{x}{4p_M}(\lambda_o - \lambda_{max}{}^M)^2 - \frac{1-x}{4p_T}(\lambda_o - \lambda_{max}{}^T)^2 + k_M + k_T \tag{15}$$

Differentiating E_{obsd} in eq 15 with respect to $f(\lambda)$ gives

$$\partial E_{obsd}/\partial\lambda = -\frac{x}{2p_M}(\lambda_o - \lambda_{max}{}^M) - \frac{1-x}{2p_T}(\lambda_o - \lambda_{max}{}^T) \tag{16}$$

Since $p_M = p_T$, setting eq 16 equal to zero (to obtain the λ_o of a mixture of melittin_{mon} and melittin_{tet}) and solving for λ_o, we can obtain eq 17.

$$\lambda_o = x\lambda_{max}{}^M + (1 - x)\lambda_{max}{}^T \tag{17}$$

(II) Derivation of Relationship between Dissociation Constant for Self-Association and Ionic Strength. Equation 18

$$K_d^\circ = \frac{a_M{}^4}{a_T} = \frac{m_M{}^4}{m_T}\frac{\gamma_M{}^4}{\gamma_T} = K_d\frac{\gamma_M{}^4}{\gamma_T} \tag{18}$$

defines the relationship between the thermodynamic equilibrium constant (K_d°) for melittin tetramer formation and the observed equilibrium constant (K_d) in terms of the activities (a), molalities (m), and activity coefficients (γ) for melittin monomers (M) and tetramers (T). The activity coefficients can be estimated from an extended Debye–Hückel treatment, which assumes a spherically symmetrical charge distribution for the protein ion (Edsall & Wyman, 1958). Thus

$$\log \gamma_M = \frac{z_M{}^2e^2}{4.606kTD}\left(\frac{1}{b_M} - \frac{\kappa}{1 + \kappa a_M}\right) \tag{19}$$

and

$$\log \gamma_T = \frac{(4z_M)^2e^2}{4.606kTD}\left(\frac{1}{b_T} - \frac{\kappa}{1 + \kappa a_T}\right) \tag{20}$$

where z_m is the charge of a melittin monomer, e is the electronic charge, k is the Boltzmann constant, D is the dielectric constant, T is the thermodynamic temperature (K), and a is the sum of the ionic radius of the protein (b) and its counterion. The term κ varies with $\Gamma/2$ according to eq 21 where N is

$$\kappa = \left(\frac{8\pi Ne^2\Gamma/2}{1000kTD}\right)^{1/2} \tag{21}$$

Avogadro's number. The values of the ionic radii of a melittin monomer, b_M, and tetramer, b_T (1.03 and 1.64 nm, respectively), were estimated from eq 22 which assumes that the ionic

$$b = \left[\frac{3M_r}{4\pi N}(\bar{v}_2 + 0.25)\right]^{1/3} \tag{22}$$

radii are equivalent to those of a sphere of the same mass and density as melittin with 25% hydration. Values of 2840 and 11 360 were used for the monomer and tetramer molecular weights (M_r), respectively, and 0.78 for the partial specific volume ($\bar{v}_2$) of melittin (Terwilliger et al., 1982). The value of the ionic radius of the counter ions was taken as 0.2 nm, the ionic radius of a chloride ion, to yield values of 1.23 and 1.84 nm for monomeric a_M and tetrameric a_T, respectively. Writing eq 18 in negative logarithmic form and substituting pK_d^0, pK_d, and eq 19 and 20 for the activity coefficient terms

gives eq 4, $pK_d = pK_d^0 - z_m^2\Phi$, where

$$\Phi = \frac{4e^2}{4.606kTD}\left[4\left(\frac{1}{b_T} - \frac{\kappa}{1 + \kappa a_T}\right) - \left(\frac{1}{b_M} - \frac{\kappa}{1 + \kappa a_M}\right)\right] \quad (23)$$

Registry No. Melittin, 20449-79-0.

References

Bates, R. G. (1964) *Determination of pH. Theory and Practice*, pp 62-94, 123-130, Wiley, New York.

Bello, J., Bello, H. R., & Granados, E. (1982) *Biochemistry 21*, 461.

Burnstein, E. A., Vedenkina, N. S., & Ivkova, M. N. (1973) *Photochem. Photobiol. 18*, 263.

DeGrado, W. F., Kezdy, F. J., & Kaiser, E. T. (1981) *J. Am. Chem. Soc. 103*, 679.

Drake, A. F., & Hider, R. C. (1979) *Biochim. Biophys. Acta 555*, 371.

Duforcq, J., & Faucon, J.-F. (1977) *Biochim. Biophys. Acta 467*, 1.

Edsall, J. T., & Wyman, J. (1958) *Biophysical Chemistry*, pp 282-514, Academic Press, New York.

Formisano, S., Johnson, M. L., & Edelhoch, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3340.

Garnier, J., Gaye, P., Mercier, J.-C., & Robson, B. (1980) *Biochimie 62*, 231.

Habermann, E. (1972) *Science (Washington, D.C.) 177*, 314.

Habermann, E., & Jeutsch, J. (1967) *Hoppe-Seyler's Z. Physiol. Chem. 351*, 884.

Haverkate, F., & Van Deenen, L. L. M. (1965) *Biochim. Biophys. Acta 106*, 78.

King, E. L. (1964) *How Do Chemical Reactions Occur?*, p 131, W. A. Benjamin, New York.

Knoppel, E., Eisenberg, D., & Wickner, W. (1979) *Biochemistry 18*, 4177.

Quay, S. C., & Tronson, L. P. (1983) *Biochemistry* (following paper in this issue).

Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190.

Talbot, J. C., Dufourcq, J., de Bony, J., Faucon, J. F., & Lussan, C. (1979) *FEBS Lett. 102*, 191.

Teale, F. W. J. (1960) *Biochem. J. 76*, 381.

Terwilliger, T. C., Weissman, L., & Eisenberg, D. (1982) *Biophys. J. 37*, 353.

Tosteson, M. T., & Tosteson, D. C. (1981) *Biophys. J. 36*, 109.

Weast, R. C., Ed. (1970) *Handbook of Chemistry and Physics*, pp D-123, D-207, The Chemical Rubber Co., Cleveland, OH.

Conformational Studies of Aqueous Melittin: Determination of Ionization Constants of Lysine-21 and Lysine-23 by Reactivity toward 2,4,6-Trinitrobenzenesulfonate†

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ABSTRACT: The reaction of monomeric and tetrameric melittin with 2,4,6-trinitrobenzenesulfonate (TNBS) was studied as a function of pH to determine ionization constants for Lys-21 and -23 in melittin. The reaction is a simple second-order process and is well described by the rate equation, $d[P]/dt = k_{obsd}[\text{melittin}]_0[\text{TNBS}]_0$. Monomeric melittin at $\Gamma/2$ 0.01, 23 °C, contains two ionization constants with values of $10^{-6.5}$ M and and $10^{-8.6}$ M. The intrinsic reactivity of these groups differs by 6-fold, probably reflecting a difference in accessibility to the bulky TNBS. In tetrameric melittin at $\Gamma/2$ 2.53, 23 °C, the data support the assignment of two K_H values with a sum of $2 \times 10^{-7.4}$ M. The location of TNBS substitution was determined by cleavage of TNBS-melittin adducts with trypsin (at Arg and unsubstituted Lys residues) or with *o*-iodosobenzoic acid (at the single Trp residue) and by purification by Sephadex LH-20 column chromatography, acid hydrolysis, and thin-layer chromatography to identify free amino acids. The results indicate that the lysine residue with the pK of 6.5 is Lys-21 while Lys-23 has a pK of 8.6 in monomeric melittin. The failure to obtain substitution at Lys-7 at the highest pH values of this study indicates this residue has an ionization constant $\geq 10^{-9.6}$ M. In addition, the α-amino of glycine reacts with TNBS over 20 times more slowly than the lysines of melittin, precluding determination of its ionization constant by this technique.

The pharmacological and biochemical actions of the neurotoxin melittin have been extensively explored [see review in Habermann (1972)]. Studies of the action of melittin fragments indicate that the C-terminal sequence, -Lys_{21}-Arg-Lys-Arg-, is essential both for binding to and for interaction with lipid membranes (Verma et al., 1976; Dawson et al., 1978).

In this paper, we determine the ionization constants of the ϵ-amino groups of lysine residues 21 and 23. The clustered basicity of these residues led us to anticipate that the K_H values for these groups would be larger than those for typical ϵ-amino groups. In fact, the K_H values, measured by reactivity with 2,4,6-trinitrobenzenesulfonate (TNBS),[1] were as much as

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