N-Terminus and Lysine Side Chain pK_a Values of Melittin in Aqueous Solutions and Micellar Dispersions Measured by ^{15}N NMR†

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ABSTRACT: Melittin (MLT) is a 26-amino acid cytolytic peptide from the *Apis mellifera* honey bee. It is known to exist as an α-helical tetramer, as an α-helical monomer, or as a monomeric random coil depending on solvent conditions. The charge state of MLT is believed to be a major factor in determining its aggregation properties and its interaction with lipids. Several, contradictory, indirect measurements of the pK_a values of the three lysine groups in MLT have been reported. In the present study, high-resolution ^{15}N NMR at 50.6 MHz was used to directly measure the pK_a values of the amino groups of the Gly-1, Lys-7, Lys-21, and Lys-23 residues of MLT. Specifically, the pH dependence of MLT ^{15}N chemical shifts was measured separately for the isotopically enriched backbone nitrogen of Gly-1 and the side chain nitrogen atoms of Lys-7, Lys-21, and Lys-23 at a MLT concentration of 1.2 mM and a temperature of 23 °C. Measurements were made for MLT in potassium phosphate buffer, in neat water, and in 1-myristoyl-2-hydroxyl-*sn*-glycero-3-phosphocholine (MMPC) lipid micelles. The experiments showed for MLT tetramer in aqueous phosphate buffer that the amino nitrogen of Gly-1 has a pK_a of 8.15, and that the Lys-7, Lys-21, and Lys-23 side chain nitrogen atoms have pK_a values of 10.21, 10.03, and 10.24 respectively. The pK_a values were somewhat lower for MLT in neat water with Gly-1 at 7.85 (in which case MLT is a monomer), and Lys-21 and Lys-23 at 9.83 and 9.70, respectively (in which case MLT is a tetramer). Similarly for MLT in 48 mM MMPC micelles and 50 mM potassium phosphate, the pK_a values are 7.90, 10.09, 9.87, and 10.10 for Gly-1, Lys-7, Lys-21, and Lys-23, respectively. These pK_a values indicate that the lysine residues are positively charged when MLT forms a tetramer at pH values between 8.5 and 10.

Melittin (MLT)[1] is a cytolytic peptide from the *Apis mellifera* honey bee. It has 26 amino acid residues and its primary sequence is G_1IGAVLK$_7$VLTTGLPALISWIK$_{21}$-RK$_{23}$RQQ. Over the past 15 years, extensive studies have characterized its different conformations in solution under various solvent conditions (Lauterwein et al., 1980; Bello et al., 1982; Quay & Condie, 1983; Dempsey, 1988, 1990; Bazzo, 1988; Weaver et al., 1989a; Buckley et al., 1993). For example, at concentrations in the micromolar to low millimolar range, MLT exists as an α-helical tetramer or as a random coil monomer depending upon the ionic strength, temperature, pH, or sulfate or phosphate content of the solution (Drake & Hider, 1979; Faucon et al., 1979; Talbot et al., 1979; Strom et al., 1980; Podo et al., 1982; Yunes, 1982; Quay et al., 1985). Furthermore, MLT adopts a primarily helical structure in lipids (Brown et al., 1982; Vogel, 1987; Inagaki et al., 1989). Because of these properties, MLT has served effectively as a model for studying peptide conformational transitions and aggregation properties, and peptide−lipid interactions.

The conformation and the aggregation state of MLT in solution are determined by several factors including hydrogen bonding networks, hydrophobic interactions, and van der Waals dispersion forces. In addition, the concentration of MLT and the charge state of MLT are apparently important determinants of oligomerization. At pH 3.5, and concentrations in the low millimolar range or below in aqueous solution, MLT contains six positive charges and no negative charges, and adopts a conformation with little discernible secondary structure when studied by either CD or NMR (Lauterwein et al., 1980; Yunes, 1982). At pH 9, MLT exists as a tetramer (Podo et al., 1982). The propensity of MLT to form a tetramer increases with increasing pH of the solution apparently due in part to decreased Coulombic repulsion consequent, presumably, on deprotonation of titratable amino moieties which favors formation of secondary structure and aggregation. There is not yet consensus regarding the order of events in tetramer formation, i.e., whether secondary structure in the form of amphiphilic helices forms first followed by hydrophobically driven interhelical interactions which result in a "stable" tetramer, or both occur simultaneously. However, available evidence suggests a highly cooperative process for oligomerization which is probably independent of the precise solution conditions that promote formation of the tetramer.

The multiple factors capable of triggering melittin aggregation make evaluation of the precise mechanisms dif-

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[1] Abbreviations: CD, circular dichroism; F-moc, 9-fluorenylmethyloxycarbonyl; FTIR, Fourier transform infrared spectroscopy; HPLC, high-pressure liquid chromatography; MLT, melittin; MMPC, 1-myristoyl-2-hydroxyl-*sn*-glycero-3-phosphocholine; NMR, nuclear magnetic resonance; TNBS, 2,4,6-trinitrobenzenesulfonate.

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Table 1: Reported pK_a Values of MLT

by reaction	Gly-1 (α-N)	Lys-7 (ζ-N)	Lys-21 (ζ-N)	Lys-23 (ζ-N)
Quay & Tronson (1983) at 23 °C				
monomer		>9.6	6.5	8.6
tetramer			7.4	7.4
Lauterwein et al. (1980) at 25 °C				
by ^{1}H NMR	Gly-1 (α-N)	Lys-7 (ζ-N)	Lys-21 (ζ-N)	Lys-23 (ζ-N)
monomer	7.7	>9	>9	>9
Wilcox & Eisenberg (1992) at 25 °C				
tetramer only	Gly-1 (α-N)	Lys-7 (ζ-N)	Lys-21 (ζ-N)	Lys-23 (ζ-N)
by CD	7.5			8.5
by calculation	7.3	9.4	9.8	8.8
Goto & Hagihara (1992) at 20 °C				
by CD	Gly-1 (α-N)	Lys-7 (ζ-N)	Lys-21 (ζ-N)	Lys-23 (ζ-N)
monomer	7.35	9.69	9.69	9.69
tetramer	6.9	9.25	9.25	9.25
Stanislawski & Rüterjans (1987) at 25 °C				
by ^{13}C NMR	Gly-1 (α-N)	Lys-7 (ζ-N)	Lys-21 (ζ-N)	Lys-23 (ζ-N)
methylated[a] tetramer		9.65	8.84	8.84
vesicle-bound		9.65	8.42	8.42
Intrinsic pK_a: Matthew et al. (1985) at 25 °C				
N-terminus			ζ-N of Lys	
8.0			10.4	

[a] Measured on MLT containing lysine residues modified by addition of two methyl groups at the Nζ-position. Lys-21 and -23 were not distinguished.

ficult. One of the most problematic is the pH-induced transition from monomer to tetramer. Although this transition is ascribed generally to deprotonation of quaternary ammonium moieties, there has to date not been any direct verification of the presumption. A crucial question is: "What are the pK_a values of the charged groups in MLT in different solvents?" From the answer to this question, the charge status of MLT can be inferred and factors governing the aggregation of MLT can be understood more clearly.

From the MLT amino acid sequence, it is apparent that only Gly-1, Lys-7, Lys-21, Lys-23, Arg-22, and Arg-24 are the residues likely to be charged at pH 7. Among these, the Arg residues with their higher intrinsic pK_a values (>12) are unlikely to play a role in the pH dependence of the physical form of MLT. Accordingly, Gly-1 and the Lys residues must be the major factors. However, thus far, contradictory pK_a values of the Lys residues of MLT appear in the literature. For instance, the pK_a value of Lys-21 in tetrameric MLT was reported to be 7.4 by Quay and Tronson (1983), measured using reaction kinetics, but 9.25 by Goto and Hagihara (1992), measured by CD. In a similar vein, the pK_a value of Lys-23 in tetrameric MLT was given as 8.6 by Quay and Tronson (1983), 8.5 by Wilcox and Eisenberg (1992), measured by CD, but again 9.25 by Goto and Hagihara (1992). Table 1 summarizes the various pK_a values given in the literature for MLT.

Measurements of pK_a values of specific charge groups in peptides or proteins can be made directly by use of NMR by monitoring the chemical shift of a pertinent nucleus as a function of pH. In particular in the case of MLT, pK_a values of Gly-1, Lys-7, Lys-21, and Lys-23 residues can be obtained from such measurements on peptide specifically isotopically enriched in ^{15}N. Because the nitrogen nuclei of these residues are bonded to labile protons, their chemical shifts are highly sensitive to the ionization state of the residue. In this study, ^{15}N chemical shifts were measured versus pH over the pH range 4−12 for MLT samples with ^{15}N isotopic enrichment at the backbone amine of Gly-1 and at the side chain ζ-position of Lys-7, -21, and -23. The resulting titration curves were analyzed, and the pK_a values of these four residues of MLT in different solutions were obtained. Overall, pK_a values near the intrinsic values of these residues were found. To corroborate these results, two sets of ^{13}C experiments were performed in addition on ^{13}C-enriched MLT samples as outlined below.

MATERIALS AND METHODS

Materials

^{15}N Experiments. MLT samples selectively enriched with ^{15}N at the amino position of the N-terminus, Gly-1, and at the side chain ζ-position of Lys-7, Lys-21, and Lys-23 were prepared by solid phase synthesis employing F-moc chemistry on a peptide synthesizer and were subsequently purified by preparative reverse phase HPLC on a Vydac C-18 column with acetonitrile and trifluoroacetic acid as eluents. Analytical HPLC served to verify peptide purity, and isotopic enrichment was verified by mass spectrometry and ^{15}N NMR. The isotopically enriched (99%) ^{15}N amino acids were purchased from Cambridge Isotope Laboratories. In order to avoid overlap of the ^{15}N NMR signals of the three Lys residues, three separate samples, each labeled at only one of the Lys residues, were synthesized. The MLT samples were dissolved in three different solvents. These were (1) aqueous (H_2O) potassium phosphate buffer with phosphate concentrations of 50 mM (Lys-7- and Lys-21-labeled MLT samples), 120 mM (the Gly-1- and Lys-21-labeled sample), or 150 mM (the Lys-23-labeled sample); (2) neat water (type I); and (3) 48 mM MMPC lipid micelles in 50 mM potassium phosphate buffer in H_2O. The MMPC was purchased from Avanti Polar Lipids, Inc., in powder form, and micelles were prepared by ultrasonication for 30 min of 10 mL of lipid suspension in water buffered with 50 mM phosphate at 25 ± 2 °C. Solvents with phosphate concentration varying from 0 to 150 mM were used (i) to allow the pK_a of the N-terminus to be determined with MLT mainly either monomeric or tetrameric, (ii) to match phosphate concentrations used in other studies (Wilcox & Eisenberg, 1992), (iii) to remove any doubt that MLT was in the tetrameric state near the Lys pK_a values, and (iv) to compare the pK_a values of Lys-21 and Lys-23 in tetrameric MLT in the presence and absence of phosphate.

^{13}C Experiments. Two differently ^{13}C-enriched MLT samples were synthesized as above, the first containing ^{13}Cα-Gly-1, ^{13}Cα-Ala-4, and ^{13}Cα-Gly-12; the second containing ^{13}Cα-Gly-3, ^{13}Cα-Leu-9, ^{13}Cα-Ala-15, and ^{13}Cε-Lys-21. The respective samples were dissolved in 50 mM potassium phosphate buffer in D_2O and in neat H_2O. These isotopically labeled amino acids also were purchased from Cambridge Isotope Laboratories.

Methods

NMR Spectroscopy. (A) ^{15}N Experiments. ^{15}N spectra at 50.653 MHz were collected on a Varian Unity 500 FT

spectrometer on samples with a MLT concentration of 1.2 mM. The spectral width was 4107 Hz with a transmitter offset of −2914 Hz. Peptide samples were placed in a 10 mm glass NMR tube and maintained at 23 ± 0.2 °C during data acquisition. The number of transients collected ranged from 5000 to 20 000 and took from 4 to 18 h for each spectrum at a given pH value to attain a signal-to-noise ratio of at least 4:1. Ammonium nitrate ($^{15}NH_4NO_3$) dissolved in D_2O in a capillary was chosen as an external chemical shift reference at 0 ppm with the D_2O serving as the lock signal. The reproducibility of the chemical shift measurements was ±0.1 ppm. Proton-coupled ^{15}N spectra were collected at most of the pH values except near pH 4 where, because of the low exchange rate of the labile protons with water protons, the spectra were very weak multiplets; for this case, proton-decoupled spectra were collected. Differences between the measured chemical shifts of the coupled and decoupled spectra were quite small (<0.1 ppm) and were neglected. Changes in ^{15}N chemical shifts for the samples in phosphate due to variations in ionic strength with pH were estimated to be ≤0.2 ppm, and did not significantly affect the measured pK_a values.

(B) ^{13}C Experiments. ^{13}C spectra of MLT at various pH values were collected with a Varian Unity 300 FT spectrometer at a temperature of 25 ± 0.2 °C on MLT samples with a concentration of 1 mM in 50 mM phosphate buffer in D_2O and in neat water at a MLT concentration of 1.2 mM. Dioxane in D_2O in a capillary served as the chemical shift reference (67.4 ppm) and as the lock signal.

pH Measurements. For both ^{15}N and ^{13}C experiments, the pH measurements were made with a Corning 320 pH meter at room temperature (24 ± 1 °C) with a 6 mm Trizma electrode purchased from Sigma Chemical Co. The pH values of the MLT solutions were adjusted by titration with HCl and NaOH solutions. Total volume changes of less than 20 μL in the MLT samples were incurred. The pH was varied from 4 to 12; there was no evidence of decomposition of MLT in the experiments, even for experiments conducted at high pH. In most cases, more than 10 different pH points were employed to determine one pK_a value with the exception of the MLT sample with Gly-1 and Lys-21 labeling in H_2O solution where 7 pH values were used. The uncertainty in the pH of the solutions was estimated as ±0.05 for samples in phosphate buffer, and ±0.1 for samples in water and lipid micelles.

For reference, a set of measurements was made on $^{15}N\zeta$-Lys free in solution. The chemical shifts of the ζ-^{15}N of Lys in neat water were measured versus pH on a Varian Unity 500 FT spectrometer at 23 ± 0.2 °C at a Lys concentration of 8 mM. The lysine side chain "intrinsic" pK_a value was found from these experiments to be 10.5 ± 0.1, which is in good agreement with the generally accepted value of 10.4 (Matthew et al. 1985). The procedure for finding the pK_a value was similar to that employed for MLT which is described below.

RESULTS AND DISCUSSION

^{15}N chemical shifts of the amino nitrogen of Gly-1 and the side chain ζ-nitrogens of the three Lys residues of MLT in the three different solutions moved upfield with increasing pH as shown in Figures 1−3. The changes in chemical shifts can be rationalized in terms of changes in the diamagnetic

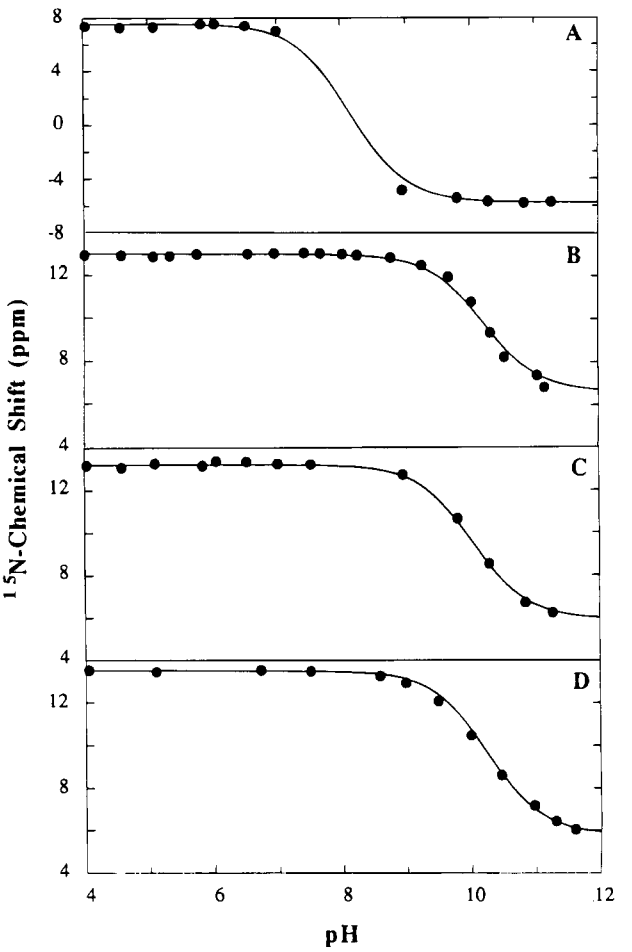

FIGURE 1: ^{15}N chemical shift vs pH for MLT in potassium phosphate buffer at 23 °C. Panel A is for the amino ^{15}N of Gly-1 in 120 mM phosphate buffer; panel B is for the ζ-^{15}N of Lys-7 in 50 mM phosphate buffer; panel C is for the ζ-^{15}N of Lys-21 in 120 mM phosphate buffer; panel D is for the ζ-^{15}N of Lys-23 in 150 mM phosphate buffer. The solid lines are fits to eq 1.

contributions to the shielding tensor. Deprotonation of the amino acid residues at increasing pH results in an increased electron density at the ^{15}N nucleus, increasing in turn the local diamagnetism, and causing an upfield shift. It follows from the Henderson−Hasselbalch equation that

$$\delta = [\delta_{acid} + \delta_{base}\, 10^{pH-pK_a}]/[1 + 10^{pH-pK_a}] \qquad (1)$$

(Forman-Kay et al., 1992; Schaller & Robertson, 1995), where δ is the ^{15}N chemical shift and δ_{acid} and δ_{base} represent the chemical shift values at the low and high extremes of pH, respectively. The influence of pH on the measured ^{15}N chemical shifts in Figures 1−3 follows the above equation where the pK_a value corresponds to the midpoint of the curve presuming negligible structural perturbation of the chemical shift values.

The pK_a values found for the four labeled residues in the different MLT preparations were obtained by nonlinear least-squares fits of the data to eq 1 using the program Mathematica and are given in Table 2. For MLT in phosphate buffer, Gly-1 has a pK_a of 8.15, and each Lys residue has a pK_a >10. Somewhat lower pK_a values prevail for MLT in water, viz., 7.85 for Gly-1, and 9.83 and 9.70 for Lys-21 and Lys-23, respectively. Similar values were found for MLT in the MMPC micelles. The uncertainties of these pK_a values,

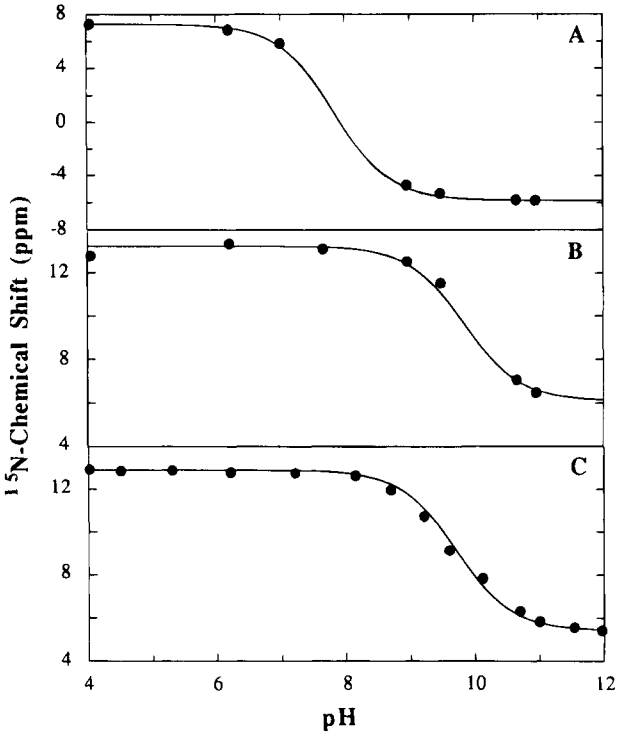

FIGURE 2: ^{15}N chemical shift vs pH for MLT in neat H_2O at 23 °C. Panel A is for the amino ^{15}N of Gly-1; panel B is for the ζ-^{15}N of Lys-21; panel C is for the ζ-^{15}N of Lys-23. The solid lines are fits to eq 1.

estimated from the fitting procedures, were ±0.09 for MLT in phosphate buffer and ±0.15 for MLT in water and lipid micelles, respectively. As a check, the pK_a of Lys-21 of tetrameric MLT was also measured in 50 mM phosphate and found to be 10.09, which agrees well with the value obtained at 120 mM phosphate (Table 2). Fits of the chemical shifts to an equation modified to include the Hill coefficient, n (Markley, 1975), were also made. The pK_a values were unchanged, and n was found to lie between 0.8 and 1.2, indicating no significant degree of cooperativity in the deprotonation events.

Since MLT in solution makes a transition from monomer to tetramer with increasing pH, the aggregation state of MLT at the measured pK_a values must be determined. This can be addressed by means of ^{13}C NMR. In conjunction with investigations of MLT conformation and dynamics (Weaver et al., 1989a,b; Buckley et al., 1993; Buckley, unpublished results), it was found that ^{13}Cα chemical shifts in MLT are sensitive indicators of the conformational and aggregation state of MLT. In particular, ^{13}C chemical shift values correlate directly and unambiguously with helicity, and therefore with tetramer formation in MLT. Their interpretation is independent of issues which potentially plague interpretation of data from more indirect methods such as CD, FTIR, or fluorescence, e.g., peptide length (CD), amino acid composition (FTIR), or spectral overlap (fluorescence). Moreover, there have been extensive CD studies of MLT conformational changes effected by ionic strength or pH which provide an effective data base for comparison with the NMR data presented here (Goto & Hagihara, 1992; Wilcox & Eisenberg, 1992).

Two sets of ^{13}C-NMR pH titration experiments were performed at 75.4 MHz and 25 °C. Figure 4 shows the proton-decoupled ^{13}C spectra obtained at six different pH

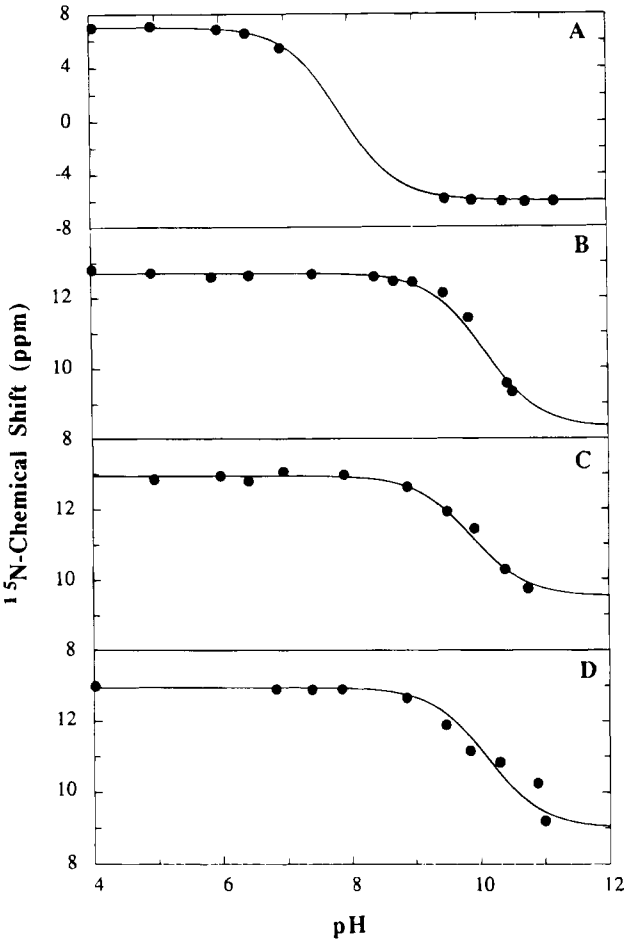

FIGURE 3: ^{15}N chemical shift vs pH for MLT in 48 mM MMPC lipid micelles and 50 mM potassium phosphate at 23 °C. Panel A is for the amino ^{15}N of Gly-1; panel B is for the ζ-^{15}N of Lys-7; panel C is for the ζ-^{15}N of Lys-21; panel D is for the ζ-^{15}N of Lys-23. The solid lines are fits to eq 1.

Table 2: pK_a Values[a] of Gly-1, and Lys-7, -21, and -23 of MLT in Different Solutions Measured in This Work

	Gly-1 (α-^{15}N)	Lys-7 (ζ-^{15}N)	Lys-21 (ζ-^{15}N)	Lys-23 (ζ-^{15}N)
	(i) MLT in Phosphate Buffer[b]			
pK_a	8.15	10.21	10.03	10.24
	(ii) MLT in H_2O			
pK_a	7.85[c]		9.83[d]	9.70[d]
	(iii) MLT in 48 mM MMPC Lipid Micelles, 50 mM Potassium Phosphate			
pK_a	7.90	10.09	9.87	10.10

[a] The uncertainties of the pK_a values of MLT are ±0.09 in (i) and ±0.15 in (ii) and (iii). The measurements were made at 23 °C. [b] MLT tetramer. [c] MLT monomer. [d] MLT tetramer.

values in the range 4−9 for a MLT sample labeled with ^{13}Cα-Gly-1, ^{13}Cα-Gly-12, and ^{13}Cα-Ala-4 and dissolved in 50 mM phosphate buffer. Two distinct chemical shift values exist for the resonances of ^{13}Cα-Gly-1, ^{13}Cα-Gly-12, and ^{13}Cα-Ala-4 with increasing pH as MLT goes from monomeric to tetrameric form as is seen clearly in Figure 4. Specifically for Ala-4, for example, at low pH ($\leq$5) the single peak at 50.0 ppm is indicative of monomer only; at pH 6, two peaks appear with similar amplitude corresponding to an admixture of monomer and tetramer; at pH 7, MLT exists predominantly in a tetrameric state with a single peak at 51.4 ppm. A similar behavior occurs for Gly-12. It then follows that the pK_a values obtained here for Gly-1 and

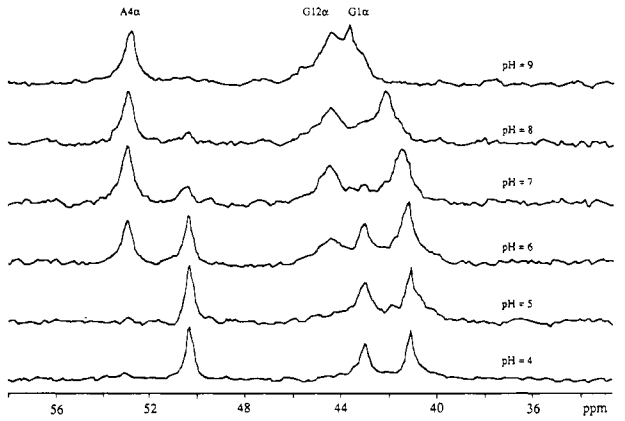

FIGURE 4: ^{13}C NMR spectra at 75.4 MHz and 25 °C of MLT enriched with $^{13}C\alpha$-Gly-1, $^{13}C\alpha$-Ala-4, and $^{13}C\alpha$-Gly-12. The MLT concentration is 1 mM in 50 mM potassium phosphate buffer in D_2O at pH values of 4, 5, 6, 7, 8, and 9, not corrected for isotope effects. The resonances of given residues are indicated in the figure.

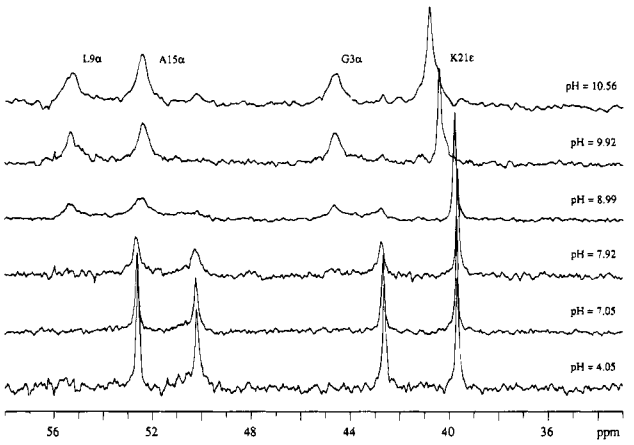

FIGURE 5: ^{13}C NMR spectra at 75.4 MHz and 25 °C of $^{13}C\alpha$-Gly-3, $^{13}C\alpha$-Leu-9, $^{13}C\alpha$-Ala-15, and $^{13}C\epsilon$-Lys-21. The MLT concentration is 1.2 mM in neat water solution at pH values of 4.05, 7.05, 7.92, 8.99, 9.92, and 10.56. The resonances of given residues are indicated in the figure.

the Lys residues in MLT in phosphate buffer are those of tetrameric MLT since (i) the experiments made use of a phosphate concentration of 120 mM for the ^{15}N-Gly peptide and the propensity of MLT to form the tetramer increases with phosphate concentration (Wilcox & Eisenberg, 1992), and (ii) the Lys pK_a values were much greater than 7. A similar titration was carried out in neat H_2O at pH values between 4 and 11 for a second MLT sample labeled with $^{13}C\alpha$-Leu-9, -Ala-15, and -Gly-3, and $^{13}C\epsilon$-Lys-21. The spectra are shown in Figure 5. Focusing on the α-^{13}C resonance of Gly-3, one can see that the monomer for this MLT sample in neat water predominates up to pH 8, whereas the tetramer is dominant at pH 10. A similar conclusion follows from the Leu-9 and Ala-15 resonances. Thus, for MLT in water, the pK_a value (7.85) of Gly-1 applies to MLT predominantly in the monomeric state, and the pK_a values (9.83 and 9.70) of Lys-21 and Lys-23 are for tetrameric MLT.

Also here and in dynamics measurements on MLT (Buckley, unpublished results), the ^{13}C chemical shifts of $^{13}C\epsilon$-Lys-7, -21, and -23 were found to be insensitive to the monomer−tetramer transition of MLT. This is apparent in Figure 5; there is no change in the chemical shift of the $^{13}C\epsilon$-Lys-21 resonance up to pH 9 while at pH 10.5 approximately a 1 ppm shift was observed. The latter shift evidently is due solely to the pH change and not to conformational effects. A similar behavior was seen for $^{13}C\epsilon$-Lys-23 in MLT in phosphate buffer solution (not shown). These observations are consistent with the Lys pK_a values found from the ^{15}N measurements.

In deriving pK_a values from the Henderson−Hasselbalch equation, we assumed that the ^{15}N chemical shifts of the titrated residues were dependent only on pH and not significantly affected by other factors such as the MLT conformation. Comparison of the ^{15}N chemical shifts of MLT in different conformations but at the same pH values (not too near the pK_a) provides an *estimate* of the effect of conformation on the measured ^{15}N chemical shifts. The chemical shifts of the ζ-^{15}N of Lys-23 in 150 mM phosphate buffer (tetrameric MLT) and water solution (monomeric MLT) at pH ~7 were 13.5 and 12.8 ppm, respectively, a difference of 0.7 ppm in the two different conformations. For Gly-1, the amino ^{15}N chemical shift was 6.97 ppm in 120 mM phosphate buffer at pH 7 (tetramer) and 5.85 ppm in water at pH 7 (monomer), a difference of 1.12 ppm. The transition from monomer to tetramer thus results evidently in changes in ^{15}N chemical shifts which are opposite in sign to and much smaller in magnitude than the chemical shift changes caused by simply altering the charges of the groups with increasing pH (see Figures 1 and 2). Conformation-dependent chemical shift changes of the same sign and of similar magnitudes were compiled by Wishart et al. (1991) for backbone amide ^{15}N nuclei in proteins.

Under the conditions of our experiments, MLT was tetrameric at pH values at least within 1 pH unit of the pK_a value of the Gly and Lys residues in phosphate solution so the derived pK_a values should not be affected at all by the conformational transition. In aqueous solution, there is, however, the potential that the apparent pK_a values were affected. To estimate the extent of the effect, we altered the chemical shift values at higher pH for glycine and at lower pH for lysine by the amounts noted in the preceding paragraph, and found a decrease in the pK_a values from the fitting procedures of ~0.1 in each case. Therefore, the influence of conformational change on the ^{15}N chemical shifts of the Gly-1 and the Lys residues of MLT both in phosphate buffer and in water does not significantly alter the pK_a values retrieved. For MLT in MMPC lipid micelles, MLT assumes mainly an α-helical conformation (Brown et al., 1982; Vogel, 1987; Inagaki et al., 1989), so again conformational perturbation to the determination of pK_a values can be neglected.

To rationalize pH-induced tetramerization of MLT, it has been proposed that for MLT at pH ≥9, the Lys side chain amino nitrogen atoms are no longer ionic, resulting in reduced Coulombic repulsion among the head groups of the four monomeric units. From several measurements (see Table 1), unusually low pK_a values for Lys-21 and Lys-23 have been inferred. Our results clearly contradict these expectations. The Lys side chain pK_a values found here for MLT in phosphate buffer are close to their intrinsic values and indicate unequivocally that the MLT tetramer forms even when the Lys residues are positively charged.

The pK_a values of the N-terminus and of the Lys residues of MLT in the MMPC micelles are slightly smaller than those of the MLT tetramer in phosphate buffer, are somewhat larger than those of MLT in water, and do not differ much from the intrinsic pK_a values (Tables 1 and 3), but are larger

than values obtained in vesicles (Stanislawski & Rüterjans, 1987). The slightly smaller values we found in the micelles versus the tetramer in phosphate might be related to interactions between the choline head groups of the MMPC and the charged groups of MLT. These head groups could either block or at least hinder the interactions between the phosphate moieties and MLT. At pH values below 9, MLT is an α-helical monomer in micelles (Brown et al., 1982; Vogel, 1987; Inagaki et al., 1989), and the α-helical conformation persists even at higher pH (Yuan, unpublished results). The aggregation status of MLT in MMPC micelles, however, is not certain above pH 9. Thus, the pK_a values of the Lys residues of MLT in MMPC micelles obtained here may apply to monomeric, tetrameric, or some other aggregate of MLT.

As noted above, the results we report here for MLT in aqueous solution are clearly disparate from reports of other groups (see Tables 1 and 2). The most striking differences are with the values of Quay and Tronson (1983) for the pK_a of the lysine amino moieties. However, interpretation of their data was intrinsically problematic because of the method employed, namely, the pH-dependent kinetics of labeling of amino moieties with 2,4,6-trinitrobenzenesulfonate (TNBS). This negatively charged and partially hydrophobic agent is very likely to partition to some (albeit quantitatively undefined) extent into or onto the cationic MLT tetramer which could then have caused anomalous reaction rates with the lysine amino groups depending on the site of partitioning. For example, a molecule analogous to TNBS in being amphiphilic with an anionic charge, namely, 8-toluidinonaphthalinesulfonate, adsorbs to the melittin tetramer (unpublished results). Additionally, one would have to be concerned about effects of initial chemical labeling on subsequent reaction rates by the agent simply because one is now dealing with a modified melittin. Lastly, an examination of the X-ray structure of tetrameric MLT (Terwilliger & Eisenberg, 1982a,b) shows that the side chains of Lys-21 and Lys-23 are somewhat differently disposed toward solvent. Although the precise positions of these side chains must be dynamically averaged, the crystal structure probably reliably reports a propensity for the side chain of Lys-23 to be somewhat less exposed on average than that of Lys-21 while that of Lys-7 is apparently always fully solvent-exposed. Overall, the differences between our results and those of Quay and Tronson (1983) are such that we can no longer accept their conclusions. As shown in Tables 1 and 2, additional disparities between our results and those of others are not as great. We believe those differences derive most likely from their use of indirect methods for determination of the pK_a values; e.g., CD by Goto and Hagihara (1992) and Wilcox and Eisenberg (1992). Finally, Stanislawski and Rüterjans (1987) employed ^{13}C NMR to probe pH effects on MLT aggregation, but their experiments were conducted on lysine side chains quaternized by methylation.

Formation of the MLT tetramer has been extensively studied by many investigators (Faucon et al., 1979; Talbot et al., 1979; Brown et al., 1980; Bello et al., 1982; Terwilliger & Eisenberg, 1982a,b; Quay & Condie, 1983; Quay & Tronson, 1983; Goto & Hagihara, 1992; Hagihara et al., 1992; Wilcox & Eisenberg, 1992), and there is consensus that oligomerization is influenced by ionic strength, specific ion interactions, pH, and peptide concentration. Goto and Hagihara (1992) and Hagihara et al. (1992) have provided probably the best overall assessment to date of these physicochemical factors and their involvement in tetramer formation. They pointed out that the key is the balance between the "unfolding forces", including electrostatic repulsion, and "folding forces", which include hydrophobic effects and stabilizing van der Waals forces consequent upon side chain−side chain interactions at the interhelical surfaces. Charge repulsion among the monomeric units is effectively reduced either by increasing pH or by anion binding. For the latter, multivalent anions are most effective in the MLT system, particularly phosphate, given its propensity for binding at the guanidinium cation of arginine residues. Examination of the crystal structure of tetrameric melittin reveals, moreover, a clustering of cationic charges at two poles of the ellipsoidal oligomer. The structure enhances the extent of electrostatic repulsion between monomeric units whose carboxy-terminals' hexapeptides (Lys-Arg-Lys-Arg-Gln-Gln) already display a substantial localization of cationic charge. Given this structure, it is easy to understand the high likelihood for anion condensation onto these highly cationic peptide surfaces. It is also easy to understand why water structure and dynamics, and Debye−Hückel screening effects all likely contribute to stabilizing the MLT tetramer. The clustering of charge and the proclivity for relatively selective anion−cation interactions argue against the use of smeared charge models to properly explain ionic effects on MLT conformation. These points were well made either implicitly or explicitly by other authors, most notably by Goto and Hagihara (1992), Hagihara et al. (1992), and Wilcox and Eisenberg (1992). However, at this juncture, we would suggest a slightly different rationalization for the effects of pH and ionic strength on melittin oligomerization than has been posited hitherto.

There are two material issues. First, from both the results we report here and the preceding work of others, it is clear that at sufficiently high ionic strength, particularly in solutions containing phosphate, even fully cationic melittin will form a tetramer. In other words, there is no need for deprotonation of any of the easily titratable cationic charges for oligomerization to occur. Second, at very low ionic strength alkalinization alone can trigger tetramerization, and under such conditions the apparent pK_a(s) of the group(s) (lysines) involved is/are 9.7 and above. However, the phase diagram for tetramer formation is unavoidably complex, determined as it is by a set of linked physiochemical properties whose individual contributions cannot be readily quantified (Hagihara et al., 1992; Goto & Hagihara, 1992). A key result we present is that there apparently are not substantive differences in the pK_a values of the α-NH_2 or ζ-NH_2 groups for monomeric and tetrameric melittin. In particular, we found a somewhat smaller pK_a value for the α-NH_2 group in the monomer than in the tetramer, and even though MLT was tetrameric at the Lys-ζ-NH_2 pK_a values in our measurements in neat water and phosphate, there is no indication in the former samples of a significant effect on the titration curves brought on by the monomer-tetramer transition at around pH 9. The somewhat larger pK_a values in the presence of phosphate are probably just due to electrostatic shielding. Thus, at any particular ionic strength and peptide concentration, there is always a balance between tetramer stabilizing and destabilizing forces, and pH titration serves simply to tip the balance one way or the other. This nuance to the model of Goto and Hagihara (1992) would

predict that if there is sufficient electrostatic screening by anions—as opposed to deprotonation—melittin tetramer formation would be independent of pH. This is precisely what is found experimentally (Wilcox & Eisenberg, 1992). Accordingly, as indicated by our experiments, there is no need to invoke molecular environment-determined pK_a changes for either the glycine or the lysine amino moieties to explain the pH effect on MLT oligomerization.

Finally, these results suggest some potentially interesting further experiments with the MLT system. For example, the lysine residues may be replaced with groups having lower pK_a values such as histidine, or with amino acids that are not ionic such as glutamine. We would predict that such derivatives will form more stable tetramers and would show pH effect curves shifted to lower pH values relative to wild-type melittin. In contrast, changing the lysine residues to arginine would tend to destabilize the tetramer. Preliminary data with a mutant form of melittin, K21H-MLT, bear out these predictions, but, interestingly, the tetramer of a second mutant form, K23H-MLT, is substantially destabilized relative to wild-type MLT (Venyaminov and Prendergast, unpublished results). We believe the latter result is attributable to steric factors, and underscores the difficulty in quantifying the individual contributions of any one physiochemical process involved in promoting tetramer formation when the causative processes are linked.

ADDED IN PROOF

Recently van Veen et al. (1995) studied three variants of melittin modified in the C-terminal region. They found that a variant in which both Lys-21 and Lys-23 were replaced by Gln formed a tetramer more readily with increasing ionic strength (phosphate) than did wild-type melittin.

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