# Conformational and Aggregational States of ω-Aminoacylmelittin Derivatives

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ABSTRACT: Melittin, a 26-residue peptide from bee venom, is known to change from a largely random to a largely α-helical conformation as a function of peptide concentration, pH, and ionic strength. In this report, we have determined the effect of displacing the positive charges of the amino groups of N-terminal glycine and lysine residues away from the backbone of melittin in coil-to-helix transitions by using ω-aminoacyl derivatives of melittin. These were prepared by acylating the amino groups of melittin with ω-amino acids to yield the melittin derivatives glycylmelittin (MLT-2), (4-aminobutanoyl)melittin (MLT-4), and (5-aminopentanoyl)melittin (MLT-5), respectively. At pH 7.2, there is a chain-length-dependent increase in helicity from MLT to MLT-5. The ω-aminoacylmelittin derivatives also show a concentration-dependent increase in helicity at pH 7.2. However, at pH 2.3, a concentration-independent, but chain length-dependent increase in helicity was observed. A hydrophilic derivative glycylglycylmelittin (MLT-GG) and a hydrophobic derivative MLT-5, which have side chains of equal length, show similar helicity, at pH 7.2, but at pH 2.3 MLT-GG shows almost no helicity, while MLT-5 is about 60% helical. The lysyl derivative (MLT-K), which has additional positive charges compared to melittin, behaves much like MLT-2. At pH 7.2, all the derivatives exhibit both cold- and heat-induced denaturation; a significant amount of residual structure is retained in the temperature range 80–100 °C. These results are discussed in terms of the electrostatic and hydrophobic interactions involving the side chains.

The helix–coil transitions of synthetic small polypeptides under different environmental conditions have been investigated, since these peptides can be used as models for studying protein folding (Brown & Klee, 1969; Marqusee & Baldwin, 1987; Fairman et al., 1989; Marqusee et al., 1989; DeGrado et al., 1989). Melittin (MLT),[1] a cationic, amphipathic, 26-residue lytic peptide from bee venom, is an example of a natural model system useful in studying formation of helices and their association in protein folding and protein stability (Knöppel et al., 1979; Bierzynski et al., 1982; Rico et al., 1983, 1984, 1986). The amino acid sequence $NH_3^+$-G-I-G-A-V-L-$K^+$-V-L-T-T-G-L-P-A-L-I-S-W-I-$K^+$-$R^+$-$K^+$-$R^+$-Q-Q-$NH_2$ contains a high proportion of hydrophobic residues in the N-terminal 20 amino acids and a concentration of six hydrophilic, including four cationic, amino acids at the C-terminus (Habermann, 1972). In the crystal structure, the melittin molecule consists of two α-helical segments (residues 1–10 and 13–26) intersecting at an angle of 120°. These are connected by a hinge reaction (11–12) to form a bent α-helical rod with the hydrophilic and hydrophobic sides facing opposite directions. Four such monomeric mellitin molecules are clustered together through hydrophobic interactions to form a tetramer (Terwilliger & Eisenberg, 1982; Terwilliger et al., 1982). The transition of the monomeric random coil to tetrameric helix in aqueous solutions at high pH, high peptide concentration, or high ionic strength, or by conversion of positive charges to neutral or negative charges by acylation makes it a very good environment-dependent model system for studying protein folding (Faucon et al., 1979; Talbot et al., 1979; Brown et al., 1980; Lauterwein et al., 1980; Bello et al., 1982; Tatham et al., 1983; Kubota & Yang, 1986). The conformational and aggregational properties of melittin in solution are influenced by two factors. Promoting self-association is the hydrophobic effect that acts to sequester the nonpolar amino acids into the interior of proteins, and opposing self-association is the high positive charge density (Knöppel et al., 1979; Brown et al., 1980; Bello et al., 1982; Tatham et al., 1983).

The following questions were posed: Does the location of the positive charges relative to the backbone influence the oligomer formation? If so, how does the displacement of the charges on the lysine residues away from the backbone affect the conformational and aggregational properties of the melittin? Does the introduction of additional positive charges affect these properties? Does the increase in hydrophobicity of the side chains, as a result of displacing the charges away from the backbone, have any effect? Elucidation of the roles of such molecular determinants in the assembly of monomers would be of considerable value in the understanding of structural relationships of peptides and proteins. We, therefore, modified the ε-amino groups of all the lysine residues and the α-amino group of N-terminal glycine using ω-amino acids. The primary structures of ω-aminoacylmelittin derivatives are shown in Figure 1. In order to assess the role of hydrophobicity in the ω-aminoacyl side chains on the aggregational properties of melittin, we also synthesized a hydrophilic derivative in which the dipeptide Gly–Gly was grafted to the side chains. Additional charges were introduced into melittin by synthesizing a lysyl melittin (MLT-K) in which the net charge is +8 to +10 compared with +5 to +6 in MLT. (The number of charges will be discussed below). We present evidence here that the conformational and aggregational properties of these ω-aminoacylmelittin derivatives are functions of the length of the side chain.

## EXPERIMENTAL PROCEDURES

*Preparation of ω-Aminoacylmelittin Derivatives.* Native melittin (Sigma Chemical Co.) was twice purified by chro-

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[1] Abbreviations: MLT, melittin; MLT-2, glycylmelittin; MLT-4, (4-aminobutanoyl)melittin; MLT-5, (5-aminopentanoyl)melittin; MLT-GG, glycylglycylmelittin; MLT-K, lysylmelittin; MLT-suc, tetrasuccinylmelittin; MLT-ac.cit, tetra(acetylcitryl)melittin; Boc, *tert*-butyloxycarbonyl.

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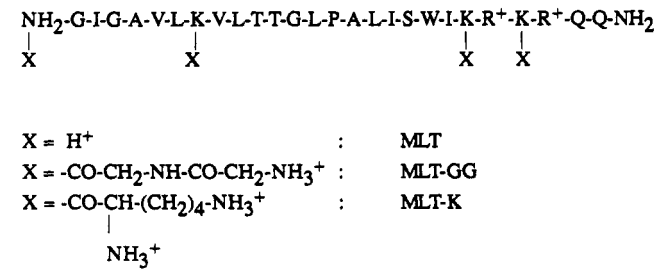


FIGURE 1: Primary structure of melittin and its ω-aminoacylmelittin derivatives. All the derivatives have a net charge of +6 except MLT-K, which has a net charge of +10. For lysine residues, X is understood to represent the substituent on the $\epsilon$-$NH_2$ group.

matography using Sephadex G-50 (1.5 × 100 cm, 20 mM acetic acid). The mass spectrum and amino acid analysis of the purified melittin were consistent with the expected values (Ramalingam et al., 1992). Other proteins and peptides (as molecular weight markers) were from Sigma, except insulin B-chain from Boeringer Mannheim. Boc-ω-aminoacylmelittin derivatives were prepared by acylating the $\alpha$- and $\epsilon$-amino groups of glycine and lysine residues of melittin (1 equiv) with Boc-ω-amino acid succinimide esters (100 equiv) at pH 8.0, 25 °C. The protecting group (Boc) was removed using *p*-toluenesulfonic acid/triphenylphosphine. All of the derivatives were purified by chromatography using a Sephadex G-15 column equilibrated with 100 mM ammonium acetate. Mass spectral analysis of all peptide derivatives showed good agreement between predicted and observed masses. (predicted mass values are given in parentheses): MLT-2 3074.6 (3074.1); MLT-4 3185.4 (3186.1); MLT-5 3242.3 (3242.3); MLT-GG 3302.1 (3302.2); MLT-K 3357.4 (3358.3). UV absorption spectra of all the derivatives were similar to that of melittin, indicating that the Trp residue did not undergo any modification. Amino acid analyses of the ω-aminoacylmelittin derivatives were consistent with the expected values. Permethylation of the ammonium groups of melittin was carried out using dimethylsulfate at about pH 9.5 following a reported procedure (Granados & Bello, 1979). The permethylated melittin (MLT-Me) was purified by chromatography as for melittin derivatives. Amino acid analysis of MLT-Me showed loss of 0.8 glycine residue, confirming the methylation of N-terminal glycine, and loss of 2.96 lysine residues (theoretical value 3) and showed the presence of $N^{\epsilon}$,$N^{\epsilon}$,$N^{\epsilon}$-trimethylated lysine residues. (Trimethylglycine would not be detectable.) The loss of 0.8 residue of glycine also shows that the terminal glycine in the purified melittin is largely free and not formylated. Permethylation of MLT-2 and MLT-5 was carried out as for melittin. Amino acid analyses of the permethylated melittin derivatives MLT-2Me and MLT-5Me showed total loss of the additional glycine and 5-aminopentanoic acid residues, indicating complete methylation.

*Molecular Weights by Gel Filtration.* Gel filtration of the ω-aminoacylmelittin derivatives was carried out using a 1 × 50 cm column of Sephadex G-50 eluted by 20 mM phosphate, pH 7.2, at a rate of 8 mL/h. One-half milliliter of a 1.0 mg/mL solution was applied to the column, and 1.0-mL fractions were collected. The concentrations of the peptides in the fractions were determined from the tryptophan absorption spectrum using an $\epsilon_{280}$ value of 5600 $M^{-1}$ $cm^{-1}$ (Bello et al., 1982). A second set of experiments were carried out using 1.0 M NaCl as the eluent.

*Circular Dichroism.* CD spectra were obtained with a Jasco-500 spectropolarimeter which was calibrated with camphorsulfonic $d_{10}$ acid, at 25 °C. Sample solutions were made in 20 mM phosphate buffer or 20 mM Tris·HCl (for CD spectra in methanol), pH 7.2. The CD spectra of the peptides were measured using either 1- or 2-mm silica cells placed in a thermostated cell holder. The temperature of the cell holder was controlled by a circulating bath. The cell was held in a snugly fitting aluminum cell holder, heated on all four sides and the bottom for uniform and rapid heating of the cell. All optical measurements at low temperature were done while flushing the sample compartment and the cell faces with dry nitrogen. Temperature inside the cell holder was measured using a platinum resistance element and a digital temperature indicator (Omega model 199P2). For the melting profile studies, the temperature of the circulating bath was increased at 1 °C/min by a Neslab ETP-3 programmer. Although at high temperatures the temperature in the cell could lag behind the temperature of the cell holder, all experiments were done under identical conditions. Melting profiles were recorded continuously by feeding the CD and thermometer outputs to an *X–Y* recorder. Data points were selected from the *X–Y* recorder output at 5 °C intervals and plotted against temperature.

The percent helicities were calculated as follows: %helix = $[\theta]_{obs}/[\theta]_{100}$, from the experimentally observed mean residue ellipticity $[\theta]$ at 222 nm and the value for $[\theta]$ corresponding to 100% helix content at this wavelength. The 100% ellipticity value was taken at −27 500 on the basis of the highest ellipticity value observed with 630 $\mu$M melittin, pH 11.5, in phosphate/glycine/NaCl (Bello et al., 1982).

## RESULTS

*Effect of Peptide Concentration and pH on Conformation.* In this work, percent helicity is based on the maximum helicity observed for melittin under the most favorable helix-forming conditions (see Experimental Procedures). The CD spectra of MLT, MLT-2, MLT-5, MLT-GG and MLT-K, at a peptide concentration of 30 $\mu$M (20 mM phosphate buffer, pH 7.2) are shown in Figure 2. MLT shows some ordered structure (18% helix), similar to the value reported earlier (Bello et al., 1982). As the length of the aminoacyl chain increases, there is promotion of helix. For MLT-2 and MLT-5, the helix contents are 45% and 58%, respectively. As the acyl chain is lengthened, the amino groups are, potentially, displaced away from the peptide backbone, and the hydrophobicity of the chain increases. MLT-GG (the glycylglycyl derivative), which has substituent acyl chains of the same length as those of MLT-5, but with a highly polar peptide group, shows 55% helicity, which is close to the value obtained for MLT-5. MLT-K, with a greater net charge than in MLT or the other derivatives, shows greater helicity than does MLT, but less than MLT-2 through MLT-5. The dependence of helix promotion on the length of the side chain of the grafted ω-amino acid is shown in Figure 3 for peptide concentrations of 8 and 70 $\mu$M at pH 7.2. The melittin derivatives show a concentration-dependent increase in helicity at pH 7.2 (Figures 3 and 4), with the largest slope below 30 $\mu$M, except for MLT, MLT-4, and MLT-K.

The increased ellipticity as the acyl chain length increases could arise from displacement of the charge away from the

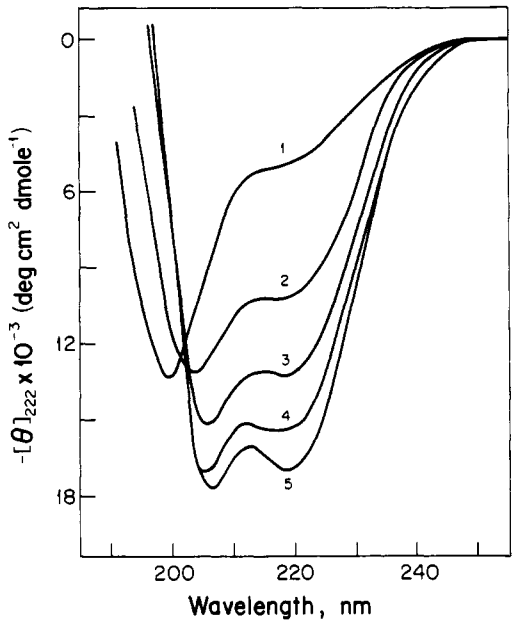


FIGURE 2: Comparison of the circular dichroic spectra of ω-aminoacylmelittin derivatives in 20 mM phosphate, pH 7.2, at a peptide concentration of 30 μM, at 25 °C. (1) MLT; (2) MLT-K; (3) MLT-2; (4) MLT-GG; (5) MLT-5.

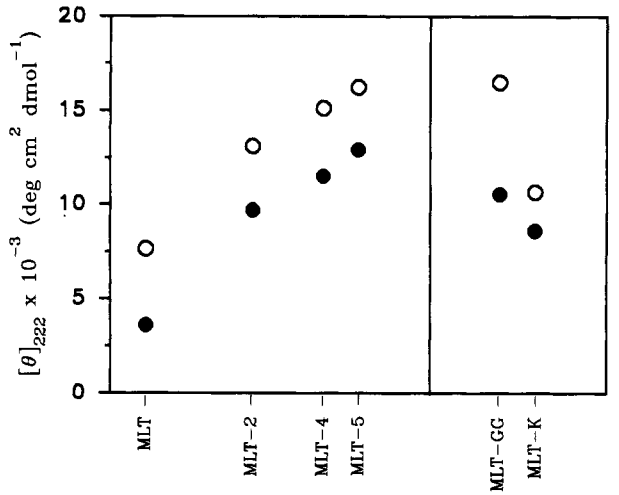


FIGURE 3: Chain-length dependence of the mean residue ellipticity at 222 nm for the ω-aminoacylmelittin derivatives in 20 mM phosphate, pH 7.2, at 25 °C. Peptide concentrations used were (●) 8 μM and (○) 70 μM. The data points corresponding to the peptides MLT, MLT-2, and MLT-4 have been displaced to take into account the increase in the chain length. MLT-GG and MLT-K have been deliberately separated from the other peptides for clarity.

main chain or from increased hydrophobicity. The role of hydrophobic interactions was investigated by measuring the ellipticity values for MLT (120 μM), MLT-5 (30 μM), and MLT-GG (30 μM) in the presence of 0.8 M Gdn·HCl). In the absence of Gdn·HCl, the ellipticity values are −11 200 deg cm$^2$ dmol$^{-1}$ for MLT (Figure 3) and −15 700 and −15 200 deg cm$^2$ dmol$^{-1}$ for MLT-5 and MLT-GG, respectively (Figure 2). In 0.8 M Gdn·HCl the ellipticity values are −4000 deg cm$^2$ dmol$^{-1}$ for MLT, −8700 deg cm$^2$ dmol$^{-1}$ for MLT-GG, and −12 500 deg cm$^2$ dmol$^{-1}$ for MLT-5.

There might also be an effect arising from p$K_a$ differences between the amino groups of the peptides, which would produce differences in net charge. Therefore, we measured the CD spectra of the melittin derivatives (30 μM) in 20 mM phosphate at pH 2.3 (using 20 mM phosphoric acid to adjust the pH), at which all amino groups are charged and all peptides have

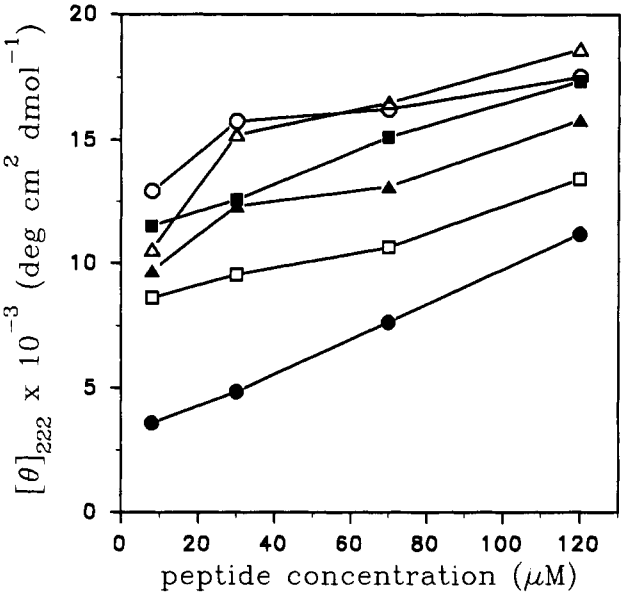


FIGURE 4: Concentration dependence of the mean residue ellipticity at 222 nm for the ω-aminoacylmelittin derivatives in 20 mM phosphate, pH 7.2, at 25 °C. (●) MLT; (▲) MLT-2; (■) MLT-4; (○) MLT-5; (△) MLT-GG; (□) MLT-K.

the same charge, +6, except +10 for MLT-K. The results (Figure 5a) show that helicity increases with acyl chain length, especially from MLT through MLT-5. MLT-GG is only slightly more helical than MLT and much less than MLT-5, and MLT-K shows a little helical content. The results for MLT-2 and MLT-GG at pH 2.3 are in sharp contrast with those at pH 7.2. At pH 7.2 the ellipticities of MLT-2 and MLT-GG are in the range of those of MLT-4 and MLT-5.

At pH 2.3 the ellipticities of MLT through MLT-5 and of MLT-GG and MLT-K are independent of concentration (from 30 to 120 μM, Figure 5a,b). In a separate set of experiments, peptide solutions (30 and 120 μM) were made using 20 mM HCl to adjust to a pH of 2.3. The ellipticities obtained for these derivatives (data not shown) are concentration-independent but showed chain-length dependence and agreed with the corresponding values obtained in 20 mM phosphate, pH 2.3 (Figure 5a,b).

The increase in ellipticity between pH 2.3 and 7.2 indicates that some amino groups are being titrated. This was supported by the results of experiments with methylated MLT, MLT-2, and MLT-5. These are designated MLT-Me, MLT-2Me, and MLT-5Me. In these derivatives the amino groups were trimethylated to quaternary ammonium groups, which are fully charged at all pH values. With MLT-Me, MLT-2Me, and MLT-5Me, there practically is little pH dependence of ellipticity from pH 2.3 to 11.2 (Table I). A small increase in ellipticity exhibited by MLT-2Me with increasing pH may have resulted from a small fraction of incompletely methylated amino groups. The ellipticities of the methylated and their respective unmethylated peptides are essentially the same at pH 2.3, at which both types have the same charge, but differ substantially at pH 7.2. Both methylated and unmethylated peptides show chain-length dependence.

At pH 11.2 and 30 and 120 μM concentration, there is a large increase in ellipticity for MLT and MLT-K compared to pH 7.2, but not for MLT-2, MLT-4, and MLT-GG. These solutions showed apparent absorption near 330 nm, well above the absorption band, indicative of turbidity. The solution of MLT-5 was turbid at pH 11.2, and no useful data could be obtained.

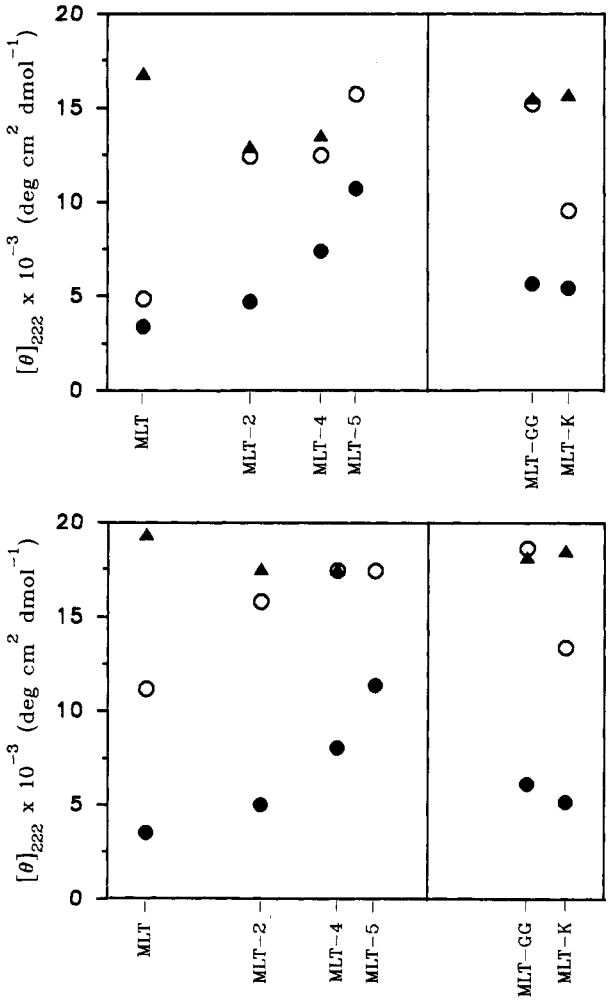


FIGURE 5: Mean residue ellipticity at 222 nm for the ω-aminoacylmelittin derivatives (in 20 mM phosphate) at various pH values at 25 °C. (●) pH 2.3; (○) pH 7.2; (▲) pH 11.2. (a, top) peptide concentration, 30 μM. (b, bottom) Peptide concentration, 120 μM. At pH 11.2, the data point for MLT-5 is omitted because of turbidity. The data points corresponding to the peptides MLT, MLT-2, and MLT-4 have been displaced to take into account the increase in the chain length. MLT-GG and MLT-K have been deliberately separated from the other peptides for clarity.

Table I: Mean Residue Ellipticity (−[θ]) at 222 nm[a] of Melittin Derivatives

| peptide[b] | pH 2.3 | pH 7.2 | pH 11.2 |
|---|---|---|---|
| MLT | 3400 | 4800 | 16800 |
| MLT-2 | 4700 | 12400 | 12900 |
| MLT-5 | 10700 | 15700 | –[c] |
| MLT-Me | 3200 | 3400 | 3400 |
| MLT-2Me | 4400 | 5500 | 6500 |
| MLT-5Me | 11100 | 12700 | 11400 |

[a] Expressed as deg cm$^2$ dmol$^{-1}$. [b] Peptide concentration is 30 μM, in 20 mM phosphate. [c] Turbidity.

*Molecular Weights by Gel Filtration.* The apparent molecular weights of the ω-aminoacyl derivatives were measured by gel filtration on a Sephadex G-50 column using 20 mM phosphate, pH 7.2, as eluent. MLT elutes with an apparent molecular weight of 4500 instead of 2800 as obtained from the amino acid composition (Figure 6a). MLT-5 elutes with an apparent molecular weight of 16 000, and MLT-4 and MLT-GG elute with an apparent molecular weight of 11 000. MLT-2 and MLT-K elute between monomer and tetramer, suggesting a slow equilibrium between monomer and tetramer or the presence of significant amounts of dimer.

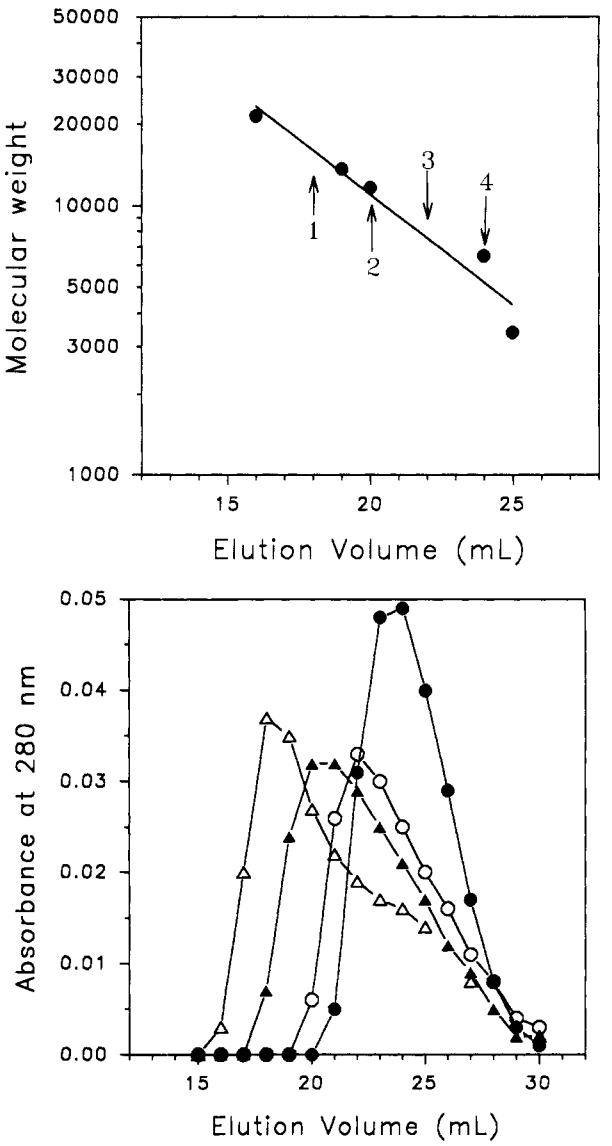


FIGURE 6: (a, top) Molecular weights by gel filtration (Sephadex G-50 1 × 50 cm). Circles are from highest to lowest molecular weight, α-chymotrypsinogen, RNase S protein, aprotinin, and insulin B-chain. The arrows are the following: (1) MLT-5; (2) MLT-4 and MLT-GG; (3) MLT-2 and MLT-K; (4) MLT. Eluent: 20 mM phosphate, pH 7.2. (b, bottom) Gel permeation profiles for melittin derivatives (Sephadex G-50, 1 × 50 cm). Eluent: 20 mM phosphate, pH 7.2. The flow rate was 9 mL/h. (●) MLT; (○) MLT-2; (▲) MLT-4; (△) MLT-5. The elution patterns of MLT-K and MLT-GG are similar to those of MLT-2 and MLT-4, respectively.

The elution patterns of MLT, MLT-2, MLT-4, and MLT-5 are shown in Figure 6b. The elution patterns of MLT-K and MLT-GG are similar to those of MLT-2 and MLT-5. The eluted peaks for MLT, MLT-2, MLT-4, MLT-GG, and MLT-K are symmetric, whereas MLT-5 is asymmetric with the advancing edge sharper than the trailing counterpart. The tailing region for MLT-5 showed a distinct second component corresponding to the elution position of melittin monomer. The asymmetry of the eluted peak was considered to be characteristic of a dissociating system (Ackers & Thompson, 1965; Cunnigham et al., 1991), suggestive of a tetramer–monomer equilibrium occurring in the column. Schubert et al. (1985) used sedimentation equilibrium experiments and subsequent mathematical analysis to determine the population of dimers in the association equlibrium of melittin. The maximum contribution of the dimer did not exceed 0.5% (w/w) of the total peptide weight. In our studies, MLT-2 and

MLT-K appear to be eluted as dimers, but this requires further study.

*Effect of Counterions on Peptide Conformation.* All the peptides exhibit high helicity (74–80%, based on $[\theta]_{222}$ of −20 400 to −21 900 deg cm$^2$ dmol$^{-1}$) in 1.0 M NaCl, $NaClO_4$, and phosphate solutions, somewhat more at 120 than at 30 μM. MLT-5 in 1.0 M $NaClO_4$ solution is turbid at both peptide concentrations studied, so that the ellipticity values are not reliable. Apparently there was no turbidity in NaCl or phosphate. As these salts are more effective salters-out than $NaClO_4$ (McDevitt & Long, 1952), the turbidity observed for hydrophobic melittin derivative, MLT-5 in 1.0 M $NaClO_4$, may involve an interaction of perchlorate with the charged groups. Goto and Hagiwawa (1992) have shown that there is a stronger interaction of ammonium groups of melittin with $ClO_4^-$ compared to $Cl^-$. The presence of tetramers was inferred from the peak positions in gel filtration (Sephadex G-50) studies using 1.0 M NaCl as eluent. RNAse (13 700 Da) eluted at 19 mL, and insulin-B chain (3500 Da) eluted at 27 mL. The ω-aminoacylmelittin derivatives eluted at volumes of 19 ± 1 mL.

The attainment of high helicity in 1.0 M salts indicates the screening of repulsive interactions in the C-terminus residues. Another probable effect of high salt is the salting-out of monomers to generate tetramers through hydrophobic bonding (Bello et al., 1982). To test whether or not helix formation is promoted by hydrophobic association in the presence of salts, the effect of Gdn·HCl on helix formation of MLT was examined. Gdn·HCl would be expected to promote helix formation by screening charges but might have the opposite effect by solubilizing hydrophobic groups. Addition of 0.8 M Gdn·HCl to 30 μM MLT in 1.0 M NaCl resulted in lowering the ellipticity by 50% from −20 500 deg cm$^2$ dmol$^{-1}$ to −10 000 deg cm$^2$ dmol$^{-1}$, suggesting the hydrophobicity-induced association of melittin in high salts.

*Effect of Methanol on Peptide Conformation.* Melittin is known to adopt a monomeric α-helical structure in methanol (Weaver et al., 1989). In 95% methanol, all the ω-aminoacylmelittin derivatives at 30 μM exhibited helical structures with ellipticity values ranging from −21 500 to −23 600 deg cm$^2$ dmol$^{-1}$, similar to the ellipticity observed for MLT. The helix-stabilizing property of methanol is effective despite the charge repulsions in MLT and MLT-K.

*Effect of Temperature on Conformation.* The thermal stability of the ω-aminoacylmelittin derivatives was investigated at pH 2.3 and 7.2. At pH 2.3, 30 μM MLT shows a continuous increase in ellipticity from 0 to 100 °C. MLT-2 and MLT-4 show a small cold-induced denaturation, followed by the maintenance of the ellipticity at high temperatures (Figure 7a); while, for MLT-5, a small amount of cold- and heat-induced denaturation occurs. The melting profiles of MLT-5 converge at high temperatures with ellipticity in the range of −10 000 deg cm$^2$ dmol$^{-1}$, whereas the melting profiles for MLT-2, MLT-4, MLT-GG, and MLT-K converge at high temperatures with ellipticity in the range of −7500 deg cm$^2$ dmol$^{-1}$. The melting profiles for the peptides at 120 μM concentration (data not shown) are similar to the corresponding profiles at 30 μM. At pH 7.2 and at 30 μM peptide concentration, all the peptides behave like natural proteins except MLT and MLT-K. MLT shows a continuous increase in ellipticity. MLT-K shows only cold denaturation and gives a constant ellipticity from 30 to 100 °C. Other derivatives show cooperative melting profiles with cold and hot denaturation. The melting profiles for the melittin derivatives converge at high temperatures. At 120 μM peptide concentration, MLT and the ω-aminoacylmelittin derivatives show cooperativity with cold and hot denaturation. Also, at 120

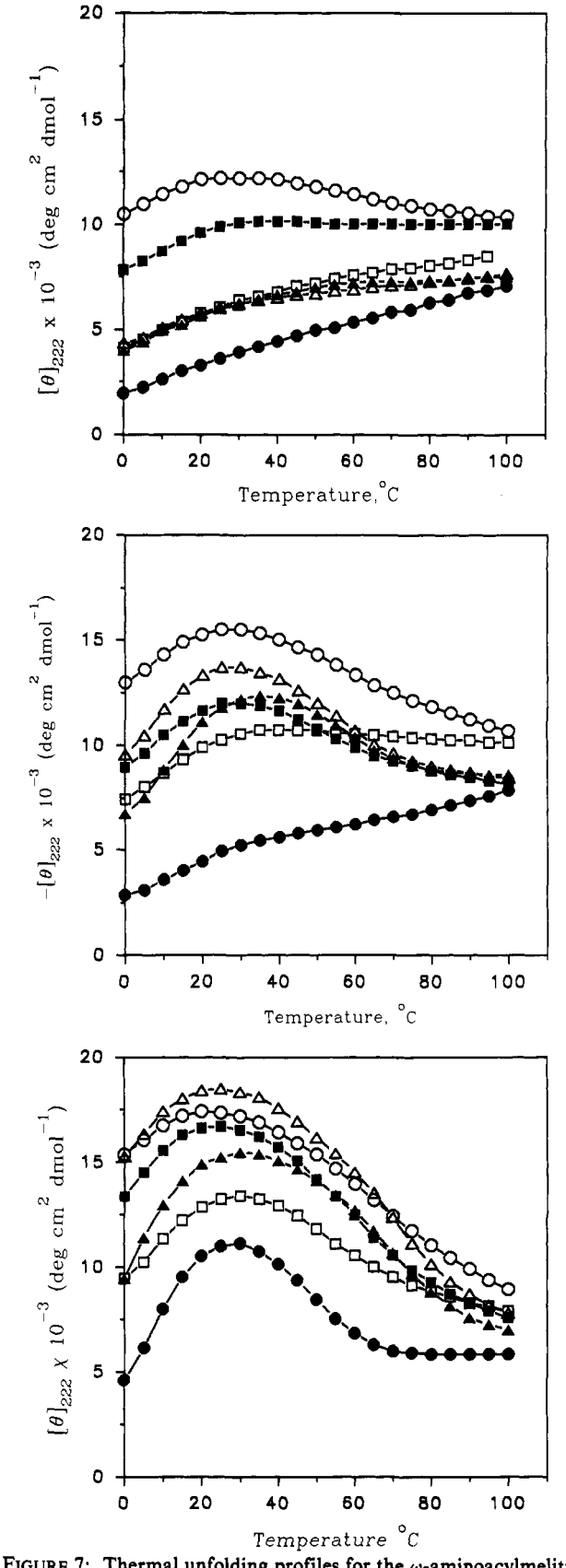


FIGURE 7: Thermal unfolding profiles for the ω-aminoacylmelittin derivatives (in 20 mM phosphate). (a, top) Peptide concentration 30 μM, pH 2.3. (b, middle) Peptide concentration 30 μM, pH 7.2. (c, bottom) Peptide concentration 120 μM, pH 7.2. (●) MLT; (▲) MLT-2; (■) MLT-4; (○) MLT-5; (△) MLT-GG; (□) MLT-K.

$\mu M$ concentration all of the peptides are more helical in the range of 0–60 °C than at 30 $\mu M$. In this study also, we see the dependence of helicity on the length of the acyl chain. The melting profile studies were not carried out at pH 11.2, because MLT-5 was turbid at pH 11.2 and the other derivatives showed turbidity upon heating.

## DISCUSSION

*Concentration and Chain Length Dependence of Conformational Transitions.* The transition of MLT from monomeric random coil to tetrameric helix is inhibited by the repulsion arising from the high net charge. This conclusion is indicated by the promotion of helix when the charge repulsions are reduced by (a) screening by salt, (b) diminution of charge by titration of the amino groups to high pH, or (c) acetylation of succinylation of the amino groups. The results reported here show that substitution of the increasingly longer amino acyl chains promotes tetrameric helix formation. There are three possible mechanisms by which this occurs: First, reduction of charge repulsions arising from greater distances between charges; second, a hydrophobic effect; third, changes in net charge arising from changes in $pK_a$. Included in the first mechanism is a reduction of the unfavorable interaction between the positive end of the helix macrodipole and the charge on Gly-1. In MLT-GG, the charge has been moved out as far as in MLT-5 (with the caveat that the peptide group in the glycylglycine chain of MLT-GG is rigid, while the pentanoyl chain of MLT-5 is more flexible), but the side chains of MLT-GG are much more polar than are those of MLT-5. Also, MLT-2 has polar substituents, the glycyl side chain residues. At pH 2.3, all of the peptides have the same charge, +6. At pH 2.3 MLT-2 and MLT-GG show little helicity, while MLT-5 is much more helical. The difference between MLT-5 and MLT-GG speaks against an effect of simply moving charges away from the main chain and supports a hydrophobic effect.

In MLT-4 and MLT-5 hydrophobic stabilization might arise from folding back of the acyl side chains onto the tetrameric core to shield partially exposed hydrophobic side chains as well as partially removing the apolar part of the acyl chain from water. The large reduction in the ellipticity value obtained for MLT and MLT-GG (from 40% to 14% and 55% to 31%) in the presence of 0.8 M Gdn·HCl is consistent with hydrophobic interactions in the core of the tetrameric helix. However, under similar conditions, MLT-5 showed a smaller reduction in ellipticity (from 58% to 45%); this might result from shielding of peptide hydrogen bonds in the helix by the folded-back acyl side chains. Gdn·HCl is a protein denaturing agent that diminishes the hydrophobic interactions in proteins by solubilizing hydrophobic groups (Wetlaufer et al., 1964). Gdn·HCl also solubilizes peptide groups (Nozaki & Tanford, 1970), which could contribute to the large loss of helix. The decrease in helicity induced by Gdn·HCl shows a large effect of denaturant even at the relatively low concentration of 0.8 M, which would normally be considered a high concentration for charge screening. Even in the presence of 1.0 M NaCl, 0.8 M Gdn·HCl has a large denaturing effect on MLT.

At pH 7.2, MLT-2 and, more so, MLT-GG show much greater helicity than at pH 2.3. MLT-5 also shows an increase, but of lesser magnitude than do MLT-2 and MLT-GG. The pH effects indicate that some amino groups are being titrated. In MLT-2 and MLT-GG, in which there are four $\alpha$-amino groups, the $pK_a$ values are expected to be below 8.0 [the $pK_a$ of the amino groups of GlyGly, GlyGlyGly, and GlyAlaAlaGly are 8.1, 7.9, and 7.9, respectively (Sober, 1968)], so that, on raising the pH from 2.3 to 7.2, an average decrease of 1–1.5 charges would ensue. This would promote helix formation. In MLT-4 and MLT-5, there must also be a decrease in charge, but of smaller magnitude, because shifting the amino groups farther out in the acyl chain should raise the $pK_a$. The $pK_a$ values of the amino groups of the 4- and 5-aminoalkanoic acids are >9.0 (Sober, 1968). However, in MLT-4 and MLT-5, one or more of the amino groups appears to have a $pK_a$ low enough to be partially titrated at pH 7.2. Quay and Tronson (1983) have estimated the $pK_a$ values of the amino groups of melittin from the reactivity of these groups toward trinitrobenzenesulfonate. The $pK_a$ values of the amino groups of monomeric MLT are 6.5 for Lys-21, 8.6 for Lys-23, and ≥9.6 for Lys-7, whereas for the tetrameric MLT, $pK_a$ for Lys-21 and Lys-23 was 7.4 for both (Quay & Tronson, 1983). Goto and Hagiwara (1992) have estimated the $pK_a$ for the N-terminal amino group of melittin to be 7.35 and for all the $\epsilon$-lysine groups of melittin to be ≥9.0 by acid titration and measuring the accompanied conformational change by CD. NMR studies of monomeric MLT yielded a $pK_a$ of 7.8 for the N-terminal amino group and ~9.0 for the $\epsilon$-amino groups of lysine residues (Lauterwein et al., 1980). $^{13}C$ NMR pH-titration studies of tetrameric melittin dimethylated (on amino groups) with [$^{13}C$]methyl groups indicate a $pK_a$ of 10.2 for Lys-7 and 9.4 for Lys-21 and 23 (Stanislawski & Ruterjans, 1987).

The change in ellipticity of MLT, at 30 $\mu M$, between pH 2.3 and 7.2 is small, suggestive of a small difference in charge. This might arise from the $pK_a$ of the terminal $\alpha$-amino group. However, a large charge difference from the titration of Lys-21 (assuming a $pK_a$ of 6.5) is not ruled out. At 120 $\mu M$ the ellipticity between pH 2.3 and 7.2 is greater. The mass action effect at 120 $\mu M$ drives the formation of helical tetramer, for which the stage is set by a charge reduction between pH 2.3 and 7.2. Since, the amino groups of MLT-4 and MLT-5 are, formally, far from the main chain and far from those on other chains in the tetramer, why are their $pK_a$ values not >9.0? A possibility is that they fold back on the tetramer and interact with it hydrophobically. This would bring the amino groups closer together and lower the $pK_a$ values. We must also consider differences in counterion binding, as between pH 2.3 and 7.2 there is a shift from shift from $H_2PO_4^-$ to $HPO_4^{2-}$. However, at pH 2.3 in HCl the results were similar to those using $H_3PO_4$. It is unlikely that $Cl^-$ and $H_2PO_3^-$ would have similar effects; therefore, the nature of the counterion may not be important in this result.

The ellipticity of MLT-K (MLT < MLT-K < MLT-2) appears to result from the combined effects of high charge density and the proximity of the $\alpha$-amino groups of the grafted lysine residues to the backbone. For MLT-K at pH 2.3 the net charge of +10 may account for the low ellipticity, despite the longer chain. Since the $pK$ values of the $\alpha$-amino groups are expected to be about 7.8 or less (because of the multiple charges), at pH 7.2 the charge would be reduced, promoting helix formation, but not as much as for MLT-2 and MLT-GG, which have smaller net charges. MLT-K at pH 7.2 would have a higher charge than MLT-5 and be less hydrophobic than is MLT-5. These factors would result in lesser helicity. MLT-K, from the structural point of view, is similar to MLT-2 if the charge on the $\alpha$-amino group of the grafted residue is considered or if a higher homolog of MLT-5 if the $\epsilon$-amino groups is considered.

In the methylated peptides (MLT-Me, MLT-2Me and MLT-5Me) there is little pH effect; this is expected, because the quaternary ammonium groups are not titratable. The increase in helicity in this series with increasing side chain

length is consistent with a reduction in charge repulsions. The hydrophobicity also increases with chain length, so that a clear decision is not evident. The relative importance of these two effects may not be the same as in the unmethylated series.

Another possible contribution to increased helicity in the aminoacyl series is an interaction between the negative pole of the helix dipole and the positive ω-ammonium groups of the ω-aminoacyl melittin derivatives. The long acyl chain would permit the positive charge to approach the negative pole. This could be promoted by hydrophobic interaction between the aminoacyl group and the tetramer.

At pH 2.3, the helix content of MLT-4 and MLT-5 is concentration-independent. Presumably, the helix content is on a plateau at 30 μM, and the transition to helical tetramer occurs at lower concentration. (A monomeric helix is unlikely, and has been observed only in alcohols, Weaver et al., 1989). At pH 2.3, the maximum ellipticity is about −11 400 deg cm$^2$ dmol$^{-1}$, less than the value of −18 600 deg cm$^2$ dmol$^{-1}$ at pH 7.2, which is less than the value of about −23 000 deg cm$^2$ dmol$^{-1}$ attained in high salt or in alcohols and −27 500 deg cm$^2$ dmol$^{-1}$ at high pH and high concentration. The results suggest that melittin may attain several levels of helicity, each determined by the conditions of the experiment. The helicity of the two halves of the molecule may be determined with some degree of independence, since one half (residues 1–10) is more hydrophobic than the other, while the second (residues 13–26) is the less hydrophobic and contains the highest charge density.

*Temperature-Induced Conformational Transitions.* The ellipticity–temperature profiles for MLT, MLT-2, MLT-GG, and MLT-K represent the monomeric, largely unfolded peptides at pH 2.3. These peptides maintain some residual structure throughout the temperature range studied, indicating that there may be some local order for these peptides. On the other hand, the more hydrophobic derivative, MLT-5, having some ordered structures at room temperature, melts at both lower and higher temperatures. All of the mellitin derivatives exhibit significant structures at temperatures near 100 °C. Such retention of structures at high temperatures (via hydrophobic interactions) have been suggested for proteins (Tanford, 1968; Bello, 1978) and has been likened to a "molten globular state" (Griko et al., 1988a,b; Privalov et al., 1986).

At pH 7.2 (30 μM), ω-aminoacylmelittin derivatives behave similarly to many proteins, exhibiting both cold-induced and heat-induced denaturation. However, the melting curves for MLT and MLT-K represent monomeric, largely unfolded peptides. At 120 μM peptide concentration, all the derivatives show cooperative melting profiles exhibiting cold- and hot-induced denaturation. All of the melting profiles (both at pH 2.3 and 7.2) approximately converge at higher temperatures with the ellpiticity values of −7000 to −10 000 deg cm$^2$ dmol$^{-1}$. It may be that these peptides adopt similar structures at high temperatures. An unresolved question is whether these peptides are monomeric or oligomeric at high temperature.

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