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Similarities in Melittin Functional Group Reactivities during Self-Association and Association with Lipid Bilayers†

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ABSTRACT: Competitive labeling of melittin over a range of concentrations in the presence and absence of liposomes provides a series of "snapshots" of the chemical reactivities of melittin's intrinsic nucleophiles. Distinct trends in apparent reactivities were observed for the Gly-1 α-amino group and the ϵ-amino groups of Lys-7 and Lys-21 and -23, over a range of concentrations, providing evidence for different forms of associated melittin in solution. The monomer–tetramer transition can be followed, in accord with structural details derived from X-ray crystallography. The reactivity behavior of the α-amino group of Gly-1 and the ϵ-amino groups of Lys-21 and Lys-23 suggests these groups undergo similar perturbations in their microenvironments during the monomer–tetramer transition in free solution. Similar changes in reactivity behavior occur upon association of melittin monomers with bilayer-forming lipids. Together, these findings suggest that the local environments of the N- and C-terminal segments have similar physicochemical properties in both the solution tetramer and the lipid-associated complex. The concentration dependence of the chemical properties of melittin is correlated with surface accessibility calculations which are used to provide a framework for interpretation. Aspects of several previously proposed models of membrane lysis can be accounted for by concentration-dependent properties of melittin.

The 26 amino acid peptide melittin is a potent α-helical cytotoxin comprising up to 50% of the dry weight of honeybee venom (King et al., 1976) and is frequently used to study protein–lipid interactions. Numerous mechanisms have been proposed to explain melittin's association with biological membranes and their consequent physical disruption, but the dynamic processes underlying these events remain poorly understood. Earlier models of melittin association with bilayers include surface adsorption (Schoch & Sargent, 1980), partial or full insertion (Terwilliger et al., 1982; Vogel et al., 1983), channel or pore formation (Tosteson & Tosteson, 1981; Vogel

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& Jahnig, 1986), and "rafts" of various sizes (Talbot et al., 1987; Raghunathan et al., 1990). However, quasielastic light scattering (Prendergast et al., 1982) and ^2H and ^{31}P NMR (Dufourcq et al., 1986) indicate that the melittin–phospholipid interactions are dynamic and heterogeneous, with reversible associations of various combinations of the component molecules, varying with temperature and concentration.

Attempts have been made to probe the role of individual residues in melittin in membrane lysis. However, introduction of reporter groups at the lysine residues (Stanislawski & Ruterjans, 1987; Altenbach et al., 1989) may affect the physicochemical properties of melittin, thereby altering its biochemical effects. A less invasive approach follows from the recent complete assignment of the 2D NMR[1] resonances of melittin as complexed with dodecylphosphocholine micelles (Ikura et al., 1991).

In the present study, we have applied the principles of competitive labeling (Kaplan et al., 1971; Young & Kaplan, 1989) to characterize the chemical reactivities of specific functional groups in melittin. In this approach, trace amounts of radiolabeled reagent are incorporated into the molecule of interest. The extent of label incorporation at each site is proportional to its apparent chemical reactivity. In this way, the functional groups act as intrinsic reporter groups. In practice, tritiated reagent is used, and the pattern of radiolabel incorporation provides a snapshot of the apparent reactivities for each modified group. In this study, we report competitive labeling studies of melittin at different concentrations in the presence and absence of liposomes with 1-fluoro-2,4-dinitrobenzene. Chemical modification with this reagent provides information on the microenvironments of the Gly-1, Lys-7, Lys-21, and Lys-23 nucleophiles at different concentrations of melittin.

Materials and Methods

Melittin, pepsin, egg phosphatidylcholine (type V-E), 1-fluoro-2,4-dinitrobenzene, all amino acids, peptides, and derivatives were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Melittin purity was assessed by amino acid analysis. It should be noted that a small amount of naturally occurring melittin is *N*-formylated at the amino terminal. We expect this to be the case in our commercial preparation, but this is not expected to influence the general validity of the results. Purity of the phosphatidylcholine was checked by thin layer chromatography using chloroform–methanol–28% ammonia (65:25:5) and found to be free of impurities. All of the above were used without further purification. Ultrapure urea (Schwarz/Mann, Cambridge, MA) was used throughout. Water used in HPLC was distilled, deionized, and filtered through 0.45-μm membranes from Millipore. The buffer used throughout was 5 mM NA_2HPO_4 and 0.1 M KCl at pH 7.5. HPLC grade acetonitrile and methanol was obtained from BDH. All other chemicals and solvents used were reagent grade or better. [^{14}C]FDNB and [^{3}H]FDNB were obtained from Amersham Corp. (Oakville, ON, Canada), and NEN Canada (Lachine, PQ, Canada) supplied the Aquasol-2 for scintillation counting.

Liposome Preparation. Liposomes were made using an HPLC pump, injector, and precolumn in-line solvent filter (Upchurch Scientific, Inc.) consisting of a passivated stainless steel frit (pore size 0.5 μm, thickness 1.6 mm) contained in a stainless steel fitting. Egg phosphatidylcholine and cholesterol (2:1, mol/mol) was deposited on the bottom of a glass test tube (16 × 125 mm) from chloroform solution and dried with a stream of dry nitrogen gas. Buffer (5 mM Na_2HPO_4, 0.1 M KCl, pH 7.5) was added to the tube and the lipid dispersed by vortexing for 1 min. A measured volume (≤2.0 mL) of this dispersion was then loaded by syringe into the HPLC sample loop and injected at a flow rate of 1.0 mL/min. Passage through the stainless steel frit breaks down the large lipid aggregates, producing multilamellar vesicles ranging in size from 300 to 400 Å in diameter.

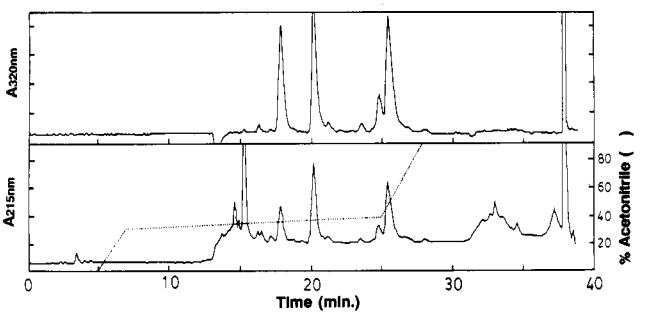

FIGURE 1: Gradient elution profile of a pepsin digest of Dnp-melittin. Eluate was monitored at 215 and 320 nm for the presence of peptide and Dnp chromophores, respectively. Peptides A, B, and C were collected, recycled, and identified by amino acid analysis. The deduced sequences of the peptides were A, residues 1–3 (Gly-Ile-Gly); B, residues 6–13 (Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu); and C, residues 20–26 (Ile-Lys-Arg-Ls-Arg-Lys-Gln-Glnamide). The radioactive purity of the radiolabeled Dnp-peptides was established via hydrolysis of the purified Dnp-peptides and purification of the constituent radiolabeled Dnp-amino acids. The specific activities of peptides A, B, and C were shown to result from radiolabeled Dnp-Gly, N^{ϵ}-Dnp-Lys, and N^{ϵ}-Dnp-Lys, respectively.

Functional Group/Peptide Mapping of Melittin. Melittin (5 mg) was dissolved in 2.0 mL of 8 M urea, to which was added 1.0 g of $NaHCO_3$, followed by 10 μL of 50% FDNB in acetonitrile (v/v). The reaction was protected from light and after 18 h was brought to pH 2 with concentrated HCl. Dinitrophenol was removed by ether extraction, and the Dnp-melittin was precipitated upon evaporation of residual ether by a nitrogen stream. The precipitated protein was pelleted on a bench-top centrifuge, washed successively with distilled water, 50% acetone, and 100% acetone, and lyophilized. Dnp-melittin (2.3 mg) was dissolved in 1.0 mL of 99% formic acid, and the solution diluted to 10% formic acid with distilled water. Pepsin (approximately 0.25 mg) was added and the solution incubated at 37 °C overnight. Two lyophilizations yielded a fluffy yellow powder. Separation of the Dnp-peptides was carried out on an Ultrasphere ODS reverse-phase C18 column (0.46 × 25 cm) using 0.01 N HCl with a gradient of AcCN. Eluate was monitored at 320 and 215 nm to detect Dnp-derivatives and peptides, respectively (Figure 1). Fractions (0.5 mL) corresponding to peaks A, B, and C were pooled from several runs, recycled with HPLC using the same solvent system, lyophilized, and hydrolyzed in 1.0 mL of 6 N HCl at 110 °C for 18 h, in vacuo). The hydrolysates were split into two portions, one of which was analyzed for the presence of Dnp-Gly and N^{ϵ}-Dnp-Lys by HPLC using 0.01 N HCl with a gradient of MeOH, while the second portion of the hydrolysate was used for amino acid analysis. On the basis of these compositions, peptides A, B, and C were assigned to the primary sequence of melittin, in accord with the peptic cleavage sites reported by Habermann (1972).

Sample Preparation. Stock solutions with equimolar concentrations of melittin and alanylalanine in buffer were made and appropriate aliquots transferred to screw cap Pyrex test tubes and made up to the desired concentrations. Aliquots

[1] Abbreviations: NMR, nuclear magnetic resonance; FDNB, 1-fluoro-2,4-dinitrobenzene; Dnp, dinitrophenyl; HPLC, high-performance liquid chromatography.

of stock liposome preparation were added to half of the samples to a final lipid concentration of 1 mg/mL, with buffer being substituted for the liposome preparation in the control (solution) studies. After liposome addition, samples were incubated for 15 min at room temperature. This was felt to be sufficient to ensure interaction between melittin and the liposome membranes. By comparison, the fast phase of melittin-induced erythrocyte lysis is complete by 15 min (DeGrado et al., 1982). After the incubation period, trace labeling was commenced (vide infra).

Competitive Labeling. (*i*) *^{3}H-Trace Labeling.* An aliquot (10 μL) of acetonitrile containing [^{3}H]FDNB (1.09 nmol, specific radioactivity 14.8 μCi/mmol) was added to duplicate samples for each concentration with vigorous stirring, and the reaction was left to proceed in the dark for 18 h with shaking. After the ^{3}H trace labeling, the reactions were left shaking in the dark overnight at either 37 °C or room temperature. Complete derivatization of all groups was accomplished by making each sample approximately 8 M in urea (through addition of 2.0 g of urea), adding 0.25 g of $NaHCO_3$ and 50 μL of 50% FDNB (v/v in acetonitrile), and reacting for 18 h at room temperature in the dark with shaking.

(*ii*) *^{14}C-Labeling.* Lyophilized stock solutions were dissolved in a minimal volume of $NaHCO_3$-saturated 8 M urea. To this was added 200 μL of a 25% [^{14}C]FDNB (v/v in acetonitrile) (2.0 mmol, 125 μCi/mmol) and reacted as above. Aliquots containing approximately 0.93 mg of ^{14}C-labeled melittin and 0.05 mg of ^{14}C-labeled alanylalanine were added to each [^{3}H]FDNB-labeled sample.

The ^{3}H/^{14}C-Dnp-labeled mixtures were brought to pH 2.0 and extracted with ether, and the chemically modified melittin was pelleted as above. The pellet was washed, lypophilized, and digested as above, and peptides A, B, and C were collected and counted. The alanylalanine in the supernatant was desalted by passage over Porapak Q and purified by HPLC using ammonium formate buffer at pH 3.0 and acetonitrile (Cockle et al., 1982).

To ensure radioactive purity of peptides A, B, and C, representative samples of each peak from different runs were divided into two portions. The first portion was counted directly, while the second was hydrolyzed. Purification of the Dnp-amino acids from these hydrolysates by HPLC and subsequent counting showed that the Dnp-peptides were sufficiently pure without recourse to a further purification step.

Liquid Scintillation Counting. All samples were dried in borosilicate counting vials, dissolved in 100 μL of 0.01 M HCl, and made up to 5 or 10 mL with Aquasol-2. Scintillation counting was done on a Beckman 1800 scintillation counter with automatic quench correction and dpm converter.

Solvent Accessibility Calculations. Solvent-accessible surface areas were calculated using molecular modeling software from Polygen (Waltham, MA). The recently refined crystal structure of melittin at 2.0 Å by Eisenberg, Gribskov, and Terwilliger (Protein Data Bank entry 2MLT; Bernstein et al., 1977) was used. Tetramers were generated from the crystallographic dimer using symmetry operations from the original report of the structure (Terwilliger & Eisenberg, 1982). A probe size of 1.4-Å radius was used.

Results

The radiolabeling of melittin and alanylalanine in the absence of liposomes provides a standard by which the amount of available [^{3}H]FDNB can be normalized from one sample to another (Kaplan et al., 1971). This procedure takes into account both variations in the amount of label added during the trace-labeling step (Young & Kaplan, 1989), as well as correcting for the effect of dilution of the nucleophiles over a range of concentrations. Each data point is the mean of duplicate experiments, with an experimental error of between 0 and 10%. The quality of the data is as good, or better, as in previous studies (Oomen & Kaplan, 1987, 1990). Functional group ^{3}H/^{14}C ratios were normalized with respect to the alanylalanine internal standard $_3$H/^{14}C ratios at each concentration. The concentration range studied spanned almost three orders of magnitude, from micromolar (1×10^{-6} M) to nearly millimolar (7×10^{-4} M).

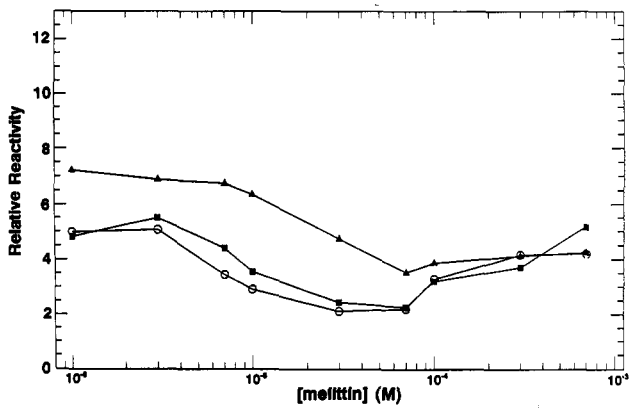

FIGURE 2: Free melittin in solution. Reactivity of melittin's functional groups relative to internal standard alanylalanine reactivity as a function of concentration. The data are for the Gly-1 N^{α}-amino group (■), Lys-7 N^{ϵ} (▲), and combined Lys-21/23 N^{ϵ}-groups (○). The values for Gly-1 have been divided by 10 to underscore their similarity to the combined Lys-21 and Lys-23 reactivities.

Melittin Free in Solution. These experiments provide information on the concentration dependence of melittin functional group reactivities when melittin is free in solution. As such, they also serve as the control for those experiments in which the influence of added liposomes were studied. The concentration-dependent reactivity profiles for the melittin functional groups relative to the internal standard are shown in Figure 2. The N-terminal glycine is the most reactive group in melittin toward FDNB, in keeping with the expected pK of α-amino groups, being an order of magnitude greater than the lysine nucleophiles. The trends in concentration dependence are very similar to the Lys-21/23 trends, however, so the Gly-1 values have been reduced by a factor of 10 for the purposes of comparison with the lysine groups. Lys-7 is the next most reactive residue, followed by the average value for lysines 21 and 23. Although differing in magnitude, the variation in Gly-1 and Lys-21/23 reactivity trends are virtually identical. In each case, there is at least a 50% reduction in reactivity as the concentration of melittin is increased from the micromolar range; most of the reactivity is then recovered at millimolar concentrations. The reactivity behavior of the Lys-7 residue is different from all other nucleophiles in that the relative reactivity is not recovered at the higher concentrations ($>5 \times 10^{-5}$ M). The absolute difference in reactivities between Lys-7 and Lys-21/23 reactivities is notable. Although the average value for Lys-21 and Lys-23 might be expected to be the same as that of the Lys-7 group, the net contributions of the Lys-21/23 groups is indicative of different microenvironments for their N^{ϵ}-amino groups as compared to Lys-7 at low concentrations of melittin. The reactivities of all of melittin's lysine N^{ϵ}-amino groups converge at higher concentrations ($>10^{-4}$ M).

Melittin in Association with Liposomes. In general, the reactivities of the lysine functional groups are significantly lowered in the presence of liposomes (Figure 2). At micromolar melittin concentrations, the reactivity profile of the lysine

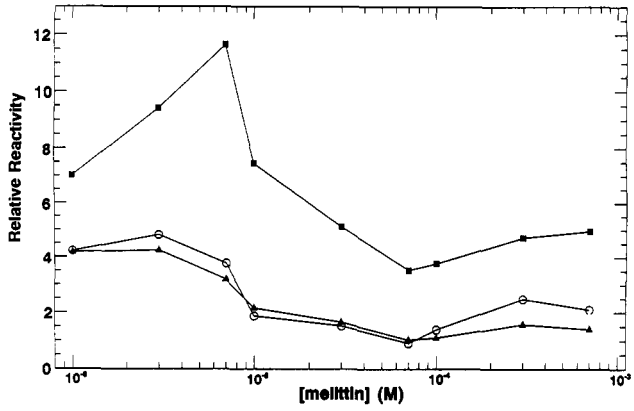

FIGURE 3: Melittin in the presence of liposomes. Gly-1 reactivity is divided by a factor of 10 as in Figure 2. Symbols are the same as in Figure 2.

residues is similar, though reduced, to that observed in the absence of liposomes. However, as millimolar concentrations are approached reactivities are depressed to a greater extent in the presence of liposomes. As with the control samples, [^{3}H]FDNB incorporation is about an order of magnitude greater in Gly-1 than the level of incorporation into the lysine residues. In contrast to the lysine residues, the reactivity of the Gly-1 N^{α}-amino group is substantially higher in presence of liposomes than in their absence, except at the highest concentrations (i.e., (1–7) × 10^{-4} M). The Gly-1 reactivity profile is more complex when liposomes are present, with a significant increase at 3 × 10^{-5} M, and decreasing at both higher and lower concentrations.

Comparison of melittin functional group reactivity in the presence and absence of liposomes can be made by calculating the L/C ratio (Oomen & Kaplan, 1987, 1990). The L/C ratio is calculated by dividing the relative reactivities obtained in the presence of liposomes by those obtained in the control (solution) studies. For an L/C ratio of 1, the reactivities are the same; L/C values greater than 1 indicate that liposomes increase the reactivity, and values less than 1 indicate a reduction in reactivity. An L/C for each of the functional groups summarizes the differential effects that liposomes had on melittin (Figure 3).

Discussion

Melittin Free in Solution. It is well established that melittin is predominantly monomeric at low (micromolar) concentrations and tetrameric at high (millimolar) concentrations and that the equilibrium position between these forms is highly sensitive to pH, ionic strength, and especially divalent cation concentration (Bello et al., 1982; Tatham et al., 1983). Tetramer association is also highly correlated with α-helix formation. In the crystal of the tetramer, about 90% of the peptide is α-helical, whereas in solution the monomer may have an α-helix content as low as 18% depending on buffer conditions (Bernheimer & Rudy, 1986). The concentrations we have studied encompass a range so that both major forms are present at the extrema. Melittin is predominantly monomeric at about 10^{-6} M and mostly tetrameric at concentrations greater than 10^{-4} M.

Previous studies using the present approach on glucagon in dilute solution have shown that excluded volume effects are detectable by competitive labeling (Oomen & Kaplan, 1990), but no attempts were made to correlate apparent reactivities with quantitative measures of local structure and microenvironment. As a first approximation to understanding the factors influencing the apparent reactivities of melittin's nucleophilic groups, the solvent-accessible surface areas of the N^{α} and N^{ϵ} atoms were calculated (Table I). Measures of solvent-accessible surfaces using this refined crystal structure provide estimates of reagent accessibility to the reactive nucleophiles in the tetrameric form. Because the monomer–tetramer transition is so strongly coupled to α-helix formation, the crystallographic dimer is a reasonable approximation to the dimer found on the tetramerization pathway (Schwarz & Beschiaschvili, 1988). However, solvent accessibility in the monomer is likely to be underestimated when based on the crystal dimer, due to the adoption of random-coil states in addition to the helical conformers present in solution. Realistic values for the Gly N^{α}-amino group in the monomer are expected to approach those of the lysine N^{ϵ} atoms (Table I).

Table I: Solvent-Accessible Surface Areas of Melittin Nucleophilic Groups

melittin form	Gly-1 (Å^2)	Lys-7 (Å^2)	Lys-21 (Å^2)	Lys-23 (Å^2)
monomer	28[a]	65	52	61
AB dimer	28	65	26	61
tetramer	25	65	26	22

[a] The value for this residue in solution is underestimated in the crystal and is expected to have a value similar to those of the lysines in the monomer. See the text for details.

The chemical reactivities of functional groups is influenced by short- and long-range interactions. Short-range effects include those due to local steric hindrance (van der Waals forces) and nonbonded interactions (electrostastic interactions). Long-range interactions include solvation and excluded volume effects (Minton, 1983). In turn, both long- and short-range interactions will influence the ionization state, and hence the reactivity, of protein nucleophiles. In the monomer, the N^{α} and N^{ϵ} amines are maximally accessible to modification reagent. Upon association to form tetramers, the accessibility of these groups to reagent is reduced due to close packing and subsequent decrease in solvent accessibility, as well as long-range excluded volume effects due to the bulk of the tetramer (Figure 5).

Another parameter influencing apparent reactivities is the microenvironment of each nucleophile within the tetramer, which may favor either the ionized or unionized (nucleophilic) amine, depending on the polarity of neighboring side chains and the local dielectric. In the monomer, solvent and counterion effects favor the ionized form of the amines, and there is little steric hindrance. In the tetramer, steric effects become more important but can be offset by an increase in the proportion of reactive nucleophile. Therefore, contributions from both ionization state and steric factors counter each other to lesser and greater extents during the monomer–tetramer transition, and the apparent reactivity is the sum of these two counterbalancing factors over all the species present in solution.

In the crystal tetramer, the Lys-7 side chain extends into solution (Figure 4). Lys-7 is the most solvent accessible of the nucleophiles, and its microenvironment is essentially unaltered upon tetramer formation from monomers (Table I, Figure 5). Consequently, changes in Lys-7 reactivity must reflect longer range influences, particularly differential changes in excluded volume effects during protomer association. These excluded volume effects are substantial, resulting in a 50% decrease in reactivity upon tetramer formation (Figure 2), with no change in solvent accessibility during this process (Table I). The effect is exactly analogous to the behavior of Lys-12 in glucagon, which is also solvent exposed and which displayed decreased reactivity upon trimerization of helical glucagon protomers (Oomen & Kaplan, 1990).

NMR studies indicate that the pK of the Gly-1 N^{α} is 7.7 in monomeric melittin (Lauterwein et al., 1980) and that it

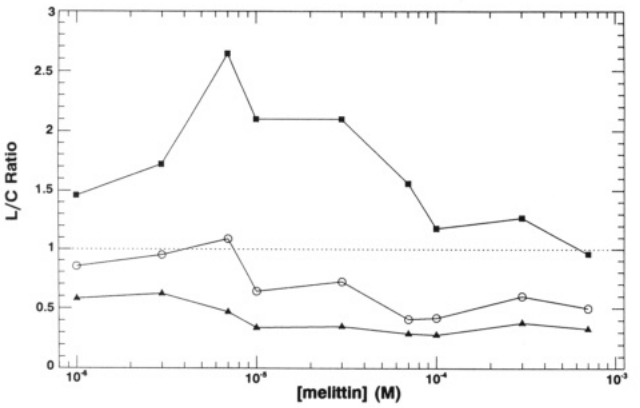

FIGURE 4: The net effect of liposomes on the chemical reactivities of melittin's nucleophiles as a function of melittin concentration. L/C is the liposome/control ratio as explained in the text. Symbols are the same as in Figure 2.

is largely unprotonated in the tetramer (Brown et al., 1980). The absence of a charge reduces repulsion between the N-terminal and the positively charged C-terminal segments of adjacent protomers. In the tetramer, Gly-1 experiences negligibly reduced solvent accessibility (Table I) but more substantial long-range steric hindrance due to the bulk of the tetramer (Figure 4). This latter effect is not seen in the monomer, but with no suppression of charge formation the amino terminal is free to ionize, shifting the equilibrium toward the less reactive protonated amine. Thus, these effects counteract one another at the extrema of the concentration range studied: during the transition from tetramer to monomer greater reagent accessibility to Gly-1 is offset by an increase in protonation, resulting in a net decrease in reactivity, whereas, in the monomer, accessibility is maximal for both solvent and FDNB. A previous chemical modification study of melittin employing trinitrobenzenesulfonate failed to detect significant modification of the Gly-1 N^{α}-amino group (Quay & Condie, 1983). We cannot explain their result, since we observe marked reactivity using FDNB.

Lys-21 and Lys-23 are more reactive in the monomer than in the tetramer (Figure 3), but one or both of these side chains becomes less reactive near the midpoint of the monomer–tetramer transition (Figure 3). Dissociation of the tetramer to the crystallographic dimers, and thence to monomers, would shift the ionization state in the tetramer in a manner similar to the Gly-1 residue. The C-terminal lysines may be expected to have increased reactivity in the tetramer, since the presence of adjacent, permanent positive charges on Arg-22 and Arg-24 would make the ionized form enthalpically unfavorable.

In the monomeric form of melittin, the side chains of Lys-21 and Lys-23 should be almost as solvent accessible as Lys-7 (Table I). Although lysines 21 and 23 are close to each other, they have different accessibilities and microenvironments upon dimer and tetramer formation. The average reactivity of these two groups changes in a manner similar to that of Gly-1, suggesting that, in the tetramer, reactivity lost due to decreased accessibility and excluded volume is recovered to some extent by a p*K* shift to favor the unionized form of at least one of these residues. Lys-21 is likely to be more reactive than Lys-23 in the tetramer, since th N^{ϵ} atom is surrounded by apolar side chains (Ile-17, also Leu-6 of an adjacent monomer) and therefore will have a lower p*K* value. Conversely, Lys-23 is surrounded by polar functional groups donated by neighboring arginine and glutamine side chains and also has structured solvent associated with it in the crystal structure, indicating a preference for the charged form of the amine.

Melittin in Association with Liposomes. The reactivity of the amino terminal is greater in the presence of liposomes than in their absence at all concentrations except the highest (Figure 4). Enhanced apparent reactivity of amines in the presence of liposomes, as seen for Gly-1, has been noted for the Lys-B29 N^{ϵ} group of insulin in a similar study (Oomen & Kaplan, 1987). Such effects may be characteristic of amines at particular locations within the bilayer–water interface. As the concentration of melittin is increased, Gly-1 reactivity increases

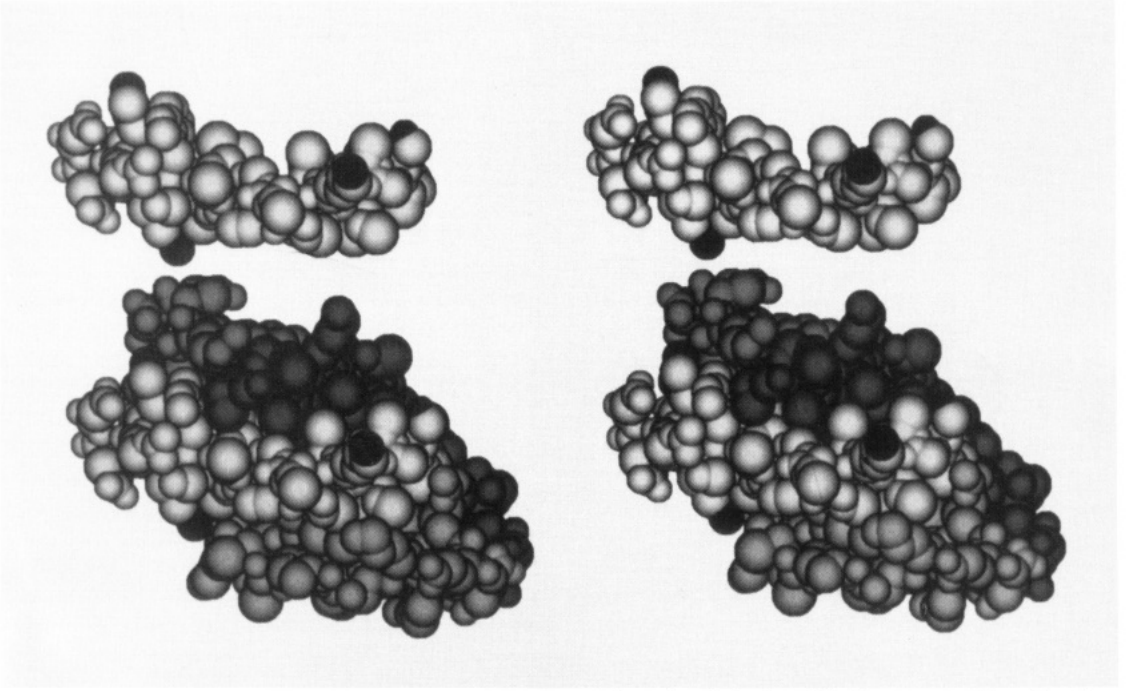

FIGURE 5: Stereopair of melittin. The crystallographic monomer is shown independently (top) and in the context of the other three protomers which make up the crystallographic tetramer (bottom). The other three protomers are in darker shades of gray. The nitrogen atoms of the primary amine nucleophiles labeled in this study are shaded black in the top monomer, and their positions are similarly located in the tetramer for comparison. They are, from left to right, Lys-23, Lys21, Lys-7, and Gly-1. Only the Lys-7 N^{ϵ} atom remains sterically unhindered upon tetramer formation from the monomer. This display was derived from Protein Data Bank file 2MLT, deposited by Eisenberg et al. (1991).

but then converges to the value found for tetrameric melittin in solution. These results suggest that the sum of all contributions that help to determine the conformation of this segment of the peptide is similar in both instances, allowing the N-terminal to associate with the bilayer in a melittin–lipid complex that closely mimics the microenvironment of the N-terminus in the tetramer. For the monomer, conformational energy calculations of melittin in an apolar environment reproduces the crystal structure quite well (Pincus et al., 1982), supporting the role of local environment in determining melittin conformation.

Melittin interaction with lipid bilayers is qualitatively different below peptide concentrations of 10^{-6} M, since lysis is not observed at these concentrations (Sessa et al., 1969) and monomeric melittin has been shown to adsorb to lipid bilayers at submicromolar concentrations (Schoch & Sargent, 1980). Adsorption would be a prerequisite for penetration of the N-terminal into the hydrocarbon phase at a shallow angle, leaving the charged C-terminus solvent-exposed (Terwilliger et al., 1982) but taking advantage of peptide amphiphilicity.

At low concentrations, the reactivity trends of all lysine groups are qualitatively similar to those of the solution studies (Figure 4). The reactivity of Lys-7 shows a parallel and proportionate decrease in reactivity at all concentrations when liposomes are present, being approximately one-third to one-half of the solution values. The magnitude of this decrease is stable at concentrations of 10^{-5} M or greater, with little variation in the L/C ratio (Figure 4). The apparent reactivity of tetramer Lys-7 residues in solution is very close to the value for the monomer in the presence of lipids, probably for the same reason, i.e., steric shielding, in this case by the lipid bilayer to which the monomer is adsorbed, rather than excluded volume effects from the tetramer. This suggests that the same conformational and environmental changes that account for the reactivity trends of Lys-7 in solution may also be present here and that Lys-7 does not enter the membrane but remains uniformly accessible throughout most of the concentration range.

At low concentrations, apparent Lys-21/23 reactivity trends run parallel to those of Lys-7 and appear relatively unaffected by the presence of liposomes. However, at higher concentrations these N^{ϵ} groups have a lower apparent reactivity. The simplest explanation for these findings is that when monomers are associated with the lipid bilayer, the C-terminal segments remain exposed to solvent, but one or both of the lysines become less ionized, thereby cancelling excluded volume effects from the lipid bilayer.

Certain structural features of the lipids may promote increased reactivity. Hydrogen bonding of the unprotonated amine to a cholesterol hydroxyl group or to one of the glycerol backbone ester carbonyls could increase the nucleophilicity of the nitrogen amine via intermolecular field effects. The dielectric constant of membrane–water interfaces changes from a value of 78 in the bulk water to 2 in the acyl chain region of the lipid bilayer, a distance of about 15 Å (Thorne & Duniec, 1983). A deprotonatd amine need not extend deeply into the bilayer to experience a significant change in effective dipole which would stabilize the reactive form of the nucleophile. By contrast, reactivities may be reduced by interactions with phospholipid headgroups as during the formation of metastable lipid–melittin complexes (Dufourcq et al., 1986), which have been observed for other amphipathic peptides.

Published studies have each measured different properties of melittin at different concentrations; the absence of standard conditions probably accounts to some extent for the many models of melittin–lipid association (Bernheimer & Rudy, 1986; Raghunathan et al., 1990). It is reasonable to assume that no single model can explain all the data unless it takes account of the dynamic properties of these peptide–lipid assemblies and the full range of possible association states. The current study clearly demonstrates the importance of concentration on the properties of melittin.

Concentration-Dependent Lysis. In the crystal structure of the melittin dimer, the apolar residues of the protomers form a contiguous hydrophobic patch which is clearly segregated from the charged residues. The pincer-like shaped dimer spans a distance of approximately 30 Å between the N-terminal Gly-1 residues, corresponding to the thickness of the hydrocarbon region of the lipid bilayer. The melittin dimer can be regarded as a bipolar amphiphile: two antipodal polar regions connected by an a polar one. The precise dimensions of these regions relative to one another provides the basis for melittin's ability to rearrange phospholipids and stabilize them in different aggregate forms. Phospholipid bilayer membranes are stable structures with isotropic physical properties that are the result of balanced forces acting on them. These forces can be altered by the adsorption of melittin and its insertion into the lipid bilayer, changing the interfacial free energy of the outer monolayer and resulting in a new equilibrium state. Changes in membrane structure following destabilization of the bilayer may result in exposed edges that can be stabilized by melittin dimers.

Edge stabilization would occur when dimers associate in the membrane to form small pores, such as the tetrameric ion channels observed at low concentrations of melittin (Tosteson & Tosteson, 1981). These pores could enlarge with the addition of melittin protomers. At higher melittin concentrations, pores would come into contact with one another, and the bilayer would break down to an equilibrium mixture of smaller vesicular structures as reported by Dufourcq et al. (1986). Analogous surface and membrane-lytic activity has been observed in staphylococcal δ-hemolysin (Morgan et al., 1986) and the 33-residue ichthyotoxic peptides from the sole, *Pardachirus pavoninus*, the pardaxins (Thompson et al., 1986). "Edge-active" detergents have been observed to mediate such disc-to-vesicle transitions in phospholipid dispersions (Fromherz et al., 1986), and amphiphilic peptides such as apolipoproteins (Segrest, 1977) and glucagon (Epand & Sturtevant, 1982), as well as melittin (Prendergast et al., 1982), have repeatedly been observed to form discoid aggregates with lipids. Melittin is able to stabilize the edge of discoid phospholipid bilayers because the peptide provides an interface that satisfies the free energy requirements of the lipid hydrocarbon region, the polar headgroup region, and the surrounding aqueous medium.

Conclusion. Competitive labeling has provided a sensitive nonperturbing approach to following the conformational transition of melittin from a mixture of monomeric conformers in dilute solution to the tetrameric form. Apparent chemical reactivities of the nucleophilic groups of melittin were concentration dependent, decreasing in a manner consistent with tetramer formation from monomers. The monomer–tetramer transition could also be followed in the presence of multilamellar liposomes in order to investigate the importance of melittin concentration on the biochemical action of melittin on membranes. Similar reactivity profiles were obtained for melittin in the presence or absence of liposomes. The microenvironment of the N-terminal segment has similar properties in the tetramer and upon association with lipid bilayers, as measured by Gly-1 α-amino group reactivity. The qualitative similarity of apparent reactivity profiles for the other

nucleophilic groups and the estimation of solvent accessible surface areas suggest that excluded volume effects are important modifiers of functional group reactivity. Thus, although decreased in magnitude, apparent reactivities suggest that the same conformational transitions take place in the presence of liposomes and that these latter, in some respects, may provide association surfaces similar to melittin's own. The results support the idea of a multistep process of bilayer adsorption, destabilization, and disruption to smaller melittin–lipid complexes that is highly sensitive to the concentration of melittin.

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