Interactions of Melittin, a Preprotein Model, with Detergents†

Eva Knöppel, David Eisenberg, and William Wickner*

ABSTRACT: Bee venom melittin is a water-soluble tetramer of identical polypeptide chains. Each chain has 26 residues. The 20 N-terminal residues are hydrophobic and the 6 C-terminal residues are basic. Melittin has been shown to integrate into natural and synthetic membranes and to lyse a wide variety of cells. To understand how a water-soluble protein can spontaneously partition into a membrane, we have studied the interaction of melittin with micelles of deoxycholate (DOC), Brij 58, and sodium dodecyl sulfate ($NaDodSO_4$). Circular dichroism spectra showed that $NaDodSO_4$, an ionic detergent, and Brij 58, a nonionic detergent, caused similar major changes in the protein's conformation. Gel filtration studies revealed that melittin forms mixed micelles with either Brij or DOC. The melittin–DOC mixed micelles have 2 mol of DOC per mol of melittin. Cross-linking studies with dimethyl suberimidate confirmed that the protein is a tetramer and showed that it becomes monomeric either in mixed micelles with Brij or DOC or in butanol. Despite this major structural change of melittin in the presence of an amphiphile, the covalently cross-linked form is as active in human erythrocyte lysis as the native protein.

Are proteins secreted across membranes or assembled into membranes by assuming two or more distinct conformations? A remarkably wide variety of globular proteins have been classified either as water soluble or as integral to cellular membranes in their single, stable, observed conformation. Water-soluble proteins have most of their hydrophobic amino acid residues buried in the interior, while their surface is comprised of polar residues (Eisenberg, 1970). With few exceptions (Goodman, 1958), soluble proteins do not bind nonionic detergents (Clarke, 1975) but will be denatured by $NaDodSO_4$[1] (Reynolds & Tanford, 1970). In contrast, integral membrane proteins can only be separated from their lipid bilayer with the aid of detergents and need detergents for continued dispersal in aqueous buffers. While many soluble proteins bind 1.4 g of $NaDodSO_4$ per g of protein (Reynolds & Tanford, 1970), integral membrane proteins bind up to 3 g/g of protein (Robinson & Tanford, 1975). When detergent is removed, the hydrophobic surfaces of integral membrane proteins can only bind to each other and aggregation results. Capaldi & Vanderkooi (1972) categorized proteins by the polarity of their amino acids and noted that proteins of both classes have broadly distributed polarities with substantial overlap between the classes. The different solubility properties were therefore presumed to be a result of the spatial distribution of these residues in the folded protein.

Melittin is the best characterized member of a small class of proteins which are both water-soluble and which can spontaneously assemble into membranes [other examples are staphylococcal α-toxin (Weissman et al., 1966; Freer et al., 1966), streptolysin S, and the Pr_{IV-VII} precursor of yeast cytochrome oxidase (Poyton & McKemmie, 1976)]. Half of the protein of bee venom is melittin. Its abundance has facilitated its isolation (Haberman & Reiz, 1965), the determination of its amino acid sequence (Haberman & Jentsch, 1967), and studies of its structure and function. Melittin is a water-soluble tetramer of identical subunits (Haberman & Reiz, 1965), each of 26 amino acid residues (Figure 1). It has a strikingly hydrophobic amino acid composition (only 27% polar residues) for a soluble protein (Haberman & Kowallek, 1970). The sequence of melittin is strongly amphipathic; 19 of the N-terminal 20 residues are hydrophobic and the C-terminal 6 residues are polar. This was thought to provide a structural rationale for the lytic activity of melittin toward cells as disparate as bacteria and erythrocytes (Haberman & Jentsch, 1967; Sessa et al., 1969; Hegner, 1968; Mollay et al., 1976). Bee venom contains other lytic factors, such as phospholipase and hyaluronidase, which may contaminate impure preparations of melittin (Haberman & Reiz, 1965). However, chemical synthesis of melittin has established that this small protein, although devoid of catalytic activity, is itself a potent lytic agent (Schröder et al., 1971). Studies using synthetic melittin analogues lacking a few residues at one or the other terminus have shown that melittin's lytic potency cannot be simply ascribed to its detergent properties (Schröder et al., 1971; Haberman & Kowallek, 1970). Melittin has been shown to assemble into both natural and synthetic membranes (Sessa et al., 1969; Williams & Bell, 1972; Mollay & Kreil, 1973; Mollay, 1976; Verma & Wallach, 1976; Dufourcq & Faucon, 1977; Dawson et al., 1978). It is of interest in this regard to note that the amino acid sequence of melittin resembles the precursor forms of secretory and membrane proteins (Blobel & Dobberstein, 1975a,b; Milstein et al., 1972; Inouye et al., 1977; Randall et al., 1978; Konings et al., 1975; Sugimoto et al., 1977; Chang et al., 1978) in having a basic and hydrophobic N terminus, followed by a polar region. Melittin and nascent preproteins may assemble into membranes by similar mechanisms, reflecting their structural similarity.

Like biological membranes, detergent micelles have a polar surface and an apolar core. They have served as useful models for membranes. We have therefore investigated the interaction of melittin with "mild" detergents and report that it undergoes a major change in secondary and quaternary structure during this interaction. Major structural changes have been described for hemoglobin, immunoglobulin, and contractile proteins (Casper & Cohen, 1969) in different steps of their function. Melittin may need to sequentially mask and expose its hydrophobic residues as it assembles into membrane bilayers.

Experimental Procedure

Materials. Melittin, Triton X-100, Brij 58 [poly(ethylene glycol) (20) cetyl alcohol], and deoxycholate were purchased

† From the Molecular Biology Institute and the Departments of Biological Chemistry and Chemistry, University of California, Los Angeles, Los Angeles, California 90024. *Received February 13, 1979.* This work was supported by grants from the National Institutes of Health, National Science Foundation, and American Cancer Society.

[1] Abbreviation used: $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/79/0418-4177$01.00/0

Residue No.	1				5					10					15					20					25		
Sequence*	$^{+}H_3N$-Gly		Ile	Gly	Ala	Val	Leu	Lys	Val	Leu	Thr	Thr	Gly	Leu	Pro	Ala	Leu	Ile	Ser	Trp	Ile	Lys	Arg	Lys	Arg	Gln	Gln-$CONH_2$
P_α		.57	1.08	.57	1.42	1.06	1.21	1.16	1.06	1.21	.83	.83	.57	1.21	.57	1.42	1.21	1.08	.77	1.08	1.08	1.16	.98	1.16	.98	1.11	1.11
$<P_\alpha>$				←	1.10					→		←	0.80		→		←	1.11				→					
P_β		.75	1.60	.75	.83	1.70	1.30	.74	1.70	1.30	1.19	1.19	.75	1.30	.55	.83	1.30	1.60	.75	1.37	1.60	.74	.83	.74	.93	1.10	1.10
$<P_\beta>$				←	1.24					→		←	1.00		→		←	1.24				→					
P_t											.96	.96	1.56	.59	1.52												
$<P_t<$												←	1.02		→												
												←	1.12		→												

FIGURE 1: The amino acid sequence of bee melittin from *Apis molifera* (Haberman & Jentsch, 1967) and the empirical parameters of its folding, as defined by Chou & Fasman (1978). A value of $\langle P_\alpha \rangle$ greater than unity for six residues or of $\langle P_\beta \rangle$ greater than unity for five residues indicates a tendency for the α or β structure, respectively. According to Chou & Fasman (1978), when regions in proteins contain both α- and β-forming residues, the region is in a β sheet if $\langle P_\beta \rangle > \langle P_\alpha \rangle$. Thus, from Figure 1, it is clear that a strict application of their rules would suggest β-sheet interactions in melittin in aqueous solution. In the scheme of Chou & Fasman (1978), a bend is predicted by the values of two parameters. One, P_t, is computed from bend frequencies of residues in four positions and must be greater than 0.75×10^{-4}. For the sequence Thr-Thr-Gly-Leu, P_t is 1.2×10^{-4}, thereby satisfying the first condition. Moreover, for melittin from *Apis dorsata*, threonine in position 10 is replaced by serine, and P_t becomes 1.7×10^{-4}, much larger than the cutoff value. On the other hand, for melittin from *Apis florea*, residue 10 is replaced by alanine, and P_t becomes 0.9×10^{-4}. The second requirement for a predicted bend is that the average of bend probability, $\langle P_t \rangle$, is greater than $\langle P_\beta \rangle$ and $\langle P_\alpha \rangle$ for a given sequence. It can be seen that $\langle P_t \rangle$ for the tetrapeptide in position 10–13 is larger than $\langle P_\alpha \rangle$ but smaller than $\langle P_\beta \rangle$. However, for the five residues Thr-Thr-Gly-Leu-Pro, $\langle P_t \rangle$ is greater than both $\langle P_\alpha \rangle$ and $\langle P_\beta \rangle$. Thus, the backbone may bend along residues 10–14.

from Sigma Chemical Co. Bee venom was purchased from the Nutritional Biochemical Corp. Sephadex G-50 fine was from Pharmacia. Dimethyl suberimidate was the generous gift of Dr. G. Gilliland and Dr. J. Collier, who purchased it from Pierce Chemicals. [^{3}H]Deoxycholate was obtained from New England Nuclear.

Melittin Isolation. All bee venom and melittin samples were dissolved in 0.02 M NaP_i (pH 7.2) at 60 mg/mL and filtered through a 5 × 45 cm column of Sephadex G-50 fine in 0.02 M NaP_i (pH 7.2) at room temperature.

A_{280} was monitored and the peak fractions were pooled (80 mL; $K_D = 0.5$; $A_{280} = 20$). Purity of melittin was established by $NaDodSO_4$ gel electrophoresis (see Results and Figure 5) and by amino acid analysis, including the absence of five amino acids. The purity of the melittin has been confirmed by crystallization (unpublished experiments).

Chemical Cross-Linking. Except where otherwise noted, cross-linking (Davies & Stark, 1970) was for 16 h at 23 °C in an 0.2-mL reaction with 1 mg of dimethyl suberimidate, 90 μg of melittin, and 0.2 M triethanolamine chloride (pH 8.5).

Gel Electrophoresis. Samples (5 μL) were mixed with 5 μL of sample buffer (50% glycerol, 5% $NaDodSO_4$, 0.05% bromophenol blue, and 0.5 mM dithiothreitol) and heated at 100 °C for 3 min. They were electrophoresed in 1-mm thick slab gels as described by Laemmli (1970) but with 6 M urea, 15% acrylamide, and 0.8% bis(acrylamide) in the resolving (lower) gel. Gels were stained for 24 h in 0.04% coomassie blue and 3.5% perchloric acid and destained in 3.5% perchloric acid.

Results

Prediction of Secondary Structure from the Amino Acid Sequence. We have applied the empirical rules for protein secondary structure developed by Chou & Fasman (1978) to melittin (Figure 1). They indicate that two regions of the melittin polypeptide (residues 2–9 and 15–20) might form either α-helical or β-sheet interactions. As shown in Figure 1, a strict application of these rules suggests that β-sheet interactions are strongly favored. Similarly, the empirical rules for β bends in proteins (Chou & Fasman, 1978) suggest that melittin may have a β bend in the region of residues 10–14.

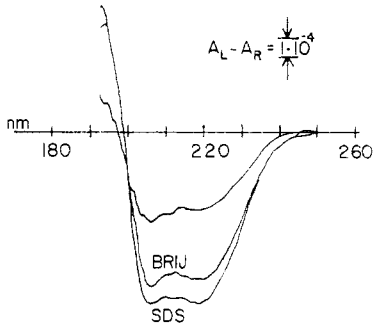

FIGURE 2: The effect of detergents on circular dichroism spectra of melittin. Spectra were recorded in an 0.2-mm cuvette. Samples contained 0.3 mg/mL melittin, 0.05 M Tris-HCl, pH 8.8, and, where indicated, 1% Brij 58 or $NaDodSO_4$.

Circular Dichroism. It has been reported (Dawson et al., 1978) that the CD spectrum of melittin in aqueous solution is characteristic of "an extended structure" and that $NaDodSO_4$ converts it to a highly helical form. We find (Figure 2) that melittin in aqueous solution (0.05 M Tris-HCl, pH 8.8) has a signal of the form that reflects significant regions of α helix. This signal is more pronounced in the presence of $NaDodSO_4$. This strongly denaturing detergent has been shown (Visser & Blout, 1971; Hunt & Jirgensons, 1973; Jirgensons, 1976; Yang et al., 1977) to convert a wide variety of proteins to α-helical structures. The nonionic detergent Brij 58 caused a similar increase in the magnitude of the circular dichroism spectrum, presumably reflecting an increased proportion of α helix. The spectrum of melittin also reflects α-helical content in other aqueous buffers (0.02 M phosphate, pH 7.2, and both the Tris and phosphate buffers with 0.15 M KCl), but the intensity of the signal varies somewhat with the composition of the buffer (data not shown). The difference between the secondary structure predictions by empirical rules based on amino acid sequence (β structure) and CD spectra (α helix) is discussed below.

Gel Filtration. Melittin is soluble to at least 60 mg/mL in phosphate buffer at neutral pH. While it is common for proteins to bind $NaDodSO_4$, it is quite rare for soluble proteins to bind nonionic detergents such as Brij 58. To determine directly whether the detergent-induced change in the circular

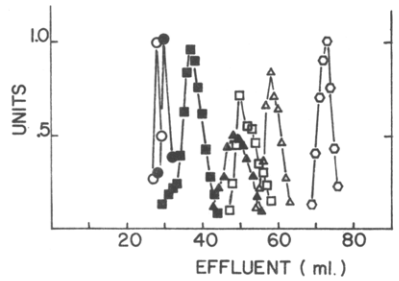

FIGURE 3: Gel filtration of melittin. Samples (1.0 mL in 0.1 M Tris-HCl, pH 8.8) were applied to a 1.5 × 46 cm Sephadex G-50 fine column in 0.1 M Tris-HCl, pH 8.8. Units of blue dextran 2000 (Pharmacia), lysozyme, and melittin were A_{700}, A_{280}, and A_{280}, respectively. Units of [^{3}H]deoxycholate or $^{32}PO_4$ were 10^4 cpm. Symbols: (○) blue dextran; (●) melittin in the presence of 1% Brij 58; (■) melittin in the presence of 1% deoxycholate; (▲) melittin in the absence of detergent; (□) lysozyme in the absence of detergent; (△) [^{3}H]deoxycholate;(○) inorganic $^{32}PO_4$. All samples contained blue dextran and $^{32}PO_4$ as markers; these always emerged within 1 mL of the indicated peak positions. Successive filtrations were as follows: (1) lysozyme (3 mg); (2) melittin (3 mg); (3) melittin (3 mg) with 1% Brij 58 in the sample and column buffer; (4) melittin (3 mg) with 1% deoxycholate in the sample and column buffer; (5) [^{3}H]deoxycholate (1%; 10^6 cpm) with 1% deoxycholate in the sample and running buffer.

Table I: Gel Filtration of Melittin[a]

sample	K_D (G-50)
melittin	0.46, 0.49
+ Brij	0.04, 0.04
+ deoxycholate	0.18, 0.19
lysozyme	0.50
deoxycholate micelles	0.64

[a] Gel filtrations were performed as described in Figure 3. Each value was obtained from an independent filtration.

dichroism spectrum reflected binding, melittin was filtered through Sephadex G-50 fine (Figure 3). When there was no detergent present, melittin (closed triangles) emerged at a K_D (fractional position from excluded volume, $K_D = 0$, to column volume, $K_D = 1$) of 0.48. The peak width and position were comparable to that of lysozyme (open squares; $K_D = 0.50$), a protein of similar molecular weight. When melittin was dissolved in a 1% solution of Brij 58 and filtered in the presence of this detergent, the protein emerged in a discrete, included peak (closed circles; $K_D = 0.04$) near the void volume of the column. This is consistent with the high micelle molecular weight of 82000 for this detergent (Helenius & Simons, 1975) which is presumably reflected in a high molecular weight of the Brij–melittin mixed micelles. In 1% deoxycholate, the protein emerged with a K_D of 0.18 (closed squares), a position distinct from that of either detergent-free melittin or deoxycholate micelles (open triangles). That this was indeed a mixed micelle species was confirmed by filtering melittin through Sephadex G-50 in the presence of [^{3}H]deoxycholate (Figure 4), a method described by Hummel & Dryer (1962). Measurement of the additional deoxycholate which emerged from the column with melittin showed that there were two deoxycholate molecules per melittin polypeptide chain. These and other gel filtration experiments are summarized in Table I.

Covalent Cross-Linking. Melittin was treated with the bifunctional cross-linking reagent dimethyl suberimidate (Davies & Stark, 1970) and electrophoresed on $NaDodSO_4$–polyacrylamide gels to measure the effect of detergents and solvents on its quaternary structure. Without cross-linking, our melittin preparations showed a single band of appropriate molecular weight relative to lysozyme (Jolles et al., 1963) and M13 coat protein (Nakashima & Konigsburg, 1974) standards (Figure 5A, left panel). Incubation of melittin with dimethyl suberimidate at concentrations of 0.1–5.0 mg/mL leads to progressive cross-linking to a tetrameric structure. This tetramer is the major species when from 10 to 60 μg of melittin was cross-linked (Figure 5B, left panel), indicating that the reaction is intramolecular. In contrast, only small amounts of multimers are seen when melittin is cross-linked in the presence of 1% Brij 58 (Figure 5A, center panel) or 1% deoxycholate (Figure 5A, right panel). When increasing concentrations of melittin were cross-linked in the

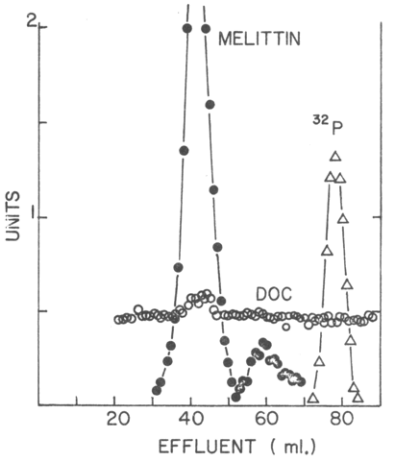

FIGURE 4: Binding of deoxycholate to melittin. A 3-mL sample (3.5 mg of melittin, 1% deoxycholate, 1.5×10^5 cpm of [^{3}H]deoxycholate, and 10^5 cpm of $^{32}PO_4$) was applied to a 1.5 × 46 cm Sephadex G-50 fine column in 0.1 M Tris-HCl, pH 8.8, 1% deoxycholate, and 5×10^4 cpm of [^{3}H]deoxycholate per mL. One-milliliter fractions were assayed for A_{280}, ^{3}H, and ^{32}P. One unit is 1 A_{280}, 10^5 cpm of ^{3}H, or 10^4 cpm of ^{32}P. Stoichiometry calculation (1% deoxycholate is 24 mM): $(5 \times 10^3$ excess cpm$)[[10^{-3}$ L$/(4.9 \times 10^4$ cpm$)](24 \times 10^{-3}$ mol/L$)] = 2.45 \times 10^{-6}$ mol of deoxycholate and 3.5 mg of melittin $\times$ [(1 mol)/$(2.84 \times 10^6$ mg$)] = 1.23 \times 10^{-6}$ mol of melittin; [$(2.45 \times 10^{-6}$ mol of deoxycholate$)/(1.23 \times 10^{-6}$ mol of melittin$)$] = 1.99 mol of deoxycholate per mol of melittin.

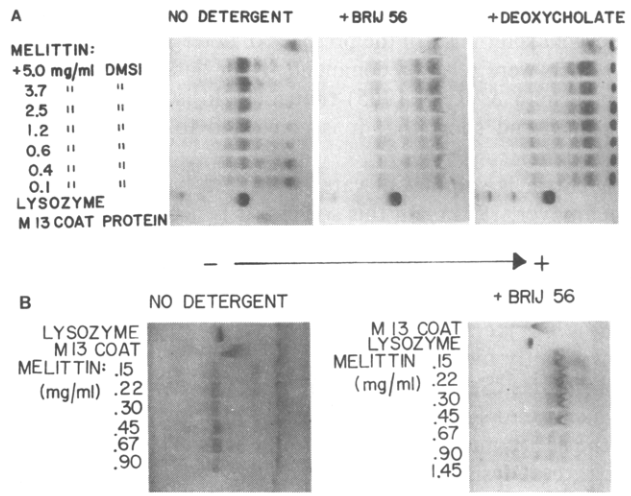

FIGURE 5: Cross-linking melittin with dimethyl suberimidate. Melittin was cross-linked and the products were analyzed on $NaDodSO_4$–polyacrylamide gels as described under Experimental Procedure. Lysozyme (2 μg) and M13 coat protein (5 μg) were applied to each gel as standards. (A) Cross-linking was done at various concentrations of dimethyl suberimidate (DMSI) as indicated. In the experiments shown in the center and right-hand panels, the melittin cross-linking incubations had 1% Brij 58 and 1% deoxycholate, respectively. (B) Effect of protein concentration on the cross-linking of melittin. Melittin was cross-linked as described under Experimental Procedure except that the concentration of protein in the cross-linking reaction was varied as indicated.

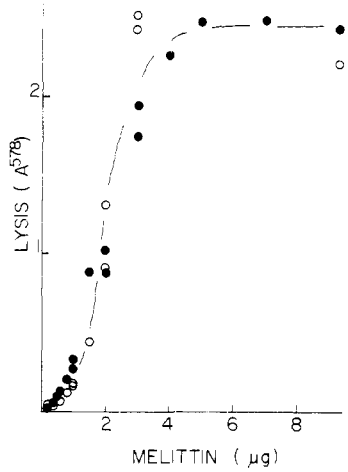

FIGURE 6: Lysis of human erythrocytes. Outdated human blood (30 mL; from the UCLA blood bank) was centrifuged (6000*g*; 10 min; 4 °C), and cells were resuspended 3 times in 30 mL of 0.68% NaCl and 0.02 M NaP_i and suspended in 750 mL of this buffer. Lysis assays (3 mL; in this same buffer) had 1 mL of erythrocyte suspension and the indicated amounts of melittin. After 30 min at 37 °C, each sample was centrifuged (5 min; 10000*g*; 23 °C) and the supernatant was assayed for A_{578}. (●) Melittin; (○) covalently cross-linked melittin.

presence of detergent (Figure 5B, right panel), monomer predominated at low protein concentrations but was replaced by higher multimers at higher protein concentrations, demonstrating that the cross-linking reagent was active in the detergent solutions. In the presence of detergent, cross-linked tetramers were never predominant. These data confirm reports (Haberman & Reiz, 1965) that melittin is a water-soluble tetramer and show that it is converted to a monomer by detergents.

Melittin is soluble in water-saturated butanol (Kreil & Bachmayer, 1971). In this solvent, it is not cross-linked by dimethyl suberimidate[2] and is therefore probably monomeric. Hemoglobin is cross-linked in this solvent, showing that the dimethyl suberimidate remains active.

Erythrocyte Lysis. The lytic activity of melittin can be conveniently monitored by the release of hemoglobin from erythrocytes (Sessa et al., 1969). As is shown in Figure 6, the covalently cross-linked tetramer is as active in erythrocyte lysis as the native melittin tetramer. Kinetic analysis showed that the rate of lysis, as well as the yield, was unaffected (unpublished experiments). Dissociation of tetrameric melittin and dispersal of its subunits in the plane of the membrane is, therefore, not a necessary step in target-cell lysis.

Discussion

Secondary and Quaternary Structure. Conflicting indications about the secondary structure of melittin are provided by circular dichroism on the one hand and the empirical rules of folding on the other. Our circular dichroism spectra all exhibit the shape usually associated with a substantial α-helical content. Taking the value of the ellipticity at 208 nm, Θ_{208}, as a measure of the fractional α-helical content of the molecule (Adler et al., 1973; Greenfield & Fasman, 1969)

$$\% \ \alpha \text{ helix} = \frac{\Theta_{208} - 4000}{34\,000 - 4000}$$

one determines that, for the three spectra of Figure 2, melittin is 107% α helical in $NaDodSO_4$, 88% α helical in Brij, and 48% α helical in aqueous Tris, pH 8.8. In passing we note that our observation of spectral shapes associated with α-helical folding for melittin in aqueous solution is in conflict with the circular dichroism results of Dawson et al. (1978), who found a very different spectral shape in aqueous phosphate buffer. While all our aqueous spectra have a shape similar to that of the aqueous curve of Figure 2, we do find that values of the ellipticity in various aqueous buffers are slightly sensitive to the composition of the solution.

As described above, mechanical application of the Chou–Fasman empirical folding rules suggests a largely β-sheet structure for melittin with a possible bend in the backbone at residues 10–14. There are two reasons for caution in accepting these predictions in the case of melittin. The empirical folding rules have been developed from a data base of globular proteins found in aqueous environments. Not only is melittin an amphipathic molecule with a highly unusual amino acid sequence but also in aqueous solution it is in tetrameric form, and the constraints of oligomerization may affect folding in a manner not encountered so far in larger, globular proteins. It seems likely that these questions of structure can be resolved only after crystallization of melittin in a form suitable for diffraction studies.

Amphipathic Properties of Melittin. Except for serum albumin, soluble proteins generally do not bind significant amounts of detergents such as Brij 58 or deoxycholate (Clarke, 1975). Melittin is clearly an exception to this rule; it binds 2 mol of deoxycholate per mol of polypeptide and dissociates from a tetramer to a monomer in a mixed micelle. Circular dichroism measurements are consistent with this change in quaternary structure being accompanied by increased α helix, a secondary structural change. The mechanism of this reaction is still unclear. Since melittin is monomeric in butanol as well as in detergents, it is likely that the water-soluble tetramer is stabilized by largely hydrophobic forces. Although the binding of deoxycholate and the melittin tetramer may be facilitated by their opposite charges, this cannot be the only means of initial detergent binding. Brij 58, which is nonionic, must also initially bind to the melittin tetramer to trigger its unfolding and the substitution of detergent–protein hydrophobic bonds for protein–protein ones. Separating the reaction of melittin and detergent or lipid into stages may be facilitated by the ability to chemically cross-link the protein.

The question of the means by which melittin assembles into micelles and membranes is important for understanding melittin's lytic action. It is undoubtedly closely related to the means by which other water-soluble toxins spontaneously assemble into membranes. It also is of perhaps broader interest for the lessons it may offer about how proteins refold in a physiological context such as an amphipathic membrane surface. The ability of secreted proteins or integral membrane proteins to initially exist in a water-soluble conformation and to then assemble into membranes (or to cross them) may be greater than is appreciated. In this regard, Highfield and Ellis have shown that the completed precursor to the small subunit of ribulosebisphosphate carboxylase can enter chloroplasts (Highfield & Ellis, 1978), and Poyton has shown that four of the subunits of yeast mitochondrial cytochrome oxidase are initially part of a soluble cytoplasmic polypeptide precursor (Poyton & McKemmie, 1976). We have found that the precursor form of bacteriophage M13 coat protein, termed procoat, is made entirely by polysomes which are not membrane-bound, that procoat is initially water soluble, and that it rapidly chases into the cytoplasmic membrane where it is cleaved to coat protein and leader peptide.[3] Many proteins

[2] E. Knöppel and W. Wickner, unpublished experiments.

[3] K. Ito and W. Wickner, manuscript in preparation.

such as these which are secreted across membranes or which assemble into them have hydrophobic amino-terminal sequences of between 15 and 30 residues. The presence of such a sequence in melittin suggests that they may share a common pathway of membrane interaction.

Acknowledgments

We thank Dr. Randy Schekman for acquainting us with melittin and Dr. Joseph Horwitz for guidance in the circular dichroism experiment. The amino acid analysis was kindly performed by Dr. Margaret E. Haberland of Dr. Emil Smith's laboratory.

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