Dissection of Antibacterial and Toxic Activity of Melittin

A LEUCINE ZIPPER MOTIF PLAYS A CRUCIAL ROLE IN DETERMINING ITS HEMOLYTIC ACTIVITY BUT NOT ANTIBACTERIAL ACTIVITY*

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Neeta Asthana‡§, Sharada Prasad Yadav‡§, and Jimut Kanti Ghosh¶

From the Molecular and Structural Biology Division, Central Drug Research Institute, Lucknow 226 001, India

Melittin, a naturally occurring antimicrobial peptide, exhibits strong lytic activity against both eukaryotic and prokaryotic cells. Despite a tremendous amount of work done, very little is known about the amino acid sequence, which regulates its toxic activity. With the goal of understanding the basis of toxic activity and poor cell selectivity in melittin, a leucine zipper motif has been identified. To evaluate the possible structural and functional roles of this motif, melittin and its two analogs, after substituting the heptadic leucine by alanine, were synthesized and characterized. Functional studies indicated that alanine substitution in the leucine zipper motif resulted in a drastic reduction of the hemolytic activity of melittin. However, interestingly, both the designed analogs exhibited antibacterial activity comparable to melittin. Mutations caused a significant decrease in the membrane permeability of melittin in zwitterionic but not in negatively charged lipid vesicles. Although both the analogs exhibited similar secondary structures in the presence of negatively charged lipid vesicles as melittin, they failed to adopt a significant helical structure in the presence of zwitterionic lipid vesicles. Results suggest that the substitution of heptadic leucine by alanine impaired the assembly of melittin in an aqueous environment and its localization only in zwitterionic but not in negatively charged membrane. Altogether, the results suggest the identification of a structural element in melittin, which probably plays a prominent role in regulating its toxicity but not antibacterial activity. The results indicate that cell selectivity in some antimicrobial peptides can probably be introduced by modulating their assembly in an aqueous environment.

Melittin, the major component of European bee venom from *Apis mellifera*, is a well studied peptide, which is known for its strong cytolytic and antimicrobial activities (1, 2). It belongs to the family of cytolytic peptides whose members are believed to be the key components of defense and offense mechanisms of all organisms (3, 4).

A considerable number of structure-function studies have been carried out to understand the molecular mechanism of the hemolytic and antimicrobial activities of melittin. The replacement of the first 20 amino acids by another helix-forming sequence did not alter the antimicrobial and hemolytic activity of melittin (5). However, the deletion of Leu-6, Lys-7, Val-8, Leu-9, Leu-13, Leu-16, Ile-17, Trp-19, and Ile-20 dramatically reduced the hemolytic activity of melittin and to a lesser extent the antimicrobial activity also (6). The crucial roles of both termini of melittin in its functional properties have been demonstrated by the design of several analogs with deletion of N-(7, 8) and C-terminal (9) sequences.

Several efforts have been made to design analogs of melittin with decreased hemolytic but similar antimicrobial activity. For example, the diastereoisomers of melittin having D-amino acid analogs (10) at a few positions, a hybrid peptide consisting of cecropin A and melittin sequences (11), and an analog designed from the C-terminal 15-residue fragment (12) exhibited similar antibacterial activity to melittin but much less hemolytic activity. The crystal structure of melittin has been solved, which indicated a tetrameric helix-bend-helix structure (13) with two helical segments, positioned between amino acid residues 1–10 and 13–26 and the bend region within residues 11–12.

Biophysical properties of melittin such as self-association and phospholipid membrane-interaction have been studied extensively to understand the mode of action of melittin (14–17). Although in water melittin exists as monomer with mostly random coil conformation, an increase in peptide concentration or the addition of salt results in the transformation of monomer to tetramer with a pronounced helical structure (18–20). Melittin has been used as a model for studying the general features of lipid interaction of membrane proteins. Membrane-interaction studies suggest that melittin forms transmembrane pores in zwitterionic lipid bilayers by a barrel-stave mechanism (21–24) and in negatively charged lipid vesicles it acts like a detergent through a carpet mechanism (24, 25).

Despite all of the work done on melittin, it is not yet clear (i) why melittin has very low cell selectivity, *i.e.* why it exerts both high antibacterial and toxic activities, and (ii) is the same amino acid sequence responsible for both the hemolytic and antibacterial activities. Toward the identification of important structural and functional elements in melittin and to gain insight about the regulation of cell selectivity in an antimicrobial peptide in general, we have found a leucine zipper motif, located within the residues 6–20, which has not been reported earlier to the best of our knowledge. Because this motif is known to play important roles in the assembly of DNA-binding proteins (26, 27) or membrane-associated viral fusion proteins (28–30), it was of interest to look into the possible roles of this

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‡ Both authors contributed equally to this work.

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¶ Recipient of a Senior Research Fellowship from the Council of Scientific and Industrial Research, India.

To whom correspondence should be addressed. Tel.: 091-522-2212412 (ext. 4282); Fax: 091-522-223405/223938; E-mail: jighosh@yahoo.com.

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motif in the structural integrity and functional activity of melittin. For this purpose, two analogs of melittin were designed by selectively replacing heptadic leucine with single and double alanine and then synthesized. Hemolytic activity of single (MM-1) and double alanine mutants (MM-2) against human red blood cells was significantly less than that of melittin. Interestingly, MM-1 and MM-2 exhibited antibacterial activity comparable to melittin. Both of the designed analogs displayed contrasting membrane permeability in zwitterionic and negatively charged lipid vesicles. The results have been discussed in terms of the role of this motif in determining the hemolytic and antimicrobial activity of melittin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rink amide 4-methylbenzhydrylamine resin (loading capacity, 0.63 mmol/g) and all the N-α Fmoc1 and side-chain protected amino acids were purchased from Novabiochem, Switzerland. Coupling reagents for peptide synthesis such as 1-hydroxybenzotriazole, N,N,N′, diisopropylcarbodiimide, 1,1,3,3-tetramethyluronium tetrafluoroborate, and N,N′-diisopropylthiacylum were purchased from Sigma. Dichloromethane, N,N-dimethylformamide, and piperidine were of standard grades and procured from reputed local companies. Acentronite, trifluoroacetic acid, and acetonitrile and water contained 0.05% trifluoroacetic acid. The puri- fication resins and precipitated. All the peptides were purified by reverse phase after sufficient labeling, the labeled peptides were cleaved from the cent probe was achieved by a standard procedure (30, 32, 35, 36), and with dry ether. Labeling at the N terminus of peptides with a fluores- dyes were prepared by a standard procedure (32, 35, 36) with required molecular mass of the peptides, detected by electrospray-mass spec- trum and after sufficient labeling, the labeled peptides were cleaved from the vesicle suspension when fluorescence of the dye exhibited a steady level, the respective peptide was added. The peptide-induced dissipation of diffusion potential, as detected by an increase in fluorescence (at 527 nm), with excitation at 467 nm, was measured in terms of percent- age of fluorescence recovery (Ft), defined by Ft = [I0 - I(f)/(I0 - I1)] × 100%, where I0 is fluorescence after the addition of a peptide at time t, I1 is fluorescence after the addition of valinomycin, and I is the total fluorescence observed before the addition of valinomycin.

**Enzymatic Cleavage Experiments**—To detect the location of the pep- tide in the membrane-brain states, enzymatic digestion experiments were performed with their NBD-labeled versions as reported earlier (30, 32). In brief, lipid vesicles made of either PC/Chol, PCPG/Chol, or PE/PG were first added to the NBD-labeled peptide. When all the peptide was bound to lipid as detected by the saturation of the fluo- rescence level, proteinase K (final concentration, 1.20 µg/ml) was added. In this experiment fluorescence of NBD-labeled peptide was recorded at 527 nm with respect to time (s) with excitation wavelength set at 476 nm.

**Hemolytic Activity Assay**—The hemolytic activity of melittin and its analogs was assayed by a standard procedure (10, 12). In brief, fresh human red blood cells (hRBCs) that were collected in the presence of an anti-coagulant from a healthy volunteer were washed three times in PBS. Peptides, dissolved in water, were added to the suspension of red blood cells (6% final in v/v) in PBS to the final volume of 200 µl and incubated at 37 °C for 45 min. The samples were then centrifuged for 10 min at 2000 rpm, and the release of hemoglobin was monitored by measuring the absorbance (Asample) of the supernatant at 540 nm. For negative and positive controls hRBCs in PBS (Ablank) and in 0.2% (final volume) Triton X-100 (Atriton) were used, respectively. The percentage of hemolysis was calculated according to the equation, Percentage of hemolysis = ([Asample - Ablank] / [Atriton - Ablank]) × 100.

**Assay of Antibacterial Activity of the Peptides**—The antibacterial activity of the peptides was assayed in LB medium under aerobic conditions (10, 12, 42). Different concentrations of each of the peptides, dissolved in water, were added in duplicate to 100 µl (final volume) of medium containing the inocula of the test organism (≈107 colony forming units) in the midlogarithmic phase of growth. The inhibition of growth of microorganisms was assessed by measuring the absorbance at 492 nm after an incubation of 18–20 h at 37 °C. The antibacterial activity of the peptides was expressed as the minimal inhibitory concentration, the concentration at which 100% inhibition of microbial growth was observed after 18–20 h of incubation. The microorganisms used were Gram-positive bacteria, Bacillus subtilis and Staphylococcus aureus, and Gram-negative bacterium, Escherichia coli.

**RESULTS**

**Design of Analogs of Melittin**—Melittin has an interesting sequence with 26 amino acids including five cationic residues. A leucine zipper motif, with every seventh amino acid as leucine/isoleucine (leucine at position 6 and 13 and isoleucine at position 20) and also leucine in two “d”positions, was identified (Fig. 1). To look into the possible role of this important structural motif, two analogs of melittin were designed. In one of which leucine at position 13 (MM-1) was replaced with alanine and in the other (MM-2) leucine at positions 6 and 13 were replaced with two alanines (Fig. 1A). Alanine was chosen for its

\[ F_a = \left\{ \begin{array}{ll} \theta_{222} & \text{if } \theta_{222} \geq 0.105 \\ \theta_{222} - \theta_{222} & \text{if } \theta_{222} < 0.105 \end{array} \right. \]
helix propensity and similar hydrophobicity to leucine so that the amphipathic character of melittin was retained in MM-1 and MM-2.

Mutation in the Leucine Zipper Motif Drastically Reduced the Hemolytic Activity of Melittin—To look into the role of the identified leucine zipper motif in the functional activities of melittin, hemolytic activity of melittin and its designed analogs were assayed. The substitution of leucine with alanine resulted in a drastic reduction of hemolytic activity of melittin against hRBCs (Fig. 2). MM-1 exhibited 10–20% hemolytic activity of melittin, whereas MM-2 showed −1–2% activity. The results indicated that the substitution of heptad amino acids by alanine markedly inhibited the hemolytic activity of melittin probably suggesting a role of this motif in maintaining the lytic activity of the peptides toward the RBCs.

The Designed Analogs Exhibited Antibacterial Activity Comparable to Melittin—Melittin and its analogs were tested for growth-inhibiting activity in liquid cultures of two Gram-positive and one Gram-negative bacteria. Tetracycline was used as a positive control. In contrast to the hemolytic activity, the designed analogs of melittin (MM-1 and MM-2) exhibited very similar antibacterial activities to that of melittin (Table I). Thus the results clearly indicated that substitution of leucine by alanine in the heptadic positions did not affect the antibacterial activity of melittin unlike its hemolytic activity.

Contrasting Difference in Membrane Permeability of the Designed Analogs of Melittin in Zwitterionic and Negatively Charged Membranes—The ability of melittin to destabilize the phospholipid bilayer is believed to be associated with its mechanism of action. Therefore, to understand the basis of the decreased hemolytic activity and almost unaltered antibacterial activity of the designed melittin analogs, the membrane permeability of melittin, MM-1, and MM-2 were examined by determining their efficacy to dissipate the diffusion potential across the phospholipid vesicles with different lipid composition. As shown in Fig. 3A, the membrane permeability (expressed as the percentage of fluorescence recovery) of MM-1 was significantly lower than melittin in zwitterionic PC/Chol vesicles, which decreased further with MM-2. However, interestingly, both MM-1 and MM-2 exhibited very similar membrane permeability to that of melittin in negatively charged PC/PG/Chol lipid vesicles (Fig. 3B). Experimental profiles for each of the peptides in both kinds of membranes are shown in the insets of Fig. 3, A and B. Moreover, the designed analogs induced membrane permeation as efficiently as melittin (not shown) in another kind of negatively charged membrane, namely PE/PG (7.3 w/w). The results indicated that although the substitution of leucine by alanine appreciably impaired the membrane permeability of melittin in the zwitterionic membrane, it had an almost negligible effect in negatively charged membrane.

The Designed Analogs Exhibited Similar Secondary Structures to Melittin in Zwitterionic Membranes but Not in Zwitterionic Membranes—Circular dichroism experiments were performed to determine the secondary structures of melittin and its analogs in aqueous environment (PBS, pH 7.4) and in the presence of zwitterionic and negatively charged phospholipid vesicles. The corresponding mean residue ellipticity values of the individual peptides at 222 nm were used to determine their helical contents in a particular environment. Although all three peptides exhibited low helical structure in PBS (Fig. 4A), the extent of helicity was slightly more in melittin (15%) than MM-1 (8.5%) and MM-2 (6.5%). However, in the presence of increasing amount of negatively charged PC/PG/Chol lipid vesicles significant amount of helicity was induced in all three peptides as shown by a representative CD spectrum for each of these peptides with a fixed lipid/peptide molar ratio (32) (Fig. 4B). Melittin (69%), MM-1 (67%), and MM-2 (68.5%) exhibited very similar extents of helical structures indicating that the substitution of leucine by alanine did not affect the secondary structure of melittin in the presence of negatively charged lipid vesicles.

However, very contrasting results were obtained in the presence of zwitterionic PC/Chol lipid vesicles (Fig. 4C). Although melittin adopted a significant amount of helical structure (47% at lipid/peptide ~24) in the presence of PC/Chol lipid vesicles, the extent of induction of helicity was much less in MM-1 (~15%) and MM-2 (8.5%) with the same amount of lipid vesicles.

Mutation in the Leucine Zipper Motif Affected the Localization of Melittin in Zwitterionic Membranes but Not in Negatively Charged Membranes—To detect the location of melittin and its analogs onto the membrane, proteolytic cleavage experiments were performed with NBD-labeled peptides in their membrane-bound states. The basis for this experiment is that NBD-labeled peptides, bound onto the membrane surface, are easily cleaved by a proteolytic enzyme like protease K, which can be monitored by the decrease in NBD-fluorescence from its
characteristic membrane-bound level. On the other hand, a membrane-inserted peptide will not be accessible to proteinase K and therefore its NBD-fluorescence will not decrease. The addition of PC/Chol vesicles to NBD-labeled melittin and its analogs (Fig. 5A) resulted in a sharp increase in fluorescence because of the binding to the lipid vesicles with fast kinetics indicating that mutations in the leucine zipper motif did not impair the membrane-binding ability of melittin. The addition of proteinase K (at time point 3) to melittin (Fig. 5A, a) in its membrane-bound state resulted in a slow decrease in NBD-fluorescence indicating that the N-terminal of NBD-melittin to some extent protects itself from the digestion by the proteolytic enzyme. The 50% decrease in NBD fluorescence, resulting from the cleavage of NBD-melittin in its membrane-bound state took place at 1800 s, suggesting that a part of the N-terminal of melittin was inside the lipid bilayer of the zwitterionic membrane and hence not cleaved easily by proteinase K. Interestingly, NBD-labeled MM-1 and MM-2 were cleaved much faster than bound to PC/Chol vesicles compared with that of NBD-melittin as evidenced by the sharp decrease in fluorescence after the addition of proteinase K (Fig. 5A, b and c). The corresponding 50% decrease in fluorescence from the membrane-bound level for NBD-MM-1 and MM-2 took place at ~120 and 40 s, respectively. The results suggest that probably the N termini of both of the analogs were more exposed on the surface of the zwitterionic membrane than melittin with MM-2 being the most exposed one. Taken together, the results indicate that although mutations in the leucine zipper motif did not inhibit the binding of melittin to zwitterionic membrane, it impaired its localization onto it. However, N termini of melittin and its analogs were appreciably protected in PC/PG Chol vesicles from proteolytic cleavage as evidenced by the slow decrease in NBD fluorescence following the proteinase K treatment (Fig. 5B). Because very similar results were obtained with PE/PG vesicles, profiles were not presented.

The localization of N-terminal of melittin and its analogs onto the membrane were also detected by recording the emission maxima of their NBD-labeled versions in the presence of lipid vesicles (Fig. 5C and D). In the presence of zwitterionic PC/Chol vesicles melittin exhibited emission maxima at 526–527 nm, which was much shorter than the 533 nm observed for the NBD probe located on the surface of the membrane (43). Interestingly, NBD-labeled MM-1 and MM-2 with zwitterionic membrane displayed fluorescence maxima at 529–530 nm (Fig. 5C), which was close to the characteristic value for the probe, located onto the membrane surface. However, in the presence of negatively charged PC/PG Chol vesicles melittin exhibited emission maxima in the range of 525–526.5 nm (Fig. 5D), indicating the location of their N-terminal slightly inside the bilayer of this kind of membrane. Altogether, supporting the enzymatic cleavage experiments, fluorescence emission maxima of NBD-labeled melittin and its analogs suggested that the localization of N-terminal of melittin was affected by the mutation of the leucine zipper motif only in zwitterionic but not in negatively charged membranes.

Substitution of Heptadic Leucine in the Leucine Zipper Motif Impaired the Assembly of Melittin in Aqueous Environment—In an aqueous environment at high ionic strength with an increase in concentration, melittin assembles in a tetrameric helical structure as studied by several biophysical techniques (15, 18–20). We have utilized CD, fluorescence, and gel filtration methods to look into the effect of alanine substitution in the assembly and secondary structure of melittin in aqueous environment. With an increase in concentration in PBS con-
Leucine Zipper Motif in Melittin

To understand the molecular basis of reduction in hemolytic activity and retention of antibacterial activity of the melittin analogs and to evaluate the structural and functional changes associated with the mutation in this motif, membrane permeability, secondary structure, localization onto the membrane with different lipid composition, and self-association in an aqueous environment were studied with melittin, MM-1, and its analogs and to evaluate the structural and functional changes associated with the mutation in this motif, membrane permeability, secondary structure, localization onto the membrane with different lipid composition, and self-association in an aqueous environment were studied with melittin, MM-1, and MM-2. Considering the lipid component and surface charge of melittin at a higher concentration and high ionic strength was disturbed as a result of the substitution of heptadatic leucine by alanine.

**DISCUSSION**

The results presented here indicate the identification of a leucine zipper motif, which plays a crucial role in the hemolytic activity of melittin. To our knowledge, this is the first report on the identification and characterization of a leucine zipper motif in this class of naturally occurring peptides. As a measure of toxic activity, the hemolytic activity of melittin and its analogs against hRBCs were assayed. The data clearly demonstrated that the designed analogs were much less active than the wild type melittin in lysing the hRBCs (Fig. 2). The synergistic effect of the replacement of heptadatic leucine by alanine was evidenced by the fact that the double alanine mutant (MM-2) was significantly more inactive than the single alanine mutant (MM-1). However, in contrast to the hemolytic activity, interestingly, both MM-1 and MM-2 exhibited comparable antibacterial activity to melittin against the tested Gram-positive and -negative bacteria (Table 1).

To understand the molecular basis of reduction in hemolytic activity and retention of antibacterial activity of the melittin analogs and to evaluate the structural and functional changes associated with the mutation in this motif, membrane permeability, secondary structure, localization onto the membrane with different lipid composition, and self-association in an aqueous environment were studied with melittin, MM-1, and MM-2. Considering the lipid component and surface charge of melittin at a higher concentration and high ionic strength was disturbed as a result of the substitution of heptadatic leucine by alanine.

**Fig. 5. Determination of localization of melittin and its analogs.** Proteolytic digestion of NBD-labeled melittin (a), MM-1 (b) and MM-2 (c) in their membrane-bound states. A and B depict the experimental profiles with PC/Chol and PC/PG/Chol lipid vesicles, respectively. 1, 2, and 3 indicate the addition of NBD-labeled individual peptides (0.20 μM), lipid vesicles (120 μM), and proteinase K (12 μg/ml, final concentration). C and D, recording of fluorescence emission spectra of NBD-labeled melittin and its analogs in the presence of PC/Chol (C) and PC/PG/Chol (D). Peptide and lipid concentrations were the same as the proteolytic cleavage experiments. Solid line, melittin; short dashed line, MM-1; short dash-dotted line, MM-2. Peaks of the spectra in C and D are marked by arrows.

**Table 1.** Hemolytic activity of melittin and its analogs.

<table>
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<tr>
<th>Peptide</th>
<th>Hemolytic Activity (%)</th>
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<tbody>
<tr>
<td>Melittin</td>
<td>100%</td>
</tr>
<tr>
<td>MM-1</td>
<td>75%</td>
</tr>
<tr>
<td>MM-2</td>
<td>5%</td>
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Supporting the previous observation (18). There was a minor peak, which corresponded to its monomer peak. However, under the same experimental conditions, both MM-1 and MM-2 eluted at higher volumes (Fig. 7, inset) slightly after aprotinin (molecular weight, 6500), indicating the presence of their dimeric masses. Also both MM-1 and MM-2 exhibited prominent peaks of their monomeric masses. Taking the gel filtration and the previous CD and fluorescence experiments together, the results suggested that the tetrameric helical assembly of melittin at a higher concentration and high ionic strength was disturbed as a result of the substitution of heptadatic leucine by alanine.
the outer membrane of RBC and bacterial membrane, experiments were performed with zwitterionic PC/Chol and negatively charged PC/PG/Chol and PE/PG lipid vesicles. Often the results obtained from the structural and functional studies of membrane-active peptides with these kinds of membranes correlate with their hemolytic and antibacterial activities (10, 44).

Replacement of heptadic leucine by alanine resulted in an appreciable reduction in the membrane permeability of melittin in PC/Chol vesicles (Fig. 3A), clearly indicating an essential role of the identified leucine zipper motif in the pore-forming activity of melittin in the zwitterionic membrane. However, in contrast, the melittin analogs exhibited a very similar permeability to melittin in negatively charged PC/PG/Chol and PE/PG membrane. The results further suggest that the amino acid sequence requirements of a peptide to permeate zwitterionic and negatively charged lipid vesicles are not the same, consistent with a recent study showing that a peptide derived from *E. coli* toxin hemolysin E efficiently induced permeation in the negatively charged but not in zwitterionic membrane (32).

The kinetics of membrane permeation induced by the designed melittin analogs in zwitterionic and negatively charged membrane showed some interesting features. For example, the

**Fig. 6.** Detection of self-association of melittin and its analogs by concentration-dependent CD and fluorescence experiments. A, B, and C are the plots of CD (mdeg, millidegree) for different concentrations of melittin, MM-1, and MM-2, respectively in PBS (pH 7.4) containing 1.5 M NaCl. D, the corresponding plots of mean residue ellipticity values at 222 nm ([θ]) versus peptide concentration (μM) of melittin (a), MM-1 (b), and MM-2 (c). E, fluorescence spectra of melittin and its analogs as marked in the figure at ~18 μM in PBS with 1.5 M NaCl. Excitation wavelength was set at 280 nm.
kinetics of the zwitterionic PC/Chol membrane permeation induced by the single alanine mutant MM-1 (Fig. 3A, inset, 1.52 μM) was only slightly slower than that of melittin, although its maximum level of dissipation of diffusion potential was significantly reduced. In contrast, in the negatively charged PC/PG/Chol membrane, the kinetics of membrane permeation induced by the designed melittin analogs were very close to that of melittin (profiles not shown). This result further suggests that whatever difference exists between the designed melittin analogs and melittin, in terms of their assembly or even in the mechanism of permeation of negatively charged membrane, is only at relatively lower peptide concentration.

Although melittin and its analogs exhibited similar α-helix contents in the negatively charged membrane, the secondary structures exhibited by the mutants were much less than that of melittin in the zwitterionic membrane (Fig. 4). The molecular basis of these observations is not clear; however, it seems that the substitution of heptadic leucine by alanine in the leucine zipper motif affects the secondary structure of melittin in zwitterionic membranes but not in negatively charged membranes.

Functional activity of a membrane-associated peptide may be influenced by its localization onto the membrane. Proteolytic cleavage experiments (Fig. 5, A and B) suggested that the substitution of heptadic leucine by alanine had a remarkable effect on the localization of melittin onto the zwitterionic membrane but not onto the negatively charged membrane. The N-terminal of melittin was located slightly inside the zwitterionic membrane, which is consistent with other studies (24, 45) whereas the mutants were exposed on its surface. Interestingly, the N termini of both melittin and its analogs were located inside the bilayer of negatively charged lipid vesicles. Similar localizations were observed for NBD-labeled dermaseptin-3 and -4, which also dissociated like melittin (46, 47) therein and have been postulated to manifest their antimicrobial activities by the carpet/detergent mechanism (48). The results of the proteolytic cleavage experiments were supported by the emission maxima of NBD-labeled peptides in the presence of zwitterionic and negatively charged lipid vesicles (Fig. 5, C and D). Although NBD-melittin in the presence of both zwitterionic and negatively charged lipid vesicles and NBD-labeled analogs in the presence of negatively charged lipid vesicles exhibited emission maxima, which were much shorter than that observed for the probe located onto the membrane surface, NBD-labeled MM-1 and MM-2 in zwitterionic membranes displayed maxima that were close to the characteristic value (43) for the probe, located on the surface of the membrane. Tryptophan fluorescence of unlabeled melittin and the designed mutants was also recorded in the presence of both kinds of lipid vesicles (data not shown). Melittin exhibited emission maxima at 336–337 and 334–335 nm in the presence of zwitterionic and negatively charged lipid vesicles, respectively, which supports previous observations (49) and indicates that the tryptophan residue was located in the shallow interface region of the membrane bilayer as already reported (50). Although MM-1 and MM-2 exhibited very similar emission maxima to melittin in a negatively charged membrane, the maxima (338–339 nm) exhibited by MM-1 and MM-2 in the presence of zwitterionic vesicles probably indicated that the location of their tryptophan residues was slightly more toward the hydrophilic region of the membrane than that of melittin.

CD, tryptophan fluorescence (Fig. 6), and gel filtration studies (Fig. 7) with melittin and its analogs clearly indicated that secondary structure and assembly of melittin in an aqueous environment were disrupted following the substitution of heptadic leucine by alanine. Although melittin adopted a tetrameric helical assembly at a relatively higher concentration and at a high ionic strength, both MM-1 and MM-2 at similar conditions exhibited only their dimeric masses (Fig. 7) with a much reduced helical structure. It is important to mention...
that, although under the same experimental conditions, both of the designed melittin analogs exhibited their dimeric masses, the single alanine mutant (MM-1) adopted an appreciably more helical structure than the double alanine mutant (MM-2) (Fig. 6, B–D). Furthermore, the results suggested that the identified leucine zipper motif is probably a key structural element that regulates the secondary structure and self-association of melittin in an aqueous environment. Also the tetrameric crystal structure of melittin is stabilized by the side-chain interaction of the hydrophobic amino acids (13) like leucine and isoleucine, which are present in the a and d positions of the identified leucine zipper motif. These observations are in agreement with studies that suggest a crucial role of this motif in the assembly of protein/peptides in aqueous and membrane environments (30, 51, 52).

A contrasting difference in the membrane permeability of melittin and its analogs in zwitterionic and negatively charged lipid vesicles probably reflects that the mechanisms of membrane permeation of melittin in these two kinds of membranes are different from each other, which is in agreement with the present notion that melittin forms a pore via a barrel-stave mechanism in a zwitterionic membrane (21–24) and acts as a detergent in a negatively charged membrane (24, 25). Probably the results suggest that the substitution of leucine by alanine disturbs only the mechanism of melittin-induced membrane permeation in zwitterionic but not in the negatively charged membrane.

Assembly or conformation of melittin-like membrane lytic peptides in aqueous environments may influence their activity in the membrane also, because most likely these peptides bind to the target membrane from the aqueous environment. The results presented here probably indicate that a strong hydrophobic force of melittin is needed to disrupt the zwitterionic lipid bilayer. Proteolytic cleavage experiments and emission spectra of NBD-labeled analogs, which were recorded with peptide concentration as low as $10^{-7}$ M, revealed that although the N-terminal of melittin could penetrate the bilayer of zwitterionic membrane, its designed mutants were localized onto the same membrane surface (Fig. 5). By disturbing the leucine zipper motif, the helical structure and self-association and thus the hydrophobic force of melittin were abrogated, which may contribute to the reduced activity of melittin analogs in the zwitterionic membrane and also against RBCs.

That the identified leucine zipper motif may play a crucial role in regulating the toxic activity of melittin can explain several results in the literature, the molecular basis of which was not clearly understood. For example, a critical look at the sequence of melittin analog, designed by DeGrado et al. (5), reveals that although it lacks appreciable sequence homology to melittin, the identified leucine zipper motif with amino acids at a and d positions remained intact in it. Probably for this, the analog (5) self-associated (53) with the helical structure and retained the hemolytic activity. The absence of proline may add to its better self-association property and helical structure and thus to the higher hemolytic activity than melittin. Probably the deletion of amino acids at 6, 9, 13, 16, and 20 of melittin showed a more drastic effect (6) on the hemolytic activity because of impairing its helical assembly as these residues are located at crucial a and d positions of the leucine zipper motif. Similarly, the reason why the deletion of the N-terminal of melittin had drastic effect on its hemolytic activity compared with its antimicrobial activity can be explained (7, 8, 12).

In conclusion, the results demonstrated a mechanistic dissection of the hemolytic and antibacterial activities of melittin. The results probably indicate the presence of different sequence elements, which control the toxic and antibacterial activity of melittin. The ability of melittin to efficiently permeate both the negatively charged and zwitterionic membranes makes it a poor cell-selective molecule and renders it active against both prokaryotic (e.g. antibacterial activity) and eukaryotic cells (e.g. hemolytic activity). The results suggested that the substitution of heptadic leucine by alanine disturbed the helical assembly of melittin in an aqueous environment, the secondary structure, membrane permeability, and localization in zwitterionic membrane, which probably caused the reduction in hemolytic activity of melittin. However, alanine substitution did not alter much the amphipathic character of melittin, showed much less effect on its membrane permeability, secondary structure, and localization in negatively charged membrane and presumably therefore did not affect its antibacterial activity.

Antimicrobial peptides are potential lead molecules for designing novel drugs. However, one of the major issues is their selective lytic activity. The results presented here probably suggest that cell selectivity in some antimicrobial peptides can be introduced by modulating their secondary structures and/or assembly in an aqueous environment.

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Leucine Zipper Motif in Melittin

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Neeta Asthana, Sharada Prasad Yadav and Jimut Kanti Ghosh

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