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Hemolytic and Antimicrobial Activities of the Twenty-Four Individual Omission Analogues of Melittin†

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Received October 10, 1990; Revised Manuscript Received February 20, 1991

ABSTRACT: Although melittin's hemolytic activity has been extensively studied, the orientation of membrane-bound melittin remains uncertain. We have investigated the effect of individually omitted amino acid residues on melittin's activity and related these results to the existing models of melittin–membrane interaction. The extent of hemolysis of the omission analogues closely followed the four known conformational regions of melittin: omission of any of the residues making up the two α-helical regions decreased the hemolytic activity relative to melittin, while omission of any of the residues making up the "hinge" or the C-terminal regions had little or no effect. Our results correlate best with a proposed model in which melittin initially forms "holes" in the membrane, resulting in an initial rapid loss of hemoglobin; the membrane-bound melittin is then internalized into the membrane, resulting in a later slow phase of hemoglobin loss. It was also found that induced structural effects caused by peptide–lipid interactions could be studied by using RP-HPLC, with an excellent correlation found between the retention times of the individual omission analogues and their hemolytic activities.

Melittin, the predominant peptide isolated from honey bee venom (*Apis mellifera*), is known for its marked cytolytic activity (Habermann & Jentsch, 1967). This peptide, consisting of 26 amino acid residues, exhibits strong amphipathic surface activity, although no simple relationship has been found between its surface activity and lytic properties (Fennell et al., 1968; Stocker & Traynor, 1986; Dorman & Markley, 1971; Schröder et al., 1971; Habermann, 1972; Dawson et al., 1978). In our continuing studies of lipid-induced changes in peptide conformations (Houghten & DeGraw, 1987; Houghten & Ostresh, 1987; Büttner et al., 1990a,b; Blondelle et al., 1991;

† Supported in part by National Science Foundation Grant DIR8713707 to R.A.H.

0006-2960/91/0430-4671$02.50/0

Büttner & Houghten, 1991; Ostresh et al., 1991), we have chosen to examine melittin in more detail. The structure of melittin has been investigated by using such varied techniques as X-ray diffraction (Anderson et al., 1980; Terwilliger & Eisenberg, 1982a,b), nuclear magnetic resonance (NMR) (Lauterwein et al., 1979; Bazzo et al., 1988; Pastore et al., 1989), circular dichroism (Lauterwein et al., 1979; Yunes, 1982), fluorescence quenching (Talbot et al., 1979; Quay et al., 1985; Vogel & Jähnig, 1986), Raman spectroscopy (Vogel & Jähnig, 1986), and infrared spectroscopy (Verma et al., 1974). The results of X-ray crystallography and NMR in methanolic solution indicate that melittin adopts a structure consisting of two amphipathic α-helices joined by a "hinge" region between threonine-10 and glycine-12 (Anderson et al., 1980; Terwilliger & Eisenberg, 1982a,b; Bazzo et al., 1988).

Furthermore, melittin, which is water-soluble, adopts different conformations and aggregation states in various aqueous solutions. Thus, it exists primarily as a tetramer in solutions of high ionic strength and/or high concentration [e.g., 0.02 M Tris–acetate + 1.5 M NaCl (Talbot et al., 1979), 57 μM melittin + 1.6 M NaCl in 0.02 M phosphate buffer (Meucci et al., 1989), or 35 mM melittin at pH 3.4 (Brown et al., 1980)]. Conversely, it exists as a monomer in solutions of low ionic strength and/or low concentration [e.g., 0.02 M Tris–acetate (Talbot et al., 1979), 3.4 μM melittin in 0.02 M phosphate buffer (Meucci et al., 1989), or 0.4–4.0 mM melittin at pH 3.3 (Lauterwein et al., 1980)]. Upon initial interaction with membrane surfaces, it has been found that the tetramer dissociates to a monomer, which retains an α-helical conformation prior to insertion into the cell membrane (Hider et al., 1983; Batenburg & de Kruijff, 1988).

The effects of the different regions of the melittin sequence on its hemolytic activities have been investigated in earlier studies using melittin-like model peptides or analogues of melittin. In these studies, the hemolytic activity required a specific cationic region located at the C-terminus (Habermann & Kowallek, 1970; Dorman & Markley, 1971; DeGrado et al., 1981; Hider et al., 1983; Boman et al., 1989; Manjunatha Kini & Evans, 1989). The length of the amphipathic helical segment that apparently binds melittin to the cell membrane also seems important to cell lysis since analogues with shortened N-terminal sequences were very poor lytic agents (Dawson et al., 1978; Gevod & Birdi, 1984).

Despite these extensive efforts, the orientation of membrane-bound melittin remains uncertain. Moreover, none of the three models proposed to explain the interaction of melittin with cell membranes has been accepted as definitive [work reviewed by Dempsey (1990)]. Therefore, we have now used a set of 24 omission analogues of melittin (threonine-10 and -11 as well as glutamine-25 and -26 omissions yield the same analogues) to investigate the effect of individually omitted amino acid residues on melittin's hemolytic activity. Each of the proposed models of melittin–membrane interaction was then examined to determine which best correlated with our data.

Materials and Methods

Peptide Synthesis. For the simultaneous multiple peptide synthesis (SMPS) method used here (Houghten, 1985), all solvents and reagents were of analytical grade and used without further purification. The protected *t*-Boc amino acids were obtained from Bachem Inc. and used as received. The side chains of serine and threonine were protected with *O*-benzyl groups, lysine with *o*-chlorobenzyloxycarbonyl, arginine with tosyl, and tryptophan with formyl. SMPS was performed by using 100 mg of *p*-methylbenzhydrylamine resin (m-BHA) (substitution = 0.54 mequiv/g) in each individual resin packet. To obtain omission analogues, individual packets were removed during coupling of the corresponding omitted residue to all of the other analogues and then returned to the common reaction vessel before completion of the synthesis. For cleavage, Tam's low–high HF procedure was used (Tam et al., 1983). All of the m-BHA resins were treated in a common vessel for the low-HF step since little peptide is lost from m-BHA during this step (unpublished results). The high-HF step was performed in a 24-vessel apparatus designed earlier in this laboratory (Houghten et al., 1986) and available from Multiple Peptide Systems (San Diego, CA). The identities of the resulting peptides were confirmed by amino acid analysis (Yale University School of Medicine, New Haven, CT) and by fast atom bombardment mass spectroscopy analyses (M-Scan Inc., West Chester, PA) and/or by time-of-flight mass spectroscopy analyses on a BIOION 20 spectrometer.

RP-HPLC. Analytical RP-HPLC was carried out by using a Vydac C-18 column (ODS 25 cm × 4.6 mm) in conjunction with a Beckman Model 421 HPLC system and dual Model 110A pumps. The solvent system was composed of 0.05% TFA/H_2O (buffer A) and 0.05% TFA/CH_3CN (buffer B) with a flow rate of 1.0 mL/min. The peptides were detected at 215 nm with a Hitachi 100-20 spectrophotometer. The chromatograms were recorded, and peak areas were integrated on a Shimadzu C-R3A Chromatopac integrator. The peptides were then purified by using a Waters Milliprep 300 preparative HPLC modified with a Gilson Model 232 preparative autosampler and preparative Foxy Fraction collector. The desired peptides typically eluted in 3 to 5 fractions. Fractions of the desired purity (determined by using analytical HPLC) were pooled and lyophilized.

Hemolytic Assays. The hemolytic activities of the peptides were determined by using human red blood cells (RBC). RBC were stored in heparin at 4 °C. The serum was removed by centrifugation just prior to the assays. The cells were then washed twice with phosphate-buffered saline (PBS/35 mM phosphate buffer/0.15 M NaCl, pH 7.0) and resuspended in PBS. Peptides dissolved in PBS were then added to 0.5 mL of a 5% solution of the stock RBC in PBS to reach a final volume of 1.0 mL. Following gentle shaking, the suspension was incubated for 1 h at 37 °C. The samples were then centrifuged at 1000*g* for 5 min. The supernatant was separated from the pellet and the optical density (OD) measured at 414 nm. Zero hemolysis (blank) and 100% hemolysis controls were determined by using a centrifugate of human RBC suspended in PBS and Triton 1%, respectively.

Antimicrobial Assays. Pseudomonas aeruginosa ATCC 27853 and *Staphylococcus aureus* ATCC 29213 were used as model Gram-negative (−) and Gram-positive (+) bacteria, respectively. Bacteria were grown overnight at 37 °C in Luria–Bertani (LB) medium. This culture was reinoculated and incubated at 37 °C to reach the exponential phase of bacteria growth, i.e., a final bacterial suspension containing 10^5 to 5×10^5 colony-forming units (CFU)/mL. The concentration of cells was established by plating 100 μL of different dilutions of the culture solution (e.g., 10^{-2}, 10^{-3}, and 10^{-4}) onto solid agar plates. Following an overnight incubation at 37 °C, the CFU thus formed were counted on each agar plate. As control blanks, eight wells per 96-well tissue culture plate contained only medium, while as a positive growth control, eight other wells contained medium plus cells. These controls were used to detect possible medium contamination and to provide a measure of uninhibited growth of the microorganisms. The plates were incubated overnight at 37 °C,

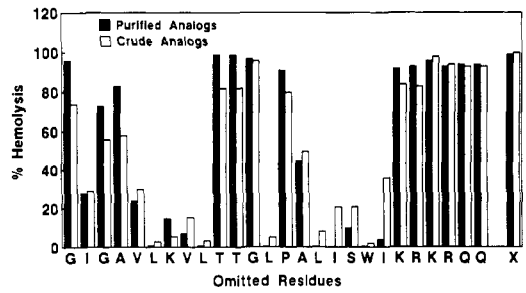

FIGURE 1: Hemolytic activity of the crude and purified forms of the melittin omission analogues. The hemolytic activity was determined at a peptide concentration of 50 μg/mL using a 2.5% RBC solution. The activities of the major impurities (dimeric forms, see text) removed during the purification of glycine-1, glycine-12, and isoleucine-20 omission analogues were 97, 99, and 90%, respectively. Values found in repeat determinations differed by less than 5%. X = full melittin sequence.

and the OD was determined at 620 nm using a Titertek Multiskan apparatus. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of peptide at which there was no change in OD between time 0 and 18 h.

Results

Synthesis and Purification of the Melittin Omission Analogues. Each position of melittin was separately omitted to generate the complete series of 24 omission analogues. These analogues were prepared by solid-phase methods (Merrifield, 1963) using SMPS (Houghten, 1985). The homogeneity of the crude peptides, determined by RP-HPLC, ranged between 50 and 60%. Only three omission analogues, corresponding to the omission of glycine-1, glycine-12, or isoleucine-20, contained significant individual impurities (17%, 18%, and 12%, respectively). These contaminants were isolated along with the correct sequences during purification by preparative RP-HPLC. The desired purified omission analogues were obtained in overall yields averaging 40%. Amino acid analyses (in which tryptophan was not determined) of the contaminants revealed residue ratios identical with those expected for the omission analogues associated with these impurities. Fast atom bombardment mass spectral analyses gave the correct mass ions for melittin and for the three purified analogues corresponding to the omission of glycine-1, glycine-12, and isoleucine-20, but no recognizable mass ions for the three associated contaminants. Mass spectral analyses performed by time-of-flight procedures on a BIOION 20 mass spectrometer gave, in contrast, clear mass ions for these three contaminants. In each case, these were the dimeric forms of the associated omission analogues [e.g., for the glycine-1 omission analogue, the molecular weight of the contaminant was 5579.2, which corresponds to two times the monomeric molecular weight (2789.8)]. The dimerization appears to occur through the tryptophan residues as reported for other peptides under acidic conditions (Omori et al., 1976).

Comparisons of the Hemolytic Activities of the Crude and Purified Forms of Melittin Omission Analogues. Melittin and its 24 individual omission analogues were tested in both crude and purified forms for hemolytic activity at 5, 10, 30, 50, and 100 μg of peptide/mL of RBC solution (1.7, 3.4, 10.2, 17.5, and 35 μM, respectively). The crude and purified peptides produced virtually the same amounts of hemolysis except for the analogue in which isoleucine-20 was omitted (Figure 1). In this instance, the crude peptide yielded a hemolytic activity of 36% at 50 μg/mL, compared to only 4% for the purified form. The contaminant, which is the dimeric form of this analogue, had 90% hemolytic activity at this concentration.

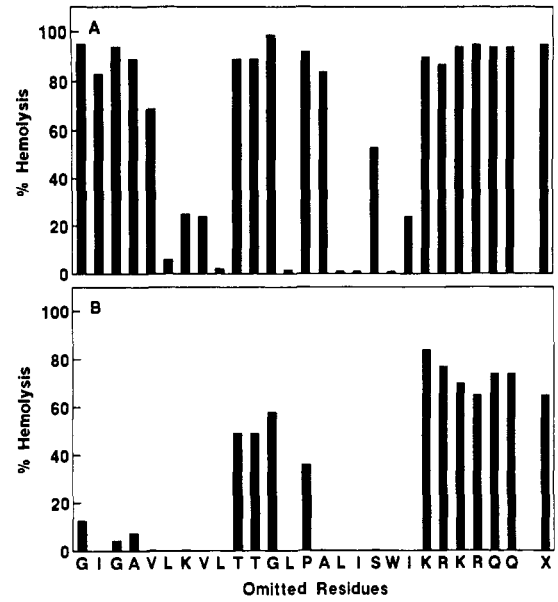

FIGURE 2: Hemolytic activity of melittin omission analogues at two different concentrations. The activities of the purified analogues were determined at a peptide concentration of (A) 100 μg/mL and (B) 10 μg/mL (conditions as in Figure 1). X = full melittin sequence.

The two other contaminants isolated by preparative RP-HPLC also showed high hemolytic activities at 50 μg/mL (97% and 99% for the dimers of glycine-1 and glycine-12 omission analogues, respectively). However, in these instances, the monomeric forms had virtually identical hemolytic activities.

Relative Hemolytic Activity of Each Purified Melittin Omission Analogue. Since the entire sequence of melittin was thought to be involved in its hemolytic activity (Manjunatha Kini & Evans, 1989), we compared the activity of each omission analogue to determine the relative importance of each residue. It should be noted that omission of a single residue from a peptide existing in a helical array can modify the relative orientation of residues throughout the helix. Figure 2 represents the hemolytic activities of each purified analogue at 100 μg/mL (Figure 2A), a concentration used to show the weakly active analogues, i.e., key positions, and at 10 μg/mL (Figure 2B), used to follow those with little or no effect (X = full sequence of melittin). The results clearly delineate the four separate regions composing the melittin molecule. Omission of any of the residues making up the two separate α-helical regions (residues 1–9 and 13–20), except proline-14, significantly decreased the hemolytic activity relative to melittin. However, individual omission of the residues making up the "hinge" and the C-terminal regions of melittin had little or no effect.

The omission of any of the hydrophobic residues from the sequence of melittin greatly decreased hemolytic activity. Thus, omission of any of the eight hydrophobic residues found between positions 6 and 9 and 15 and 20 and at position 13 yielded a decrease in activity ranging from 75 to 99% at the highest concentration tested (100 μg/mL, Figure 2A). In contrast to the hemolytic activity that remained following omissions of lysine-21 or lysine-23 (relative activity to melittin: 0.94 and 0.99, respectively), the omission of lysine-7 led to a 74% loss in activity at 100 μg/mL (Figure 2A).

Omission of threonine-10, threonine-11, or glycine-12, all thought to be in the "hinge" between the two α-helices (Anderson et al., 1980; Terwilliger & Eisenberg, 1982a,b; Bazzo et al., 1988), as well as omission of proline-14, resulted in a modest decrease in hemolytic activity relative to melittin (10–25% loss for threonine-10 and -11 and glycine-12 at 10

Table I: HD_{50} (μg/mL) of Melittin Omission Analogues[a]

Peptide sequence	Omitted residue	HD_{50}	$\frac{HD_{50}\ (analog)}{HD_{50}\ (melittin)}$
IGAVLKVLTTGLPALISWIKRKRQQ	G-1	29	4
G GAVLKVLTTGLPALISWIKRKRQQ	I-2	65	9
GI AVLKVLTTGLPALISWIKRKRQQ	G-3	40	6
GIG VLKVLTTGLPALISWIKRKRQQ	A-4	35	5
GIGA LKVLTTGLPALISWIKRKRQQ	V-5	76	11
GIGAV KVLTTGLPALISWIKRKRQQ	L-6	537	77
GIGAVL VLTTGLPALISWIKRKRQQ	K-7	207	30
GIGAVLK LTTGLPALISWIKRKRQQ	V-8	174	25
GIGAVLKV TTGLPALISWIKRKRQQ	L-9	1027	148
GIGAVLKVL TGLPALISWIKRKRQQ	T-10	11	1.5
GIGAVLKVLT GLPALISWIKRKRQQ	T-11	11	1.5
GIGAVLKVLTT LPALISWIKRKRQQ	G-12	9	1.3
GIGAVLKVLTTG PALISWIKRKRQQ	L-13	1776	254
GIGAVLKVLTTGL ALISWIKRKRQQ	P-14	15	2
GIGAVLKVLTTGLP LISWIKRKRQQ	A-15	56	8
GIGAVLKVLTTGLPA ISWIKRKRQQ	L-16	1016	145
GIGAVLKVLTTGLPAL SWIKRKRQQ	I-17	737	105
GIGAVLKVLTTGLPALI WIKRKRQQ	S-18	97	14
GIGAVLKVLTTGLPALIS IKRKRQQ	W-19	867	124
GIGAVLKVLTTGLPALISW KRKRQQ	I-20	215	31
GIGAVLKVLTTGLPALISWI RKRQQ	K-21	6	0.9
GIGAVLKVLTTGLPALISWIK KRQQ	R-22	5	0.7
GIGAVLKVLTTGLPALISWIKR RQQ	K-23	7	1
GIGAVLKVLTTGLPALISWIKRK QQ	R-24	8	1.1
GIGAVLKVLTTGLPALISWIKRKR Q	Q-25	6	0.9
GIGAVLKVLTTGLPALISWIKRKRQ	Q-26	6	0.9
GIGAVLKVLTTGLPALISWIKRKRQQ	No	7	1

[a] The HD_{50}'s were calculated as described in the text, and related to the HD_{50} for native melittin. Numbers greater than 1 indicate lower activity relative to melittin.

μg/mL, 25% and 45% loss at 30 and 10 μg/mL, respectively, for proline-14).

In contrast to the other regions, single-residue omissions from the C-terminal region (lysine-21, arginine-22, lysine-23, arginine-24, glutamine-25, or glutamine-26) resulted in a small, but significant, increase in activity compared to native melittin at 10 μg/mL (Figure 2B). These results appear to indicate that the individual residues making up the C-terminal region are not individually essential to the hemolytic activity of melittin. The hemolytic activity was also retained in this region when lysine-21 through glutamine-26 were individually replaced by leucine (Blondelle & Houghten, 1991).

HD_{50} of Melittin Omission Analogues. Kinetic studies have shown that monomeric melittin binds to RBC within seconds, which induces the release of hemoglobin (DeGrado et al., 1982). We therefore analyzed the hemolytic activity of each omission analogue using five different concentrations and compared the resulting "Hemolytic Dose" necessary to lyse 50% of the cells (HD_{50}). This yields a more accurate measurement of each analogue's relative activity (Table I and Figure 3). The percentage of hemolysis was determined at peptide concentrations of 5, 10, 30, 50, and 100 μg/mL, and the HD_{50}'s were calculated for each omission analogue by using the software program *Graphpad* developed by Harvey Motulsky, M.D. (University of California, San Diego). Among the hydrophobic residues, the importance of the leucine residues, particularly leucine-13, is more dramatically illustrated by calculating the HD_{50}'s of the omission analogues than by

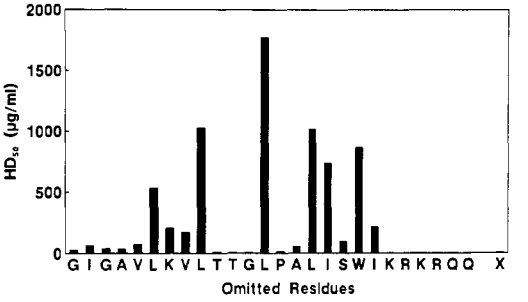

FIGURE 3: Concentration of peptide required to cause 50% lysis of RBC (HD_{50}). HD_{50}'s were calculated by using purified omission analogues at concentrations of 5, 10, 30, 50, and 100 μg/mL. X = full melittin sequence.

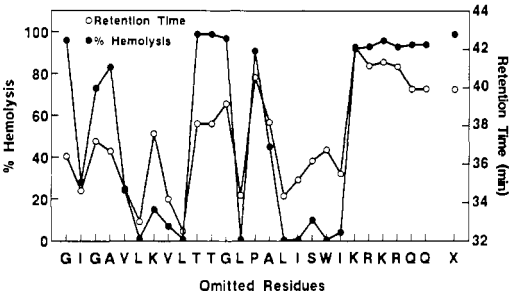

FIGURE 4: Correlation between the retention times found during RP-HPLC and the hemolytic activity of melittin omission analogues. The hemolytic activity of the purified analogues was determined at a peptide concentration of 50 μg/mL as in Figure 1. The retention times were determined by using a 1% gradient increase (10–75% B in 65 min). The correlation coefficient was $r = 0.815$ with $p < 0.001$. X = full melittin sequence.

comparing their hemolytic activities at a fixed concentration. In this instance, the relative HD_{50} of the analogue resulting from omitting leucine-13 was found to be 254-fold greater than the HD_{50} of melittin. As with the leucine residues, tryptophan-19 played a key role in the hemolytic activity of melittin with an HD_{50} 124-fold greater than the HD_{50} of melittin.

Relationship between the Hemolytic Activity of Melittin Omission Analogues and Their Retention Times Found during RP-HPLC. RP-HPLC has been found to be a useful model system for the study of induced conformational effects of peptides at aqueous/lipid interfaces (Melander & Horvâth, 1980; Houghten & DeGraw, 1987; Houghten & Ostresh, 1987; Büttner et al., 1990a,b; Blondelle et al., 1991; Büttner & Houghten, 1991; Ostresh et al., 1991). An example of this is found in peptides which have identical amino acid compositions but different linear sequences, in which decreased amphipathicity was correlated with early retention times and increased amphipathicity with late retention times. In the present study, as shown in Figure 4, decreases in experimental retention times found for the omission analogues of melittin during RP-HPLC correlated well with decreased hemolytic activity (correlation coefficient $r = 0.815$, $p < 0.001$), supporting the role of amphipathicity in the hemolytic activity of melittin.

Antimicrobial Activity of Melittin Omission Analogues. Since melittin possesses antimicrobial properties against both Gram (+) and Gram (−) bacteria (Fennell et al., 1968; Stocker & Traynor, 1986), the omission analogues used in this study were examined for their effects of *P. aeruginosa* [Gram (−) bacteria] and *S. aureus* [Gram (+) bacteria] (Table II). Although many of the analogues inhibited the growth of these two bacteria, only the glycine-12 omission analogue exhibited an increase in activity against *S. aureus* relative to melittin. This analogue was also one of the most hemolytic peptides of

Table II: Minimum Inhibitory Concentration (MIC in μg/mL) of Melittin Omission Analogues[a]

Peptide sequence	Omitted residue	MIC against P.aeruginosa	MIC against S.aureus
IGAVLKVLTTGLPALISWIKRKRQQ	G-1	50	25
G GAVLKVLTTGLPALISWIKRKRQQ	I-2	100	50
GI AVLKVLTTGLPALISWIKRKRQQ	G-3	100	50
GIG VLKVLTTGLPALISWIKRKRQQ	A-4	100	100
GIGA LKVLTTGLPALISWIKRKRQQ	V-5	100	50
GIGAV KVLTTGLPALISWIKRKRQQ	L-6	>100	>100
GIGAVL VLTTGLPALISWIKRKRQQ	K-7	>100	>100
GIGAVLK LTTGLPALISWIKRKRQQ	V-8	100	50
GIGAVLKV TTGLPALISWIKRKRQQ	L-9	>100	>100
GIGAVLKVL TGLPALISWIKRKRQQ	T-10	50	25
GIGAVLKVLT GLPALISWIKRKRQQ	T-11	50	25
GIGAVLKVLTT LPALISWIKRKRQQ	G-12	50	12.5
GIGAVLKVLTTG PALISWIKRKRQQ	L-13	>100	>100
GIGAVLKVLTTGL ALISWIKRKRQQ	P-14	100	25
GIGAVLKVLTTGLP LISWIKRKRQQ	A-15	100	25
GIGAVLKVLTTGLPA ISWIKRKRQQ	L-16	100	>100
GIGAVLKVLTTGLPAL SWIKRKRQQ	I-17	100	>100
GIGAVLKVLTTGLPALI WIKRKRQQ	S-18	100	100
GIGAVLKVLTTGLPALIS IKRKRQQ	W-19	>100	>100
GIGAVLKVLTTGLPALISW KRKRQQ	I-20	50	50
GIGAVLKVLTTGLPALISWI RKRQQ	K-21	50	25
GIGAVLKVLTTGLPALISWIK KRQQ	R-22	50	50
GIGAVLKVLTTGLPALISWIKR RQQ	K-23	50	50
GIGAVLKVLTTGLPALISWIKRK QQ	R-24	50	25
GIGAVLKVLTTGLPALISWIKRKR Q	Q-25	50	25
GIGAVLKVLTTGLPALISWIKRKRQ	Q-26	50	25
GIGAVLKVLTTGLPALISWIKRKRQQ	No	50	25

[a] The MIC was defined as the lowest concentration of peptide at which there was no change in OD at 620 nm between times 0 and 18 h.

the series (Figure 2). Those analogues in which a leucine was omitted were poorly hemolytic (Figure 2) and had minimal activity against either bacteria.

Discussion

Variations in the hemolytic activities of the single-omission analogues of melittin have provided new information on the specific role of each residue. Melittin can be envisioned as containing four regions: the N-terminal α-helix (residues 1–9), the flexible "hinge" region (residues 10–12), the central α-helix (residues 13–20), and the C-terminal positively charged region (residues 21–26). Overall, the general consensus is that melittin must exist as an amphipathic α-helix for lytic activity to occur. The discussion below is structured in the order of these regions.

Formylation of the N-terminus of melittin results in the retention of 80% of melittin's hemolytic activity (Lübke et al., 1971), which indicates that a free N-terminal α-amino group is not essential for hemolytic activity. Analogues prepared having shortened N-terminal sequences such as Mel(7–26) (Habermann & Kowallek, 1970) and Mel(8–25) (Dawson et al., 1978; Gevod & Birdi, 1984) were poor lytic agents. Our data (Figure 2) do not definitively clarify the importance of the individual residues in the N-terminal region. Thus, for residues 1–6 at 100 μg/mL (Figure 2A), only the omission of leucine-6 dramatically decreased the hemolytic activity, while at 10 μg/mL (Figure 2B) omission of any of residues 1–6 yielded poor lytic agents compared to melittin.

The positive charge on lysine-7 appears to have a specific role on the hemolytic activity of melittin. The result found upon omission of lysine-7 is in agreement with the loss of activity brought about by succinylation of the ε-amino groups of the lysine residues of melittin (Habermann & Kowallek, 1970; Manjunatha Kini & Evans, 1989). This result was explained by the formation of intramolecular salt bridges between the carboxylate of the succinate of the altered lysines at positions 21 and/or 23 and the guanidino groups of the arginines (Hider et al., 1983). Our results suggest that the loss in activity resulting from succinylation of all three lysine residues may develop primarily from the absence of a positive charge in position 7. Similarly, increased hemolytic activity was reported for homoarginine melittin (relative activity, 3.5; Habermann & Kowallek, 1970), in which the ε-amino groups of all of the lysines were converted into guanidino groups. Thus, in this case, lysine-7 although chemically modified remained positively charged. These results are, however, in strong contrast to those found upon N-acetylation of all lysine residues and the N-terminal group of melittin in which hemolytic activity was reported to be retained (relative activity, 0.8; Habermann & Kowallek, 1970). The differences in these results are not readily reconciled.

The three "hinge" region residues, consisting of threonine-10, threonine-11, and glycine-12, appear to be flexible "connecting linkers" between the two helices. The small decrease in hemolytic activity found when individually omitting any of these residues indicates that these omissions, while shortening the length of the "hinge" region, do not substantially alter the overall effective orientation of melittin necessary for hemolysis.

An amphipathic α-helical model peptide, which is homologous to melittin in only five positions (the leucines at positions 6, 9, 13, and 16 and the tryptophan at position 19), and the C-terminal hexapeptide region, and composed solely of leucine on the hydrophobic side of the helix and of serine and glutamine as hydrophilic residues, has been reported to have greater hemolytic activity than melittin (HD_{50} 2.5-fold lower than the HD_{50} of melittin; DeGrado et al., 1981, 1982). Since the difference in hydrophilicity between the two faces of the helix is greater than that of melittin, and since no helix-breaking or perturbing residues are present in this model peptide, it has a higher overall potential to form an amphipathic α-helix than melittin. The model peptide's greater hemolytic activity indicates that increasing the length of melittin's helix should enhance its hemolytic activity. However, we have found that omission of proline-14 led to only a small decrease in hemolytic activity. The extension of the helical region by omitting a residue in this manner would be expected to change the region of amphipathicity by altering the juxtaposition of the amphipathic segments of melittin, as best illustrated in the helical wheel format (Figure 5A,B). The necessity for amphipathicity is further emphasized by the increased hemolytic activity reported when proline-14 was replaced with alanine, which increased the helicity and/or amphipathicity of this analogue relative to melittin (Dempsey, 1990).

The omission of any of the hydrophobic residues throughout the sequence of melittin, and in particular omission of any of the leucine residues, dramatically decreased hemolytic activity. These omissions would be expected to alter the overall amphipathicity and to decrease the hydrophobicity relative to melittin, which in turn would affect the strength of its interaction with the membrane surface. On the contrary, if one assumes that the tryptophan-19 omission analogue remains

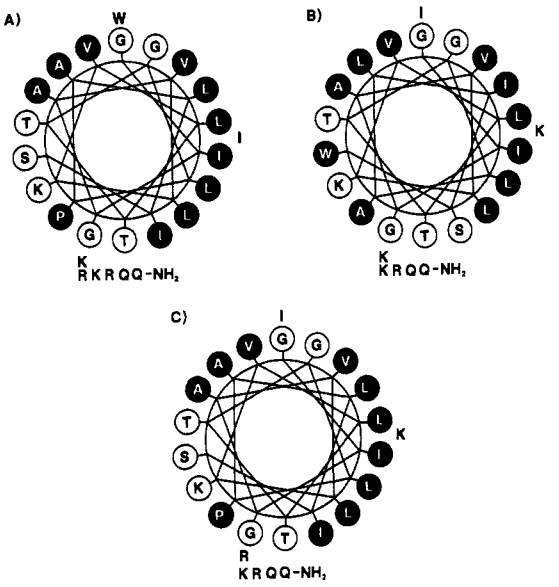

FIGURE 5: Helical wheel representations of the conformation of (A) native melittin, (B) the corresponding P-14 omission analogue, and (C) the corresponding W-19 omission analogue.

in a helical conformation upon interaction with the cell wall, then this omission at the extreme C-terminal end of this helical region would be expected to minimally change the analogue's overall amphipathicity compared to native melittin (Figure 5C). Yet, shortening of the region between the amphipathic and the cationic regions may prevent the analogue's insertion into the membrane, since fluorescence quenching showed that membrane penetration involves the entire hydrophobic side of melittin (Tosteson & Tosteson, 1981; Chen & Lin-Shiau, 1985). The greatly reduced activity of this omission analogue does, however, concur with the reported decrease in activity following photooxidative destruction of the tryptophan indole group of melittin (relative activity; $\ll$0.05; Habermann & Kowallek, 1970). The specific importance of the tryptophan indole group for the hemolytic activity of melittin is substantiated by the decrease in hemolytic activity that we found upon substituting tryptophan-19 for a leucine (the HD_{50} of the leucine-19 substitution analogue is 3.66-fold greater than the HD_{50} of melittin; Blondelle & Houghten, 1991). Furthermore, this agrees with the increase in hemolytic activity obtained when the imino group of the indole moiety of tryptophan-19 is modified with the 2-hydroxy-5-nitrobenzyl group (relative activity, 2.15; Habermann & Kowallek, 1970).

Schröder et al. (1971) found that Mel(1–20), which is missing the entire basic C-terminal region of melittin, retains its surface tension lowering activity but does not cause lysis. The retention of hemolytic activity found upon individual omission of any of the positively charged C-terminal residues agrees with that of tetraacetylmelittin (acetylation of the N-terminal amino group and three ϵ-lysine amino groups), which retains two positive charges due to the arginines in the C-terminal region (Habermann & Kowallek, 1970). However, the loss in activity upon succinylation of the ϵ-amino groups of the lysine residues of melittin (Habermann & Kowallek, 1970; Hider et al., 1983), as noted above, may reflect primarily the succinylation of lysine-7. These results indicate that the C-terminal segment must be sufficiently cationic to bind to the phospholipid head groups of the membrane if lysis is to result.

It should be noted that omission of any residue within an α-helix results in the modification of the relative orientation of the other residues throughout the helix. Such a perturbation may affect the binding of the omission analogues to the membrane, which in turn would cause a change in their lytic properties. We have initiated studies to establish whether those peptides with decreased activities fail to bind to membranes, or bind to the membrane but fail to cause hemolysis. The radiolabeled forms of melittin and the above analogues are being used to determine their relative binding capabilities. The action of each analogue, when bound to the membrane, is also being studied through determination of the kinetics of red blood cell lysis. As determined by CD spectroscopy (Beschiaschvili & Seelig, 1990), melittin has been found to adopt an α-helical conformation in the presence of lipid membranes. In a similar manner to the relative binding of the omission analogues, the induced conformation of melittin in the presence of vesicles would be expected to be affected by the omission of a single residue and, as suggested by RP-HPLC (Figure 4), to correlate with its hemolytic activity. Comparison of the ability of melittin and its omission analogues to adopt an α-helical conformation when bound to vesicles is being investigated by using CD spectroscopy.

Our results agree with the common feature of all the models proposed for the orientation of melittin on the bilayer membrane: that amphipathicity is required for melittin to bind to erythrocytes. One such model involves the formation of ion-permeable channels through the membrane upon a tetrameric association of melittin monomers (Tosteson & Tosteson, 1981; Vogel & Jähnig, 1986). The loss in activity upon omitting lysine-7 is in agreement with this model, since these channels exhibit selectivity for anions over cations (Tosteson & Tosteson, 1981; Gevod & Birdi, 1984; Tosteson et al., 1985). Thus, besides altering its amphipathicity, omission of lysine-7 may alter the access of ions into cells be making the channel of entry more hydrophobic or decreasing its internal diameter. Supporting this hypothesis is the loss of activity we found upon substituting lysine-7 for a leucine, which will not alter the relative juxtaposition of other residues making up the overall amphipathic regions of melittin as will occur upon omission of lysine-7 (the HD_{50} of melittin is 21-fold less than the HD_{50} of the leucine-7 substitution analogue; Blondelle & Houghten, 1991). In contrast, either substitution of lysine-7 for an asparagine (Tosteson et al., 1988) or acetylation of lysine-7, along with the acetylation of the other two lysines and the N-terminal (Hanke et al., 1983), has been reported to show identical voltage-dependent ionic permeability. No data on hemolysis of the asparagine-7 substitution analogue were presented.

In another proposed model, melittin forms a wedgelike conformation, thereby weakening the overall membrane structure and allowing leakage of hemoglobin (Dawson et al., 1978; Terwilliger et al., 1982; Stanislawski & Rüterjans, 1987). Both helices partially penetrate the membrane, while the positively charged residues and the N-terminus remain accessible to the solvent and act as anchors on the membrane surface. A flexible "hinge" between the two helices purportedly allows partial penetration of the molecule into the membrane. However, our results suggest that neither the "hinge" region nor the flexibility supplied by proline-14 is essential to the hemolytic activity of melittin or, alternatively, a two-residue "hinge" may be sufficient for a wedgelike conformation to occur.

Kinetic studies have shown that the release of hemoglobin resulting from lysis of the cells follows a biphasic pattern (DeGrado et al., 1982). During an initial rapid phase, discrete "holes" were postulated to form upon interaction of melittin with the membrane, causing the observed initial release of

hemoglobin. Melittin is then thought to become internalized, resulting in the closing of the "holes", followed by a reopening of the membrane from the inside. This results in a second, slower phase of hemoglobin loss after reorganization and association of melittin and the membrane. This third theoretical model requires an amphipathic α-helix with a highly hydrophobic face to account for the binding of melittin on the membrane surface and for the penetration of melittin into the membrane by hydrophobic forces. The loss in activity found in the present study upon omission of any of the most hydrophobic residues, i.e., leucine, isoleucine, or tryptophan, agrees with this requirement. A positively charged region is also necessary for this model, since the internalization of melittin into the membrane would be driven by the secondary electrostatic attraction of such a charged region with the inner negative charges of the membrane (DeGrado et al., 1982). Since lysine-7 has a key role in the hemolytic activity of melittin, and the loss of individual positive charges in the C-terminal region does not affect activity, lysine-7 may also be involved in the internalization of melittin. Our results, which indicate that the presence of the "hinge" region and proline-14 is not essential, further support this model.

In summary, the activities of the omission analogues of melittin indicate that specific elements are necessary for the lytic properties of melittin to be significant: (a) the peptide must be capable of induction into an overall amphipathic helical structure; (b) the amphipathic region(s) must be of sufficient length to allow the penetration of the peptide into the membrane; (c) the key residues are leucine-6, lysine-7, valine-8, leucine-9, leucine-13, leucine-16, isoleucine-17, tryptophan-19, and isoleucine-20, which appear necessary to assure the length of the two amphipathic regions, and/or their overall hydrophobicity, as well as the necessary positive charge at position 7 in the N-terminal α-helix. No significant increase in the antimicrobial activity of melittin followed omission of single residues throughout its sequence. As found previously for melittin-like hybrid peptides (Boman et al., 1989), the small differences found in the inhibition of microbial growth caused by our individual omission analogues clearly indicate that the antimicrobial activity of melittin is less sequence-dependent than the lysis of red cells.

Finally, we have found that induced structural effects caused by peptide–lipid interactions can be estimated by using RP-HPLC and that retention times correlate well with hemolytic activities for the omission analogues of melittin. This correlation also substantiates the essential role of the amphipathicity of melittin in its hemolytic activity. Although the results of the present studies do not preclude any of the earlier models proposed, they are best reconciled with the proposed model in which melittin initially forms "holes" in the membrane and is then internalized into the membrane. To further our understanding of the role each residue plays in the hemolytic activity of melittin, detailed studies involving shortened sequences, as well as leucine and lysine substitution analogues, are in progress.

ACKNOWLEDGMENTS

We thank Phyllis Minick, Peggy Totzke, and Jackie Caltabellotta for their excellent editing and preparation of the manuscript.

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Peptidyl (Acyloxy)methyl Ketones and the Quiescent Affinity Label Concept: The Departing Group as a Variable Structural Element in the Design of Inactivators of Cysteine Proteinases†

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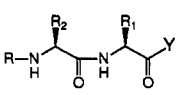
Received October 18, 1990; Revised Manuscript Received January 25, 1991

ABSTRACT: (Acyloxy)methyl ketones, of general structure Z-[AA_2]-[AA_1]-CH_2OCOAr, are potent inactivators of the cysteine proteinase cathepsin B. These reagents have been designed as affinity labels in which the dipeptidyl moiety serves as an affinity group (complementary to the S_1 and S_2 sites of the enzyme), while the (acyloxy)methyl ketone unit ($-COCH_2OCOR$), containing a weak leaving group in the form of a carboxylate nucleofuge, functions as the potentially reactive entity that labels the enzyme. The inhibition is time dependent, active site directed, and irreversible. The apparent second-order rate constant k_{inact}/K_{inact}, which characterizes the inhibition of cathepsin B by this series, spans several orders of magnitude and in certain cases exceeds 10^6 M^{-1} s^{-1}. The activity of this series of inhibitors was found to be exquisitely sensitive to the nature of the carboxylate leaving group as well as the affinity group. A strong dependence of second-order inactivation rate on leaving group pK_a was uncovered for Z-Phe-Ala (acyloxy)methyl ketones [$\log(k/K) = -1.1\ (\pm 0.1) \times pK_a + 7.2\ (\pm 0.4)$; $r^2 = 0.82$, $n = 26$]. Heretofore in constructing affinity labels the choice of leaving group was quite restricted. The aryl carboxylate group thus offers considerable variation as a design element in that both its binding affinity and reactivity can be controlled by substituent effects. Specific peptidyl (acyloxy)methyl ketones thus represent prime examples of highly potent, chemically stable enzyme inhibitors with variable structural elements in both the affinity and departing groups.

Affinity labeling of specific amino acid residues has been an invaluable technique for elucidating key aspects of enzyme structure, function, and reaction mechanisms (Jakoby & Wilchek, 1977). As originally conceived (Baker et al., 1961; Lawson & Schramm, 1962; Schoellmann & Shaw, 1962; Wofsy et al., 1962), affinity labels were attempts to concentrate "group-specific reagents" at the active sites of target enzymes in order to obtain reagents of enhanced specificity capable of establishing specific stable covalent linkages. In the realm of proteinase inhibition over the past 35 years, the reagents have evolved from analogues of functionalized amino acids bearing a reactive group to more specific alkylating agents possessing complex peptide-binding determinants complementary to the target enzyme active site (e.g., **1a-c**) (Shaw, 1980, 1990).



1a, Y = CH_2Cl **1d**, Y = CH_2F
1b, Y = CHN_2 **1e**, Y = CH_2OCOAr
1c, Y = $CHS(CH_3)_2$

† Contribution No. 322 from the Institute of Bio-Organic Chemistry.

0006-2960/91/0430-4678$02.50/0