Design of Model Amphipathic Peptides Having Potent Antimicrobial Activities†

Sylvie E. Blondelle and Richard A. Houghten*

Torrey Pines Institute for Molecular Studies, 3550 General Atomics Court, San Diego, California 92121

Received May 13, 1992; Revised Manuscript Received October 7, 1992

ABSTRACT: Induced amphipathic α-helical conformations play an important role in the biological activity of peptides. By using reversed-phase high-performance liquid chromatography (RP-HPLC) as a means to study the secondary structure of peptides at aqueous/lipid interfaces, a sequence (Ac-LKLLKKLL-KKLKKLLKKL-NH_2) was found to readily adopt an amphipathic α-helical conformation upon interacting with the lipid groups of the stationary phase during RP-HPLC. This peptide exhibited potent antimicrobial activities against both Gram-positive and Gram-negative bacteria. We have prepared a complete set of omission, as well as of leucine and lysine substitution, analogs of this sequence. These analogs were used to investigate the effects of such alterations on the parent sequence's antimicrobial and hemolytic activities relative to each analog's behavior during RP-HPLC. The potential for the formation of ion channels through cell membranes by this amphipathic model peptide was also evaluated through preparation of analogs which varied in length from 8 to 22 residues, while maintaining their amphipathicity.

Since Fleming discovered penicillin (Fleming, 1929), antibiotics have been widely used for the cure of innumerable, potentially fatal infectious illnesses. However, the widespread use of these agents has caused numerous antibiotic-resistant strains to develop, resulting in the continuous need for new antibiotics. Isolation from biological sources (bacteria, insects, amphibians, mammals, etc.) has served as a common means for the discovery of novel antimicrobial peptides such as the gramicidins, magainins, defensins, and cecropins [reviewed in Blondelle and Houghten (1992a)]. Studies directed toward understanding the relationships between the secondary structure and biological activities of these "natural" peptides indicate that the amphipathic α-helical conformation plays an important role in their biological activities [reviewed in Kaiser and Kézdy (1987)]. In an effort to further understand the necessary conformational properties of biologically active peptides, a number of research groups have designed synthetic peptides capable of forming idealized amphipathic structures (DeGrado et al., 1981; DeGrado & Lear, 1985; DeGrado, 1983; Taylor, 1988).

It is generally thought that the biological activity of amphipathic α-helical peptides results from their ability to form ion channels through membrane bilayers (Anzai et al., 1991; Lear et al., 1988; Agawa et al., 1991; Tosteson & Tosteson, 1981; Christensen et al., 1988). Such channels can form by the self-aggregation of peptide monomers in which the hydrophilic residues face inward and the hydrophobic residues interact with the phospholipid groups of the cell wall. A length of approximately 20 residues has generally been reported to be necessary to provide an α-helix capable of spanning the hydrocarbon portion of the lipid bilayer and, in turn, causing the formation of an ion channel through the membrane (Lear et al., 1988). It was found, however, that short cationic α-helical amphipathic model peptides (8–12 residues in length), composed solely of leucine, arginine, and alanine residues, were also able to form ion channels (Anzai et al., 1991). An oligomeric bundle of head to tail dimers was envisioned as an explanatory channel model (Anzai et al., 1991; Agawa et al., 1991). The potent antimicrobial activity against Gram-positive bacteria observed for these amphipathic cationic model peptides correlated well with their propensity to form ion channels (Lee et al., 1986). Their lack of activity against Gram-negative bacteria was not explained.

Reversed-phase high-performance liquid chromatography (RP-HPLC)[1] has been found to have significant potential for the study of the secondary structures of peptides induced at aqueous/lipid interfaces. Using closely related model peptides, composed solely of lysine and leucine residues, each having a sequence capable of being induced into a different structural motif, it was observed in this laboratory that the secondary structures of peptides which are induced when bound to the C-18 of the stationary phase influenced the retention times of these peptides (Houghten & Ostresh, 1987; Ostresh et al., 1991; Büttner et al., 1992; Houghten & De Graw, 1987; Büttner & Houghten, 1991). Separation of peptides during RP-HPLC is known to be primarily due to the different hydrophobic interactions of the individual peptides with the lipid groups of the stationary phase (Hancock & Sparrow, 1983). Such interactions can in many respects be considered to be comparable to the hydrophobic interactions occurring during biological processes. For example, good correlations were found between retention times and the hemolytic activities of series of peptide analogs of the bee venom peptide melittin (Blondelle & Houghten, 1991; Blondelle et al., 1992). An inverse correlation was also obtained between retention times and antimicrobial activity against *Escherichia coli* in a series of peptides in which proline was used to individually replace each residue of the amphipathic α-helical model peptide Ac-LLKLLKKLLKKLKK-NH_2 (Büttner et al., 1992).

In an extension of these RP-HPLC studies, we have now investigated in greater detail the biological activities of model peptides composed of leucine and lysine residues. In particular, we have systematically examined the biological activities of leucine or lysine substitution analogs of Ac-LKLLKKLL-KKLKKLLKKL-NH_2 in relation to their behavior during RP-HPLC, as well as its omission analogs. This peptide was found to be induced into an amphipathic α-helical conformation during RP-HPLC in earlier studies (Ostresh et al., 1991; Büttner & Houghten, 1991). The potential for ion

† This work was funded in part by NSF Grant DIR8713707.

[1] RP-HPLC, reversed-phase high-performance liquid chromatography; HF, hydrogen fluoride; TFA, trifluoroacetic acid; MIC, minimum inhibitory concentration; OD, optical density; RBC, red blood cells; PBS, phosphate-buffered saline; CFU, colony-forming units.

0006-2960/92/0431-12688$03.00/0 © 1992 American Chemical Society

channel formation of this model amphipathic peptide upon interaction with bacterial cell walls was also evaluated by varying the number of residues from 8 to 22 while maintaining the peptide's inducible amphipathic nature.

MATERIALS AND METHODS

Peptide Synthesis. The methodology of simultaneous multiple peptide synthesis (Houghten, 1985) was used to prepare the peptides studied. To obtain omission or substitution analogs, individual packets were removed or coupled separately during synthesis of all of the other analogs and then returned to the common reaction vessel before completion of the synthesis. For cleavage, low–high hydrogen fluoride (HF) was used (Tam et al., 1983). Twenty-four peptides were individually cleaved at the same time with HF using a multiple-vessel cleavage apparatus (Houghten et al., 1986; Multiple Peptide Systems, San Diego, CA). The identities of the resulting peptides were confirmed by time-of-flight mass spectroscopy analyses on a BIOION 20 spectrometer.

RP-HPLC. Analytical RP-HPLC was carried out using a Vydac C-18 column (ODS 25 cm × 4.6 mm) in conjunction with a Beckman-Altex Model 421 HPLC system and two Model 110A pumps. Samples were monitored at 215 nm using a Hitachi Model 100-20 variable-wavelength spectrophotometer. Buffer A consisted of 0.05% TFA in water (pH 3.2) and buffer B of 0.05% TFA in acetonitrile. Peptides were purified using a Waters Milliprep 300 preparative HPLC modified with a preparative Foxy Fraction collector. Fractions of the desired purity (>95% as determined using analytical HPLC) were pooled and lyophilized.

Antimicrobial Assays. Escherichia coli ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were chosen as model Gram-negative bacteria and *Staphylococcus aureus* ATCC 29213 as Gram-positive bacteria. Bacteria were grown overnight at 37 °C in Mueller–Hinton broth, reinoculated, and incubated at 37 °C to reach the exponential phase of bacterial growth [i.e., a final bacterial suspension containing from 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. In 96-well tissue culture plates, peptides dissolved in H_2O were added to the bacterial suspension at concentrations varying from 256 to 0.25 μg/mL derived from serial 2-fold dilutions, and then incubated overnight at 37 °C. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of peptide at which there was no change in optical density (OD), between time 0 and 18 h at 620 nm using a Titertek Multiskan Plus apparatus (Flow Laboratories, Mc Lean, VA).

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

RESULTS

Peptide Design. The structural effects responsible for variation in antimicrobial and hemolytic activities were initially evaluated using three peptides composed solely of lysine and leucine residues synthesized in earlier studies (Ostresh et al., 1991). Lysine and leucine have strong helix-forming potential in globular proteins but quite dissimilar physical properties (Chou & Fasman, 1974). The three peptides were blocked at their N- and C-termini with an acetyl and amide group, respectively, to minimize charge effects at both chain termini. On the basis of a regular α-helical structure of 3.6 residues per turn, an initial length of 18 residues was chosen so that 5 complete turn of an α-helix were possible. The primary sequences (Figure 1) were chosen to represent three different structural motifs: β-sheet (peptide 1), segmentally amphipathic α-helix (peptide 2), and classically amphipathic α-helix (peptide 3). Figure 1 illustrates these sequences in their lateral projections and helical wheel formats (Schiffer & Edmundson, 1967) to show the distribution of the two different side chains of the amino acid residues. Despite their identical composition (i.e., nine leucine and nine lysine residues), the peptides eluted over a 20-min range (Table I). The differences in retention times were attributed to the induced conformational effects upon interaction with the C-18 of the RP-HPLC stationary phase. The postulated induced conformational effects vary according to the juxtaposition of the amino acids along the peptide backbone (Ostresh et al., 1991).

The antimicrobial activity against *E. coli*, *P. aeruginosa* [Gram(−) bacteria], and *S. aureus* [Gram(+) bacteria] as

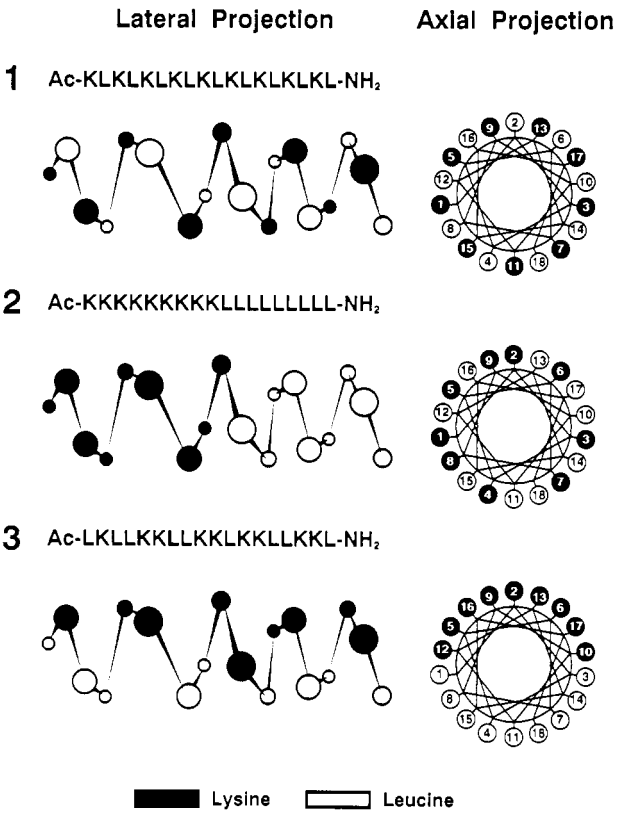

FIGURE 1: Lateral and helical wheel representation of the three model peptides.

Table I: Biological Activity and RP-HPLC Retention Times of Model Peptides Representing Different Structural Motifs

peptide	retention time (min)	MIC (μg/mL) against *S. aureus*	*E. coli*	*P. aeruginosa*	% hemolysis at 100 μg/mL
1	24.85	>256	128	>256	0.1
2	39.10	>256	256	256	3.6
3	43.97	64	64	128	19

Table II: MIC and Hemolytic Activity of the Omission Analogs of Ac-LKLLKKLLKKLKKLLKKL-NH_2

peptide sequence	omitted residue	MIC (μg/mL) against *S. aureus*	*E. coli*	*P. aeruginosa*	% hemolysis at 100 μg/mL
Ac-LKLLKKLLKKLKKLLKKL-NH_2	None	64	64	128	19
Ac- KLLKKLLKKLKKLLKKL-NH_2	L-1	32	16	16	10
Ac-L LLKKLLKKLKKLLKKL-NH_2	K-2	32	8	16	15
Ac-LK LKKLLKKLKKLLKKL-NH_2	L-3/4	32	4	8	6
Ac-LKLL KLLKKLKKLLKKL-NH_2	K-5/6	8	32	32	50
Ac-LKLLKK LKKLKKLLKKL-NH_2	L-7/8	8	16	4	13
Ac-LKLLKKLL KLKKLLKKL-NH_2	K-9/10	64	8	16	50
Ac-LKLLKKLLKK KKLLKKL-NH_2	L-11	256	64	8	0.6
Ac-LKLLKKLLKKL KLLKKL-NH_2	K-12/13	8	16	8	32
Ac-LKLLKKLLKKLKK LKKL-NH_2	L-14/15	32	8	8	7
Ac-LKLLKKLLKKLKKLL KL-NH_2	K-16/17	32	8	16	25
Ac-LKLLKKLLKKLKKLLKK -NH_2	L-18	32	16	8	11

well as the hemolytic activity of the three peptides was determined. Peptide 3 was found to be induced into an amphipathic α-helical array during RP-HPLC [i.e., it has a longer retention time than predicted using retention coefficients (Meek & Rosetti, 1981; Büttner & Houghten, 1991)] and showed the highest activity, in particular against *S. aureus* (Table I). Peptide 3 was also found to exhibit the greatest hemolytic activity (Table I).

To study the mechanism of action of peptide 3 (Ac-LK-LLKKLLKKLKKLLKKL-NH_2) on bacterial and red blood cell walls, we determined the biological activity of three series of analogs of this peptide in relation to their behavior during RP-HPLC. Peptide 3 is termed the parent peptide in the following text.

Omission Analogs of Ac-LKLLKKLLKKLKKLLKKL-NH_2. To evaluate the importance of the amphipathicity (i.e., the different presenting faces of the peptide) on the biological activity of the parent peptide (Ac-LKLLKKLLKKLKKLL-KKL-NH_2), we prepared a complete series of single-omission analogs of this sequence. Thus, each position was separately omitted to generate a set of 11 distinct analogs [omission of any 2 consecutive identical residues yielded the same analog (Table II)]. All analogs except omission of leucine-11 were found to exhibit improved antimicrobial activity relative to the parent peptide against *E. coli*, *P. aeruginosa*, and *S. aureus*. No improvement in activity was seen for the leucine-11 omission analog except against *P. aeruginosa*. Only the omission of the leucine residues, however, yielded a decrease in hemolytic activity (Table II). The leucine-11 omission analog shows the lowest hemolytic activity of this series (0.6% at 100 μg/mL). Illustrated in a helical wheel format (Figure 2), this analog shows a hydrophobic region of no longer than three spatially contiguous residues, which may, in the case of RBCs, result in a weakened interaction between the peptide and cell wall lipids, and in turn in a decrease in activity.

Role of the Hydrophobic Region on the Biological Activity of Ac-LKLLKKLLKKLKKLLKKL-NH_2. To study the role of the hydrophobic face or region of our model amphipathic peptide on its biological activity, we investigated the effect of individually substituting each leucine residue for a lysine. We therefore prepared a set of nine analogs in which a single lysine was "walked" through the leucines of Ac-LKLLKK-

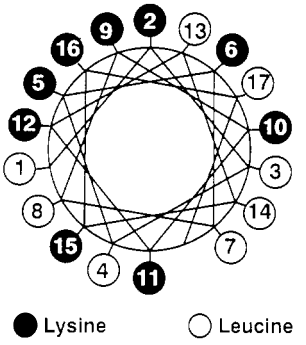

FIGURE 2: Helical wheel representation of the leucine-11 omission analog.

LLKKLKKLLKKL-NH_2 (i.e., each leucine was separately replaced by a lysine). As shown in Table III, in every case these substitutions yielded an increase in antimicrobial activity as compared to the parent sequence.

In order to establish the relative importance of the hydrophobic face as compared to the hydrophilic face of our amphipathic α-helix in the antimicrobial activity of the parent sequence, we synthesized the equivalent set of nine analogs in which a single leucine was walked through the lysine residues. Perturbation of the hydrophobic face upon replacing a single leucine with a lysine resulted in up to a 16-fold increase in antimicrobial activity (Table III). Less effect was found upon perturbing the hydrophilic face, i.e., upon replacing a single lysine for a leucine (2–4-fold enhancement; Table III).

In contrast to the antimicrobial activity, the greatest increases in hemolytic activity (up to 84% hemolysis) were found upon replacing individual lysine residues with leucine. Decreased hemolysis was observed in every case upon replacing any of the original leucine residues with lysine (Table III). In particular, the greatest increase in hemolytic activity was found upon substituting lysine-6, -10, or -12 for a leucine. As shown on a helical wheel representation, the distribution of the residues on the helix of the two analogs having a leucine at position 10 or 12 is such that the hydrophobic face consists of 10 contiguous leucine residues (Figure 3). The increased hemolytic activity observed upon substituting lysine-6 with a leucine cannot be attributed to its position relative to the lipid/

Table III: MIC and Hemolytic Activity of Lysine and Leucine Substitution Analogs

peptide sequence	substituted residue	MIC (μg/mL) against *S. aureus*	*E. coli*	*P. aeruginosa*	% hemolysis at 100 μg/mL
Ac-LKLLKKLLKKLKKLLKKL-NH_2	none	64	64	128	19
Ac-**K**KLLKKLLKKLKKLLKKL-NH_2	L-1	8	32	16	4
Ac-LK**K**LKKLLKKLKKLLKKL-NH_2	L-3	8	32	16	5
Ac-LKL**K**KLLKKLKKLLKKL-NH_2	L-4	4	8	8	4
Ac-LKLLKK**K**LKKLKKLLKKL-NH_2	L-7	4	4	4	3
Ac-LKLLKKL**K**KKLKKLLKKL-NH_2	L-8	8	8	8	5
Ac-LKLLKKLLKK**K**KLLKKL-NH_2	L-11	8	8	4	3
Ac-LKLLKKLLKKLKK**K**LKKL-NH_2	L-14	4	8	8	6
Ac-LKLLKKLLKKLKKL**K**KL-NH_2	L-15	4	8	8	10
Ac-LKLLKKLLKKLKKLLKK**K**-NH_2	L-18	8	8	8	17
Ac-LLLLKKLLKKLKKLLKKL-NH_2	K-2	16	32	32	47
Ac-LKLL**L**KLLKKLKKLLKKL-NH_2	K-5	32	64	32	29
Ac-LKLL**L**LLKKLKKLLKKL-NH_2	K-6	32	32	32	79
Ac-LKLLKKLLL**K**LKKLLKKL-NH_2	K-9	32	32	64	26
Ac-LKLLKKLLK**L**LKKLLKKL-NH_2	K-10	32	64	64	84
Ac-LKLLKKLLKKLL**K**LLKKL-NH_2	K-12	64	64	128	79
Ac-LKLLKKLLKKLKLLL**K**KL-NH_2	K-13	32	32	64	ND[a]
Ac-LKLLKKLLKKLKKLLL**K**L-NH_2	K-16	16	32	64	48
Ac-LKLLKKLLKKLKKLLKL**L**-NH_2	K-17	64	64	128	49

[a] ND, not determined.

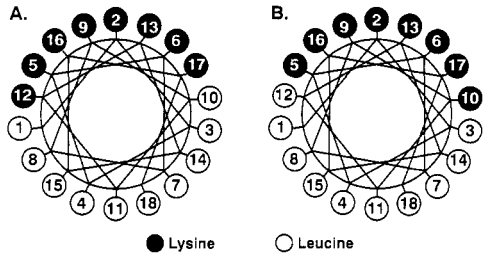

FIGURE 3: Helical wheel representation of the leucine substitution analogs at positions 10 (A) and 12 (B).

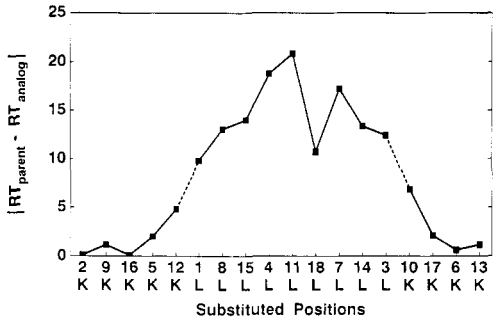

FIGURE 4: RP-HPLC of the lysine and leucine substitution analogs as compared to the parent sequence. The absolute values of the differences between the retention times of each peptide analog and the parent peptide are plotted as a function of each substituted position. The retention times were determined by using a 1% gradient increase starting at 5% buffer B.

aqueous interfaces as seen on a helical wheel representation. Thus, if one does not consider lysine-17 as being part of the helix, then lysine-6 would be the second residue from the interface. Substitution of lysine-5, which is the second residue from the N-terminal interface on the helical wheel representation, did not lead to a similar increase in hemolytic activity.

Introduction of a lysine into the hydrophobic face of the amphipathic α-helix not only makes the peptide more hydrophilic but also would be expected to disrupt the continuity of the hydrophobicity of this face. Both of these factors, in turn, should weaken the hydrophobic interactions between the peptide and a cell wall or the C-18 of the RP-HPLC stationary phase. As expected, all of the analogs in which a leucine was replaced by a lysine eluted earlier than the parent

Table IV: Experimental RP-HPLC Retention Times of Lysine and Leucine Substitution Analogs

peptide sequence	substituted residue	retention time (min) found	Δ-(parent − analog)
Ac-LKLLKKLLKKLKKLLKKL-NH_2	none	55.33	0
Ac-**K**KLLKKLLKKLKKLLKKL-NH_2	L-1	45.48	9.85
Ac-LK**K**LKKLLKKLKKLLKKL-NH_2	L-3	42.86	12.47
Ac-LKL**K**KLLKKLKKLLKKL-NH_2	L-4	36.52	18.81
Ac-LKLLKK**K**LKKLKKLLKKL-NH_2	L-7	38.07	17.26
Ac-LKLLKKL**K**KKLKKLLKKL-NH_2	L-8	42.23	13.10
Ac-LKLLKKLLKK**K**KLLKKL-NH_2	L-11	34.45	20.88
Ac-LKLLKKLLKKLKK**K**LKKL-NH_2	L-14	41.93	13.40
Ac-LKLLKKLLKKLKKL**K**KL-NH_2	L-15	41.28	14.05
Ac-LKLLKKLLKKLKKLLKK**K**-NH_2	L-18	44.58	10.75
Ac-LLLLKKLLKKLKKLLKKL-NH_2	K-2	55.42	−0.09
Ac-LKLL**L**KLLKKLKKLLKKL-NH_2	K-5	57.31	−1.98
Ac-LKLL**L**LLKKLKKLLKKL-NH_2	K-6	55.94	−0.61
Ac-LKLLKKLLL**K**LKKLLKKL-NH_2	K-9	54.19	1.14
Ac-LKLLKKLLK**L**LKKLLKKL-NH_2	K-10	62.21	−6.88
Ac-LKLLKKLLKK**L**LLKKL-NH_2	K-12	60.14	−4.81
Ac-LKLLKKLLKKLKLLL**K**KL-NH_2	K-13	54.18	1.15
Ac-LKLLKKLLKKLKKLLL**K**L-NH_2	K-16	55.40	−0.07
Ac-LKLLKKLLKKLKKLLKL**L**-NH_2	K-17	57.42	−2.09

peptide during RP-HPLC (Table IV). It should be noted that all of the lysine substitution analogs would be expected to have eluted identically, as should all of the leucine analogs, if conformation was not playing a role in elution behavior (Meek & Rosetti, 1981). However, substantial differences were found in the retention times of the two sets of analogs. Larger variations between the retention times of the analogs and the peptide were observed upon replacing the leucine residues with lysine rather than the lysine residues with leucine (Table IV). An informative way to plot these data relates the absolute values of the differences between the retention times of the individual analogs and the parent peptide as a function of each substituted position. If one envisions the parent peptide as having been induced into an amphipathic α-helical conformation upon interaction with the C-18 of the stationary phase, then one can plot the data beginning at lysine-2, the furthest position from the expected helical interface (see helical wheel representation in Figure 1), and moving counterclockwise along the helix (Figure 4). Thus, the further away the

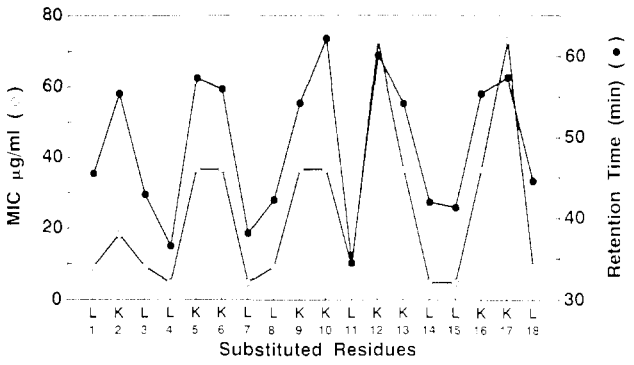

FIGURE 5: Correlation between RP-HPLC retention times and MICs of lysine and leucine substitution analogs. The MICs against *S. aureus* were defined as the lowest concentration of peptide at which there was no change in the OD at 620 nm between times 0 and 18 h. The retention times were determined as described in Figure 4. The correlation coefficient was $r = 0.825$ with $p < 0.001$.

substitution on the hydrophobic face is from the helix interface (i.e., substitution of leucine-4, -7, and -11), the greater the variation in retention times, and in turn the greater the perturbation in the peptide/lipid interactions. These substitution analogs showed the highest antimicrobial activities as well as the lowest hemolytic activities. On the other hand, the substitution of an existing lysine with a leucine did not significantly alter the RP-HPLC behavior of the analog as compared to the parent peptide, except upon replacement of the lysines at positions 10 or 12, which are the closest to the expected leucine/lysine C-18 interface. We believe that the longer retention times of these two analogs resulted from stronger peptide/lipid interactions due to an extended contiguous hydrophobic face being presented to the C-18 (Figure 3). These analogs also exhibited the highest hemolytic activity. Good correlations were found between decreased hydrophobicity (i.e., early retention time) and increased antimicrobial activity, as well as between decreased hydrophobicity and decreased hemolytic activity [r:0.825 for *S. aureus* (Figure 5); r:0.853 for *E. coli*; r:0.777 for *P. aeruginosa*; r:0.833 for hemolysis].

Effect of the Length of the Amphipathic α-Helix. Many known antimicrobial peptides have been postulated to lyse bacterial or RBC membranes through the formation of ion channels (Anzai et al., 1991; Lear et al., 1988; Agawa et al., 1991; Tosteson & Tosteson, 1981; Christensen et al., 1988). We have investigated the effect of the length of a potential amphipathic region on the biological activity of the parent peptide by varying the number of residues while maintaining the peptide's amphipathic nature. Thus, 14 peptides were prepared in which the 18-residue parent sequence was either shortened or lengthened from the N-terminus to generate peptides varying in length from 8 to 22 residues (Table V).

The antimicrobial activities of these peptides against the 3 bacteria tested were low for those peptides having up to 10 residues or more than 18 residues (Table V). The analogs composed of 14 or 15 residues exhibited the highest antimicrobial activities, although the hemolytic activity of the 14-residue peptide was much lower than that of the 15-residue analog (1% versus 15%; Table V). Only those peptides that were more than 14 residues in length caused significant RBC lysis (Table V).

DISCUSSION

Using RP-HPLC as a means to contrast the secondary structure of peptides at aqueous/lipid interfaces to their biological activity, we have been able to design synthetic amphipathic peptides with potent antimicrobial activity. The highest activity was found for the classically amphipathic α-helical sequence (Ac-LKLLKKLLKKLKKLLKKL-NH_2, peptide 3, Figure 1), which confirmed the role of an amphipathic α-helical conformation on biological activity. However, the large difference in the hemolytic activities of the three model peptides indicates that the hemolytic activity is more sequence-dependent than the lysis of bacterial cells, and suggests that two different mechanisms of action are involved in the lysis of bacteria and RBCs.

It was found that alterations in the above parent sequence could be made which resulted in an enhancement of its antimicrobial activity coupled with a decrease in hemolytic activity. For instance, the omission of any residue making up an amphipathic α-helix is expected to alter the peptide amphipathicity by effectively rotating one part of the amphipathic region relative to the other. If classical amphipathicity is essential for biological activity, such omissions should result in a decrease in activity. The unexpected increase in antimicrobial activity observed in the majority of the omission analogs indicates that a hydrophobic presenting face of five contiguous residues (resulting from omission of either L-7, K-9, K-12, or L-14), as compared to a presenting face of nine contigous residues (in the parent sequence), is sufficient for the peptide to interact with the lipids and/or disrupt the organization of the lipids of the bacterial cell wall. This is supported by the higher antimicrobial activity observed for those analogs in which the leucine at position 4, 7, or 11 was replaced by a lysine. Since these leucine residues are located in the central region of the hydrophobic face of the induced amphipathic α-helix, their substitution with a lysine should result in a signficantly smaller continuous hydrophobic face, and in turn weaker peptide/lipid interactions. Such peptide/lipid interactions can be seen by RP-HPLC since RP-HPLC separations occur primarily due to hydrophobic interactions between the peptides and the C-18 of the stationary phase. Thus, the perturbation resulting from replacing a leucine residue with a lysine would be expected (and was found) to result in early retention times during RP-HPLC for these analogs. The results above are contrary to a mechanism of lysis involving the formation of an ion channel in which a longer length of the hydrophobic region would be necessary to span the lipid bilayer (Lear et al., 1988).

For the series of amphipathic peptides prepared, which varied in length from 8 to 22 residues, the highest antimicrobial activities were found for the 14- and 15-mer sequences. The 14-mer showed little hemolytic activity. When compared to the magainins and cecropins, this 14-residue sequence has higher antimicrobial activity and a similarly low hemolytic activity [MICs against *S. aureus*, *E. coli*, and *P. aeruginosa* are for magainin 2-amide 32–64, 8–16, and 128 μg/mL, respectively, and for cecropin A >256, 8–16, and 8–16 μg/mL, respectively; 0% hemolysis was found at 100 μg/mL for both peptides]. It is interesting to note that both the 14-residue and the 15-residue peptides contain a larger hydrophilic than hydrophobic face (8 lysines versus 6 and 7 leucines, respectively). Fifteen-residue peptides made up from segments of cecropin and melittin were recently found to have higher antimicrobial activity than the cecropins (Andreu et al., 1992). These peptides were strongly α-helical in the presence of hexafluoroisopropyl alcohol as determined by circular dichroism. Similar to our results, these findings did not reconcile with a mechanism for antimicrobial activity proposed for the cecropins that involved ion channel formation (Wade et al., 1990). Several other examples of helix formation in aqueous

Table V: Effect of Peptide Chain Length on MIC and Hemolytic Activity

peptide sequence	peptide length	MIC (μg/mL) against *S. aureus*	MIC (μg/mL) against *E. coli*	MIC (μg/mL) against *P. aeruginosa*	% hemolysis at 100 μg/mL
Ac-LKKLLKKL-NH_2	8	128	128	128	0
Ac-KLKKLLKKL-NH_2	9	256	64	128	0
Ac-KKLKKLLKKL-NH_2	10	256	128	64	0
Ac-LKKLKKLLKKL-NH_2	11	32	16	8	0
Ac-LLKKLKKLLKKL-NH_2	12	8	32	8	4
Ac-KLLKKLKKLLKKL-NH_2	13	8	16	8	0.7
Ac-KKLLKKLKKLLKKL-NH_2	14	4	16	8	1
Ac-LKKLLKKLKKLLKKL-NH_2	15	4	16	8	15
Ac-LLKKLLKKLKKLLKKL-NH_2	16	32	64	128	16
Ac-KLLKKLLKKLKKLLKKL-NH_2	17	8	32	16	14
Ac-LKLLKKLLKKLKKLLKKL-NH_2	18	64	64	128	19
Ac-LLKLLKKLLKKLKKLLKKL-NH_2	19	256	256	256	7
Ac-KLLKLLKKLLKKLKKLLKKL-NH_2	20	256	256	128	27
Ac-KKLLKLLKKLLKKLKKLLKKL-NH_2	21	256	64	128	25
Ac-LKKLLKLLKKLLKKLKKLLKKL-NH_2	22	256	256	128	33

solution have been reported for short alanine-based peptides (Marqusee et al., 1989), as well as for similar peptides composed of lysine and leucine residues (DeGrado & Lear, 1985). These latter peptides were also found to bind to calmodulin through both electrostatic and hydrophobic interactions (O'Neil et al., 1987; O'Neil & DeGrado, 1990).

All of the analogs having more than 10 residues were found to elute from 10 to 23 min later than would be predicted using retention coefficients (Meek & Rosetti, 1981; data not shown). These retention times increase progressively with the length of the hydrophobic face. The later than predicted retention times found point to the formation of strong peptide/lipid interactions resulting from the induction of peptides into an amphipathic α-helical conformation. If peptide/lipid interactions are the single or major phenomenon involved in the biological process, then longer peptides would be expected to have the highest biological activity. However, those analogs having more than 18 residues exhibit a lower antimicrobial activity against the 3 bacteria tested. These results show that although strong peptide/lipid interactions are required for antimicrobial activity to occur (as evidenced by RP-HPLC), it is highly probable that a number of other factors are involved as well.

A large number of contiguous hydrophobic residues in an amphipathic peptide appear to be necessary for significant hemolysis to occur. Thus, shortening the hydrophobic region upon omitting any of the leucine residues, or reducing the length of the peptide to 14 residues (resulting in 6 leucines on the hydrophobic side), yielded a decrease in hemolytic activity. Replacement of individual leucine residues for lysine, in particular at position 4, 7, or 11 (resulting in 4–6 contiguous leucine residues), also resulted in substantially weaker hydrophobic interactions as evidenced by their behavior during RP-HPLC (Table IV). Such reduced hydrophobic interactions may also be occurring between the peptides and RBC membranes, resulting, in turn, in lower hemolytic activities. Similarly, a larger hydrophobic face, expected to occur upon leucine substitution at position 10 or 12, yielded increased hemolytic activity. Such increased hydrophobicity could be expected to result in a deeper penetration into, and/or a stronger interaction of, the peptides with the membrane. These results are in agreement with the dramatic decrease in hemolytic activity found upon replacement by a lysine (Blondelle & Houghten, 1992b; Blondelle et al., 1992) or omission (Blondelle & Houghten, 1991) of any of the hydrophobic residues of the 26-residue bee toxin, melittin.

In conclusion, lysis of bacterial cells by the model peptides studied here seems to involve initial electrostatic interactions between the lysines and the negatively charged phospholipid head groups of the cell wall. These interactions may then allow partial penetration of the peptides into the membrane driven by hydrophobic forces between the leucines and the phospholipids of the bilayer. This perturbation of the arrangement of the phospholipids may weaken the bacterial cell wall, resulting, ultimately, in cell lysis. Stronger hydrophobic interactions appear to be necessary for the lysis of RBCs, which may be occurring through the formation of ion channels as found for cationic peptides (Anzai et al., 1991; Agawa et al., 1991; Lear et al., 1988). Although the mechanisms of action are as yet not completely understood, these studies indicate that potent synthetic antimicrobial peptides showing very low hemolytic activity can be designed from the general knowledge of the secondary structure of bioactive peptides. Furthermore, RP-HPLC appears to be a useful tool for the study of such biologically active compounds and peptide/lipid interactions in general. From the above studies, such interactions appear to be involved to a greater extent in the lysis of RBCs than of bacterial cells. However, since such interactions are unlikely to be the only phenomena involved in cell lysis, other means of investigation are required to more fully understand the mechanism of such disruptions.

ACKNOWLEDGMENT

We thank David Burcin for his technical assistance and Eileen Silva for the editing and preparation of the manuscript.

REFERENCES

Agawa, Y., Lee, S., Ono, S., Aoyagi, H., Ohno, M., Taniguchi, T., Anzai, K., & Kirino, Y. (1991) *J. Biol. Chem. 266(30)*, 20218.

Andreu, D., Ubach, J., Boman, A., Wåhlin, B., Wade, D., Merrifield, R. B., & Boman, H. G. (1992) *FEBS Lett. 296(2)*, 190.

Anzai, K., Hamasuna, M., Kadono, H., Lee, S., Aoyagi, H., & Kirino, Y. (1991) *Biochim. Biophys. Acta 1064*, 256.

Blondelle, S. E., & Houghten, R. A. (1991) *Biochemistry 30*, 4671.

Blondelle, S. E., & Houghten, R. A. (1992a) in *Annual Reports in Medicinal Chemistry* (Bristol, J. A., Ed.) pp 159–168, Academic Press, San Diego.

Blondelle, S. E., & Houghten, R. A. (1992b) in *Innovation and Perspectives in Solid Phase Synthesis* (Epton, R., Ed.) pp 121–127, Intercept, Andover, England.

Blondelle, S. E., Burcin, D. E., Salazar, N., & Houghten, R. A. (1992) in *Peptides, Proceedings of the Twelfth American Peptide Symposium* (Smith, J. A., & Rivier, J. E., Eds.) pp 433–434, Escom, Leiden, The Netherlands.

Büttner, K., & Houghten, R. A. (1991) in *Peptides 1990, Proceedings of the Twenty-First European Peptide Symposium* (Giralt, E., & Andreu, D., Eds.) pp 478–480, Escom, Leiden, The Netherlands.

Büttner, K., Blondelle, S. E., Ostresh, J. M., & Houghten, R. A. (1992) *Biopolymers 32*, 575.

Chou, P. Y., & Fasman, G. D. (1974) *Biochemistry 13(2)*, 222.

Christensen, B., Fink, J., Merrifield, R. B., & Mauzerall, D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5072.

DeGrado, W. F. (1983) in *Peptides: Structure and Function, Proceeding of the Eighth American Peptide Symposium* (Hruby, J. H., & Rich, D. H., Eds.) pp 195–198, Pierce Chemical Co., Rockford, IL.

DeGrado, W. F., & Lear, J. D. (1985) *J. Am. Chem. Soc. 107*, 7684.

DeGrado, W. F., Kézdy, F. J., & Kaiser, E. T. (1981) *J. Am. Chem. Soc. 103*, 679.

Fleming, A. (1929) *Br. J. Exp. Pathol. 10(3)*, 226.

Hancock, W. S., & Sparrow, J. T. (1983) in *High-Performance Liquid Chromatography, Advances and Perspectives* (Horváth, C., Ed.) pp 49–85, Academic Press, New York.

Houghten, R. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5131.

Houghten, R. A., & De Graw, S. T. (1987) *J. Chromatogr. 386*, 223.

Houghten, R. A., & Ostresh, J. M. (1987) *BioChromatography 2*, 80.

Houghten, R. A., Bray, M. K., De Graw, S. T., & Kirby, C. J. (1986) *Int. J. Pept. Protein Res. 27*, 673.

Kaiser, E. T., & Kézdy, F. J. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 561.

Lear, J. D., Wasserman, Z. R., & DeGrado, W. F. (1988) *Science 240*, 1177.

Lee, S., Mihara, H., Aoyagi, H., Kato, T., Izumiya, N., & Yamsaki, N. (1986) *Biochim. Biophys. Acta 862*, 211.

Marqusee, S., Robbins, V. H., & Baldwin, R. L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5286.

Meek, J. L., & Rosetti, Z. L. (1981) *J. Chromatogr. 211*, 15.

O'Neil, K. T., & DeGrado, W. F. (1990) *Trends Biochem. Sci. (Pers. Ed.) 15*, 59.

O'Neil, K. T., Wolfe, H. R., Jr., Erickson-Vitanen, S., & DeGrado, W. F. (1987) *Science 236*, 1454.

Ostresh, J. M., Büttner, K., & Houghten, R. A. (1991) in *HPLC of Peptides and Proteins: Separation, Analysis, and Conformation* (Mant, C., & Hodges, R. S., Eds.) pp 633–642, CRC press, Boca Raton, FL.

Schiffer, M., & Edmundson, A. B. (1967) *Biophys. J. 7*, 121.

Tam, J. P., Heath, W. F., & Merrifield, R. B. (1983) *J. Am. Chem. Soc. 105*, 6442.

Taylor, J. W. (1988) in *Recent Progress in Chemistry and Biology of Centrally Acting Peptides* (Dhawan, B. N., & Rapaka, R. S., Eds.) pp 25–41, Central Drug Research Institute, Lucknow, India.

Tosteson, M. T., & Tosteson, D. C. (1981) *Biophys. J. 36*, 109.

Wade, D., Boman, A., Wåhlin, B., Drain, C. M., Andreu, D., Boman, H. G., & Merrifield, R. B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4761.