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Structure-Activity Analysis of an Antimicrobial Peptide Derived from Bovine Apolipoprotein A-II

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We previously showed that bovine apolipoprotein A-II (apoA-II) has antimicrobial activity against *Escherichia coli* in PBS, and its C-terminal residues 49–76 are responsible for the activity using synthetic peptides. In order to understand the structural requirements of peptide 49–76 for the antimicrobial activity, the N- or C-terminus was truncated and then the charged (Lys or Asp) or Ser residues were replaced by Ala. Deletion of the first or last three amino acids and replacement of Lys-54/55 or 71/72 by Ala caused a substantial decreases in α-helical content in 50% TFE, showing the possible presence of helices in N- and C-terminal regions, respectively. The anti-*Escherichia coli* activity of the peptide correlated with its liposome-binding activity. Replacement of Lys-54/55 or 71/72 by Ala resulted in an almost complete loss of anti-*E. coli* activity with a substantial decrease in liposome-binding activity. Moreover, deletion of the last three amino acids caused a reduction to 1/17 of the original anti-*E. coli* activity with a moderate decrease in liposome-binding activity. In contrast, replacement of Ser-65/66, Asp-59, or Asp-69 by Ala hardly affected the anti-*E. coli* activity. These findings suggest that Lys-54/55 and Lys-71/72 on the putative helices are critical for antimicrobial activity, and the C-terminal 3 amino acids are important for the structural integrity of the C-terminal region for effective antimicrobial activity.

Key words: antimicrobial peptide, apolipoprotein A-II, circular dichroism, helix.

A number of antimicrobial peptides have been isolated from many organisms, including animals and plants (*1–7*). These peptides vary considerably in length, amino acid sequence and secondary structure, but have two distinctive features: they are amphipathic and cationic (a net positive charge of +2 or more with at most a single negatively charged amino acid) (*5–7*). Most are considered to act on the cell membranes as a primary target, destroying the barrier function and killing the bacterium, although the exact mode of action of the peptides is not clearly understood (*8–10*). Some have been studied with a view to the possible therapeutic use as antimicrobial compounds with new mechanisms of action (*11, 12*) because increasing resistance of microorganisms to conventional antibiotics has become one of the main problems in human health (*13–16*).

Apolipoprotein A-II (apoA-II) is a protein component of high-density lipoprotein (HDL) and modulates the ability of HDL to promote cholesterol efflux from cells (*17, 18*). We have recently found that bovine apoA-II shows anti–*Escherichia coli* activity (*19*) and its C-terminal region (residues 49–76) is critical for this activity (*20*). The target site of peptide 49–76 appears to be the cytoplasm because electron micrographs of *E. coli* cells treated with peptide 49–76 reveal morphological changes only in the cytoplasm (*20*). Several peptides are also considered to have mechanisms of action other than membrane permeabilization (*21–25*).

In the previous study, we showed that peptide 49–76 has an amphipathic helix in its N-terminal region and that Phe-52/53 are important for lipid binding (*20*). In order to gain further insight into the structural requirements for the anti–*E. coli* activity, we synthesized several analogues of varying lengths or with amino acid replacements, measured the anti–*E. coli* activities of these peptides, and investigated the secondary structures of the peptides by circular dichroism (CD) in a membrane-mimicking environment. Truncation and modification provided information about the essential region and the charged or helix-breaking amino acids necessary for anti–*E. coli* activity.

MATERIALS AND METHODS

Materials—L-Phosphatidyl-ethanolamine (PE), L-phosphatidyl-DL-glycerol (PG) and cardiolipin (CL) were purchased from Sigma.

Microorganism and Culture Medium—E. coli ATCC 25922 was used in this study. Growth medium consisted of LB medium (1% peptone, 0.5% yeast extract, 1% NaCl) for

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Abbreviations:apoA-II, apolipoprotein A-II; CFU, colony-forming unit; CL, cardiolipin;PE, L-phosphatidyl-ethanolamine; PG, L-phosphatidyl- DL-glycerol; TFE, trifluoroethanol.

E. coli.

Synthetic Peptides—The following synthetic peptides were used (Table I): peptides 49–76, 52–76, and 49–73 corresponding to residues 49–76, 52–76, and 49–73 of apoA-II, respectively; S65A/S66A with replacement of Ser-65/66 by Ala; K54A/K55A with replacement of Lys-54/55 by Ala; K71A/K72A with replacement of Lys-71/72 by Ala; D59A with replacement of Asp-59 by Ala; D69A with replacement of Asp-69 by Ala. The eight peptides were synthesized using an Applied Biosystems 433A automated peptide synthesizer, and purified by reverse-phase HPLC on a C_{18} column. The identity of each peptide was confirmed by MS on a LCQ Benchtop ESI/MS/MS mass spectrometer (Finnigan Mat, San José, CA, USA).

Antimicrobial Assay—The antimicrobial activity of the synthetic peptides was tested as previously described (*20*). Briefly, 5×10^4 colony-forming units (CFU) of cells collected during exponential growth were incubated for 2 h at 37°C in 50 μl of phosphate-buffered saline (PBS) in the presence or absence of the peptides. Thereafter, the samples were appropriately diluted, plated in duplicate on agar plates and incubated for 20 h to determine the number of viable colonies. To test the influence of ionic strength on anti–*E. coli* activity, the cells were also exposed for 2 h to peptides in 9 mM sodium phosphate (pH 7) with increasing NaCl concentrations.

Electron Microscopy—*E. coli* cells (10^8 CFU/ml) were incubated with a peptide for 1 h in PBS at 37°C. Each peptide was used at a concentration that caused a reduction of cell viability of 80–90% except peptides K54A/K55A and K71A/K72A, which were used at the same concentration as peptide 49–76. After being treated with a peptide, the cells were fixed in 2.5% glutaraldehyde/0.1 M cacodylate buffer, pH 7.4, and post-fixed in 1% OsO_4. The specimens were embedded in Spurr (TAAB, Aldermaston, England), sectioned, stained with uranyl acetate, and examined with a Hitachi electron microscope (H-7500).

Affinity of Peptides for Phospholipid Vesicles—A peptide and liposomes were mixed under the same conditions as used for the CD measurements, and the mixture was chromatographed on a gel-permeation column (Superose 12) in PBS. The two main peaks of peptide bound to or free from the liposomes were detected in the effluent. The ratio of peptide bound to liposomes was calculated from the peak size of the free peptide.

Circular Dichroism (CD) Measurements—CD spectra were recorded with a J-720 spectropolarimeter (JASCO) using a 1 mm optical path cuvette. The CD spectra of the peptides in 10 mM sodium phosphate buffer (pH 7.4), 50% (v/v) TFE, or phospholipid vesicles were recorded at room temperature in the 200–250 nm wavelength range. The phospholipid vesicles (liposome) were prepared by sonication of multilamellar vesicles composed of PE/PG/CL in a molar ratio of 7:2:1, resembling the *E. coli* cell membrane (*26*). A mixture of peptide and liposome solution (peptide/lipid molar ratio 1:30) was incubated for 4 h at room temperature. CD data are expressed in terms of mean residue ellipticity, [θ], using the mean residue molecular mass from the primary structure. The helicity was estimated from the equation: Percentage α-helix $=\{-([\theta]_{222} + 2340)/30300\} \times 100$ (*27*).

RESULTS

Design of Synthetic Peptides—All the peptides used in this study are summarized in Table I. First, we synthesized two peptides with truncated sequences from the N- or C-terminus of peptide 49–76 to determine the region of the molecule essential for activity. Secondly, we synthesized four analogues with modified sequences to evaluate the role of charged amino acids in the activity. In general, the positive charge on cationic antimicrobial peptides is thought to be important for the interaction of the peptide with the cell membrane (*28*). We replaced Lys-54/55, Lys-71/72, or Asp-59/69 with Ala to maintain the helical structure. In fact, analogues of helical peptides such as histatin, cecropin and magainin, with substitutions of Lys or His with helix-forming amino acids (Ala, Glu, and Leu) often show a substantial decrease in activity, but they show mostly similar CD spectra in organic solvent, compared with the parent peptides (*29–32*). Thirdly, we synthesized an analogue to assess the contribution of Ser-65/66 to the stabilization of the secondary structure. The helical structure of antimicrobial peptides often comprises a helix-turn-helix arrangement (*5*).

Antimicrobial Activity—To determine the importance of the N- or C-terminus for activity, we synthesized length analogues and tested their anti–*E. coli* activity (Table I). When 3 amino acids were removed from the N-terminus of peptide 49–76, most of the activity was retained. In contrast, removal of 3 amino acids from the C-terminus caused a reduction to 1/17 of the original activity. A previous study

TABLE I. **Amino acid sequences, anti–*E. coli* and liposome-binding activities, and α-helicities of peptide 49–76 and its analogues.**

Peptide	Sequence	IC50	Liposome binding	α-helicity TFE	α-helicity Liposome
	50 60 70				
49 - 76	LTPFFKKAGTDLLNFLSSFIDPKKQPAT	3	95	42	36
52 - 76	FFKKAGTDLLNFLSSFIDPKKQPAT	6	55	30	21
49 - 73	LTPFFKKAGTDLLNFLSSFIDPKKQ	50	44	23	17
S65A/S66A	LTPFFKKAGTDLLNFL**AA**FIDPKKQPAT	10	50	20	17
K54A/K55A	LTPFF**AA**GTDLLNFLSSFIDPKKQPAT	>300	25	25	12
K71A/K72A	LTPFFKKAGTDLLNFLSSFIDP**AA**QPAT	>300	20	14	11
D59A	LTPFFKKAGT**A**LLNFLSSFIDPKKQPAT	3	83	28	11
D69A	LTPFFKKAGTDLLNFLSSFI**A**PKKQPAT	3	93	37	16

IC50: the concentration (μM) of peptide that reduces cell viability to 50%. Liposome-binding: the ratio (%) of peptide bound to PE/PG/CL vesicles. α-helicity: the percentage α-helix in 50% TFE and with liposomes.

indicated that replacing Phe-52/53 with Ala abolishes the anti-*E. coli* activity of peptide 49–76 (*20*).These observations indicate that residues 52–76 are essential region for the anti-*E. coli* activity of bovine apoA-II.

To assess the role of a helix-breaking amino acid (Ser) and charged amino acids (Lys and Asp), we synthesized replacement analogues and tested their anti-*E. coli* activity (Table I). The replacement of Ser-65/66 with Ala caused a decrease to about one-third in the anti-*E. coli* activity. However, the substitution of Lys-54/55 or Lys-71/72 by Ala resulted in an almost complete loss of activity. In contrast, the replacement of Asp-59 or Asp-69 with Ala had no effect on the activity. These results suggest that Lys-54/55 and Lys-71/72 are critical for the anti-*E. coli* activity of peptide 49–76, and the last three amino acids are necessary for effective activity.

In the previous study, we observed that the anti-*E. coli* activity of peptide 49–76 is little affected by the addition of NaCl and the cytoplasmic morphology of *E. coli* cells is changed by treatment with peptide 49–76 (*20*). The NaCl effect on the anti-*E. coli* activity of all the analogues was similar to that of peptide 49–76 (not shown). Furthermore, electron micrographs of *E. coli* cells treated with peptides 49–73, 52–76, 49–73, S65A/S66A, D59A, or D69A were identical to that of cells treated with peptide 49–76 (Fig. 1B). These observations suggest that peptide 49–76 and all of its active analogues have the same mode of action. However, treatment with inactive analogues, peptides K54A/K55A or K71A/K72A, produced cells showing very similar morphologies to untreated *E. coli* cells (Fig. 1A).

Liposome-Binding Activity—To assess the affinity of the peptides for lipid, we measured the binding activities of the peptides to liposomes with a lipid composition resembling the *E. coli* cell membrane (Table I). The removal of 3 amino acids from the N- or C-terminus, or the replacement of Ser-65/66 with Ala produced a moderate reduction in the activity. Also the replacement of Lys-54/55 or Lys-71/72 with Ala caused a substantial decrease in the activity. In contrast, the replacement of Asp-59 or Asp-69 with Ala had no effect on the activity. The liposome-binding activity of a peptide correlates with its anti-*E. coli* activity, indicating that the anti-*E. coli* activity depends on the number of bound and penetrating peptide as well as on the number of intracellular peptides. These findings suggest that the electrostatic attraction of Lys-54/55 and Lys-71/72 to anionic lipid is important for the binding of peptide 49–76 to the *E. coli* cell membrane.

CD Measurements—The CD spectra were measured to characterize the secondary structure of the synthetic peptides in membrane-mimetic environments such as TFE or liposomes. Peptide 49–76 and its all examined analogues seemingly showed no ordered secondary structure such as α-helix in 10 mM sodium phosphate buffer as judged from the CD spectra, but formed a well-defined α-helical structure in hydrophobic environments (Fig. 2). All the peptides showed a higher α-helical content in 50% TFE than in liposome solution (Table I). When peptides 52–76 and D59A were added to the liposome solution, slight turbidity appeared. This may affect the accuracy of the determination of the α-helical content of these peptides in liposome solution.

The removal of 3 amino acids from the N- or C-terminus of peptide 49–76 caused a 29–50% decrease in α-helical content in 50% TFE. Furthermore the replacement of Lys-54/55 or Lys-71/72 with Ala caused a 40–70% decrease in 50% TFE. Although truncation or replacement of amino acid residues at the N- or C-terminal regions may affect the overall helical conformation, it is possible that peptide 49–76 forms α-helices at the N- and C-termini, and that these helices are stabilized through electrostatic interactions of Lys with neighboring negative charges. To assess electrostatic interactions, Asp-59/69 was replaced with Ala. The level of the decrease in α-helical content of peptide D59A in 50% TFE was almost the same as that of peptide K54A/K55A, whereas the α-helical content of peptide D69A was closer to that of peptide 49–76 or 52–76 than peptide K71A/K72A. These findings suggest that a salt-bridge is present

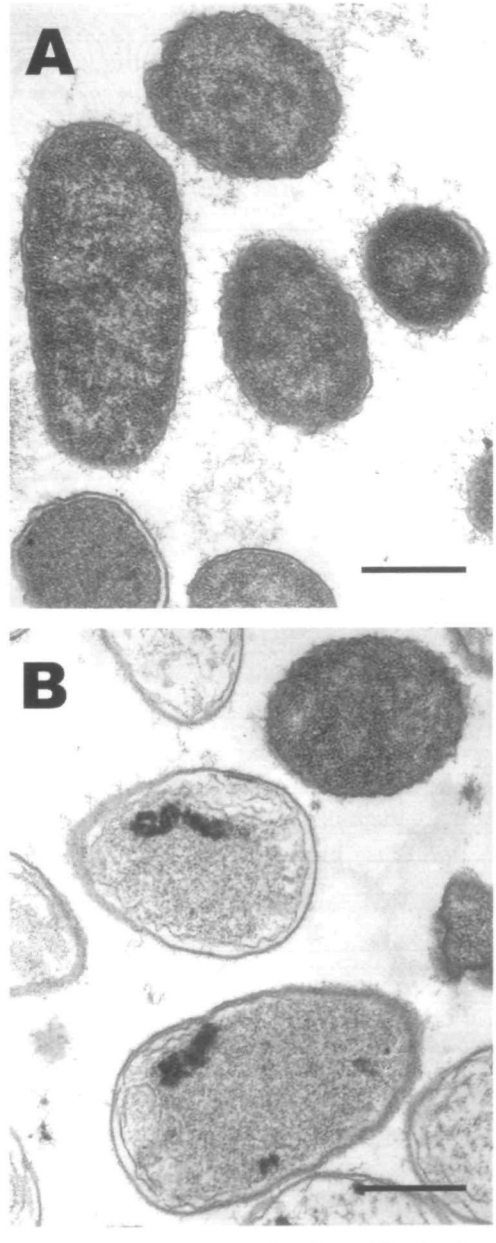

Fig. 1. **Ultrastructure of untreated and peptide 49–76–treated *E. coli* cells.** (A) Untreated control; (B) bacteria treated with peptide 49–76 (0.2 mg/ml) for 60 min. Bars represent 0.5 μm.

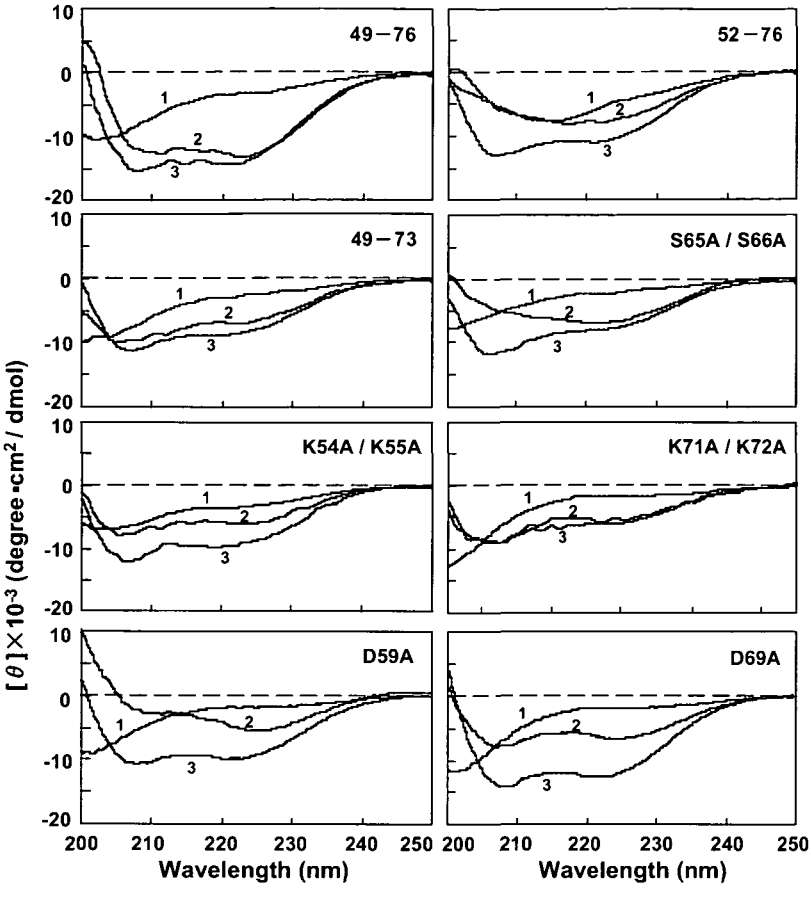

Fig. 2. **CD spectra of peptide 49–76 and its analogues in 10 mM sodium phosphate, pH 7.4 (spectrum 1), with PE/PG/CL vesicles (spectrum 2), and in 50% TFE (spectrum 3).**

in the N-terminal helix but not in the C-terminal helix. Also the replacement of Ser-65/66 with Ala caused about a 50% decrease in α-helical content in 50% TFE. These results suggest that the helical structure of peptide 49–76 in 50% TFE is stabilized by a salt-bridge between Lys-55 and Asp-59, and by Ser-65/66.

In liposome solution, the helical structure of peptide 49–76 was induced to an extent similar to that in 50% TFE. It is most likely that peptide 49–76 binds to the liposome surface, and then the helical structure is formed on the lipid. The α-helical contents of peptide 49–76 and its analogues correlate with their liposome-binding activities except for peptides D59A and D69A, suggesting that the overall helix content depends on the amount of membrane-bound peptide, and the α-helical content of membrane-bound analogues is almost equal to that of peptide 49–76. These findings suggest that Lys-54/55 and Lys-71/72 are important for the binding of peptide 49–76 to membranes, and the last three amino acids are necessary for the effective binding of Lys-71/72 to membranes. Overall, it is possible that the secondary structure of peptide 49–76 contains two helices in organic solvent or on liposomes.

DISCUSSION

Structure-activity studies have been made on various antimicrobial peptides to understand their mechanisms action and also to improve their antibacterial activity. In the previous study, we showed that peptide 49–76 has an amphipathic helix in its N-terminal region, and that Phe-52/53 is important in lipid binding (*20*). Here, we synthesized several analogues of peptide 49–76, replacing the charged amino acids (Lys, Asp) or helix-breaking amino acid (Ser) with Ala, and carried out a study of the structure–activity relationships.

In a membrane-mimicking environment, peptide 49–76 is assumed to comprise two helices with an amphipathic α-helix around residues 54–59 and a cationic helix around residues 71–72. The helical structure is affected by various factors (*10*). We have shown that a salt-bridge between Lys-55 and Asp-59 stabilizes the N-terminal helix of peptide 49–76 in 50% TFE. Moreover, the replacement of Ser-65/66 with Ala causes about a 50% decrease in the α-helical content in 50% TFE. It is known that the replacement of helix-breaking amino acids with Ala results in a significant increase in the helical content of antimicrobial peptides, suggesting that helix-breaking amino acids form the turn structure (*33, 34*). It remains unclear whether Ser-65/66 form a turn. Possibly the interaction between the N- and C-terminal regions separated by Ser residues stabilizes the helical structure of peptide 49–76. Such consideration may explain why the α-helical content of peptide 49–73 was substantially decreased. Also, in liposome solution, peptide S65A/S66A shows about a 50% decrease in α-helical content with a similar decrease in lipid affinity. Possibly Ser-65/66 are necessary for the appropriate orientation of the N- and C-terminal helices of peptide 49–76 on the membrane. In contrast, peptides D59A and D69A exhibit a sub-

stantial decrease in α-helical content in liposome solution, although their liposome-binding activities are similar to peptide 49–76. The causes for the decrease remain unclear in this study. In the case of peptide D59A, the CD measurement may be incorrect because the peptide caused turbidity in the liposome solution. However, peptide K71A/K72A showed a remarkable decrease in α-helical content in 50% TFE. This decrease in α-helical content could not be explained by the interruption of a salt-bridge. The replacement of K-71/72 with Ala may increase solvation of the backbone CO and NH groups in the C-terminal helix, thereby destabilizing the α-helical hydrogen bonds of the backbone CO and NH groups in 50% TFE. Vila *et al.* have reported that Lys introduced into Ala-based polypeptides stimulates the formation of α-helices (*35*).

We have previously shown that peptide 49–76 appears to penetrate the *E. coli* membrane and exerts antimicrobial activity in the cytoplasm (*20*). In this study, we have shown that Lys-54/55 and 71/72 are active sites for the anti–*E. coli* activity of peptide 49–76. Plotting these residues on the helix axial projection wheel showed that K54 and K72 are at almost the same position, which seems to be the border between the hydrophobic and hydrophilic surfaces. Since these two residues are split near both ends of the helical segment, these positive charges would interact with the phospholipid head groups to stabilize the amphipathic helix with a relatively large hydrophobic surface (approximately 50%) at a position deeply penetrating the membrane. Moreover, peptides K54A/K55A and K71A/K72A did not change the cytoplasmic morphology of *E. coli* cells. These findings support the hypothesis that the positive charges are necessary for the binding of the peptide to the *E. coli* cell membrane.

In summary, the results support a model in which peptide 49–76 is composed of two α-helices. The Lys residues on these helices are important for the binding of the peptide to lipid bilayers. Antimicrobial peptides have been proposed as blueprints for the design of novel antimicrobial agents (*12*). Further detailed study of the structure-activity relationships of peptide 49–76 may provide information relevant to the development of useful antibacterial substances.

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