Lipid-binding and antimicrobial properties of synthetic peptides of bovine apolipoprotein A-II

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We previously showed that bovine apolipoprotein A-II (apoA-II) had antimicrobial activity against *Escherichia coli* and the yeast *Saccharomyces cerevisiae* in PBS. We have characterized here the active domain of apoA-II using synthetic peptides. A peptide corresponding to C-terminal residues Leu⁴⁹–Thr⁷⁶ exhibited significant antimicrobial activity against *E. coli* in PBS, but not against *S. cerevisiae*. Experiments using amino-acid-substituted peptides indicated that the residues Phe⁵²–Phe⁵³–Lys⁵⁴–Lys⁵⁵ are required for the activity. Peptide Leu⁴⁹–Thr⁷⁶ induced the release of calcein trapped inside the vesicles whose lipid composition resembles that of *E. coli* membrane, suggesting that peptide Leu⁴⁹–Thr⁷⁶ can destabilize the *E. coli* membrane. CD measurements showed that the α -helicity of peptide Leu⁴⁹–Thr⁷⁶ increased from 3.5 to 36% by addition of the vesicles. When *E. coli* cells were incubated with peptide

Leu⁴⁹–Thr⁷⁶, some proteins were released to the external medium, probably owing to membrane destabilization caused by the peptide. In electron micrographs of *E. coli* cells treated with peptide Leu⁴⁹–Thr⁷⁶, transparent nucleoids and granulated cytoplasm were observed. Amino acid substitutions, Phe⁵²→Ala (Phe^{52,53}→Ala) in peptide Leu⁴⁹–Thr⁷⁶ caused the loss of antimicrobial activity against *E. coli*, although protein-releasing activity was retained. Electron micrographs of the cells treated with peptide Leu⁴⁹–Thr⁷⁶(Phe^{52,53}→Ala) revealed morphological change only at the nucleoids. Therefore peptide Leu⁴⁹–Thr⁷⁶ appears to primarily target the cytoplasm rather than the membrane of *E. coli* cells.

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

INTRODUCTION

Previously, we purified apolipoprotein A-II (apoA-II) from fetal-calf serum as a substance which inhibits the growth of the yeast *Saccharomyces cerevisiae*. Purified apoA-II inhibits the growth of *Escherichia coli* more than that of yeasts [1]. ApoA-II is a protein component of high-density lipoprotein (HDL) and interacts with phospholipids through its amphipathic α -helix [2]. Human apoA-II, and especially its C-terminal domain [3,4], has a high affinity for lipids and is able to displace apolipoprotein A-I (apoA-I) from HDL [5]. The main function of lipoprotein is to transport lipids and lipid-soluble materials throughout the body. Additionally, human apoA-I and A-II have been shown to interact with many cellular systems, including spermatozoa, neutrophils, complements, placental tissue and viruses [6–10]. It is postulated that the amphipathic α -helix plays a central role for lipid binding and many biological functions mediated by apolipoproteins [11].

Numerous antimicrobial proteins and peptides of various structures have been identified and characterized from many organisms, including bacteria, fungi, insects, amphibians, reptiles and mammals [12]. These proteins and peptides are believed to play important roles in innate immunity [13,14] and many of them are studied to develop new therapeutic approaches for infectious diseases [15,16]. Antimicrobial peptides possess various

structural characteristics [13,17], including amphipathic α -helices and β -sheets, and are usually cationic and membrane active. Many cationic antimicrobial peptides, such as cecropins and magainins, permeate the outer membrane of bacteria and form channels or pores in the cytoplasmic membrane [12,16,18]. The amphipathic helices in human apoA-I have been postulated to stabilize membrane bilayers [19,20]. In contrast, Lambert et al. have demonstrated that residues 53–70 of human apoA-II form oblique-orientated helices at the lipid/water interface and induce membrane destabilization [21].

Therefore, the aim of the present study was to examine the structural features of apoA-II which induces antimicrobial activity and to determine the antimicrobial domain using synthetic peptides. We have shown that the peptide Leu⁴⁹–Thr⁷⁶ was responsible for the anti-*E. coli* and lipid-binding activities of apoA-II. We have also shown that peptide Leu⁴⁹–Thr⁷⁶ forms an amphipathic α -helix at its N-terminal region that is required for the activity.

EXPERIMENTAL

Materials

Bovine apoA-II was isolated from fetal-calf serum as described previously [1]. L-Phosphatidylethanolamine (PE), L-phosphatidyl-DL-glycerol (PG), cardiolipin (CL), L-dimyristoylglycerol-

Abbreviations used: ApoA-I, apolipoprotein A-I; ApoA-II, apolipoprotein A-II; calcein, 2',7'-bis(carboxymethyl)aminomethylfluorescein; CL, cardiolipin; CFU, colony-forming unit; HDL, high-density lipoprotein; MLV, multilamellar vesicles; Myr₂GroPCho, L-dimyristoylglycerophosphocholine; PC, phosphatidylcholine; PE, L-phosphatidylethanolamine; PG, L-phosphatidyl-DL-glycerol; SUV, small unilamellar vesicles; TFE, trifluoroethanol; P/L, peptide/lipid.

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Figure 1 Schematic representation of synthetic peptides used in this experiment and amino acid sequence of bovine apoA-II

Numbers refer to amino acid positions in apoA-II. Peptide Leu⁴⁹–Thr⁷⁶ analogues have the substitution Ala⁵²–Ala⁵³ for Phe⁵²–Phe⁵³ or Ser⁵⁴–Ser⁵⁵ for Lys⁵⁴–Lys⁵⁵.

phosphocholine (Myr₂GroPCho) and 2',7'-bis(carboxymethyl)-aminomethylfluorescein (calcein) were purchased from Sigma.

Micro-organisms

Micro-organisms used in this study were *Escherichia coli* A.T.C.C. 25922 and *Saccharomyces cerevisiae* A364A. Growth media consisted of YPD medium [1% (w/v) yeast extract/2% (w/v) peptone/2% (w/v) glucose] for *S. cerevisiae* and Luria-broth medium for *E. coli* [1].

Synthetic peptides

The following synthetic peptides were used (Figure 1): peptides Glu¹–Lys³⁰, Gly³¹–Leu⁶⁰, Leu⁴⁹–Thr⁷⁶ and Thr⁵⁸–Thr⁷⁶ corresponding to the residues 1–30, 31–60, 49–76 and 58–76 of apoA-II respectively; peptide Leu⁴⁹–Thr⁷⁶(Phe^{52,53} → Ala) bearing the substitution Ala–Ala for Phe–Phe at positions 52 and 53; peptide Leu⁴⁹–Thr⁷⁶(Lys^{54,55} → Ser) with a substitution of Ser–Ser for Lys–Lys at positions 54 and 55. The six peptides were synthesized on resin with a model 433A automated peptide synthesizer (Perkin–Elmer Applied Biosystems Division, Foster, CA, U.S.A.), using the 9-fluorenylmethylcarbonyl ('Fmoc') strategy. They were purified by reverse-phase HPLC using a C₁₈ column with a linear gradient of 5–65% solution B (0.075% trifluoroacetic acid in acetonitrile) in solution A (0.1% trifluoroacetic acid in water). The identity of each peptide was confirmed by MS on a LCQ Benchtop ESI/MS/MS mass spectrometer (Finnigan Mat, San José, CA, U.S.A.).

Antimicrobial assay

The antimicrobial activity of synthetic peptides and apoA-II was tested as previously described [22]. Briefly, 5 × 10⁴ colony-forming units (CFU) of mid-exponential-phase cells were incubated for 2 h at 37 °C in 50 µl of PBS [9 mM sodium phosphate (pH 7)/150 mM NaCl] in the presence or absence of the peptides. Thereafter samples were appropriately diluted, plated in duplicate on agar plates and incubated for 20–48 h to determine the number of viable colonies. To test the influence of ionic strength on anti-*E. coli* activity, cells were also exposed for 2 h to peptide Leu⁴⁹–Thr⁷⁶ (10 µM) or apoA-II (80 µM) in 9 mM sodium phosphate (pH 7) with increasing NaCl concentrations (10–150 mM).

Peptide concentrations were determined by means of quantitative amino acid analysis.

Interaction of peptides with phosphatidylcholine

The association of synthetic peptides with lipids was assessed by monitoring the rate of clearance of the turbidity of Myr₂GroPCho

multilamellar vesicles (MLV) due to the solubilization of Myr₂GroPCho MLV, as described previously [23]. Briefly, a chloroform solution of Myr₂GroPCho was transferred to a test tube and the solvent was removed under a stream of N₂. The test tube was vortex-mixed to obtain MLV in PBS. The mixture of peptide and Myr₂GroPCho MLV was incubated at 25 °C for 10 min and the absorbance was measured spectrophotometrically at 325 nm.

Leakage of liposome contents

The PE/PG/CL (7:2:1, molar ratio) small unilamellar vesicles (SUV) encapsulating calcein were prepared as described by Lambert et al. [21]. After a 10 min incubation of the peptides with the SUV solution (60 µM phospholipids) at 25 °C in 10 mM Tris/HCl, pH 8, containing 150 mM NaCl and 0.1 g/l Na-EDTA, the release of calcein into the external medium was monitored with a Hitachi F-2000 fluorescence spectrophotometer; 100% leakage was achieved by lysis of the vesicles with 5 g/l Triton X-100. The percentage release of calcein was calculated from the following equation:

$$\text{Percentage calcein release} = (a - b) / (c - b) \times 100$$

where *b* = initial fluorescence, *c* = total fluorescence observed after addition of Triton X-100 and *a* = fluorescence at time *t*.

Electron microscopy

E. coli cells (10⁸ CFU/ml) were incubated with peptide Leu⁴⁹–Thr⁷⁶ (0.2 mg/ml) for different times in PBS at 37 °C. They were fixed with 2.5% glutaraldehyde/0.1 M cacodylate buffer, pH 7.4, and post-fixed in 1% OsO₄. The cells were embedded in Spurr, (an epoxy resin produced by TAAB Laboratory Equipment Limited, Aldermaston, Berks., U.K.), stained with uranyl acetate and lead citrate and examined under a Hitachi electron microscope (H-600).

CD measurements

CD spectra of the peptides were recorded with a J-600 automatic recording dichrograph (JASCO) at room temperature. Far-UV CD spectra were measured between 200 and 250 nm in a 1-mm-optical-pathlength cuvette at a peptide concentration of 0.2 mg/ml either in a 10 mM sodium phosphate buffer, pH 7.4, or in a 1:1 (v/v) mixture of trifluoroethanol (TFE) and buffer. TFE was used as a structure-promoting solvent. CD spectra were also measured in the presence of phospholipid vesicles [peptide/lipid (P/L) molar ratio 1:30]. The phospholipid vesicles were

prepared by sonication of Myr₂GroPCho or PE/PG/CL MLV, as described previously [24]. CD data are expressed in terms of mean residue ellipticity at 222 nm ($[\theta]_{222}$). The helicity was estimated by the equation:

$$\text{Percentage } \alpha\text{-helix} = \{ -([\theta]_{222} + 2340) / 30300 \} \times 100 \text{ [25].}$$

RESULTS

Identification of antimicrobial domain of bovine apoA-II

Purified apoA-II affected the viability of the Gram-negative bacterium *E. coli* and the yeasts *S. cerevisiae* and *Candida albicans* [1]. To determine the active site of apoA-II, peptide fragments covering various regions of apoA-II were synthesized (Figure 1) and their antimicrobial activities were tested. Among them, peptide Leu⁴⁹–Thr⁷⁶ showed significant antimicrobial activity against *E. coli* (Figure 2a), but not against yeasts (results not shown). The concentration of peptide Leu⁴⁹–Thr⁷⁶ with reduction of cell viability to 50% (IC₅₀) was $\approx 3 \mu\text{M}$ at a cell density of 10^6 CFU/ml (Figure 2a), and that of IC₅₀ at 10^8 CFU/ml was $\approx 30 \mu\text{M}$ (results not shown).

The effect of NaCl on anti-(*E. coli*) activity of both peptide Leu⁴⁹–Thr⁷⁶ and apoA-II was similar (Figure 2b), suggesting that peptide Leu⁴⁹–Thr⁷⁶ is responsible for the anti-(*E. coli*) activity of apoA-II. Since peptide Thr⁵⁸–Thr⁷⁶ does not show anti-(*E. coli*) activity, residues Leu⁴⁹–Gly⁵⁷ are thought to play an important role for the activity. To assess the role of residues Leu⁴⁹–Gly⁵⁷,

Figure 2 Antimicrobial activity of synthetic peptides

(a) Anti-(*E. coli*) activity of synthetic peptides of apoA-II. (b) Effect of NaCl on the anti-(*E. coli*) activity of peptide Leu⁴⁹–Thr⁷⁶ and apoA-II. Antimicrobial activity was tested as described in the Experimental section. Data shown are for peptides Glu¹–Lys³⁰ (□), Gly³¹–Leu⁶⁰ (△), Leu⁴⁹–Thr⁷⁶ (●) and Thr⁵⁸–Thr⁷⁶ (×), apoA-II (○) and control (◆). The molecular masses of peptide Leu⁴⁹–Thr⁷⁶ and apoA-II are 3126 Da and 8549 Da respectively.

Figure 3 Interaction of synthetic peptides with phospholipids

(a) Turbidity decrease of a mixture of the peptides with Myr₂GroPCho liposome. The turbidity was monitored by absorption at 325 nm as described in the Experimental section. (b) Leakage of liposomal contents induced by the peptides. Peptides and PE/PG/CL SUV in which calcein was encapsulated, were incubated at 25 °C for 10 min as described in the Experimental section. Data shown are for peptides Leu⁴⁹–Thr⁷⁶ (●), Leu⁴⁹–Thr⁷⁶(Phe^{52,53} → Ala) (■) and Leu⁴⁹–Thr⁷⁶(Lys^{54,55} → Ser) (▲) and apoA-II (○).

Figure 4 The CD spectra of peptide Leu⁴⁹–Thr⁷⁶

CD spectra of peptide Leu⁴⁹–Thr⁷⁶ were determined in 10 mM sodium phosphate at pH 7.4 (spectrum 1), with Myr₂GroPCho vesicles (spectrum 2), with PE/PG/CL vesicles (spectrum 3) and in 50% TFE (spectrum 4) as described in the Experimental section.

Table 1 Ellipticity of synthetic apoA-II peptides

The mean residue ellipticity at 222 nm ($[\theta]_{222}$) was calculated from:

$$[\theta]_{222} = \text{MRW} \cdot \theta_{222} / Pc$$

where c is the peptide concentration, P is the cuvette pathlength, MRW is the mean residue weight of the peptide and θ_{222} is the measured ellipticity angle at 222 nm. Values in parentheses indicate percentage α -helix.

Peptide	Mean residue ellipticity ($[\theta]_{222}$, in degrees $\cdot$ cm ² $\cdot$ dmol ⁻¹)			
	Aqueous	With TFE	With Myr ₂ GroPCho	With PE/PG/CL
Gly ³¹ -Leu ⁶⁰	-610 (0)	-5360 (10)	-1060 (0)	n.d.*
Thr ⁵⁸ -Thr ⁷⁶	-2790 (1.5)	-8230 (19)	-3070 (2.4)	n.d.*
Leu ⁴⁹ -Thr ⁷⁶	-3390 (3.5)	-14600 (41)	-11300 (29)	-13200 (36)
Leu ⁴⁹ -Thr ⁷⁶ (Phe ^{52,53} → Ala)	-3510 (3.9)	-12600 (34)	-5640 (11)	-10500 (27)
Leu ⁴⁹ -Thr ⁷⁶ (Lys ^{54,55} → Ser)	-2630 (1.0)	-7770 (18)	-6250 (13)	-6120 (13)

* n.d., not determined.

Figure 5 Analyses of *E. coli* viability and protein composition

(a) Exponentially growing cells (10^8 CFU/ml) were incubated with 0.2 mg/ml of the peptides in PBS at 37 °C. At the indicated time, cell suspensions were assessed for viability as described in the Experimental section. Data shown are for peptides Leu⁴⁹-Thr⁷⁶ (●), Leu⁴⁹-Thr⁷⁶(Phe^{52,53} → Ala) (■) and Leu⁴⁹-Thr⁷⁶(Lys^{54,55} → Ser) (▲). (b) After assays for (a), the rest of samples were centrifuged and proteins from cells (P) and supernatant (S), which had been incubated with peptide Leu⁴⁹-Thr⁷⁶ for 15 min (lanes 1 and 2), 30 min (lanes 3 and 4), and 60 min (lanes 5 and 6) or without peptide for 60 min (lanes 7 and 8), were analysed by SDS/PAGE.

peptide Leu⁴⁹-Thr⁷⁶ analogues, peptides Leu⁴⁹-Thr⁷⁶(Phe^{52,53} → Ala) and (Lys^{54,55} → Ser) were synthesized (Figure 1). The two peptides did not show any anti-*(E. coli)* activity at concentrations up to 100 μ M (results not shown). These results suggest that residues Leu⁴⁹-Gly⁵⁷ are important in the anti-*(E. coli)* activity of apoA-II.

Lipid-binding properties of peptides

Previous studies have indicated that the C-terminal region of human apoA-II (residues Thr⁵⁰-Gln⁷⁷) binds phosphatidylcholine (PC) [26], which is the major lipid of HDL [27]. Therefore, we tested whether peptide Leu⁴⁹-Thr⁷⁶ shows this activity. The formation of complexes between the peptides and PC was monitored by measuring the turbidity of Myr₂GroPCho multilamellar vesicles (MLV) with increasing peptides. The apolipoprotein and many synthetic amphipathic peptides were shown to spontaneously form small particles in the size range of HDL,

when exposed to PC dispersions [28]. Among the peptides tested, peptides Leu⁴⁹-Thr⁷⁶ and Leu⁴⁹-Thr⁷⁶(Lys^{54,55} → Ser), had high lipid-binding activity similar to apoA-II (0.03–0.04 P/L molar ratio), whereas peptide Leu⁴⁹-Thr⁷⁶(Phe^{52,53} → Ala) showed low activity (Figure 3a). Other peptides, Glu¹-Lys³⁰, Thr⁵⁸-Thr⁷⁶ and Gly³¹-Leu⁶⁰ had very low activity, dissolving MLV at 0.36, 0.41 and > 1 (P/L molar ratio) respectively (results not shown). These data suggest that residues Leu⁴⁹-Thr⁷⁶, especially Phe⁵²-Phe⁵³, play an important role in the interaction of apoA-II with HDL. As in human apoA-II, Leu⁵²-Ile⁵³ has been shown to be important in PC binding [26].

Since peptide Leu⁴⁹-Thr⁷⁶ binds PC liposomes, it may interact directly with phospholipids in *E. coli* membranes. To test this possibility, small unilamellar vesicles (SUV) trapping calcein were prepared from a mixture of PE, PG and CL in a molar ratio of 7:2:1, resembling *E. coli* membranes [29], and tested for susceptibility to the peptides (Figure 3b). Peptide Leu⁴⁹-Thr⁷⁶ and Leu⁴⁹-Thr⁷⁶(Phe^{52,53} → Ala) released calcein from SUV more

Figure 6 Electron micrographs of representative *E. coli* cells treated with peptide Leu⁴⁹-Thr⁷⁶

E. coli cells (10^8 CFU/ml) were treated with or without 0.2 mg/ml (64 μ M) of peptide Leu⁴⁹-Thr⁷⁶ as described in the Experimental section. Data shown are for 60 min incubation without the peptide (**a** and **e**), for 15 min incubation with the peptide (**b**), for 30 min incubation with the peptide (**c** and **f**) and for 60 min incubation with the peptide (**d**). Bars represent 1 μ m for (**a**)–(**d**) and 0.1 μ m for (**e**) and (**f**).

effectively than did peptide Leu⁴⁹-Thr⁷⁶(Lys^{54,55} → Ser). Also these peptides could dissolve PE/PG/CL MLV at nearly the same P/L molar ratio (results not shown). These results suggest that Lys⁵⁴-Lys⁵⁵, but not Phe⁵²-Phe⁵³, are important in membrane destabilization causing the release of contents, although

both Phe⁵²-Phe⁵³ and Lys⁵⁴-Lys⁵⁵ function in the interaction of peptide Leu⁴⁹-Thr⁷⁶ with membrane phospholipids.

Conformation of synthetic peptides

The secondary structures of the peptides were determined by CD spectroscopy. The α -helicity of peptide Leu⁴⁹-Thr⁷⁶ was increased from 3.5 to 29 and to 36 % by the addition of a Myr₂GroPCho and PE/PG/CL vesicles respectively, whereas those of peptides Gly³¹-Leu⁶⁰ and Thr⁵⁸-Thr⁷⁶ were not changed (Figure 4 and Table 1). This is in a good agreement with a previous report that the α -helicity of human apoA-II (residues Thr⁵⁰-Gln⁷⁷) was increased from 17 to 41 % when the peptide was associated with lipid [26]. The α -helix formation in peptide Leu⁴⁹-Thr⁷⁶ is predictable at residues Leu⁴⁹-Ala⁵⁶ by Chou-Fasman analysis [30] and at Leu⁴⁹-Lys⁵⁵ and Leu⁶⁰-Phe⁶⁴ by the GOR (Garnier/Osguthorpe/Robson) method [31]. It is likely peptide Leu⁴⁹-Thr⁷⁶ can form α -helices at its N-terminal region. The helical content of peptide Leu⁴⁹-Thr⁷⁶ (Lys^{54,55} → Ser) was lower than that of peptide Leu⁴⁹-Thr⁷⁶ (Table 1), probably because Ser tends to form β -turns, whereas Lys promotes helical formation [30].

Analyses of proteins released by synthetic peptides from *E. coli*

When *E. coli* cells were treated with peptide Leu⁴⁹-Thr⁷⁶, the cells were not lysed, as the cell number of the mixture was unchanged during the incubation (results not shown), but lost their proteins into the medium as determined by SDS/PAGE (Figure 5b). The proteins were recovered from the supernatant of the mixture and had molecular masses of 85, 71, 58, 50, 48 and 16 kDa (Figure 5b, lanes 2, 4 and 6). Peptide Leu⁴⁹-Thr⁷⁶(Phe^{52,53} → Ala), but not peptide Leu⁴⁹-Thr⁷⁶(Lys^{54,55} → Ser), showed protein-releasing activity similar to peptide Leu⁴⁹-Thr⁷⁶ (results not shown). However *E. coli* cells treated with peptide Leu⁴⁹-Thr⁷⁶(Phe^{52,53} → Ala) did not lose their proliferative activity during the incubation (Figure 5a), although they formed very small colonies on Luria broth/agar plates, compared with those treated with peptide Leu⁴⁹-Thr⁷⁶(Lys^{54,55} → Ser) or PBS (results not shown). Thus protein-releasing activity did not correlate well with anti-*E. coli* activity, though it did cause recoverable damage to the target *E. coli* cells.

Ultrastructural changes of *E. coli* cells

We tested the morphological effects of these peptides on the target *E. coli* cells. Peptide Leu⁴⁹-Thr⁷⁶ caused some drastic effects on the ultrastructure of *E. coli* cells (Figure 6). After a 30 min exposure of cells to the peptide, filamentous structures on the cell surface, which might be pili (fimbriae, fibrillae) [32,33], were completely removed, and electron-light nucleoids, which are normally scattered throughout the cytoplasm (Figure 6a), were gathered together to form clear clusters containing fine fibrillar materials (Figures 6b and 6c). The cytoplasm was packed together, increasing electron density (Figures 6b and 6c). These structural changes were confirmed by the electron micrographs taken at a higher magnification (compare Figure 6f with Figure 6e). At this stage many cells lost proliferative activity (Figure 5a). At 60 min, the nucleoids were enlarged, becoming electron-lucid by the loss of fibrillar materials. In contrast, the cytoplasm was extensively granulated and was compressed between the nucleoid and the outer membrane. The cytoplasmic membrane was

Figure 7 Electron micrographs of representative *E. coli* cells treated with peptides Leu⁴⁹-Thr⁷⁶(Phe^{52,53} → Ala) (a) and Leu⁴⁹-Thr⁷⁶(Lys^{54,55} → Ser) (b)

E. coli cells were treated with the peptides for 60 min under the conditions described in Figure 6. The bars represent 1 μ m.

entangled in part and frequently fragmented (Figure 6d). On the other hand, peptide Leu⁴⁹-Thr⁷⁶(Lys^{54,55} → Ser) did not induce these morphological changes (compare Figure 7a with Figure 6d), while peptide Leu⁴⁹-Thr⁷⁶(Phe^{52,53} → Ala) caused clear clusterization of nucleoids without compression of the cytoplasm and fragmentation of the cytoplasmic membrane (compare Figure 7b with Figure 6d). These data suggest that the cytoplasmic changes leading to compression of the cytoplasm found only in cells treated with peptide Leu⁴⁹-Thr⁷⁶ (Figure 6c), rather than changes of the nucleoid, are important to anti-(*E. coli*) activity as the activity was lost with peptide Leu⁴⁹-Thr⁷⁶ analogues, but not with peptide Leu⁴⁹-Thr⁷⁶ (Figure 5a).

DISCUSSION

We determined the antimicrobial domain of apoA-II using synthetic peptides, and showed that peptide Leu⁴⁹-Thr⁷⁶ is responsible for anti-(*E. coli*) activity of apoA-II and that Phe⁵²-Phe⁵³-Lys⁵⁴-Lys⁵⁵ is required for the activity.

As described in the Introduction, the target of antimicrobial peptides is often cytoplasmic membrane [12,14,17]. However, our present data suggest that peptide Leu⁴⁹-Thr⁷⁶ primarily targets the cytoplasm rather than the membrane of *E. coli* cells. The evidence supporting this conclusion is as follows.

(a) Amino acid substitutions Phe^{52,53} → Ala in peptide Leu⁴⁹-Thr⁷⁶ diminished its anti-(*E. coli*) activity, though it failed to affect its membrane-destabilizing activity. In peptide Leu⁴⁹-Thr⁷⁶, the membrane-destabilizing activity did not correlate well with the anti-*E. coli* activity.

(b) Peptide Leu⁴⁹-Thr⁷⁶ caused morphological changes mainly at the nucleoid and cytoplasm (Figure 6c). These morphological changes are unlikely post-mortem events, since peptide Leu⁴⁹-Thr⁷⁶(Phe^{52,53} → Ala) can cause clear clustering of nucleoid (compare Figure 7b with Figure 6c). However, it remains to be clarified whether the ultrastructural consequences result from a triggering of a cellular stress response.

(c) Additionally, electron micrographs of bacteria treated with membrane-acting antimicrobial peptides such as polymyxin [34–36], sarcotoxin I [37], defensins [38–40] and platelet microbicidal protein [41] show blebbing of the outer membrane or damage of the cytoplasmic membrane. But no blebs on the outer membrane were seen in a cell treated with peptide Leu⁴⁹-Thr⁷⁶ (Figure 6f). Therefore it appears that the cytoplasmic changes leading to compression of the cytoplasm are important in cell death induced by peptide Leu⁴⁹-Thr⁷⁶, although the mechanism of what happened in the cytoplasm has not been defined.

The origin of proteins released from *E. coli* with peptide Leu⁴⁹-Thr⁷⁶ was not clarified in the present study, but it is unlikely that they leaked from the cytosol of *E. coli* cells, as cytosolic β -galactosidase (EC.3.2.1.23) activity was not detected (results not shown). Electron-microscopic studies showed that filamentous structures on the *E. coli* cell surface were mostly removed after the treatment with peptide Leu⁴⁹-Thr⁷⁶ for 30 min (compare Figure 6c with Figure 6a). Although the structures were not well characterized here, they might be pili (fimbriae, fibrillae), which are usually revealed by scanning electron microscopy [32,33]. Pili are thread-like protein polymers resistant to dissociation into monomers by various denaturing agents such

as SDS, which readily disrupts most protein polymers [32]. Therefore it is likely that most of the released proteins detected by SDS/PAGE originated from the outer membrane and/or periplasm of the target *E. coli* cells.

CD measurement and secondary-structure prediction suggest that peptide Leu⁴⁹–Thr⁷⁶ forms an α -helix at its N-terminal region, which is important in the lipid-binding and anti-(*E. coli*) activity. An Edmundson-wheel [41a] representation of peptide Leu⁴⁹–Thr⁷⁶ shows that residues Leu⁴⁹–Ala⁵⁶ can form a cationic amphipathic α -helix. Amphipathic α -helices have been shown to play an important role in protein–lipid and protein–protein interactions [42]. The amphipathic α -helix in peptide Leu⁴⁹–Thr⁷⁶ may be necessary at least for the permeation of the *E. coli* cell membrane to reach the cytoplasm, since peptide Leu⁴⁹–Thr⁷⁶(Lys^{54,55} → Ser) with less helical content failed to cause membrane destabilization and any morphological changes in *E. coli* cells. The inability of peptide Leu⁴⁹–Thr⁷⁶(Phe^{52,53} → Ala) to cause changes of the cytoplasm suggests that Phe⁵²–Phe⁵³ on the hydrophobic face of the amphipathic α -helix are important in the lethal event, namely the changes of cytoplasm induced by peptide Leu⁴⁹–Thr⁷⁶. On the other hand, the importance of positive charges of Lys⁵⁴–Lys⁵⁵ remains unclear. It is known that a broad range of cationic antimicrobial peptides show marked decrease in activity at increasing ionic strength [43–49]. This phenomenon is generally explained by the shielding of charged groups on the peptide and the membrane, thereby preventing interaction between them [50]. In contrast, the anti-(*E. coli*) activity of peptide Leu⁴⁹–Thr⁷⁶ was slightly decreased by the addition of NaCl. Therefore the positive charge of Lys⁵⁴–Lys⁵⁵ may play a minor role, at least for the penetration of the *E. coli* cell membrane.

The present study demonstrates that the target of peptide Leu⁴⁹–Thr⁷⁶ is in the cytoplasm and that Phe⁵²–Phe⁵³ and α -helical conformation are the major structural requirements for the anti-(*E. coli*) activity. Antimicrobial peptides are at an early stage of therapeutic drug development for infectious diseases [15]. Further structure–activity analysis of apoA-II may better contribute to the design of antimicrobial peptides which have novel modes of action.

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