Characterization of the unique function of a reduced amide bond in a cytolytic peptide that acts on phospholipid membranes

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The incorporation of a reduced amide bond, $\psi(\text{CH}_2\text{NH})$, into peptide results in an increase in the net positive charge and the perturbation of α -helical structure. By using this characteristic of the reduced amide bond, we designed and synthesized novel pseudopeptides containing reduced amide bonds, which had a great selectivity between bacterial and mammalian cells. A structure–activity relationship study on pseudopeptides indicated that the decrease in α -helicity and the increase in net positive charge in the backbone, caused by the incorporation of a reduced amide bond into the peptide, both contributed to an improvement in the selectivity between lipid membranes with various surface charges. However, activity results *in vitro* indicated that a perturbation of α -helical structure rather than an increase in net

positive charge in the backbone is more important in the selectivity between bacterial and mammalian cells. The present result revealed that the backbone of membrane-active peptides were important not only in maintaining the secondary structure for the interactions with lipid membranes but also in direct interactions with lipid membranes. The present study showed the unique function of a reduced amide bond in cytolytic peptides and a direction for developing novel anti-bacterial agents from cytolytic peptides that act on the lipid membrane of micro-organisms.

Key words: anti-bacterial peptide, haemolytic activity, selectivity.

INTRODUCTION

Infectious diseases caused by micro-organisms are today increasing phenomena mainly as a result of an increase in antibiotic-resistant pathogens (reviewed in [1]). The recent development of resistant strains caused by the widespread use of current antibiotics demands the exploration of novel anti-microbial molecules with unexploited mechanisms of action (reviewed in [2]). Many host defence peptides have been discovered from various natural sources and their functions have been characterized (reviewed in [3]). Most of them are positively charged and adopt an α -helical structure in a membrane-mimicking environment. Although the detailed mode of action of this class of peptides is not fully understood, the peptides are expected to exert their biological function by enhancing the permeability of lipid membranes of pathogenic cell membranes in two steps [4–6]. In the first step the positively charged peptides bind to the negatively charged lipid membrane of the pathogen mainly by charge interactions; they then adopt a mostly α -helical or β -sheet structure. In the next step the peptides increase the permeability of the lipid membranes either by ion channel formation or by perturbation of the structure of the bilayer, resulting in the death of target cells.

However, the membrane-perturbing peptides themselves, like other biologically active peptides, cannot be used as therapeutic agents owing to their low stabilities *in vivo*; amide bonds in peptides are easily cleaved by protease. Many amide bond surrogates have been developed for improving the bioavailability of peptides (reviewed in [7]) and applied to various biologically active peptides. However, amide bond surrogates have not been intensively applied into cationic anti-microbial peptides that act

on lipid membranes [8–10] because secondary structure, such as an amphipathic α -helix or a β -sheet, has been regarded as a requirement for anti-bacterial activity and it has been accepted that maintaining secondary structure in the use of amide bond isosteres would be a difficult task.

In many amino acid replacement studies [11–17] on various cationic anti-microbial peptides, high α -helicity and/or high hydrophobicity have been regarded as the major factors critically related to mammalian cell toxicity rather than anti-bacterial activity. Other studies [18,19] indicated that the increase in the net positive charge of membrane-active peptides increased their binding to liposomes, which consist of negatively charged lipid membranes, resulting in an improvement in perturbatory activity and selectivity for liposomes. Recent studies [20–22] by using diastereomers (peptides containing D-amino acid residues) devoid of α -helical or β -sheet structure indicated that if the peptides had sufficient net positive charge and hydrophobicity for activity, a net positive charge and hydrophobicity were sufficient for controlling anti-bacterial activity and selectivity between bacterial and mammalian cells.

In the present study we focused our attention on elucidating the function of a reduced amide bond, $\psi(\text{CH}_2\text{NH})$, in the various amide bond surrogates for cytolytic peptides for the following reasons. Because this bond is protonated at physiological pH [23,24], the incorporation of such a bond increases the net positive charge of the peptide by the introduction of a positively charged group into the peptide backbone. Because $\psi(\text{CH}_2\text{NH})$ does not have carbonyl group to act as a proton-accepting site in hydrogen bonding [25], its incorporation into the peptide must perturb α -helical structure. We expected that the changes in structural parameters caused by the introduction of a reduced

Abbreviations used: ANTS, 8-aminonaphthalene-1,3,6-trisulphonic acid disodium salt; DPX, *p*-xylene-bispyridinium bromide; ESI, electrospray ionization; Fmoc, fluorenyl-methoxycarbonyl; MIC, minimum inhibitory concentration; PAL, 5-(4-aminomethyl-3,5-dihydroxyphenoxy)valeric acid; PtdGro, phosphatidylglycerol; PtdCho, phosphatidylcholine; TFA, trifluoroacetic acid; TFE, trifluoroethanol.

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amide bond into membrane-active peptides would improve bioavailability as well as selectivity between bacterial and mammalian cells.

To investigate the function of a reduced amide bond in membrane-active peptides, we incorporated one or more into a cytolytic peptide and studied the effect on the activity and structural parameters such as α -helicity, hydrophobicity and net positive charge. As expected, the novel synthetic pseudopeptides showed a great selectivity, not only between liposomes of various surface charges but also between bacterial and mammalian cells. The present study on the change in structural parameters caused by the incorporation of one or more reduced amide bonds demonstrated the unique function of this bond in membrane-active peptides; it also showed the function of the backbone of membrane-active peptides in direct interactions with lipid membranes.

MATERIALS AND METHODS

Materials

Fluoren-9-ylmethoxycarbonyl (Fmoc) amino acids for solid-phase peptide synthesis were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA, U.S.A.). 5-(4-Aminomethyl-3,5-dihydroxyphenoxy)valeric acid (PAL) resin was purchased from PerSeptive Biosystems GmbH (Hamburg, Germany). Piperidine, acetic anhydride, methanol, dicyclohexylcarbodi-imide, *N*-methylpyrrolidone and *N*-hydroxybenzotriazole were obtained from Applied Biosystems (Foster City, CA, U.S.A.). Phospholipid, NaCl, KCl, EDTA, calcein and Tris were purchased from Sigma (St Louis, MO, U.S.A.). 8-Aminonaphthalene-1,3,6-trisulphonic acid disodium salt (ANTS) and *p*-xylene-bispyridinium bromide (DPX) were purchased from Molecular Probes (Eugene, OR, U.S.A.). RPMI 1640 was purchased from Gibco BRL (Gaithersburg, MD, U.S.A.). Other chemicals were purchased from Aldrich (Milwaukee, WI, U.S.A.). All chemicals were of reagent grade and were used without further purification.

Synthesis of peptide and pseudopeptides

Peptides were prepared by stepwise solid-phase synthesis on an Applied Biosystems model 431A automatic peptide synthesizer. The peptide chain was assembled on PAL resin by Fmoc chemistry [26]. The protection of lysine residue side-chain groups was performed with *t*-butoxycarbonyl. A reduced amide bond was introduced by reductive alkylation of a protected amino aldehyde with the free N-terminus of the resin-bound peptide in the presence of excess NaBH_3CN in *N,N*-dimethylformamide containing 1% (v/v) acetic acid [27]. The reaction was repeated until no colour change in the resin-bound peptide was observed in a ninhydrin test. Deprotection was achieved by treatment with trifluoroacetic acid (TFA)/water/thioanisole (9.0:0.5:0.5, by vol.) at room temperature for 3–4 h. After filtration of resin and washing with TFA, a gentle stream of nitrogen was used to remove the excess TFA. The crude peptide and pseudopeptides were triturated with diethyl ether chilled at -20°C and then centrifuged at 3000 *g* for 10 min. Diethyl ether was decanted and crude peptide was dried under nitrogen. The peptide and pseudopeptides were purified by HPLC on a C_{18} column (21.2 mm $\times$ 250 mm; Phenomenex, Torrance, CA, U.S.A.). The homogeneity of the peptide and pseudopeptides (more than 96%) was checked by capillary electrophoresis (Hewlett Packard) with an unfused silica capillary column (50 μm $\times$ 60 mm) and analytical HPLC with a Waters Delta Pak C_{18} column (3.9 mm $\times$ 150 mm). The elution of peptide and pseudopeptide was monitored by measuring A_{214} . Solvent A consisted of 0.1%

(v/v) TFA in water and solvent B 0.1% (v/v) TFA in acetonitrile. The peptide and pseudopeptides were analysed with a linear gradient of 0–60% B over 60 min. MS on a Platform II mass spectrometer (Micromass, Manchester, U.K.) was used to measure the mass of the purified peptide and pseudopeptide. S1 [electrospray ionization (ESI)-MS: calc. 1434.06, obs. 1434.94 $[\text{M} + \text{H}]^+$], SR1 (ESI-MS: calc. 1420.05, obs. 1420.73 $[\text{M} + \text{H}]^+$), SR2 (ESI-MS: calc. 1420.05, obs. 1421.04 $[\text{M} + \text{H}]^+$), SR3 (ESI-MS: calc. 1448.06, obs. 1448.46 $[\text{M} + \text{H}]^+$), SR4 (ESI-MS: calc. 1406.06, obs. 1406.52 $[\text{M} + \text{H}]^+$).

Anti-microbial assay

Antifungal assays were performed *in vitro* by the broth microdilution method in accordance with the recommendation of the National Committee for Clinical Laboratory Standards [28]. RPMI 1640 (Gibco BRL) was used as the assay medium. *Candida* cells freshly grown on slopes of Sabouraud dextrose agar (exponential phase) were suspended in physiological saline and the cell concentration was adjusted to 10^4 cells/ml of doubly concentrated medium for use as the inoculum. Sample solution was added to the wells of a 96-well plate (100 μl per well) and serially diluted 1:1. The final concentration of sample ranged from 0.2 to 500 $\mu\text{g/ml}$. After inoculation (100 μl per well, 5×10^3 cells/ml), the 96-well plate was incubated at 30°C for 48 h and the A_{620} was measured with an enzyme-linked immunosorbent assay reader (SLT, Salzburg, Austria) to assess cell growth. The minimum inhibitory concentration (MIC) was defined as the lowest concentration exhibiting no visible growth compared with the control cells. Each MIC was determined from three independent experiments performed in duplicate. An antibacterial assay was performed *in vitro* by the same method as for the antifungal assay, except that the assay medium and the incubation temperature were different. In the anti-bacterial assay, antibiotic medium 3 (pH 7.0 at 25°C ; Difco, Detroit, MI, U.S.A.) was used and cells were incubated at 37°C for 24 h.

Haemolytic assay

The detailed method is described elsewhere [29]. Packed mouse erythrocytes were washed three times with buffer [150 mM KCl/5 mM Tris/HCl (pH 7.4)] and then suspended in 10 vol. of the same buffer (stock cell suspension). For treatment with antibiotic, the stock cell suspension was diluted 1:24 with the same buffer and preincubated in a water bath at 37°C for 15 min; the test sample was then added. After incubation for 1 h, the sample was centrifuged at 4000 *g* for 5 min and the A_{540} of the supernatant was determined. The haemolysis effected by 0.1% (v/v) Triton X-100 was taken as 100% haemolysis.

Measurement of CD spectra

CD spectra were recorded on a Jasco J-715 spectropolarimeter (Jasco, Tokyo, Japan) by using a quartz cell of 1 mm path length, at wavelengths ranging from 190 to 250 nm. CD spectra were recorded at room temperature and were obtained with a 0.5 nm bandwidth and a scan speed of 10 nm/min. Two scans were averaged to improve the signal-to-noise ratio. CD spectra were measured in the presence of 50% (v/v) trifluoroethanol (TFE), 25 mM SDS or phosphatidylcholine/phosphatidylglycerol (PtdCho/PtdGro, 3:1) liposomes. The liposomes were prepared by using a stream of nitrogen gas to evaporate the solvent from a solution of lipid mixture (PtdCho/PtdGro) in chloroform. The resulting film was hydrated in a buffer (pH 7.4), vortex-mixed vigorously for 10 min and then sonicated. The suspension was extruded through polycarbonate filters (five times

through two stacked 200 μm pore-size filters followed by ten times through two stacked 100 μm pore-size filters). The diameter of vesicles was measured with a Zetasizer (Malvern Instrument, Southborough, MA, U.S.A.). The mean diameter of vesicles was 103.1 nm (polydispersity index 0.09). CD spectra are expressed as mean residue ellipticity, $[\theta]$; the percentage α -helicity was calculated from $[\theta]$ at 208 and 222 nm [30,31].

Leakage assay of liposomes containing calcein

PtdGro or PtdCho (Sigma) (10 mg) was dissolved in chloroform and dried with a stream of nitrogen gas to form a thin lipid film on the wall of a glass tube. The resulting film, after being dried under vacuum, was hydrated with 2 ml of buffer [10 mM Tris/HCl (pH 7.4)/154 mM NaCl/0.1 mM EDTA] containing 70 mM calcein. The suspension was vortex-mixed for 10 min. This turbid liposome solution was sonicated (under nitrogen, in ice-water) five times, each for 10 min, with a titanium-tipped sonicator. The solution was freeze-thawed for six cycles. Calcein trapped in vesicles was separated from free calcein on a Sephadex G-50 (Pharmacia, Uppsala, Sweden) column. The concentration of liposomes was determined on the basis of the method described by Vaskovsky et al. [32]. A liposome solution (10 μl) was added to 2 ml of 10 mM Tris/HCl buffer, pH 7.4, containing 154 mM NaCl and 0.1 mM EDTA in the cuvette. To the mixture was added an appropriate concentration of peptides. When leakage occurred, calcein was released from the liposomes and emitted fluorescence. Leakage was therefore measured directly by determining the relative change in fluorescence, with excitation at 490 nm and emission at 520 nm, on a Jasco J-777 spectrofluorometer (Jasco, Tokyo, Japan). For the determination of 100% dye release, 20 μl of 10% (v/v) Triton X-100 solution was added to the liposome solution. The percentage of dye released in the sample was calculated as dye release (%) = $100 \times (F - F_0) / (F_t - F_0)$, where F is the fluorescence intensity achieved with peptides, and F_0 and F_t are fluorescence intensities without peptides and with Triton X-100 respectively.

Leakage assay of liposomes containing ANTS-DPX

Liposomes were prepared by evaporation of the lipid mixture in chloroform with nitrogen gas. The resulting thin film was hydrated in a buffer that contained 12.5 mM ANTS, 45 mM DPX, 68 mM NaCl and 10 mM Hepes, then agitated for 30 min and vortex-mixed for 10 min. The resulting multilamellar vesicles were sonicated and shaken for 1 h at room temperature. The suspension was freeze-thawed for five cycles and then centrifuged at 10000 g for 1 h. The assay was based on the quenching of ANTS fluorescence by DPX. Leakage was measured with liposomes containing both ANTS and DPX by the change in fluorescent emission by ANTS [33]. The fluorescence from liposomes containing 12.5 mM ANTS and 45 mM DPX was set as the baseline and that from liposomes lysed with Triton X-100 was set as 100% leakage. Fluorescence (excitation at 360 nm, emission at 535 nm) was measured with a Jasco FP-777 spectrofluorometer.

Dye release was measured as described above for calcein.

RESULTS

Design and synthesis of the pseudopeptides

To investigate the function of a reduced amide bond in the selectivity of membrane-active peptides, we chose a cytolytic peptide, S1 (KLLKLLKLLK-NH₂; single-letter amino acid codes), as a model peptide and synthesized the pseudopeptides

containing one or more reduced amide bonds in different positions. A cytolytic peptide was selected because S1 was reported to be active against both bacteria and mammalian cells and to have high hydrophobicity and high α -helicity in membrane-mimicking condition [18]. As shown in Table 1, a reduced amide bond was incorporated into the N-terminus of S1 (to form SR1) to increase the net positive charge without changing the secondary structure. In view of the fact that one turn of an α -helix contains 3.6 amino acid residues, one reduced amide bond was incorporated into Leu³-Leu⁴, forming SR2, to increase the net positive charge and simultaneously to decrease α -helicity. In addition, to investigate the effect of multiple reduced amide bonds, they were incorporated into Leu³-Leu⁴ and Leu⁸-Lys⁹ (SR4). In SR3, two such bonds were incorporated into the same positions as SR4 and the reduced amide bond in Leu⁸-Lys⁹ was acetylated to yield a different net positive charge on the backbone.

The pseudopeptides were synthesized by a solid-phase synthesis with Fmoc-based chemistry. The reduced amide bond was introduced as follows: the Fmoc-amino aldehyde corresponding to the Fmoc-amino acid was used in a solid-phase peptide synthesis in the presence of NaBH₃CN and acetic acid. Cleavage, ether precipitation and preparative HPLC provided the product. ESI-MS revealed that the isolated compound was the desired product; the purity was more than 96% as measured by analytical HPLC and by capillary electrophoresis.

Characterization of the peptide and corresponding pseudopeptides

Generally, anti-bacterial peptides bind initially to the lipid membranes of bacteria, adopt a secondary structure such as an α -helical or β -sheet structure and then enhance the permeability of the lipid membranes. Thus the secondary structure in lipid membrane rather than in phosphate buffer should be well correlated with activity against bacterial and mammalian cells. To investigate the secondary structure of the peptide and the pseudopeptides in lipid membranes, the CD spectrum of each compound was measured in various membrane-mimicking conditions [34–36]. Figure 1(A) shows CD spectra measured in TFE/water (50:50, v/v). The CD spectrum of S1 exhibited double minimum bands at 208 and 222 nm, which indicated that peptide S1 must adopt a well-defined α -helical structure in this condition. The CD spectrum of each pseudopeptide was also measured in the presence of TFE/water (50:50, v/v) and the α -helicity was calculated. As shown in Table 1, SR1, containing one reduced amide bond at the N-terminus of the primary structure, had a similar α -helicity to that of peptide S1, whereas the α -helicity of SR2, containing a reduced amide bond in Leu³-Leu⁴, was much lower. This result indicated that the perturbatory effect of this bond on α -helical structure depended on its position in the primary structure of the peptide. Both SR3 and SR4, containing two such bonds, adopted a random structure as a major conformation in the same condition.

As shown in Figure 1(B), we also measured CD spectra in phosphate buffer containing SDS micelles. SDS, which consists of an aliphatic tail and a negatively charged head group, mimicked the lipid membranes of bacteria [35]. The fractional α -helicity was calculated and is summarized in Table 1. The α -helicities of the peptide and the pseudopeptides measured in the presence of SDS micelles were, except for SR1, in agreement with those measured in the presence of 50% (v/v) TFE. Interestingly, SR1, which had one reduced amide bond, had a slightly higher α -helicity than the model peptide in the presence of SDS micelles.

As shown in Table 1, the peptide and its pseudopeptides were characterized by HPLC with a C₁₈ reverse-phase column, with

Table 1 Characteristics of peptide S1 and its derived pseudopeptides containing one or more reduced amide bonds

The C-terminus of S1 and its pseudopeptides was amidated. The α -helicity was calculated from $[\theta]$ at 208 nm as described in [31] and from $[\theta]$ at 222 nm as described in [30]. Retention time was determined on a C₁₈ column as described in the Experimental section.

Name	Sequence (single-letter amino acid codes)	Net charge at pH 7.4	α -helicity (%)		Retention time (min)
			$[\theta]$ at 208 nm	$[\theta]$ at 222 nm	
S1	KLLKLLKLLK-NH ₂	+5	57*/80†	44*/51†	47.6
SR1	K γ [CH ₂ NH]LLLKLLKLLK-NH ₂	+6	75/84	52/50	45.0
SR2	KLL γ [CH ₂ NH]LKLLKLLK-NH ₂	+6	29/47	16/27	38.6
SR3	KLL γ [CH ₂ NH]LKLL γ [CH ₂ NAc]KLLK-NH ₂	+6	n.a.‡	n.a.‡	36.6
SR4	KLL γ [CH ₂ NH]LKLL γ [CH ₂ NH]KLLK-NH ₂	+7	n.a.‡	n.a.‡	34.9

* CD spectra were measured in the presence of 25 mM SDS.
† CD spectra were measured in 50% (v/v) TFE.
‡ α -helicity was not calculated because SR3 and SR4 had mainly random conformations.

Figure 1 Conformations of S1 and its derived pseudopeptides in the presence of TFE or SDS

CD spectra were measured at a sample concentration of 100 μ M in 10 mM sodium phosphate buffer, pH 7.4, containing 50% (v/v) TFE (A) or 25 mM SDS (B). Samples were as follows: solid line, S1; dot-dashed line, SR1; dotted line, SR2; broken line, SR3; double-dot-dashed line, SR4.

acetonitrile/water as eluent. The retention time, which reflected the hydrophobic interactions between the peptides and the C₁₈ stationary phase, has been reported to parallel the hydrophobicity of the peptides or the amphipathic α -helicity of the peptides that had the same amino acid compositions [37]. From the retention time, S1 must be the most hydrophobic compound in this series. Even though SR1 had a similar α -helicity to that of the peptide, or even a slightly higher one, the retention time of SR1 was shorter than that of S1, which must have been due to the fact that the positively charged group in the amide backbone of SR1 decreases the hydrophobic interactions between the pseudopeptide and the stationary phase. Even though SR1 and SR2 had the same primary structure apart from the position of the reduced amide bond, the retention time of SR1 was a little longer than that of SR2, owing to the relatively low α -helicity of SR2. On the basis of the CD spectrum, SR3 and SR4 had random conformations. SR3 had a longer retention time than SR4, which must have been due to the increase in hydrophobicity and/or the decrease in net positive charge caused by the acetylation of the amino group in the backbone.

Fluorescence study

The membrane-permeabilizing ability of S1 and the pseudopeptides have been investigated by dye release from liposomes with various surface charges. Various concentrations of the

peptide and the pseudopeptides were mixed with vesicles that had contained fluorescent dye. Fluorescence was measured as a function of peptide concentration. The large unilamellar vesicles consisting of PtdCho served as a lipid membrane model with neutral charged surfaces and the large unilamellar vesicles consisting of PtdGro were used as a phospholipid membrane model with negatively charged surfaces. Because the Gram-positive bacterial membrane consists mainly of PtdGro and cardiolipin [38], liposomes consisting of PtdGro were used as a model to mimic the lipid membranes of Gram-positive bacteria. As shown in Figure 2(A), the peptide and the corresponding pseudopeptide had a potent perturbatory activity for PtdGro vesicles; the peptide and the pseudopeptides induced 100% leakage at concentrations between 1 and 5 μ g/ml. However, as shown in Figure 2(B), the peptide and the corresponding pseudopeptides had different perturbation activities for PtdCho vesicles. The control peptide, S1, induced 100% leakage of entrapped dye at concentrations above 2 μ g/ml, whereas 5 μ g/ml of pseudopeptide SR1 did not induce 100% leakage. The decrease in perturbatory activity was more clearly observed with SR2, SR3 and SR4: SR2 induced just under 40% leakage of entrapped dye at a concentration of 5 μ g/ml; at this concentration SR3 and SR4 did not induce even 10% leakage. We also prepared liposomes resembling the phospholipid compositions of fungal membranes and human erythrocyte membranes [39,40] and measured the release of the dye from

Figure 2 Leakage of calcein from liposomes with different surface charges

(A) Liposomes consisting of PtdGro were prepared and incubated with samples for 1 h at 25 °C. (B) Liposomes consisting of PtdCho were prepared and incubated with samples for 1 h at 25 °C. Symbols in both panels: ●, S1; ○, SR1; ▼, SR2; ▽, SR3; ■, SR4.

Figure 3 Leakage of dye from liposomes resembling the phospholipid compositions of the membranes of fungi and erythrocytes

(A) The phospholipid composition of liposomes resembled that of fungal membrane (PtdCho/phosphatidylethanolamine/phosphatidylserine/PtdIns/cerebroside/sphingomyelin/cholesterol, 30:30:10:10:5:20, molar ratios). The sample was incubated with liposomes for 1 h at 25 °C. (B) The phospholipid composition of liposomes resembled that of human erythrocyte membrane (PtdCho/phosphatidylethanolamine/phosphatidylserine/sphingomyelin/cholesterol, 25:22:10:18:25, molar ratios). The sample was incubated with liposomes for 1 h at 25 °C. Symbols in both panels: ●, S1; ○, SR1; ▼, SR2; ▽, SR3; ■, SR4.

Table 2 Anti-microbial activities of peptide S1 and its derived pseudopeptides containing one or more reduced amide bonds

MIC values were determined from three independent experiments performed in duplicate.

Name	MIC (µg/ml)				
	<i>Escherichia coli</i> ATCC 2592	<i>Pseudomonas aeruginosa</i> ATCC 9027	<i>Staphylococcus aureus</i> ATCC 6538	<i>Micrococcus luteus</i> ATCC 9341	<i>Candida albicans</i> ATCC 36232
S1	6.25–12.5	6.25–12.5	6.25–12.5	3.12–6.25	3.12–6.25
SR1	6.25–12.5	6.25–12.5	6.25–12.5	3.12–6.25	6.25–12.5
SR2	3.12–6.25	3.12–6.25	3.12–6.25	3.12–6.25	12.5–25
SR3	6.25–12.5	6.25–12.5	6.25–12.5	6.25–12.5	25–50
SR4	6.25–12.5	12.5–25	12.5–25	6.25–12.5	25–50

them caused by the peptide and the pseudopeptides. As shown in Figure 3(A), the perturbation activities of the peptide and pseudopeptides were different for these liposomes. Specifically, SR1, which had a similar α -helicity to the model peptide, caused slightly less leakage than the model peptide, S1. This must have been because the increase in net positive charge of SR1 decreased the hydrophobic interactions between the pseudopeptide and the lipid membranes, resulting in a decrease in leakage potency.

Interestingly, SR1 and SR2, which had the same primary structures apart from the position of the reduced amide bond, had clearly different perturbation activities towards the liposomes. SR2, with a lower α -helicity than SR1, had a lower perturbatory activity than SR1; SR3 and SR4, which had an increased net positive charge and random conformations in membrane-mimicking conditions, had very low perturbation activities.

Figure 4 Haemolytic activities of S1 and its derived pseudopeptides

Erythrocytes were incubated in phosphate buffer with various concentrations of sample for 1 h at 37 °C. Symbols: ●, S1; □, melittin; ○, SR1; ▼, SR2; ▽, SR3; ■, SR4.

We also performed the leakage experiment with liposomes mimicking the human erythrocyte membrane. As shown in Figure 3(B), the perturbatory activities of the peptide and the pseudopeptides were in agreement with those of the peptide and the pseudopeptides for the liposomes resembling fungal membrane.

Anti-microbial activity and haemolytic activity

As shown in Table 2, the MIC values of S1 and its pseudopeptides were measured for bacteria and fungi. The model peptide, S1, was active against bacteria and fungi at concentrations ranging from 6.25 to 12.5 µg/ml. The single or double incorporation of reduced amide bonds had little effect on anti-bacterial activity but had an effect on antifungal activity. Specifically, SR3 and SR4 had similar anti-bacterial activities to that of S1 but had an 8-fold lower antifungal activity than S1. This result was consistent with the perturbatory activity measured with liposomes resembling the lipid membrane composition of fungi. This result also suggested that the incorporation of a reduced amide bond into a peptide could increase the specificity between bacteria and fungi by enhancing the ability to discriminate between bacterial and fungal lipid membranes.

To check the cytotoxicity against mammalian cells, S1 and its pseudopeptides were added to mouse erythrocytes and the level of lysis was measured. As shown in Figure 4, the cytolytic peptide S1 and a well-known haemolytic peptide, melittin, caused 100% lysis at a concentration of 20 µg/ml, whereas SR1, which contained one reduced amide bond, caused 100% lysis at 60 µg/ml. The other pseudopeptide containing one such bond, SR2, caused 100% lysis at more than 180 µg/ml. Interestingly, SR3 and SR4 did not show haemolytic activity at concentrations up to 200 µg/ml. The trend of haemolytic activity in the peptide and the pseudopeptides was in agreement with that of the perturbatory activity for the liposomes resembling human erythrocyte membranes. The activity *in vitro* indicated that the incorporation of a reduced amide bond into a cytolytic peptide enhanced selectivity between bacterial and mammalian cells without a loss of anti-bacterial activity.

DISCUSSION

Many structure–activity relationship studies on cationic anti-microbial peptides have revealed that structural parameters such as α -helicity, hydrophobicity and net positive charge are im-

portant to biological activity, whereas high α -helicity and hydrophobicity are critically related to cytotoxic activity against mammalian cells. However, these studies were performed only with amino acid replacements. The effect of backbone modification of the cationic anti-microbial peptide on the structural parameters for the activity have not been extensively studied. Specifically, the function of the backbone of membrane-active peptides in direct interactions with lipid membranes has not been studied.

In the present study, we concentrated on the function of the reduced amide bond out of various amide bond surrogates and investigated the function of this bond in a cytolytic peptide. We also expected that this study would show the function of the backbone of membrane-active peptides in activity. The incorporation of one or more such bonds resulted in an increase in net positive charge on the backbone and the perturbation of α -helical structure, which subsequently affected activity. As expected, the structural parameter change caused by the introduction of the reduced amide bond improved selectivity between bacterial and mammalian cells. We wished to elucidate the function of this bond in improving the selectivity of the cytolytic peptide from following viewpoints: the secondary structural change caused by introducing such a bond and the change in the direct interaction of the backbone of the peptide with lipid membranes.

Because the incorporation of a reduced amide bond into peptides could simultaneously affect the net positive charge on the backbone and the peptide's secondary structure, we designed and synthesized a pseudopeptide series containing such bonds in different positions on the peptide to investigate each change separately.

Because membrane-active peptides exert their biological actions through lipid membranes, we examined activities via secondary structures in lipid membranes rather than in phosphate buffer. Thus the secondary structure of the peptide was characterized in the presence of lipid membranes or membrane-mimicking solvent. In this study, we characterized the secondary structure of the peptide and pseudopeptides under conditions mimicking lipid membrane, for convenience.

On the basis of the CD spectra of S1 and SR1 in the presence of 50% (v/v) TFE, SR1, containing one reduced amide bond, has a similar α -helicity to that of the model peptide. Several studies on peptides with D-amino acid replacements also indicated that the terminal amino acid was less important in maintaining α -helical structure [36,41]. Interestingly, the CD spectra of S1 and SR1 in the presence of SDS micelles revealed that the latter had the slightly higher α -helicity under these conditions. This kind of abnormal large increase in α -helicity in the presence of negatively charged vesicles has also been observed previously with D-amino acid replacements at the beginning of the α -helical region of peptides [36]. The fact that S1 and SR1 had similar α -helicities in the presence of TFE could explain the abnormally large α -helicity of SR1 in the presence of SDS micelles. The real α -helicity of SR1 might be similar to that of S1 in the presence of SDS micelles; however, the increase in net positive charge in SR1 should enhance the binding of SR1 to the negatively charged surface of SDS micelles by an increase in charge–charge interactions, resulting in an increase in ellipticity. By comparing the change in the structural parameters between S1, SR1 and SR2 we could elucidate the function of the reduced amide bond in selectivity through the change in net positive charge on the backbone and through the change in secondary structure. The fluorescence study on S1 and SR1 revealed that the increase in net positive charge on the backbone contributed to an improvement in selectivity for lipid membranes depending on the

surface charge of the lipid membrane. However, the effect of net positive charge on the backbone was less significant than α -helicity; this was confirmed by an activity study *in vitro*. For example, SR1, which had a similar α -helicity to S1, had slightly less haemolytic activity than S1, whereas SR2, which had a lower α -helicity than S1 and SR1, had much less haemolytic activity than S1. Double replacements were also performed. In SR3, one reduced amide bond was acetylated to monitor the effect of net positive charge on the backbone in the double replacements. SR3 and SR4, which had predominantly random structures in lipid-membrane-mimicking conditions, showed no perturbatory activity towards neutrally charged lipid membranes. In addition, neither compound had cytotoxicity towards mammalian cells. Thus we observed no effect of net positive charge in the multiple replacements. However, the result also indicated that α -helicity was a more important factor in selectivity than net positive charge on the backbone.

The lipid membrane-perturbatory activity of S1 and SR1 indicated that the increase in net positive charge on the backbone improved the selectivity between lipid membranes with different surface charges. In this case there are two possible effects of the net positive charge on the backbone: it might improve selectivity by increasing charge–charge interactions or by decreasing hydrophobic interactions between the backbone of the pseudopeptide and the lipid membranes. When we reviewed the perturbatory activity of SR1 for liposomes with various surface charges, the activity of SR1 towards neutrally charged liposomes was lower than that of S1, whereas the activity of SR1 towards negatively charged liposomes was similar to that of S1. This result indicated that the positively charged group in the backbone did not improve the activity towards negatively charged lipid membranes but decreased the activity towards neutrally charged lipid membranes. In addition, the activity towards neutrally charged lipid membranes was correlated with the retention time rather than with the net positive charge of the pseudopeptides. The retention time has been reported to parallel the hydrophobic interactions between peptides and the stationary phase [41]. We therefore expected that hydrophobic interactions rather than charge–charge interactions between the backbone and lipid membranes were important for selectivity.

We wondered why the increase in net positive charge on the backbone did not improve the perturbatory activity towards negatively charged lipid membranes. We suggest two possible explanations. First, the model peptide, S1, already has sufficient net positive charge for binding to negatively charged lipid membranes, so an increase in net positive charge does not affect the activity. Secondly, when the peptide initially binds to the lipid membranes the positively charged group in the backbone, unlike the group in the side chain of the amino acid, cannot easily access the lipid membranes, so the increase in net positive charge does not contribute to an increase in activity.

In conclusion, the incorporation of a reduced amide bond perturbed α -helical structure and increased net positive charge by the introduction of a positively charged amino group into the backbone, which removed the cytotoxicity of cytolytic peptide towards mammalian cells without diminishing anti-bacterial activity. Our results suggest that the backbone of membrane-active peptides are important in the maintenance of secondary structure for interactions with lipid membranes as well as in direct interactions with lipid membranes. The hydrophobic interactions rather than the charge–charge interactions between the pseudopeptides and lipid membranes are therefore more important in the specificity of the pseudopeptides. The present study has shown the unique function of the reduced amide bond in cytolytic peptides that act on lipid membranes and demon-

strates a method for developing novel anti-bacterial agents from cytolytic peptides.

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