Antibacterial Activity of Bactenecin 5 Fragments and Their Interaction with Phospholipid Membranes

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Abstract: Bactenecin 5 (Bac 5) is an antibacterial 43mer peptide isolated from bovine neutrophils. It consists of an Arg-rich *N*-terminal region and successive repeats of Arg-Pro-Pro-Ile (or Phe). We synthesized Bac $5_{1\text{-}23}$ and several related peptides to clarify the roles these regions play in antibacterial activity. An assay of antibacterial activity revealed that such activity requires the presence of Arg residues at or near the *N*-terminus, as well as a chain length exceeding 15 residues. None of the peptides exhibited haemolytic activity. Polyproline II-like CD curves were observed for most of the peptides. Measurements of the membrane perturbation and fusion indicated that the perturbation and fusogenic activities of the peptides were, generally, parallel to their antibacterial activities. Amino acid substitution in the repeating region had some effect on antibacterial activity. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antibacterial activity; bactenecin 5; circular dichroism; membrane perturbation; Pro/Arg-rich peptide

INTRODUCTION

Neutrophils play an important role in vertebrate defence against microbial infection by their production of antibacterial peptides and proteins [1]. Two highly cationic peptides, bactenecin 5 (Bac 5) and Bac 7, were isolated from large granules of bovine neutrophils [2]. These peptides primarily exhibit antibacterial activities against gram-negative bacteria.

Both Bac 5 and Bac 7 consist of an Arg-rich *N*-terminal region and successive repeats of short peptide units: Arg-Pro-Pro-Ile (or Phe) for Bac 5 (Figure 1) and Pro-Arg-Pro for Bac 7 [3,4]. Peptides known as the Pro/Arg-rich family, including Bac 5 and Bac 7, have been identified, e.g. abaecin [5], apidaecin [6], and PR-39 [7,8]. It has been reported that, as with many other cationic peptides, bactenecins interact with bacterial cells by electrostatically binding to negatively charged cell surfaces, thereby causing a rapid increase in membrane permeability [9].

The structure–activity relationship of Bac 5 has been widely studied, resulting in the synthesis of a variety of fragments covering the entire sequence and model peptides, including the examination of their properties and antibacterial activities. Gennaro *et al.* [10] reported that a minimum length of 18–20 residues from the *N*-terminus is required for antibacterial activity, whereas it is claimed by Raj and Edgerton [11] that 7-22 residues constitute a functional domain for antifungal activity.
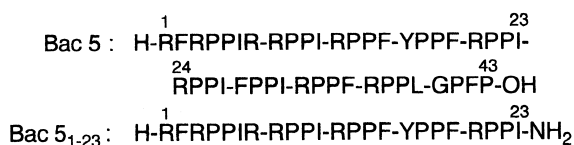
Bac 5 : H-RFRPPIR-RPPI-RPPF-YPPF-RPPI-¹ ²³
RPPI-FPPI-RPPF-RPPL-GPFP-OH²⁴ ⁴³

Bac 5₁₋₂₃ : H-RFRPPIR-RPPI-RPPF-YPPF-RPPI-NH₂¹ ²³

Figure 1 Amino acid sequences of Bac 5 and Bac 5₁₋₂₃.

Conformational analyses by NMR and CD measurements indicated that Bac 5 has a poly-L-proline II conformation [11,12]. We also previously synthesized two series of Bac 5 model peptides, Ac-(Arg-Pro-Pro-Phe)$_n$-NHCH$_3$ ($n = 2, 4, 6, 8,$ and 10; PR$_2$, P$_4$, P$_6$, PR$_8$, and PR$_{10}$) and H-Arg-Phe-Arg-Pro-Pro-Ile-Arg-(Arg-Pro-Pro-Phe)$_n$-NH$_2$ ($n = 2$–5 and 7; BM$_2$-BM$_5$ and BM$_7$) [13,14]. From the CD study on the PR series of peptides, we found that the repeating region of Bac 5 makes a polyproline II-like conformation in an aqueous environment [13]. Use of the BM series of peptides led us to presume that the polyproline II-like structure plays a significant role in antibacterial activity, and that the *N*-terminal region may play an important role in strengthening the activity [14].

To further clarify the structure–activity relationship, we selected an *N*-terminal 23-residue peptide of Bac 5, i.e. Bac 5₁₋₂₃ (Figure 1), as a standard sample on the basis of the reported data [10,11]. We then synthesized Bac 5₁₋₂₃ and its related peptides. The properties were examined by CD measurement, membrane perturbation and membrane fusion, in addition to assays of antibacterial and haemolytic activity. The results demonstrated that antibacterial activity requires the presence of Arg residues at or near the *N*-terminus, and a chain length in excess of 15 residues. Amino acid substitution in the repeating region of Bac 5₁₋₂₃ had some effect on the biological activity.

MATERIALS AND METHODS

Peptide Synthesis

Peptide synthesis was achieved by the solid-phase method using Fmoc-amino acids and SAL resin [15]. The final protected peptides were treated with TFA in the presence of m-cresol and thioanisole for 1 h at 25°C. After evaporation, the residues were solidified with ether, and chromatographed by RP-HPLC on a YMC-Pack ODS-A (10 × 250 mm), with a linear gradient of 100% H$_2$O/0.1% TFA and 100% acetonitrile/0.08% TFA. The fractions containing the desired peptides were lyophilized. The purity of the final products was evaluated by analytical RP-HPLC, MALDI-TOF-MS, and amino acid analysis, and was found to exceed 95%. The results are shown in Table 1.

Antibacterial Activity

The serial solution dilution method [14] was used to determine the MIC values. The cell suspension was diluted with a tryptic soy broth (TSB) medium to 10^4 cells mL^{-1}. Several concentrations of the peptide solution were placed in the test tubes, made up to 20 μL with the medium, and 180 μL of cell suspension was added. After incubation for 20 h at 37°C, absorbance at 620 nm was measured. The results are shown as MIC values in Table 2.

Haemolytic Activity

PBS solution (pH 7.4, 1 mL) was added to 1 mL of fresh rabbit blood. The resulting mixture was centrifuged at 2600 rpm for 5 min, and the precipitates were collected. After being washed with PBS solution three times, the obtained precipitates were suspended in four-fold volumes of the PBS solution. An assay of haemolytic activity was conducted as described by Niidome *et al.* [14]. PBS solution (pH 7.4, 1 mL) was added to the rabbit erythrocyte solution (5 μL), followed by the peptides. The resulting suspension was incubated for 20 min at 37°C, then centrifliged. The supernatant was monitored at 413 nm.

Preparation of Phospholipid Vesicles

SUVs of DPPC and DPPC/DPPG (3:1) were prepared for the CD, calcein leakage, and membrane fusion experiments [13,14]. Phospholipid (10 μmol) was dissolved in CHCl$_3$/MeOH (2:1 v/v, 2 mL), then dried under a stream of N$_2$ gas. The dried lipid was hydrated in 20 mM Tris HCl buffer (pH 7.4, 10 mL) using a Branson bath-type sonicator. The suspension was sonicated for 20 min at 50°C using a Titech Ultrasonic Processor Model VP-ST at an intensity of 10 W. The vesicles were allowed to stand for 30 min at 25°C before the measurements were made. The lipid concentration was 1 mM. The calcein-entrapped vesicles were similarly prepared. The dried lipid (27 μmol) was hydrated in 20 mM Tris HCl buffer (pH 7.4, 2.0 mL) containing 0.1 M

Table 1 Amino Acid Analysis and Molecular Weight of Synthetic Peptides

	Amino acid analysis					MALDI-TOF-MS	
	Arg	Phe	Pro	Ile	Tyr	Obs	Calcd$[M+H]^+$
Bac 5_{1-23}	5.5(6)	3.1(3)	10.0(10)	2.7(3)	1.1(1)	2870.0	2870.5
Bac 5_{2-23}	4.7(5)	3.2(3)	10.0(10)	2.9(3)	1.0(1)	2714.9	2714.4
Bac 5_{3-23}	4.5(5)	2.1(2)	10.0(10)	2.6(3)	1.2(1)	2566.7	2567.2
Bac 5_{4-23}	3.8(4)	2.2(2)	10.0(10)	2.8(3)	1.2(1)	2410.9	2411.0
Bac 5_{5-23}	3.8(4)	2.2(2)	9.0(9)	2.7(3)	1.1(1)	2314.2	2313.9
Bac 5_{6-23}	4.3(4)	2.4(2)	8.0(8)	3.2(3)	1.2(1)	2216.0	2216.7
Bac 5_{7-23}	4.4(4)	2.3(2)	8.0(8)	2.2(2)	1.2(1)	2104.3	2103.6
Bac 5_{8-23}	3.3(3)	2.2(2)	8.0(8)	2.2(2)	1.1(1)	1947.8	1947.4
Bac 5_{1-19}	4.8(5)	3.0(3)	8.0(8)	1.7(2)	1.0(1)	2407.5	2407.0
Bac 5_{1-15}	4.5(5)	2.0(2)	6.0(6)	2.1(2)		1902.1	1902.4
Bac 5_{1-7}	2.9(3)	1.2(1)	2.0(2)	0.9(1)		940[a]	940
Bac 5_{24-43}	2.9(3)	3.2(3)	10.0(10)	2.0(2)	Leu 1.2(1), Gly 0.8(1)	2297.8	2296.8
R-Bac 5_{1-23}	6.6(7)	3.1(3)	10.0(10)	3.0(3)	1.1(1)	3027.3	3026.7
RR-Bac 5_{1-23}	7.6(8)	3.0(3)	10.0(10)	2.6(3)	1.1(1)	3182.7	3182.9
$[R \rightarrow K]$ Bac 5_{1-23}		3.0(3)	10.0(10)	2.6(3)	1.1(1) Lys 5.7(6)	2703.0	2702.5
$[Y^{16}R]$ Bac 5_{1-23}	6.7(7)	3.0(3)	10.0(10)	2.7(3)		2863.7	2863.6
$[I^{11,23}F]$ Bac 5_{1-23}	5.9(6)	4.9(5)	10.0(10)	0.8(1)	1.2(1)	2939.2	2938.6

[a] Molecular weight determined by fast atom bombardment mass spectrometry (FAB-MS).

Table 2 Antibacterial Activity of Peptides

Peptide	MIC (μg mL^{-1})[a]			
	S. aureus IFO 12 732	*B. subtilis* IFO 3134	*E. coli* IFO 12 734	*P. aeruginosa* IFO12 582
Bac 5_{1-23}	4	32	4	>128
Bac 5_{2-23}	16	64	8	>128
Bac 5_{3-23}	32	>128	4	>128
Bac 5_{4-23}	>128	>128	>128	>128
Bac 5_{5-23}	>128	>128	>128	>128
Bac 5_{6-23}	>128	>128	>128	>128
Bac 5_{7-23}	32	>128	128	>128
Bac 5_{8-23}	>128	>128	>128	>128
Bac 5_{1-19}	8	128	16	>128
Bac 5_{1-15}	128	>128	128	>128
Bac 5_{1-7}	>128	>128	>128	>128
Bac 5_{24-43}	>128	>128	>128	>128
R-Bac 5_{1-23}	2	8	8	128
RR-Bac 5_{1-23}	2	4	4	128
$[R \rightarrow K]$ Bac 5_{1-23}	32	>128	64	>128
$[Y^{16}R]$ Bac 5_{1-23}	2	16	4	>128
$[I^{11,23}F]$ Bac 5_{1-23}	8	32	8	64
magainin 2	16	8	16	16
gramicidin S	1	1	16	16

[a] Method, serial solution dilution; medium, TSB medium (pH 7.4); innoculom size 10^4 cells mL^{-1}.

calcein. The calcein-entrapped vesicles were separated from the free calcein by gel filtration using Sephadex G-75 (1.1×24 cm) with the same buffer (pH 7.4).

Circular Dichroism Measurement

CD spectra were recorded on a JASCO Model J-720W spectropolarimeter with a thermostatted cell holder, using a quartz cell, having a path length of 1.0 mm. The peptide concentrations were 20 μM in 20 mM Tris HCl buffer (pH 7.4) and in the presence of the DPPC SUVs, and 10 μM in the presence of the DPPC/DPPG (3:1) SUVs, respectively. The percentage helicity of the peptides was calculated with the following equation: % helicity $= 100 \times [\theta]_{222}/$ $(40\,000 \times (1-2.5/n) + 100 \times t)$, where $[\theta]_{222}$ is the experimentally observed mean residue ellipticity at 222 nm, n is the number of residues, and t is the temperature (°C) [16].

Calcein Leakage

Calcein leakage measurements were conducted as reported in the literature [14,17]. Twenty μL of the vesicles containing 0.1 M calcein was added to a cuvette containing 20 mM Tris HCl buffer (pH 7.4, 1.0 mL) to give a resultant vesicle solution with a lipid concentration of 70 μM. Twenty μL of an appropriate dilution of the peptides in the buffer was added to a cuvette placed in a holder (25 or 50°C). The intensity of calcein fluorescence was monitored at 515 nm (excited at 470 nm) and measured 2 mm after the peptides were added. To determine the fluorescence intensity for 100% calcein release, 10 μL of 10% Triton® X-l00 (10% in Tris HCl buffer) was added to dissolve the vesicles. The percentage of calcein release caused by the peptides was evaluated by the equation $100 \times (F - F_0)/(F_t - F_0)$, where F is the fluorescence intensity produced by the peptides, F_0 is the intensity observed without the peptides, and F_t is the intensity after the Triton® X-100 treatment.

Membrane Fusion

Two kinds of vesicles, one containing NBD-PE, and the other containing Rh-PE (2.0 mol % each), were prepared by sonication, as described by Ohmori *et al.* [17]. Equimolar amounts of the vesicles were mixed at 25°C, and the final lipid concentration was adjusted to 70 μM. The peptides were added to the mixed vesicles, and the decrease in NBD fluorescence was recorded continuously at an emission wavelength of 530 nm (excited at 450 nm). The intensity (F_t) of the fluorescence was measured 5 min after adding the peptides. The intensity (F_t) of the fluorescence for 100% fusion was obtained using vesicles in which NBD-PE and Rh-PE were premixed. To check the degree to which the peptides quenched the NBD fluorescence, a control experiment was conducted by combining NBD-PE and the peptides without Rh-PE. The lipid-mixing percentage was evaluated by the equation $100 \times (F_0 - F)/(F_0 - F_t) - 100 \times (F_0' - F')/(F_0 - F_t')$, where F is the fluorescence intensity achieved by the peptides for NBD-PE and Rh-PE, F_0 the intensity observed with NBD-PE and Rh-PE, F' the intensity achieved by the peptides for NBD-PE, and F_0' the intensity observed only with NBD-PE.

RESULTS AND DISCUSSION

Peptide Design and Synthesis

Using many Bac 5 model peptides, i.e., Ac-(Arg-Pro-Pro-Phe)$_n$-NHCH$_3$ ($n = 2$, 4, 6, 8, and 10) [13] and H-Arg-Phe-Arg-Pro-Pro-Ile-Arg-(Arg-Pro-Pro-Phe)$_n$-NH$_2$ ($n = 2$–5 and 7) [14], we have found that the *N*-terminal Arg-rich portion and a certain peptide chain length of the repeating region in Bac 5 are important factors in determining antibacterial activity [14]. To further clarify the structure–activity relationship, we designed and synthesized a series of *N*- and *C*-terminal truncated Bac 5 peptides and amino acid-substituted peptides (Table 1). Bac 5$_{1-23}$, which contains the *N*-terminal Arg-rich portion and four repeats of the tetrapeptide unit (Figure 1), was chosen as a standard peptide because 18–20 residues from the *N*-terminus have been reported as a requirement for antibacterial activity [10]. All of the peptides were synthesized by the solid phase method on SAL resin with Fmoc-amino acids. The crude products were then purified by RP-HPLC; the final products were identified by amino acid analysis and MALDI-TOF-MS. The results were satisfactory, as shown in Table 1.

Antibacterial and Haemolytic Activities of Peptides

The serial solution dilution method [14] was employed to examine the antibacterial activity of the peptides. Two gram-positive bacteria, *S. aureus* and *B. subtilis*, and two gram-negative bacteria, *E. coli* and *P. aeruginosa* were chosen as the test bacteria. The results are shown as MIC values in Table 2.

Although it has been reported that Bac 5 is active against gram-negative bacteria [3], the synthetic fragments were active against both gram-positive and gram-negative bacteria. The difference in the observed activity this time might be owing to differences in the methods of antibacterial assay. Bac 5_{1-23} exhibited antibacterial activities of 4 μg mL^{-1} against *S. aureus* and *E. coli*, and 32 μg mL^{-1} against *B. subtilis*. However, even at 128 μg mL^{-1}, no activity was observed against *P. aeruginosa*. For the *N*-terminal truncated peptides, Bac 5_{2-23}-Bac 5_{8-23}, the activity decreased with a decrease in the peptide chain length except for Bac 5_{7-23}, which exhibited weak activity for both *S. aureus* and *E. coli*. In a previous paper [14], we pointed out the importance of the *N*-terminal Arg-rich portion for antibacterial activity, which is in accord with the present results. To further confirm this importance, we prepared two additional Arg-rich peptides, RR-Bac 5_{1-23} and R-Bac 5_{1-23}. These peptides definitely demonstrated activities stronger than the activity of Bac 5_{1-23}; they were especially effective against *B. subtilis*.

We have found that a certain chain length in the repeating region is also required for the activity to occur [14]. To further clarify our findings, we examined the antibacterial activity of some Bac 5 fragments that lacked the *C*-terminal portion. Bac 5_{1-19} showed moderate activity, although its antibacterial spectrum was slightly different from that of Bac 5_{1-23}–5_{3-23}. Bac 5_{1-15} was very weak against *S. aureus* and *E. coli*, and Bac 5_{1-7} had no activity. These results indicate that the *N*-terminal Arg-rich non-repeating portion alone is insufficient in producing the antibacterial activity, but it does play an important role in longer peptides such as Bac 5_{1-23}. As with Bac 5_{4-23}, the *C*-terminal peptide, Bac 5_{24-43}, exhibited no activity. This may be owing to lack of the Arg cluster or the total basicity of the peptides. Interestingly, Bac 5 has many Arg residues but no Lys residue. We also examined the antibacterial activity of an analogue, [R→K] Bac 5_{1-23}, which has six Lys residues instead of Arg. The substitution of all of the Arg residues with Lys residues caused a drastic decrease in activity, indicating that the antibacterial activity requires strong basicity.

Bac 5_{1-23} has no antibacterial activity at 128 μg mL^{-1} against *P. aeruginosa*, whereas the BM series of peptides do. For instance, BM$_4$, i.e. [Y^{16}R, I^{11,23}F] Bac 5_{1-23}, shows activity of 64 μg mL^{-1} [14]. These results suggest that substitution of either Tyr→Arg or Ile→Phe is effective for activity against *P. aeruginosa*. We, therefore, synthesized [Y^{16}R] Bac 5_{1-23}

and [I^{11,23}F] Bac 5_{1-23}. Only [I^{11,23}F] Bac 5_{1-23} exhibited any activity, indicating, together with our previous results [14], that periodic, or at least, the presence of the Phe residues in Bac 5_{1-23} increases activity against *P. aeruginosa*. Table 2 shows that magainin 2 exhibits moderate activity against all of these bacteria. The activity of gramicidin S is strong against gram-positive bacteria and moderate against gram-negative bacteria. The antibacterial spectra of magainin 2, gramicidin S and Bac 5_{1-23} are quite different from each other. These phenomena may reflect differences between the amphipathicity and basicity of these three peptides.

None of the peptides exhibited haemolytic activity for rabbit red blood cells up to a peptide concentration of 100 μM (data not shown). Bac 5_{1-23} and its analogues are very basic, and rather hydrophilic. The erythrocytes contain a high amount of cholesterol, but little acidic phospholipid in the outer monolayer [18]. A high degree of hydrophobicity of the peptides is known to be necessary for the interaction of peptide-neutral phospholipid, strongly suggesting the lack of the ability of 5_{1-23} to interact with rabbit erythrocytes.

Circular Dichroism Measurements of Peptides

To examine the secondary structures of the peptides, CD measurements were performed in 20 μM Tris HCl buffer (pH 7.4) in the presence of either the DPPC or DPPC/DPPG (3:1) SUVs. Some of the results are shown in Figure 2. In the buffer, at 25°C, the peptides had a negative band at 204 nm, with a slight shoulder around 230 nm (Figure 2(A)). Bac 5_{1-7} showed a slightly different CD curve from the CD curves of the other peptides, although it also had a negative band at 204 nm (data not shown). The order of [θ] values at 204 nm of the peptides was Bac 5_{4-23} > Bac 5_{7-23} > Bac 5_{1-23} > Bac 5_{1-19} and was dependent on the Pro contents. These results are reasonable, because the Pro-rich peptides with the polyproline II conformation show a strong negative band at 204 nm, and a shoulder at 227 nm [8,12,13,19]. No noticeable changes in the CD curves were observed in the presence of the DPPC SUVs at 25°C and 50°C, suggesting that there is very little interaction between the peptides and the vesicles under these conditions. The DPPC/DPPG (3:1) SUVs caused a slight change in the CD curves at 25°C, but changes were found at 50°C for [peptide] = 10 μM and [lipid] = 1 mM (Figure 2(B)). The negative bands, at 204 nm, were fairly shallow, and the order of negative band depth was Bac

Bac 5_{7-23} > Bac 5_{4-23} > Bac 5_{1-23} > Bac 5_{1-19}. The ratios of $[\theta]_{lipid}/[\theta]_{H_2O}$ at 204 nm were 0.71, 0.72, 0.73, and 0.83 for Bac 5_{1-23}, Bac 5_{1-19}, Bac 5_{4-23}, and Bac 5_{7-23}, respectively. This result shows that the peptides having an *N*-terminal portion and longer chain length interact strongly with the acidic SUVs. The intrinsic structures of the peptides may be retained in the presence of the lipid, because the negative band at 204 nm remained.

The amino acid-substituted analogues had similar CD patterns (Figure 2(C) and 2(D)) to those of Bac 5_{1-23} and its truncated peptides. The ratios of $[\theta]_{lipid}/[\theta]_{H_2O}$ at 204 nm were 0.67, 0.71, 0.72, and 0.76 for $[I^{11,23}F]$ Bac 5_{1-23}, Bac 5_{1-23}, $[Y^{16}R]$ Bac 5_{1-23} and $[R \rightarrow K]$ Bac 5_{1-23}, respectively. The analogues containing Phe residues instead of Ile showed somewhat reduced CD curves, suggesting that the presence of aromatic residues is favourable for binding with the acidic phospholipid membrane.
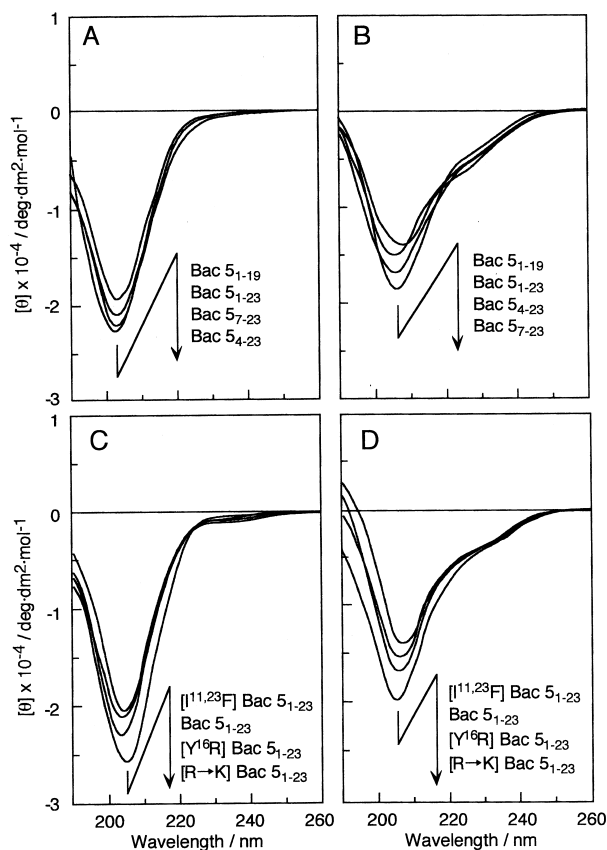

Figure 2 CD curves of Bac 5_{1-23} and its truncated peptides (A, B) and the amino acid-substituted analogues (C, D) in 20 mM Tris HCl buffer (pH 7.4) at 25°C (A, C) and in the presence of DPPC/DPPG (3:1) SUVs at 50°C (B, D). [Peptide] = 20 µM (A, C) or 10 µM (B, D), [lipid] = 1 mM.

Calcein Leakage Activities of Peptides

Basic amphiphilic peptides generally cause membrane perturbation, which is responsible for bacterial cell lysis [20–22]. Therefore, to evaluate the interaction of the peptides with the lipids, the leakage of a dye calcein from 70 µM DPPC and DPPC/DPPG (3:1) SUVs was examined by changing the [peptide]/[lipid] ratio. A negligible amount of calcein leakage or none at all was found for the DPPC and DPPC/DPPG (3:1) SUVs at 25°C (data not shown). Probably, the peptides had very little interaction with the lipids, as presumed from the results that the changes in the CD patterns of the peptides were also very small. This lack of interaction may be due to the rigid structure of the DPPC and DPPC/DPPG (3:1) SUVs at 25°C, which is below their phase transition temperature.

In contrast, calcein leakages were observed for both neutral and acidic lipid SUVs at 50°C (Figure 3). In the presence of the DPPC SUVs, the leakage activities of Bac 5_{1-23} and $[Y^{16}R]$ Bac 5_{1-23} were about 7% at a peptide concentration of 20 µM, while $[I^{11,23}F]$ Bac 5_{1-23} had a leakage activity of 15% (Figure 3(A)), indicating that the Ile → Phe substitution is effective in increasing the leakage activity of the peptides. The peptides, Bac 5_{4-23}, Bac 5_{1-19}, and so on, had weak activities of less than 5% (data not shown). Use of the DPPC/DPPG (3:1) SUVs
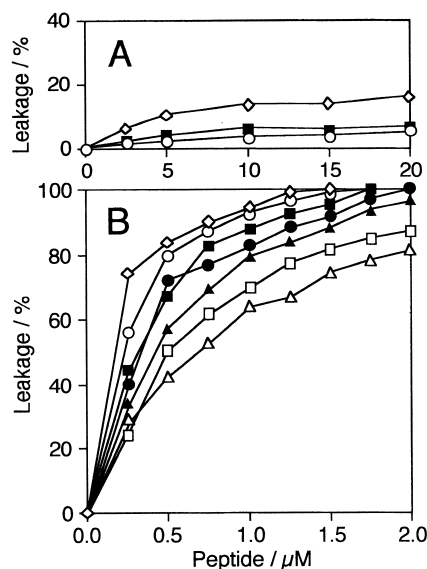

Figure 3 Profiles of calcein leakage as a function of peptide concentration for 70 µM DPPC (A) and DPPC/DPPG (3:1) SUVs at 50°C (B). Bac 5_{1-23} (○), Bac 5_{4-23} (●), Bac 5_{7-23} (△), Bac 5_{1-19} (▲), $[R \rightarrow K]$ Bac 5_{1-23} (□), $[Y^{16}R]$ Bac 5_{1-23} (■), and $[I^{11,23}F]$ Bac 5_{1-23} (◇).

produced drastic changes in the calcein leakage curves at 50°C. All the peptides showed strong activity, even at low peptide concentrations (Figure 3(B)). Of the 23-residue peptides, the leakage activity of $[I^{11,23}F]$ Bac $5_{1\text{-}23}$ was stronger than that of $[Y^{16}R]$ Bac $5_{1\text{-}23}$ and Bac $5_{1\text{-}23}$. Bac $5_{1\text{-}23}$ exhibited full leakage activity at 1.75 μM. The activity decreased with a decrease in the length of the peptide chain, i.e. Bac $5_{1\text{-}23}$ > Bac $5_{4\text{-}23}$ > Bac$5_{1\text{-}19}$ > Bac $5_{7\text{-}23}$. It should be noted that Bac $5_{4\text{-}23}$ had strong leakage activity, even though it lacked antibacterial activity. The membrane consisting of the DPPC/DPPG (3:1) SUVs is far more unstable than that of bacterial cells, and hence, is sensitive to many kinds of basic peptides. Bac $5_{4\text{-}23}$ might have sufficient basicity and amphipathicity to interact with acidic vesicles consisting of DPPC and DPPG, although it could not perturb the membrane of bacterial cells. We thus believe that the leakage activity of a peptide is generally parallel to its antibacterial activity, although there are some exceptions. We previously obtained similar results using Bac 5 model peptides [14].

Membrane Fusion Activity of Peptides

Membrane perturbation by amphiphilic peptides often accompanies membrane fusion [20,23]. To evaluate the membrane fusion activity of the peptides, we examined the intermixing of phospholipids between vesicles containing either NBD-PE or Rh-PE for Bac $5_{1\text{-}23}$, Bac $5_{4\text{-}23}$, and Bac $5_{7\text{-}27}$ (Figure 4). In the presence of the DPPC SUVs at 25°C, fusion activities of ca. 20% were found for Bac $5_{1\text{-}23}$ and of ca. 10% for Bac $5_{4\text{-}23}$ and Bac $5_{7\text{-}23}$. These peptides exhibited no leakage activity for the D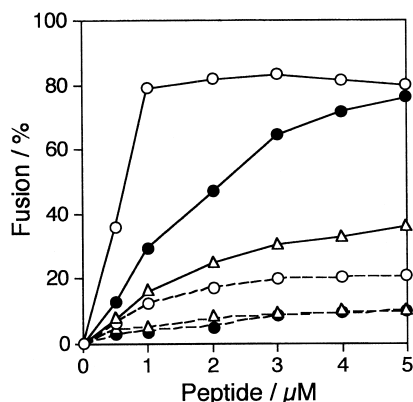PPC SUVs at 25°C, clearly indicating that membrane fusion occurs far more easily than does leakage. In the presence of the DPPC/DPPG (3:1) SUVs at 25°C, the activity order was Bac $5_{1\text{-}23}$ > Bac $5_{4\text{-}23}$ > Bac $5_{7\text{-}23}$ indicating that fusion activity depends on the length of the peptide chain and is parallel to calcein leakage activity. Using electron microscopy, we found that the Bac 5 model peptides caused membrane fusion, rather than vesicle aggregation [14], and hence, believe that Bac $5_{1\text{-}23}$ and other peptides would also produce the same phenomenon.

CONCLUSIONS

Details of the structure-activity relationship of Bac 5 are not well known. Using the Bac 5 model peptides, we have shown that a repeating region is essential for antibacterial activity and that the *N*-terminal region increases the activity [14]. In the present study, we examined the effect of the *N*-terminal region on antibacterial activity in more detail. We found that antibacterial activity requires the presence of Arg residues at or near the *N*-terminus, as well as a chain length of more than 15 residues. Together with the previous results [13,14], we concluded that the repeating region of Bac 5 is the minimum determinant of antibacterial activity because it forms a framework to hold a polyproline II-like conformation. An Arg-rich *N*-terminal region acts to enhance the activity. In analogous results, the discrepancy in the role of a functional domain of Bac 5 by Gennaro *et al.* [10] and Raj and Edgerton [11] may be the result of a difference in the methods used for biological assays. Bac 7 and PR-39, Arg-Pro-rich antibacterial peptides, also have Arg-rich *N*-terminal regions [4,7]. Hence, it is likely that these peptides share similar structure–activity relationships.

Figure 4 Membrane fusion activities of the peptides for 70 μM DPPC (----) and DPPC/DPPG (3:1) SUVs (—) at 25°C. Bac $5_{1\text{-}23}$ (○) Bac $5_{4\text{-}23}$ (●), Bac $5_{7\text{-}23}$ (△).

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