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Effect of Proline in Basic and α-Helical Amphipathic Peptides on Acidic Liposomes and Their Antimicrobial Activity

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A helix-breaker amino acid, proline, was introduced into an antimicrobial peptide, Ac-(L-Leu-L-Ala-L-Arg-L-Leu)$_3$-NHCH$_3$ (4_3). The peptides [Pro^6]4_3 (1), [Pro^{2,6}]4_3 (2), and [Pro^{2,6,10}]4_3 (3) were able to form α-helix in order of 1 > 2 > 3 in the presence of acidic liposomes, indicating that proline does not always behave as a strong helix-breaker in specific surroundings. The antimicrobial activity was found to be connected closely with their amphipathic property.

Currently, there is growing interest in conformation on binding sites of biologically active peptides.[1] In this connection, studies on lipid-peptide interactions in model systems provide basic information about the function of biological membranes.[2] We have reported that a basic model peptide, Ac-(L-Leu-L-Ala-L-Arg-L-Leu-)$_3$-NHCH$_3$ (4_3), took a strongly amphipathic α-helical structure in the presence of liposomes in contrast with a slightly α-helical structure in buffer solution.[3,4] The peptide caused perturbation of biomembrane structures to exhibit several biological activities, e. g., antimicrobial activity was related with the amphipathic property of the peptide.[4, 5]

It is well known that proline is the strongest α-helix-breaker as well as glycine.[6] Recently, Scheraga and coworkers[7] reported that the disrupting effect of a prolyl residue in α-helix depends on the location of the prolyl residue in sequence, e. g., the proline inside α-helix acts on distortion of the residue preceding proline only and keeps a whole structure as α-helix with a slightly distorted conformation. This suggests that the helix-breaking ability of

	1	2	3	4	5	6	7	8	9	10	11	12			
Ac-	Leu-	Ala-	Arg-	Leu-	Leu-	Ala-	Arg-	Leu-	Leu-	Ala-	Arg-	Leu-	$NHCH_3$	4_3	
Ac-						-Pro-							$NHCH_3$	[Pro^6]4_3	(1)
Ac-		-Pro-				-Pro-							$NHCH_3$	[Pro^{2,6}]4_3	(2)
Ac-		-Pro-				-Pro-				-Pro-			$NHCH_3$	[Pro^{2,6,10}]4_3	(3)

Fig. 1. Designed and synthesized peptides.

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proline may be reduced considerably under strongly helix-inducing environments. Therefore, it is interesting whether proline introduced in 4_3 works as a helix-breaker or not in the amphipathic surroundings, and if it works, what effects appear on a biological activity of 4_3 analogs containing proline. We designed and synthesized three analogs of 4_3 with one to three prolines in the hydrophobic side of its α-helical structure (Fig. 1). We also present herein the relationship between the conformation under several conditions and the antimicrobial activity.

Peptides were designed as follows: (1) all the prolines were introduced into the hydrophobic part of the amphipathic structure of 4_3, when it takes an α-helical structure; (2) peptide 1 possesses a proline in the middle of 4_3; (3) peptide 2 has two prolines at the N-terminus and in the middle of 4_3; (4) peptide 3 has three prolines, one of which is located at the C-terminus in addition to two prolines in 2 (Fig. 1). Peptides 1-3 were synthesized by the solution method. Their synthetic route was essentially the same as that of 4_3 which was reported previously.[4] The purity of the final products was confirmed by thin layer chromatography, paper electrophoresis, and amino acid analysis. Their elemental analysis gave data supporting the following formulae: $1 \cdot 4AcOH \cdot 6H_2O$; $2 \cdot 5AcOH \cdot 6H_2O$; $3 \cdot 5AcOH \cdot 6H_2O$.

The predicted secondary structures of the peptides and their hydrophobic moments are listed in Table 1. Peptide 4_3 is predicted obviously to take an α-helical structure and highly hydrophobic moment. As expected, proline-containing peptides can not take the α-helical structure or decrease their α-helical structure. Interestingly, 2 takes a mixture of the α-helical structure and a β-structure. Hydrophobic moments of the analogs are very similar to that of 4_3, assuming that proline is involved in α-helix.

In order to estimate the conformation of the peptides experimentally under several conditions, CD spectra were measured and the results are shown in Fig. 2. All the analogs of 4_3 showed a random structure in the aqueous buffer solution. In the presence of neutral liposomes, 1 showed a broad and negative band between 200 and 230 nm, suggesting the presence of some ordered structure such as an α-helical structure. However, no evidence of ordered structure was found in the spectra of 2 and 3. Since 4_3 took obviously the α-helical structure slightly in buffer and strongly in DPPC-liposomes as reported previously,[4] it is clear that proline is

Table 1. Prediction of secondary structure of the peptides and their hydrophobic moments

	4_3	1	2	3
Prediction[a]	LARLLARLLARL hhhhhhhhhhhc[c]	LARLLPRLLARL ccceeeeecccc[c]	LPRLLPRLLARL eeeeehhhhhhc[c]	LPRLLPRLLPRL eeeeeeeeeeee[c]
Hydrophobic moment[b]	0.54	0.52	0.49	0.51

a) The prediction was performed by the Chou-Fasman method.[6] b) The moment was calculated by the Eisenburg method.[8] c) The predicted structures are symbolized as follows : h, α-helix; e, extended structure; c, coil.

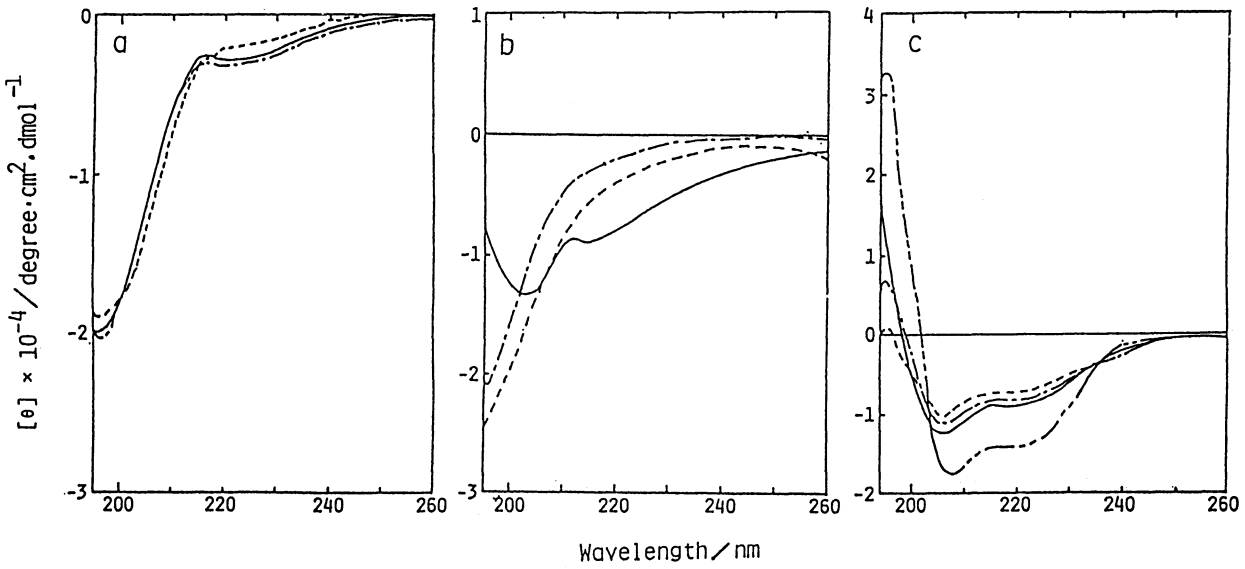

Fig. 2. CD spectra of 1-3 and 4_3 in buffer solution (a), in the presence of DPPC liposomes (b) and DPPC-DPPG (3:1) liposomes (c). Each peptide is symbolized as follows; 1 (——), 2 (— - —), 3 (- - - -) and 4_3 (— - - —). Spectra in Hepes buffer were measured at the peptide concentration of 100 μM. Maltilamellar vesicles were prepared by sonication for 30 min at 50°C in 20 mM Tris-HCl buffer solution (pH 7.4), and the peptides were dissolved at the concentration of 10-20 μM in 20 mM Tris buffer solution containing 0.9 mM liposomes.

certainly the α-helix-breaker. However, in acidic liposomes, all the peptides exhibited a positive band down to 200 nm and a double minimum at 206-208 and 222 nm responsible for the α-helix although the band intensity decreased in order of 4_3 > 1 > 2 > 3, that is, the helical content of 4_3 obviously decreased with the increase of proline residue. Surprisingly 3, which contains three prolines, still kept α-helix-like structure in acidic liposomes. These results indicate that the predicted conformations of the peptides are not consistent with those in acidic liposomes. These mean that the proline introduced into 4_3 is not the strong helix-breaker in the amphipathic surroundings of acidic lipid bilayer. We proposed previously that 4_3 took an amphipathic α-helix on the surface of the membrane, and the hydrophilic side in the helix interacted with the acidic moiety of phospholipid in the membrane and the hydrophobic side immersed into the membrane holizontally.[9] It is likely that 1-3 interact with lipid similarly because of their α-helix-like structure similar to that of 4_3 in the presence of liposomes. Proline is considered as the helix-breaking residue for the reason that it prevents the formation of a hydrogen bond with the preceding turn of the helix and/or causes a distortion of the preceding turn of the helix with steric hindrance by the bulky proline ring. However, the helix-breaking tendency of proline may be much reduced by the following interactions : (i) electrostatic interaction between arginine side chains and phospholipid head group and (ii) hydrophobic interaction between lipophilic side of the peptide helix and phospholipid alkyl chains. The fact that α-helical structure of 1-3 was induced in acidic liposomes implies that the electrostatic interaction between the cationic arginine residues in the peptides and the anionic head groups in acidic

Table 2. Antimicrobial activity of synthetic peptides

Organism	Minimum inhibitory concentration (μg/ml)[a]			
	4_3	1	2	3
S. aureus 1840	6.25	12.5	25	>100
B. subtilis PCI 219	3.13	6.25	6.25	50
E. coli O-111	>100	>100	>100	>100
S. flexneri EW-10	50	25	50	>100
S. sonnei EW-33	>100	>100	100	100

a) Inhibition of growth of microorganisms was determined by the dilution method using a trypticase soy agar medium.

phospholipids is very important to keep α-helical structure in bilayer.

Our results suggest that one should be careful in applying the empirical secondary structure prediction methods to the peptide conformation in the amphipathic surroundings, e.g., peptide and protein bound to biomembrane.

The antimicrobial activity of synthetic peptides is shown in Table 2. Minimum inhibitory concentration for *S. aureus* and *B. subtilis* decreased in order of 4_3 > 1 > 2 > 3, which is highly consistent with that of their α-helical intensity in acidic liposomes. These results support strongly the previous conclusion that the effect of the peptides on biological activity is correlated to the ability of taking the amphipathic structure by α-helix formation (4).

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