Biochimica et Biophysica Acta, 984 (1989) 174–182
Elsevier

BBAMEM 74541

The spectroscopic analysis for binding of amphipathic and antimicrobial model peptides containing pyrenylalanine and tryptophan to lipid bilayer

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(Received 29 March 1989)

Key words: Amphipathic antimicrobial peptide; Alpha-helical peptide; Fluorescent peptide; Pyrenylalanine; Tryptophan fluorescence; Peptide–lipid interaction; Peptide–peptide interaction

The binding of basic amphipathic fluorescent peptides to lipid bilayers was studied in relation to their antimicrobial activity. Four fluorescent peptides containing pyrenylalanine or tryptophan in an amphipathic basic peptide (4_4) consisting of four repeated units of tetrapeptide, -L-Leu-L-Ala-L-Arg-L-Leu-, were found to have antimicrobial activities against Gram-positive bacteria and to take conformations with fairly high α-helical content both in aqueous solutions and liposomes. The fluorescence spectroscopic data suggested that the pyrenylalanine-peptide existed as a monomer in methanol or liposomes but as an oligomer in aqueous solutions to form an excimer beteen pyrenylalanyl residues. Upon binding with liposomes, the fluorescence spectra of the tryptophan-containing peptide shifted to a shorter wavelength, indicating the change in the state of tryptophan from hydrophilic environment to hydrophobic one. The analytical data for the quenching of tryptophan fluorescence by I^- anion suggest that the tryptophan residue in the peptide is not deeply buried in the hydrophobic core of the bilayers. Based on these findings, it is suggested that the peptides may interact with liposomes in such a manner that they lie parallel to the surface of the lipid bilayers with their hydrophobic regions shallowly in the amphipathic moiety of the bilayers.

Introduction

The interaction of a variety of basic and amphipathic peptides found in natural sources with lipid bilayers has been extensively studied with regard to their antimicrobial activity [1–4]. Some of the basic model peptides designed from a common feature of the extension peptides of mitochondrial protein precursors exhibit potent antimicrobial activities against Gram-positive bacteria, and such biological activities have been attributed to the amphiphilicity of the peptides [5,6]. Recently, attention has been directed to the unique properties of the synthetic model peptides: the model peptides so far examined can not solubilize membrane-bound enzymes from mitochondria and microsomes [7]. In this respect, the amphipathic peptides differ from detergents. Such a difference in the properties of the model peptides and detergents has been advantageously utilized as a criterion to discriminate the membrane-bound enzymes from the soluble protein molecules [8]. There is also evidence indicating that the peptides perturb the structure of the lipid bilayer, resulting in the fusion of liposomes [9]. Thus, the amphipathic peptides with an antimicrobial activity have attracted a great deal of interest because of their unique characteristics. However, the mode of the interaction of the peptides with lipid bilayers has not been understood completely. To shed further light on the structure-function relationship of the amphipathic peptides, in the present study, four fluorescent peptides (Fig. 1) in which pyrenylalanine

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Abbreviations: AcONSu, *N*-acetoxysuccinimide; Boc, *t*-butyloxycarbonyl; DPPC, dipalmitoyl-D,L-α-phosphatidylcholine; EDC, 1-ethyl-(3-dimethylaminopropyl)carbediimide; Et_3N, triethylamine; HOBt, 1-hydroxybenzotriazole; Pac, phenacyl; Pya, L-1-pyrenylalanine; TFE, trifluoroethanol; Tos, *p*-toluenesulfonyl; TFA, trifluoroacetic acid.

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                    5       7       9
Ac-Leu-Ala-Arg-Leu-Leu-Ala-Arg-Leu-Leu-Ala-Arg-Leu-Leu-Ala-Arg-Leu-NHCH3  4
Ac-                                 -Pya-                          -NHCH3  1
Ac-                 -Pya-           -Pya-                          -NHCH3  2
Ac-                         -Pya-                                  -NHCH3  3
Ac-                                 -Trp-                          -NHCH3  4
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Fig. 1. Structures of the antimicrobial peptide 4_4 and its fluorescent derivatives. All amino acids are of the L-configuration.

(Pya) or tryptophan was introduced into the amphipathic and antimicrobial peptide, Ac-(L-Leu-L-Ala-L-Arg-L-Leu-)$_4$-$NHCH_3$ (4_4), were synthesized. Their binding to liposomes and their orientation in lipid bilayers were analyzed by CD and fluorescent spectroscopy.

Materials and Methods

Materials. DPPC was purchased from Sigma Chemical Co., St. Louis. All other reagents were of the highest analytical grade.

Synthesis of the peptides. Synthetic route for peptide 2 is shown in Fig. 2. The fully protected peptide 14 was obtained through fragment condensation of the octapeptide acid 10 and the octapeptide amide 11 as described previously [10]. Protecting group of 14 was removed with sodium/liquid ammonia as follows. To a stirred solution of 14 (70 mg, 24 μmol) in liquid ammonia was added sodium metal until a dark color was stable for more than 3 min. The ammonia was removed by blowing nitrogen stream. To the residue was added 10% acetic acid. The solution was passed through the Sephadex G-25 column. The fractions containing the peptide were collected and lyophilized to give peptide 2 as a yellow powder. Yield, 44 mg (63%). Compounds 1, 3 and 4 were prepared by essentially the same procedure as described for peptide 2. Chemical and physical constants of final products are listed in Table I.

Preparation of liposomes. A mixture of uni- and multi-lamellar liposomes was prepared as follows. A lipid film obtained by evaporation of a chloroform solution of DPPC (20 mg) was hydrated in Tris-HCl buffer (pH 7.4) (2 ml) by repeated vortexed-mixing at 50 °C for 30 min, followed by a sonication using a Tomy Seiko ultrasonic disrupter model UR-200P at 50 °C for 30 min. The liposomes obtained were diluted to desired concentrations by Tris-HCl buffer.

Spectroscopic experiments. Circular dichroism (CD) spectra were recorded on a JASCO J-50A spectropolarimeter connected with a JASCO data processor Model J-dry using a quartz cell of 1 mm pathlength. For measurements of the CD spectra of peptides in DPPC liposomes, the peptides were dissolved directly in 20 mM Tris-HCl buffer (pH 7.4) containing 0.9 mM DPPC liposomes. To scan a scattering due to liposomes, the CD spectrum of liposomes was subtracted from the spectrum of the peptide in the presence of liposomes.

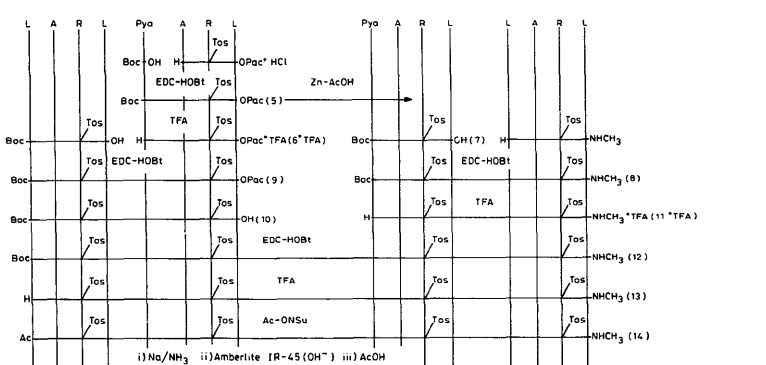

Fig. 2. Synthetic route for peptide 2. The longitudinal lines show each amino acid residue and the horizontal lines show the sequence of peptides.

All measurements were performed at 20°C and the data were expressed in terms of mean residue ellipticites. The α-helical content was calculated on the basis of the theoretical value of $[\theta]_{222}$ for a 100% α-helix of the number of amino acid residues in each peptide as described by Chen et al. [11]. There is a possibility that Pya has some effect on the CD band in the far ultraviolet region. In the present case, however, contribution of the Pya residue in peptides to the CD band was negligible since CD spectra showed only the characteristics of α-helical band but not any other band due to Pya.

Fluorescence spectra were recorded on a JASCO FP-550A spectroflurometer equipped with a thermostatted cell holder. The spectra were measured with excitation at 342 nm for the Pya-containing peptides and 280 nm for the Trp-containing peptide. All measurements were done at 25°C.

Titration curves for binding of Pya-containing peptides to liposomes. To attain the titration curves for binding of peptides to liposomes, appropriate aliquots of DPPC liposomes (1.0 mM) were successively added to a solution (2.5 ml) of peptide (0.6 μM) in 20 mM Tris-HCl buffer (pH 7.4). After each addition of liposomes, the mixture was kept at 25°C for 10 min to achieve the equilibration. Fluorescence intensities were corrected for blank measurements from suspension of liposomes in buffer. Corrections were made for dilution of peptide. The data were analyzed according to Eqn. 1:

$$1 - \varepsilon = (1 - \varepsilon_b) - K_d(1 - \varepsilon)/nm \qquad (1)$$

where K_d is the dissociation constant of lipid-peptide complex, m denotes the lipid concentration, and n is the number of binding sites per lipid. The quantity ε characterizes the relative change of the spectrsocopic parameter accompanying binding of the peptide to the liposomes at a particular lipid concentration, m. This quantity is defined as the relative change for intensity ratio of fluorescence of pyrene monomer and excimer, I_E/I_M, according to Eqn. 2:

$$\varepsilon = (I_E/I_M)/(I_E/I_M)_0 \qquad (2)$$

where the parameters $(I_E/I_M)_0$ are the intensity ratio of the initial excimer and monomer fluorescences, respectively. The fluorescence intensities for pyrene monomer (I_M) and excimer (I_E) were obtained at 376 and 450 nm, respectively. The parameter ε_b characterizes the spectral properties of lipid-bound peptide. It is equal to the limiting value of ε when all peptides present in the solution are bound to the membrane. This parameter will have a specific value for each peptide and lipid system. As derived from Eqn. 1, the slope of a plot of $(1 - \varepsilon)$ versus $(1 - \varepsilon)/m$ yields K_d/n. The $1 - \varepsilon_b$ value is given by the ordinate intercept in the plot of $(1 - \varepsilon)$ versus $(1 - \varepsilon)/m$.

Quenching of tryptophan fluorescence by KI. Quenching of tryptophan fluorescence of peptide 4 by KI was performed in Tris-HCl buffer (pH 7.4) in the absence and presence of 200 μM DPPC liposomes. Aliquots of 4 M sodium iodide solution in the buffer containing 1 mM Na_2SO_3 were added to the DPPC liposomes to prevent the formation of I^{3-}. The quenching data were analyzed by Stern-Volmer plot using Eqn. 3:

$$F_0/F = 1 + K_q[Q] \qquad (3)$$

where F_0 and F are the fluorescent intensities at 280 nm in the absence and presence of quencher at concentration [Q], respectively. K_q is the Stern-Volmer quenching constant.

Antimicrobial activity. The antimicrobial activities of the peptides were measured by the serial dilution method using *Bacillus subtilis* IFO 3007 and *Escherichia coli* B as described previously [6].

Results and Discussion

Design and synthesis of the peptides

As well as tryptophan, the fluorescent amino acid, Pya, has been advantageously employed as a fluorescent probe for elucidation of the structure-function relationship of biologically active peptides, particularly for analysis of the peptide–lipid and peptide–peptide interactions because of its high quantum yield and long life time [13–17]. It has also been known that Pya can form an excimer between an excited-state and ground-state [13]. As previously reported [5], 4_d is a basic and amphipathic peptide and has an antimicrobial activity against Gram-positive bacteria, but this peptide has no fluorescence suitable for spectroscopic analysis of peptide–peptide or peptide–lipid interactions. Therefore, introduction of the fluorescent probe to 4_d is expected to facilitate the studies on the structure-function relationship of this peptide. Four fluorescent analogs of 4_d, in which Leu and Arg residues were replaced by Pya or Trp, were designed as shown in Fig. 1. As shown in Fig. 3, peptides 1 and 2 in which Leu^9 and $Leu^{5,9}$, respectively, are replaced by Pya are considered to retain the lipophilic region of the mother peptide 4_d. Replacement of Arg^7 by Pya yields a less amphipathic peptide 3 compared to 4_d, assuming that it adopts the α-helical structure as well as peptides 1 and 2. It is reasonable to consider that the amphiphilicity of the mother peptide 4_d is retained in peptide 4, in which Leu^9 is replaced by Trp.

Optically active pyrenylalanine was prepared through the stereoselective hydrogenation of a dehydrodiketopiperazine derivative [18]. As shown in Fig. 2, synthesis of the peptides are essentially based on the convenient

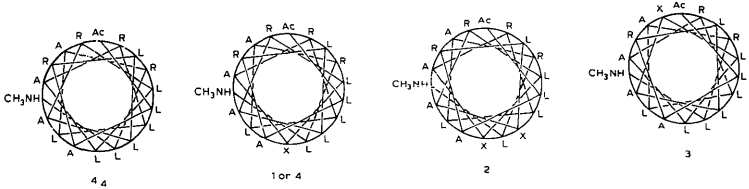

Fig. 3. α-Helical wheels of the fluorescent peptides. On peptide 1, as an example, the projection of amino acid residues in α-helical segment shows the distribution of the charged residues on the top surface and the hydrophobic residues on the bottom surface. One-letter symbols of amino acids are used for concise representation. Pyrenylalanine is represented by X.

condensation of peptide fragments. The protected tetrapeptides were prepared stepwise and then the fragment peptides were coupled with EDC-HOBt to give the hexadecapeptide. When the peptides was treated with HF [19] or methanesulfonic acid method [20] to deprotect the $N^{guanidino}$-tosyl group, the resulting peptide showed fluorescence spectra different from that of pyrenyl chromophore, indicating that the treatment of the peptide with the strong acid was accompanied by a side reaction attributable to modification of pyrenyl chromophore by the tosyl cation (unpublished data). Therefore the peptides were deprotected by the sodium/liquid ammonia reduction method [21] to yield the final products. The yields and physical constants of the fluorescent analogs of the peptide 4_4 are summarized in Table I. These peptides were obtained in fairly high yields, and all the analytical data confirmed the structure of the designed peptides.

Antimicrobial activities of synthetic peptides

The growth inhibitory potencies of the synthetic peptides against both *B. subtilis* and *E. coli* are summarized in Table II. All the peptides showed antimicrobial activities, but their potencies to inhibit the growth of bacteria were lower compared to the mother peptide, 4_4. Lower activities of peptides 1 and 2 compared with those of 4 and 4_4 may be attributable to the bulkiness of Pya residue and/or to the less α-helical nature of the peptides in lipid bilayers (see below). It should be noticed that peptide 3 shows higher antimicrobial activity relative to peptides 1 and 2, despite its lower amphiphilicity. We previously reported that amphipathic peptide Ac-(Leu-Ala-Arg-Leu)$_2$-NHCH$_3$ (4_2) consisting of two units of tetrapeptide had an ability to inhibit the growth of Gram-positive bacteria [5]. Taking these facts into account, the fairly high antimicrobial activity of peptide 3 may be interpreted as being due to the conserved sequence in the C-terminal segment between peptides 3 and 4_2. Peptide 4 was the most potent of fluorescent peptides tested, and its ability to inhibit the growth of bacteria was almost identical to that of 4_4. It appears that the replacement of Leu^9 by Trp does not have much effect on the antimicrobial activity of 4 because of the less bulky side chain of the Trp residue than the Pya side chain at position 9.

These findings directed us to carry out a spectro-

TABLE I

Yield and physical constants of model peptides

Peptide	Formula [a] (mol. wt.)	Yield (%)	R_{Arg} [b]	Amino acid analysis [c]		
				Ala	Leu	Arg
1·4AcOH	$C_{100}H_{165}O_{17}N_{29}\cdot 4C_2H_4O_2\cdot 11H_2O$ (2502.0)	68	0.82	3.96	7.00	3.98
2·4AcOH	$C_{113}H_{167}O_{17}N_{29}\cdot 4C_2H_4O_2\cdot 11H_2O$ (2624.1)	63	0.80	3.92	6.00	4.24
3·3AcOH	$C_{100}H_{164}O_{17}N_{26}\cdot 3C_2H_4O_2\cdot 6H_2O$ (2290.8)	63	0.74	4.08	8.00	2.57
4·4AcOH	$C_{92}H_{162}O_{17}N_{30}\cdot 4C_2H_4O_2\cdot 6H_2O$ (2308.8)	77	0.87	4.43	7.00	3.77

[a] The reasonable compositions are considered from the results of elemental analysis.

[b] Paper electrophoresis was carried out a Toyo Roshi No. 52 paper with the solvent system of HCOOH-AcOH-MeOH-H_2O for 2 h at 600 V. Arginine was used as a reference.

[c] Amino acid analyses were performed on a Hitachi KLA-5 amino acid analyzer after the hydrolysis in 6 M HCl in sealed tubes at 110 °C for 24 h. The values of pyrenylalanine could not be calculated for compounds 1, 2 and 3 because no elution took place under the conditions of standard amino acid analysis. The value of tryptophan was 0.87 for compound 4.

TABLE II

Antimicrobial activity of synthetic peptides and 4_4

Peptide	MIC [a] (μg/ml)	
	B. subtilis 1FO 3007	*E. coli* B
4_4	0.5 – 1.25	5–10
1	2.5 – 5	25
2	5 –12.5	25
3	2.5 – 5	25
4	1.25– 2.5	5–12.5

[a] Minimum inhibitory concentration.

TABLE III

α-helical contents of peptides

Peptide	α-helical content (%) [b]		
	Buffer [a]	TFE	DPPC
4_4	38	39	58
1	49	38	52
2	54	38	49
3	37	36	46
4	40	32	58

[a] At the concentration of 20 mM Tris-HCl (pH 7.4).
[b] These values are given with an experiment error of a few percent.

scopic analysis for the interaction of the fluorescent peptides with liposomes as a model system for the binding of 4_4 to lipid bilayer.

Spectroscopic properties of peptides

CD spectra of the peptide 1 measured in Tris-HCl buffer (pH 7.4) and TFE are shown in Fig. 4, compared with the spectrum taken in the presence of DPPC liposomes. The spectrum measured in the buffer solution had a CD band of double minimum at 206 and 222 nm, which is characteristic of α-helix. The CD spectrum of the peptide in TFE was similar in shape to that measured in the buffer solution, but was characterized by a decreased molar ellipticity and a shift of CD band to shorter wavelengths. Similar spectra were obtained for other peptides (data not shown). By analyzing the

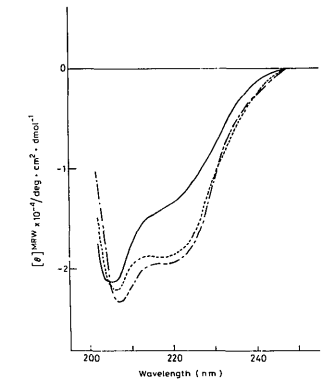

Fig. 4. CD spectra of peptide 1 in various solvents. The spectra were measured in 20 mM Tris-HCl buffer (pH, 7.4) in the absence (·····) and the presence of 100 μM DPPC liposomes (·–·–·), and in trifluoroethanol (———) at 21°C. The concentration of the peptide was 10 μM.

CD spectra according to Chen et al. [11], the helical contents were calculated for individual peptides and summarized in Table III. It is evident that the helical contents of peptides 1, 2 and 4 in the buffer solution were higher compared to those in TFE, a solvent capable of increasing the helicities of peptides, while the mother peptide, 4_4, and 3 had an almost identical helical content in the buffer solution and TFE. These findings suggest that the introduction of the fluorescent side chain of Pya and Trp into the hydrophobic regions in peptide 4_4 may induce peptide–peptide interactions which favor an increase in α-helical structure. In the buffer solution, there may be a self-association of peptides owing to the lipophilic region of amphipathic structure assuming that these peptides adopt the α-helices.

Such a view can be supported by the fluorescence spectroscopic data of Pya-containing peptides. The fluorescence spectrum of peptide 1 in methanol as excited at 342 nm had emission maxima at 376 nm and 396 nm, similar to that given for a model compound, Boc-Pya-OH, as shown in Fig. 5. Similar spectrum was also obtained for peptide 3. However, the fluorescent spectrum of peptide 1 taken in Tris-HCl buffer (pH, 7.4) had an additional emission maximum at 450 nm. Such an emission maximum at 450 nm is characteristic of the excimer and can only be due to the proximity of two pyrenyl side chains. Since peptide 1 possesses only one pyrenyl group in the sequence, it is plausible that peptide 1 is self-associated in the buffer but exists as a monomer in methanol and in DPPC liposomes. On the other hand, the fluorescence spectrum of peptide 2 in methanol exhibited a small but distinct shoulder at 450 nm, suggesting that the excimer is present even in the organic solvent. It is reasonable to consider that peptide 2 can display such an excimer fluorescence due to the proximity of the two pyrenyl groups in the α-helical structure of the peptide. In buffer peptide 2 exhibited the fluorescence spectrum with an unusually high intensity of 450 nm. There is a possibility that the self-association of the peptide in buffer may stabilize the intramolecular or intermolecular interaction between pyrenyl residues in

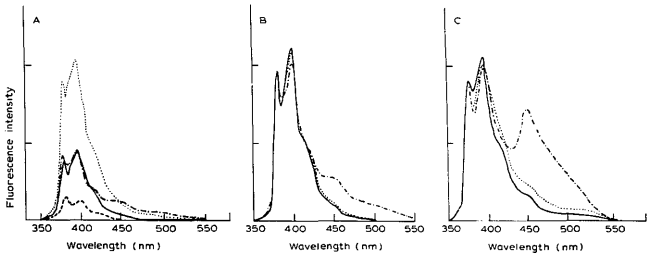

Fig. 5. Fluorescence spectra of the synthetic peptides and Boc-Pya-OH. Panel A shows the fluorescence spectra of peptide 1 in 20 mM Tris-HCl buffer (pH 7.4) in the absence (·–·–·) or presence of DPPC liposomes (·····), and in methanol (———). The fluorescence spectrum of Boc-Pya-OH (0.1 μM) in methanol (– – –) is shown as a reference. Measurements were done with 0.6 μM of the peptide and 100 μM of DPPC liposomes at 25 °C with excitation at 342 nm, and the emission spectra normalized to the same intensity at a Pya emission maximum at 376 nm are shown in panel B. The normalized emission spectra of peptide 2 are shown in panel C.

the peptide. It has been reported that amphipathic helical peptides such as melittin and mastoparan self-associate in buffer solution [22,23].

Binding of peptides to DPPC liposomes

As shown in Fig. 4, upon binding to DPPC liposomes, the CD band of peptide 1 shifted to longer wavelength by 2 nm, accompanied by the increase in the molar ellipticity. It is noteworthy that only a slight increase in the α-helical contents was obtained for peptides 1 and 3 * on the addition of DPPC liposomes, while the α-helical content of peptide 4, with a fairly high antimicrobial activity (Table II), increased substantially on binding with DPPC-liposomes, as did the mother peptide, 4_4. On the contrary, the α-helical content of peptide 2 with a low antimicrobial activity did not increase even by the addition of DPPC liposomes. It is likely that the bulky side chain of two Pya residues present at the α-helical region of peptide 2 may weaken the peptide-lipid interaction and this may decrease the antimicrobial activity of peptide 2.

The binding of DPPC liposomes to peptide 1 led to a great increase in the fluorescence intensities at 376 and 396 nm concomitantly with the decrease in the excimer fluorescence at 450 nm (Fig. 5A). The spectrum of 1 in methanol is almost identical with that in liposomes as seen in Fig. 5B, suggesting that in DPPC liposomes peptide 1 may exist as a monomer. The fluorescence spectrum of peptide 2 in liposomes was also characterized by a decrease in the fluorescence intensity at 450–550 nm which is representative of the excimer.

To attain a better understanding of the nature of the peptide–lipid interaction, the binding of peptides to DPPC liposomes was quantitatively analyzed by fluorescence spectroscopy. The fluorescence intensities at 376 and 396 nm increased with increasing the DPPC concentration (Fig. 6B). The values of K_d/n for the binding of peptides to lipid bilayers were obtained from plots of $(I_E/I_M)/(I_E/I_M)_0$ versus concentration of DPPC (Fig. 6A). The K_d/n value was 2.0 μM for peptide 1, 1.4 μM for peptide 2, and 13 μM for peptide 3 at pH 7.4 and 25 °C. These K_d/n values were smaller than those reported for other oligopeptides [12], suggesting that peptides 1–3 interact with DPPC liposomes with strong binding abilities. Peptides 1 and 2 gave lower K_d/n values than peptide 3. Considering the fact that peptides 1 and 2 are more amphipathic as compared to peptide 3, it can be concluded that the hydrophobic region in the peptide principally contributes to the peptide–lipid interaction.

Fig. 7 shows the fluorescence spectra of peptide 4 in the absence and presence of DPPC liposomes. The tryptophan fluorescence spectrum measured in Tris-HCl buffer (pH 7.4) in the absence of DPPC had an emission maximum at 352 nm. Upon addition of DPPC liposomes, the fluorescence spectrum shifted to shorter wavelengths by 8 nm. Such a fluorescence spectrum of peptide 4 induced by binding with DPPC liposomes can be explained by the change in the state of Trp-9 from a hydrophilic to a hydrophobic environment. Such a view is not inconsistent with the fluorescence quenching data. The fluorescence intensity of peptide 4 decreased with increasing concentration of KI. The quenching data

* Peptide 3 was almost but not totally bound to lipid at the concentrations in CD measurement as can be seen in the lipid/peptide ratio on Fig. 6.

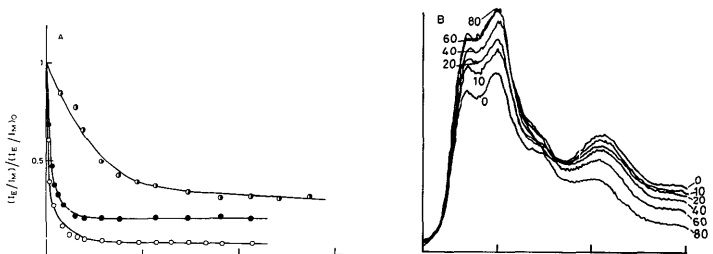

Fig. 6. (A) Titration curves for binding of DPPC liposomes to the peptides. Fluorescence spectra were measured under the same conditions described in Fig. 5 except that the concentration of DPPC was varied. The $(I_E/I_M)/(I_E/I_M)_0$ values were plotted against the concentration of DPPC. The plot was given for peptide 1 (○), 2 (●) and 3 (◑). (B) Variation of fluorescence intensity of peptide 2 with increasing concentrations of DPPC (μM) is shown. Details were described in Materials and Methods.

were analyzed according the Stern-Volmer relationship (Eqn. 3). As shown in Fig. 8, the plots both in the absence and presence of DPPC gave straight lines, and the slope of the plot in the presence of DPPC liposomes was smaller than that in the absence of DPPC. These observations indicate that the binding of the peptide to lipid bilayers decreased the accessibility of I^- anion to Trp-9 of the peptide. The consensus is that I^- anion cannot have access to the tryptophan residue buried in the interior of the protein molecule or in the core of the lipid bilayer, but has an ability to quench tryptophan present at or relatively near the surface of the protein or lipid bilayer. As demonstrated here, Trp-9 in peptide 4

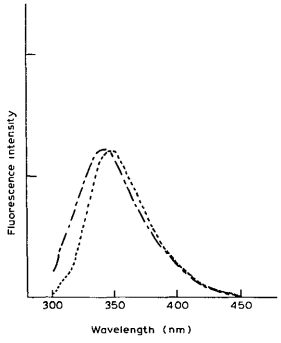

Fig. 7. The fluorescence spectra of peptide 4. The spectra were recorded in Tris-HCl buffer (pH 7.4), in the absence (– – –) and presence of DPPC liposomes (·–·–·). Spectroscopic measurements were done at 25 °C with excitation of 280 nm. The concentrations of peptide and DPPC were 6 μM and 100 μM, respectively.

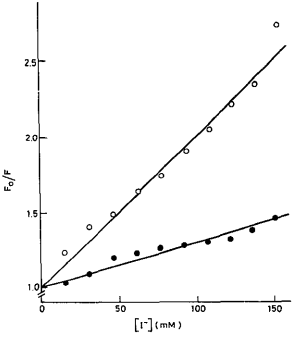

Fig. 8. Stern-Volmer plots of the quenching of tryptophan fluorescence in peptide 4 by KI. The fluorescence spectrum of peptide 4 was recorded in Tris-HCl buffer (pH 7.4) at 25 °C in the absence (○) and presence (●) of 200 μM DPPC liposomes. The concentration of the peptides was 6 μM and the excitation wavelength was 280 nm. Data for quenching of free peptide and the complex with DPPC were plotted according to Eqn. 3. Details are described in Materials and Methods.

is still quenched by I^- even in the presence of DPPC liposomes although the accessibility of this residue to the quencher decreased. The fluorescence maximum of peptide 4 (344 nm) in lipid bilayer was at a longer wavelength than that found for the tryptophan at the hydrophobic core of the lipid bilayer (330 nm), as reported by Voges et al. [24]. It is reasonable to consider that in the complex with DPPC liposomes the tryptophan residue in the peptide is not deeply buried in the hydrophobic core of the lipid bilayer. In the lipid bilayer, Trp-9 in peptide 4 may be present at or near the boundary of the ester link (H belt) [25] and the hydrophobic alkyl chain moiety of DPPC. It has been suggested that α-helical and amphipathic peptides lie parallel to the membrane phase where the peptides penetrate only the hydrophobic region into lipids [26,27]. There is a possibility that peptides form channel oligomers by spanning lipid bilayer and exposing the hydrophobic side to the lipid and hydrophilic phase to the aqueous pore [28]. In the present case, however, this possibility can be disregarded since the Trp-9 residue is in the middle in the chain of peptide 4, which means that Trp-9 would be located at hydrophobic core of the bilayers in order to form the pore. This model dose not agree with the results which suggest that the tryptophan residue penetrates only slightly into the lipid bilayer.

The results represented here provide some insight into the structure-function relationships of the antimicrobial peptide 4_a. Introduction of the pyrene or indole group into the amphipathic peptide 4_a facilitates the analysis of features of the peptide–peptide and peptide–lipid interaction. As demonstrated here, spectroscopic data strongly suggest that the model peptides are self-associated in aqueous solution but are dissociated into monomer upon binding with DPPC liposomes. In aqueous solution, intermolecular interactions due to the hydrophobic sides of the α-helical regions of peptide may form an aggregation site, shielding these sides from the aqueous phase, while the positively charged side chain of the arginine residue may be still exposed to the aqueous phase. However, in DPPC liposomes, the interaction of polar amino acid side chains of the peptide with the polar head group of the lipid may release the peptide–peptide interaction, allowing the hydrophobic regions of monomeric peptide to penetrate into the surface of the phospholipid bilayer. Such a shallow penetration of these peptides into the phspholipid bilayers might produce a difference in the action profile between the present peptides and active-surface detergents: the peptides do not perturb the cell-membrane so drastically as detergents do. There is a possibility that peptides lead to the fragmentation of liposomes into discs and micelles at high ratios of peptide to lipid (= 5) as has been suggested for melittin [29]. Our model peptides, however, do not induce such morphological changes in liposomes [9]. Although the mode of action of the model peptides is still not clear, it can be postulated that the peptides perturb the structure of the lipid bilayer of the cell membrane and hence they inhibit the growth of Gram-positive bacteria.

Acknowledgments

We thank Prof. H. Nishikawa, Fukuoka University, for CD measurements. We also thank Prof. H. Yonezawa, Kagoshima University, for the microbial assays.

References

1 Haberman, E. (1972) Science 177, 314–322.
2 Steiner, H. (1982) FEBS Lett. 137, 283–287.
3 Comte, M., Malnoe, A. and Cox, J.A. (1986) Biochem. J. 240, 567–573.
4 Zasloff, M. (1987) Proc. Natl. Acad. Sci. USA 84, 5449–5453.
5 Lee, S., Mihara, H., Aoyagi, H., Kato, T., Izumiya, N. and Yamasaki, N. (1986) Biochim. Biophys. Acta 862, 211–219.
6 Mihara, H., Kanmera, T., Yoshida, M., Lee, S., Aoyagi, H., Kato, T. and Izumiya, N. (1987) Bull. Chem. Soc. Jpn. 66, 697–706.
7 Ito, I., Ogishima, T., Ou, W., Omura, T., Aoyagi, H., Lee, S., Mihara, H. and Izumiya, N. (1985) J. Biochem. 98, 1571–1582.
8 Harano, T., Miyata, T., Lee, S., Aoyagi, H. and Omura, T. (1988) J. Biochem. 103, 149–155.
9 Suenaga, M., Lee, S., Park, N.G., Aoyagi, H., Kato, T., Umeda, A. and Amako, K. (1989) Biochim. Biophys. Acta 981, 143–150.
10 Yoshida, M., Lee, S., Mihara, Aoyagi, H. and Kato, T. (1986) Mem. Fac. Sci., Kyushu Univ. Ser. C. 16, 161–166.
11 Chen, Y.-H., Yang, J.T. and Chan, K.H. (1974) Biochmistry 13, 3350–3359.
12 Surewicz, W.K. and Epand, R.M. (1984) Biochemistry 23, 6072–6077.
13 Goedeweeck, R. and De Schryver, F.C. (1984) Photochem. Photobiol. 39, 515–520.
14 Goedeweeck, R., Van der Auweraer, M. and De Schryver, F.C. (1985) J. Am. Chem. Soc. 107, 2334–2341.
15 Egusa, S., Sisido, M. and Imanishi, Y. (1985) Macromolecules 18, 882–889.
16 Mihara, H., Lee, S., Shimohigashi, Y., Aoyagi, H., Kato, T., Izumiya, N. and Costa, T. (1985) FEBS Lett. 193, 35–38.
17 Mihara, H., Lee, S., Shimohigashi, Y., Aoyagi, H., Kato, T., Izumiya, N. and Costa, T. (1986) Biochem. Biophys. Res. Commun. 136, 1170–1176.
18 Mihara, M., Lee, S., Shimohigashi, Y., Aoyagi, H., Kato, T., Izumiya, N. and Costa, T. (1987) Int. J. Pept. Prot. Res. 30, 605–612.
19 Sakakibara, S., Shimonishi, Y., Kishida, Y., Okada, M. and Sugihara, H. (1967) Bull. Chem. Soc. Jpn. 40, 2164–2167.
20 Yajima, H. and Fujii, N. (1983) in The Peptide: Analysis, Synthesis, Biology (Gross, E. and Meienhofer, J., eds.), Vol. 5, pp. 65–109, Academic Press, New York.
21 Schnabel, E. and Li, C.H. (1960) J. Am. Chem. Soc. 82, 4576–4579.
22 Talbot, J.C., Dufourcq, J., De Bony, J., Faucon, J.F. and Lussan, C. (1978) FEBS Lett. 102, 191–193.
23 Higashijima, T., Wakamatsu, K., Saito, K., Fujino, M., Nakajima, T. and Miyazawa, T. (1984) Biochim. Biophys. Acta 802, 157–161.
24 Voges, K.-P., Jung, G. and Sawyer, W.H. (1987) Biochim. Biophys. Acta 896, 64–76.
25 Chatterjie, N. and Brockerhoff, H. (1978) Biochim. Biophys. Acta 511, 116–119.

26 Dawson, C.R., Drake, A.F., Helliwell, J. and Hider, R.C. (1978) Biochim. Biophys. Acta 510, 75–86.
27 DeGrado, W.F., Kézdy, F.J. and Kaiser, E.T. (1981) J. Am. Chem. Soc. 130, 679–681.
28 Bernheimer, A.W. and Rudy, B. (1986) Biochim. Biophys. Acta 864, 123–141.
29 Dufourcq, J., Faucon, J.F., Fourche, G., Dasseux, J.L., Le Maire, M. and Gulik-Krzywicki, T. (1986) Biochim. Biophys. Acta 859, 33–48.