Conformation of Tachyplesin I from *Tachypleus tridentatus* When Interacting with Lipid Matrices

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ABSTRACT: The mode of action of tachyplesin I, an antimicrobial cationic heptadecapeptide amide isolated from the hemocyte debris of a horseshoe crab, *Tachypleus tridentatus*, toward lipid matrices was studied with synthetic tachyplesin I, its analogs with Phe in place of Trp or Tyr, a linear analog with no disulfide bonds, and two linear short fragments. Circular dichroism spectra showed that tachyplesin I took an antiparallel β-structure in buffer solution and a certain less ordered structure in acidic liposomes composed of egg phosphatidylcholine and egg phosphatidylglycerol (3:1). Spectrophotometric titration of the peptides with laurylphosphorylcholine revealed that both Trp and Tyr residues orient toward the inside of lipid matrices, suggesting that they are on the same side of the peptide backbone. The carboxyfluorescein leakage experiment and fluorescence data indicated that tachyplesin I interacted strongly with neutral and acidic lipid bilayers and an aromaticity-rich hydrophobic part of the peptide was embedded in lipid membranes. All the peptides except for the short fragments were almost equally active in lipopolysaccharide binding. The energy-transfer experiment showed that a conformational change occurred such that the Tyr and Trp residues are positioned more closely to each other in acidic liposomes than in buffer solution. The present study strongly suggested that amphipathic lipid bilayers induced a conformational change of tachyplesin I from an energetically stable β-structure to a less ordered, probably more amphipathic structure.

Insects, amphibians, reptilians, and vertebrates produce in some cases cationic polypeptides which show a cytolytic effect in vitro on bacteria and parasites, presumably contributing to the defense mechanisms of the host against invading microorganisms. Cationic polypeptides isolated so far are classified into two groups from the primary structures: (1) amphipathic linear peptides such as magainins (Zasloff, 1987), cecropins (Steiner et al., 1988), and sarcotoxins (Okada & Natori, 1985) and (2) cyclic peptides restricted by disulfide bond(s) such as defensins (Eisenhauer et al., 1989) and bactenesin (Romeo et al., 1988). For group 1, it has been reported that a mode of action is attributable to an increase in permeability of bacterial membrane in which peptides would take an amphipathic α-helical structure. On the other hand, there have been few such investigations for group 2 peptides.

Recently, a series of cationic antimicrobial peptides with two intramolecular disulfide bonds that belong to group 2 have been isolated from the hemocyte debris of several horseshoe crab species such as *Tachypleus tridentatus* and *Limulus polyphemus* and named tachyplesins I–III (Nakamura et al., 1988; Miyata et al., 1989) and polyphemsines I and II (Muta et al., 1990), respectively. The tachyplesin family has been shown to inhibit the growth of a broad range of microorganisms, including Gram-positive and -negative bacteria and fungi, at a minimum concentration of 0.8–12 μg/mL (Nakamura et al., 1988). Interestingly, they can bind to lipopolysaccharide (LPS)[1] and significantly inhibit the LPS-mediated activation of factor C, an initiation factor in the *Limulus* clotting cascade. The concentration of tachyplesins in hemocytes is extremely high, approximately 10 mg in hemolymph of an individual horseshoe crab, suggesting that the peptides contribute a defense of arthropoda. The structure of tachyplesin I is shown in Figure 1.

Conformational analysis of tachyplesin I by two-dimensional nuclear magnetic resonance (NMR) spectroscopy indicated that the peptide takes a fairly rigid conformation, i.e., an antiparallel β-sheet conformation connected by a β-turn and two disulfide bonds. The peptide has two clusters: one composed of the hydrophobic bulky side chains and the other the charged basic amino acid residues (Kawano et al., 1990). This amphipathic structure may have a crucial role in the interactions of tachyplesin I with LPS or bacterial membranes. In the present study, we investigated the mode of action of tachyplesin I with an amphipathic β-structure on lipid matrices with the aid of its synthetic derivatives.

Tachyplesin I contains Trp at position 2 being located in a hydrophilic cluster and two Tyr residues at positions 8 and 13 in a hydrophobic cluster. We recently reported that the Trp and one or two Tyr residues are affected when interacting with laurylphosphorylcholine (LPC) micelles (Shieh et al., 1989). However, the details of the interactions, especially the conformational change induced by lipid matrices, are unknown. The use of derivatives of tachyplesin I appears to be effective for clarification of such problems. For these

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[1] Abbreviations: CD, circular dichroism; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EYPC, egg yolk phosphatidylcholine; EYPG, egg yolk phosphatidylglycerol; HOBt, 1-hydroxybenzotriazole; LPC, laurylphosphorylcholine; LPS, lipopolysaccharide; 4-Me-Bzl, 4-methylbenzyl; NMR, nuclear magnetic resonance; OPac, phenacyl ester; Acm, acetamidomethyl; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane. All amino acids are of the L-configuration.

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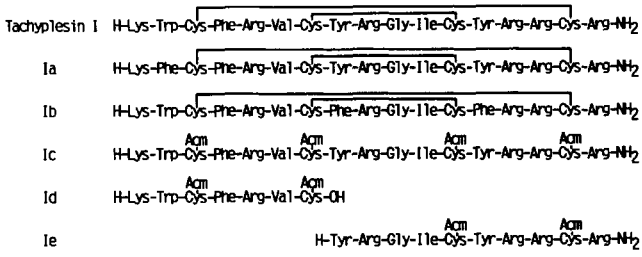

FIGURE 1: Structures of tachyplesin I and its analogs.

reasons, we synthesized Ia with Phe in place of Trp at position 2 and Ib with Phe in place of Tyr at positions 8 and 13 by the solution method. Phenylalanine was selected because it has an aromaticity similar to those of Trp and Tyr. Peptide Ic with no disulfide bonds and Id and Ie which are the N-terminal heptapeptide and C-terminal decapeptide fragments, respectively, of Ic were also synthesized. The structures of the derivatives are shown in Figure 1. The interactions of tachyplesin I and its analogs with phospholipid matrices were investigated by means of circular dichroism, ultraviolet difference spectroscopy, leakage of carboxyfluorescein encapsulated in liposomes, fluorescence spectroscopy, and LPS-binding assay.

Tachyplesin I may permit the measurement of resonance excitation energy transfer between Tyr at positions 8 and 13 and Trp at position 2 and thus the estimation of the average distance between them. Efficiency of energy transfer is dependent on both distance between chromophores and their mutual orientations. Energy transfer in tachyplesin I was studied in the absence and presence of acidic liposomes with Ia and Ib as references. In Ib no intramolecular energy transfer occurs because two Tyr residues are replaced by Phe. The distances were calculated with the aid of the Forster theory (Forster, 1966). As will be shown later, this experiment gave valuable information for the mode of action of tachyplesin I.

MATERIALS AND METHODS

General. Egg yolk phosphatidylcholine (EYPC) and egg yolk phosphatidylglycerol (EYPG) were purchased from Sigma Chemical Co., St. Louis. Boc-amino acids were supplied by Peptide Institute, Osaka. Laurylphosphorylcholine was synthesized as described by Hirt and Berchtold (1958) and purified as reported previously (Yoshida et al., 1988). Carboxyfluorescein from Eastman Kodak Co., Rochester, was further purified by recrystallization from ethanol. Other reagents used were of the highest grade available. Amino acid analyses were conducted on a Hitachi 835 amino acid analyzer after hydrolysis with 5.7 M HCl in sealed tubes at 110 °C for 24 h.

Peptide Synthesis. Tachyplesin I was synthesized by the classical solution method. Four fragments, Boc-Lys(Z)-Trp-(CHO)-Cys(4-Me-Bzl)-Phe-OPac, Boc-Arg(Tos)-Val-Cys-(4-Me-Bzl)-OPac, Boc-Tyr(OBzl)-Arg(Tos)-Gly-Ile-Cys(4-Me-Bzl)-OPac, and Boc-Tyr(OBzl)-Arg(Tos)-Arg(Tos)-Cys(4-Me-Bzl)-Arg(Tos)-NH_2, were synthesized by stepwise elongation from the C-terminus and by the active ester or mixed anhydride method. The Boc group was cleaved with HCl in dioxane or trifluoroacetic acid (TFA), and the phenacyl ester was removed with zinc powder in AcOH (Wang et al., 1977). The final protected peptide was obtained by condensation of Boc-heptapeptide-OH and H-decapeptide-NH_2 with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide and 1-hydroxybenzotriazole (König & Geiger, 1970) and then treated with HF/anisole/1,2-ethanedithiol (8:1.2:0.8) for 3 h to eliminate all the protecting groups. The product showed a UV-absorption spectrum characteristic of tryptophan. The crude product was subjected to gel filtration on a Sephadex G-10 column with 0.05 M HCl, and the fraction containing the peptide was lyophilized. The peptide was then subjected to reduction and oxidation under the conditions described previously (Shieh et al., 1989) and purified by HPLC. The yield was 4%. Amino acid analysis gave the reasonable values. Analogs Ia and Ib were synthesized in the same manner. The cysteine residues were protected with the acetamidomethyl (Acm) group in Ic, Id, and Ie.

Preparation of Liposomes. A mixture of uni- and multilamellar liposomes was prepared as follows. A lipid film obtained after evaporation of a chloroform solution of EYPC or EYPC–EYPG (3:1) (20 mg) was hydrated in 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl (2 mL) by repeated vortex-mixing at room temperature for 30 min, followed by sonication using a Tomy Seiko ultrasonic disrupter Model UR-200P at 50 °C for 30 min. The liposomes obtained were immediately used after dilution to desired concentrations by 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl.

Circular Dichroism Spectra. Circular dichroism (CD) spectra were recorded on a JASCO J-600 spectropolarimeter connected with a JASCO data processor Model J-dry using a quartz cell of 1-mm path length. For measurements of CD spectra of peptides in EYPC or EYPC–EYPG (3:1) liposomes, peptides were dissolved directly in 20 mM Tris-HCl buffer (pH 8.0) containing 0.9 mM EYPC or EYPC–EYPG (3:1) liposomes. To compensate for scattering due to liposomes, the CD spectrum of liposomes alone was subtracted from that of peptide in the presence of liposomes. All measurements were conducted at 23 °C, and the data were expressed in terms of mean residue ellipticity.

Spectroscopic Titrations of Peptides with Laurylphosphorylcholine. Difference spectra were recorded on a Hitachi 100-60 spectrophotometer by titrating native tachyplesin I and its analogs (89–97 μM) with increasing concentrations of LPC (0.17–2.32 mM) at pH 8.0 (20 mM Tris-HCl containing 0.1 M NaCl). The dissociation constants (K_d) for the binding of LPC in a monodispersed state were computed according to

$$\Delta A = \Delta\epsilon[\mathrm{LPC}][\mathrm{P}]/(K_d + [\mathrm{LPC}]) \quad (1)$$

where ΔA is the difference absorbance, $\Delta\epsilon$ the difference molar absorption coefficient, and [P] the concentration of peptide.

Fluorescence Spectra. Fluorescence spectra were recorded on a JASCO FP-550A spectrofluorometer equipped with a thermostated cell holder. Spectra were measured with excitation at 280 nm for tryptophan-containing peptides at 25 °C. Quenching of tryptophan fluorescence of peptides by titrating with I^- was performed in 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl in the absence and presence of 200 μM EYPC–EYPG (3:1) liposomes as described previously (Lee et al., 1989). The quenching data were analyzed by the Stern–Volmer equation:

$$f_0/f = 1 + K_{sv}[\mathrm{Q}] \quad (2)$$

where f_0 and f are the fluorescence intensities at 350 nm in the absence and presence of a quencher at concentration [Q], respectively. K_{sv} is the Stern–Volmer quenching constant.

Leakage of Carboxyfluorescein from Liposomes. Leakage of carboxyfluorescein from liposomes was determined by the procedure of Weinstein et al. (1977) with a minor modification (Suenaga et al., 1989). Liposomes containing 0.1 M 5(6)-carboxyfluorescein prepared above were passed through a

Sepharose 4B column (1 × 20 cm) to remove untrapped carboxyfluorescein. Fractions (2 mL) were collected, and small unilamellar vesicles collected in the fraction 7 (2.8 mM phospholipids) were used for the leakage experiment. Fraction 7 (50 μL) was mixed with Tris-HCl buffer (pH 8.0, 2 mL) to give a final concentration of 70 μM phospholipids. An appropriately diluted solution (50 μL) of peptide in Tris-HCl buffer was then added to the liposome solution at 25 °C. The increase in fluorescence of 5(6)-carboxyfluorescein due to its dilution upon leaking out of liposomes was monitored at 515 nm with excitation at 470 nm. The change in fluorescence intensity was measured for 3 min after addition of peptide. Complete release of carboxyfluorescein was achieved by adding Triton X-100 (0.1% v/v). The percentage of dye release was evaluated by the equation $100(f-f_0)/(f_t-f_0)$, where f is the fluorescence intensity caused by peptide and f_0 and f_t are those with buffer alone and with 0.1% Triton X-100, respectively.

Intramolecular Energy Transfer between Tyrosine and Tryptophan Residues in Tachyplesin I. The solutions of tachyplesin I and Ib were prepared at concentration of 10 μM in Tris-HCl buffer (pH 8.0). The intramolecular energy-transfer efficiency in membrane was determined with increasing concentrations of EYPC–EYPG (3:1) liposomes at 25 °C.

On the basis of kinetic analysis of the relaxation process of the excited singlet state of Trp and Tyr, the efficiency (E) of intramolecular energy transfer (from Tyr to Trp) is related to fluorescence intensities in the presence (f) and absence (f_0) of an energy-transfer process:

$$f/f_0 = [(OD_\lambda^{Y(W)} + OD_\lambda^{Y(Y)}E)/OD_\lambda^{F(W)}] \times (\phi^{Y(W)}/\phi^{F(W)}) \quad (3)$$

where $OD^{F(W)}$ and $OD^{Y(W)}$ are the fractional absorptions of Trp residue in the absence (Ib) and presence (tachyplesin I) of Tyr, and $OD^{Y(Y)}$ is the fractional absorption of Tyr in Ia. $\phi^{Y(W)}$ and $\phi^{F(W)}$ are the fluorescence quantum yields of the acceptor (Trp) in tachyplesins I and Ib, respectively. Equation 3 can be reduced to eq 4 similarly with usual intermolecular energy transfer if $\phi^{F(W)}$ is identical to $\phi^{Y(W)}$ and $OD^{F(W)} = OD^{Y(W)}$, where $K^* = OD^{Y(Y)}/OD^{F(W)}$.

$$(f-f_0)/f_0 = K^*E \quad (4)$$

Estimation of the intramolecular donor–acceptor distance was based on the Forster theory (Forster, 1966):

$$E = R_0^6/(R_0^6 + R^6) \quad (5)$$

where R is the distance between donor (Tyr) and acceptor (Trp) and R_0 is the distance when the energy-transfer rate is equal to the deexcitation rate of an energy donor in the absence of an acceptor. This critical distance R_0^6 is expressed as

$$R_0^6 = (8.8 \times 10^{-25})\kappa^2\phi_D n^{-4} J_{DA} \quad (6)$$

where κ^2 is the orientational factor, ϕ_D the fluorescence quantum yield of a donor, n the index of refraction of the intervening medium, and J_{DA} the overlap integral. J_{DA} is given by

$$J_{DA} = \int_0^\infty F_D(\lambda)\, e_A(\lambda)\, \lambda^4\, d\lambda \quad (7)$$

where $F_D(\lambda)$ is the normalized fluorescence spectrum of a donor and $e_A(\lambda)$ the molar absorption coefficient of an acceptor. Eisinger et al. (1969) have discussed the problems for evaluating R_0^6. Although the orientational factor κ^2 depends on mutual orientation of transition moments of the hydroxyphenyl and indole side chains, an assumption that κ^2 is 2/3, a dynamic random average value, is employed in practice (Eisinger et al., 1969). Using this value, R_0 for Tyr and Trp residues in tachyplesin I was computed to be 13.0 Å.

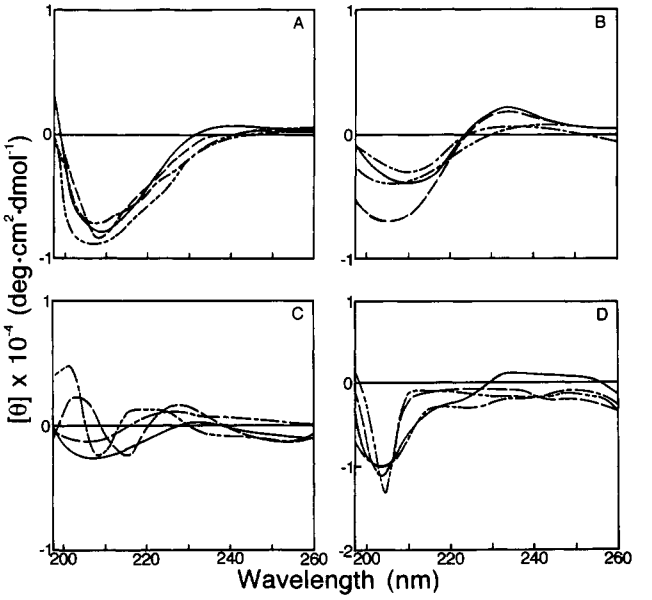

FIGURE 2: CD spectra of tachyplesin I and its analogs in buffer solution (A) and in the presence of LPC micelles (B), EYPC liposomes (C), and EYPC–EYPG (3:1) liposomes (D). Tachyplesin (—), Ia (– –), Ib (– · –), and Ic (– ·· –). The concentrations of peptides were 10–40 μM. The concentrations of LPC micelles and EYPC and EYPC–EYPG (3:1) liposomes were 1.5, 0.9, and 0.9 mM, respectively.

Lipopolysaccharide Binding Assay. Tachyplesin I and its synthetic analogs were measured as to the inhibitory effect on the LPS-mediated activation of factor C (Nakamura et al., 1988). One unit of tachyplesin I and its analogs was defined as the amount that inhibits 50% of the factor C activation mediated by 2 μg of LPS.

RESULTS

Circular Dichroism Study. Circular dichroism spectra were measured in Tris-HCl buffer and in the presence of LPC micelles and of EYPC or EYPC–EYPG (3:1) liposomes (Figure 2). In buffer solution, tachyplesin I showed a broad negative minimum around 210 nm (Figure 2A), indicating that this peptide forms a certain ordered structure. As tachyplesin I in water was reported to have an antiparallel β-sheet structure (Kawano et al., 1990), the spectrum is considered to reflect this structure. It is known that the β-pleated sheet structure shows a characteristic negative CD band near 216–218 nm (Greenfield & Fasman, 1969). It is likely that tachyplesin I has a nearly complete, but not ideal, antiparallel β-structure. In this connection, a similar spectrum was observed for gramicidin S which takes a β-structure and β-turn conformation and has broad negative bands at 208 and 217 nm with an ellipticity value on the mean residue basis of -3×10^4 deg·cm²/dmol (Quadrifoglio & Urry, 1967). Tachyplesin I analogs yielded similar CD curves as tachyplesin I (Figure 2A). It is known that in aromatic amino acid-rich peptides contribution of aromatic groups to CD cannot be neglected (Trudelle, 1975). The small differences in curve shapes and intensities might reflect slight differences in conformation and/or CD contributions of different aromatic groups in tachyplesin I and its analogs.

Tachyplesin I in LPC micelles showed a negative minimum shallower than that in buffer solution and a weak positive band around 235 nm (Figure 2B). A similar spectrum was

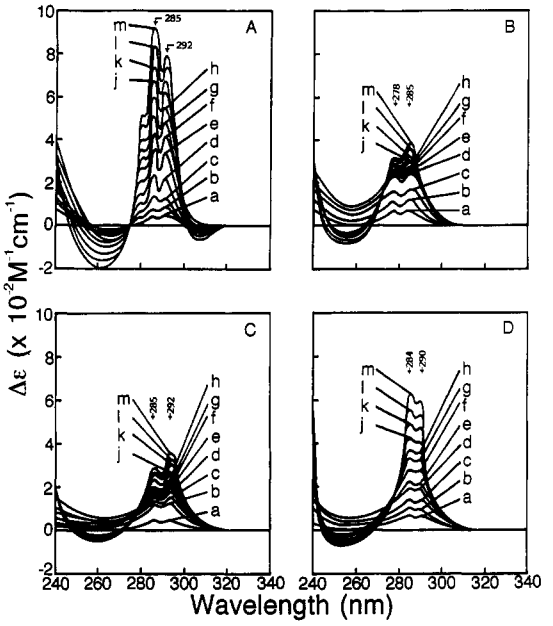

FIGURE 3: Ultraviolet difference spectra of tachyplesins I (A), Ia (B), Ib (C), and Ic (D) when titrated with increasing concentrations of LPC in buffer solution (pH 8.0). Peptide concentrations (in μM): tachyplesins I 89, Ia 97, Ib 89, and Ic 92. LPC concentrations (in mM): a 0.17, b 0.34, c 0.51, d 0.67, e 0.84, f 0.99, g 1.15, h 1.30, j 1.45, k 1.59, l 1.88, and m 2.15 (A), and a 0.19, b 0.37, c 0.55, d 0.73, e 0.90, f 1.07, g 1.24, h 1.40, j 1.56, k 1.72, l 2.03, and m 2.32 (B–D). Magnitudes of difference spectra are expressed by the difference molar absorption coefficient ($\Delta\epsilon$).

observed for Ib though a minimum around 210 nm is deeper than that of tachyplesin I, whereas Ia and Ib, a linear analog, showed similar CD spectra but without a positive band around 230 nm. Why tachyplesins I and Ia show a significant positive band near 230 nm is unknown. Tachyplesin I and its analogs in micelles appear to take structures similar to those in buffer solution.

In neutral liposomes, CD curves of tachyplesin I and its analogs showed no characteristic feature for the peptide backbone structures (Figure 2C), suggesting that the contribution of aromatic side chains in each peptide might be large.

In acidic liposomes, tachyplesins I and Ia gave a broad negative band at 205 nm with a weak band around 215 nm and Ib and Ic a rather sharp band at 205 nm (Figure 2D). These CD patterns may indicate that tachyplesin I and its analogs underwent conformational changes by being transferred into acidic liposomes from antiparallel β-sheet structures to somewhat less ordered structures, perhaps β-structures partially containing random structures.

Spectroscopic Titration of Peptides with Laurylphosphorylcholine. The interactions of tachyplesin I and its analogs with LPC were investigated by means of ultraviolet difference spectroscopy at pH 8.0. Difference spectra for tachyplesin I and its analogs are shown in Figure 3. As previously reported, maxima at 285 and 292 nm for tachyplesin I could be ascribed to both Tyr and Trp residues interacting with LPC. Positive signals in the near-ultraviolet region indicate that both Trp and Tyr residues are in a hydrophobic environment (Shieh et al., 1989). The dissociation constants of tachyplesins I, Ia, Ib, and Ic for the binding to LPC in a monodispersed state were computed to be (4.4–4.6) $\times 10^{-4}$, 1.02×10^{-3}, 1.1×10^{-3}, and 8.1×10^{-4} M, respectively. The results suggest that the Trp or Tyr residues in tachyplesin I are not necessarily critical in terms of the binding to LPC. Interestingly, the binding of a linear analog Ic was comparable to those of the cyclic analogs.

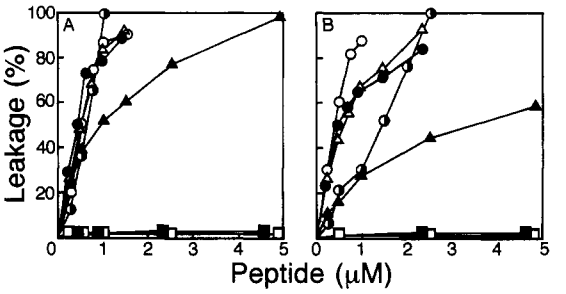

FIGURE 4: Release of encapsulated carboxyfluorescein from EYPC liposomes (A) and EYPC–EYPG (3:1) liposomes (B) as a function of the peptide concentration. Tachyplesins I (○), Ia (●), Ib (△), Ic (▲), Id (□), Ie (■), and 4_3 (◑). The concentrations of EYPC liposomes and EYPC–EYPG (3:1) liposomes were 70 μM.

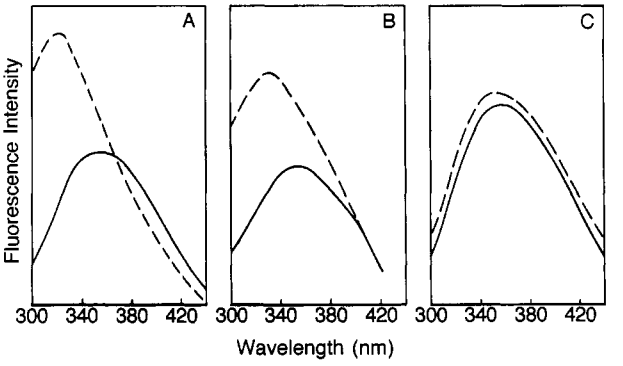

FIGURE 5: Fluorescence spectra of tachyplesins I (A), Ic (B), and Id (C) in the absence (—) and presence (– – –) of EYPC–EYPG (3:1) liposomes. The concentrations of peptides and liposomes were 11 and 220 μM, respectively.

The plots of the difference molar absorption coefficients at 285 nm against LPC concentrations gave biphasic curves (data not shown) which could be ascribed to a critical micellar concentration (0.6–0.7 mM) of LPC (Yoshida et al., 1988). Short peptides, Id and Ie, showed no remarkable affinity to LPC (data not shown).

Leakage of Carboxyfluorescein from Liposomes. In order to test whether bacterial membranes could be the target for action of tachyplesin I and its analogs, the dye leakages from EYPC or EYPC–EYPG (3:1) liposomes were examined. Dose–response curves for EYPC liposomes are shown in Figure 4A. A basic amphipathic α-helical peptide, Ac-(Leu-Ala-Arg-Leu)$_3$-NHCH$_3$ (4_3), was employed as a reference. This peptide has a strong antibacterial activity against Gram-positive bacteria but a weak activity against Gram-negative bacteria and can induce a complete dye release at an extremely low concentration (1 μM) (Lee et al., 1986). Tachyplesins I, Ia, and Ib induced a complete dye release at comparable low peptide concentrations (about 1.5 μM). Peptide Ic also showed a fairly high leakage ability and caused a 100% leakage at a concentration only 3-fold higher than that of tachyplesin I. Peptides Id and Ie showed no sign of leakage even at the high concentration (5 μM) (Figure 5A). The abilities of the peptides to release the dye from neutral liposomes were in the order: 4_3 > tachyplesin I, Ia, and Ib > Ic ≫ Id and Ie.

In the dye release from acidic liposomes, tachyplesin I was most active compared with its analogs and 4_3 (Figure 4B). It appears to be more active for acidic liposomes than for neutral liposomes. The abilities of Ia and Ib were similar to that of 4_3. Peptide Ic showed a lower leakage ability when compared with the cyclic analogs and 4_3. The fragments Id and Ie showed no ability. The abilities of the peptides to release the dye from acidic liposomes were in the order: tachyplesin I > Ia, Ib, and 4_3 ≫ Ic ≫ Id and Ie.

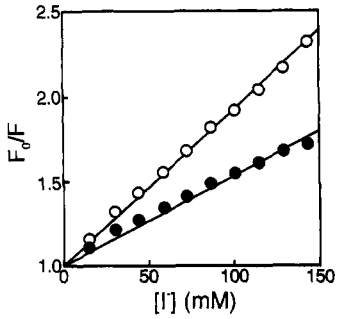

FIGURE 6: Stern–Volmer plots of the quenching of tryptophan fluorescence in tachyplesin I by KI in the absence (○) and presence (●) of EYPC–EYPG liposomes. The concentrations of the peptide and liposomes were 6 and 200 μM, respectively.

Fluorescence Study of Tachyplesin I and Its Analogs. In an attempt to disclose the location of peptide in lipid bilayers, the interactions of tachyplesin I and its analogs containing Trp with acidic liposomes were investigated by fluorescence spectroscopy. Fluorescence spectra of tachyplesins I, Ic, and Id in buffer solution showed emission maxima at 356, 354, and 358 nm, respectively (Figure 5). Upon the addition of acidic liposomes, emission maxima of tachyplesin I and Ic shifted to shorter wavelengths by 34 and 22 nm, respectively (Figure 5, Panels A and B, respectively). Such blue shifts indicate two possibilities: that the Trp residue in these peptides is deeply embedded in lipid bilayers or translocated into a highly apolar amino acid cluster. The latter is, however, unlikely because the Trp residue is located in a hydrophilic cluster when tachyplesin I takes a β-structure (Kawano et al., 1990). No blue shift was observed for Id (Figure 5C), suggesting that this short peptide cannot interact with liposomes.

The quenching effect of I^- toward tryptophan fluorescence was studied in the absence and presence of acidic liposomes. The slopes of the Stern–Volmer plots of tachyplesin I in acidic liposomes (K_{sv} = 5.4 M^{-1}) were about half that for buffer solution (K_{sv} = 9.3 M^{-1}) (Figure 6). Similar plots were also obtained for Ib and Ic. Since I^- anion can quench fluorescence due to surface-localized Trp residue, the plots indicate that in liposomes the Trp residue in tachyplesin I and its analogs may be embedded in a hydrophobic moiety of lipid bilayers.

Intramolecular Energy Transfer between Tyrosine and Tryptophan Residues in Tachyplesin I. Energy transfer from the Tyr residue(s) to the Trp residue in tachyplesin I was measured by absorption and fluorescence emission spectroscopy using tachyplesins I and Ib, the latter of which contains Phe instead of the Tyr residues. Diluted solutions (10 μM) were employed because in this concentration range intermolecular interactions of peptides can be negligible (Chiu & Berson, 1977).

When excited at 285 nm, the resulting fluorescence spectra of tachyplesins I and Ib showed maxima around 365 nm. However, the fluorescence intensity of tachyplesin I was greater than that of Ib. This suggests that the excitation energy absorbed by the Tyr residue(s) is transferred to the Trp residue in tachyplesin I. When excited at 305 nm, tachyplesins I and Ib gave the almost identical fluorescence quantum yield which was estimated to be 0.05 by using *N*-acetyl-L-tryptophan amide as a fluorescence standard. Absorption spectra of tachyplesins I, Ia, and Ib at the same concentration were measured accurately in order to obtain the values of $OD^{Y(W)}$, $OD^{F(W)}$, and $OD^{Y(Y)}$. At 285 nm, $OD_{285}^{Y(W)}$ was equal to $OD_{285}^{F(W)}$ and the ratio of $OD_{285}^{Y(Y)}$ to $OD_{285}^{F(W)}$ was 0.37. These results show that the energy-transfer efficiency (E) of tachyplesin I is given

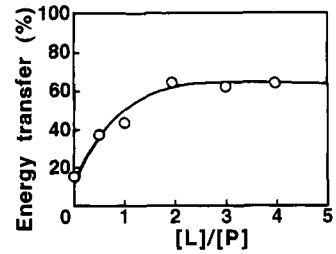

FIGURE 7: Energy-transfer efficiency of tachyplesin I in acidic liposomes. The experimental details are described under Materials and Methods.

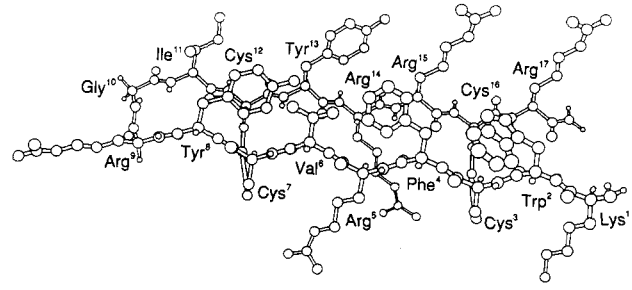

FIGURE 8: Structure of tachyplesin I in aqueous solution presumed on the basis of the NMR experiments.

by eq 4, and then K^* was estimated to be 0.37. By measuring the fluorescence intensity excited at 285 nm, the energy-transfer efficiency (E) of tachyplesin I in buffer solution was calculated to be 0.15.

The E value for tachyplesin I in acidic liposomes was next determined. The values for $(f - f_0)/f_0$ were recorded by titrating with increasing concentrations of acidic liposomes. The E value increased with increase of concentrations of acidic liposomes (Figure 7). The curve was saturated at [L]/[P] = 4. This pattern shows that the energy transfer in acidic liposomes is greater than that in aqueous solution. At [L]/[P] = 4, the E value was estimated to be about 0.62.

When the donor–acceptor distance is calculated by using the experimental values obtained and eq 5, special care should be paid to the effects of the Tyr–Tyr interaction and the changes in J and κ^2. However, the 1/6th power dependence of R_0 on the three parameters attenuates the effects of their variations. The distances between Trp and Tyr residues in free and membrane-bound tachyplesin I were estimated to be approximately 18 and 12 Å, respectively.

Lipopolysaccharide Binding Assay. The Gram-positive cells have an LPS-containing outer envelope surrounding peptidoglycan layers and cytoplasmic membranes. Affinities of tachyplesin I and its analogs to LPS were tested as described under Materials and Methods. The ID_{50} values for neutralizing 2.5 pg of LPS were 1.05 and 0.92 μg for native and synthetic tachyplesins I, respectively. Those for peptides Ia, Ib, and Ic were 0.90, 1.65, and 1.50 μg, respectively. Substitutions of Tyr or Trp by Phe appeared to give no significant effect for LPS-binding activity. It is worth noting that linear peptide Ic retains a high binding affinity to LPS. However, peptides Id and Ie showed no neutralizing activity. The entire sequence of tachyplesin I appears to be required for eliciting affinity to LPS.

DISCUSSION

The previous NMR study (Kawano et al., 1990) indicated that tachyplesin I took an antiparallel β-sheet structure with a β-turn (Figure 8)in aqueous solution, i.e., an amphipathic structure constructed by a hydrophobic cluster (residues 6–13) and a hydrophilic cluster (residues 14–17). The disulfide bonds

appear to constrain tachyplesin I to a rigid conformation. The present CD study suggested that tachyplesin I in buffer solution takes such a β-structure. On the other hand, different conformations were observed in the presence of neutral micelles and of neutral or acidic liposomes, indicating that the conformation of tachyplesin I can be affected by the organization and ionizing state of lipids. Particularly in the milieu of acidic liposomes it appeared to take a somewhat less ordered structure, being not greatly different from β-structure. This implies that a stable antiparallel β-structure reinforced by the disulfide bonds can be changed to somewhat less ordered but particular conformations in micelles and in lipid bilayers. It is noted that linear derivatives Ic can take a conformation similar to those of the cyclic peptides in all media tested except for neutral liposomes. The linear sequence of tachyplesin I is likely to encode information of its secondary structure regardless of the presence or absence of the disulfide bonds.

Carboxyfluorescein leakages from neutral or acidic liposomes induced by tachyplesin I and its analogs reflect their abilities to perturb membrane bilayers. Replacement of Trp or Tyr by Phe yielded no great effect on the ability. The fact that peptide Ic can perturb lipid bilayers although less effectively when compared with the cyclic peptides, while peptides Id and Ie, the components of Ic, cannot (Figure 4), may support that peptide Ic has a structure similar to those of tachyplesin I and its cyclic analogs. However, peptide Ic did not show antimicrobial activity against Gram-positive and -negative bacteria (data not shown). The lower binding ability of Ic to lipid bilayers appears to correlate with the absence of antimicrobial activity. In this sense, the disulfide bonds are crucial for eliciting activity toward bacterial membranes.

The fluorescence spectra (Figure 5) and the fluorescence quenching experiments (Figure 6) in the presence of neutral or acidic liposomes indicated that the Trp residue in tachyplesin I and its analogs was embedded in lipid bilayers. Therefore, tachyplesin I is tightly bound to lipids to perturb bilayers regardless of the charge of the lipid head groups. Thus, it is suggested that the interaction of a hydrophilic part containing Trp^2 with the hydrophobic core in lipid bilayers makes the peptide penetrate into lipid bilayers. Since in the dye release experiment a burst was observed immediately after addition of tachyplesin I (data not shown), the dye leakage might be ascribed to penetration of the peptide into lipid bilayers.

As has been shown in Figure 4, a basic α-helical peptide 4_3, which is highly amphipathic and hydrophobic, can release the dye by interacting with both neutral and acidic liposomes. This is due to penetration of its hydrophobic part into the hydrophobic core of liposomes (Lee et al., 1986). On the other hand, a basic β-structural peptide, Ac-(Ser-Val-Lys-Val)$_2$-$NHCH_3$, composed of alternating hydrophilic and hydrophobic residues, interacted only with acidic liposomes and released the dye only weakly (Ono et al., 1990). The fact that tachyplesin I with a β-sheet structure can interact strongly with neutral liposomes might be explained by the presence of the highly hydrophobic "head" group composed of the four hydrophobic amino acid residues (Val^6, Tyr^8, Ile^{11}, and Tyr^{13}) positioned on the same side of an antiparallel β-sheet structure (Figure 8). Such a hydrophobic cluster is endowed with an increased affinity to neutral lipid bilayers as in 4_3.

The NMR study showed that Trp^2 is nearly in a hydrophilic "tail" because Trp^2, Phe^4, Arg^{15}, and Arg^{17} are all located on the same side of an antiparallel β-structure (Kawano et al., 1990). However, in the interaction with LPC micelles, Trp^2 interacted with the hydrophobic core of micelles together with the Tyr residue(s) (Figure 3), indicating that Trp^2 is on the same side as the Tyr residue(s). All the other evidence indicated that Trp^2 is embedded in an apolar region when interacting with lipid bilayers. Taking account of such observations, it might be required for the two Arg residues to be exlcluded from the hydrophobic interaction with the lipid core although these residues are favorable for an initial interaction with the anionic head groups of lipids. Such a requisite may be one of the driving forces to bring about the conformational change from a β-structure to a somewhat less ordered structure.

Interaction of tachyplesin I with lipid bilayers was accompanied by its conformational change as has been discussed above. This was also supported by the fluorescence energy transfer experiment that allows us to estimate the donor (Tyr)–acceptor (Trp) distance. By transferring tachyplesin I from aqueous buffer to acidic liposomes, the distance between Trp^2 and Tyr^8 or Tyr^{13} or both was changed from 18 to 12 Å. It could be inferred that the conformational change involves two elements. First, the backbone conformation changes from a β-structure to a somewhat less ordered structure possibly as reflected in the CD spectra. Second, orientations of the side chains of the Trp and Tyr residues are changed by interacting with lipid bilayers, and thus these side chains are brought closer to each other.

Many cytolytic peptides act on biomembranes which are amphipathic in nature (Kaiser & Kezdy, 1987). In the amphipathic environments, the peptide is forced to take particular secondary structures such as α-helix, 3_{10}-helix, and β-structure. In the nonaqueous interior of lipid bilayers, a sterically allowed secondary structure of the peptide is more stable than that in aqueous media, so the lipid environments are extremely unfavorable for unfolding a polypeptide (Engelman et al., 1986). However, this is not the case for tachyplesin I. The present study showed that lipid bilayers induced the conformational change of tachyplesin I from a β-structure to a somewhat less ordered structure. This means that strong hydrophobic interactions between tachyplesin I and lipid bilayers convert an energetically stable secondary structure to a somewhat less stable one.

As for a model of action of cytolytic peptides, Kini and Evans (1989) proposed that they have a common region in their structures to interact with cell membranes; the cationic region is involved in the binding to cell surface, whereas the hydrophobic region determines a lytic activity. All the experimental results obtained for tachyplesin I suggest that the hydrophilic side of an amphipathic β-structure interacts with an acidic moiety of phospholipids in membranes and then the hydrophobic side penetrates deeply into a hydrocarbon moiety of membranes which is accompanied by the conformational change of the peptide. It seems likely that an antiparallel β-sheet conformation and an appropriate hydrophobicity of tachyplesin I cause an expansion of the membrane structure, resulting in an increase in the permeability of the membrane and death of the bacteria.

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