Conformations and Orientations of Aromatic Amino Acid Residues of Tachyplesin I in Phospholipid Membranes†

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ABSTRACT: Tachyplesin I, an antibacterial and antiviral heptadecapeptide from the hemocyte debris of *Tachypleus tridentatus*, contains four aromatic amino acids (Trp^2, Phe^4, Tyr^8, and Tyr^{13}) which have been shown to be crucial for activity. In order to investigate conformations and orientations of the aromatic amino acid residues of tachyplesin I in lipid bilayers, its analogs, [Phe^8]- and/or [Phe^{13}]-tachyplesin(s) I in which Tyr^8 and Tyr^{13} are replaced by Phe, were synthesized. Circular dichroism spectral studies showed that three peptides are considerably different in conformation in aqueous solution at pH 8.0 whereas they take similar conformations in the presence of neutral egg yolk phosphatidylcholine (EYPC) liposomes. Energy transfer kinetics showed that the distances of Trp^2-Tyr^8 and Trp^2-Tyr^{13} are 16 Å (max of 18.3 Å, min of 15.1 Å) and 18 Å (max of 20.2 Å, min of 16.6 Å) in buffer but are 12 Å (max of 15.2 Å, min of 8.6 Å) and 18 Å (max of 22.9 Å, min of 12.9 Å), respectively, in the presence of acidic EYPC/EYPG (3:1) liposomes. Decay kinetics for Trp^2 fluorescence indicated that Trp^2 takes at least three conformations in buffer and in acidic liposomes where fractions of three Trp^2 conformers vary by changing the medium from buffer to acidic liposomes. Although tachyplesin I is not in amphiphilic structure in buffer in spite of its rigid antiparallel β-conformation, its interaction with lipid bilayers appears to induce amphiphilic structure *via* minor alteration of peptide backbone and side chain orientations, resulting in shortening the distance of Trp^2-Tyr^8.

A series of cationic antimicrobial peptides with two intramolecular disulfide bonds such as tachyplesin I−III (Nakamura et al., 1988; Miyata et al., 1989) and polyphemsins I and II (Muta et al., 1990) have been isolated from the hemocyte debris of horseshoe crab species, *Tachypleus tridentatus* and *Limulus polyphemus*, respectively. The tachyplesin family exhibits a variety of biological activities such as suppression of growth of both Gram-positive and -negative bacteria and fungi (Nakamura et al., 1988), the inhibition of lipopolysaccharide-mediated activation of factor C which is an initiation factor in the Limulus clotting cascade, and binding to DNA (Yonezawa et al., 1992). Tachyplesins are also known to have antiviral activity against human immunodeficiency virus (HIV)-1 (Morimoto et al., 1991).

Conformational analysis of tachyplesin I and its analogs by nuclear magnetic resonance (NMR)[1] spectroscopy indicated that the peptides take fairly rigid conformations, i.e., antiparallel β−sheet conformation connected by a β−turn and two disulfide bonds. Tachyplesin I has two clusters, one composed of hydrophobic bulky side chains and the other composed of charged basic amino acid residues (Kawano et al., 1990; Tamamura et al., 1993). This amphiphilic structure may play a crucial role in interaction of tachyplesin I with bacterial membranes.

In addition, the recent structure−activity relationship studies based on the synthesis of tachyplesin analogs have shown that aromatic amino acids of tachyplesin I are very important for antiviral activity (Akaji et al., 1989; Shieh et al., 1989; Nakashima et al., 1992). Particularly, Trp^2 is essential for inhibiting viral replication. It was noted that an aromatic amino acid-rich compound, T^{22} ([$Tyr^{5,12}$, Lys^7]-polyphemsin II), has strong anti-HIV activity and relatively low cytotoxic activity in vitro (Nakashima et al., 1992). Thus, the conformations of tachyplesins, particularly the orientations of aromatic amino acids, in lipid bilayers are very attractive for investigation in relation to their action mode. In this connection, using tachyplesin I and its two synthetic analogs, [Phe^2]- and [$Phe^{8,13}$]tachyplesins I, the following results have previously been seen. (1) When tachyplesin I interacts with acidic liposomes, a hydrophobic moiety is embedded in lipid bilayers. (2) The Trp−Tyr distance changes greatly in the absence and presence of lipid bilayers. (3) The energy donor (Tyr^8 or Tyr^{13} or both)−acceptor (Trp^2) distance in tachyplesin I is 18 Å in buffer and 12 Å in acidic EYPC/EYPG (3:1) liposomes (Park et al., 1992). However, we could not specify clearly which Tyr in two tyrosines is the energy donor. In the present study, therefore, tachyplesin I analogs, [Phe^8]- and [Phe^{13}]-tachyplesins I (Figure 1), were newly designed and synthesized in order to gain a further

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[1] Abbreviations: CD, circular dichroism; DMPC, dimyristoylphosphatidylcholine; DMPS, dimyristoylphosphatidylserine; EYPC, egg yolk phosphatidylcholine; EYPG, egg yolk phosphatidylglycerol; NMR, nuclear magnetic resonance; SVR, serial variance ratio; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane. All amino acids are of the L-configuration.

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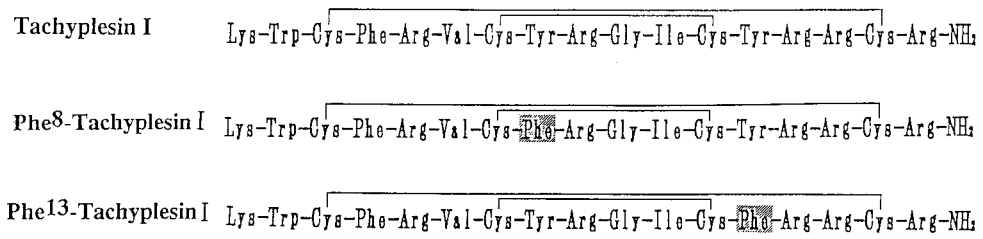

FIGURE 1: Structures of tachyplesin I and its synthesized analogs.

insight into the orientation of aromatic residues of tachyplesin I in the absence and presence of lipid bilayers. [Phe[8]]- and [Phe[13]]-tachyplesins I are in cyclic form in which either of two tyrosines is substituted by Phe. The interactions of tachyplesin I and its analogs with phospholipid matrices were investigated by means of circular dichroism and time-resolved fluorescence measurements.

MATERIALS AND METHODS

General. DMPC, DMPS, EYPC, and EYPG were purchased from the Sigma Chemical Co. (St. Louis). Boc amino acids were supplied by the Peptide Institute (Osaka, Japan). Carboxyfluorescein from the Eastman Kodak Co. (Rochester, NY) was further purified by recrystallization from methanol. Other reagents used were of the highest grade available.

The peptides were similarly synthesized by the solution method similarly as described previously (Park et al., 1992). The fully protected peptides were finally treated with the HF method. The crude peptides obtained were treated by mercaptoethanol to reduce partially oxidized peptides and then oxidized using dimethyl sulfoxide in 30% acetic acid (Tam et al., 1991). The crude products were subjected to gel filtration on a Sephadex G-15 column with 30% acetic acid. The fractions corresponding to the oxidized monomer were collected for lyophilization. The peptides obtained were further purified by HPLC with a C18 preparative column with a gradient of acetonitrile in 0.1% trifluoroacetic acid. The purity were confirmed by analytical HPLC on a Hitachi 3056 ODS column. The purified peptides and their mother solutions were stocked at −30 °C. Amino acid analyses were conducted on a Hitachi 835 amino acid analyzer. Peptides were hydrolyzed with 5.7 M HCl in evacuated, sealed tubes at 110 °C for 24 h. Analytical data of peptides were as follows: [Phe[8]]-tachyplesin I, Gly, 1.30 (1); Cys, 1.51 (2); Val, 1.00 (1); Ile, 0.94 (1); Phe, 1.81 (2); Tyr, 0.91 (1); Lys, 0.88 (1); Arg, 5.24 (5); and [Phe[13]]tachyplesin I. Gly, 1.34 (1); Cys, 1.64 (2); Val, 1.00 (1); Ile, 1.01 (1); Phe, 1.96 (2); Tyr, 1.00 (1); Lys 0.84 (1); Arg, 5.33 (5). The phospholipid concentration was determined by an assay using the phospholipids-test Wako reagent purchased from the Wako Pure Chemical Ind. (Osaka) and was expressed in terms of phosphorus concentration. Peptide concentrations were determined by amino acid analysis and from molar absorption coefficients.

Preparation of Liposomes. A mixture of uni- and multilamellar liposomes was prepared as follows. A lipid film obtained by evaporation of a chloroform solution of EYPC or EYPC/EYPG (3:1) (20 mg) was hydrated in 20 mM Tris-HCl (2 mL, pH 8.0) containing 0.1 M NaCl by repeating Vortex mixing at 25 °C for 30 min, followed by sonication using a Tomy Seiko model UR-200P ultrasonic disrupter for 30 min at 25 °C. The liposomes obtained were immediately used after dilution to desired concentrations with 20 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl.

Circular Dichroism Spectra of Tachyplesin I and Its Analogs. CD spectra of tachyplesin I and its analogs were recorded on a JASCO J-720 spectropolarimeter using a quartz cell with a 0.5 mm path length in 20 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl and in the presence of phospholipids at 25 °C. Peptide stock solution (0.1 mL, 2 mg/mL) dissolved in 20 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl was added to 0.4 mL of Tris-HCl with or without neutral or acidic liposomes. CD spectra of tachyplesin I were also measured as a function of its concentration in 5 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl using quarts cells with 0.5, 1.0, and 10 mm path lengths at 25 °C. The data were expressed in terms of mean residue ellipticity. The spectral data were analyzed by the J720/98 system program (version 1.20C) on an NEC PC-9801 personal computer.

Steady States of Fluorescence Measurements. Fluorescence spectra of tachyplesin I and its analogs were recorded on a Hitachi 850 spectrofluorometer equipped with a thermostated cell holder. Fluorescence spectra were measured in 20 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl and in the presence of DMPC, DMPS, and EYPC/EYPG (3:1) liposomes at 25 °C with excitation at 285 or 295 nm for tryptophan-containing peptides. The spectral data were transported to an NEC PC-9801 personal computer by RS-232C port and were analyzed. The steady state fluorescence anisotropy of tachyplesins was measured using Glane-Taylor polarizers. The excitation wavelength was set at 285 nm, and the emission was monitored at 320 or 350 nm for tyrosine or tryptophan. Band-passes were 5 nm.

Time-Resolved Fluorescence. Time-resolved fluorescence measurements were conducted using the technique of time-correlated single-photon counting with laser/microchannel plate-based instrumentation. The instrumentation and the procedures for time-resolved measurements have been given elsewhere (Szabo et al., 1989). A Spectra Physics sync-pumped argon ion dye laser and cavity damper system served as the excitation source operating at 825 kHz and with a pulse of 15 ps. The emission was detected after passing through a polarizer set at 54.5° for vertical excitation and a monochrometor, with a 4 nm band path, on a Hamamatsu 1564U microchannel plate photomultiplier. The channel width of a multichannel analyzer was 21.0 ps/channel, and the fluorescence emission was collected through 2048 channels. Each decay curve typically contained $1-2 \times 10^6$ total counts. The instrument response function was determined from Raman scattering of excitation by water (Willis et al., 1990) and had a full width half-maximum (FWHM) of 60 ps. The function describing fluorescence decay following excitation is given by a sum of exponentials, $I(t) = \sum[\alpha_i \exp(-t/\tau_i)]$, where τ_i is the decay time of the ith component and α_i is the corresponding pre-exponential

factor. The data were analyzed using the nonlinear least-squares iterative convolution method based on the Marquardt algorithm (Marquardt, 1963). The adequacy of the fluorescence decay fitting was judged by inspection of the plots of weighted residuals and the serial variance ratio (SVR) and the reduced χ^2.

Evaluation of the Energy Transfer Rate, Efficiency, and Distance of the Energy Donor/Acceptor Pair. The energy transfer rate (k_T) was obtained with the curve fitting procedure based on the Marquardt−Levenberg algorithm (Marquardt, (1963) using an energy transfer kinetic equation for an energy acceptor fluorescence decay

$$F(t) = [k_T D_0/(1/\tau_A - 1/\tau_{DA})] \times [\exp(-t/\tau_{DA}) - \exp(-t/\tau_A)] + A_0 \exp(-t/\tau_A) \quad (1)$$

where k_T is the energy transfer rate, D_0 and A_0 are the initial concentrations of the donor and acceptor, respectively, and τ_A is the fluorescence lifetime of the acceptor. τ_{DA} is the fluorescence lifetime of the donor in the presence of the energy acceptor and was given by the equation $1/\tau_{DA} = k_T + 1/\tau_D$, using the donor's lifetime in the absence of the acceptor (τ_D). The energy transfer efficiency was evaluated by eq 2

$$E = k_T/({\tau_D}^{-1} + k_T) \quad (2)$$

where τ_D is the lifetime of the energy donor in the absence of the acceptor. The distance (R) between the donor and acceptor was related to the energy transfer efficiency (E) with equation $1/E - 1 = (R/R_0)^6$, where R_0 is the critical distance. Theoretically, k^2 ranges from 0 to 4. Therefore, the upper ($\langle k^2\rangle^{max}$) and lower ($\langle k^2\rangle^{min}$) limits were calculated for the orientation factor based on Dale et al. (1979).

$$\langle k^2\rangle^{max} = {}^2/_3[1 + \langle {d_D}^x\rangle + \langle {d_A}^x\rangle + 3\langle {d_A}^x\rangle\langle {d_D}^x\rangle] \quad (3)$$

$$\langle k^2\rangle^{min} = {}^2/_3[1 + (\langle {d_D}^x\rangle + \langle {d_A}^x\rangle)/2] \quad (4)$$

The polarization factor, $\langle d^x\rangle$, is related to anisotropy (r) by $r/r_0 = \langle d^x\rangle^2$, where r_0 is the anisotropy at $t = 0$ in the anisotropy decay.

RESULTS

Circular Dichroism Spectra of Tachyplesin I and Its Analogs. Conformations of tachyplesin I and its analogs in 20 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl and in the presence of neutral or acidic liposomes prepared from egg yolk phospholipids were monitored by circular dichroism spectra. In buffer at pH 8, tachyplesin I showed maxima at 232 and below 200 nm and a negative minimum at 208 nm with a shoulder at 212 nm (Figure 2a). The spectra did not change in the peptide concentration range from 4.5 to 90 μM (data not shown), indicating that the structural β-conformation of tachyplesin I is independent of peptide concentration. The curves for tachyplesin I and its analogs were generally similar in shape, but the maximum intensities and their wavelengths were slightly different from one another. Replacement of Tyr by Phe in tachyplesin I reduced the ellipticity at 235 nm. Interestingly, replacement of Tyr[8] by Phe led to an increase in intensities of double minima without a shift of the wavelength, and that of Tyr[13] by Phe led to the increase in intensity and the shift to shorter wavelengths

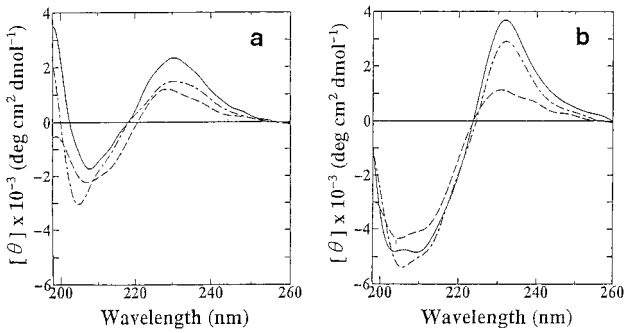

FIGURE 2: CD spectra of tachyplesin I and its analogs at pH 8.0 (20 mM Tris-HCl containing 0.1 M NaCl) in the absence (a) and presence (b) of 1 mM neutral EYPC liposomes: tachyplesin I (—), [Phe[8]]tachyplesin I (− −), and [Phe[13]]tachyplesin I (−·−). The concentration of tachyplesins was 90 μM.

about 3 nm of a minimum peak at 208 nm but without considerable change of ellipticity at 213 nm. These suggest that replacement of Tyr[8] or Tyr[13] by Phe exerts a somewhat different effect on different conformations of peptides.

In neutral EYPC liposomes, tachyplesin I and [Phe[8]]- and [Phe[13]]-tachyplesins I revealed fundamentally similar spectra having double minimum peaks of almost the same intensities at 205 and 213 nm, although a little difference was observed at a positive peak around 235 nm, indicating that they take a similar backbone conformation (Figure 2b). On the other hand, the considerable differences in shape and in intensity in buffer and in liposomes were observed for tachyplesin I and [Phe[13]]tachyplesin I, indicating that both peptides take different conformations in buffer and in neutral liposomes. It should be noted that double-minimum peaks of these three peptides at 205 and 213 nm in the presence of EYPC are very similar to that of [Phe[8]]tachyplesin I in buffer, although the intensities in lipid bilayers are 2.5 times deeper than that in buffer.

In acidic EYPC/EYPG (3:1) liposomes, the observed wavelengths and intensities of double-minimum and positive peaks around 200−240 nm for three peptides were almost the same as those in EYPC liposomes, indicating that the peptides have almost the same backbone conformations and interact in a similar manner in both media (data not shown). However, their intensities in acidic liposomes were considerably reduced because of the increasing turbidity of the solution due probably to peptide-induced aggregation or fusion of liposomes.

Fluorescence Study of Tachyplesin I and Its Analogs. The fluorescence spectra of tachyplesin and its analogs excited at 295 nm are shown in Figure 3. The tryptophan residue of tachyplesins gave a maximum at 350 nm in buffer solution and shifted to about 340 nm by binding to EYPC/EYPG (3:1) liposomes. The fluorescence intensity of maximum peak decreased to about 80% at a concentration ratio [L]/[P] of 200. The fluorescence spectra of tachyplesin I excited at 285 nm were almost the same as those at 295 nm except for fluorescence intensities (data not shown). These results indicate that Trp residues in tachyplesins are translocated from hydrophilic to hydrophobic surroundings by increasing lipid concentrations. The steady state fluorescence anisotropy values of tachyplesins in buffer and membranes are summarized in Table 1. Since tachyplesins are small, the fluorescence anisotropy was small in buffer solution. In membranes, large increases in anisotropy values were

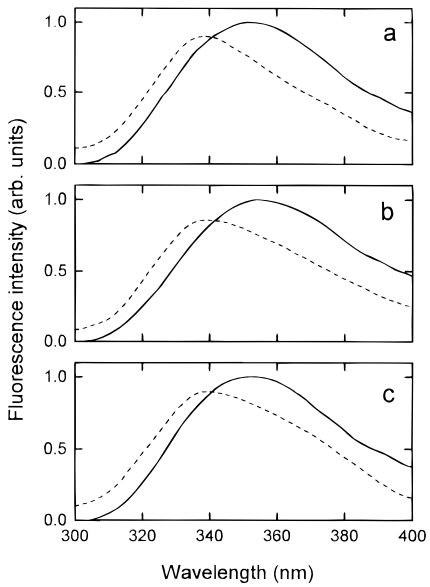

FIGURE 3: Fluorescence spectra of tachyplesin I (a), [Phe[8]]-tachyplesin I (b), and [Phe[13]]tachyplesin I (c) in 20 mM Tris-HCl buffer (pH 8.0) in the presence of EYPC/EYPG (3:1) liposomes. The excitation wavelength was 295 nm, andthe peptide concentration was 6 μM and the lipid concentration 1.2 mM: In buffer (—) and in liposomes (– –).

observed but the differences by replacement of Tyr with Phe were not recognized.

Next, time-resolved fluorescence properties of tachyplesin I and [Phe[8]]- and [Phe[13]]tachyplesins I were studied. The qualities of the fluorescence decay analysis for tachyplesin I in buffer are shown in Figure 4. No remarkable improvement was attained for the χ^2 value, the serial variance ratio (SVR), and residual plots between triple- and quadruple-exponential analyses. Therefore, it was concluded that the fluorescence decay of tachyplesin I was described with triple-exponential functions. In all the studies conducted in buffer and in the presence of EYPC/EYPG, DMPC, and DMPS liposomes, three decay components were required for description of the decay of tryptophan fluorescence of tachyplesins. Since three decay times characterizing individual decay components were independent on the emission wavelength through the whole spectral region of tryptophan fluorescence (300−400 nm), the global analysis was performed by assuming that the fluorescence decay function of tachyplesins would be constructed as eq 5

$$I(\lambda, t) = \sum_{i=1}^{3} \alpha_i(\lambda) \exp(-t/\tau_i) \tag{5}$$

where τ_i is the emission wavelength-independent decay time of the ith component and $\alpha_i(\lambda)$ is the corresponding pre-exponential factor at emission wavelength λ. The global analysis gave an excellent fit to the multiple-emission wavelength data in all cases. The results are presented in Table 2. The emission spectra associated with each individual decay time component (DAS, decay-associated spectra; Knutson et al., 1982) are given by

$$I_i(\lambda) = I_{ss}(\lambda)[\alpha_i(\lambda)\tau_i/\sum[\alpha_i(\lambda)\tau_i]] \tag{6}$$

where $I_i(\lambda)$ is the DAS of the ith component, $I_{ss}(\lambda)$ is the steady state spectrum, and its multiplier is the fractional fluorescence, f_i $[=\alpha_i(\lambda)\tau_i/\sum[\alpha_i(\lambda)\tau_i]]$, of the ith component at wavelength λ. The fractional DAS of tachyplesin I obtained in buffer, in DMPC liposomes, and in DMPS liposomes were shown in Figure 5. In buffer, DAS of the longest decay time component gave a maximum at 350 nm. Upon the binding with DMPC liposomes and with DMPS liposomes, maxima of fluorescence spectra shifted to 340 and 335 nm, respectively (Figure 5b,c)). The maximum of the shortest decay time component of DAS exhibited a large blue shift from 340 to 320 nm in the presence of DMPS liposomes (Figure 5c). The fluorescence decay properties of tachyplesins I given by eq 5 suggest a probability of the presence of ground state conformational heterogeneity, and the individual decay components characterized by three decay times may correspond to conformers of Trp[2] (Dahms et al., 1995). It is well-confirmed from crystallographic studies on protein (Ponder & Richards, 1987) and oligopeptide (Benedetti et al., 1983) that the side chains of the amino acid residues exhibit a few preferential configurations corresponding to the fully staggered rotamers.

Recent NMR studies have verified the existence of rotamers for conformational states of the tryptophan residue and showed that the population of rotamers is closely related to the pre-exponential factor of fluorescence decay kinetics (Ross et al., 1992). In fact, Willis et al. (1992) showed that the time-resolved fluorescence properties of parathyroid hormone could well be explained by three $C_\alpha-C_\beta$ rotamers of the tryptophan indole side chain. Trp[2] of tachyplesin I and its analogs is adjacent to Lys[1] which is one of the strongest quenchers of tryptophan fluorescence. Therefore, it is possible that conformers have different decay times according to configurations of the indole ring against the ϵ-amino group of Lys[1]. The indole ring of a conformer, which corresponds to the shortest decay time component, is in closest proximity to the ϵ-amino group and *vice versa*. Although the relative population of conformers can be estimated from DAS through the following relations,

$$A_i = \int I_i(\lambda)\, d\lambda \tag{7}$$

$$A_i \propto \tau_i C_i \tag{8}$$

$$C_i = (A_i/\tau_i)/[\sum(A_i/\tau_i)] \tag{9}$$

where A_i is the area under the DAS of the ith component and C_i is the relative concentration of the ith component. The relative populations of ground state conformers C1, C2, and C3 in buffer and in liposomes, which are attributable to longest, intermediate, and shortest decay time components, respectively, are given in Table 3. When the data in acidic DMPS liposomes were compared with those in buffer, decreases in of C1 and C2 components and a large increase in the C3 component were seen. Although the same trend was seen for neutral liposomes, the increase in the C3 component was not so large. These suggested that tachyplesin I in neutral liposomes tends to take a conformation in which the Trp[2] side chain is in proximity to the ϵ-amino group of adjacent Lys[1], and the population of such a conformer is increased by increasing acidity of lipids in membranes.

It is well-known that, when there is no interaction between the tryptophan residue and other amino acid residues, the fluorescence quantum yield and decay characteristics of tryptophan show no dependence on the excitation wave-

Table 1: Steady State Fluorescence Anisotropy of Tachyplesins[a]

	tachyplesin I	[Phe[8]]tachyplesin	[Phe[13]]tachyplesin	[Phe[2]]tachyplesin[b]
Tris-HCl buffer (pH 8.0)	0.04	0.04	0.04	0.05
EYPC/EYPG (3:1)	0.22	0.21	0.22	0.23

[a] Excitation and emission wavelengths: tachyplesin I, [Phe[8]]tachyplesin I, and [Phe[13]]tachyplesin I, 295 and 350 nm, respectively; and [Phe[2]]tachyplesin I, 285 and 320 nm, respectively. [b] This compound was synthesized previously (Park et al., 1992).

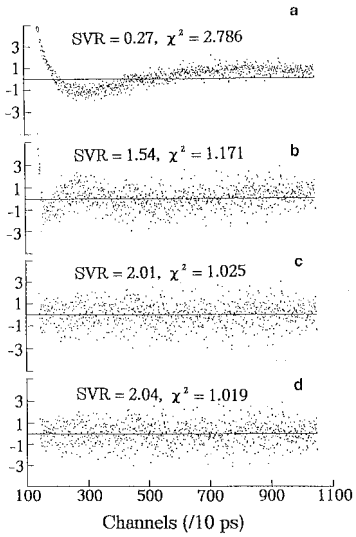

FIGURE 4: Plots of weighted residuals/root mean square of residuals for (a) one-exponential, (b) double-exponential, (c) triple-exponential, and (d) quadruple-exponential fits to the fluorescence decay of tachyplesin I in 20 mM Tris-HCl buffer (pH 8.0). Excitation was at 295 nm and emission at 350 nm.

length. However, the present study showed that the fluorescence intensities of tachyplesin I and its analogs, [Phe[8]]- and [Phe[13]]tachyplesins I, excited at 285 nm were greater than those excited at 295 nm where the Trp residue is exclusively excited. The fluorescence decay characteristics of tachyplesin I and its analogs also changed when excited at 285 nm instead of at 295 nm. Fluorescence decay parameters of tachyplesin I and its analogs, [Phe[8]]- and [Phe[13]]tachyplesins I, in acidic EYPC/EYPG (3:1) liposomes and buffer solution obtained when excited at 285 and 295 nm are listed in Table 4. The fluorescence of tachyplesin I in EYPC/EYPG liposomes excited at 295 nm decayed faster than when excited at 285 nm. It is also noticeable that, in the decay curves produced by excitation at 285 nm, [Phe[13]]-tachyplesin I showed a slower decay than [Phe[8]]tachyplesin I, indicating that excitation energy transfer from Tyr^8 to Trp^2 is more efficient than that from Tyr^{13} to Trp^2. Based on the energy transfer kinetics, the fluorescence decay rate of the energy acceptor, the Trp residue, can be given by eq 1. By using the decay kinetics of tyrosine and tryptophan residues determined experimentally and by optimizing the energy transfer rate (k_T) and A_0/D_0 as parameters in eq 3, fluorescence decay curves of tachyplesin I and [Phe[8]]- and [Phe[13]]-tachyplesins I when excited at 285 nm could be satisfactorily regenerated. The decay parameters of the energy donor, Tyr, of tachyplesins in buffer and in acidic EYPC/EYPG (3:1) in the absence of the Trp acceptor were determined from the fluorescence decay of [Phe[2]]tachyplesin I, the Trp^2 residue of which was replaced with Phe (Park et al., 1992), and are given in Table 5. The energy transfer rates of tachyplesin I and [Phe[8]]- and [Phe[13]]tachyplesins I are listed in Table 6.

Energy transfer efficiencies (E) of tachyplesins can be evaluated with energy transfer rates and fluorescence lifetimes. The energy transfer efficiency of tachyplesin I (E = 26%) was nearly similar to that of [Phe[13]]tachyplesin I (E = 21%) but greater than that of [Phe[8]]tachyplesin I (E = 13%). These facts suggested that the excitation energy donor to Trp^2 is not Tyr^{13}, but Tyr^8 in tachyplesin I. Furthermore, it should be noted that the energy transfer efficiency from Tyr^8 to Trp^2 was enhanced 5 times by changing the medium of tachyplesin I or [Phe[13]]tachyplesin I from buffer to liposomes, although that from Tyr^{13} was affected little. These indicate that the distance and/or mutual orientation of Trp^2 and Tyr^8 of tachyplesin I in buffer are shortened by interacting with lipid bilayers, but those between Trp^2 and Tyr^{13} remain unchanged.

DISCUSSION

In an attempt to analyze the orientations of the aromatic groups in tachyplesin I using synthetic analogs, they are required to take the same (or at least similar) conformation as tachyplesin I. The present CD spectral study showed that there are considerable differences in conformation among tachyplesin I and [Phe[8]]- and [Phe[13]]tachyplesins I in buffer at pH 8 (Figure 2b), but they take very similar conformations in the presence of neutral liposomes or acidic liposomes. These results allow us to compare small structural changes between the side chains of the peptides in lipid bilayers.

The NMR studies by two groups (Kawano et al., 1990; Tamamura et al., 1993) have indicated that tachyplesin I took a rigid antiparallel β-sheet structure with a type I β-turn and two disulfide bonds in aqueous solution, whereupon the charged residues of Lys and Arg are distributed around the molecule. The NMR and distant geometry calculations (Tamamura et al., 1993) revealed that the side chains of Phe^4, Val^6, Tyr^8, Arg^9, Ile^{11}, Tyr^{13}, Arg^{15}, and Arg^{17} are directed to one side of the β-sheet and those of Arg^5, Arg^{14}, and two Cys bonds to the other side. Trp^2 and Arg^{17} are present in flexible NH_2- and COOH-terminal tails, respectively. According to the space filling average structural model, a hydrophobic cluster formed by Val^6, Tyr^8, Ile^{11}, and Tyr^{13} is located in a ravine formed by the side chains of Arg^9, Arg^{15}, and Arg^{17}. Thus, tachyplesin I in aqueous solution takes a soluble protein-like structure rather than a typical amphiphilic structure in which hydrophilic and hydrophilic parts are separated by a β-sheet plane. This was further supported by our following experiment. When CD spectra of tachyplesin I were recorded by changing its concentration from 4.5 to 89.6 μM, no remarkable changes were observed (data not shown). This means that no aggregation occurs in tachyplesin I even at high concentrations in buffer because the aggregation behavior of amphiphilic substances depends on their concentrations in aqueous solution.

The fluorescence decay kinetics of tachyplesin I was given by linear combination of three components whose decay

Table 2: Time-Resolved Fluorescence Decay Parameters for Tachyplesin I with the Excitation Wavelength at 295 nm

medium	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	f_1[a]	f_2	f_3	λ_{1max}[b]	λ_{2max}	λ_{3max}	SVR	χ^2
buffer[c]	2.69	1.27	0.16	0.61	0.37	0.02	350	355	330	2.20	1.016
EYPC/EYPG	2.75	0.70	0.08	0.77	0.20	0.03	335	330	325	1.95	1.026
DMPC	2.20	0.84	0.15	0.65	0.31	0.04	340	340	330	1.90	1.050
DMPS	2.75	0.70	0.10	0.59	0.35	0.06	340	335	320	1.91	1.064

[a] The fractional fluorescence of three decay time components at an emission wavelength of 350 nm. [b] Approximate emission maximum of the spectrum associated with a specified decay. [c] Tris-HCl (20 mM, pH 8.0) containing 0.1 M NaCl.

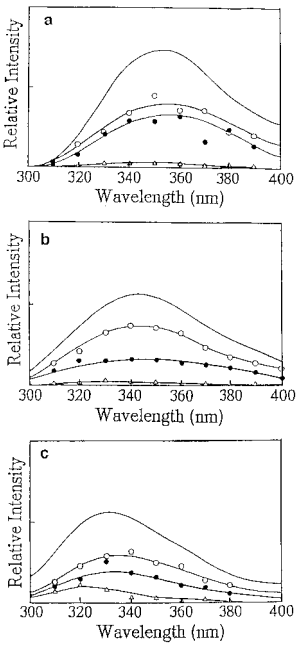

FIGURE 5: Decay-associated emission spectra of tachyplesin I excited at 295 nm in 20 mM Tris-HCl buffer (pH 8.0) (a) and in the presence of neutral DMPC (b) and acidic DMPS (c) liposomes. The steady state spectrum is indicated by a solid line with no symbol. The other spectra are the longest decay time component (○), the intermediate decay time component (●), and the shortest decay time component (△).

Table 3: Emission Wavelength-Independent Fractional Concentrations of the Three Decay Time Components[a]

	C1	C2	C3
buffer	0.35	0.45	0.20
EYPC/EYPG	0.30	0.30	0.40
DMPC	0.32	0.39	0.29
DMPS	0.16	0.38	0.46

[a] C1, C2, and C3 were calculated using DAS based on eqs 3−5.

curves were characterized by emission wavelength-dependent pre-exponential factors and the corresponding wavelength-independent decay times. Such complex fluorescence decay kinetics can be well rationalized by ground state heterogeneity resulting from the presence of three distinct conformers of the Trp^2 side chain, each of which has a different decay time, indicating that Trp^2 takes at least three conformations in buffer and in neutral and acidic liposomes. The fractions of three Trp^2 conformers changed by changing the medium from buffer to acidic liposomes, and the conformation of Trp^2 was also sensitive to electrostatic interaction in membranes. Since the fractional distribution of Trp^2 conformers changed depending on the types of membranes, special care should be paid to the orientational factor (k), when evaluating the energy transfer distance from the experimentally determined energy transfer efficiency. Tachyplesin I and its analogs gave the small anisotropy value in buffer but the large values in membranes. Therefore, R_0 values based on the maximum ($\langle k^2 \rangle^{max}$) and minimum ($\langle k^2 \rangle^{min}$) were spread over a wide range. The corresponding uncertainties of the Tyr−Trp distance were about 3.1−3.6 and 6.6−10.0 Å in the buffer and in membranes, respectively. The results are summarized in Table 6. If the random distribution approximation for the orientation factor, $k^2 = {}^2/_3$, was used, the critical distance (R_0) between Trp and Tyr was estimated to be 13 Å (Eisinger et al., 1969). Thus, from the energy transfer efficiency (E in Table 6) obtained for tachyplesin I and [Phe^{13}]tachyplesin I in EYPC/EYPG (3:1) membranes, the donor−acceptor distance (R) of tachyplesin I was calculated to be 12 Å. This value is perfectly in accord with that obtained in the previous experiment (Park et al., 1992). Our present results also demonstrated that this value corresponds to the distance between Trp^2 and Tyr^8 because the energy transfer rates in tachyplesin I and [Phe^{13}]tachyplesin I are identical. Furthermore, our energy transfer analysis indicated more delicate conformational change of tachyplesin I when interacting with membranes. When tachyplesin I was relocated from buffer to acidic membranes, the Tyr^8-−Trp^2 distance was shrunk about 4 Å but the Tyr^{13}−Trp^2 distance remained unchanged (18 Å).

From our previous and present studies using tryptophan fluorescence and the energy transfer formula, it is evident that translocation of tachyplesin from aqueous solution to lipid bilayers is accompanied by its conformational change. Taking account of such observations, we have previously proposed two elements: (a) the backbone conformation changes from a β-structure to a less-ordered structure and (b) the side chains of Trp^2 and Tyr^8 or Tyr^{13} are brought closer to each other by interacting with lipid bilayers. In the present study, the distance of Trp^2−Tyr^8 was determined to be 12 Å in lipid bilayers and 16 Å in aqueous solution. We consider here where such conformational change in buffer and in lipid bilayers comes from. Relating to this, we have previously proposed a model based on the energy donor (Tyr^8, Tyr^{13}, or both)−acceptor (Trp^2) distance in tachyplesin I in buffer. From the present results, we newly present a refined model in which the distance data obtained in buffer and in lipid bilayers were input as shown in Figure 6. Their conformational energies were calculated using the ECEPP/2 computer program (Némethy et al., 1983) and minimized by using the algorithm of Powell (1964). The typical β-sheet structure was selected as a starting conformation. Their energy values after minimization were almost identical (−106 kcal/mol in lipid bilayers and −103 kcal/mol in buffer). The peptide backbone conformation in lipid was slightly skewed from that of β-structure, but the hydrophobic residues (Trp^2, Phe^4, Val^6, Tyr^8, Ile^{11}, and Tyr^{13}) are packed more closely and the aromatic residues are lined up more straightly than those in buffer. The hydrophilic residues are not oriented to the side opposite from the

Table 4: Fluorescence Decay Parameters of Tachyplesin I, [Phe[8]]Tachyplesin I, and [Phe[13]]Tachyplesin I in Acidic EYPC/EYPG (3:1) Liposomes and in 20 mM Tris Buffer (pH 8.0)[a]

peptide	α_1	α_2	α_3	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_{av} (ns)	SVR	σ
in liposomes									
excited at 285 nm									
tachyplesin I	0.31	0.37	0.32	2.91	0.84	0.13	1.26	2.20	1.02
[Phe[8]]tachyplesin	0.29	0.36	0.35	2.72	0.75	0.09	1.09	1.87	1.05
[Phe[13]]tachyplesin	0.29	0.33	0.38	2.55	0.67	0.08	0.95	1.96	1.00
excited at 295 nm									
tachyplesin I	0.32	0.33	0.35	2.75	0.70	0.08	1.14	1.95	1.03
[Phe[8]]tachyplesin	0.27	0.32	0.41	2.55	0.69	0.10	0.94	1.92	1.06
[Phe[13]]tachyplesin	0.29	0.28	0.43	2.31	0.54	0.06	0.85	1.94	1.03
in buffer									
excited at 285 nm									
tachyplesin I	0.24	0.56	0.21	2.30	1.05	0.15	1.17	1.91	1.03
[Phe[8]]tachyplesin	0.16	0.53	0.32	2.44	1.13	0.26	1.07	1.88	1.08
[Phe[13]]tachyplesin	0.25	0.43	0.32	1.99	0.85	0.10	0.90	1.82	1.11
excited at 295 nm									
tachyplesin I	0.36	0.45	0.17	2.69	1.27	0.16	1.00	2.01	1.03
[Phe[8]]tachyplesin	0.24	0.35	0.42	2.61	1.03	0.08	1.01	1.84	1.10
[Phe[13]]tachyplesin	0.12	0.54	0.34	1.99	1.06	0.24	0.89	1.97	1.05

[a] Emission wavelength, 350 nm.

Table 5: Fluorescence Decay Parameters of [Phe[2]]tachyplesin I[a]

medium	α_1	α_2	α_3	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_{av} (ns)	SVR	σ
buffer[b]	0.07	0.58	0.36	1.54	0.58	0.39	0.53	1.08	1.107
EYPC/EYPG	0.64	0.32	−	0.99	0.20	−	0.70	1.80	1.001

[a] Excitation and emission wavelengths are 285 and 320 nm, respectively. [b] Tris-HCl (20 mM, pH 8.0) containing 0.1 M NaCl.

Table 6: Energy Transfer Parameters for Tyr to Trp in Tachyplesin I and Its Analogs[a]

		tachyplesin I	[Phe[8]]tachyplesin	[Phe[13]]tachyplesin
20 mM Tris-HCl (pH 8.0)	k_T (ns^{-1})	0.55	0.29	0.50
	E (%)	26	13	21
	R_0^{max} (Å)	14.7	14.7	14.7
	R_0^{RDA} (Å)	$^2/_3$	$^2/_3$	$^2/_3$
	R_0^{min} (Å)	12.1	12.1	12.1
	R^{max} (Å)	17.5	20.2	18.3
	R^{RDA} (Å)	15.5	17.8	16.2
	R^{min} (Å)	14.4	16.6	15.1
EYPC/EYPG (3:1)	k_T (ns^{-1})	2.77	0.24	2.60
	E (%)	66	14	65
	R_0^{max} (Å)	16.9	16.9	16.9
	R_0^{RDA} (Å)	$^2/_3$	$^2/_3$	$^2/_3$
	R_0^{min} (Å)	9.5	9.5	9.5
	R^{max} (Å)	15.1	22.9	15.2
	R^{RDA} (Å)	11.6	17.6	11.7
	R^{min} (Å)	8.5	12.9	8.6

[a] R_0^{max} and R_0^{min} were evaluated with $\langle k^2\rangle^{max} = 1.4$ and $\langle k^2\rangle^{min} = 0.43$ in buffer and $\langle k^2\rangle^{max} = 3.24$ and $\langle k^2\rangle^{min} = 0.10$ in EYPC/EYPG liposomes.

hydrophobic cluster to take an amphiphilic structure. However, when interacting with lipid bilayers, cationic charges of Arg residues may be energetically oriented to the side opposite from the hydrophobic cluster.

An NMR study (Tamamura et al., 1993) indicated that the side chains of Tyr[8] and Arg[9] in tachyplesin I in water are oriented to the same direction of β−sheet structure and in close proximity by being surrounded by water molecules. In Figure 6a, the Arg[9] side chain can be rotated in the same direction as Tyr[8] residues. Thus, the hydrophilic surrounding of Tyr[8] and Arg[9] may keep the conformation of tachyplesin I to retain the distance of Trp[2] and Tyr[8] at 16 Å. When the hydrophobic cluster in tachyplesin interacts with lipid bilayers, the orientation of Tyr[8] and Arg[9] may be released by hydrophobic interaction between Tyr[8] and the lipid hydrophobic region, charge interaction between the head group of lipids and the guanidino group of Arg[9], and so on, resulting in conformational change making the distance of Tyr[8]−Trp[2] shorter. Interestingly, the shapes of double-minimum peaks around 200−230 nm of CD spectra were very similar among [Phe[8]]tachyplesin in buffer (Figure 2a) and the three peptides in lipid bilayers (Figure 2b), although the intensities were considerably different from one another. In [Phe[8]]tachyplesin, the increase of hydrophobicity by replacement of Tyr[8] with Phe may make the proximity of the side chains of Arg[9] and Phe[8] in the hydrophilic surrounding impossible, resulting in a conformation similar to that of tachyplesin I in lipid bilayers as observed in CD studies.

In cell-lytic and antimicrobial peptides, some of them show random structure in aqueous solution and amphiphilic α-helical structure in membrane surface (Kaiser & Kézdy,

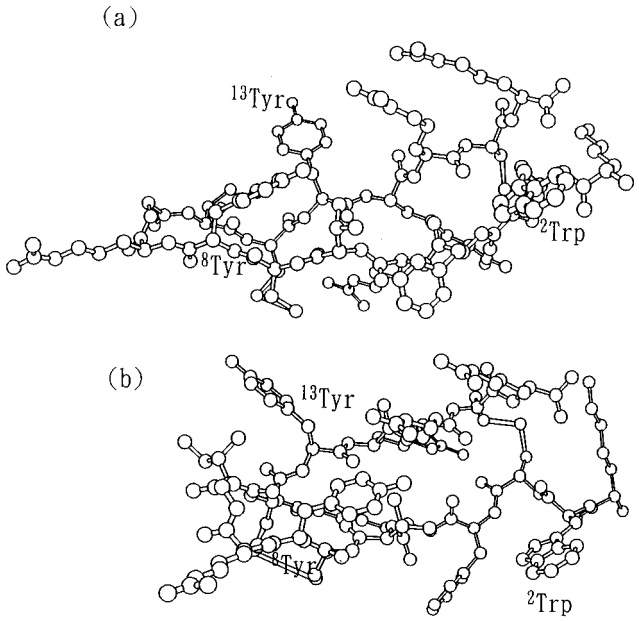

FIGURE 6: Presumed structural models of tachyplesin I in aqueous solution (a) and in lipid bilayers (b). Their energies were calculated by starting from the typical backbone conformation of β-sheet structure (ϕ, and $\psi = -139$ and 135°, respectively) and type I β-turn (ϕ_1, ψ_1, ϕ_2, and $\psi_2 = -60$, -30, -90, and 0°) using the ECEPP/2 computer program (Némethy et al., 1983) and the Powell minimization algorithm (Powell, 1964). In calculation, the conformations of all the side chains were fixed as *trans* and the distances between Tyr^{13} and Trp^{2} and Tyr^{8} and Trp^{2} (central points of the aromatic moieties) were fixed at 18 and 16 Å in buffer and at 18 and 12 Å in lipid bilayers, respectively.

1987; Saberwal & Nagaraji, 1994). It is well-known that such conformational changes are crucial for biological activity. On the contrary, β-structural antimicrobial peptides isolated from natural sources such as gramicidin S, defensin (Eisenhauer et al., 1989), and bactenecin (Romeo et al., 1988) possess amphiphilic structure restricted by cyclic structure. The tachyplesin is not amphiphilic in aqueous solution in spite of the rigid antiparallel β-conformation, implying that it can disperse as a monomer in aqueous solution. When interacting with biomembranes, it can take amphiphilic structure with a minor structural alteration in the peptide backbone and side chains by hydrophobic interaction, resulting in perturbation of the membrane organization or activation−deactivation of membrane surface receptors.

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