Binding of the Antimicrobial Peptide Temporin L to Liposomes Assessed by Trp Fluorescence*

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The structure and membrane topology of the antimicrobial peptide temporin L (FVQWSFKFLGRILN\textsubscript{H\textsubscript{2}}) were studied using liposomes as model bilayers. Circular dichroic spectra revealed temporin L to adopt an \(\alpha\)-helical conformation when bound to liposomes. Binding of temporin L to liposomes induced significant blue shifts of the emission spectra of the single Trp residue (Trp\(^{4}\)) and also changed its quantum yield. The observed changes in the characteristics of the Trp\(^{4}\) fluorescence are in keeping with the insertion of this residue into the hydrophobic region of the liposomal bilayers. Access of the aqueous quencher acrylamide to Trp\(^{4}\) decreased in the sequence 1-stearyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC)/cholesterol (X\(_{\text{cholesterol}} = 0.1\)) > SOPC > SOPC/1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG, X\(_{\text{POPG}} = 0.1\)) > SOPC/POPG (X\(_{\text{POPG}} = 0.2\)) > SOPC/POPG (X\(_{\text{POPG}} = 0.4\)), where X represents molar fraction of the indicated lipid. Whereas quenching of Trp\(^{4}\) by brominated phospholipids was significant in SOPC liposomes, the quenching efficiency was enhanced when the vesicles contained POPG. The depth of insertion of Trp\(^{4}\) into lipid bilayers was calculated by both the parallax method and distribution analysis and revealed this residue to reside at an average distance of \(d \approx 8.0 \pm 0.5\) Å from the center of both SOPC and SOPC/POPG bilayers. However, in the presence of cholesterol, \(d\) was increased to \(9.5 \pm 0.5\) Å, thus revealing Trp\(^{4}\) to become accommodated more superficially in the bilayer. The above data suggest the presence of two populations of temporin L in SOPC- and POPG-containing membranes with parallel and perpendicular orientation with respect to the plane of the membrane surface.

More than 500 antimicrobial peptides have been discovered so far from animals as well as plants (a good collection of sequences, besides a wealth of other useful information, can be found on the World Wide Web at www.bbcm.univ.trieste.it/~tossi/pag1.htm). These peptides are important components of innate defense mechanisms and are thought to kill bacteria by permeabilizing and/or disrupting their membranes. Experimental evidence suggests that direct peptide-lipid interactions are important for the expression of their antibiotic activity instead of specific association with a chiral receptor (1). Accordingly, the mechanisms of action and the significance of specific lipid-peptide interactions have been addressed by studying the interactions of antimicrobial peptides with model membranes (2–11). Yet, the detailed molecular events remain unknown.

Temporins represent a family of cationic, 10–13-residue-long antimicrobial peptides isolated from the skin of Rana temporaria (red frog), found in many regions of central Europe (12). Temporins are related to peptides isolated from wasp venoms (13), which have both hemolytic and chemotactic activities. Most temporins and in particular those containing a net positive charge are active against Gram-positive and -negative bacteria (12). Temporins induce the release of liposome-entrapped fluorescent probes, confirming direct interaction with lipid bilayers (14). However, the nature of the structural changes in the bilayer, possible specific interactions with lipids, and the location of temporins in phospholipid membranes remain poorly understood. In our previous study, we could show that temporins B and L readily insert into lipid monolayers and that the intercalation was enhanced by increasing contents of the negatively charged bacterial phospholipid PG\(^{1}\) in the membranes (11). Likewise, both peptides increased acyl chain order and induced lipid segregation in binary bilayers, composed of PG and PC.

Several studies have revealed common features in most antimicrobial peptides to be an amphipathic character and a net positive charge (for a review, see Ref. 15). Bacterial lipid membranes with net negative charge are thought to represent the putative molecular targets for the antimicrobial peptides (2, 3, 16). Accordingly, several of the antimicrobial peptides preferentially bind to anionic lipids (2, 3, 10, 11, 17). This could provide a potential mechanism for microbial specificity, since most of the anionic lipids of mammalian membranes are sequestered on the cytoplasmic side of the membrane (18), whereas in microbial membranes they are exposed to the external medium. In this study, we used liposomes composed of zwitterionic 1-stearyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC), acidic phospholipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), and cholesterol to study the membrane association of temporin L. Temporin L is the only temporin described so far containing a single tryptophan residue in its sequence (FVQWSFKFLGRILN\textsubscript{H\textsubscript{2}}), thus allowing us to use fluorescence spectroscopy to study its association with bilayers. Analysis of the emission spectra and fluorescence quenching of temporin L was used to characterize peptide-membrane inter-

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actions and to investigate the topology of this peptide in liposomes. Depth-dependent quenching of fluorescence by phosphatidylcholines brominated at different positions along the acyl chains (BrB-PCs) provides a sensitive technique to determine the depth of the Trp residue in bilayers (19, 20) and was used to study the location of temporin L in phospholipid liposomes. The structure, orientation, and depth of penetration of temporin L into membranes provided insight into the mode of its action and the effect of different lipids on its function and selectivity.

EXPERIMENTAL PROCEDURES

Materials—Hepes and EDTA were from Sigma, SOPC, POPG, 1-palmitoyl-2-(6,7-dibromostearoyl)phosphatidylcholine (16,7)-Br2-PC, 1-palmitoyl-2-(9,10-dibromostearoyl)phosphatidylcholine (9,10)-Br2-PC, 1-palmitoyl-2-(11,12-dibromostearoyl)phosphatidylcholine (11,12)-Br2-PC, and β-cholesterol were from Avanti Polar Lipids (Alabaster, AL). Concentrations of lipids were determined gravimetrically with a high precision electrobalance (Cahn,崇田, CA). The purity of the lipids was checked by thin layer chromatography on silicic acid-coated plates (Merck) developed with chloroform/methanol/water (65:25:4, v/v/v). Examination of the plates after iodine staining and, when appropriate, upon UV illumination revealed no impurities. Temporin L was purchased from Synept (Dublin, CA). The purity of the peptide was >94% as verified by HPLC and mass spectrometry (11).

Preparation of Small Unilamellar Vesicles (SUVs)—Appropriate amounts of the lipid stock solutions were mixed in chloroform to obtain the desired compositions and then dried under a stream of nitrogen followed by high vacuum for a minimum of 2 h. The lipid residues were subsequently hydrated at 50 °C with 5 mM Hepes, 0.1 mM EDTA, pH 7.0, to yield a lipid concentration of 5 mM. The suspension was sonicated in a bath sonicator (Bedford, MA) until achieving clear solutions. The vesicles were used within the same day.

Preparation of Large Unilamellar Vesicles (LUVs)—Appropriate amounts of the lipid stock solutions were mixed in chloroform to obtain the desired compositions. The solvents was removed under a stream of nitrogen, and the lipid residue was subsequently maintained under a reduced pressure for at least 2 h. The dry lipids were hydrated at 50 °C in 5 mM Hepes, 0.1 mM EDTA, pH 7.0, to yield a lipid concentration of 1 mM. The resulting dispersions were extruded through a stack of two polycarbonate filters (100-nm pore size; Millipore Corp., Bedford, MA) using a Lipofast low pressure homogenizer (Avestin, Ottawa, Canada) to obtain large unilamellar vesicles.

Circular Dichroism Measurements—UV CD spectra from 250 to 190 nm were recorded with a CD spectrophotometer (Olis R1F 1000F; On-Line Instrument Systems Inc., Bogart, GA) with the temperature maintained with a circulating bath. A 1-mm path length quartz cell was used at final concentrations of 50 μM and 3 μM of the peptide and liposomes, respectively, in 5 mM Hepes, 0.1 mM EDTA, pH 7.0. Interference by circular differential scattering by liposomes was subsequently hydrated at 50 °C with 5 mM Hepes, 0.1 mM EDTA, pH 7.0, to yield a lipid concentration of 5 mM. The suspension was sonicated in a bath sonicator (Bedford, MA) until achieving clear solutions. The vesicles were used within the same day.

Fluorescence Spectroscopy—All fluorescence measurements were performed in quartz cuvettes with 1-cm path length. LUVs were added to a solution of temporin L (5 μM final concentration) in 5 mM Hepes, 0.1 mM EDTA, pH 7.0, maintained at 25 °C with continuous stirring in a total volume of 2 ml. After 3 h of equilibration, fluorescence spectra were measured with a Perkin-Elmer LS 50B spectrometer with both emission and excitation band passes set at 5 nm. The tryptophan residue of temporin L was excited at 280 nm, and emission spectra were recorded from 290 to 450 nm, averaging five scans. Spectra were recorded as a function of the lipid/peptide molar ratio and corrected for the contribution of light scattering in the presence of vesicles. Blue shifts were calculated as the differences in wavelength of the maxima in emission spectra of lipid- peptide and peptide samples. S.D. in the blue shift was less than 0.5 nm.

Quenching of Trp Emission by Acrylamide—To reduce absorbance by acrylamide, excitation of Trp at 295 nm instead of 280 nm was used (22). Aliquots of the 3.0 mM solution of this water-soluble quencher were added to the peptide in the absence or presence of liposomes at a peptide/lipid molar ratio of 1:100. The values obtained were corrected for dilution, and the scatter contribution was derived from acrylamide titration of a vesicle blank. The data were analyzed according to the Stern-Volmer equation (23),

\[
F_0/F = 1 + K_{sv}[Q]
\]  

(Eq. 1)

where \( F_0 \) and \( F \) represent the fluorescence intensities in the absence and the presence of the quencher \( Q \), respectively, and \( K_{sv} \) is the Stern-Volmer quenching constant, which is a measure of the accessibility of Trp to acrylamide. On the premise that acrylamide does not significantly partition into the membrane bilayer (22), the value for \( K_{sv} \) can be considered to be a reliable reflection of the bimolecular rate constant for collisional quenching of the Trp residue present in the aqueous phase. Accordingly, \( K_{sv} \) is determined by the value of non-vegetable-associated free peptide as well as the fraction of the peptide residing in the surface of the bilayer.

Quenching of Trp by Brominated Phosphatidylcholines—Collisional quenching of Trp by brominated phospholipids (BrB-PCs) was introduced to assess the localization of this residue in bilayers (22, 24). BrB-PCs are considered to be well suited for this purpose, since they should introduce insignificant perturbation into the membrane (24, 25). The indicated LUVs were added to a temporin L solution (final concentration of 5 μM in 5 mM Hepes, 0.1 mM EDTA, pH 7.0). After 3 h at 25 °C, emission spectra were recorded, averaging five spectra. The differences in the quenching of Trp fluorescence by (6,7)-, (9,10)-, and (11,12)-Br2-PC were used to calculate the probability for location of the fluorophore in the membrane using two methods (viz. the parallax method (26) and distribution analysis (27)). In the first method, the depth of the Trp residue is calculated as follows,

\[
Z = L_{3/4} \times \left( \frac{I_0 - I}{I_0} \right) + L_{3/4}
\]  

(Eq. 2)

where \( Z \) represents the distance of the fluorophore from the center of the bilayer, \( L_{3/4} \) is the distance of the shallow quencher from the center of the bilayer, and \( L_{3/4} \) is the distance between the shallow and deep quencher. \( F_0 \) is the fluorescence intensity in the presence of the shallow quencher, \( F_1 \) is the fluorescence intensity in the presence of the deep quencher, and \( C \) is the concentration of quencher in molecules/Å². In distribution analysis, the depth of the Trp residue is calculated by fitting the data to the equation,

\[
\ln\left(F/F_0\right) + \left(h - h_m\right)/\sigma = \left(\frac{I_0 - I}{I_0}\right) + \frac{1}{\sigma}
\]  

(Eq. 3)

where \( F_0 \) represents the intensity in the absence of the brominated phospholipids, \( F \) is a set of intensities measured as a function of vertical distance from the bilayer center to the quencher in the presence of the shallow quencher, \( h \) is the fluorescence intensity in the presence of the shallow quencher, \( F_1 \) is the fluorescence intensity in the presence of the deep quencher, \( F_2 \) is the fluorescence intensity in the presence of the deep quencher, \( C \) is the concentration of quencher in molecules/Å². When equal concentrations of the Br- lipid residues were used, the value for \( h_m \) is unity (28).

RESULTS

CD Spectroscopy—Several studies have indicated that a specific secondary structure is required for the biological activity of antimicrobial peptides (for a review, see Ref. 29). The structure of temporin L in zwitterionic (SOPC)- and negatively charged phospholipid (POPG)-containing bilayers was investigated using circular dichroism. In an aqueous solution, temporin L adopts a random coil conformation, revealed by the single minimum at 200 nm (Fig. 1). However, in the presence of small unilamellar vesicles, the peptide is α-helical, with the characteristic double minima at 210 and 222 nm. Compared with the zwitterionic SOPC small unilamellar vesicles, the helical conformation is more pronounced in the presence of the negatively charged phospholipid POPG, with the calculated helical content increasing from 69 to 89% at \( \chi_{TOPG} = 0 \) and 0.4, respectively.

Binding of Temporin L to Liposomes—The sensitivity of the fluorescence emission of the Trp residue to its environment allows us to monitor the binding of temporin L to liposomes. Temporin L in buffer has a fluorescence emission maximum at 352 nm (Fig. 2A), typical for Trp in a polar environment (19).
A blue shift was smaller, whereas the decrement in F spectra saturated at a lipid/peptide molar ratio of 60 and was 2). In the presence of SOPC, the blue shift in the Trp emission affinity of temporin L for liposomes containing the acidic phospholipid, was used. This neutral water-soluble quencher has the advantage that no electrostatic interactions take place with the head group of negatively charged phospholipids. Stern-Volmer plots for the quenching of Trp by acrylamide, recorded in the absence and presence of lipid vesicles, are depicted in Fig. 3. Fluorescence of Trp4 decreased in a concentration-dependent manner by the addition of acrylamide to the peptide solution both in the absence and presence of liposomes, without other effects on the spectra (data not shown). However, in the presence of liposomes, less decrement in fluorescence intensity was evident, thus revealing that Trp4 is less accessible to the quencher in the presence of LUVs. Compared with the measurements in the absence of liposomes, the values for Ksv were decreased in the presence of SOPC and SOPC/POPG LUVs (Table I), suggesting that Trp4 was buried in the bilayers, becoming inaccessible for quenching by acrylamide. Comparison of Ksv values for POPG-containing and neat SOPC liposomes indicates that the binding of temporin L to liposomes is enhanced by POPG. Accordingly, Ksv is slightly lower at XPOPG = 0.1 than in the presence of SOPC LUVs. However, at XPOPG = 0.2 and 0.4, the values for Ksv were decreased further with essentially no difference between these two contents of POPG. Interestingly, Ksv for cholesterol-containing liposomes is only slightly less than in the absence of liposomes yet significantly higher than the values measured for the other lipid compositions. These data thus suggest the affinity of temporin L for liposomes to increase in the order SOPC/cholesterol (Xched = 0.1) < SOPC < SOPC/POPG (XPOPG = 0.1) < SOPC/POPG (XPOPG = 0.2) < POPG (XPOPG = 0.4).

Quenching of Trp by Br2-Phosphatidylcholines—The decreased access of the aqueous quencher acrylamide to Trp4 upon interaction of temporin L with lipid vesicles should be accompanied by an increased accessibility to quenchers present in the hydrocarbon phase of the bilayers. The intercalation depth of Trp4 into the hydrophobic core of the bilayer can be assessed from the quenching efficiency of bromine-labeled lipids. Whereas this Trp was quenched significantly in a SOPC vesicle, less decrement in fluorescence intensity was evident, thus revealing that Trp4 is less accessible to the quencher in the presence of LUVs. Compared with the measurements in the absence of liposomes, the values for Ksv were decreased in the presence of SOPC and SOPC/POPG LUVs (Table I), suggesting that Trp4 was buried in the bilayers, becoming inaccessible for quenching by acrylamide. Comparison of Ksv values for POPG-containing and neat SOPC liposomes indicates that the binding of temporin L to liposomes is enhanced by POPG. Accordingly, Ksv is slightly lower at XPOPG = 0.1 than in the presence of SOPC LUVs. However, at XPOPG = 0.2 and 0.4, the values for Ksv were decreased further with essentially no difference between these two contents of POPG. Interestingly, Ksv for cholesterol-containing liposomes is only slightly less than in the absence of liposomes yet significantly higher than the values measured for the other lipid compositions. These data thus suggest the affinity of temporin L for liposomes to increase in the order SOPC/cholesterol (Xched = 0.1) < SOPC < SOPC/POPG (XPOPG = 0.1) < SOPC/POPG (XPOPG = 0.2) < POPG (XPOPG = 0.4).

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creasing concentration of liposomes (Fig. 5A). From these data, we calculated the penetration depth of Trp 4 in bilayers by the parallax method (26) as well as by distribution analysis (27, 28, 31). The results reveal the Trp residue to be buried in the membrane, residing in both SOPC and SOPC/POPG liposomes at a distance of $\frac{90}{2} \pm 0.5$ Å from the center of the bilayer (Fig. 6). In addition, the insertion took place at all lipid/peptide molar ratios tested, and except at $X_{\text{POPG}} = 0.1$ there were only insignificant differences in the penetration depth. At $X_{\text{POPG}} = 0.1$, the depth of bilayer penetration of Trp 4 was shallow at a lipid/peptide molar ratio of 10, whereas the peptide was immersed deeper upon increasing the concentration of liposomes (Fig. 6). Least effective quenching by brominated lipids was evident in the presence of cholesterol, thus suggesting that temporin L was associated only with the membrane surface, in keeping with the observed efficient quenching by acrylamide. For SOPC/cholesterol LUVs ($X_{\text{cholesterol}} = 0.1$), quenching of Trp by Br$_2$-PCs saturated at a lipid/peptide molar ratio of 100 Å, and the increase in tryptophan fluorescence intensity (●) and the blue shift (*) in the maximum emission wavelength as a function of $X_{\text{POPG}}$ are shown in B. Blue shifts in Trp emission maxima and changes in fluorescence intensity as a function of lipid/peptide molar ratio are depicted in C and D, respectively.
of the Trp residue from the center of the bilayer is $9.5 \pm 0.5$ Å (Fig. 6).

**DISCUSSION**

The characteristics of quenching of the Trp residue of temporin L by acrylamide and brominated phospholipid in the presence of SOPC liposomes demonstrate that this peptide inserts in part into the hydrophobic lipid phase of the bilayer. The fluorescence intensities of temporin L decrease upon its transfer from the buffer into a SOPC membrane. Temporin L could aggregate upon binding to SOPC LUVs. Accordingly, in these aggregates, the Trp residues may be in close proximity so as to result in Trp self-quenching and quenching by the positive charges of the peptide, as suggested for nisin, another antimicrobial peptide (19). A similar interpretation may apply to the decrement of fluorescence in POPG-containing membranes at lipid/peptide molar ratio of $<10$. However, quenching could also be due to the charged head group of POPG interacting directly with the π-orbitals of Trp (22). Third and perhaps the most likely possibility is π-orbital-cation interaction (30), which is suggested by the close proximity of Trp^4 and K7 in α-helical temporin L (Fig. 7). The observed decrement in the maximal $λ_{max}$ of Trp emission upon interaction with SOPC liposomes reveals that Trp^4 becomes accommodated in a more hydrophobic environment. In this context, it is useful to note that the bilayer is highly dynamic. Accordingly, the interface between the polar aqueous phase and the nonpolar hydrocarbon region of the bilayer is not sharp but rather a transition zone, which accounts for $\sim 50\%$ of the bilayer thickness (for a review, see Ref. 32). Due to its polarizable π-orbitals, Trp has been suggested to favor partitioning into this zone with an averaged polarity gradient (33). Above a lipid/peptide stoichiometry of 10, the fluorescence of Trp^4 increases with increasing contents of POPG in the liposomes. Insertion of temporin L in α-helical conformation into POPG-containing membranes at the above stoichiometries is suggested by the observed blue shifts in the Trp emission maxima $λ_{max}$ and the quenching profile by brominated PCs. At $X_{POPG} = 0.2$ and 0.4, the highest Trp fluorescence is evident at a lipid/peptide molar ratio of 20. However, slight attenuation of fluorescence intensity was in evidence above this stoichiometry and could be due to reduced cooperative insertion of temporin L when the lipid/peptide molar ratio is increased. In the presence of cholesterol, Trp^4 fluorescence intensity was decreased by 30%. Accordingly, the environment of Trp^4 appears to become more polar, and the fluorescence quantum yield decreases. Quenching by acrylamide and brominated lipids suggests that in the presence of cholesterol most of the temporin L bound to the membrane resides in the surface. Aggregation of the peptides in the surface of the cholesterol-containing membranes and self-quenching of Trp could provide another mechanism for the decrement of Trp fluorescence. Notably, the effects of temporin L on membranes were significantly decreased in the presence of cholesterol. Cholesterol increases membrane acyl chain order (34) and stabilizes the bilayer structure (35), which could prevent the penetration of temporin L into the lipid bilayer and decrease the extent of membrane perturbation by this peptide.

The blue shifts of the emission maxima in the presence of SOPC and SOPC/POPG vesicles suggest that Trp^4 inserts into the hydrophobic region of the bilayer. Compared with zwitterionic membranes, larger blue shifts in Trp emission and higher quenching efficiency by brominated PCs are evident for membranes containing the negatively charged POPG. These differences are most likely due to a larger amount of peptides being inserted or deeper penetration of the peptide into bilayers. The data on the quenching by brominated lipid suggest

**TABLE I**

| Buffer       | 9:1 PC:cholesterol | PC/PG |
|--------------|--------------------|-------|
| $K_{sv}$     | 13.53              | 11.44 |
| $X_{POPG}$   | 10:0               | 15:0  |
| $X_{POPG}$   | 15:0               | 16:1  |
| $X_{POPG}$   | 20:0               | 21:1  |
| $X_{POPG}$   | 25:0               | 26:1  |
| $X_{POPG}$   | 30:0               | 31:1  |

**FIG. 3.** Stern-Volmer plots for the quenching of the fluorescence of the Trp residue of temporin L by acrylamide in an aqueous buffer (C) and in the presence of liposomes composed of SOPC with $X_{POPG} = 0$ (○), 0.1 (●), 0.2 (△), and 0.4 (□) and with $X_{POPG} = 0.4$ (○). The final concentrations of temporin L and liposomes were 5 μM and 500 μM, respectively, in a total volume of 2 ml of 5 μM Hepes, 0.1 mM EDTA, pH 7.0. The temperature was maintained at 25°C with a circulating water bath.

**FIG. 4.** Depth-dependent quenching of tryptophan fluorescence by liposomes containing brominated phosphatidylcholines ($X = 0.30$) at a lipid/temporin L molar ratio of 100. The concentration of temporin L was 5 μM in a total volume of 2 ml of 5 μM Hepes, 0.1 mM EDTA, pH 7.0. The temperature was maintained at 25°C with a circulating water bath. Each data point represents the mean of triplicate measurements, with the error bars indicating ± S.D.
that the depth of insertion of Trp is insignificantly affected by POPG. Accordingly, the amount of temporin L inserting into bilayers should increase for POPG-containing membranes. Amphipathic α-helical peptides such as magainin, cecropin B, cecropin P, dermaseptin B, and dermaseptin S bind to and permeate into zwitterionic membranes, although 10-fold higher concentrations are needed compared with negatively charged membranes (2, 36, 37). Similarly, whereas temporin L binds to zwitterionic membranes, it has a higher affinity for POPG-containing membranes. These results further indicate that membrane penetration as well as cooperative interactions and aggregation of temporin L in bilayers are involved in the disruption of the bilayer by this antimicrobial peptide.

Quenching by acrylamide reveals that Trp 4 of temporin L is shielded in the presence of liposomes. However, although at a lipid/peptide molar ratio of 100 the data indicate the peptide to be quantitatively membrane-bound, this residue was not completely inaccessible to acrylamide. Experiments with brominated phospholipids further demonstrate the most efficient

FIG. 5. Depth-dependent quenching of tryptophan fluorescence by phospholipids brominated at different positions along the acyl chains and incorporated at X = 0.30 in liposomes composed of SOPC with X_{POPG} = 0 (○), 0.1 (●), 0.2 (△), and 0.4 (▼) and with X_{chol} = 0.1 (●). The data are shown as a function of the lipid/peptide molar ratio. The concentration of temporin L was 5 μM in a total volume of 2 ml of 5 mM Hepes, 0.1 mM EDTA, pH 7.0. The temperature was maintained at 25 °C with a circulating water bath. The brominated lipids were (6,7)-Br_2-PC (A), (9,10)-Br_2-PC (B), and (11,12)-Br_2-PC (C).

FIG. 6. Distance d of the single tryptophan residue of temporin L from the bilayer center in the presence of liposomes composed of SOPC with X_{POPG} = 0 (○), 0.1 (●), 0.2 (△), and 0.4 (▼) and with X_{chol} = 0.1 (●). Values for d were calculated by the parallax method (A) and by distribution analysis (B).
formation of multimeric pores has been suggested to be responsible for membrane permeabilization. However, also alternative models, in which the peptides destroy the membrane barrier function without pore formation, have been proposed (for a review, see Ref. 38). For temporins as well as other short antimicrobial peptides, it would be particularly intriguing to discriminate between the different models proposed or to investigate whether they act by different mechanisms. Our studies reveal that temporin L inserts into the hydrophobic core of the membranes in a cooperative manner. Either a "toroidal" or "barrel stave" model would thus be feasible (39). One α-helical monomer of temporin L could span one half-leaflet of a bilayer. Accordingly, pore formation by this peptide is likely to require dimerization (14). The toroidal model suggested for magainin 2 involves five peptide helices together with several surrounding phospholipids, which form a membrane-spanning pore (40). For the latter mechanism, the concentration of bromines near the peptide would be rather different than in the bulk of the bilayer, particularly in POPG-containing membranes. This can be rationalized by the Br₂-PCs being partially excluded from the predominantly negatively charged lipid domains where the peptide is preferentially bound. Such variation in the local distribution of the lipid probes could affect the analysis of the quenching data by the parallax method, whereas distribution analysis would remain unaffected (28). However, distance calculation of the distance of Trp from the bilayer center by the parallax method and distribution analysis gave similar values for SOPC- and POPG-containing membranes, thus suggesting that partial segregation of lipids caused by temporin L (11) has only insignificant effects on the quenching profiles by the brominated lipids. In combination with the data revealing that temporin L readily inserts into and perturbs zwitterionic SOPC bilayers (11), the barrel stave model (39) could be the most likely mechanism for its action, as suggested for other temporins (14).

Our data show that temporin L inserts into lipid bilayers as a well defined structure. The membrane is thus perturbed by the supramolecular complex formed by the peptide with its associated phospholipids. Association of α-helical peptides is likely to be primarily driven by van der Waals forces (for a review, see Ref. 41). Alternatively, the antimicrobial peptides could lack specific knob-into-hole packing, which is a general characteristic of helical bundle membrane proteins (42) and causes promiscuous aggregation. The latter may induce local phase transitions, such as the separation of lipid-peptide micelles from the bilayers or local changes of the membrane curvature due to the intercalating peptides, which would cause transient collapse of the barrel structure. These processes could relate to the vesiculation of giant vesicles induced by temporin B and L observed in our previous study (11).

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