Chemistry and Physics of Lipids 165 (2012) 365–373

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

EFFECT OF HEAD GROUP AND CURVATURE ON BINDING OF THE ANTIMICROBIAL PEPTIDE TRITRPTICIN TO LIPID MEMBRANES

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ARTICLE INFO

Article history:
Received 30 September 2011
Received in revised form 7 December 2011
Accepted 9 December 2011
Available online 22 December 2011

Keywords:
tritrpticin, antimicrobial peptide, micelles, bilayers, spectroscopic techniques, toroidal pore

ABSTRACT

In this work we examine the interaction between the 13-residue cationic antimicrobial peptide (AMP) tritrpticin (VRRFPWWWPFLRR, TRP3) and model membranes of variable lipid composition. The effect on peptide conformational properties was investigated by means of CD (circular dichroism) and fluorescence spectroscopies. Based on the hypothesis that the antibiotic acts through a mechanism involving toroidal pore formation, and taking into account that models of toroidal pores imply the formation of positive curvature, we used large unilamellar vesicles (LUV) to mimic the initial step of peptide-lipid interaction, when the peptide binds to the bilayer membrane, and micelles to mimic the topology of the pore itself, since these aggregates display positive curvature. In order to more faithfully assess the role of curvature, micelles were prepared with lysophospholipids containing (qualitatively and quantitatively) head groups identical to those of bilayer phospholipids. CD and fluorescence spectra showed that, while TRP3 binds to bilayers only when they carry negatively charged phospholipids, binding to micelles occurs irrespective of surface charge, indicating that electrostatic interactions play a less predominant role in the latter case. Moreover, the conformations acquired by the peptide were independent of lipid composition in both bilayers and micelles. However, the conformations were different in bilayers and in micelles, suggesting that curvature has an influence on the secondary structure acquired by the peptide. Fluorescence data pointed to an interfacial location of TRP3 in both types of aggregates. Nevertheless, experiments with a water soluble fluorescence quencher suggested that the tryptophan residues are more accessible to the quencher in micelles than in bilayers. Thus, we propose that bilayers and micelles can be used as models for the two steps of toroidal pore formation.

1. Introduction

The widespread use (and often misuse) of conventional antibiotics in human and animal healthcare have elevated the occurrence of antibiotic resistance in the last decades (Lohner, 2009). Indeed, in 2001 the World Health Organization (WHO) classified antibiotic resistance as a priority disease (World Health Organization, 2001). One strategy that has been considered to solve this problem is the use of antimicrobial peptides (AMPs) as a new class of antibiotics.

AMPs are important constituents of the first line of defense of the innate immune system (Brogden, 2005). Since the isolation/characterization of cecropins, magainins, and defensins in the 1980's, 1,800 AMPs have been identified in different organisms, such as fungi, bacteria, plants, invertebrates, and vertebrates. Generally, the main function of AMPs is to kill or decrease the growth of a pathogenic microorganism, however recent studies showed that they can also modulate the innate and adaptive immune systems (Yang et al., 1999; Jenssen et al., 2006). While conventional antibiotics normally disable or kill bacteria over a period of days, AMPs act within minutes (Blondelle et al., 1999). Furthermore, AMPs present a wide spectrum of activity, acting against Gram-positive and Gram-negative bacteria, protozoa, fungi, virus, and mammalian cells (Brogden, 2005).

It was originally believed that AMPs act at the membrane level by the insertion and/or damage/permeabilization of the cytoplasmic membrane of the microorganism cell (Lehrer et al., 1989; Wade et al., 1990; Shai, 2002; Huang et al., 2004; Lohner and Blondelle, 2005). These peptides are usually short, cationic, and acquire an amphipathic conformation upon interaction with the cell membrane. Three models are often considered to explain membrane permeabilization (Brogden, 2005; Jenssen et al., 2006; Bechinger, 2011): *barrel-stave* (Ehrenstein and Lecar, 1977), *carpet* (Pouny et al., 1992), and *toroidal pore* (Ludtke et al., 1996). More recently, it has also been proposed that in some cases, some cationic antimicrobial peptides might act by promoting clustering of negatively charged phospholipids on the bacterial membrane, in particular, the abundant lipid phosphatidyl glycerol (Epand and Epand, 2009).

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doi:10.1016/j.chemphyslip.2011.12.005

Discussions of the mechanism of action of AMPs have also brought up considerations about the role of lipid composition (Lee et al., 2004; Su et al., 2011) and about the peptide:lipid ratio (Lee et al., 2004) on this mechanism. In addition, recent evidences also suggest that at least some of these can act at intracellular targets (Brogden, 2005). However, even in this case, an AMP would have to cross the cell membrane to reach the cytoplasm, which implies an initial interaction at the membrane level. Thus, the study of peptide-membrane interactions and the elucidation of the effects these peptides exert at the cell membrane are important to understand their biological activity.

In this work we focus on the AMP tritrpticin (VRRFPWWW-PFLRR, TRP3), a 13-residue cationic peptide, which belongs to the cathelicidin family. The high Arg (30%) and Trp (23%) content, together with three consecutive Trp residues, make this peptide unique (Chan et al., 2006; Sitaram, 2006). TRP3 has broad antimicrobial activity against both Gram-positive and Gram-negative bacteria, and some fungi (Lawyer et al., 1996; Cirioni et al., 2006; Ghiselli et al., 2006), in addition to hemolytic activity. However, the hemolytic effect only takes place at concentrations much higher than those required for antimicrobial activity (Yang et al., 2002). The existing studies show that TRP3 acts at the membrane level, but its detailed mechanism of action is not known.

NMR studies showed that, while TRP3 interconverts between different conformations in solution, upon binding to SDS (sodium dodecyl sulfate) micelles it acquires an amphipathic conformation with two adjacent turns around Pro^5 and Pro^9, and the three Trp clustered together. This conformation positions a cluster of hydrophobic residues at the micelle-water interface while the flexible N- and C-termini containing two positively charged Arg residues each are located on the other side of the structure, facing the aqueous phase (Schibli et al., 1999). Similar conformations were found for several TRP3 analogues bound to dodecylphosphocholine (DCP) micelles (Schibli et al., 2006; Andrushchenko et al., 2006). Studies with model membranes showed that TRP3 binds preferentially to negatively charged bilayers, and, to a lesser extent, to zwitterionic bilayers. It also promotes composition-dependent leakage from the inner compartment of lipid vesicles (Schibli et al., 2002). In addition we showed that the peptide displays ion channel-like activity in planar lipid bilayers and we proposed that the channel structure is that of a toroidal pore (Salay et al., 2004).

According to the Shai-Matsuzaki-Huang (SMH) model (Zasloff, 2002), initially the peptide binds to the membrane interface, acquiring an amphipathic conformation and aligning its long molecular axis parallel to the membrane surface. This localization pushes the polar head groups of the lipids, leading to a local induction of positive curvature (Matsuzaki, 2001). The unfavorable energy of membrane deformation reaches a critical value, leading to a dynamic pore formed by a supramolecular peptide-lipid complex (Matsuzaki et al., 1996), or a toroidal pore (Ludtke et al., 1996; Yang et al., 2000) whose polar surface is formed by the polar face of amphipathic peptides and the polar head groups of lipids.

Because of the high complexity of biological membranes, one usually resorts to biomimetic model systems in order to investigate the effects of composition and physical properties of the phospholipid matrix in the membranes. These models include planar lipid bilayers, multilamellar and unilamellar vesicles, micelles, and Langmuir monolayers (Schreier et al., 2000; Jelinek and Kolusheva, 2005; Seddon et al., 2009). The interaction between TRP3 and lipid model membranes has been investigated (Nagpal et al., 1999; Schibli et al., 1999; Nagpal et al., 2002; Schibli et al., 2002; Yang et al., 2002; Yang et al., 2003; Salay et al., 2004; Schibli et al., 2006; Andrushchenko et al., 2006; Andrushchenko et al., 2007; Andrushchenko et al., 2008; Nguyen et al., 2011). The aim of this work is to contribute to the understanding of the mechanism of action of TRP3 at the molecular level. To achieve this goal, we performed CD (circular dichroism) and fluorescence spectroscopic studies of the peptide in solution and upon interaction with model membranes–micelles and bilayer vesicles - of variable lipid composition. In particular we focus on the effect of membrane curvature on the conformational behavior of the peptide. Since it has been proposed that the mechanism of action of TRP3 involves formation of a toroidal pore, which, like micelles, presents positive curvature, we studied the interaction with micelles in order to mimic the molecular arrangement of peptide and lipids in the toroidal pore structure.

2. Materials and Methods

2.1. Reagents

Acrylamide and sodium dodecyl sulfate (SDS) were from Bio-Rad, Hercules, CA. L-α-tryptophan and cardiolipin of bovine heart (CL) were from Sigma Chemical Co., St. Louis, MO. 1-palmitoyl-2-hidroxy-*sn*-glycero-3-phosphocholine (LPC), 1-palmitoyl-2-hidroxy-*sn*-glycero-3-phosphoethanolamine (LPE), 1-palmitoyl-2-hidroxy-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (LPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (POPG), and phosphatidyl ethanolamine transphosphatidylated from egg phosphatidyl choline (ePE) were from Avanti Polar Lipids, Alabaster, AL.

2.2. Peptide synthesis

TRP3 was synthesized manually according to the standard N^{α}-tert-butyloxycarbonyl (Boc) protecting group strategy (Merrifield, 1963; Stewart and Young, 1984) using p-toluenesulfonyl (TOS) and formyl (For) Boc-amino acid derivatives of Arg and Trp, respectively. The synthesis was performed as described by Malavolta et al. (2002). Analytical HPLC (Waters), LC/MS (electrospray-Micromass)-mass spectrometry (theoretical m/z = 1902.28; found: 1902) and amino acid analysis (Biochrom 20 Plus, from Amersham Biosciences) were used to check the homogeneity of purified TRP3.

2.3. Preparation of model membranes

2.3.1. Large Unilamellar Vesicles (LUV)

Phospholipids were dissolved in chloroform and lipid films with the desired molar ratios (pure POPC, POPC:ePE 2:1, POPC:POPG 2:1, POPC:ePE:POPG 1:1:1, ePE:POPG 2:1, and ePE:POPG:CL 67:23:10; the latter mimics the polar lipid composition of *E. coli* B (ATCC 11303) membranes) were prepared in glass tubes after solvent evaporation with a stream of nitrogen; final traces of solvent were removed in a vacuum chamber for at least 2 h. The dried lipid film was suspended by vigorous vortexing in MilliQ water, pH 7.19 (± 0.02), at room temperature. The lipid suspensions were submitted to five cycles of freeze-thawing followed by thirty passages through two stacked 100 nm polycarbonate filters using an Avestin extruder at room temperature. Lipid phosphorous was determined according to Rouser et al. (1970).

2.3.2. Micelles

Stock solutions of lysophospholipids (LPC, LPC:LPE 2:1, LPC:LPG 2:1, LPC:LPE:LPG 1:1:1), and SDS were prepared by solubilizing weighed amounts in MilliQ water, pH 7.19 (± 0.02), to obtain the desired concentration.

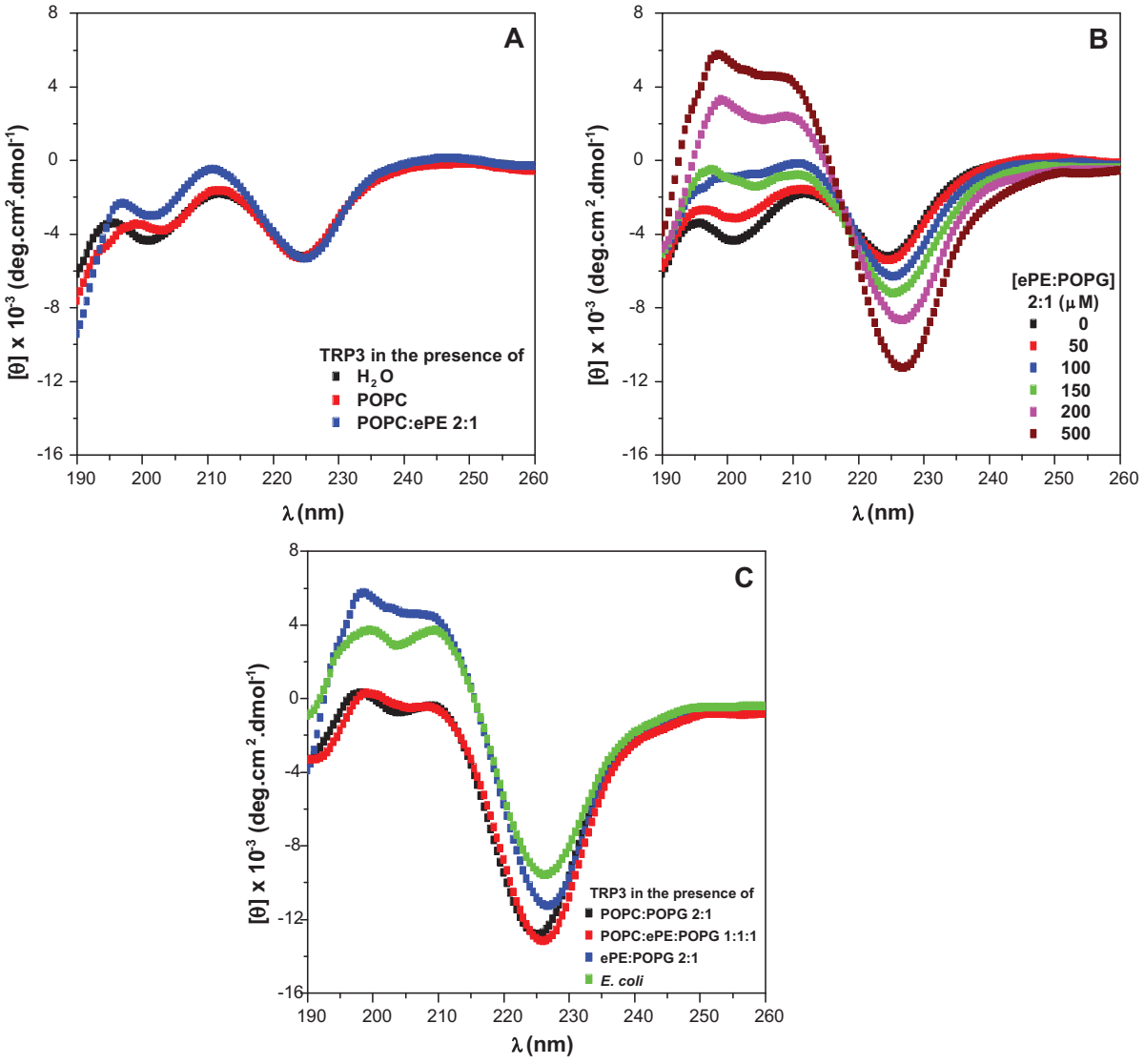

Figure 1. Far-UV CD spectra of 25.6 ± 1.3 μM TRP3 (A) in aqueous solution and in the presence of 500 μM POPC and POPC:ePE 2:1; (B) in the presence of increasing ePE:POPG 2:1 concentration (μM): 0; 50; 100; 150; 200; 500; (C) in the presence of LUV of variable lipid composition, POPC:POPG 2:1; POPC:ePE:POPG 1:1:1; ePE:POPG 2:1; ePE:POPG:CL 67:23:10 (*E.coli*); lipid:peptide ratio 20:1. pH 7.19 ± 0.02.

2.4. Circular dichroism (CD) studies

2.4.1. Titration with model membranes

CD spectra were acquired at room temperature in a Jasco J-720 spectropolarimeter. Samples were placed in 1.00 mm optical length quartz cells. The final spectra were the average of 6 scans, after subtracting the spectrum obtained under the same conditions of a sample without peptide. Spectra were scanned from 190 to 260 nm at 50 nm.s^{-1} using a 2 nm slit. The initial peptide concentration was 25.6 ± 1.3 μM. The lipid concentration varied from 0 to 500 μM and from 0 to 2.0 mM in LUV and micelles, respectively.

2.5. Fluorescence studies

2.5.1. Titration with model membranes

Fluorescence spectra were acquired at room temperature in a Hitachi F-4500 equipment interfaced with a computer. The excitation wavelength was 280 nm and the emission was scanned from 305 to 450 nm, at 60 nm.min^{-1}, with the photomultiplier at 700 V, using excitation and emission slits of 2.5 nm. Spectra taken under the same conditions without peptide were subtracted from spectra of the samples, and dilution effects were corrected. The initial peptide concentration was 10.2 ± 0.9 μM for studies with micelles and 5.9 ± 0.6 μM for studies with LUV. The lipid concentration varied from 0 to 300 μM and from 0 to 2.0 mM in LUV and micelles, respectively.

2.5.2. Fluorescence quenching by acrylamide

The intrinsic fluorescence of Trp residues in TRP3 was quenched by the titration with acrylamide in the concentration range 0–200 mM. Stern-Volmer constants (Lakowicz, 2006) were determined by means of equation (1):

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of variable acrylamide concentrations, respectively, K_{SV} is the Stern-Volmer constant, and [Q] is the acrylamide concentration.

3. Results

3.1. Binding of TRP3 to LUV

Figure 1A presents the far-UV CD spectra of TRP3 in solution and in the presence of LUV composed of zwitterionic phospholipids (pure POPC and POPC:ePE 2:1). The spectrum of TRP3 in solution is mainly characterized by the presence of a negative band centered at 225 nm. This band is ascribed to the coupling of the ^1B_b transition of tryptophan side chains with transitions of the peptide backbone and/or with transitions of other spatially close aromatic residues (Grishina and Woody, 1994). The presence of this band in the far-UV region is a dominating feature in the CD spectrum of TRP3 and precludes the analysis of the spectra in terms of secondary structure. For this reason, the CD spectra were analyzed by comparing the spectral features observed under different conditions, namely, in solution, and upon binding to bilayers or micelles. Based on the notion that the partition of membrane-active peptides into membrane interfaces is generally followed by the acquisition of secondary structure (Wimley and White, 1996), the spectral changes observed upon interaction of the peptide with the model membranes were interpreted as being due to the acquisition of secondary structure by the peptide. Moreover, this approach is reinforced by the NMR determination of the secondary structure achieved by TRP3 and some of its analogues upon binding to SDS and DPC micelles. Such kind of analysis has already been performed in CD studies of TRP3-model membrane interaction (Schibli et al., 1999; Yang et al., 2002; Andrushchenko et al. 2006).

Essentially no spectral changes were observed in the presence of LUV of POPC or POPC:ePE 2:1 (Fig. 1A), suggesting that TRP3 did not interact with zwitterionic membranes. In contrast, upon titration with increasing concentrations of LUV containing a negatively charged phospholipid (ePE:POPG 2:1), spectral changes were observed in the far-UV CD spectrum of TRP3: the negative band at 225 nm became more intense and two positive bands appeared at *ca.* 200 nm and 210 nm (Fig. 1B). Based on the considerations above, these spectral changes were taken as an indication of acquisition of secondary structure by TRP3, as a consequence of peptide-bilayer interaction. This interaction was examined as a function of membrane composition. In all cases, the negatively charged phospholipid POPG was included in view of the highly frequent presence of the phosphoglycerol head group in the membranes of bacteria (Goldfine, 1984; Lohner, 2001; Epand and Epand, 2009). Figure 1 C presents the far-UV CD spectra of TRP3 in the presence of LUV of POPC:POPG 2:1, POPC:ePE:POPG 1:1:1, ePE:POPG 2:1, and ePE:POPG:CL 67:23:10. The latter system corresponds to the qualitative and quantitative composition of polar lipids in the membranes of *E. coli* B (ATCC 11303). The Figure shows that the main spectral features in the spectra of TRP3 are similar, suggesting that the peptide acquires similar conformations upon binding to these bilayers.

Fluorescence spectra also monitored the binding of TRP3 to bilayers of variable lipid composition. The shift of the Trp residues from the polar aqueous phase to the less polar membranous environment caused an increase in peptide fluorescence intensity, as well as a decrease in the wavelength of maximum emission (λ_{em}^{max}). In contrast with the CD results, fluorescence data indicated a weak interaction between TRP3 and pure POPC and POPC:ePE 2:1. However, under the experimental conditions used, saturation was not achieved, precluding further analysis of these data. Similar to what was found in the CD studies, the fluorescence spectra provided clear evidence for the interaction between TRP3 and bilayers containing negatively charged phospholipids. For this reason, only the results obtained with these membranes were analyzed further. The binding isotherms of TRP3 to LUV containing negatively charged phospholipids indicate that the fluorescence intensity increased with increasing lipid concentration until saturation (Fig. 2). Table 1 presents the values of $(I-I_0/I_0) \times 100$ at saturating lipid concentrations, where I_0 and I represent the fluorescence intensities at λ_{em}^{max} (in the membrane) in the spectra of TRP3 in the absence and in the presence of LUV. The Table also informs the values of λ_{em}^{max} for L-α-tryptophan in aqueous solution and for TRP3 in the absence and presence of LUV. The value of λ_{em}^{max} for L-α-tryptophan is that reported in the literature (Lakowicz, 2006); the smaller value for TRP3 in aqueous solution suggests that the Trp residues in the peptide chain experience an environment on the average less exposed to solvent. A criterion for the extent of penetration of Trp residues into the membrane is the blue shift in the emission spectrum as a consequence of partitioning in a more hydrophobic environment. Table 1 shows that the values of λ_{em}^{max} decreased in the presence of membranes. Furthermore,

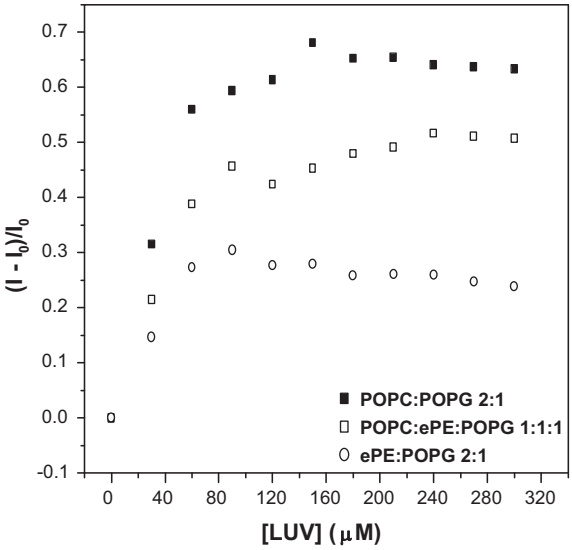

Figure 2. Increase of fluorescence intensity ($I-I_0/I_0$, see text) as a function of lipid concentration, for POPC:POPG 2:1, POPC:ePE:POPG 1:1:1, and ePE:POPG 2:1. pH 7.19 ± 0.02.

Table 1
Increase in intensity, $(I - I_0)/I_0 \times 100$, and λ_{em}^{max} in the fluorescence spectra of L-α-tryptophan and TRP3 in the absence and presence of LUV and micelles of variable lipid composition. pH 7.19 ± 0.02.

	$[(I - I_0) \times 100/I_0]$ (%)[a]	λ_{em}^{max} (nm)
In solution		
L-α-tryptophan	-	350.0
TRP3	-	347.0
LUV		
POPC:POPG 2:1	59[b]	344.2
POPC:ePE:POPG 1:1:1	48[b]	342.6
ePE:POPG 2:1	26[b]	345.8
ePE:POPG:CL 67:23:10 (*E. coli*)	-[c]	345.2
Micelles		
LPC	53[d]	344.8
LPC:LPE 2:1	28[d]	346.2
LPC:LPG 2:1	41[d]	341.0
LPC:LPE:LPG 1:1:1	28[d]	346.2
SDS	-[e]	346.0

[a] I_0 and I represent the fluorescence intensity at λ_{em}^{max} in the absence and in the presence of membrane, respectively.
[b] Values obtained at 345 nm in the presence of 210 μM lipid.
[c] It was not possible to evaluate the fluorescence increase due to light scattering effects.
[d] Values obtained at 345 nm in the presence of 1000 μM lipid.
[e] The increase in fluorescence was not evaluated due to the interaction of the peptide with SDS both below and above the detergent critical micelle concentration.

Table 2
Stern-Volmer constants, K_{SV}, M^{-1}, for acrylamide quenching of the fluorescence of L-α-tryptophan and of TRP3 in the absence and presence of LUV and micelles of variable lipid composition. pH 7.19 ± 0.02.

	K_{SV} (M^{-1})
In solution	
L-α-tryptophan	17.7
TRP3	10.4
LUV	
POPC:POPG 2:1	2.5
POPC:ePE:POPG 1:1:1	2.1
ePE:POPG 2:1	2.1
ePE:POPG:CL 67:23:10 (*E. coli*)	2.8
Micelle	
LPC	3.9
LPC:LPE 2:1	3.4
LPC:LPG 2:1	3.4
LPC:LPE:LPG 1:1:1	3.3
SDS	8.1

the small blue shifts observed suggest that the Trp residues of TRP3 are located on the average close to the bilayer-water interface.

We also evaluated the accessibility of tryptophan residues to the water soluble quencher acrylamide. The extent of accessibility was measured by the Stern-Volmer constant (K_{SV}), which decreases with the decrease in accessibility (Table 2). The value of K_{SV} for free L-α-tryptophan in aqueous solution was similar to that reported in literature (Tallmadge et al., 1989); the smaller value for TRP3 in aqueous solution is in agreement with the smaller value of $\lambda_{em}{}^{max}$ (Table 1) and indicates that the Trp residues in the peptide are less accessible to acrylamide than the free amino acid. In the presence of negatively charged bilayers, the values of K_{SV} decrease even further (Table 2), once more evincing the binding of the peptide to the negatively charged bilayers.

3.2. Binding of TRP3 to micelles

In order to analyze the role of curvature on TRP3-model membrane interaction, we examined the effect of micelles on peptide conformational properties. The composition of the aggregates was chosen so that the head group composition would be qualitatively and quantitatively the same as that of some of the bilayers studied; thus, micelles were prepared from lysophospholipids. In addition, we used SDS to compare with data already reported in the literature. Micelles containing more than 50 mol % LPE could not be prepared, probably due to the fact that this lipid has a Kraft temperature of approximately 60 °C (Slater et al., 1989).

The far-UV CD spectra of TRP3 undergo changes upon titration with increasing concentrations of LPC micelles (Fig. 3A). The intensity of the negative band around 225 nm increased and a positive band appeared at *ca.* 198 nm with a shoulder around 210 nm. These spectral changes, suggested that the interaction with LPC micelles induced acquisition of conformation. Furthermore, the spectra suggest that TRP3 acquires similar conformations upon binding to micelles of different lipid composition (Figure 3B). The results showed that, in contrast to what was found in the case of bilayers, besides binding to negatively charged micelles, TRP3 also binds to zwitterionic micelles. In addition, the spectra were different from those obtained in bilayers, as seen in Figure 4. Finally, it should be noticed that the spectra of micelle-bound TRP3 were similar to that obtained in the presence of SDS micelles, which was similar to those reported in the literature (Schibli et al., 1999; Yang et al., 2002).

Fluorescence spectra also monitored the interaction of TRP3 with micelles. The intensity increased and the wavelength of maximal emission decreased in the presence of micelles. Figure 5 presents the binding isotherms for the different lipid compositions. Also in this case, a saturation behavior was observed. The values of $(I\text{-}I_0/I_0) \times 100$ at saturation as well as those of $\lambda_{em}{}^{max}$ are given in Table 1. In addition the blue shifts resulting from binding were also small, indicating that the tryptophan residues are located close to the micelle-water interface. Acrylamide quenching studies yielded K_{SV} values (Table 2) lower than in solution, again reflecting peptide binding to the micelles. Interestingly, the Trp residues seem to be more accessible to the quencher in micelles than in bilayers. The much higher value of K_{SV} in the presence of SDS micelles when compared to the lysophospholipid micelles could be due to a greater exposure of the peptide on the micelle surface, possibly due to a larger effect of electrostatic of peptide-detergent interactions, and/or to a higher partitioning of acrylamide in these micelles.

4. Discussion

TRP3 has broad antimicrobial activity against Gram-positive and Gram-negative bacteria, as well as some fungi (Lawyer et al., 1996; Cirioni et al., 2006; Ghiselli et al., 2006), in addition to hemolytic activity. However, the hemolytic effect only takes place at concentrations much higher than those required for antimicrobial activity (Yang et al., 2002). The existing studies show that TRP3 acts at the membrane level, although the detailed mechanism of action is still not fully understood.

An increasing amount of experimental data demonstrates the preferential interaction of positively charged AMPs with negatively charged phospholipids (White et al., 1995; Wenk and Seelig, 1998, Sitaram and Nagaraj, 1999; Zasloff, 2002; Jenssen et al., 2006; Lohner, 2009). This preference would be responsible for the selectivity of AMP towards microorganisms, in view of the differences between the lipid composition of mammalian and bacterial membranes. The main phospholipids in the outer leaflet of bacterial membranes are PE (zwitterionic), PG, and cardiolipin (both negatively charged) (Goldfine, 1984; Lohner, 2001; Epand and Epand, 2009); the latter two impart negative charge on the membrane surface. In contrast, the major lipids in the outer leaflet of mammalian membranes are zwitterionic PC and sphingomyelin, and neutral cholesterol, which leads to a net surface charge of approximately zero (Rothman and Leonard, 1977).

Fluorescence studies ($\lambda_{em}{}^{max}$ blue shifts, red-edge effects, fluorescence quenching by water soluble and membrane embedded quenchers) showed that TRP3 binds preferentially to negatively charged membranes and, to a lesser extent, to zwitterionic membranes (Schibli et al., 2002). Isothermal titration calorimetry (ITC) studies corroborated these findings (Andrushchenko et al., 2008), pointing to the role of electrostatic interactions for peptide binding. With regard to the effect on bilayer permeability, Schibli et al. (2002) demonstrated that TRP3 induces leakage of the contents from the inner compartment of lipid vesicles. In addition, Salay et al. (2004) showed the channel-like activity of TRP3 in planar lipid membranes (BLM) and Schibli et al. (2006) provided evidence for TRP3-induced lipid flip-flop. These data have been taken as evidences in favor of a toroidal pore mechanism for TRP3.

It is widely acknowledged that the partitioning of membrane active peptides into membranes is generally followed by the acquisition of secondary structure (Wimley and White, 1996). NMR studies showed that, in the presence of anionic SDS and zwitterionic DPC micelles, TRP3 acquires an amphipathic conformation with two adjacent turns around Pro^5 and Pro^9, and the three Trp clustered together. This conformation positions a cluster of hydrophobic residues at the micelle-water interface and the flexible N- and C-termini containing two positively charged Arg residues each on the other side of the structure, facing the aqueous phase (Schibli et al., 1999). Such topography is in agreement with the known preference of Trp and other aromatic residues for the

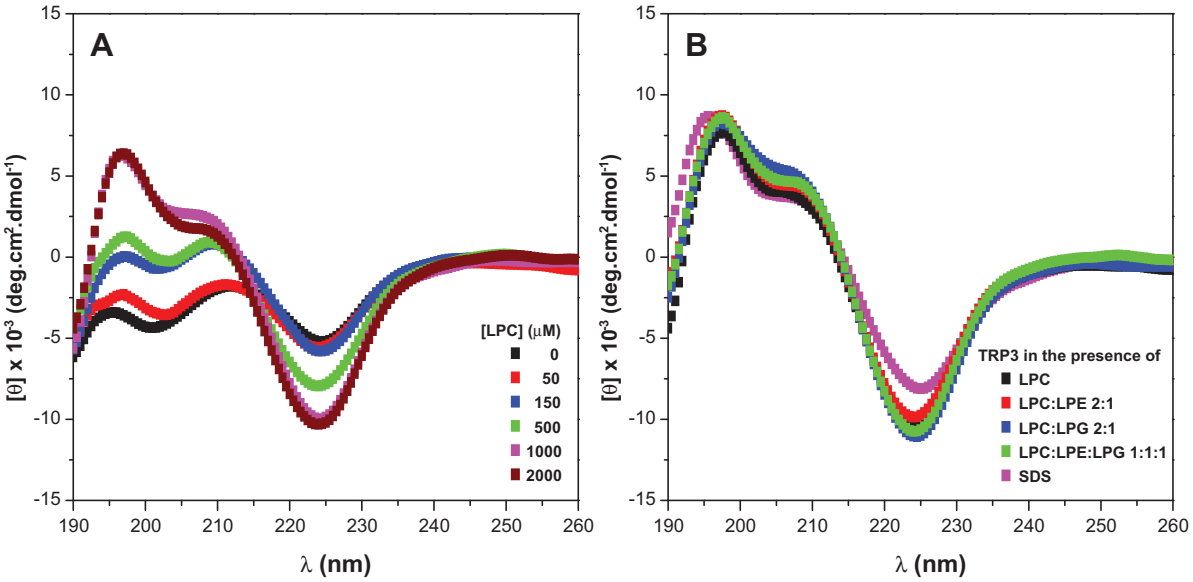

Figure 3. Far-UV CD spectra of 25.6 ± 1.3 μM TRP3 (A) in the presence of increasing LPC concentration (μM): 0; 50; 150; 500; 1000; 2000; (B) in the presence of micelles of variable lipid composition: LPC; LPC:LPE 2:1; LPC:LPG 2:1; LPC:LPE:LPG 1:1:1; SDS. Lipid:peptide ratio 60:1, except SDS where the ratio was 1000:1. pH 7.19 ± 0.02.

membrane-water interface (Wimley and White, 1996; Yau et al., 1998).

Discussions concerning the mechanism of action of AMP, in particular when the mechanism under consideration is the formation of toroidal pores, have increasingly focused on the role of curvature, more specifically positive curvature, since the models for toroidal pore formation imply that the participating lipids acquire positive curvature. In this context, one should take into account both the propensity of lipids to acquire positive curvature and the propensity of peptides to induce positive curvature. Since it has been proposed that TRP3 acts by a toroidal pore mechanism (Vogel et al., 2002; Schibli et al., 2002; Salay et al., 2004), the main goal in this work was to investigate the effect of curvature on the interaction between TRP3 and model lipid membranes. For this purpose, we examined the conformational properties of the TRP3 with bilayers (LUV) and micelles of variable lipid composition by means of CD and fluorescence spectroscopies. The reasoning underlying the choice of micelles was that these aggregates display positive curvature, therefore, these structures would, to some extent, mimic the molecular organization of lipids and peptides in the toroidal pore. In order to compare the data obtained for bilayers and micelles, the latter were prepared with lysophospholipids carrying the same head groups as those of phospholipids in LUV, at the same molar proportions. Thus, the only difference between the two types of aggregates was the molecular organization of the components.

The presence of three tryptophan residues posed a problem with respect to the use of CD to obtain information about peptide secondary structure. These residues gave rise to a band ascribed to the coupling of the ^1B_b transition of the Trp side chains with transitions of the peptide backbone and/or with transitions of other aromatic residues side chains spatially close (Grishina and Woody, 1994) which precluded analysis of the far-UV CD spectra in terms

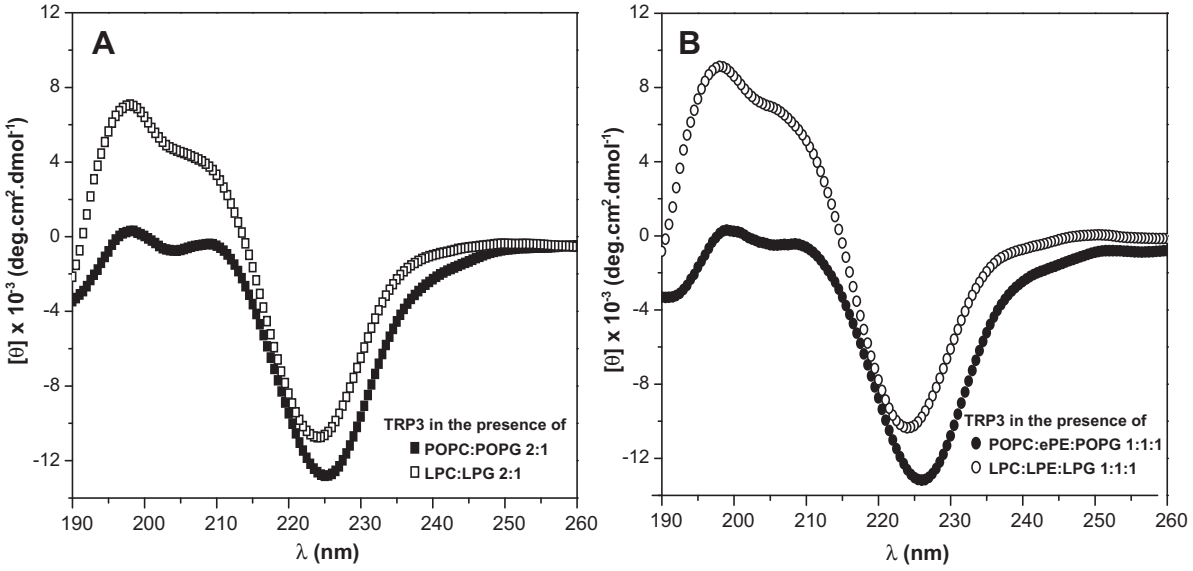

Figure 4. Far-UV CD spectra of 25.6 ± 1.3 μM TRP3 (A) in the presence of POPC:POPG 2:1 and LPC:LPG 2:1; (B) in the presence of POPC:ePE:POPG 1:1:1 and LPC:LPE:LPG 1:1:1. Lipid:peptide ratio of 20:1. pH pH 7.19 ± 0.02.

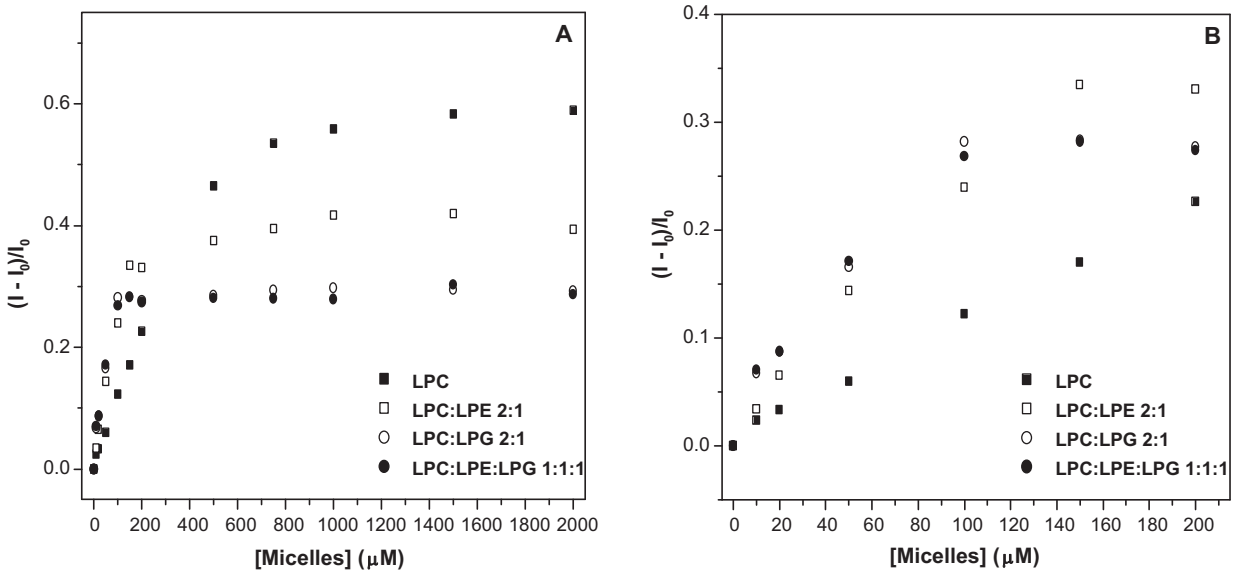

Figure 5. Increase of fluorescence intensity ($I-I_0/I_0$, see text) as a function of lipid concentration. (A) LPC, LPC:LPE 2:1, LPC:LPG 2:1, and LPC:LPE:LPG 1:1:1. pH 7.19 ± 0.02. In (B) the concentration axis was expanded to allow the comparison between data for micelles and bilayers.

of secondary structure. Nevertheless, upon binding of TRP3 to the model membranes, spectral changes were observed, suggesting the acquisition of conformation.

Thus, it was possible to observe differences between bilayers and micelles: while TRP3 interacted with the former mostly when the membranes contained negatively charged phospholipids (Fig. 1), revealing the importance of electrostatic interactions for peptide binding, the peptide was able to interact with both zwitterionic (LPC, LPC:LPE 2:1) and negatively charged micelles (Fig. 3). Similar results were found in other studies, where the interaction of peptides with micelles was seen to be independent of the charge at the polar head group (Pertinhez et al., 1995; Pertinhez et al., 1997; Salinas et al., 2002). Such findings suggest that other interactions (hydrophobic, hydrogen bonding, van der Waals interactions, hydration forces) have a greater contribution to the energetics of binding in micelles. Several studies point to the formation of hydrogen bonds between the guanido group of Arg residues in peptides and phospholipid phosphate groups (Epand and Vogel, 1999; Tang et al., 2007; Tang and Hong, 2009). It has been proposed that these hydrogen bonds are the driving force for pore formation. Recently, Nguyen and coworkers (2011) demonstrated the occurrence of such hydrogen bonds upon interaction of TRP3 with model membranes.

The CD data also suggested that the conformation of TRP3 is similar in bilayers, irrespective of lipid composition. The same holds for micelles. Moreover, the similarity of the spectra in lysophospholipid micelles and in SDS suggests that the conformation of the peptide in these aggregates is that found by NMR in SDS and DPC micelles (Schibli et al., 1999; Schibli et al., 2006). However, the conformations are different in micelles and bilayers, even when both share identical head groups (Fig. 4). These results strongly suggest that the different conformations acquired by TRP3 are predominantly modulated by membrane curvature and that the conformation in micelles could be related to that displayed by the peptide in toroidal pores. Moreover, the data also illustrate the mutual influence of lipid and peptide with regard to pore formation: while the peptide promotes the acquisition of curvature, the lipid organization modulates peptide conformation.

The fluorescence data corroborate the CD results in showing that, while TRP3 binds to bilayers only when they contain negatively charged phospholipids (Fig. 2 and Table 1), the interaction with micelles is independent of surface charge (Fig. 5 and Table 1). The results also point to the location of TRP3 close to the membrane-surface interface in both bilayers and micelles, as evinced by the small blue shifts in the values of $\lambda_{em}{}^{max}$ with respect to that in water (Table 1). These results are in agreement with previous observations (Schibli et al., 1999; Schibli et al., 2002). Nevertheless, the higher values of the Stern-Volmer constants (Table 2) suggest that the location of TRP3 in micelles is more superficial. This conclusion, however, should be taken with some caution, since it should be considered that the quencher might have a greater degree of partitioning into micelles, as discussed by Eftink and Ghiron (1976) in the case of SDS.

Since the experimental approaches used in this study focused on the peptide, it was not possible to examine the question of whether the peptide-membrane interaction promoted lipid clustering; this question will be dealt with in future work.

Possible implications of the present data for the mechanism of toroidal pore formation by TRP3

The results obtained in this work provide subsidies for the elucidation of the mechanism of pore formation by TRP3. Salay and coworkers (2004) demonstrated the ion channel-like activity of TRP3 and proposed that the mechanism of action of the peptide involves the formation of a toroidal pore. The following sequence of events is thought to lead to pore formation: initially, the peptide binds parallel to the membrane surface at the membrane-water interface; subsequently, accumulated peptide molecules induce formation of local positive curvature with the participation of lipids that have a propensity to form positive curvature (*e.g.*, PG and PC, Lohner and Blondelle, 2005). The cartoon in Figure 6 depicts this sequence of events.

Both bilayers and micelles are model membranes formed by amphiphilic molecules; however, due to differences in their geometry, these molecules form different aggregates: while micelles display positive curvature, bilayers can be envisioned as planar structures at the molecular level; in addition, micelles have a looser molecular packing, and their polar head groups are more hydrated. We propose that the results obtained with bilayers correspond to

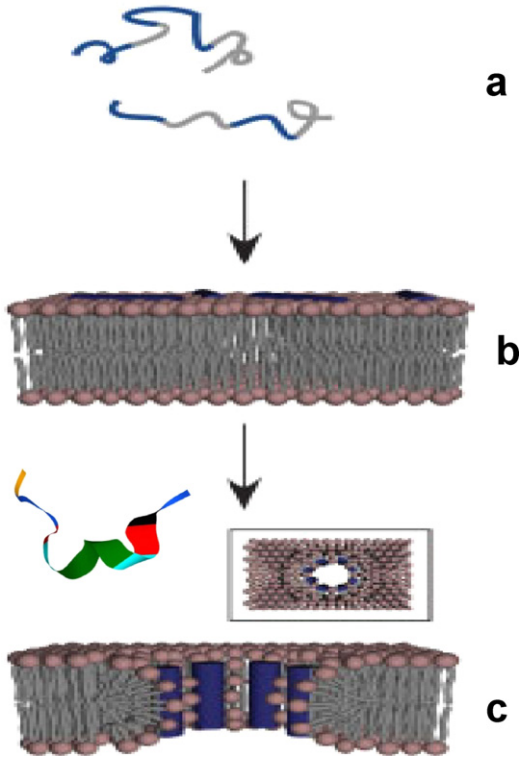

Figure 6. Cartoon depicting (a) a generic peptide in solution, (b) peptide bound to the bilayer surface, (c) toroidal pore showing the micelle-like lipid organization. Between (b) and (c), on the right, top view of the toroidal pore. Reproduced from Toke, 2005, with permission of John Wiley & Sons. On the left, the SDS-bound ribbon conformation of TRP3 (pdb1d6x), as determined by NMR by Schibli et al., 1999.

the initial step of TRP3 binding to the membrane, while the results for micelles are related to the geometry of both lipid and peptide molecules in the pore. As a consequence, we suggest that micelles can be used to mimic the molecular arrangement of lipids and peptides in the toroidal pore.

Acknowledgements

This research was supported by grants from FAPESP. J.C.B.Jr. had CNPq M.Sc. fellowship and presently has a Ph.D. fellowship, M.R.S.P. is a M.Sc. student, E.T.S. had a PIBIC undergraduate student fellowship, C.R.N. and S.S. are CNPq research fellows.

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