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Conformation and Ion Channel Properties of a Five-helix Bundle Protein

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Abstract: The primary amphipathic peptide Ac-Met-Gly-Leu-Gly-Leu-Trp-Leu-Leu-Val-Leu[10]-Ala-Ala-Ala-Leu-Gln-Gly-Ala-Lys-Lys-Lys[20]-Arg-Lys-Val-NH-CH_2-CH_2-SH called SPM was able to induce formation of ion channels into planar lipid bilayers with main conductance values of 75 and 950 pS in 1 M KCl. The 75 pS value can be attributed to an aggregate composed of five monomers since the corresponding five-unit bundle (5-SPM) also presented a 70 pS channels under the same conditions. The upper 950 pS level would be generated by a hexameric aggregate. Ion channels induced by both SPM and its pentameric bundle are slightly cation selective but not voltage-dependent. The structural studies showed that the SPM and 5-SPM possess mainly an α-helical structure ($\sim$40%) and are strongly embedded in the bilayer. This behaviour and the strong hydrophobic interactions occurring between helices in the bundle induce a strong stabilization of 5-SPM in the bilayer and would be responsible for the stepwise current fluctuations observed during the incorporation of 5-SPM into the membrane. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: amphipathic peptide; conformation; five-helix bundle; ion channel

INTRODUCTION

The last few years have seen a large increase in the use of peptides as shuttles to facilitate the cellular uptake of drugs [1–11]. Within the various peptide groups described so far, amphipathic peptides appear the most promising tools. In this area, we have previously shown that primary amphipathic peptides, when incubated with fibroblast cells, were able to rapidly transfer a probe into the cells with a final localization which was mainly nuclear [12–14]. These peptides presented the particularity to have a sequence composition derived from a hydrophobic signal peptide from *Caiman crocodylus* Ig (v) light chain and a polar nuclear localization sequence from the SV40 large T antigen. However, at high concentrations, these peptides became toxic for the cells and displayed antibacterial activity [15] suggesting the formation of ion channels in the cell membrane as described for melittin [16], peptaibols or other related peptides [17,18]. Reconstitution experiments performed with the primary amphipathic peptide Ac-Met-Gly-Leu-Gly-Leu-His-Leu-Leu-Val-Leu[10]-Ala-Ala-Ala-Leu-Gln-Gly-Ala-Lys-Lys-Lys[20]-Arg-Lys-Val-NH-CH_2-CH_2-SH (SP-NLS) showed that it forms ion channels in planar lipid bilayers or *Xenopus laevis* oocyte plasma membranes [19]. This amphipathic peptide which adopts an α-helical conformation also displayed a voltage-triggered insertion into lipid bilayers while the induced conductances were almost voltage-independent. The ion channels were selective for monovalent cations and did not display the typical multi-level channel behaviour involving the barrel stave model found with alamethicin [20,21]. In contrast, its ion

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channel properties suggested a magainin-like behaviour with the formation of pre-aggregates on the membrane surface [22]. According to the number of monomers, the size of the channel fluctuated and induced variations of the conductance values. In order to study the interactions between these primary amphipathic peptides and the lipid membrane, we decided (i) to introduce an intrinsic fluorescent probe in the hydrophobic part of the peptide to investigate the position of the peptide with respect to the membrane, (ii) to stabilize the helical state and (iii) to block the number of monomers making up the channel by building a template associated peptide.

Thus, the histidine in position 6 in the hydrophobic part of SP-NLS was replaced by a tryptophan giving the peptide Ac-Met-Gly-Leu-Gly-Leu-Trp-Leu-Leu-Val-Leu^{10}-Ala-Ala-Ala-Leu-Gln-Gly-Ala-Lys-Lys-Lys^{20}-Arg-Lys-Val-NH-CH_2-CH_2-SH called SPM (Scheme 1). Further, a strategy developed by Mutter's group (the template-assembled synthetic protein (TASP) approach, [23–26]) was applied for building a template-associated peptide. SPM has been covalently linked to a cyclic template containing 20 residues, designed especially to accommodate five of the above mentioned SPMs. Furthermore, owing to the tetrameric repeat unit of the cyclic template, all linkages are expected to point into the same orientation. The synthesis of the linear peptide together with that of the template-assembled construct has been described elsewhere [27].

In this paper, we describe the conformational consequences induced by the replacement of His^6 by Trp^6 in the hydrophobic part of SPM and we compare this behaviour with the pentameric aggregate (5-SPM) (Scheme 2). The fluorescent studies allowed us to localize SPM and its pentameric aggregate, 5-SPM, with respect of the membrane. Finally, a comparison of the ion channel properties of both compounds shows different behavioural characteristics when incorporated in planar lipid bilayers.

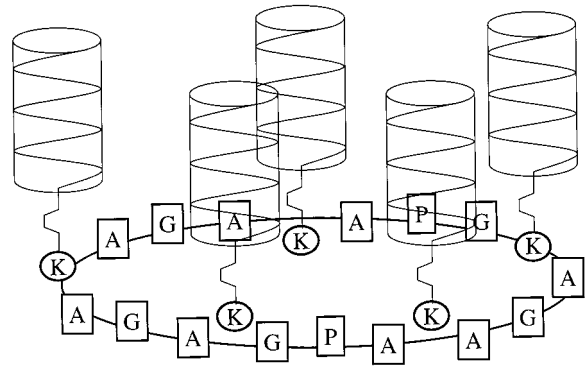
MATERIALS AND METHODS

Peptides

The synthesis of 5-SPM has been described elsewhere [27]. Briefly, SPM was synthesized from H_2N-

Ac-M-G-L-G-L-W-L-L-V-L^{10}-A-A-A-L-Q-G-A-K-K-K^{20}-R-K-V-NH-CH_2-CH_2-SH

Scheme 1 Chemical sequence of SPM.



Scheme 2 Representation of the assembled template 5-SPM.

AEDI-Expansin resin and purified by reversed phase high performance liquid chromatography (RP-HPLC) and the cyclic peptidic template obtained by cyclization of the lysine Boc protected peptide. The cyclic peptide was then submitted to side chain deprotection and to iodoacetylation. The final 5-SPM peptide was obtained after reacting the pentaiodoacetylated peptide and the linear SPM. Amino acid analysis and matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectra unambiguously identified the 5-SPM.

Spectroscopic Measurements

Circular dichroism (CD) measurements were carried out on a Jasco Model 810 dichrograph at a scan speed of 20 nm min^{-1} using quartz cells with 1 mm path length. The spectra shown are the results of two scans. Peptide concentrations in water were in the 0.1–0.2 mg ml^{-1} (40 to 80 μM) range.

Fluorescence spectra were recorded on a spectrofluorimeter Spex fluorologue Model 1681 (Jobin–Yvon, Paris, France). The excitation wavelength was adjusted to 280 nm corresponding to the absorption maximum of tryptophan residues. The emission spectra were recorded in the 300–420 nm range with a band pass of 4 nm. Phospholipid vesicles (small unilamellar vesicles) were prepared by a 10 min sonication procedure and aliquots of vesicle suspensions were progressively added to the peptide dissolved in water and which had an optical density of 0.1 at 290 nm.

Nuclear magnetic resonance (NMR) experiments were recorded on 400 or 600 MHz Bruker AMX spectrometers. All data were acquired using procedures described previously [28].

The theoretical analysis of the helical content was achieved using the AGADIR algorithm available from the web site: http://www.embl-heidelberg.de/Services/serrano/agadir/agadir-start.html [29].

Peptide Reconstitution in Planar Lipid Bilayers

For single-channel recordings, peptide reconstitution was made with Montal–Mueller type bilayers [30] or with bilayers made at the tip of patch clamp pipettes [31]. The Montal and Mueller method used large membrane area formed over a hole ($\varnothing = 150$ μm) in a Teflon film (thickness: 10 μm) pretreated with hexadecane/hexane (1:40, v/v) separating two half glass cells [19]. The current fluctuations were recorded using a BLM 120 amplifier (Bio-logic, Claix, France). In 'tip-dip' experiments, the tip of the pipette could be estimated between 0.5–1 μm. Transmembrane currents were amplified through a Bio-logic RK300. The different recordings were stored on a digital tape recorder DTR-1200 (Bio-logic) and analysed with Satori 3.01 software (Intracell, Royston, UK). The lipid used for both methods was diphytanoylphosphatidylcholine (DPhPC) from Avanti Polar Lipids (Alabaster, USA) dissolved in 1% hexane for large bilayers or 0.1% in hexane in the pipette configuration. The electrolyte was 1 M KCl or 1 M NaCl buffered with 10 mM sodium phosphate at pH 7.4.

Selectivity experiments were carried out by imposing a KCl gradient through the lipid bilayers: from 0.1 M on the *cis* side to 1 M on the *trans* side. The zero-current potential (E_{rev}) was corrected by subtracting the asymmetric potential due to the salt gradient ($E_{rev} = V_{i=0} - V_{asymmetric}$).

RESULTS

Conformational Structure of SPM and 5-SPM

When dissolved in water, the CD spectrum of SPM showed a minimum at 205 nm with a shoulder around 220 nm and a maximum at about 190 nm (Figure 1). Such a spectrum is characteristic of a major α-helical structure. The amount of helical form is slightly increased upon the addition of 0.5% octylpolyoxyethylene (OPOE) (see Figure 1), the latter being the detergent used for all further lipid bilayer experiments. From the intensity of the 220 nm band, the maximum helical content can be estimated to be about 40% [32]. The CD spectrum of 5-SPM in water revealed an enhancement of the helical content compared to SPM (Figure 1). Taking into account the cyclic template contribution ($\sim$ 1300 deg cm^{-2} mol^{-1} at 222 nm as deduced from figure 8 in [27] but now calculated on the basis of a peptide containing 145 residues), it can be stated that the helical content is increased upon going from SPM to 5-SPM. Again, addition of 0.5% OPOE induced a slight increase of the helical content (Figure 1).

In water, the high concentration of SPM required for the NMR spectroscopy induced strong interactions and the resonance lines were too broad to allow confident interpretations. Nevertheless, in presence of 20% acetonitrile, the identification of the precise localization of the α-helical domain could be achieved. The various sequential and medium range nuclear Overhauser effects (NOEs; Figure 2) showed mainly (i, $i+3$) αN and $\alpha\alpha$ contacts covering the entire hydrophobic domain. These NOEs confirmed the existence of an α-helical structure, covering the *N*-terminal part of the peptide. Unfortunately, NMR on 5-SPM could not give any information about the localization of the α-helix. Indeed the required concentration of 5-SPM for these experiments provoked a severe line broadening, preventing any interpretation whatsoever.

Lipid Binding Properties

To study the lipid–peptide interactions, we used the fluorescence properties of the Trp[6] residue present within the hydrophobic sequence. In water, the fluorescent emission of SPM presented a maximum centered at 357 nm. The addition of DOPG (dioleoylphosphatidylglycerol) or DOPC (dioleoylphosphatidylcholine) vesicles induced a blue shift of the fluorescence emission from 357 to about 330 nm and increased the fluorescence intensity (Figure 3). This behaviour characterizes a Trp residue going from a polar environment to a non-polar one indicating for the latter situation a large embedding of the Trp residue in the hydrophobic domain of the lipid phase [33]. Furthermore, from the evolution of the fluorescence spectra (Figure 3(A and B)) which show a progressive shift in the case of DOPC while this shift occurs at very low lipid/peptide ratios for DOPG, it can be stated that the affinity for DOPG is higher than for DOPC, which agreed with the charges carried by the phospholipids and the peptide. For 5-SPM in water, however, the fluorescence spectrum shows a maximum at 342 nm. This result showed that the Trp residue in 5-SPM was in a less polar environment, which is in agreement with the design of the peptide which leads to associated

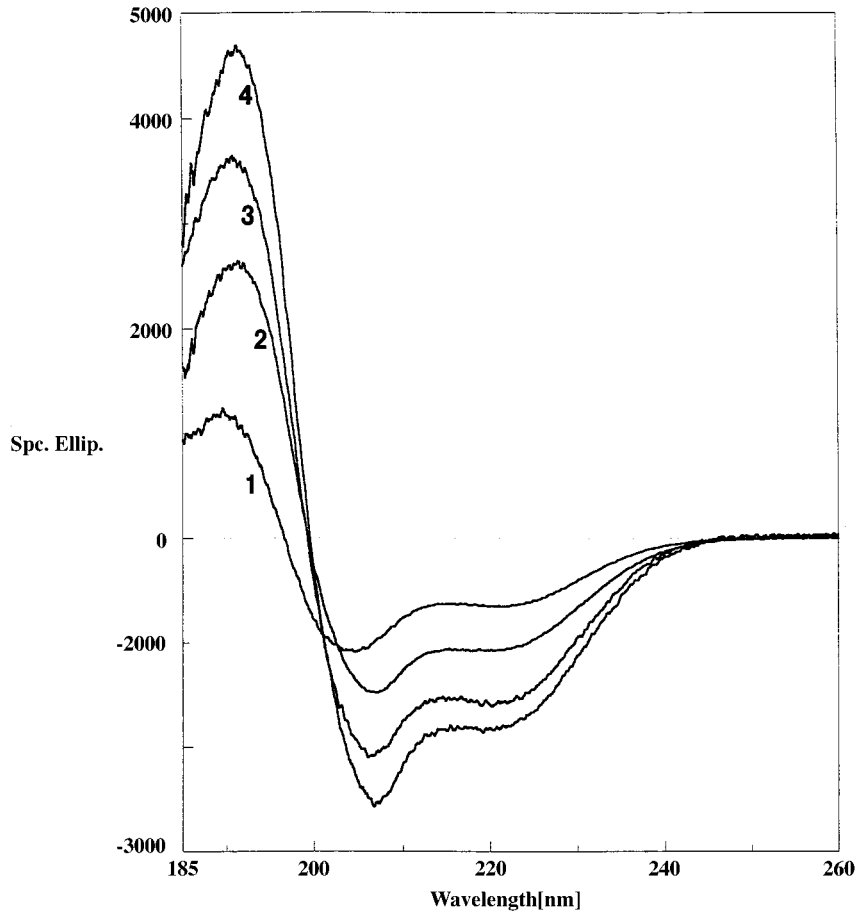


Figure 1 CD spectra of (1) SPM in water, (2) SPM in 0.5% OPOE, (3) 5-SPM in water and (4) 5-SPM in 0.5% OPOE. Peptide concentrations were 0.15 mg ml^{-1}. The ellipticity is in deg cm^{-2} mol^{-1}. Note that the contribution at 220 nm of the cyclic peptides as deduced from figure 8 in [27] is 1500 deg cm^{-2} mol^{-1} which is much lower than the difference between the spectra of SPM and 5-SPM. Taking into account this contribution the helical content in 5-SPM can be estimated at 55%.

strands. The addition of either DOPG or DOPC vesicles induced a blue shift down to 330 nm (Figure 4) of the maximum of fluorescence and an increase of the fluorescence emission. Again, as was observed for SPM, the affinity for DOPG is higher than that for DOPC (data not shown).

Ion Channel Properties

Reconstitution experiments in planar lipid bilayers were carried out with both SPM and its pentameric aggregate 5-SPM at 10^{-13} M in 0.5% OPOE, detergent which allowed a better incorporation of these peptides. When a Montal–Mueller bilayer type was doped by SPM, discrete current fluctuations were generated by potential application (Figure 5). The associated amplitude histogram allowed the calculation of two corresponding major conductance values of (75 ± 10) pS and (950 ± 70) pS in 1 M KCl. When the electrolyte KCl was replaced by NaCl, the peptide displayed a very similar behaviour and the major conductance values were (70 ± 10) pS and (900 ± 70) pS. SPM induced the same channels

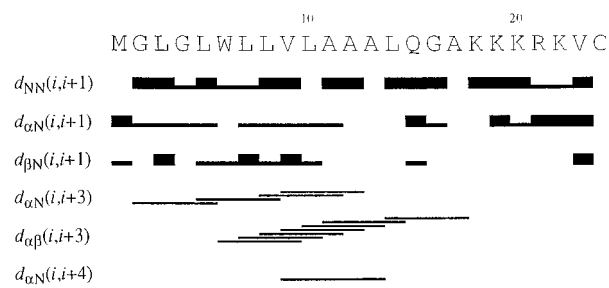


Figure 2 Medium-range NOE contacts of SPM (2 mM) in water containing 20% acetonitrile. The thickness of the horizontal bars is indicative of the NOEs' relative intensities.

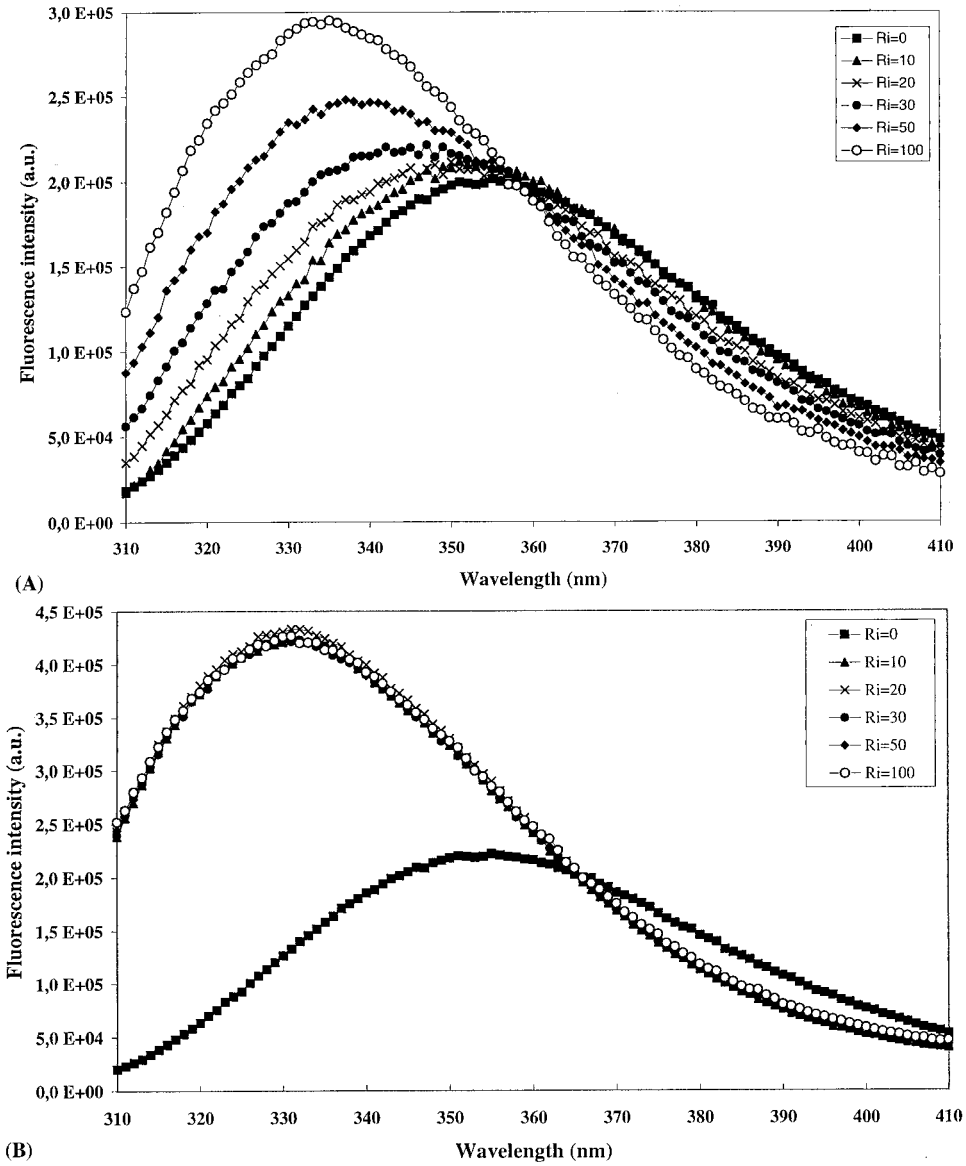


Figure 3 Fluorescence spectra of SPM (all intensities are given in arbitrary units): (A) in the presence of vesicles of DOPC; (B) in the presence of vesicles of DOPG. The lipid/peptide ratios are given in the insets.

when bilayers were made at the tip of a patch clamp pipette (data not shown). No channel activity was observed when the detergent (0.5% OPOE) alone was added to the bathing solution.

In the 'tip-dip' configuration, the template (10^{-13} M in 0.5% OPOE) induced stepwise current fluctuations which remained open for a long period of time (Figure 6). These channels present a single conductance value of (70 ± 6) pS in 1 M KCl against (60 ± 5) pS in 1 M NaCl.

The ionic selectivities of the channels formed by both SPM and 5-SPM were investigated by measuring the zero-current potential after installation of a salt gradient (0.1 M/1 M KCl, *cis*/*trans*) through a DPhPC bilayer doped by 10^{-12} M of peptide. The permeability ratios P_K/P_{Cl} were calculated according to the Goldman–Hodgkin–Katz method [34]. This ratio was 1.5 (with $E_{rev} = 8$ mV) and 1.7 (with $E_{rev} = 10$ mV) for SPM and 5-SPM, respectively, indicating that both peptides form cation selective channels.

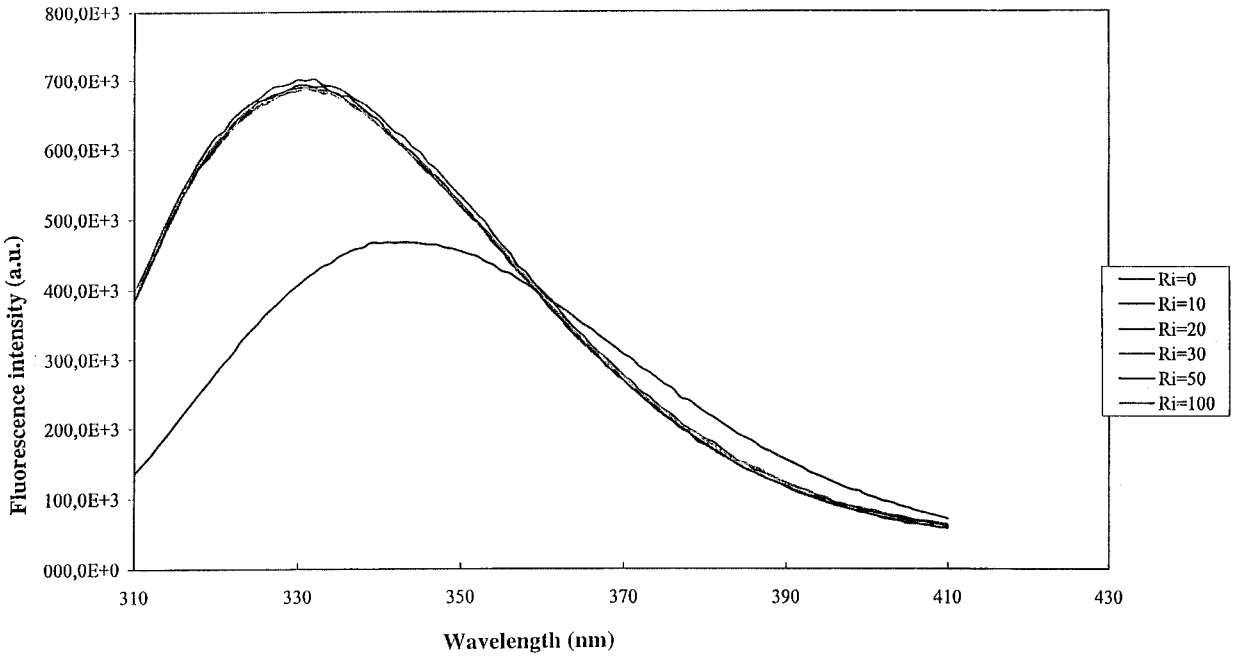


Figure 4 Fluorescence spectra of 5-SPM in the presence of DOPG vesicles.

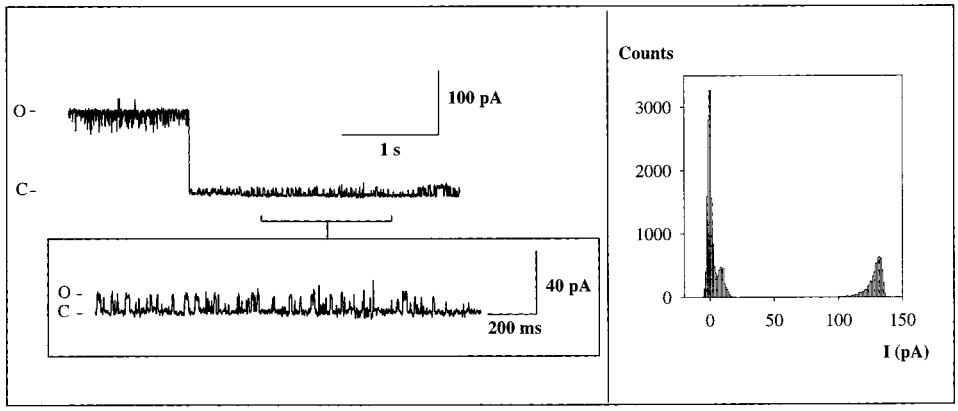


Figure 5 Single-channel current induced by SPM into DPhPC planar bilayers. Peptide concentration was 10^{-13} M in the measurement compartments containing buffered 1 M KCl. The applied voltage was 140 mV. The insert shows a part of the upper trace with an enlarged scale. The associated amplitude histogram allowed the calculation of different conductance values of (75 ± 10) pS and (950 ± 70) pS. 'C' denotes the closed state and 'O' the open state.

DISCUSSION

In a previous work [12] using CD, fourier transform infra red (FTIR) and NMR, we have demonstrated that the SP-NLS peptide is non structured in water. The substitution of His^6 by Trp induced strong modifications to the structure since SPM shows in water a CD spectrum suggesting the presence of about 40% α-helix. This amount is still increased by the presence of OPOE. NMR experiments shows the α-helical domain covers the hydrophobic domain.

These results were corroborated by a theoretical study using the AGADIR algorithm, which predicts the α-helical content of short peptides. A virtual Trp scan covering the hydrophobic region (Table 1) suggests that the crucial position for a Xxx→Trp substitution is found at position 6, namely His in SP-NLS. Such a substitution would strongly enhance the propensity to induce a α-helical structure

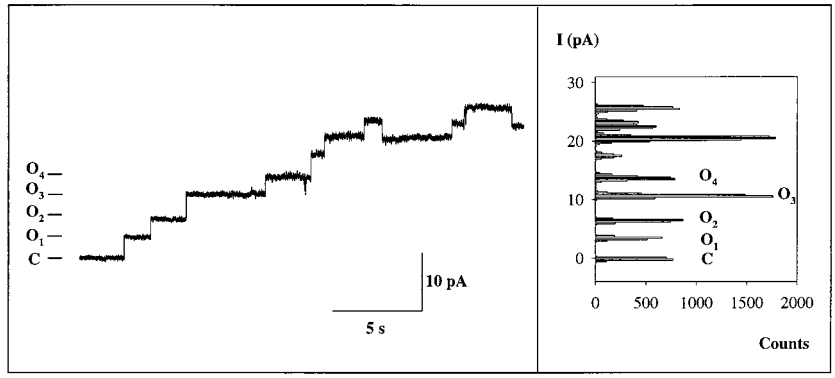


Figure 6 Single-channel trace induced by 5-SPM at 10^{-13} M in a DPhPC bilayer. The electrolyte was buffered 1 M KCl and the applied voltage was 50 mV. The corresponding amplitude histogram allowed the calculation of a mean conductance value of (70 ± 6) pS. 'C' denotes the closed state and 'O' the open state.

Table 1 Variations of the Predicted Helical Content Restricted to the Hydrophobic Domain as a Function of the Position of the Trp Residue

W position	SP-NLS	2	3	4	5	**6**	7	8	9	10	11	12	13	14	15	16	17
Helical content	23	25	18	42	10	**49**	10	11	21	13	28	24	10	13	38	20	11

since an average of 40% was obtained with SPM compared with 17% for SP-NLS in water. In addition, this analysis confirms that the strong enhancement of the α-helical form extends over nearly all the hydrophobic domain of the peptide while the charged *C*-terminal NLS sequence remains non-structured.

CD experiments showed that the conformational state of 5-SPM in water is almost identical to the one of SPM obtained in the presence of OPOE. This result indicates that the hydrophobic interactions between the neighbouring peptides in 5-SPM favour the folding of the aggregate and the α-helical structure. This result agrees with previous observations made on the four-melittin bundle where the linking to a template enhances the helical content [26].

About the embedding of SPM and 5-SPM into lipid bilayers, fluorescence measurements indicate that both insert into the membrane. Indeed, the blue shift accompanied by an enhancement of the fluorescence intensity for SPM and 5-SPM is indicative of Trp embedded in the lipid core [34], suggesting an anchoring of the peptides into lipid bilayers through their hydrophobic domains.

The ability for the peptide SPM to induce channel formation in artificial lipid bilayers was analysed under the same conditions as already carried out for the parent peptide SP-NLS [19]. Typical traces (Figure 5) clearly showed that SPM did not induce the typical multi-level behaviour previously observed with alamethicin, i.e. 'the barrel-stave model' with increasing conductance increments between sub-states [20,21]. On the contrary, this figure shows two different non-cooperative levels of conductance (75 pS and 950 pS) reminiscent of the behaviour observed with magainin [22]. Indeed, the latter also induces two main conductance levels occurring independently in separate trials, suggesting the formation of conducting aggregates by the association of a constant number of helices. Here if the 75 pS channel can be attributed to the aggregation of five monomers since the same conductance value is found with 5-SPM, the upper 950 pS channel would be due to a hexameric aggregate. This result is in agreement with the 900 pS channels attributed to the hexameric aggregate from alamethicin dimers [35]. Nevertheless, it is difficult to attribute unambiguously the number of monomers constituting an aggregate because the nature of the monomer influences strongly the auto-association of peptides. For example, the conductance values found for the four melittin TASP-bundle (600 pS in 1 M KCl [26]), for the four alamethicin TASP-bundle (250 pS in [36]) and for the tethered tetrameric M2δ synporin (20 pS in 0.5 M KCl), [37] are completely different although these bundles are composed of four α-helices. We can note that two major single-channel conductances (250 pS and 875 pS) were also determined for SP-NLS in OPOE [19] and they

could represent the conductance of the pentameric and hexameric aggregats of SP-NLS (even if the replacement of His^6 by Trp^6 seems to decrease the ionic conductance of the smallest aggregate).

Surprisingly, 5-SPM presented a behaviour in planar lipid bilayers totally different from SPM. 5-SPM induced regular stepwise current fluctuations commonly described for bacterial outer membrane proteins called porins [38]. This unusual behaviour for a template-assembled protein indicates that the incorporation of 5-SPM molecules is continuous, stabilized and that each channel remains open during the incorporation of other 5-SPMs. The strong hydrophobic interactions between the SPM monomers bound to the template inducing the embedding of the aggregate and the absence of voltage-dependence would provoke a stabilization of the 5-SPM in the membrane and the constant opening of the channel. Each step presents a similar conductance value of 70 pS in 1 M KCl, a value very close to the 40 pS found in 0.5 KCl with the pentameric channel protein mimicking the pore structure of the nicotinic cholinergic receptor [39].

Interestingly, the cation/anion permeability ratio of 1.5 and 1.7 for SPM and 5-SPM, respectively, can be compared of the weak cationic selectivity of the neutral alamethicin peptide [40]. This result seems to indicate that the strong positively charged *C*-terminus of both compounds is not involved in the channel activity.

In conclusion, we have shown that the replacement of His^6 in the hydrophobic sequence of SP-NLS by a tryptophan increased the α-helical structure of this peptide (SPM). The α-helical content is still slightly enhanced when SPM is engaged in a synthetic pentameric bundle 5-SPM. Both SPM and 5-SPM are strongly embedded into lipid bilayers and induce no voltage dependent ion channels into planar lipid bilayers but with a particular behaviour for 5-SPM where a stepwise incorporation involving a strong stabilization of the pore forming aggregate in the membrane was observed. These results agree with a mechanism where the channels formed by aggregates of SPM and 5-SPM, are already functional and do not require a previous adsorption on the membrane surface before embedding.

Acknowledgements

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