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Tilted peptides: a structural motif involved in protein membrane insertion?‡

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Abstract: Tilted peptides are short hydrophobic protein fragments characterized by an asymmetric distribution of their hydrophobic residues when helical. They are able to interact with a hydrophobic/hydrophilic interface (such as a lipid membrane) and to destabilize the organized system into which they insert. They were detected in viral fusion proteins and in proteins involved in different biological processes involving membrane insertion or translocation of the protein in which they are found. In this paper, we have analysed different protein domains related to membrane insertion with regard to their tilted properties. They are the *N*-terminal signal peptide of the filamentous haemagglutinin (FHA), a *Bordetella pertussis* protein secreted in high amount and the hydrophobic domain from proteins forming pores (i.e. ColIa, Bax and Bcl-2). From the predictions and the experimental approaches, we suggest that tilted peptides found in those proteins could have a more general role in the mechanism of insertion/translocation of proteins into/across membranes. For the signal sequences, they could help the protein machinery involved in protein secretion to be more active. In the case of toroidal pore formation, they could disturb the lipids, facilitating the insertion of the other more hydrophilic helices. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: signal sequence; hydrophobicity; pore formation; membrane translocation; molecular modelling

INTRODUCTION

Many important biological processes involve the partitioning of peptides into lipid membranes. Such peptides are typically quite hydrophobic. A few years ago, we discovered a peculiar property of hydrophobic fragments in viral fusion proteins [1]. When we modelled those short peptides (10–20 residues) as α-helices, we found that they had a gradient of hydrophobic amino acids along their axis [2]. They are not only amphipathic (hydrophobic on one side, less hydrophobic on the other side), but also their net hydrophobicity increases from one end of the helix to the other. Because of this 'top-to-bottom' hydrophobic asymmetry, we predicted that such peptides would have an equilibrium tilt when they are at a hydrophobic/hydrophilic interface, such as the lipid/water interface [3]. We call them 'tilted' or oblique peptides. The importance of the helical conformation was notably assumed by using mutants. For many tilted peptides, we have designed mutants without the hydrophobic gradient, i.e. non-tilted [4–7]. Experimentally, those mutants induced significantly less lipid perturbation, strongly suggesting a correlation between the tilt and lipid destabilization. This correlation between calculations and experimental results exists only when the peptides are assumed as α-helices, not as β-strands.

Neutron diffraction experiments as well as NMR and electron paramagnetic resonance (EPR) approaches have confirmed the existence of tilted peptides in bilayers [8,9] and the importance of the tilted configuration for the fusogenic activity [10].

Tilted peptides were notably found in viral fusion proteins, such as the gp32 fusion protein of the simian immunodeficiency virus (SIV), where they are involved in the first steps of the fusion between the host cell and the viral membrane [4]. They were also detected in proteins involved in lipid metabolism and in different biological processes involving membrane insertion or translocation of the protein in which they are found [11–13]. All those processes need the destabilization of the membrane organization. As mentioned, we have shown that the hydrophobicity gradient is related to the lipid-destabilizing activities of tilted peptides, by using mutants [4,6].

In this paper, we analyse the sequence of different proteins inserting or traversing the membrane with regard to the presence of a potential tilted fragment. We suggest that such motifs could have a more general role in the mechanism of insertion of proteins into membranes.

MATERIALS

Egg phosphatidylcholine (PC), phosphatidylglycerol (PG) and cardiolipin (CL) were purchased from Sigma (St Louis, USA).

Abbreviations: FHA, filamentous haemagglutinin; ColE1, colicin E1; ColIa, colicin Ia; pf, pore-forming.

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Egg phosphatidylethanolamine (PE) was from Lipid Products (Nr. Redhill, Surrey). Octadecyl rhodamine chloride (R18) was from Molecular Probes (Eugene, OR).

Peptides were synthesized by conventional solid phase peptide synthesis, using Fmoc for transient NH_2-terminal protection, and were characterized using mass spectrometry. Peptide purity was between 80 and 85%, as indicated by analytical HPLC. *N*-and *C*-extremities were acetylated and amidated, respectively.

METHODS

Molecular Modelling of Peptides

Peptides were constructed as three-dimensional α-helices using Hyperchem 6.0 (Hypercube Inc.). Their conformation was minimized by the Polak–Ribiere algorithm in an AMBER force field with a gradient delta less than 0.1 kcal/(Å mol).

Membrane Insertion

We inserted peptides into an implicit bilayer using the Integral Membrane Protein and Lipid Association (IMPALA) method developed by Ducarme *et al.* [14]. It simulates the insertion of any molecule (protein, peptide or drug) into a bilayer by adding energy restraint functions to the usual energy description of molecules [15–17].

The lipid bilayer is defined by C(*z*), which represents an empirical function describing membrane properties. Two restraints simulate the membrane, one the bilayer hydrophobicity (E_{pho}), and the other, the lipid perturbation (E_{lip}). The environment energy (E_{env}) applied on the peptide that inserts into the membrane is equal to the sum of both restraints. The method is described in detail in Refs 14 and 16.

A systematic procedure was performed to insert and orient the peptide into the membrane. During this process, the peptide systematically crosses the force field of the membrane from −40 to +40 Å with respect to the membrane centre in steps of 1Å. For each position along the *z*-axis, 2000 random orientations were tested. From among these 2000 positions, the orientation of minimum energy was selected. At the end of the systematic analysis, the procedure selects the position and the orientation of minimum energy from among all selected minima.

Calculations were performed on an Intel Pentium 4 processor with CPU 3.80 GHz and 4.00 Gb of RAM.

Liposome Preparation

Large unilamellar vesicles (LUV) were prepared by the extrusion technique [18] using an extruder (Lipex Biomembranes Inc., Vancouver, Canada). In brief, dry lipid films, which are mixtures of PE/PC/CL/PG (6 : 2 : 1 : 1 w/w), were hydrated for 1 h at 37 °C with a Tris buffer (10 mM Tris, 150 mM NaCl, 0.01% EDTA, 1 mM NaN_3, pH 8). The resulting suspension was submitted to five successive cycles of freezing and thawing and thereafter extruded 10 times through two stacked polycarbonate filters (pore size 0.08 μm) under a nitrogen pressure of 20 bars.

The phospholipid concentrations were determined by phosphorus analysis [19].

Lipid-mixing Experiments

Mixing of liposome membranes was followed by measuring the fluorescence increase of R18, a lipid soluble probe, occurring after the fusion of labelled and unlabelled liposomes, as described [6]. Labelled liposomes were obtained by incorporating R18 in the dry lipid film at a concentration 6.3% of the total lipid weight. Labelled and unlabelled liposomes were mixed in a weight ratio 1 : 4 and a final concentration of 50 μM in 10 mM Tris, 150 mM NaCl, 0.01% EDTA, 1 mM NaN_3, pH 8. Incubation of labelled and unlabelled vesicles in buffer alone did not modify the fluorescence intensity. Fluorescence was recorded at room temperature (λ_{exc}: 560 nm, λ_{em}: 590 nm) on an LS-50B Perkin Elmer fluorimeter.

Core-mixing Experiments

The mixing of liposome contents was monitored using the core-mixing assay of Kendall and MacDonald [20]. Liposomes (LUV) were prepared as described above in 10 mM Tris-HCl buffer, 150 mM NaCl, 1 mM NaN_3 (pH 8.0) and containing calcein at 0.8 mM and $CoCl_2$ at 1.0 mM or EDTA at 20 mM. Untrapped solutes were removed by one elution on a Sephadex G-75 column with 10 mM Tris-HCl, 150 mM NaCl, 1 mM NaN_3 buffer, pH 8.0. In a standard experiment, calcein, Co^{2+}- and EDTA-containing vesicles were mixed at 1 : 1 molar ratio in a 10 mM Tris-HCl buffer, pH 8.0 (150 mM NaCl, 1 mM NaN_3). When peptides were added, the calcein fluorescence was monitored at room temperature (λ_{exc}: 490 nm, λ_{em}: 520 nm) as a function of time on an LS-50B Perkin Elmer fluorimeter. Co^{2+} (0.4 mM chelated with citrate at 1 : 1 mol/mol) was present in the medium to avoid fluorescence due to leakage of vesicle contents. The maximum fluorescence was determined in presence of Triton X-100 0.5% (EDTA 10 mM).

Leakage of Liposome Vesicle Contents

The 8-hydroxypyrene-1,3,6-trisulfonic acid/*p*-xylylene bis(pyridinium) bromide (HPTS/DPX) assay of Ellens *et al.* [21] was used to monitor vesicle leakage. The assay is based on the quenching of HPTS by DPX. HPTS and DPX are both encapsulated in the aqueous phase of the same liposomes. Leakage of vesicles was followed by measuring the dequenching of HPTS released into the medium. Fluorescence was recorded at room temperature (λ_{exc}: 360 nm, λ_{em}: 520 nm) on an LS-50B Perkin Elmer fluorimeter.

Infrared Spectroscopy (FTIR) Measurements

Attenuated total reflection (ATR) infrared spectroscopy was used to determine the secondary structure of the lipid-bound peptides.

Spectra were recorded at room temperature on a Brüker Equinox 55 spectrometer equipped with a liquid-nitrogen-cooled mercury–cadmium telluride (MCT) detector at a resolution of 2 cm^{-1}, by averaging 512 scans. Lipid-bound peptides (see preparation below) were spread out on a germanium ATR plate (50 × 20 × 2 mm, Aldrich Chimica, with an aperture of 45° yielding 25 internal reflections) and slowly dried under a stream of N_2. Reference spectra of a germanium plate were automatically recorded after purge for 15 min with dry air and substracted from the recently run sample

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spectra. The plate was sealed in a universal sample holder and rehydrated by flushing the holder with N_2 satured with D_2O for 3 h at room temperature.

The determination of the secondary structure was carried out from the deconvolution of the amide I band (1700–1600 cm^{-1}) as described [22].

Peptide/lipid sample preparation. Twenty micrograms of the peptide and 100 μg lipids (same composition as for liposome preparation) were incubated for 1 h at room temperature in a 10 mM Tris-HCl, 150 mM NaCl buffer at pH 7.5. After incubation, the lipid–peptide mixture was filtered through an anisotropic hydrophilic YM membrane (cut-off 10 kDa) of a Centrifree micropartition system (Amicon) to separate lipid associated from free peptides. Phospholipid concentration was determined as mentioned above.

RESULTS

Since we have seen evidences of tilted peptides in eukaryotic signal sequences [12] and in proteins that spontaneously insert in membranes such as ColE1 [13], we asked ourselves whether such motifs could be retrieved in other related proteins.

Colicins are water soluble bacterial proteins that insert into membranes through conformational changes. They are composed of three domains of which the *C*-terminal domain, also called the pf domain, is responsible for the lipid insertion. Structurally, this domain is a 10-helix bundle. We have shown that the hydrophobic hairpin lying in the core of the soluble form of the pf domain is formed by two helices that have the properties of tilted peptides [13].

On the other hand, signal sequences of yeast invertase and apolipoprotein B-100 were previously shown to be tilted peptides, their secretory activity being related to the presence of a hydrophobicity gradient [12].

To extend these studies, we analysed the sequence of proteins related either to ColE1 or to signal peptides. For the former, ColIa and two members of the Bcl family, i.e. Bcl-2 and Bax, involved in apoptosis and structurally close to colicins [23], were chosen. For the signal peptides, we studied the extension of the FHA signal sequence. *Bordetella pertussis* FHA, an adhesive factor secreted in large amount, possesses an unusual signal sequence, notably characterized by a conserved *N*-terminal (*N*-ter) extension of 22 residues [24]. This extension is thought to be involved in the export rate at the bacterial inner membrane [25].

Table 1 shows the sequence and the properties of the fragments that were predicted as tilted on the basis of sequence analysis and on similarity with ColE1. They were then modelled as α-helices and their membrane insertion was calculated using the IMPALA method. Figure 1 shows that the *N*-ter extension of FHA (Figure 1(A)), helix 9 of ColIa (Figure 1(B)) and helix 5 of Bcl-2 (Figure 1(C)) and Bax (Figure 1(D)) insert obliquely in the model IMPALA membrane; the insertion

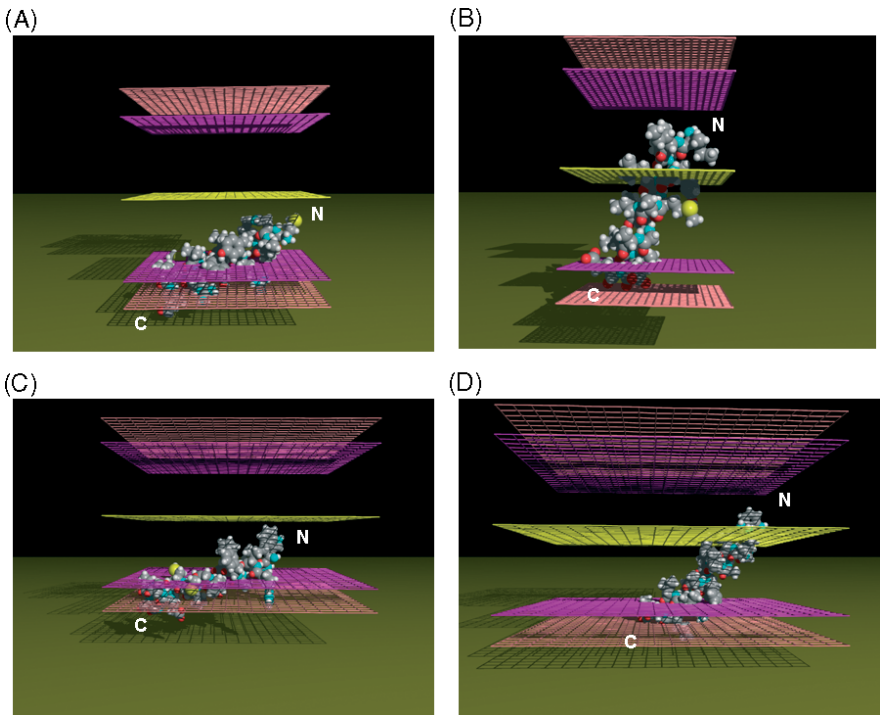

Figure 1 Most stable position of FHA *N*-ter peptide (A), ColIa helix 9 (B), Bcl-2 helix 5 (C) and Bax helix 5 (D) in the implicit membrane after IMPALA simulations. *N*-and *C*-ends of the peptides are indicated. Mid-plane is the bilayer centre ($z = 0$); first upper (beneath) plane is the lipid acyl chain/polar headgroup interface at 13.5 Å from the centre; second upper (beneath) plane is the lipid/water interface ($z = 18$ Å).

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angles are reported in Table 1. Since tilted peptides are defined as having an angle of insertion into the lipids between 30 and 60° towards the membrane, we predicted that the different fragments should belong to this class.

The FHA and the Bcl-2 peptides were tested experimentally for their lipid-destabilizing activities. The Bcl-2 fragment is highly similar to the Bax H5 (Table 1), sharing 37% identity and 80% similarity and the ColIa fragment is related to ColE1 H9 (88% similarity), for which we have clearly shown that it is able to disturb the lipid organization and hence has tilted properties [13].

As shown on Figure 2(A) and (B), respectively, the FHA peptide is able to induce lipid- and core mixing of liposomes containing phosphatidylglycerol and cardiolipin, mimicking the composition of the inner bacterial and the mitochondrial membranes. Lipid mixing induced by Bcl-2 H5 is similar to that obtained with the FHA peptide, with relative fluorescence intensity around 200 units after 15 min (data not shown). The disturbing properties of FHA are further confirmed by leakage assays (Figure 2(C)). The secondary structure of the FHA and Bcl-2 peptides was determined by ATR-FTIR in the presence of lipids. The amide I peak of both peptides has a maximum around 1650 cm^{-1}, where the helix structure absorbs (data not shown). The deconvolution of the spectra indicates, for both peptides, a helical conformation ($70 \pm 5\%$ for FHA and $60 \pm 5\%$ for Bcl-2).

DISCUSSION

Tilted peptides are short hydrophobic protein fragments that are able to disturb the organization of the molecular system into which they insert. They are characterized by an asymmetric distribution of their hydrophobic residues when helical, which induces a tilted orientation (around 45°) towards the membrane plane [27].

Peptides other than the tilted ones can induce lipid perturbation, such as lytic peptides (mellitin, pardaxin, LL-37, CM15, etc.) [28–31]. Those peptides are generally helical amphipathic peptides that induce lipid perturbation by making pores through a carpet or barrel–stave model [32]. Those peptides are oriented parallel to the membrane plane; oligomerization can induce a transmembrane configuration, in the case of the barrel–stave mechanism.

Tilted peptides are clearly involved in viral fusion. Hence, we have recently shown that the fusogenicity of the fusion peptide of different glycoproteins from enveloped viruses, such as gp41 from human immunodeficiency virus (HIV) [33], gp30 from bovine leukemia virus (BLV) [34] or gp 32 from SIV (Lorin *et al.*, *J. Pept. Sci.*, accepted), is related to its oblique lipid insertion. They are thought to be involved in the first steps of the fusion process, notably by facilitating the appearance of negatively curved lipid domains that help the formation of lipid toroidal pores [3].

Tilted peptides are assumed to be involved in other biological processes implying protein/membrane interactions. We have previously shown that the distribution of hydrophobic residues, and hence the orientation towards lipids, in some signal peptides is related to the secretion efficiency of the protein from which they originate [12].

On the other hand, we have recently suggested that the tilted properties of the hydrophobic helices of the pf domain of ColE1 should help insertion and formation of the toroidal pore [13].

In this paper, we have analysed different protein domains related to membrane insertion with regard to their tilted properties, i.e. the *N*-ter extension of the FHA signal peptide and the hydrophobic helix of colIa pf domain (H9), Bax (H5) and Bcl-2 (H5), the latter three being structurally related to ColE1. ColIa H9, Bax H5 and Bcl-2 H5 are helical in the 3D structure of the whole protein; for FHA and Bcl-2, this was confirmed

Table 1 Sequence and properties of the fragments studied. Mean hydrophobicity is calculated according to the consensus hydrophobicity scale from Eisenberg *et al.* [26]. Insertion angle is the orientation of the helix axis after IMPALA simulations and is measured between the helix axis and the membrane plane. The sequence of ColE1 is indicated for sake of comparison, and the insertion angle is from Ref. 13

Protein fragment	Sequence	Mean hydrophobicity (kcal/mol)	Insertion angle (°)
FHA 1–18	MNTNLYRLVFSHVRGMLV	0.15	30
ColE1 H9	IWGIAIVTGILCSYIDK	0.56	60
ColIa H9	IIGYGLLMAVTGALIDE	0.61	61
Bcl-2 144–162	WGRIVAFFEFGGVMCVESV	0.30	35
Bax 107–125	WGRVVALFYFASKLVLKAL	0.41	50

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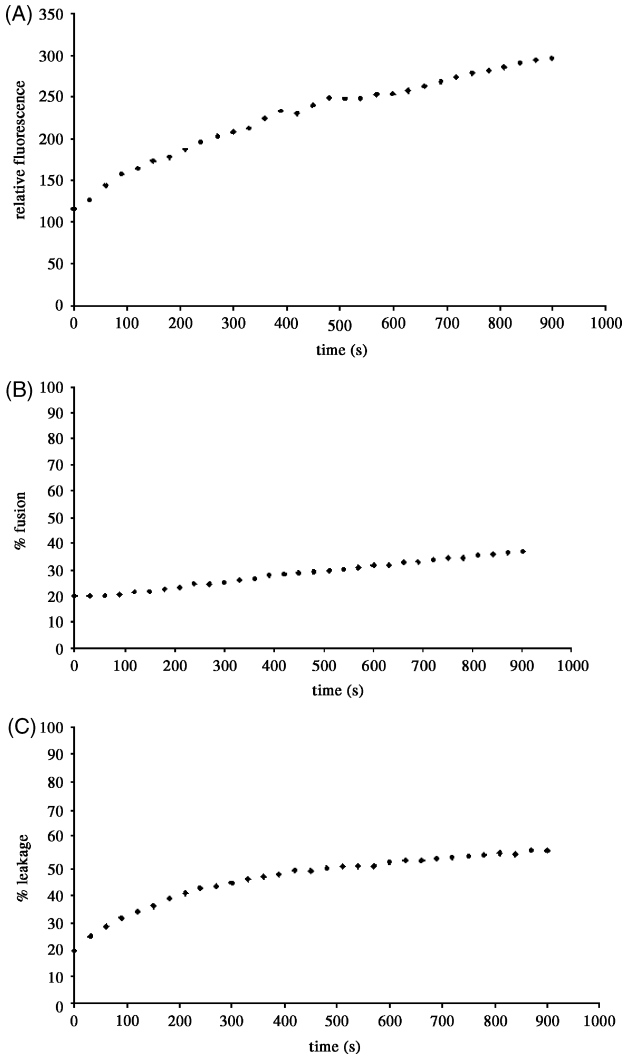

Figure 2 *In vitro* destabilizing effects of the FHA peptide dissolved in TFE at 150 μM on liposomes composed of PE/PC/CL/PG (6 : 2 : 1 : 1 w/w), corresponding to a peptide/lipid molar ratio of 1/10. In the three experiments, the contribution of TFE (1.6% final concentration) is subtracted from the curves. (A) Lipid-mixing assays: The peptide is added to a mixture (1 : 4 w/w ratio) of R18-labelled and unlabelled LUV and R18 fluorescence is followed at room temperature during 15 min. (B) Core-mixing assays: the peptide is added to a mixture of calcein and Co^{2+}- and of EDTA-containing vesicles mixed at a 1 : 1 molar ratio (10 mM Tris-HCl, 150 mM NaCl, 1 mM NaN_3 buffer, pH 8.0). The calcein fluorescence is monitored at 520 nm at room temperature as a function of time. Leakage (100%) is established by lyzing the vesicles with Triton X-100 0.5% (EDTA 10mM). (C) Leakage of liposomal contents induced by the FHA peptide monitored with the HPTS/DPX assay at room temperature. Peptide (dissolved in 100% TFE) is added to the liposome suspension as described in section 'Methods'. The increase of the HPTS fluorescence is monitored at 520 nm as a function of time. Leakage (100%) is established by lyzing the vesicles with Triton X-100 0.5% (EDTA 10 mM).

by FTIR measurements. Hence, a helical conformation was assumed in the calculations.

The molecular modelling approach predicts that the four peptides present tilted properties, i.e. an insertion angle into the model membrane between 30 and 60°, their mass centre being at the level of the phospholipid acyl chain/headgroup interface or below and a mean hydrophobicity between 0.1 and 0.9 [17].

The lipid-destabilizing activity was further validated for the FHA and Bcl-2 peptides by *in vitro* fluorescence assays. The other two peptides were not experimentally tested since they are closely related either to Bcl-2 H5 or to the colE1 H9 which we have recently characterized extensively [13]. Furthermore, a helical fragment encompassing the Bax peptide studied here was shown to bind lipids and to disturb their organization, releasing liposome-entrapped calcein [35].

The FHA and Bcl-2 peptides are able to induce liposome fusion and to perturb the physical integrity of the membrane as other tilted peptides do (such as the H9 colE1 fragment). The presence of cardiolipin and phosphatidylglycerol increases the effects of the peptides on the lipids, since the percentage of fusion or leakage is less marked in presence of PC/PE/SM/Chol liposomes usually used to mimic eukaryotic cell membranes (data not shown). This can be related to the fact that the bacterial inner membrane, which is the target of the FHA signal peptide, and the mitochondrial membrane, for Bcl-2, contains PG and CL. We have already observed that the lipid composition could influence the fusogenic activity of the tilted peptides. In particular, it has been shown that the presence of lipids inducing negative curvature (such as PE or PA) favours fusogenicity and lipid perturbation [36,37]. The influence of lipid composition on orientation and destabilizing activity has also been reported for lytic peptides, such as pardaxin [30,31].

As in the case of ColE1 H9, we suggest that the tilted peptides from colIa, Bax and Bcl-2 could be involved in the formation of a toroidal pore, which was evidenced for the whole proteins [38–40]. It should be noted that all tilted peptides are not thought to make toroidal pores, and all peptides that are described as making such structures are not necessarily tilted peptides (like the LL-37 peptide) [29]. Actually, signal peptides having tilted properties (i.e. from FHA (this study), yeast invertase or apo B-100 [12]) are not believed to make toroidal pores. It is also worth noting that some cell-signalling helical peptides, such as SA and SKP have a more transmembrane orientation. The tilt (between 15 and 30°) towards the membrane normal depends on the bilayer thickness but does not significantly influence the lipid organization [41].

From the predictions and the experimental approaches, we assume that tilted peptides could have a more general role in the mechanism of insertion of proteins into membranes. For the signal sequences, they could help the protein machinery involved in protein secretion (such as secA) to be more active by,

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for example, facilitating the interaction of the secreted protein with the membrane. In the case of toroidal pore formation, they could disturb the lipids creating fracture, facilitating the insertion of the other more hydrophilic helices that will form the pore, as already suggested for the hydrophobic hairpin of ColE1.

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