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Conformation and orientation of penetratin in phospholipid membranes

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Abstract: The binding, conformation and orientation of a hydrophilic vector peptide penetratin in lipid membranes and its state of self-association in solution were examined using circular dichroism (CD), analytical ultracentrifugation and fluorescence spectroscopy. In aqueous solution, penetratin exhibited a low helicity and sedimented as a monomer in the concentration range ~50–500 μM. The partitioning of penetratin into phospholipid vesicles was determined using tryptophan fluorescence anisotropy titrations. The apparent penetratin affinity for 20% phosphatidylserine/80% egg phosphatidylcholine vesicles was inversely related to the total peptide concentration implying repulsive peptide–peptide interactions on the lipid surface. The circular dichroism spectra of the peptide when bound to unaligned 20% phosphatidylserine/80% egg phosphatidylcholine vesicles and aligned hydrated phospholipid multilayers were attributed to the presence of both α-helical and β-turn structures. The orientation of the secondary structural elements was determined using oriented circular dichroism spectroscopy. From the known circular dichroism tensor components of the α-helix, it can be concluded that the orientation of the helical structures is predominantly perpendicular to the membrane surface, while that of the β-type carbonyls is parallel to the membrane surface. On the basis of our observations, we propose a novel model for penetratin translocation. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: vector peptides; circular dichroism; oriented circular dichroism; peptide–lipid interactions

INTRODUCTION

Certain peptides are capable of crossing the plasma membranes of mammalian cells [1–5]. What is particularly intriguing is the ability of some of these peptides to carry covalently linked hydrophilic molecules across the hydrophobic membrane barrier [2,5,6]. An understanding of how these peptides work will improve our understanding of peptide–membrane interactions in general, but may also be useful in the development of new drug delivery vectors [7].

Penetratins, a class of peptides derived from the third helix of the Antennapedia homeodomain (residues 43–58 RQIKIWFQNRRMKWKK), exhibit unique membrane translocation properties, and have attracted widespread interest because of their ability to directly target attached oligopeptides and oligonucleotides to the cytoplasmic and nuclear compartments of cells [6]. The internalization of penetratin into cells occurs at both 4 and 37 °C, is highly efficient and is non-cell-type specific. Sophisticated recognition mechanisms such as receptor-mediated endocytosis or specific interaction with chiral receptors and enzymes are not involved in the translocation of penetratin into cells, thus directing attention to whether the membrane translocating property of penetratin can be understood in terms of its interaction with the phospholipid bilayer. Indeed, recent fluorescence microscopic evidence suggests that penetratin can translocate across vesicle bilayers prepared from pure phospholipids [8]. However, the detailed mechanism of action is at present unknown.

The structure and orientation of penetratin has been determined in organic solvent/water mixtures, in sodium dodecylsulphate (SDS) micelles and in phospholipid monolayers at the air–water interface. Penetratin adopts an amphipathic helical structure in 30% (by vol) hexafluorisopropanol, in perfluoro-*tert*-butanol and in the presence of SDS micelles but is considerably less helical in water [9]. NMR measurements of a penetratin analogue (biotinyl-5-aminopentanoic acid-(43–58)) in SDS micelles show that penetratin is at least 50% helical with residues in the *N*-terminal half of the molecule (residues 44–55 or 43–51) adopting an α-helical conformation [9]. In a later study, penetratin was found to be oriented as a straight helix positioned with its *C*-terminus deep inside the SDS micelle and its *N*-terminus near the surface of the micelle [10]. An infrared spectroscopy study of penetratin at the air–water interface of phospholipid monolayers suggested that penetratin was mainly β-sheet and was oriented parallel to the membrane surface [11]. However, all these studies used model systems that cannot closely mimic the ordered and anisotropic environment of a lipid bilayer. Conformational studies in lipid bilayer

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vesicles have shown that penetratin can adopt both α-helical and β-sheet conformations dependent on phospholipid composition and peptide : lipid ratio [12,13]. By comparison with other translocating peptides with differing secondary structure, it was suggested that secondary structure is not the sole determinant of peptide translocation.

To define a specific mechanism of translocation for penetratin, determination of the orientation of penetratin with phospholipid bilayer membranes is highly desirable. In this connection, circular dichroism (CD) spectroscopy of helical peptides bound to aligned phospholipid multilayers can reveal the orientational distribution of helices with respect to the light propagation direction and can provide an unambiguous assignment of transmembrane or surface-associated helical orientations [14–18].

Here we use a combination of ultracentrifugation, fluorescence spectroscopy and oriented CD to examine the state of penetratin self-association in solution, its binding to membranes and its secondary structure and orientation in aligned phospholipid multilayers. A model for translocation is proposed on the basis of these investigations.

MATERIALS AND METHODS

Penetratin (RQIKIWFQNRRMKWKK) was kindly provided by Dr H. C. Cheng. It was judged to be greater than 95% pure from analytical HPLC and MALDI-TOF mass spectrometry. Egg lecithin phosphatidylcholine (egg PC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3[phospho-L-serine] (POPS) were from Lipid Products (UK) and used without further purification. Small unilamellar phospholipid vesicles (SUVs) were prepared by sonication as described previously [19]. Phospholipid multilayers were prepared, aligned and hydrated on a quartz slide from a suspension of sonicated vesicles according to established procedures [14,15].

Experiments were conducted under conditions in which all the peptide are bound to lipid, as determined by fluorescence anisotropy titration. The use of fluorescence anisotropy to determine biomolecular interactions has been described previously [20]. Fluorescence anisotropy (r) was recorded with a SPEX Fluorolog Tau-2 spectrometer using excitation and emission wavelengths of 280 and 350 nm respectively. Corrections for instrumental G-factors and scattered light were performed. Titrations were carried out using a fixed concentration of peptide (three initial concentrations: 2, 5 and 10 μM) and variable lipid concentration, ([L], range: 0–500 μM). The apparent partition coefficient, K_p, which quantitates the relative affinity of the peptide for the lipid and aqueous phase was determined from fitting the anisotropy titration data to the equation [21]

$$1/r = b + 1(/bK_p[L]) \quad (1)$$

where b is the y-intercept (equal to the reciprocal of the anisotropy in the limit of infinite lipid concentration) and $1/bK_p$ is the gradient of a $(1/r)$ versus $(1/[L])$ plot. The units of K_p are $(\text{mM})^{-1}$.

CD spectra of the vesicle suspensions were recorded in 1-mm pathlength cells. For the oriented films, the quartz plate was normal to the light propagation direction. An Aviv Model 62 DS spectrophotometer was used for all CD measurements (data interval of 1 nm, bandwidth of 2 nm, integration time 4 s per point). Five spectra of each sample were taken, averaged and smoothed. The concentration of peptide was determined by measuring the absorbance at 280 nm, as described previously [18].

Two methods for the analysis of CD spectra [17] were used to determine the orientation of penetratin helix in the lipid bilayer. The first method uses a qualitative comparison of the rotational strengths associated with α-helical and β-type transitions of the oriented film and vesicle solution spectrum. For this purpose, the CD spectra of penetratin in the vesicle suspension and in the aligned phospholipid multilayers were fitted to the sum of four Gaussian components. The parameters obtained from individual fitting of each spectrum were poorly defined. Therefore, the spectra of the aligned and unaligned samples were fitted globally. The Gaussian centers (wavelength) and widths (bandwidths) were common to both spectra and linked in the analysis, while the amplitudes were independent for the film and solution spectra, thus allowing a unique set of amplitudes to be extracted from the data. Inferences on the orientation of the secondary structural elements were made by comparing the amplitude (proportional to the rotational strength) of the vesicle–penetratin sample to that of the aligned multilayer sample. This method is similar to that employed by Woody in analyzing the structure and orientation of cytochrome oxidase in lipid membranes [17]. This analysis assumes that any changes in wavelength position of the component bands between multilayer and vesicle CD spectra are small in comparison to changes in the amplitudes of the component bands. An alternative method of analyzing the CD spectra, which does not rely on spectral deconvolution, was therefore examined.

The second method of assessing the orientation of the helical parts of penetratin was made by analysis of the CD difference spectrum, obtained by subtraction of the vesicle solution spectrum from that of the aligned multilayer sample [14]. The rationale behind this method is that only structures with CD transitions that are ordered with respect to the membrane plane carry rotational strength in the CD difference spectrum. This eliminates CD transitions from disordered or unstructured regions. For helical peptides, the CD difference spectrum can be used to extract a helix orientational order parameter (S_h), which describes the space and time-averaged orientational distribution of helices with respect to the light propagation direction. S_h is particularly sensitive to the orientation of peptides and proteins in membranes and is positive for transmembrane helices and negative for surface-associated helices. S_h was determined from the difference CD spectrum between the oriented film sample ($[\theta]^o$) and the vesicle sample ($[\theta]^v$): [14]

$$S_h = S^{ref} \times [f_{ref}/f_h] \times ([\theta]^v - [\theta]^o)/([\theta]^v - [\theta]^o)^{ref} \quad (2)$$

where f_h is the fractional helicy of the peptide. The published data for melittin were used to give estimates for the reference helicy (f_{ref}), reference order parameter (S^{ref}) and reference

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CD difference spectrum $([\theta]^{v} - [\theta]^{o})^{ref}$, since both the helicy and order parameter of melittin in bilayers are known independently from infrared spectroscopy measurements [14]. Equation (2) assumes that nonhelical segments have no preferred orientation with respect to the membrane plane. The ellipticity values at 208 nm were deemed to be most reliable for the order parameter calculation since the polarization of this band is known [15].

Sedimentation equilibrium experiments were conducted using Beckman Optima XL-A analytical ultracentrifuge equipped with absorption optics (An-Ti60 rotor, 12 mm, charcoal-filled epon centerpieces). Sedimentation experiments were conducted at 20 °C and 40 000 rpm for approximately 16 h. The equilibrium distributions were determined by scanning the solution columns at 300 nm. Samples were prepared in 100 mM Tris buffer, pH 7.4, at three different starting concentrations (45, 134 and 445 μM). To obtain the molecular weight and the contribution of nonsedimenting species to the baseline absorbance for each sample, the rotor speed was increased to 60 000 rpm to allow for simultaneous fitting of data at both 40 000 and 60 000 rpm using the program SEDEQ1B (kindly provided by Dr A. Minton, NIH, Bethesda). The theoretical partial specific volume for the penetratin peptide ($0.755\ cm^3\ g^{-1}$) and the solvent density ($1.001\ g\ ml^{-1}$) were calculated using the program SEDNTERP [22].

RESULTS

Sedimentation equilibrium analysis of the penetratin peptide was used to examine its state of self-association in aqueous solution. Figure 1 represents sedimentation equilibrium distributions at three starting concentrations (45, 134 and 445 μM) in Tris–HCl buffer at pH 7.4. The global fit of the data provides a molecular weight of 2200 Da, which agrees with the calculated value (2246 Da) for the penetratin monomer. Thus, there is no concentration-dependent self-association of penetratin under these conditions. Experiments conducted using sodium phosphate/sodium hydrogen phosphate buffer (pH 7.4, PBS) yielded a higher weight average molecular weight which was attributed to the binding of phosphate ions to the positively charged regions of the peptide (results not shown). Consequently, the experiments to be described were carried out in the presence of Tris–HCl buffer at pH 7.4.

The sensitivity of the two intrinsic tryptophan fluorophores in penetratin to the environment was exploited to determine the association of penetratin to bilayer vesicles. Penetratin associated with vesicles composed of 80% egg PC/20% POPS with affinities in the range 3 to $99 \times 10^3\ M^{-1}$ (Figure 2). Of particular note is the dependence of peptide affinity on initial peptide concentration suggesting the presence of a more complex interaction than a simple peptide partition. Since peptide–peptide self-associations in free solution are excluded from the sedimentation data, the results must be due to the presence of repulsive peptide–peptide interactions on the lipid

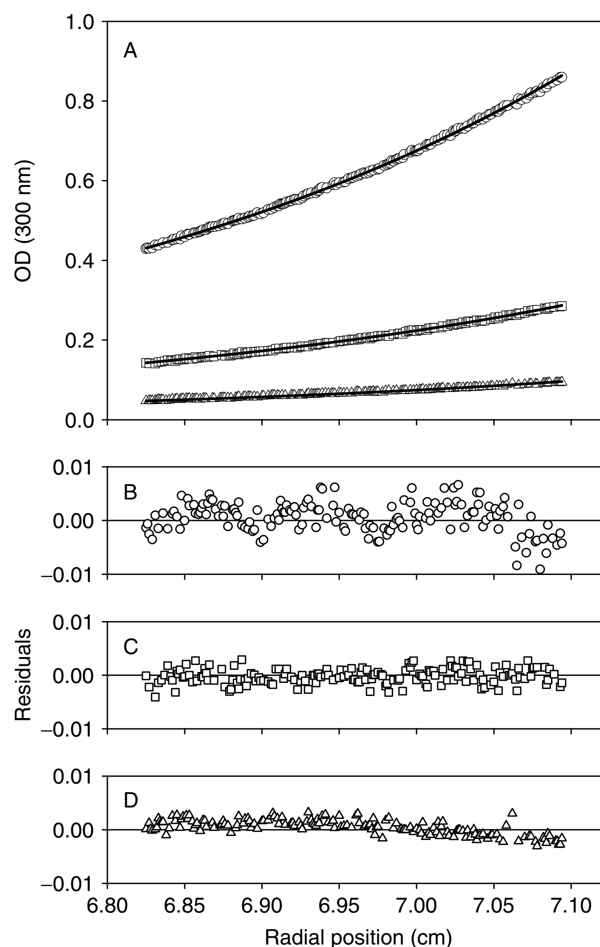

Figure 1 Sedimentation equilibrium distributions of the penetratin peptide. Samples were centrifuged at 20 °C for 16 h at 40 000 rpm and the absorbance at 300 nm recorded as a function of radial distance (Panel A). The experimental data refer to starting concentrations for the three samples of penetratin: 45 μM (triangles), 134 μM (squares) and 445 μM (circles). The best fit for each sample was obtained from global fitting of data obtained at 40 000 and 60 000 rpm. Panels B, C and D are the residuals for the fitted lines of the experimental data.

bilayer. Peptide–peptide repulsive interactions have been reported previously for charged, membrane-associating peptides [23].

The conformation and orientation of penetratin in solution, in vesicles and in lipid multilayers was examined using CD spectroscopy. In agreement with previous reports [9], we find the CD spectrum of penetratin in aqueous solution to be typical of an unordered conformation (results not shown). Addition of sonicated vesicles (80% egg PC/20% POPS, lipid-to-peptide mole ratio 50 : 1) results in a CD spectrum typical of a peptide with a significant degree of α-helical content as evidenced by the double minimum near 210 and 220 nm, and the positive CD at wavelengths lower than 205 nm (Figure 2, filled

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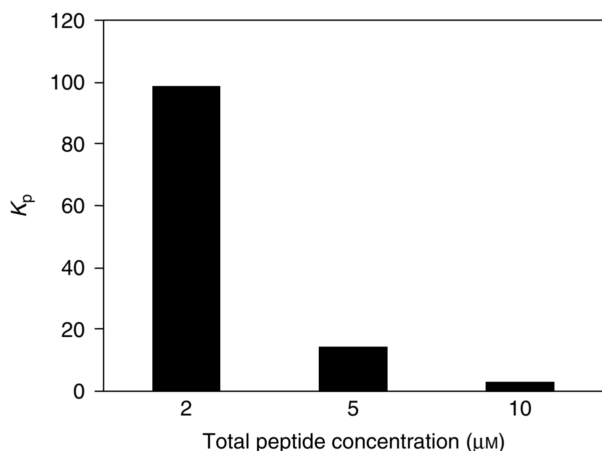

Figure 2 Influence of peptide concentration on the binding affinity of penetratin for egg PC/POPS (80/20%) vesicles. The partition coefficients (units: $(\text{mM})^{-1}$) obtained from tryptophan anisotropy titrations are plotted against total concentration of peptide.

Table 1 Band Parameters from the Parameter-linked Gaussian Deconvolution of Penetratin/egg PC/20% POPS CD Spectra

Linked Global Parameters		Floated Amplitudes, ($\times 10^3$ degree cm^2 dm^{-1})	
λ^a(nm)	Δ^b(nm)	$[\theta]^{\text{vesicle}}$	$[\theta]^{\text{multilayer}}$
192.4 ± 0.4	7.0	42.2 ± 0.2	30.3 ± 0.2
206.9 ± 0.1	6.0	-13.7 ± 0.4	1.3 ± 0.4
214.6 ± 0.4	8.0	-6.1 ± 0.5	-7.9 ± 0.2
223.4 ± 0.4	11.0	-7.9 ± 0.4	-2.7 ± 0.2

[a] Wavelength maximum of Gaussian component spectrum.
[b] Half of the bandwidth at e^{-1} times the peak height.

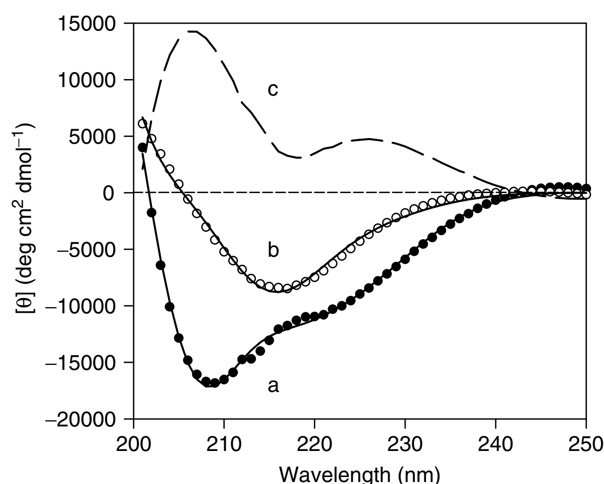

Figure 3 Circular dichorism spectra of penetratin in an aqueous suspension of vesicles (a) and in hydrated multilayers (b) of egg PC/POPS (80/20%) at a molar lipid/peptide ratio of 50 at 20 °C. (c) Difference spectrum (b–a).

symbols). This spectrum is similar to spectra of penetratin and its analogs in SDS reported by Berlose *et al.* [9]. By comparing the penetratin–vesicle complex ellipticity value at 222 nm with that of the penetratin–SDS complex and using the NMR-derived penetratin–SDS complex helicity value (56%) as a reference, we estimate that penetratin is approximately 52% helical in SUVs. Gaussian band analysis of the penetratin–vesicle complex CD spectrum shows that in addition to the α-helical transitions observed near 207 and 223 nm, there is some evidence for the presence of rotational strength due to the β-sheet near 215 nm (Table 1). The wavelength positions and widths of the Gaussian components are similar to those reported for cytochrome oxidase, a protein possessing both α-helix and β-sheet [17]. The CD spectrum of penetratin measured with the light incident normal to the plane of aligned penetratin–phospholipid multilayers is also displayed in Figure 3 (hollow symbols). Compared to the solution spectrum, the 207-nm band is absent, the 223-nm band amplitude is diminished and blue-shifted, and the point at which the spectrum crosses the wavelength axis is shifted to longer wavelength. These changes are represented by the difference CD spectrum, (i.e. that obtained by subtraction of the vesicle spectrum from that of the oriented film spectrum), as shown in Figure 3 (broken line).

A transmembrane orientation of the helical part of penetratin can be concluded on the basis of three observations. First, the absence of the long-axis polarized 207-nm π–π^* helix band and the diminished contribution of the long-axis polarized 223-nm n–π^* band in the CD spectrum of penetratin in aligned multilayers as compared to the unaligned complexes are consistent with a helical orientational distribution parallel to the light propagation direction and to the membrane normal, based on theories of CD and observations from other laboratories (Table 1, Figure 2) [14–16]. Second, the CD difference spectrum is similar in sign and shape to that of a poly-γ-methyl L-glutamate helix aligned with its long axis parallel to the light propagation direction [24]. Third, a quantitative analysis of the difference CD near 210 nm yields a positive value for the helix order parameter ($S_h = 0.54$), consistent with a transmembrane helix orientation (Eqn 1). In contrast, helices lying parallel to the membrane surface have a negative order parameter, and helices with a random orientation have an order parameter of zero.

The orientation of β-type structures is less defined than that of the α-helical segments of penetratin because of the reduced symmetry of the β-type structure as compared to the α-helix and the correspondingly more complex CD tensor [25]. Moreover, the β-type structures make a relatively minor contribution to the

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vesicle–peptide CD spectrum compared to the helical structures. As a consequence, only general qualitative conclusions regarding the orientation of β-type structures can be made. However, two observations are consistent with the presence of β-type carbonyls aligned parallel to the membrane surface. First, the ellipticity of the β-type n–π^* transition at 215 nm, whose magnetic dipole transition moment is directed along the carbonyl bond [26] is decreased in the aligned as compared to the solution of unaligned penetratin–lipid complexes (Table 1). Second, in the CD difference spectrum there is a trough in the region 215–220 nm, as compared with the gradual monotonic decrease seen in the difference spectrum of the transmembrane melittin helix (Figure 5 (c) of Ref. 14). These results are satisfied by any orientational distribution favouring an in-plane orientation of β-type carbonyls.

DISCUSSION

A number of alternative models have been considered for the mode of action of peptides with model membranes. The models can be divided into two major categories:

Pore Models

Barrel–stave model. This model involves the formation of transmembrane pores by the aggregation of several peptide monomers. The transmembrane helices are proposed to form bundles in which outwardly directed hydrophobic surfaces interact with the lipid constituent of the membrane, while inward facing hydrophilic surfaces produce an aqueous pore.

Toroidal pore model. In this model, a transmembrane helix orientation is obtained but the hydrophilic and the charged regions of the peptide monomers interact with the phospholipid head-groups that line the pore. Peptide–peptide association is not required in this model, in contrast to the barrel–stave model.

Non-pore Models

This model involves the formation of a layer of peptide monomers lying parallel to the surface of the membrane. The formation of a peptide layer in such a 'carpet-like' manner is thought to disrupt the phospholipid packing leading to disintegration of the membrane.

The major difference between the two alternative models is the orientation of the peptide relative to the lipid surface; perpendicular to the membrane surface if a transmembrane pore is formed, *versus* parallel to the membrane surface if a layer of peptide monomers is formed. Analysis of the oriented circular dichroism spectrum of penetratin in aligned phosphatidylcholine–phosphatidylserine multilayers demonstrated that the long axis of penetratin helix is inserted perpendicular to the membrane surface. This would be at odds with the hypothesis that the active translocating form of the molecule involves a large population of helices lying parallel to the membrane surface. With respect to other non-pore models, Prochaintz [6] has proposed that penetratin induces changes in lipid structure leading to reverse micelle formation to form an aqueous compartment within the lipid bilayer. Although such a spherical cavity is an attractive proposal, it is not consistent with the present experimental results which require that penetratin has an anisotropic and transmembrane orientation.

The results obtained here suggest that a transmembrane pore model should be considered. A barrel–stave model is compatible with the presence of transmembrane helices but incompatible with the monomeric nature of the peptide in solution, its reduced apparent affinity for membranes at higher peptide concentration (which suggests peptide–peptide repulsion on the lipid surface).

We propose an alternative two-state transient lipidic pore model [23] that relies on the observation of surface and inserted peptide states (Figure 4). First, monomeric penetratin binds preferentially to the charged head-group region of the bilayer causing a transition from random coil to β-turn (Figure 4a). As more peptides accumulate on the membrane, the membrane becomes locally deformed (thinned) and the net negative charge density on the membrane surface becomes neutralized; subsequently peptide binding becomes more difficult as charged peptide monomers are repelled from each other on the lipid surface. At a critical lipid–peptide ratio, the peptide monomers begin to form transmembrane helices drawing the membrane head-groups into a transient hydrophilic

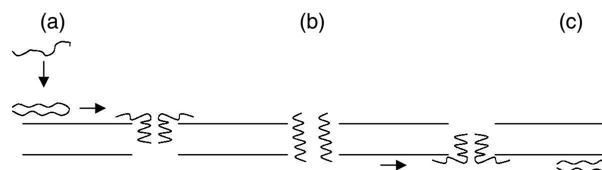

Figure 4 Model for penetratin translocation in model membrane bilayers. (a) Penetratin monomers bind to negatively charged phospholipid head-groups on the surface of membrane bilayers and undergo random-coil to β-turn conformational transition. Accumulation causes membrane thinning and a partial neutralization of membrane surface charge resulting in unfavourable energetics of peptide association on the lipid surface. A peptide transmembrane helix state becomes populated leading to the (b) transient formation of lipidic pores. (c) A transmembrane-to-surface orientational transition to the net negatively charged inner leaflet of the membrane completes the translocation process.

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toroidal pore (Figure 4b). The translocation process is completed by a transmembrane-to-surface transition to the net negatively charged inner leaflet of the bilayer (Figure 4c).

The existence of structural and orientational plasticity as observed here is an interesting property of penetratin which sets it aside from other helical peptides examined thus far. While we believe this model explains most of the experimental data obtained with model membranes, the question of the actual translocation mechanism in complex cellular membranes, in which active mechanisms of protein internalization are operative, requires further investigation [25].

Acknowledgements

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