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The design, synthesis and characterisation of channel-forming peptides

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Synthetic peptides designed using criteria derived from statistical analyses of β-barrel membrane proteins form channels in synthetic membranes at very low peptide:lipid ratios.

In recent years there has been an increasing interest in characterisation of the mechanisms by which antimicrobial peptides insert into membranes to form ion channels.[1] In spite of this, high-resolution structural data are restricted to a limited number of peptides in their monomeric forms. We were interested in developing synthetically amenable peptide templates that would demonstrate ion channel formation in liposomal membranes, to enable us to systematically probe the structural and energetic parameters controlling membrane activity. In order to achieve this, we performed a statistical exercise on the most widely represented membrane protein family in the Protein Data Bank, the β-barrel membrane proteins.[2] These prokaryotic proteins form particularly stable structures, and can be readily reconstituted into synthetic membranes. We reasoned therefore, that peptides designed around these proteins would demonstrate an equal capacity for membrane insertion, leading to the formation of stable ion channels that could be characterised by high-resolution crystallographic techniques at a later stage. Our preliminary findings are presented here.

In the first stage of development of a peptide template, a series of 26 regular non-homologous type 2:2 β-hairpins[3] were selected from the available β-barrel membrane proteins in the PDB.‡ All of the hairpins selected had similar hydrogen bonding patterns and were free from any regions of irregular structure such as β-bulges. The hairpins were modelled as N- and C- terminal strands of 13 amino acids each (residues 1–13 and 14–26 respectively) with respect to a 4-residue β-turn (residues T1–T4). A statistical analysis of amino acid identity at each position of the hairpin was performed to search for conserved residues. Sequence alignments were not considered due to the low pairwise sequence conservation of these membrane proteins, and the desire to search for amino acids of structural significance, both with respect to the interactions of the protein with the membrane, and the formation of stable β-structures through pairwise non-covalent interactions between residues distal in the primary amino acid sequence.

This exercise highlighted four amino acids in the strand regions that were found at a specific position in more than 30% of the hairpins examined. These were all hydrophobic and present on the same (lipid-facing) face of the hairpin, namely tyrosine at positions 12 (38%), 19 (43%) and 21 (33%), and leucine at position 15 (43%). Residues 12 and 15 form a cross-strand pair, probably reflecting a conserved hairpin-stabilising interaction.[4] All of the residues of the opposite (pore-facing) face of the hairpin were poorly conserved, though tended to be hydrophilic (53% of residues). In the turn, a small number of residues were identified as occurring frequently at each position: T1 (Asn, 16%; Thr, 16%; Ala, 16%), T2 (Gly, 22%; Asp, 22%), T3 (Asn, 25%; Gly, 19%; Asp, 16%) and T4 (Val, 22%; Ile, 16%).

These results permitted the design of a symmetrical backbone-cyclic peptide template that would maintain all of the key residues found in the above analysis. This was particularly appealing, as it would simplify peptide synthesis, and facilitate interpretation of the details of the biophysics of the interaction of the peptides with membranes at a later stage. Cyclic peptides have been shown to adopt a more robust β-structure than their linear counterparts,[5] which would favour assembly of the peptides into ion channels on entropic grounds.

Cyclic peptides **1** and **2** and their linear counterparts **3** and **4** were synthesised to test the validity of this approach. The hydrophobic amino acids found to be well conserved by the statistical exercise were included, and the residues of the turn were selected from the pool of residues found in the same exercise. The sequence chosen for the turn (NGNV) was screened against the PDB, producing 15 matches in non-homologous proteins, 9 of which were in β-turn motifs. All of the other residues were selected to maintain the hydrophobic and hydrophilic character of the membrane- and pore-facing surfaces of the hairpins respectively.

The peptides were synthesised in parallel on a Rink-amide resin, with the cyclisation of peptides **1** and **2** performed on the solid-phase using established methodology[6] in which the resin was initially derivatised through the side chain of N-Fmoc aspartic acid α-allyl ester. Allyl deprotection following completion of peptide chain assembly then allowed cyclisation to be

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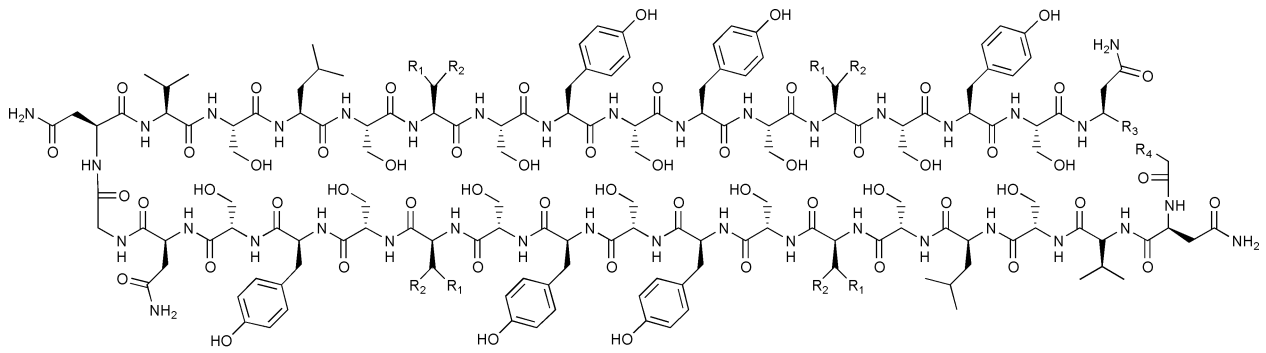

1. R_1=R_2=Me; R_3-R_4=CO-NH; **2**. R_1=^{i}Pr, R_2=H; R_3-R_4=CO-NH; **3**. R_1=R_2=Me; R_3=CO_2H, R_4=NH_2; **4**. R_1=^{i}Pr, R_2=H; R_3=CO_2H, R_4=NH_2

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performed using standard coupling methodology. In order to shorten synthesis times and circumvent peptide aggregation, pseudoproline dipeptides[7] were used to couple all Ser-Xxx residues (Xxx = Tyr, Val, Leu). The final peptides were cleaved form the resin and purified to homogeneity by reversed-phase HPLC.

In order to determine whether the peptides were capable of insertion into lipid membranes, unilamellar liposomes were prepared containing the entrapped fluorescent marker carboxyfluorescein (CF) at a concentration of 35 mM.‡ CF displays fluorescence self-quenching at concentrations > 30 mM, and therefore the formation of suitably sized channels in the liposomal membranes would be indicated by an increase in fluorescence following dilution of CF into the external medium.[8] Fig. 1 shows the results for the most active peptide of the four, peptide **1**. Following addition of the peptides, an extremely rapid release of CF was observed that slowed down after a few minutes. At the highest peptide concentrations used (peptide : lipid = 1 : 400), almost complete release of liposomal contents was achieved within 10 min, making these peptides extremely potent channel formers. Efflux was observed down to a peptide : lipid ratio of 1 : 2900, 1–2 orders of magnitude better than previously characterised peptide antibiotics.[9] At the lower peptide concentrations, the rate of release of CF slowed almost to zero before 100% had been released. This apparent inactivation implies that at least one of the key steps in pore formation is essentially thermodynamically irreversible under these experimental conditions, 100% release only being achieved when sufficient pore-forming peptide inserts into the membrane of every liposome. Addition of fresh aliquots of peptide to samples showing less than 100% release at the end of the experiment produced an immediate further release of CF, suggesting that liposome integrity was maintained throughout. This was confirmed by vesicle sizing using laser light scattering, which in the absence of peptides indicated an average liposome hydrodynamic diameter of 126.9 nm, with a low polydispersity (0.084). In the presence of peptide **1** at 0.5 μM, the vesicle size and polydispersity were essentially unchanged (144.3 nm and 0.177 respectively).

In order to compare the relative efficacy of the peptides, the concentration, in terms of the molar peptide : lipid ratio, required for 50% release of liposome contents was determined. These were found to be 0.0018, 0.0027, 0.0032 and 0.0051 for peptides **1**–**4** respectively. Both of the cyclic peptides were the most potent at allowing CF effusion, with **1** being the more potent. A similar order of activity was observed in the corresponding linear analogues, but in both cases, these peptides were less active than the cyclic peptides.

Preliminary structural investigation of the peptides in aqueous solution by CD spectroscopy indicated a high β-structure content in all cases (Fig. 2). Cyclic peptide **1** displayed a maximum at 196 nm and a minimum of approximately equal strength at 221 nm, consistent with the spectra observed for

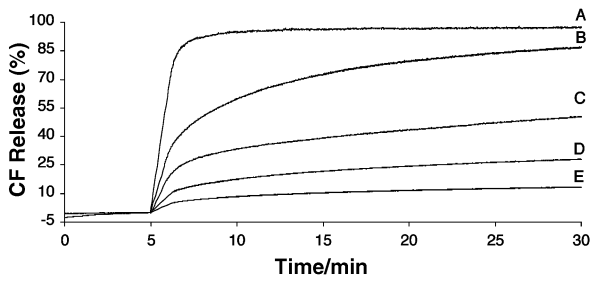

Fig. 1 Carboxyfluorescein (CF) effusion from liposomes (0.2 mM lipid) containing 35 mM CF–0.075 M NaCl mediated by peptide **1** at concentrations of 0.5 (A), 0.3 (B), 0.2 (C), 0.1 (D), 0.07 (E) μM.‡

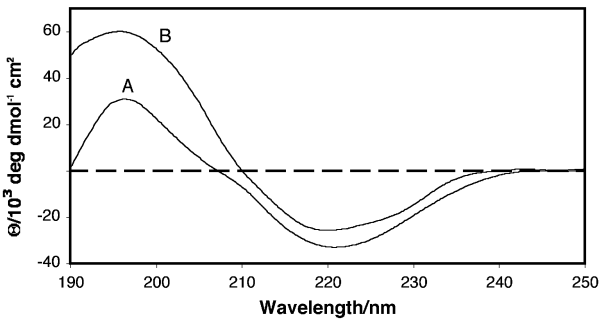

Fig. 2 CD spectra of peptides **1** (A) and **3** (B). Spectra were recorded at 22 °C in 10 mM tris buffer (pH 7.4) containing 1% trifluoroethanol.

membrane β-barrels[10] and smaller synthetic β-peptides.[11] The linear analogue **3** displayed a stronger and broader maximum at 195 nm than peptide **1**, but a weaker minimum at 219 nm.

The preferential adoption of a solution conformation suitable for channel formation by the cyclic peptides is consistent with their higher activity in promoting CF effusion. Together, the results are entirely consistent with the formation of channels by the designed peptide templates in the form of β-barrels. In order to facilitate the diffusional release of CF from the liposomes, these channels must have an internal diameter of at least 1 nm. Both the cyclic peptides and their linear analogues are extremely potent channel-formers, with both of the cyclic peptides showing the highest activity. We have therefore developed a rationale for the design of synthetically accessible channel-forming β-peptides of extremely high potency. These peptides should allow a detailed biophysical characterisation of the membrane insertion and assembly processes, and enable the subsequent design of chemically novel non-natural peptides with tailored properties.

Notes and references

‡ PDB filenames of the proteins used: 1A0T, 1AF6, 1OSM, 1OPF, 1QD6 and 1QJ8. Unilamellar liposomes were prepared by extrusion of egg phosphatidylcholine (EPC) through 100 nm laser-etched polycarbonate membranes, and purified by gel filtration on Pharmacia PD-10 columns. All solutions were isotonic (adjusted using NaCl) and buffered at pH 7.4 using 10 mM tris. Fluorescence emission at 517 nm was monitored following excitation at 480 nm. Percentage release was determined according to the equation $\% = 100(F_t - F_o/F_{max} - F_o)$, where F_t is the fluorescence intensity at time 't', F_o is the fluorescence intensity before addition of the peptide and F_{max} is the fluorescence observed following disruption of the vesicles with Triton X-100.

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