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Molecular mechanism of pore formation by actinoporins

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ABSTRACT

Actinoporins are effective pore-forming toxins produced by sea anemones. These extremely potent, basic 20 kDa proteins readily form pores in membranes that contain sphingomyelin. Much has been learned about the molecular basis of their pore-forming mechanism in recent years. Pore formation is a multi-step process that involves recognition of membrane sphingomyelin, firm binding to the membrane accompanied by the transfer of the N-terminal region to the lipid–water interface and finally pore formation after oligomerisation of three to four monomers. The final conductive pathway is formed by amphipathic α-helices, hence actinoporins are an important example of so-called α-helical pore-forming toxins. Actinoporins have become useful model proteins to study protein-membrane interactions, specific recognition of lipids in the membrane, and protein oligomerisation in the lipid milieu. Recent sequence and structural data of proteins similar to actinoporins indicate that they are not a unique family restricted to sea anemones as was long believed. An AF domain superfamily (abbreviated from actinoporin-like proteins and fungal fruit-body lectins) was defined and shown to contain members from three animal and two plant phyla. On the basis of functional properties of some members we hypothesise that AF domain proteins are peripheral membrane proteins. Finally, ability of actinoporins to form transmembrane pores has been exploited in some novel biomedical applications.

1. Introduction

Sea anemones are soft-bodied sedentary animals that rely on their venomous secretions not only to paralyse and prepare prey for digestion, but also to defend themselves from predators (Burks and Lodge, 2002; Sher et al., 2005). They produce a large number of peptide and protein toxins including ion channel modifiers (Norton, 1991), enzymes (Lotan et al., 1996) and cytolysins (Anderluh and Maček, 2002). Cytolytic proteins are important part of the sea anemone venom. They target and permeate cell membranes by forming transmembrane pores, which effectively cause cell lysis. The most common and most studied group are highly potent actinoporins, shown to be lethal to small crustaceans, molluscs and fishes (Kem, 1988; Turk, 1991; Maček, 1992; Anderluh and Maček, 2002).

Actinoporins are 20 kDa cysteineless proteins, most of them positively charged at neutral pH. One of the hallmarks of actinoporins is their sphingomyelin specificity, as they efficiently make pores in lipid membranes containing this lipid. Two most studied representatives are Equinatoxin II (EqtII) from the sea anemone *Actinia equina* and Sticholysin II (StII) from *Stichodactyla helianthus*. The primary structure

Abbreviations: EqtII, Equinatoxin II; $MTSEA^+$, (2-aminoethyl)methane thiosulphonate hydrobromide; $MTSES^-$, sodium (2-sulfonatoethyl)methanethiosulphonate; PFT, pore-forming toxins; POC, phosphocholine; StII, Sticholysin II.

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comparison between different members of the family has shown that actinoporins are an extremely conserved protein family with high sequence similarity. Since their discovery in the mid-1970s, many studies were made to understand their molecular mechanism of action. However, their true biological role is still inconclusive. Do sea anemones use them to prey other organisms? Are they expressed constitutively or upon some specific environmental signal? Are they actually a component of nematocyst, Cnidarian intracellular structures that are used in some species to deliver toxic components to the prey? Despite this, there was a considerable interest in the scientific community to study the molecular mechanism of pore formation due to various intriguing aspects of their structure and function. Much of the relevant work on actinoporins molecular mechanism of action has been published in the last five years, underlining the growing interest in this topic.

Review papers and book chapters well describe the biology of pore-forming toxins from sea anemones, which also includes the actinoporin family (Anderluh and Maček, 2002; Anderluh and Maček, 2003; Anderluh and Lakey, 2005; Alegre-Cebollada et al., 2007b). Certain specific aspects of actinoporins will be covered in other papers of this special issue. Here, we will focus on several interesting aspects of their mechanism of action at the molecular level, which make them a unique family of pore-forming proteins and that need to be further addressed in the future: the structural features of actinoporins that are being recognised in other distantly related proteins, the sphingomyelin specificity, the individual steps in the pore-forming process and the structural features of the final transmembrane pores.

2. Actinoporin fold is more conserved than previously thought

Up to now, the 3D structures of EqtII and StII have been determined in solution. They display an extremely similar structural organisation (Athanasiadis et al., 2001; Hinds et al., 2002; Mancheño et al., 2003). The molecule is composed of a tightly folded β-sandwich core flanked on two sides by α-helices (Fig. 1). The first 30 amino acids encompass one of the helices. This is the only part of the molecule able to undergo a conformational change without any structural modification of the β-sandwich. A prominent patch of aromatic amino acids is located on the bottom of the molecule. It comprises a completely exposed Trp112 (in EqtII), which was shown to participate in the initial binding of the toxin to the lipid membrane (Fig. 1) (Malovrh et al., 2000; Hong et al., 2002).

Initially, it was thought that actinoporins were an isolated family of proteins occurring only in sea anemones. However, a haemolytic toxin from the salivary gland of a marine gastropod with a high sequence similarity to actinoporins was recently identified (Kawashima et al., 2003). Moreover, a remarkable structural similarity of novel fungal lectins (Pfam code PF07367) to actinoporin structure was revealed, despite less than 15% sequence identity (Birck et al., 2004; Carrizo et al., 2005). Members of the lectin family have insecticidal and anti-proliferative properties (Trigueros et al., 2003; Marty-Detraves et al., 2004) and selectively bind Thomsen-Friedenreich antigen (Galβ1–3GalNAc) present on surfaces of some malignant cells (Carrizo et al., 2005; Damian et al., 2005). Both protein families have almost identical secondary structure elements. The binding site for the sphingomyelin headgroup of actinoporins (see below) and a binding site for cell-surface T-antigen in lectin from *Agaricus bisporus* (Carrizo et al., 2005) are positioned on the same side of the β-sandwich. These binding sites are formed by structurally equivalent amino acids. The main difference between both families is the N-terminal region of actinoporins, which is absent in fungal lectins (Fig. 1).

A detailed search of public databases with EqtII sequence as a probe yielded a number of sequences similar to actinoporins (Gutiérrez-Aguirre et al., 2006). They originate from three animal and two plant phyla and were classified in three families according to phylogenetic analysis. Most of the sequences were retrieved from fishes. The sequence similarity is confined to a region from the C-terminal half of the actinoporin molecule and roughly corresponds to one half of the β-sandwich. This region comprises the actinoporin membrane binding site with a highly conserved P-[WYF]-D pattern, located on a broad loop at the bottom of the molecule. Such conservation of a membrane-binding region suggested that the found homologues should be membrane-binding proteins. To test this hypothesis, a homologue from zebrafish was cloned, expressed in *Escherichia coli* and purified. It displayed membrane-binding behaviour, but did not have permeabilising activity or sphingomyelin specificity (Gutiérrez-Aguirre et al., 2006).

On the basis of these similarities it was proposed that actinoporin-like proteins and the fungal fruit body lectin family comprise a novel superfamily of membrane binding proteins, the so-called AF (Actinoporin-like and Fungal fruit body lectin) domains (Gutiérrez-Aguirre et al., 2006). The biological role of many of these sequences remains to be determined, however, it is clear that the archetypal actinoporin fold is widespread and used for specific binding to various molecules on the plasma membrane surface.

3. Actinoporins are sphingomyelin-binding proteins

One of the actinoporins hallmarks is that their permeabilising activity towards membranes is strongly enhanced by the presence of sphingomyelin. This feature was first described by Bernheimer and Avigad more than 30 years ago for actinoporin from *Stichodactyla helianthus* (Bernheimer and Avigad, 1976). They showed that sphingomyelin inhibits the lysis of red blood cells, that pretreatment of red blood cells with sphingomyelinase renders erythrocytes insensitive to lysis and that toxin is not active towards bacterial spheroplasts, which lack sphingomyelin in membranes. The authors proposed that sphingomyelin acts as a specific receptor for the toxin (Bernheimer and Avigad, 1976). Their isolated toxin was later shown to consist largely of two major isotoxins (Kem and Dunn, 1988), later named sticholysins I and II (Lanio et al., 2001). However, their conclusions have since been corroborated using the purified sticholysins and other actinoporins, e.g.

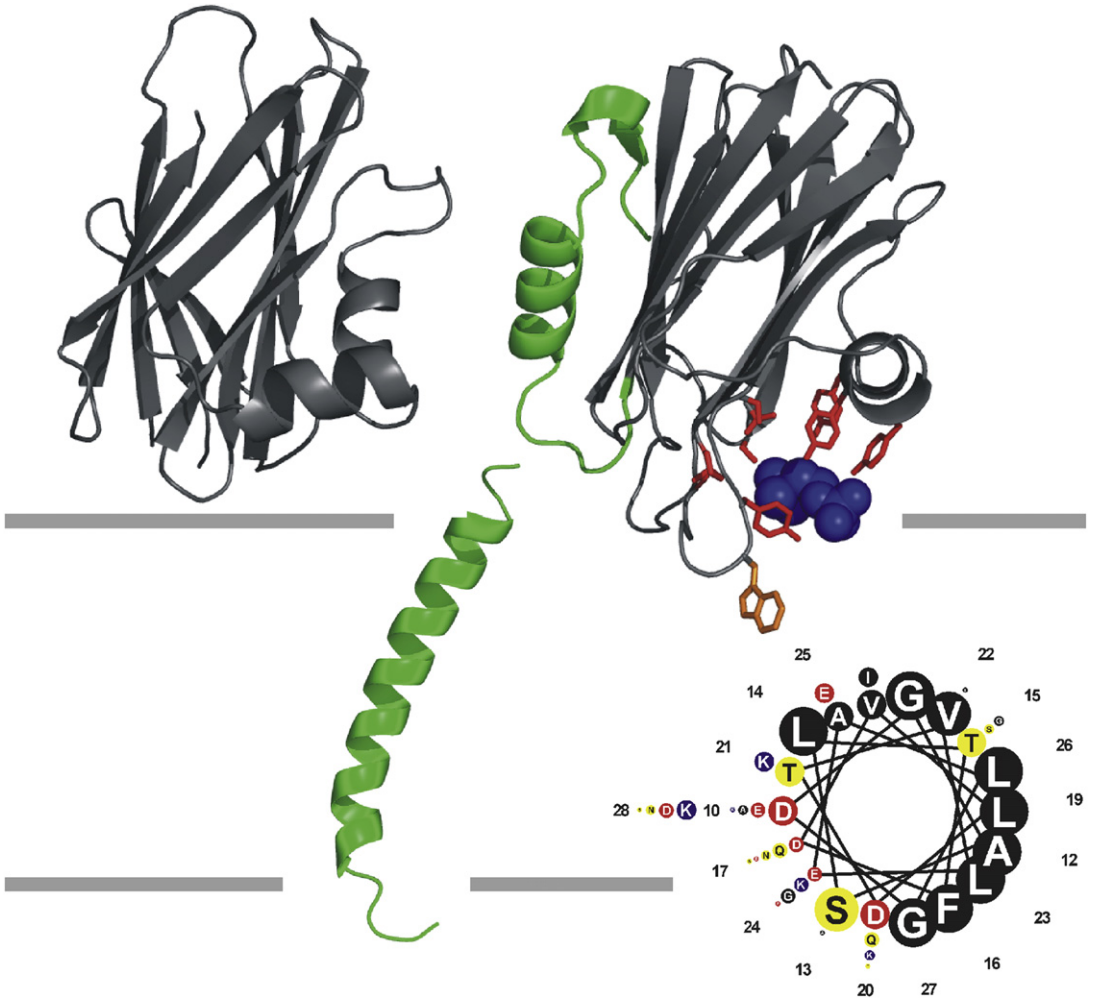

Fig. 1. Structural features of actinoporins. The structure of EqtII (PDB code 1IAZ) is shown on the right side. The N-terminal region, residues 1–32, that forms part of the walls of the final pore, is shown in green. The residues that participate in the binding of phosphocholine (blue) according to the crystal structure of StII (Mancheño et al., 2006) are shown in red. The exposed Trp112 is labelled orange. The structure (PDB 1Y2T) of a fungal lectin from *Agaricus bisporus* is shown on the left for comparison (Carrizo et al., 2005). The NMR structure of a peptide corresponding to residues 1–32 (Drechsler et al., 2006) is shown below with indicated position of membrane boundaries (thick grey lines). The structure was determined in the presence of dodecylphosphocholine micelles. It clearly shows that in the lipid environment this segment prefers an α-helical conformation. The conserved residue helical wheel analysis is shown on the bottom right. The size of the amino acid corresponds to the degree of conservation of that particular amino acid in the whole family of actinoporins (Malovrh et al., 2003). The images were prepared by PyMol (DeLano, 2002). Part of the figure is adapted from Malovrh et al. (2003) with permission.

EqtII. It was demonstrated that EqtII does not form pores in pure phosphatidylcholine planar lipid membranes and does not cause any release of fluorescence probes encapsulated in liposomes, while the presence of sphingomyelin enables it (Belmonte et al., 1993). More recently, structural studies have provided a context for the specific binding of sphingomyelin. Mancheño et al. defined a binding site for phosphocholine (POC), the headgroup of sphingomyelin and phosphatidylcholine, on the surface of StII (Mancheño et al., 2003). The POC binding site is formed by side chains of Ser54, Val87, Ser105, Pro107, Tyr133, Tyr137 and Tyr138 (Fig. 1). These residues are almost completely conserved in the actinoporin family, indicating that POC binding may proceed in the same way in all actinoporins. In addition, by introducing a ^{19}F label on EqtII tryptophans it was shown that Trp112, which is located in the vicinity of POC binding site and is the most important residue for initial contact with the membrane, participates in sphingomyelin recognition. This residue exhibited specific NMR chemical shift changes upon addition of sphingomyelin to phosphatidylcholine micelles (Anderluh et al., 2005). The preferential engagement of sphingomyelin by the toxin was inferred also from solid-state NMR data (Bonev et al., 2003). A strong support for the hypothesis that sphingomyelin acts as a receptor came also from the study of Meinardi et al. (1995), where they showed that the haemolytic activity of actinoporin from *Phymactis clematis* was inhibited by sphingomyelin but not by sphingolipids purified from the sea anemone. This toxin does not permeabilise liposomes containing sphingolipids extracted from sea anemone, it only worked when sphingomyelin was added to the liposomes. Cnidarian (coelenterate) sphingolipids contain large

proportions of phosphonosphingolipids, which have a different phospholipid headgroup. Such a difference assures that the toxin acts specifically towards membranes of prey, which contain sphingomyelin in the outer leaflet of the plasma membrane, and not towards membranes of sea anemones.

However, this view was challenged by some recent reports, which showed that membranes without sphingomyelin might also be targeted by the toxin (Doyle et al., 1989). For instance, StII was shown to permeabilise phosphatidylcholine membranes containing cholesterol (De los Rios et al., 1998; Alegre-Cebollada et al., 2007a; Martínez et al., 2007). The coexistence of different lipid phases was proposed to have an important role also on EqtII action (Barlič et al., 2004). This is not surprising, because it is generally accepted that physical state of the membrane is an important factor for membrane protein functionality (Findlay and Booth, 2006) and can have, in the case of pore-forming proteins, important consequences on the later steps of the pore-forming mechanism, i.e. on the insertion of the protein region that forms the ion-conductive pathway as shown for EqtII (Poklar et al., 1999). Therefore, it is important to distinguish between direct and specific recognition of the lipid by the protein, which leads to the specific attachment of the protein to particular membranes, as shown for many signalling domains (Cho and Stahelin, 2005), and effects that originate from the physical properties of membranes when particular lipids are used. The increased permeabilising potency of actinoporins observed against phosphatidylcholine liposomes in the presence of cholesterol is possibly explained by the cholesterol-induced microdomains formation, which may alter the accessibility of the phosphorylcholine group for the toxin binding (Barlič et al., 2004).

Recently, the hypothesis that EqtII can specifically recognise sphingomyelin was tested by direct binding assays and mutagenesis (Bakrač et al., 2008). Lipid dot blot assays and surface plasmon resonance experiments showed that EqtII specifically binds sphingomyelin or its analogue, but not other lipids, particularly phosphatidylcholine or cholesterol (Fig. 2). The structural basis for this specificity was also examined. Since phosphatidylcholine and sphingomyelin have the same phosphorylcholine headgroup, the toxin needs to recognise sphingomyelin elsewhere, most likely in the region with distinct functional groups just below the headgroup. The two closest residues that are within hydrogen bonding distance and could participate in direct recognition of sphingomyelin by their side chains are Trp112 and Tyr113 (Fig. 1). Mutations of these two residues to alanine diminished direct binding of sphingomyelin, and prevented insertion and binding into sphingomyelin containing lipid monolayers and liposomes, respectively (Bakrač et al., 2008). These results demonstrate the crucial function of these two side chains for direct lipid recognition. In agreement with this model, in StII a mutation of the tyrosine equivalent to Tyr113 (mutant Y111N) largely abolished haemolytic activity (Alegre-Cebollada et al., 2004). The sphingomyelin requirement for the binding of EqtII was recently studied also in a giant unilamellar vesicles system. It was shown that sphingomyelin strongly enhanced binding of EqtII, but was not sufficient for membrane permeabilisation. However, EqtII formed pores in sphingomyelin membranes only when liquid ordered and disordered phases coexisted (Schoen et al., 2008).

Sphingomyelin specific proteins are major virulence factors of bacteria (Ago et al., 2006; Openshaw et al., 2005) and eukaryotic protein toxins, such as actinoporins and lysenin from earthworms (Yamaji et al., 1998). These proteins use sphingomyelin as a receptor to attach effectively to eukaryotic cell membrane rafts. However, no high-resolution structural details of sphingomyelin recognition are available. It will be interesting to compare the mode of sphingomyelin recognition by these unrelated proteins, as discussed by Bernheimer (Bernheimer, 1996). The actinoporin POC binding site, together with strategically placed bulky or aromatic amino acids at positions 112 and 113, may represent a generic 3D-structural motif able to bind a single sphingomyelin molecule, either in solution or in a lipid bilayer.

4. Actinoporins pore formation is a multi-step process

Structural information on the actinoporins coupled with many functional studies allow us to propose the following steps in pore formation by actinoporins: (i) toxin first attaches to the membrane by the specific recognition of sphingomyelin using aromatic rich region and adjacent POC binding site as described above (Malovrh et al., 2000; Hong et al., 2002; Mancheño et al., 2003; Bakrač et al., 2008); (ii) the N-terminal segment is transferred to the lipid–water interface (Hong et al., 2002; Malovrh et al., 2003; Gutiérrez-Aguirre et al., 2004); (iii) toxin oligomerises on the surface of the membrane and α-helices of 3 or 4 monomers insert into the membrane and form the ion conductive pathway (Belmonte et al., 1993; Tejuca et al., 1996; Malovrh et al., 2003; Alegre-Cebollada et al., 2007a). This last step includes an important contribution by membrane lipids and is most likely arranged in a so-called toroidal pore arrangement (Valcarcel et al., 2001; Anderluh et al., 2003a) (see below).

The N-terminus was shown to be involved in the final pore formation step, as actinoporins with a removed N-terminus do not lyse red blood cells (Anderluh et al., 1997). Moreover, various tags added to the N-terminal part of the protein diminish toxin activity (Anderluh et al., 2003b; Kristan et al., 2007). Finally, a membrane binding step could be uncoupled from the pore formation by an engineered disulfide bond between the N-terminal α-helix and the main body of the protein (Hong et al., 2002; Kristan et al., 2004). Such a mutant in oxidised state was able to bind to the membrane, as the aromatic region with POC site was still accessible, however, the N-terminal region could not move freely away from the β-sandwich core impeding membrane insertion and final pore formation.

When a high enough concentration of monomers is bound to the membrane, i.e. at high toxin/lipid molar ratios, the oligomerisation occurs and the N-terminus is inserted through the membrane to form the functional pore (Maček et al., 1995). Mancheño et al have presented a model of the StII pore by docking the atomic model of

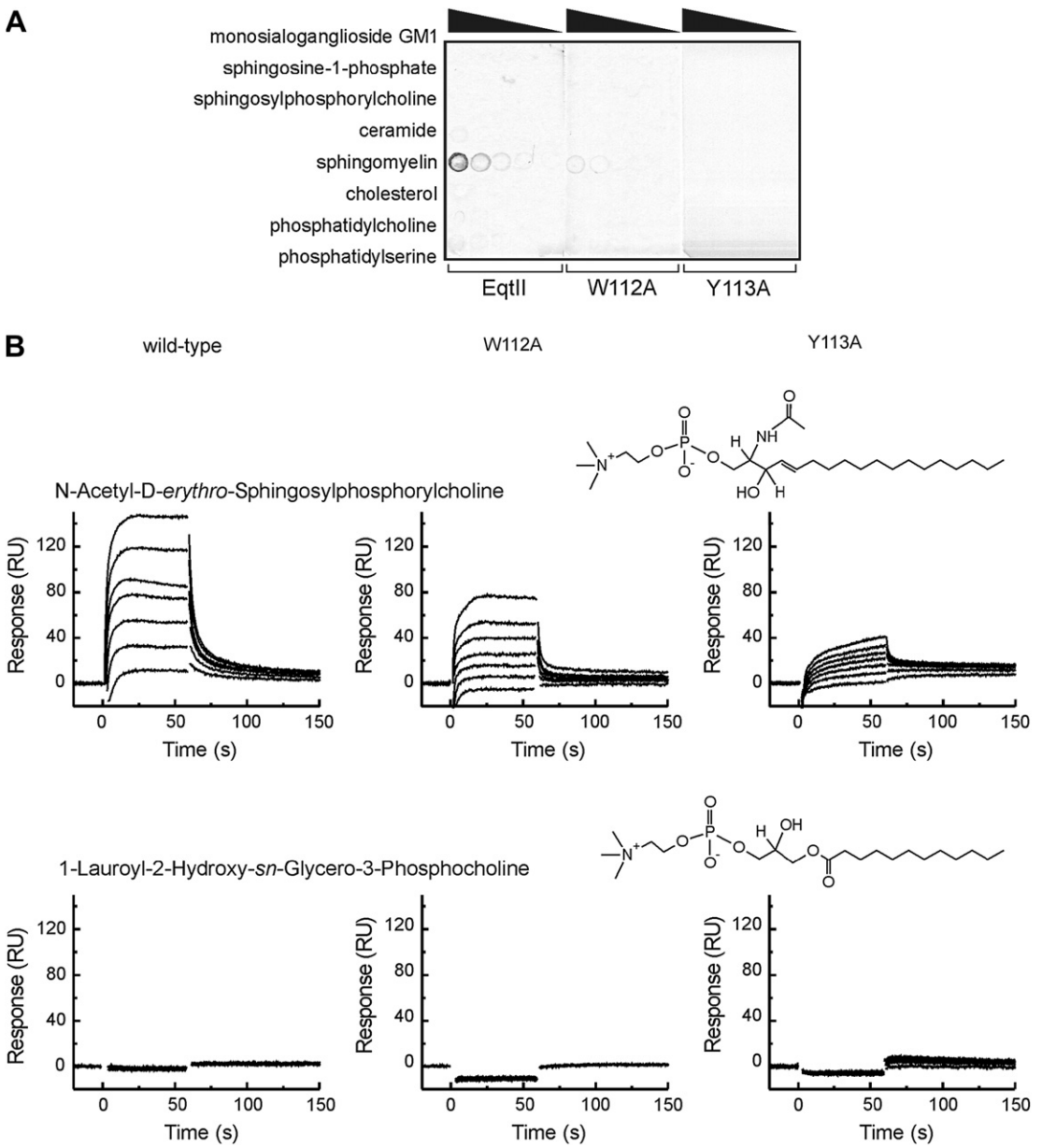

Fig. 2. EqtII specifically binds sphingomyelin and its water soluble short-chain analogue. The dot-blot lipid assay (A) and surface plasmon resonance experiments (B) show that EqtII can specifically bind sphingomyelin or its water soluble short-chain analogue, but not PC or its analogues. EqtII also did not bind to dipropionyl phosphatidylcholine and phosphocholine (not shown). Adapted from (Bakrač et al., 2008). The mutations of two critical residues for the recognition of sphingomyelin at positions 112 and 113 are also shown for comparison. Adapted from Bakrač et al. (2008) with permission.

soluble StII into 3D reconstruction of the micrograph images derived from 2D crystals of the toxin grown on phosphatidylcholine monolayers (Mancheño et al., 2003). The N-terminus was rotated away from the protein core and the proposed oligomer was formed by four monomers. N-terminal regions cross the lipid membrane in α-helical conformation with the most N-terminal residues facing the cytoplasm. This orientation was confirmed by a planar lipid bilayer study on an EqtII single cysteine mutant. Upon chemical modification of the mutant with Cys at position 1, changes in electrophysiological pore properties were observed only when charged reagents were added to the *trans* side of the pore, i.e. the other side of the planar lipid membrane from where the toxin was added. This result not only showed lumen exposure of residue 1 but also suggested that the actinoporin N-terminus crosses the membrane (Kristan et al., 2007). The very N-terminal part is also involved in the stabilisation of the final pore. Deletion of the first five amino acids produced unstable pores in planar lipid membranes, demonstrating that the first few residues in the N-terminus help to stabilise the helix in the final pore (Kristan et al., 2007).

Tryptophan mutagenesis also helped to identify a membrane-bound, but non-lytic state of EqtII. The

localisation of the N-terminal region after interaction with the membrane was studied with additional tryptophans as "natural probes" (Gutiérrez-Aguirre et al., 2004). Three mutants were designed in the N-terminal region (I18W, V22W and A25W). Residues I18W and A25W were inserted into the lipid milieu after toxin binding to the membrane according to fluorescence spectroscopy and quenching experiments. Surprisingly, the haemolytic and permeabilising activities of the V22W mutant were markedly reduced. This mutant was not able to insert the N-terminal helix deeply in the membrane, but was proposed to be stabilised in a non-lytic state in which the N-terminal helix lies parallel to the plane of the membrane (Gutiérrez-Aguirre et al., 2004). In summary, by using mutagenesis it is possible to control functional pore formation by blocking individual steps in the mechanism of action.

5. Actinoporins are α-helical pore-forming toxins

According to the helical wheel analysis, the conserved N-terminal region (10–28) of actinoporins is amphipathic and is, therefore, expected to localise in an interfacial surface region of the membrane, rather than inside the hydrophobic core (Fig. 1). The polar face of the N-terminal region in α-helical conformation contains negatively charged residues (Asp10, Asp17 and Asp24), which account for cation selectivity of the pore formed by actinoporins. Single cysteine scanning mutagenesis of the N-terminal segment 10–28 has shown that it is transferred to the lipid–water interface during pore formation and that it adopts an α-helical arrangement. Thus, the lipid membrane environment induces additional folding of this segment, effectively extending the C-terminal end of the helix from residue 17 to residue 26 in solution (Malovrh et al., 2003). Moreover, peptides corresponding to the N-terminal sequence of actinoporins adopted α-helical conformation in the presence of lipids (Fig. 1), although they lack secondary structure in a non-lipid environment and have a higher propensity to aggregate in solution (Casallanovo et al., 2006; Drechsler et al., 2006). A peptide derived from residues 1–32 of EqtII was found to be in α-helical conformation from residues 6–28 and, therefore, is long enough to span the membrane (Drechsler et al., 2006). These peptides were shown to have markedly decreased lytic activity relative to the native actinoporin and also sphingomyelin specificity was lost. The diminished activity may be due to their inability to bind the membrane and to orient correctly. These findings suggest that the rest of the protein plays a key role in pore formation, even though without a direct structural contribution to the pore. The β-sandwich of the protein contributes to the pore formation by facilitating initial sphingomyelin-specific membrane binding and it is required to anchor the N-terminal peptide in correct orientation in the final pore by providing contacts in the final oligomeric assembly.

The α-helical arrangement of the N-terminus was identified also in the final functional pore. In order to obtain structural information on the N-terminal organisation in the pore, the electrophysiological properties of single cysteine mutants modified with methanethiosulphonates were studied. After chemical modification, the comparison of the permeability of cations over anions through the pore, enabled the identification of residues exposed to the lumen (Fig. 3) (Malovrh et al., 2003). Remarkably, a clear α-helical pattern was observed as well as a decrease of charge effect from residue 10 to 28, indicating that Asp10 is at the restriction of the pore and that helices are not placed perpendicularly across the membrane but tilted 21° with respect to the normal of the membrane. This was confirmed by Fourier transform infrared spectroscopic analysis of membrane bound sticholysins (Menestrina et al., 1999; Alegre-Cebollada et al., 2007a). Altogether, these findings clearly demonstrate that actinoporins belong to an α-helical pore-forming toxins (PFT).

All PFT are able to induce transmembrane pores via a multi-step process. The shared strategy involves binding to the membrane, oligomerisation in the plane of the membrane and insertion, which leads to the functional pore. One of the most useful classifications of PFT is according to the structural element of the final pore (Gouaux, 1997). Transmembrane pores are either formed by a cluster of α-helices (α-PFT) or by stable transmembrane β-barrels (β-PFT). Examples of α-PFT are colicins (Lakey and Slatin, 2001) or, as demonstrated, actinoporins (see above), while β-PFT include staphylococcal α-toxin (Song et al., 1996), protective antigen of anthrax toxin (Benson et al., 1998), the aerolysin family (Iacovache et al., 2006), and the family of cholesterol-dependent cytolysins (CDC) (Tweten, 2005).

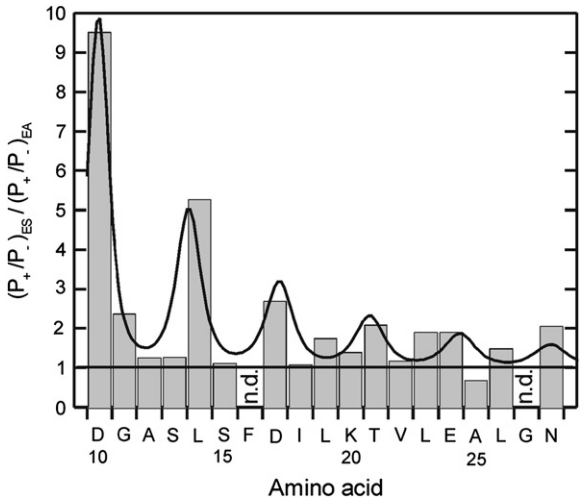

Fig. 3. Alpha-helical arrangement of the pore-lining segment of EqtII. The ratio of the selectivity indices were determined from the planar bilayer experiments after single-cysteine mutants were chemically modified with either sodium (2-sulfonatoethyl) methanethiosulphonate ($MTSES^-$) or (2-aminoethyl) methanethiosulphonate hydrobromide ($MTSEA^+$) to introduce a negative or a positive charge, respectively. Mutants exposed to the pore lumen have positive value, as they were more cation selective upon introduction of negative charge (i.e. $MTSES^-$) and less cation selective when a positive charge (i.e. $MTSEA^+$) was introduced. No changes in the ratio of selectivity indices (i.e. ratio 1, denoted by a straight line) were observed for the mutants that were not exposed to the lumen of the pore. The solid line is the fit of the model of the pore with the radius of 1.3 nm and tilt angle of the helices of 21° (Malovrh et al., 2003). Adapted from Malovrh et al., 2003 with permission.

Pore formation by actinoporins is particularly interesting because it is quite distinct from the mechanism of other bacterial PFTs, in particular from β-PFTs. The pore walls, formed by different secondary structures, account for different pore features and properties. No role of lipids in the pore wall of β-PFT is necessary. Furthermore, the high stability of β-barrel is assured by interstrand hydrogen bonds. The membrane bound oligomers formed by β-PFT are indeed usually SDS stable and allow a much more precise characterisation of the oligomer architecture and of its intermediates by several techniques (Walker and Bayley, 1995; Fang et al., 1997; Tilley et al., 2005). For example, the β-barrel pores formed on planar lipid membranes are stable and display a narrow conductance distribution (Fig. 4). On the other hand, α-helical pores of actinoporins are not rigid structures, impeding direct visualisation of the pores or easy estimation of their molecularity (number of monomers in the pore complex). Since protein and lipids form the pore walls (see below) of EqtII, it is not surprising that its pores are noisy, less stable than pores of β-PFT and characterised by a typical broad conductance distribution (Fig. 4). Usually, pores formed by actinoporins and other α-PFT do not require more than 3–4 monomers, while β-PFT need instead a higher number of monomers, which ranges from 6 to 8 in the case of staphylococcal pore-forming toxins, aerolysin and anthrax protective antigen, to more than 40 monomers in the case of CDC (Parker and Feil, 2005). Another important difference between α- and β-PFTs is the formation of a prepore with the latter toxins, i.e. the typical non-lytic intermediate, which is formed on the surface of the membrane after binding and oligomerisation, and just before β-barrel insertion through the membrane (Heuck et al., 2001). Such an intermediate has not been yet identified in actinoporins. However, a pre-pore intermediate has been observed in Cry toxin, an α-PFT from *Bacillus thuringiensis* (Pardo-Lopez et al., 2006). Notably, in this toxin the prepore formation needs the presence of a protein receptor and the interaction with a second receptor to insert into the lipid membranes.

In conclusion, it is clear that actinoporin pore formation is distinctively different from β-PFT, hence actinoporins represent an important family of α-PFTs and are a good

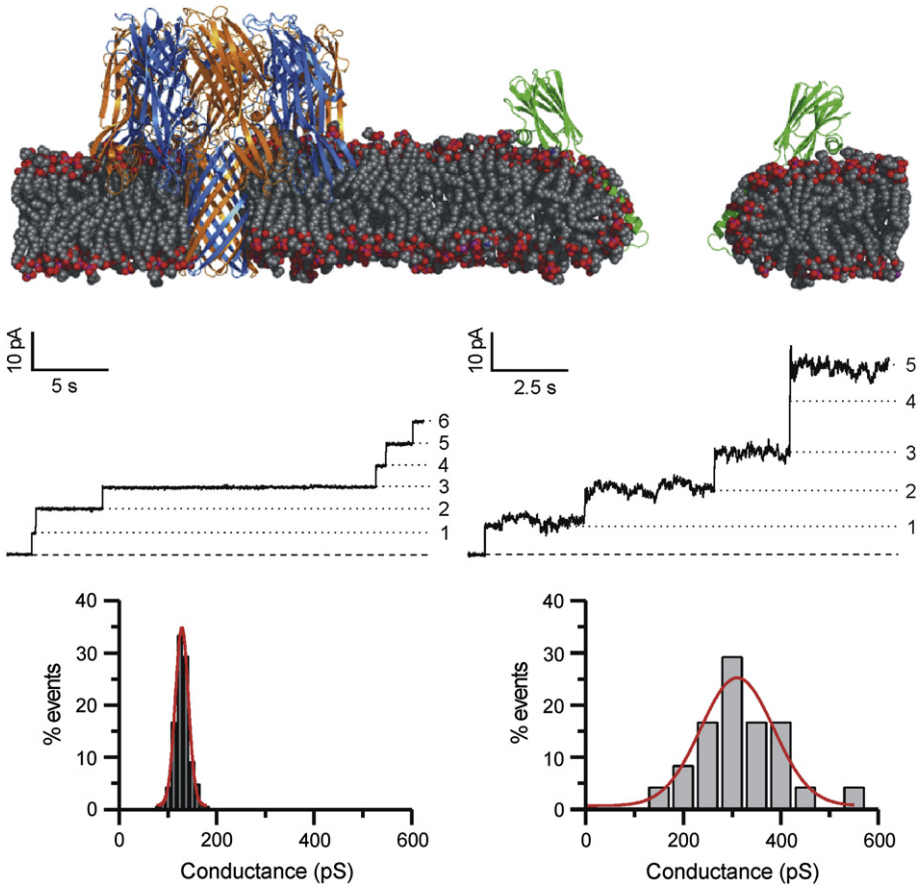

Fig. 4. Comparison of pores formed in planar lipid membranes by staphylococcal β-PFT and EqtII. The upper panel shows a structural model of a pore formed by a β-PFT, γ-haemolysin (left; here HlgA and HlgB monomers are denoted by different colours; Joubert et al., 2006) and α-PFT EqtII (right). The ionic current flowing through the membrane increases step-wise after addition of nanomolar toxin concentration (middle panel). In both cases the protein was added to the *cis* side when a constant voltage (+40 mV) was applied. Only the initial few steps of pore formation are presented. Measurements have been performed on diphytanoylphosphatidylcholine membranes in 100 mM KCl, 20 mM Hepes, 1 mM EDTA pH 7 for γ-haemolysin and diphytanoylphosphatidylcholine/sphingomyelin (80%:20%, mol/mol) in 100 mM KCl, 20 mM Tris, pH 8 for EqtII. The dashed line represents the membrane basal current before the addition of the toxins; dotted lines mark each single channel aperture. Histograms on the bottom panel are showing the distribution of pore conductances. The distribution was fitted with Gaussian curves (red curves), giving the average conductances of 128 pS and 310 pS for γ-haemolysin and EqtII, respectively.

model of how membrane may be damaged by α-helical pore-forming toxins.

6. Actinoporins pore walls are formed by protein and lipids

The data obtained with extensive double cysteine scanning mutagenesis (Kristan et al., 2004) imply that no changes of the β-sandwich structure are necessary for the pore formation. Instead, the core of the protein should remain compactly folded in order for the protein to be fully active. Indeed, mutations that destabilise one side of the actinoporin sandwich core do not increase activity as might be expected, if considerable unfolding of the β-sandwich occurred during the pore formation. Actinoporins EqtII and StII form cation selective pores which consist of 3–4 monomers as judged by cross linking reactions (Belmonte et al., 1993) and kinetic analysis (Tejuca et al., 1996). The hydrodynamic diameter of the pore is about 2 nm (Belmonte et al., 1993; Tejuca et al., 1996; De los Rios et al., 1998; Tejuca et al., 2001; Mancheño et al., 2003). Therefore, the radius of the pore is too large to be solely formed by four α-helices, suggesting that other parts of the protein should be involved. Two possibilities are feasible, either other regions of the protein undergo conformational change and insert in the membrane to form the walls of the final pore or actinoporins form toroidal pores where lipids fill spaces between the helices. The first possibility seems to be unlikely, as the conformational changes are restricted solely to the N-terminal part of the molecule and the core of the protein needs stability for the optimal toxin's activity (Kristan et al., 2004). Some experimental evidence actually exists that is consistent with the toroidal pore model and lipid involvement in pore formation. Actinoporins are able to induce lipid flip-flop between internal and external leaflets of liposome membranes and to permeabilise liposomes in the presence of phosphatidic acid, a strong inducer of negative membrane curvature (Valcarcel et al., 2001). Moreover, negatively charged lipids are able to increase the cationic selectivity of the pore supporting the proposition that lipids are part of the pore lumen (Anderluh et al., 2003a). Finally, an isotropic component was observed in ^{31}P NMR, consistent with lipid disordering (Anderluh et al., 2003a; Bonev et al., 2003). The participation of lipids in the final pore seems to be a common trait in α-helical peptides (Yang et al., 2001; Allende et al., 2005), pore-forming proteins, such as colicins (Sobko et al., 2004) and apoptotic regulator Bax (García-Sáez et al., 2006).

7. Perspectives

PFTs were found to be a useful models to study many processes occurring at the membrane level and have provided clues on structural features of membrane proteins, their folding and protein-protein interactions in the membrane environment. In the last decade, research on these toxins has rapidly expanded, with the aim of providing more profound insights into their structure, molecular mechanism and role in disease. Basic research may greatly contribute to the use of many toxins to probe numerous functions and make possible their future application to biotechnology or biotherapeutics. For instance, they were used for selective killing of parasites (Tejuca et al., 1999) and cancer cells (Panchal et al., 1996; Tejuca et al., 2004; Potrich et al., 2005; Liu et al., 2008), with built-in biological "triggers" that would activate in response to specific biological stimuli, or as biosensors (Braha et al., 1997; Astier et al., 2005). Recently, EqtII was used to selectively permeabilise red blood cells in order to efficiently deliver antibodies for efficient staining of parasites in malaria research (Jackson et al., 2007). Further applications are possible due to their ability to selectively bind sphingomyelin; for instance, they could be used for selective labelling of cellular sphingomyelin. However, some aspects of actinoporin pore formation will still need to be addressed in the future. The structure of the final functional pore need to be described in a more detail, i.e. what and where are contacts between the monomers in the final pore.

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Conflict of interest

None declared.

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