Chapter

Molecular Mechanism of Sphingomyelin-Specific Membrane Binding and Pore Formation by Actinoporins

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Abstract

Actinoporins are potent pore-forming toxins produced by sea anemones. They readily form pores in membranes that contain sphingomyelin. Molecular mechanism of pore formation 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 oligomerization of three to four monomers with accompanying pore formation. Actinoporins are an important example of α-helical pore forming toxins, since the final conductive pathway is formed by amphipathic α-helices. Recent structural data indicates that actinoporins are not restricted to sea anemones, but are present also in other organisms. They are becoming an important tool and model system, due to their potency, specificity and similarity to other proteins.

Introduction

Actinoporins are pore-forming toxins from sea anemones. It is believed that these toxins are used by sea anemones for preying and defence, but their biological role is not yet completely understood.[1-3] They are soluble in water at high concentration, but are able to undergo a conformational change, which allows tight membrane binding and creation of transmembrane pores. These events are dependent on the presence of the membrane lipid sphingomyelin and are enhanced in the presence of lipid domains.[4-6] Their activity is, therefore, tightly regulated and directed mostly to animal cells. Many actinoporin-like proteins have been found in different organisms by sequence[7,8] or structure comparisons.[9,10] Of particular interest is a family of fungal lectins, which shares similar structure and ligand-binding site.[9,11,12] Due to these properties actinoporins have become an important model system and have recently attracted a considerable attention. In this review we will summarise the current knowledge of their molecular mechanism of action and discuss how it relates to other similar proteins. The interested reader may find additional information in other reviews of actinoporins, their properties, biological roles, mechanism of action and their use in biotechnological and biomedical applications.[1,3,13-16]

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Structural Properties of Actinoporins

A hallmark of actinoporins (Pfam code PF06369) is that they are an extremely conserved protein family. The most distant members still share more than 60% of identical residues.[5] The most information about their structure and mechanism of action derives from the studies of equinatoxin II (EqtII) and sticholysins I (StI) and II (StII) from the sea anemones *Actinia equina* and *Stichodactyla helianthus*, respectively. They are 20 kDa proteins and possess no cysteines. The functional parts that enable formation of pores are largely conserved in the family and most of the members are highly basic proteins with pI above 9,[1] although one acidic actinoporin was also described.[17]

Actinoporins are single-domain proteins composed of a tightly folded 12 strand ß-sandwich flanked on two sides by α-helices (Fig. 1).[18-20] The C-terminal α-helix is attached at both ends to the ß-sandwich, whereas N-terminal α-helix is attached only at its C-terminal end one side. The helical wheel analysis of the N-terminal region, from residues 10 to 30, encompassing the N-terminal helix, revealed it to be amphipathic and that it showed weak sequence similarity to melittin, a 26-residue peptide from the honey-bee venom.[21,22] This region is the only part of the molecule that can detach from the core without disrupting the general fold of the protein and the flexibility of the N-terminal region was shown to be crucial for the formation of pores (see below).

Another interesting feature of actinoporin structure is a cluster of exposed aromatic amino acids residues at the bottom of the molecule, which were shown to provide the initial contact of the protein with the membrane.[5,23-25] Co-crystallisation of StII with phosphocholine (POC), a headgroup of lipids phosphatidylcholine and sphingomyelin, enabled the definition of the POC binding site,[20] which was later shown to be crucial for the specific recognition of sphingomyelin.[5] Residues involved in POC binding (StII numbering; Ser-52, Val-85, Ser-103, Pro-105, Tyr-111, Tyr-131, Tyr-135 and Tyr-136) are strictly conserved in actinoporins and imply that the same mechanism of lipid headgroup recognition is followed by other members of the family.[5]

Actinoporins Specifically Bind Sphingomyelin as the First Step in Pore Formation

Pore forming toxins form transmembrane pores in several discrete steps[26,27] and actinoporins are no exception to this general rule (Fig. 1). Available functional and structural data imply that this process involves binding to the lipid membrane by specifically recognising sphingomyelin, transfer of the N-terminal region to the lipid-water interface and oligomerization of three to four monomers that finally leads to pore formation (Fig. 1).[22,24,28]

The membrane lytic activity of actinoporins is highly sphingomyelin dependent (reviewed in Anderluh and Maček[1]). It was proposed that sphingomyelin has a major role in the binding,[29] which was later supported by a definition of a POC binding site on the surface of StII[20] and recent description of sphingomyelin recognition by EqtII.[5] The initial attachment to the membrane is achieved by the aromatic amino acid cluster, which includes five tyrosines and two tryptophans and POC binding site (Figs. 2 and 3). Mutations of the most important residues from the aromatic cluster, Trp-112 and Trp-116 and of the residues that form POC binding site abolished binding and consequently pore formation (Fig. 2).[23-25,28,30] Combination of POC binding site and exposed tryptophan at position 112 enable specific binding of sphingomyelin, but not other lipids, as shown recently by Bakrač et al.[5] Dot-blot assays showed that EqtII binds to sphingomyelin in a concentration-dependent manner (Fig. 2) and does not bind to any other lipid tested, i.e., cholesterol, phosphatidylcholine, ceramide, monosialoganglioside GM1, etc. Surface plasmon resonance analysis of chip-immobilized EqtII additionally showed that it is not able to bind a water soluble phosphatidylcholine analogue, but it bound a comparable sphingomyelin analogue. Actinoporins must specifically recognize regions below the choline headgroup which itself is common to both phosphatidylcholine and sphingomyelin. Residues Trp-112 and Tyr-113, both located on a broad exposed loop at the bottom of the molecule, are the closest residues to the binding site and are within hydrogen bonding distance of the distinctive hydroxyl and amido groups of the sphingomyelin backbone (Fig. 3). All other amino acids are too distant to directly participate in sphingomyelin

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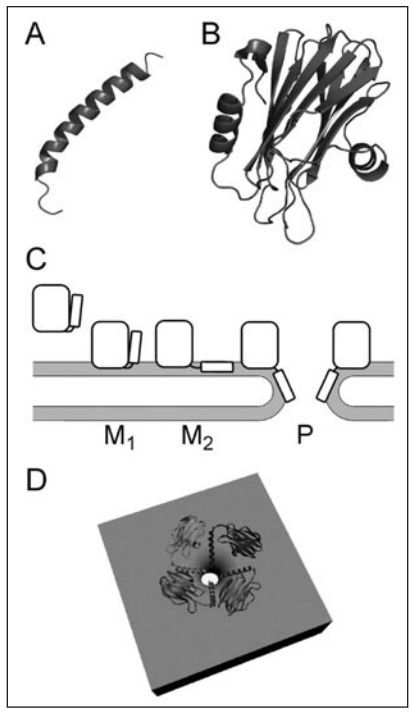

Figure 1. Structural properties of actinoporins and the mechanism of pore formation. (A) The NMR structure of the first 32 amino acids of EqtII in the presence of the dodecylphosphocholine micelles.[37] (B) The crystal structure of EqtII (PDB code 1IAZ). (C) The current model of actinoporin pore formation. It is a multistep process that involves the binding of the soluble monomer to the membrane by a cluster of aromatic amino acids and POC-binding site (M1), translocation of the N-terminal segment to the lipid-water interface (M2) and oligomerization and formation of the final transmembrane pore (P). Adapted from Malovrh et al.[22] (D) Final transmembrane pore as viewed from the above. It is composed of four monomers, of which each contributes one helix and membrane lipids.

recognition. Functional analysis of mutants with changes at these two positions confirmed this hypothesis and showed that Trp-112 and Tyr-113 are crucial for the binding and recognition of a single sphingomyelin molecule (Fig. 3).[5]

This mechanism of sphingomyelin recognition puts some previously published data on actinoporins in a clearer structural context. The importance of tyrosyl side chains for the toxin function was shown by Turk et al,[31] where chemical modification of three tyrosines in EqtII almost completely abolished hemolytic activity. Further, by introducing ^{19}F label on EqtII tryptophans, it was recently shown by NMR that Trp-112 is important for sphingomyelin recognition, as it exhibited changes in NMR chemical shift upon addition of sphingomyelin to phosphatidylcholine micelles.[32] Finally, sea anemones are protected against the action of actinoporins by the absence of sphingomyelin in their membranes. Instead, they possess a phosphonosphingolipids that have an altered phosphorylcholine headgroup.[33]

Some recent publications, however, show that addition of cholesterol to phosphatidylcholine liposomes enhance activity of actinoporins, by modulating physical properties of the membrane or by inducing membrane microdomains.[4,34,35] Recently, giant unilamellar vesicles (GUVs) have been used to investigate the role of sphingomyelin for the binding of EqtII and shown that it bound preferentially to the sphingomyelin enriched liquid ordered phase than to the liquid disordered

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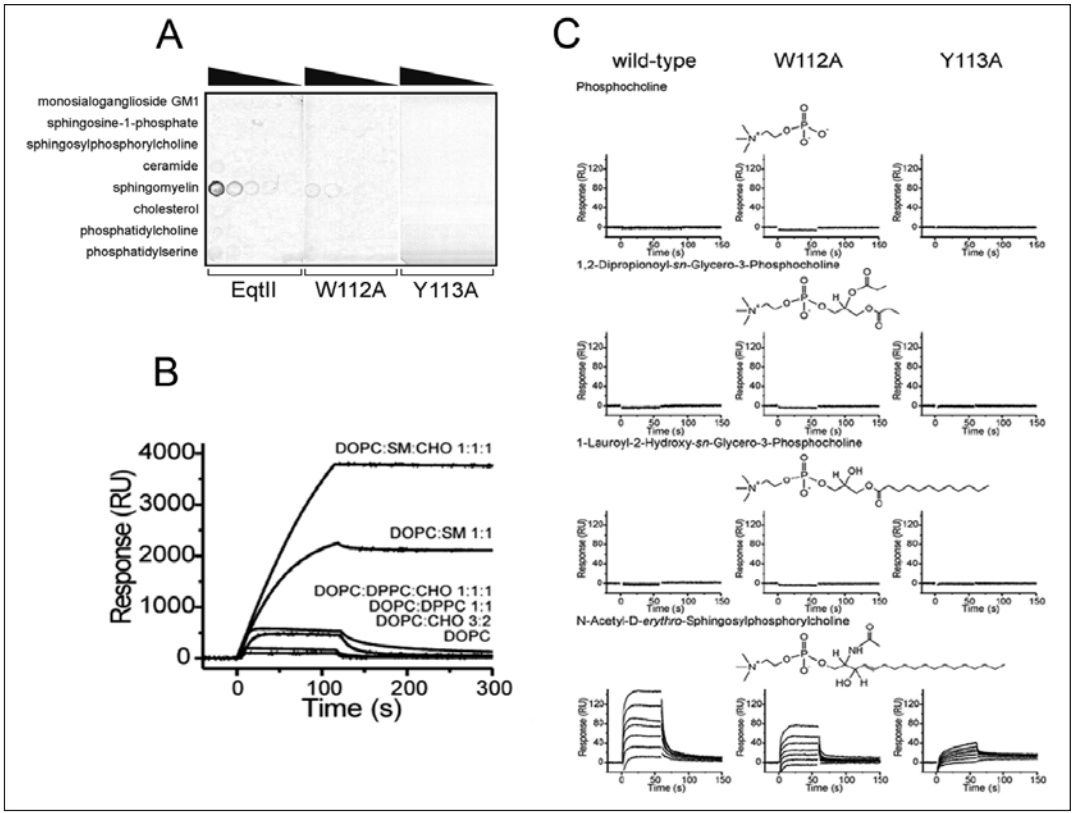

Figure 2. EqtII is a sphingomyelin-binding protein. (A) Dot-blot lipid binding assay with a series of most common membrane lipids. The first spot contains 100 pmol of lipid and then two-fold serial dilutions are followed. (B) Surface plasmon resonance analysis of EqtII binding to liposomes of different composition as indicated. (C) Surface plasmon resonance analysis of lipid analogues binding to the wild-type EqtII or its mutants.[5]

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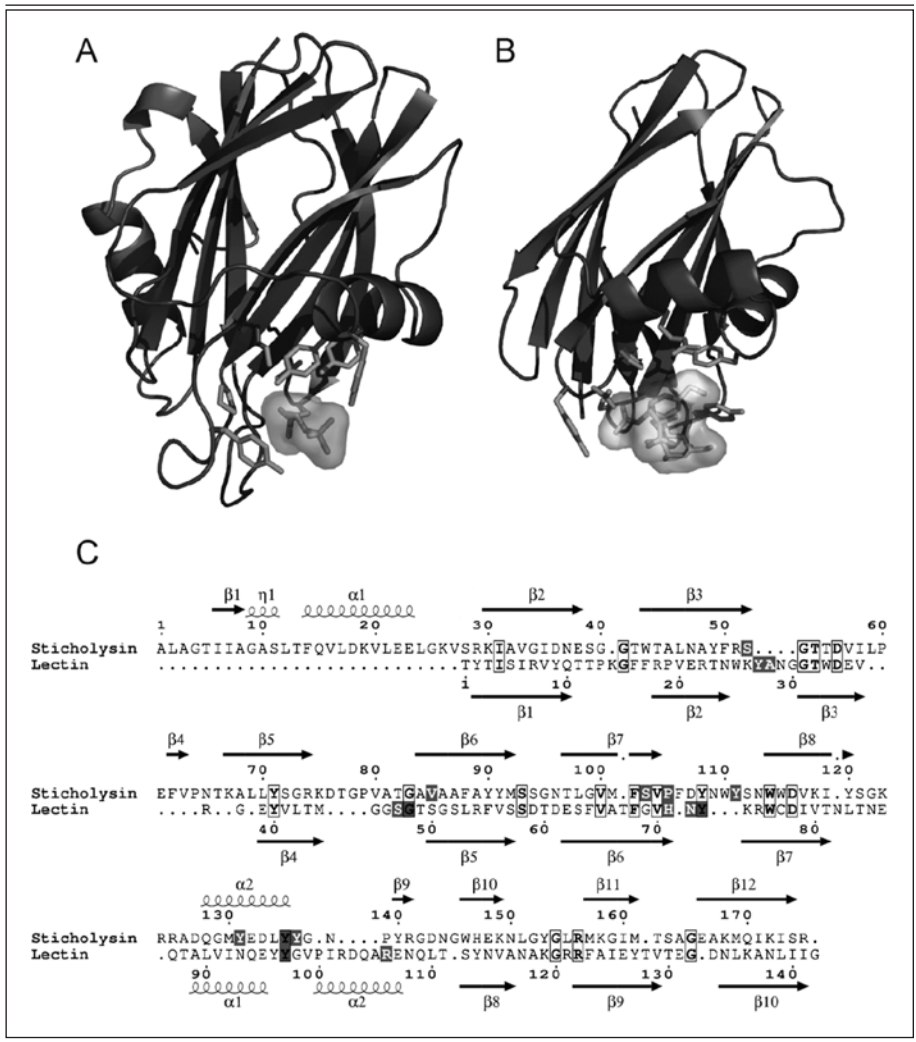

Figure 3. The similarity between actinoporins and fungal lectins. (A) Structure of actinoporin StII (PDB: 1O72).[20] (B) A fungal lectin from *Agaricus bisporus* (PDB:1Y2V).[11] The amino acids that participate in binding of cognate ligand in both proteins are shown with side chains (actinoporins bind phosphocholine and the fungal lectin binds the disaccharide Galβ1-3GalNAc). Both ligands are shown with sticks and surface representation. (C) An alignment based on the structural elements. Amino acids that enable binding of ligands are shown shaded. Amino acids that are shared between both proteins are boxed. The secondary structures of StII and lectin are shown above and below the alignment, respectively. α-helices are shown as coils and β-strands are shown as arrows. Adapted from: Anderluh G, Lakey JH, Trends Biochem Sci 2008; 33:482-490;[56] with permission from Elsevier.

phase.[6] The presence of sphingomyelin strongly promoted membrane binding, but EqtII was able to permeabilize sphingomyelin-containing GUVs only when both phases coexisted. There was no permeabilization when sphingomyelin was only in membranes of only one phase. So, sphingomyelin can also indirectly modulate the activity of actinoporins, by affecting the physical properties of lipid membranes.[6]

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Flexibility of the N-Terminal Region is Required for Pore Formation

In the next step of pore formation the N-terminal segment translocates to the lipid-water interface[22,24,36] and flexibility of this region is mandatory for the permeabilising activity. The transfer of this segment to the membrane was monitored by double cysteine mutants, where cysteines were introduced at such positions to allow formation of intramolecular disulphide bond and consequently restrict movement of this region.[24,28] Mutants within the N-terminal region were completely inactive, but were still able to bind to membranes, since the membrane binding site was not affected. The placement of the N-terminal region in a more hydrophobic membrane milieu was further confirmed by cysteine scanning mutagenesis.[22] Single cysteine mutagenesis of the N-terminal region from Asp-10 to Asn-28 has shown that the whole region is transferred to the lipid-water interface during pore formation and that it is in an α-helical conformation, which implies that the lipid environment induces additional folding of this segment and prolongs the α-helix, which extends from Ser15 to Leu26 in solution.[18,19] The NMR structure of the peptide, corresponding to the first 32 residues of EqtII, has shown that it lacks ordered secondary structure in water. However, residues 6-28 form a helix in dodecylphosphocholine micelles, thus clearly showing propensity of this part to spontaneously fold when in membranes.[37] Finally, the interaction of the N-terminal helix with the membranes was confirmed by introducing tryptophan residues at various positions along this region.[36] This approach allowed measurements of changes in intrinsic tryptophan fluorescence upon membrane interactions and was particularly instructive, since an intermediate in the pore formation was revealed.[36] A mutant which possesses tryptophan instead of valine at position 22 had reduced haemolytic and permeabilizing activities, while lipid monolayer insertion at the air water interface was not different to the wild-type protein or other mutants studied. Decreased rates of hemolysis and permeabilization activity arise from the inability to insert the α-helix in the perpendicular orientation that would give rise to the oligomeric pores in membrane bilayers. So this mutant is locked in a membrane-bound topology, where the α-helix lies parallel to the plane of the membrane in a nonlytic state.

Of particular interest for the understanding of actinoporins functioning is the fact that the peptides that correspond to the actinoporin N-terminal region do not exhibit the same hemolytic or permeabilizing activity as the intact molecule.[37,38] They showed some residual activity, mostly to negatively charged liposomes, but they lacked the selectivity for sphingomyelin containing membranes.[37,38] Hence, the actinoporin β-sandwich has an important role in the mechanism of pore formation, by enabling sphingomyelin-specific binding and stability of the final pore, where it probably helps to stabilize slightly tilted helices,[20] as discussed below.

Pore Formation Involves Nonlamellar Lipid Structures

In the final step of pore formation toxin monomers bound to the membrane oligomerize and the N-terminal helical part is inserted deeply across the membrane to form the ion-conductive pathway. The secondary structure of the actinoporins does not change much after the binding to the lipid membranes and formation of pores, according to circular dichroism (CD)[39,40] and Fourier-transform infra red (FTIR) spectroscopy.[41,42] This was inferred also from the electron microscopy images of 2D crystals of StII.[20] The reconstructions enabled to provide the model of pore formed by four molecules of StII, with minimal adjustments of the β-sandwich, which sits on the membrane, while α-helices are slightly tilted with respect to the membrane normal.[20] Such arrangement of helices was already proposed by FTIR spectroscopy[41] and later confirmed by cysteine scanning mutagenesis.[22] It was recently proposed that the N-terminal part extends to the trans side of the membrane in the final pore, i.e., to the side opposite to the rest of the membrane-bound protein.[43] The terminal five amino acids were proposed to act as an anchor, similar to the mechanism recently described for aerolysin, where it was proposed that loops of the β-barrel stabilize it in the membrane in a rivet-like fashion.[44]

Pores formed by actinoporins are 2 nm in diameter[45-47] and, hence, cannot be simply formed by four helices. Either other parts of the molecule contribute to the final oligomeric conductive pore, or the pore is composed partially of lipid molecules from the bilayer (Fig. 1). The first possibility

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requires considerable unfolding of the β-sandwich and its rearrangements in such a way that remaining space between helices is filled with the polypeptide chain. A disulfide scanning mutagenesis was employed to show that apart from the N-terminal segment there are no other parts, specifically β-sandwich and the C-terminal α-helix, that undergo gross conformational changes.[28] These results support a model where the final pore is formed by α-helices and bilayer lipids, as no other part of EqtII inserts sufficiently deeply into the membrane to fill the remaining gaps between the helices.[48,49] Such, protein-lipid, so called, toroidal pores were also proposed for smaller pore-forming peptides such as melittin[50] or larger proteins such as apoptotic Bax proteins.[51,52]

Some experimental evidence is consistent with the toroidal pore model and lipid involvement in pore formation of actinoporins. StI and StII were able to induce lipid flip-flop between internal and external leaflets of liposome membranes and inclusion of small proportions of phosphatidic acid, a strong inducer of negative membrane curvature, markedly increase the release of fluorescent markers from liposomes.[48] Negatively charged lipids were able to increase the cationic selectivity of the EqtII pore, thus supporting the proposition that lipids are part of the pore lumen.[49] Finally, an isotropic peak was observed in ^{31}P NMR, which was interpreted to occur from lipid disordering.[49] The reorientation of lipid acyl chains was also observed by FTIR.[41,42] In conclusion, it is clear that the actinoporin pore formation is unique and distinctively different from other pore forming toxins. They are a good model of how membrane may be damaged by α-helices.

Similarity to Other Proteins

For many years it was believed that actinoporins is isolated family of pore-forming toxins present only in sea anemones. However, first a haemolytic toxin, echotoxin 2, from the salivary gland of the marine gastropod *Monoplex echo* was described and found to be homologous to actinoporins.[7] Recently, a detailed search of public databases with EqtII sequence as a probe yielded a number of sequences similar to actinoporins.[8] They were from three animal (chordates, cnidarians and molluscs) and two plant (mosses and ferns) phyla. However, the majority of the sequences were from teleost fishes. The similarity to EqtII is confined to the C-terminal region from residue 83 to residue 179, which is roughly half of the β-sandwich and comprises membrane binding site with a highly conserved P-[WYF]-D pattern, located on the broad loop at the bottom of the molecule. Such conservation of a membrane-binding region suggested that these homologues should be membrane-binding proteins. To test this hypothesis, a homologue from zebrafish was cloned, expressed in *E. coli* and purified. It displayed membrane-binding behaviour, but did not have permeabilising activity or sphingomyelin specificity.[8]

Novel homologues of actinoporins were found in recent years also by structural analysis.[9-12] A novel family of fungal lectins revealed a remarkable similarity to actinoporins despite having less than 15% sequence identity.[9,11,12] The structural similarity is confined to the β-sandwich and the most important difference in the structures of both groups is that fungal lectins lack the N-terminal amphipathic region of actinoporins (Fig. 1). The lectins from *Xerocomus chrysenteron* (XCL) and *Agaricus bisporus* (ABL) have antiproliferative properties[53,54] and they both selectively and with high affinity bind the Thomsen-Friedenereich antigen (TF antigen),[11] a disaccharide (Galβ1-3GalNAc) expressed by about 90% of all human carcinomas.[55] The binding site for TF-antigen in ABL corresponds to the POC-binding site in actinoporins, the residues used for the binding are located on equivalent sites to the actinoporin residues used for the binding of the phosphocholine headgroup of sphingomyelin (Fig. 3).[56]

Just recently a novel actinoporin-like protein family was described at the structural level.[10] Many bacterial, fungal and oomycete species produce necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) that trigger leaf necrosis and immunity associated responses in various plants. The crystal structure of a Nep1-like protein from phytopathogenic oomycete *Pythium aphanidermatum* was determined and showed to possess a fold that exhibits structural similarities to actinoporins. All of these examples indicate that actinoporins fold is widespread and used by many different protein families primarily for the specific binding to various molecules of the plasma membrane.

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Conclusion

The lipid cell membrane is the first obstacle that needs to be overcome and the creation of transmembrane pores is a very efficient way of killing cells, so pore forming toxins are a very important group of natural toxins.[27,56] In recent years they have been used to study fundamental biological processes such as protein-membrane and protein-protein interactions within the lipid bilayer milieu, as well as conformational changes associated with the change of environment from polar to hydrophobic, as encountered within the core of lipid membranes. While β-PFTs, e.g., *Staphylococcus aureus* α-toxin or cholesterol dependent cytolysins,[27,57] form structurally stable transmembrane pores, those formed by α-PFTs are not stable. Consequently, there is less structural information available. Final functional pore of EqtII have still not been visualized. It needs to be unambiguously determined what is number of monomers in the final pore and what the interactions between the monomers in the final pore are.

Due to their properties, there may be many opportunities to use actinoporins in biotechnological and biomedical applications. They were used for selective killing of parasites[58] or cancer cells.[59] Recently, EqtII was used to selectively permeabilise red blood cells in order to efficiently deliver antibodies for efficient staining of parasites in malaria research.[60] Due to its sphingomyelin-binding capacity, EqtII could be a useful probe to detect and study the distribution of sphingomyelin within the cells. Most cellular sphingomyelin resides in the outer leaflet of the plasma membrane, but is synthesized de novo by a sphingomyelin synthase1 in the Golgi complex.[61] Sphingomyelin has an important role in the lipid membrane, by being a main constituent of so-called lipid-rafts, microdomains enriched with cholesterol and sphingomyelin.[62] But sphingomyelin also serves as a reservoir for lipid signalling molecules, i.e., ceramide, sphingosine and sphingosine 1-phosphate.[63] They are critical regulators of cell proliferation, differentiation and apoptosis. All these facts indicate the importance of sphingomyelin, hence a probe to detect and study its distribution and synthesis at the cellular level is crucially needed.

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