Structural foundations of sticholysin functionality

Palacios Ortega, Juan; Garcia-Linares, Sara; Rivera-de-Torre, Esperanza; Heras-Márquez, Diego; Gavilanes, José G.; Slotte, J Peter; Martínez-del-Pozo, Álvaro

Published in:
BBA - Proteins and Proteomics

DOI:
10.1016/j.bbapap.2021.140696

Published: 01/01/2021

Document Version
Final published version

BBA - Proteins and Proteomics 1869 (2021) 140696

Contents lists available at ScienceDirect

BBA - Proteins and Proteomics

journal homepage: www.elsevier.com/locate/bbapap

Structural foundations of sticholysin functionality

Juan Palacios-Ortega [a,b,*], Sara García-Linares [a], Esperanza Rivera-de-Torre [c], Diego Heras-Márquez [a], José G. Gavilanes [a], J. Peter Slotte [b], Álvaro Martínez-del-Pozo [a]

[a] Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas, Universidad Complutense, Madrid, Spain
[b] Biochemistry, Faculty of Science and Engineering, Åbo Akademi University, Turku, Finland
[c] Department of Biochemistry and Biotechnology, Technical University of Denmark, Kongens Lyngby, Denmark

ARTICLE INFO

Keywords:
actinoporins
structure-function relationship
lipid membranes

ABSTRACT

Actinoporins constitute a family of α pore-forming toxins produced by sea anemones. The soluble fold of these proteins consists of a β-sandwich flanked by two α-helices. Actinoporins exert their activity by specifically recognizing sphingomyelin at their target membranes. Once there, they penetrate the membrane with their N-terminal α-helices, a process that leads to the formation of cation-selective pores. These pores kill the target cells by provoking an osmotic shock on them.

In this review, we examine the role and relevance of the structural features of actinoporins, down to the residue level. We look at the specific amino acids that play significant roles in the function of actinoporins and their fold. Particular emphasis is given to those residues that display a high degree of conservation across the actinoporin sequences known to date. In light of the latest findings in the field, the membrane requirements for pore formation, the effect of lipid composition, and the process of pore formation are also discussed.

1. Introduction

Toxicity is widespread in life. All kinds of organisms, from bacteria to mammals, produce toxic compounds. The nature and targets of these compounds, however, can be highly varied. Toxins can be as simple as small molecules or as intricate as a large protein complex. Their targets can be structures inside cells, such as ribosomes, or on their outside, such as membrane receptors. The cellular membrane particularly stands out as toxin target for its easy availability, its universally similar structure, and the vital role it plays for all organisms.

The plasma membrane is of capital importance to all cells, providing the barrier between the self and the non-self, while fulfilling many other functions. In eukaryotes, for example, membranes also provide internal compartmentalization, helping optimize cellular processes [1]. The basic structure of cell membranes, the lipid bilayer, is very similar in all organisms, and most cells have lipid membranes that are entirely exposed to the environment.

Cell membranes are thus the target of a wide variety of toxins, many of which are pore-forming toxins (PFTs) [2,3]. PFTs are usually produced by bacteria, but many other organisms produce them as well. In many animals, PFTs are a constituent of the immune system [4–7]. In many other organisms, they are a fundamental venom component. Such is the case with many sea anemones, in whose venom actinoporins play a major role [8].

Sea anemones have been known to be toxic since ancient times. However, it was not until after the coming of the molecular sciences that the attention of a part of the scientific community was drawn to venomous animals in order to systematically find within them compounds and substances that might have potential therapeutic uses. This attention first focused on coelenterates in the 1960s and early 1970s [9–11], which eventually led to the discovery of actinoporins. It was as early as 1975, when Bernheimer and co-workers at the New York University at New York, NY, reported the specific binding of a *Stichodactyla helianthus* (then *Stoichactis helianthus*) toxin fraction to sphingomyelin (SM) [12]. In that article, Bernheimer et al. also presented the first estimates of the molecular weight (M_w), amino acid composition, and p*I* of the toxin. Not only that, but they also pointed out the resemblance of the purified toxin with "the nematocysts toxin of the sea anemone *Actinia*

Abbreviations: Cer, ceramide; Chol, cholesterol; Eqt, equinatoxin; Fra, fragaceatoxin; H_{II}, inverted hexagonal phase II; PA, phosphatidic acid; PE, phosphatidylethanolamine; PFT, pore-forming toxin; PnSL, phosphonosphingolipids; POC, phosphocholine; SM, sphingomyelin; Stn, sticholysin; TCDB, Transporter Classification Database; tPA, *trans*-parinaric acid..

* Corresponding author at: Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas, Universidad Complutense, Madrid, Spain.
E-mail address: juan.palaciosb1a@gmail.com (J. Palacios-Ortega).

https://doi.org/10.1016/j.bbapap.2021.140696
Received 19 May 2021; Received in revised form 5 July 2021; Accepted 6 July 2021
Available online 8 July 2021

equina" [12], which would be referred to as equinatoxin. Follow-up studies were published by the same group, focusing mainly on the SM-specificity displayed by this toxin and its potential interaction with the choline moiety of SM [13,14]. One of them presents the earliest report of toxin-induced release of aqueous contents by an *S. helianthus* toxin, performed using ^{14}C-labelled glucose [14]. In this other article, a toxin-ferritin conjugate was used to highlight the toxin specificity for SM by taking electron micrographs of liposomes with and without SM. They observed that liposomes were only labeled if SM was present. However, the authors apparently missed what was their most remarkable result: the first micrographs of the pore structures. These can be clearly appreciated in Fig. 8A of the article, shown here in Fig. 1 [13]. If interested in more details of the early days of research on actinoporins, the reader is referred to the following papers on the overall characterization of these toxins [15–32]. Some other interesting papers are the aforementioned first identification of SM selectivity and the importance of the choline moiety [12–14], the discovery of the cation-selective character of the transmembrane channels, which showed the preference for monovalent cations [33–35], the sequencing of an *S. helianthus* toxin (StnII, then called "Cytolysin III") [36,37] and *Actinia equina* EqtII [38], the description of the functional properties of EqtII [26], the discovery of "sequence variants" (isotoxins) for both actinoporins [39–42], the production of actinoporins on heterologous systems [43,44] and actinoporin-based immunotoxins [45,46], and the first complete sequence of StnII [47] and EqtII [28]. The first reports on FraC, now one of the four best-characterized actinoporins, did not appear until 2009 [48,49]. For other sea anemone toxins (both actinoporins and other) from different species such as *Anemonia sulcata*, *Heteractis magnifica*, *Heteractis crispa*, and *Actinia tenebrosa*, to name a few, the reader is referred to the following sources [50–60].

As stated, various actinoporins from many different sea anemones have been found and described so far. Nevertheless, the soluble structure of only five of them (sticholysin I (StnI) and StnII, EqtII, FraC, and FraE), has been resolved in atomic detail [49,61–67]. StnI and StnII are both produced by the same sea anemone, *Stichodactyla helianthus*, which occurs in the Caribbean Sea [68], while FraC and FraE are produced by the Atlantic sea anemone *Actinia fragacea* [69], and EqtII is produced by *A. equina*, which is only missing in the Pacific Ocean [70]. These structures provide an invaluable tool to gain insight into the functionality of these proteins.

Actinoporins are small, most often basic proteins that specifically bind to their target membranes by recognizing SM, often considered the actinoporin receptor [71–76]. However, other physicochemical features of the membrane can greatly influence actinoporin functionality [32,35,76–83]. The presence and effect of cholesterol (Chol) in the membrane is possibly the most remarkable of these [75,77,80,81,83–87]. The pores made by these toxins, which are cation-selective, are formed once the oligomers are bound to the membrane [35]. However, the oligomerization steps and the stoichiometry of the pore complex remain to be solved in full detail.

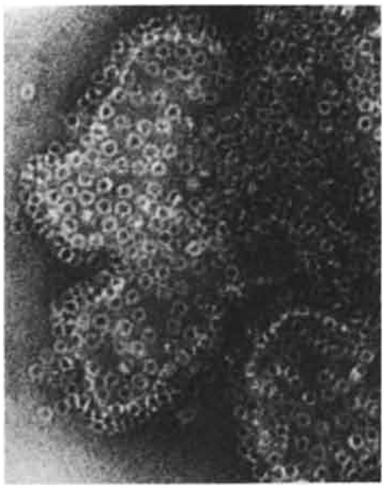

Fig. 1. Electron micrograph of *S. helianthus* toxin's membrane-bound structures, now known to be pores, as shown by Linder et al., 1977 [13]. Reproduced here under license number 5071180188343 (Elsevier).

2. Classification and general characteristics of sticholysins

Sticholysins are part of the actinoporin family. Therefore, these proteins exhibit the general features of actinoporins: small-sized cysteineless proteins with basic p*I* that form cation-selective pores of 1–2 nm in diameter on their targeted cells, provoking cell death by osmotic shock [27,31,32,35,71–73,88,89]. These proteins are said to be hemolytic since they can disrupt erythrocytes, which is the basis of a widely used activity assay [26,42,60].

2.1. Channels, Pore-Forming Toxins, and the actinoporin family

The Transporter Classification Database (TCDB) has organized all membrane transport proteins by class, subclass (usually related to the energy source used by the transporter), family, and subfamily. PFTs are classified as channels under code 1.C and, in this classification, comprise 128 different families. Actinoporins, which in the TCDB are referred to as "the Pore-forming Equinatoxin Family," are classified in a single subfamily of PFTs under code 1.C.38.1 [90].

The traditional way of classifying PFTs, however, ranks actinoporins as one of the three major families of α-PFTs [3]. The basis of this classification, unlike that of the TCDB, lies on the secondary structure lining the walls of the pores of the corresponding PFT [91–99]. Actinoporins, as colicins and cytolysin A-related toxins, form pores whose walls are lined with α-helices. The remaining PFTs, which comprise the families of bacterial hemolysins, cholesterol-dependent cytolysins, and aerolysins, are β-PFTs, based on their pore walls lined by β-strands [3]. Despite sharing a category, each of the families displays its own fold and pore formation mechanism. Membrane specificities can differ within the families, with the receptors being as varied as specific sugar moieties, lipids, such as Chol or SM, and specific proteins of the target organism, which can be the case of some pathogen-produced toxins.

2.2. Actinoporins in sea anemones and other organisms

Actinoporins, which include sticholysins, are cytolysins produced by sea anemones. These proteins are the main component of their venom, used for hunting purposes. Yet since they can be found not only in the venom but also in the coelomic fluid and the surroundings of these animals as well, they are also considered to play a role in the defense of these organisms [71,100]. The reason for the existence of several actinoporin isoforms within a single species of sea anemones is not known. However, it has been proposed that it could, among other things, broaden the range of targets [101,102].

At least 20 different species of sea anemones have been observed to produce actinoporins, though not all actinoporins have been sequenced [48,71,73,103]. Despite having multiple actinoporin sequences encoded in their genome, most sea anemones generally produce only a small number of these toxins in detectable quantities [8,44,71,73,102,104]. If a basic local alignment (with the basic local alignment search tool, BLAST) is performed on UniProtKB [105] using the sequence of StnII as the query, 31 of the obtained results (as of November 2020) are proteins produced by species in the Cnidaria phylum, of which 24 come from the Actinaria order (sea anemones), and 8 from the Scleractinia order (stony corals). All sequenced and characterized actinoporins display high sequence identity (57-90 %, Fig. 2). If the 20 Cnidarian sequences that have been reviewed in UniProtKB (i.e., manually annotated and reviewed by expert curators of the database) are selected and aligned,

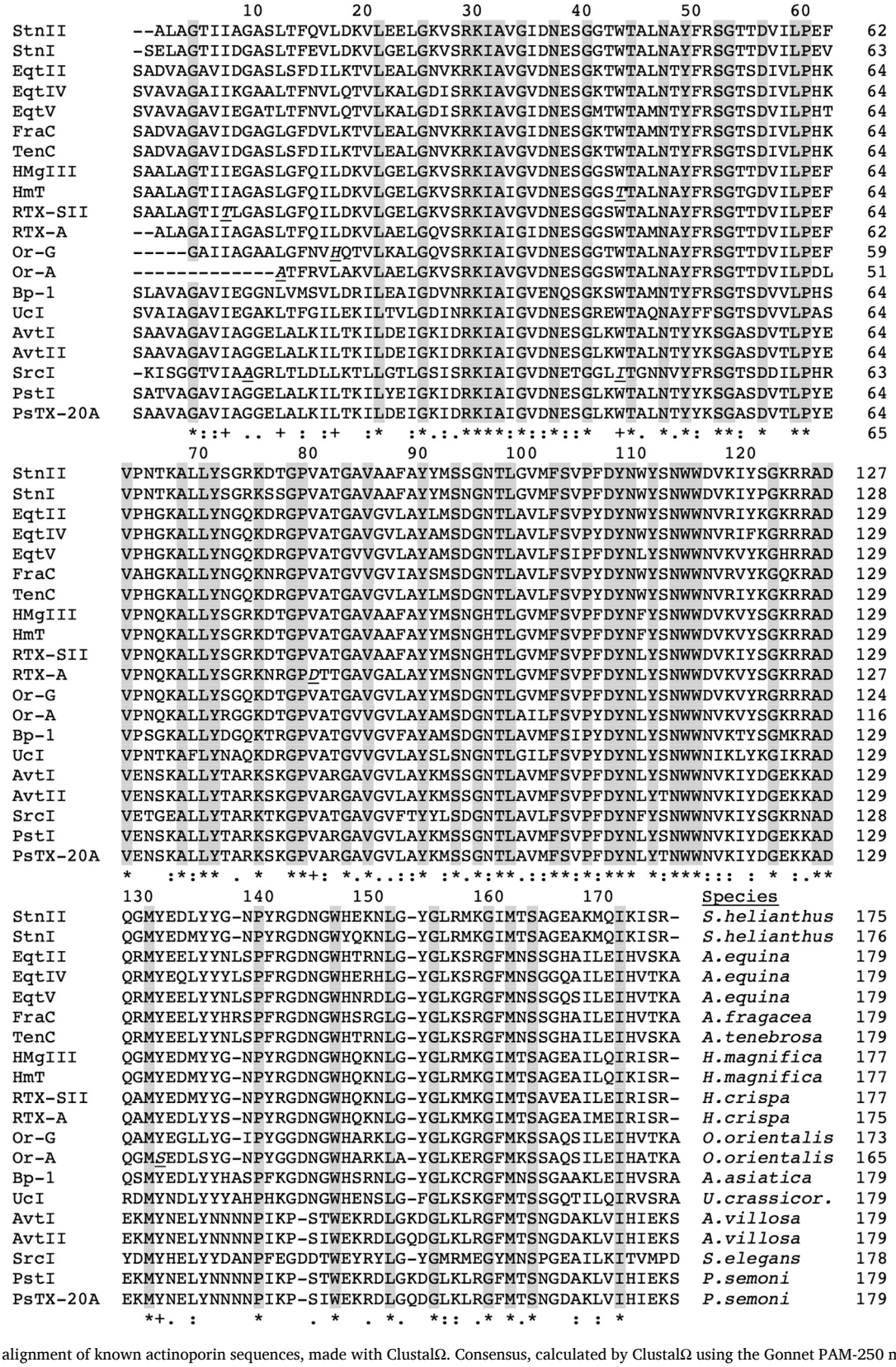

Fig. 2. Sequence alignment of known actinoporin sequences, made with ClustalΩ. Consensus, calculated by ClustalΩ using the Gonnet PAM-250 matrix, is indicated in the last line: asterisk (*) indicates total conservation, colon (:), strong conservation of similar properties, and period (.), conservation of weakly similar properties. A plus (+), added afterwards, outside of the analysis, indicates that the residue is conserved in all but one or two of the aligned sequences. The numbering is that of StnII. *P. semoni*, which produces the last two proteins, is a stony coral.

the result over all sequences reveals 55 conserved residues, 36 that have "strongly similar properties," and 19 that have weakly similar properties, according to the scoring they obtain in the Gonnet PAM 250 matrix. There are still five more positions that are conserved in at least 18 of the sequences, adding up to 115 out of the 175 residues that make StnII (~66%).

Interestingly, among the reviewed sequences gathered by BLAST, there was a protein, bryoporin (UniProtKB entry Q5UCA8), from *Physcomitrella patens* (a moss), which shows some of the conserved motifs considered to be landmarks in the actinoporin fold. The presence of such a protein in a moss has been hypothesized as a result of convergent evolution or horizontal gene transfer [106].

The alignment readily reveals that actinoporins are cysteineless (all but one in the alignment), with lengths between 165 and 179 residues, most being closer to the latter number (Fig. 2). A basic p*I* is also a prevalent feature among these toxins [71–73,88]. The existence of some possible sequence motifs is also brought to light by the alignment. According to StnII numbering, the most important motifs are at positions 29 to 40, at 67 to 71, and the region between residues 92 and 127, in which the sequence P[F/I]DYN[W/L/F]Y[S/T]NWW (residues 105 to 115), a fundamental part of the actinoporin aromatic cluster, is commonly used to identify new potential actinoporins [8,106,107]. The high degree of conservation of these residues is indicative of their importance in the folding and function of these proteins.

3. The structure of sticholysins and the actinoporin fold

The most remarkable ability of these proteins, and the basis for their function, is that they can adopt two different folds. One is water-soluble, whereas the other is triggered by the encounter with a membrane with suitable properties. While most of the three-dimensional structure of actinoporins remains the same in both folds [63,66], nearly 20% of the residues significantly change their relative position as a consequence of this metamorphosis. Furthermore, a concomitant oligomerization process is started. This is a clear confirmation that the three-dimensional structure adopted by a protein depends not only on its sequence but on the environment that it is exposed to as well.

To date, the water-soluble structures of StnI, StnII, EqtII, FraC, and most recently, FraE. have been resolved in atomic detail, either by X-ray crystallography or by nuclear magnetic resonance [49,61–67]. In all cases, the fold consists of a β-sandwich flanked by two α-helices (Fig. 3). The α-helices rest on the exterior surface of each of the two β-sheets, accommodated in the shallow valleys created by the β-sheet's warp (Fig. 4). The first β-sheet is made up of five to six β-strands, mostly antiparallel (a pair of strands at the center of the β-sheet are parallel, see Fig. 4). The first of those β-strands is just four residues long and a part of

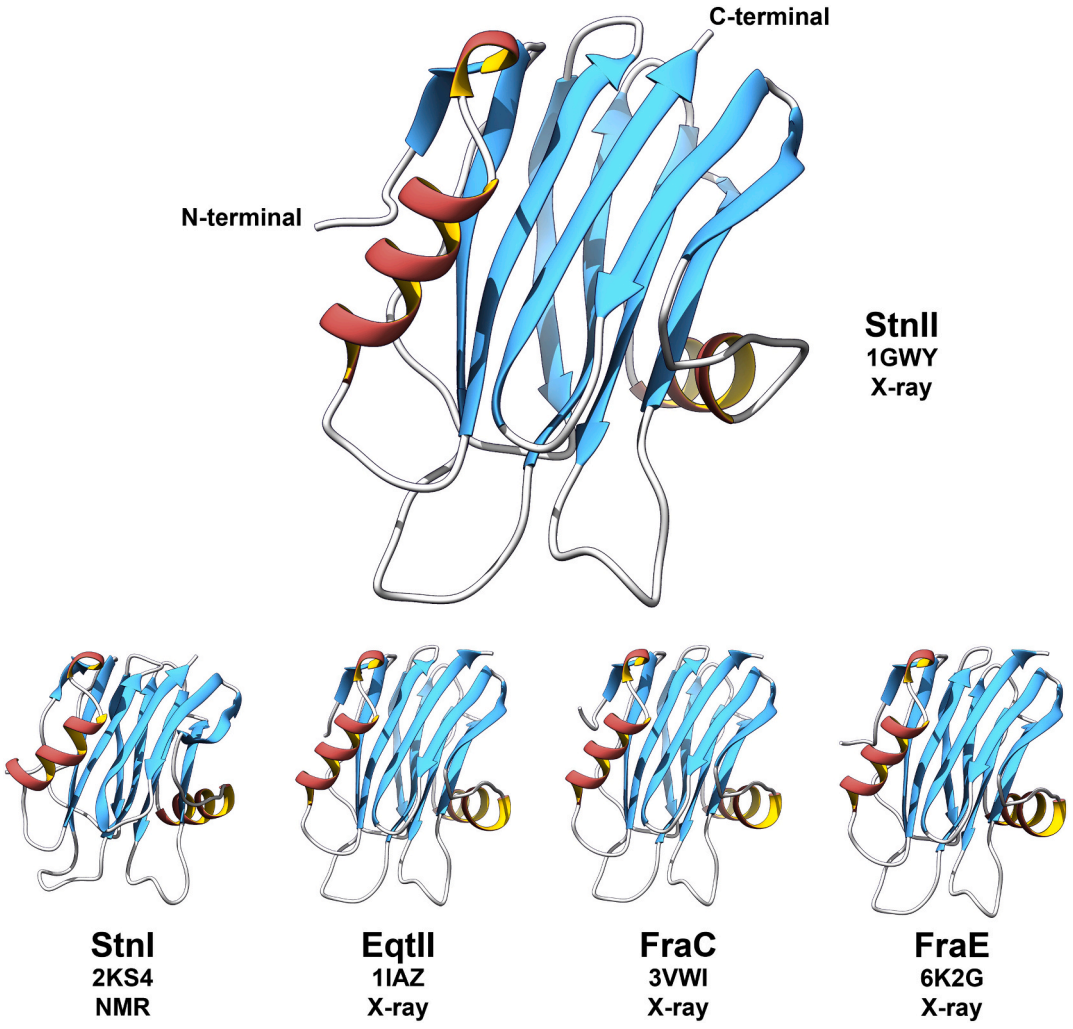

Fig. 3. The three-dimensional structures of actinoporins resolved to date. The name of each protein, along with its PDB ID and method used to obtain the structure is indicated. α and 3_{10} helices are depicted in red and yellow. β-strands are in light blue. Regions with non-periodic secondary structures are in light grey. The PDB structure of StnII (PDB ID 1GWY) is used in subsequent figures, unless otherwise indicated. All figures are made with UCSF Chimera, unless otherwise indicated [108]. Although not shown in the figure, there is also an NMR solved structure of EqtII (PDB ID 1KD6) [64].

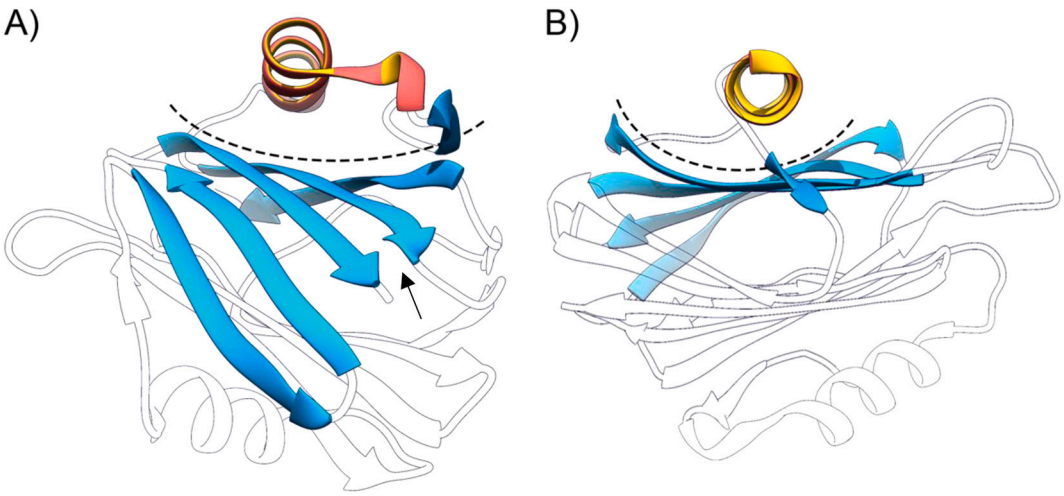

Fig. 4. A) N-terminal view of the N-terminal α-helix of StnII lying on the surface of the β-sheet. Notice (arrow) that the two middle β-strands (second and last on the sequence) are the only parallel β-strands in the structure of these proteins. B) C-terminal view of the second α-helix in the structure of StnII, lying on the surface of the other β-sheet that makes the actinoporin β-sandwich. The space observed between the helices and the sheets is filled by the sidechains of the corresponding amino acids

the protein segment that is eventually responsible for membrane penetration. Likewise, the other β-sheet is made up entirely of antiparallel β-strands.

From a structural and functional point of view, there are four significant regions/features in the structure of these toxins: the N-terminal α-helix, the exposed cluster of aromatic residues, the phosphocholine (POC) binding site, and an array of basic amino acids.

3.1. *The N-terminal α-helix and the β-strands holding it*

The first 30 residues of the protein are folded into a short β-strand, followed by a 3_{10} helix and a 10-residue-long α-helix [61,63–66,109,110]. This stretch has the highest conformational freedom of these proteins. It detaches from the β-sandwich and can fold into a longer amphipathic α-helix. While the first 30 residues contain most of the sequence variability in these proteins, the β-sheets on which the helix lies in the soluble fold have two of the most conserved sequences: the 29-RKIA[I/V]G[V/I][D/E]N[E/Q][S/T]G-40 and the 67-KA[L/F]LY-71 stretches (Fig. 2 and Fig. 5a). The N-terminal α-helix, by changing its extension and relative orientation to the rest of the protein, is responsible for membrane penetration and lining the pore lumen [109,111–114].

All actinoporins have polar residues among their first 30 residue stretch. In the sequence, they are sorted so that they face the solvent in the soluble conformation of the protein, and in the oligomer, they face the pore lumen. The identity of these residues, however, is quite varied. In the first half of this stretch, some present only uncharged polar amino acids, such as Thr and Ser. This is the case with StnII. In turn, StnI has three acidic residues in addition to the polar amino acids. Thus, its helix has a much more marked negative character [115–117] (Fig. 5b). At positions 18 and 19, all actinoporins have polar, complementary residues, most often charged. The sequence in StnI and StnII is Asp-Lys, while in EqtII and FraC it is Lys-Thr. The sidechains of those residues might establish interactions among different subunits to stabilize the final oligomer. Another pair of acid-basic residues appears at positions 22/23 and 26. There is no evidence that these residues interact between them in any of the folds.

Nevertheless, it is interesting to note that some actinoporins have a Glu-Lys pair, while others have a Lys-Asp pair, positioned the other way around. In all actinoporins, those two residues have different charges. These residues might interact with the head groups of lipids in the fenestrations observed in the available pore structures [66].

The hydrophobic residues that form the apolar part of the helix, on the contrary, are, in general, much more conserved, with Leu12 and Phe14 particularly standing out. In fact, they appear to play a double role: they are the part of the helix that stays in contact with the lipids when the helix penetrates the membrane, but they are also responsible, for the most part, for keeping the helix attached to the β-sandwich when the protein is in its soluble form. Phe14 has also been said to be relevant in oligomerization in membranes with tightly packed lipids, such as those with a high Chol content [118].

Residues Arg29 and Lys30 are conserved among all actinoporins as well. In the soluble form of these proteins, Arg29 is likely involved in a cation-π interaction with Phe106 (Tyr in some actinoporins) (Fig. 5c) [119]. It is possible that, upon membrane binding, the loop at which Phe106 is located is displaced, releasing the sidechain of Arg29, triggering the deployment of the N-terminal α-helix. Lys30 appears to also play a role in this process. In all resolved structures except that of StnI (obtained by NMR), Lys30 forms a hydrogen bond with the carbonyl oxygens of Leu21 and Gly25, a residue that is also always conserved (Fig. 5c). This hydrogen bond might be affected by Arg29 as part of the mechanisms triggering N-terminal helix deployment.

These last two residues are part of the first significantly conserved segment of the sequence, expanding from positions 29 to 40. Most of this and the following conserved segment (positions 67 to 71) are the part of the β-sandwich responsible for keeping the N-terminal helix attached to the main body of the protein. Ala32 fills a small gap between residues in the helix, and Gly34, in turn, leaves space for the sidechains of Leu12 and Leu17 (Fig. 5d). These two Leu residues are also conserved in all but one (a different one each) of the proteins shown in the alignment (Fig. 2).

The residues in between the ones mentioned, whose hydrophobic character and overall volume are conserved, are facing the inside of the β-sandwich and are thus essential for the overall folding of the protein. The remaining residues, from 35 to 40, are also highly conserved, but their roles are difficult to decipher.

Lys67 has been shown to be related to the hemolytic activity of actinoporins. It can establish a salt bridge with the residue in position 8, as long as it is of acidic nature. This is the case with StnI, but not with StnII. This way, the energy required to release the N-terminal helix is increased by the contribution of this salt bridge. In fact, the StnII mutant A8D, which can form the salt bridge, had its hemolytic activity reduced to that of StnI [120]. Meanwhile, the reverse StnI mutant, D9A, which can no longer form that bond, presented increased hemolytic activity,

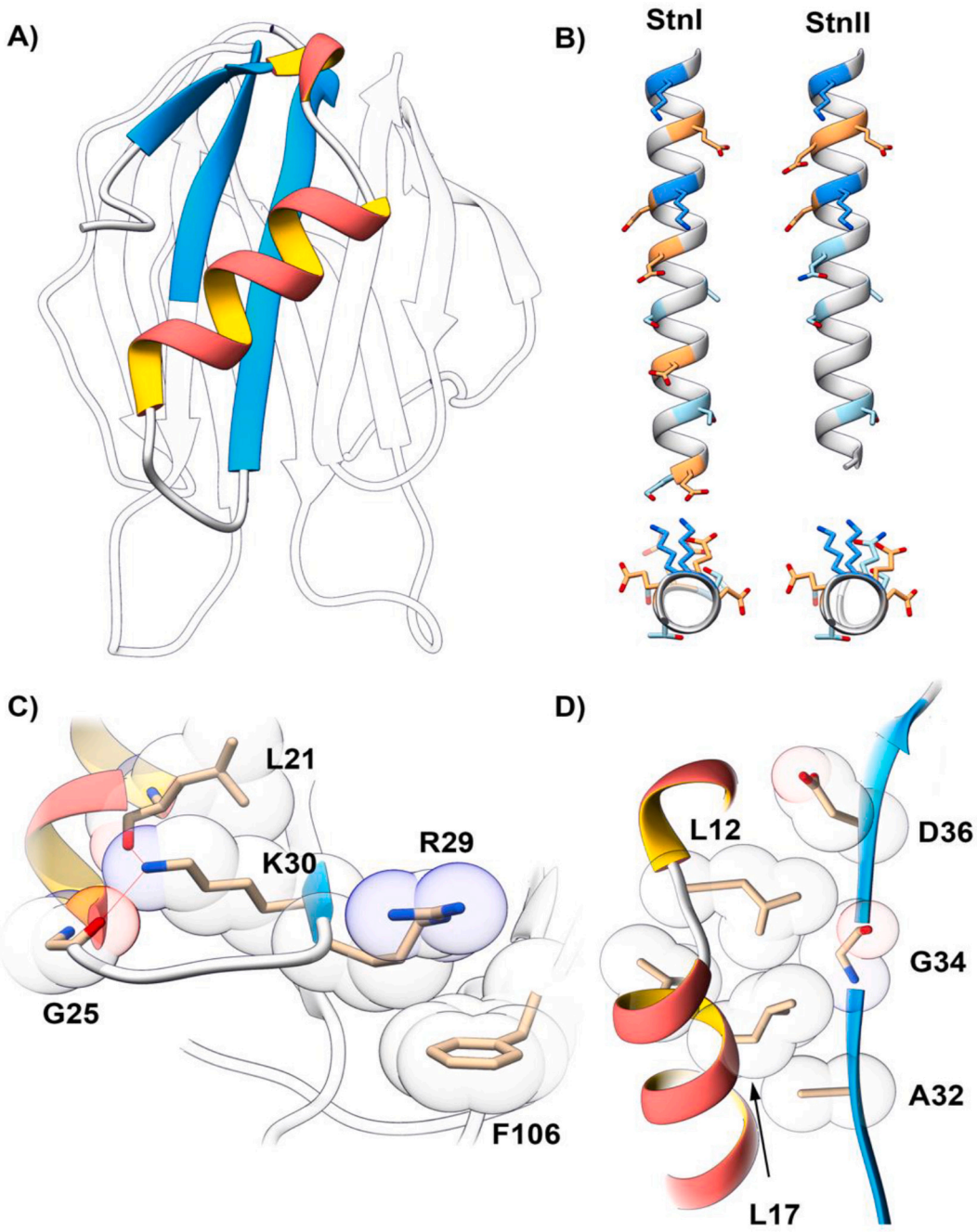

Fig. 5. A) N-terminal α-helix and the β-strands that keep it attached to the rest of the protein. Color code as in Fig. 2. B) Top: Stretched helices (C-terminal up) of StnI and StnII, from the N-terminal to V27, made in silico with UCSF Chimera using the Richardson rotamer library for the amino acid sidechains. Negatively charged residues in orange, positively charged, in blue. Other polar residues, in light blue. Hydrophobics are hidden for clarity. Bottom: C-terminal view of the helices. Note that all polar residues face the same side of the helix. C) Close up on the structure of StnII. On the left, hydrogen bonds between the carbonyl oxygens of G25 and L21 and the ε-amino group of K30. On the right, R29 is in close range of F106, involved in a cation-π interaction. D) Close up on StnII showing hydrophobic contacts between the N-terminal α-helix and the first β-strand of the actinoporin fold (see main text for details).

almost that of StnII [120]. The same effect was observed regarding calcein release [120]. This effect could also explain the differences in hemolytic activity between StnII and FraC and EqtII, which, like StnI, have an acidic residue at that position [84]. As in the previous highly conserved section, the next four residues also fulfill folding duties. Ala68 is required to be small and hydrophobic to allow the helix to be properly folded on the surface of the β-sheet. Leu 70 plays an essential role in holding the helix folded by filling the void among Leu2, Ile7, Leu12, Val16, and Leu17, giving them a hydrophobic surface to interact with, reducing the likelihood of spontaneous helix deployment by means of the hydrophobic effect.

It should be noted that the small β-strand at the beginning of the sequence also plays a role in holding the helix folded onto the β-sandwich, by being part of that same β-sheet that the helix lies on. To that regard, Gly9, a residue that is also highly conserved (in all but two of the aligned sequences), appears to play a fundamental role, providing the flexibility required to properly place both that β-strand and the N-terminal α-helix relative to the β-sheet.

Finally, the first 30-residue stretch of sticholysins has been produced and assayed independently of the whole protein (Fig. 5b). The results

show that those peptides alone are still able to induce hemolysis and can adopt helical structures in the presence of lipid or lipid-mimetics [115,121–124]. Similar experiments have also been conducted using the peptides derived of the N-terminal stretch of EqtII, using residues 11-29, 11-32, and 1-32 (EqtII numbering here). Interestingly, these peptides display limited hemolytic activity [125].

3.2. The exposed aromatic cluster

Most of the exposed aromatic cluster is part of the highly conserved sequence between positions 92 and 127, which also contains residues that are included in the other functional regions of these proteins. Briefly, it can be mentioned that most residues in this sequence are conserved, with the remaining being conservatively substituted. This stretch makes up two of the β-strands that constitute the β-sandwich, one of the most exposed and flexible loops in the actinoporin structure, and two other connecting loops. In fact, residues 97 to 104 make the β-strand that is complementary to the aforementioned conserved strand made by the residues 30 to 38 (Fig. 6, arrow).

The complete list of residues in the aromatic cluster is Phe106, Trp110, Trp111, Trp114, Tyr131, Tyr135, and Tyr136 (Fig. 6). Amino acids with aromatic sidechains in many proteins have been previously shown to be distributed preferentially at the water-membrane interface [126]. The study of several mutants evidenced that the residues in this aromatic cluster were involved in membrane recognition [114,127].

In EqtII, aromatic residues, and particularly Trp residues, have also been shown to be essential for the process of membrane binding [109,128]. Trp112, equivalent to Trp110 of StnII, was demonstrated to be key for SM-recognition [129,130]. However, the residue in this position is only Trp in six of the 20 sequences aligned in Fig. 2. In fact, the residue that appears most often in that position is Leu. Nevertheless, the EqtII W112L mutant still retained the specificity for SM [130]. Accordingly, it has also been shown that substitution of StnII Trp110 by Phe yields a fully functional mutant [131]. This indicates that this amino acid is only required to have a hydrophobic and large sidechain. The interactions in which it is involved, thus, are presumably nonspecific.

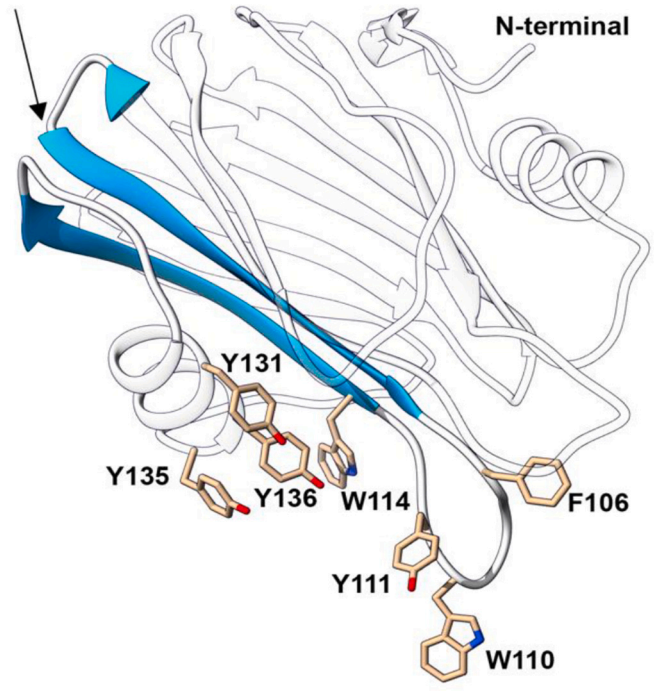

Fig. 6. The exposed aromatic cluster of actinoporins and the conserved sequence between residues 92 and 127. The sidechains of the amino acids that make up the aromatic cluster are shown. Ribbon is shown in color for positions between 92 (arrowhead in blue at the top) and 127 (loop connecting with the second α-helix of the structure). The black arrow is pointing at a β-strand whose role is discussed in the text.

Phe106, in turn, is much more conserved. The residue in this position is Phe in 13 of the aligned sequences and Tyr in the remaining ones, denoting that what is important here, at least from a functional point of view, is the presence of a phenyl-ring. In terms of folding, substitution of Phe106 by Leu resulted in a mutant whose T_m barely varied from that of the WT toxin, indicating that a large, hydrophobic residue provides the chemical character required for folding [114]. However, from a functional point of view, the capability to establish cation-π interactions seems to be essential, such as indicated before with Arg29 (Fig. 5d). Nevertheless, the hydrophobic character of Leu again seems to be capable of providing some compensation since the affinity constant of that mutant was only about a third of that of the WT protein [114].

In the resolved structures of actinoporins, Tyr111 is always exposed, in principle not interacting with any other residue in the protein. It belongs to one of the loops with the highest conformational flexibility of these proteins [123,132]. However, this amino acid is conserved in all actinoporin sequences. In contrast with most of the residues reviewed this far, Tyr111 does not seem to play a role in protein folding, as suggested by the thermostability of the StnII Y111N mutant being identical to that of the WT protein [127]. Instead, it is most likely essential for protein functionality. In fact, when StnII is co-crystallized with POC, Tyr111 is observed to interact with that moiety [61]. Its importance is further highlighted if it is mutated. The mutant Y111N of StnII is essentially incapable of membrane binding [114,127,132,133].

The remaining residues of the exposed aromatic cluster, Trp114, Tyr131, Tyr135, and Tyr136, are conserved in most aligned sequences. As with Tyr111, it appears that these amino acids are more related to protein functionality than to folding. In fact, all the aforementioned Tyr residues, including Tyr111, play an essential part as constituents of the POC-binding site. Trp114, which is conserved in all the aligned sequences, but is not considered part of the POC-binding site, is essential for membrane binding in absence of Chol, as shown by the results obtained with a StnII variant that included the mutation Trp114Phe [131]. This Trp residue has also been identified as the closest to Chol when StnII is bound to the membrane, likely playing a part in the simultaneous interaction of these toxins with SM and Chol [87].

3.3. The phosphocholine-binding site

The POC-binding site plays a fundamental role in actinoporin functionality. It is responsible for the direct interaction with the head group of SM [61]. The amino acids that form the site are Arg51, Ser52, Val85, Ser103, Pro105, Tyr111, Tyr131, Tyr135, and Tyr136 (Fig. 7a). Of these, the Tyr residues are also part of the exposed cluster of aromatic residues, and their importance in membrane folding has been discussed above. The remaining amino acids of the POC-binding site, except for Arg51, are conserved in all sequences (Fig. 2). Arg51 is preserved in most sequences or conservatively substituted by a Lys residue.

The POC-binding site was revealed by StnII co-crystallization with POC [61]. Careful inspection of that structure reveals that Tyr111 is involved in a cation-π interaction with the trimethylamine moiety of the POC molecule. Computer simulations have predicted that Tyr111 also interacts with the phosphate moiety of SM (POC is the head group of SM) via its phenolic hydroxyl group [134] (Fig. 7a). Tyr111, with its high conformational flexibility, has been proven to be fundamental for SM recognition regardless of the presence of Chol in the membrane [119].

Similarly, Tyr136 is both predicted and shown to form a hydrogen bond with that same phosphate group [61,134]. Tyr135 is predicted to interact with the 2NH and 3OH groups of SM, but in the POC-containing crystal, which does not include those groups, it helps maintain a network of solvating waters around the POC alongside Arg51, Ser52, Ser103, and Tyr131 [61]. Mutation of Tyr135 showed that this residue is also essential for membrane recognition in the absence of Chol. However, the

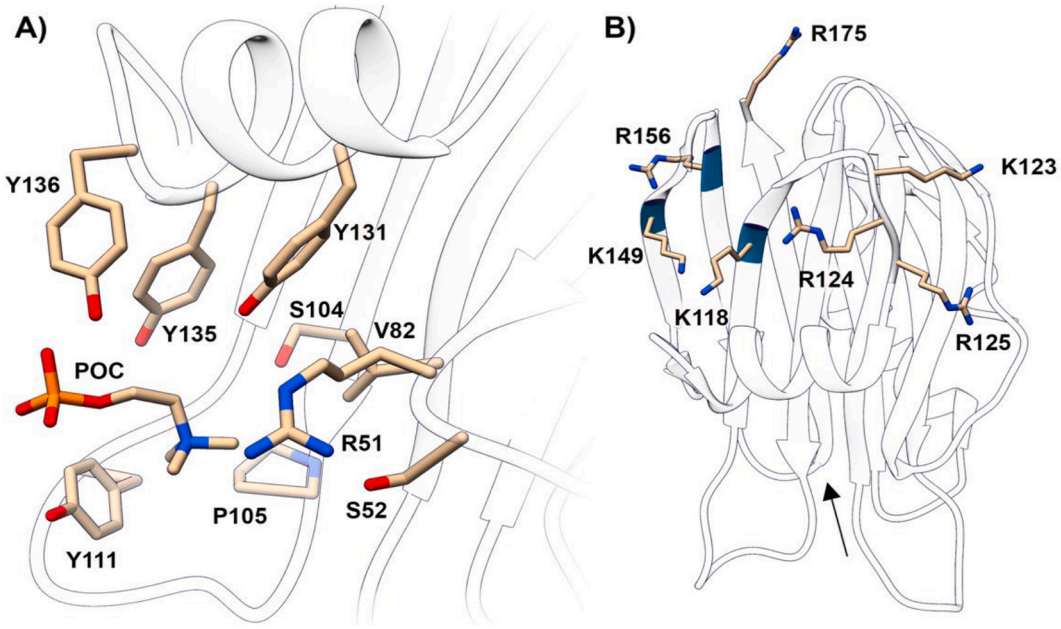

Fig. 7. A) Residues that conform the POC-binding site. Close-up at the structure of StnII complexed with POC (PDB ID 1O72). All identified hydrogen bonds and solvating waters are omitted for clarity. B) Positioning of the residues that make up the array of basic amino acids within the complete structure of StnII. The arrow indicates the location of the POC-binding site, shown in A).

presence of this sterol seems to compensate for this substitution, both in terms of activity and membrane binding [119]. Finally, and still according to the simulations, the ∂-guanidinium group of Arg51 is predicted to interact with the phosphate moiety as well [134]. This residue was shown to be, like Tyr111, fundamental for the activity of these proteins [119]. However, the mutant in which Arg51 is substituted by a Gln residue is still capable of binding Chol-containing membranes [119].

The side chain of Val85 is hydrophobic. The sidechain of Pro105 can be considered to be essentially hydrophobic as well. Both of these residues appear to be in close proximity with the methyl groups of the trimethylamine moiety of POC. In the three-dimensional structure of the StnII-POC complex, there is no major displacement of the sidechains of these amino acids when compared to the orientation they present in the POC-free StnII structure. Nevertheless, it is possible that the interaction with a complete SM molecule does cause a change in the conformation of these residues. This is especially relevant for Pro105 since it is adjacent to Phe106, which was previously mentioned to be involved in a cation-π interaction with Arg29. It is possible that the movement of Pro105, which in turn would modify the interaction of Phe106 with Arg29, acts as the trigger for the deployment of the N-terminal α-helix. These conformational changes might be induced by the overall change of that protein region in response to the presence of SM. Some of the neighboring residues of Pro105 would be drawn to interact with this molecule, whereas some others, like presumably Pro105, would most likely avoid it due to the charged character of the SM headgroup.

POC binding has been shown to be essential for membrane recognition and binding when facing Chol-lacking bilayers. The presence of Chol compensates for the single substitutions of one of these amino acids. However, even though membrane binding still occurs, Arg29 (which is not part of this site) and Tyr135, and especially Arg51 and Tyr111, have been shown to be crucial for membrane permeabilization [119,133].

Finally, Arg29, which has already been mentioned to be important for the activity of these proteins (see Section 3.1), has also been observed to play a role in terms of electrostatic surface potential [132]. The electrostatic potential of the StnII mutant R29Q is, in fact, much less positive than that calculated for the wild type (WT) variant of the protein, most likely unpairing the interaction with the phosphate moieties of the lipid in the membrane [132].

3.4. The array of basic amino acids

All characterized actinoporins display an array of basic amino acids. These are Lys118, Lys123, Arg124, Arg125, Lys149, Arg156, and Arg175. This array has been shown to be involved in the early steps of membrane recognition in EqtII [64]. In the aligned sequences shown in Fig. 2, these residues are conserved or conservatively substituted in most cases. In EqtII, for example, and compared to sticholysins, most of these amino acids are reversed, substituting Lys by Arg and vice versa. It has been hypothesized that this difference could be responsible for the different lipid selectivity displayed by StnII and EqtII [44,113,114,130,135]. However, it is not clear what the role of these residues is at the detailed molecular level since, though helpful, negatively charged lipids in the membrane are not a strict requirement for actinoporin activity.

3.5. Structural differences between StnI and StnII

StnI and StnII are the main actinoporins produced by *S. helianthus* [8]. StnII is made by 175 amino acids, while StnI has an extra residue at the N-terminal. Only 12 amino acids, plus the additional residue at the N-terminal, differ between these two proteins (Fig. 8). Of those differences, five are located at the N-terminal α-helix in the region that is exposed in the soluble fold. Two are found in one of the exposed loops that penetrate the membrane upon membrane interaction, and two more are at one side of the structure, which might be involved in monomer-monomer interactions.

In spite of sharing 93.7% sequence identity, the comparative hemolytic activity of these proteins is substantially different [84]. In fact, their membrane binding, as measured by isothermal titration calorimetry (ITC), and their induced calcein release are also unequal and dependent on the membrane composition [84]. Nevertheless, it has been shown that the difference between StnI and StnII at position 8, as mentioned in the section regarding the N-terminal stretch, is

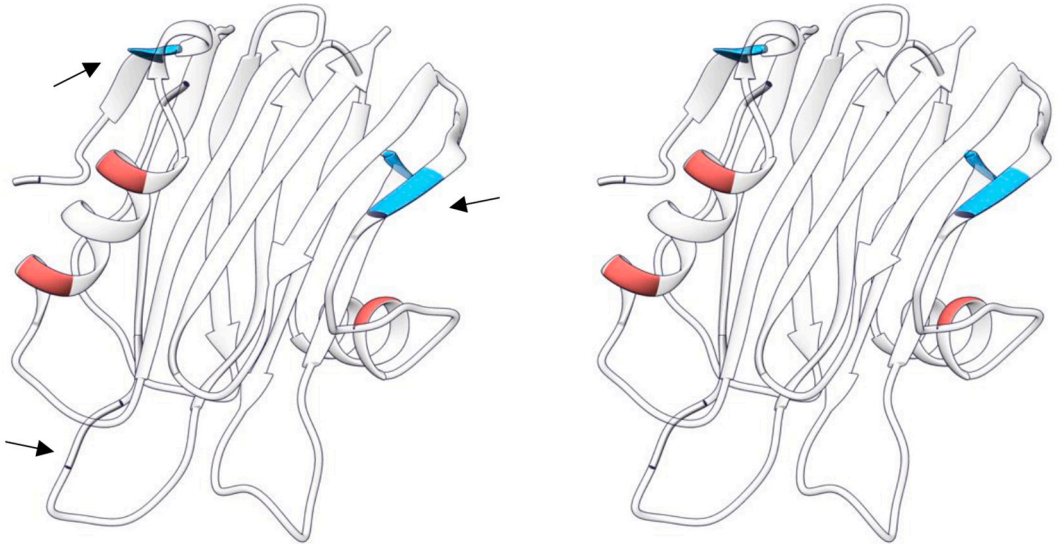

Fig. 8. Cross-eye stereo projection showing the positions differing between StnI and StnII. Arrows point at the residues whose importance is highlighted in the main text, namely position 8 (top left), which is Ala in StnII and Asp in StnI, positions 76 and 77 (lower left), which are Ser-Ser in StnII and Asp-Thr in StnI, and positions 147 and 148 (right), which are His-Glu in StnII and Tyr-Gln in StnI.

responsible, to a very large degree, for the behavior differences between StnI and StnII [120]. The role of the remaining differences is not clear and, so far, it has not been studied systematically, though hydrophobicity of the final helices lining the pore also seems to be important [84,122–124,136,137]. However, these different residues must also be responsible for the observed synergy that these two proteins display when assayed together [101].

4. Influence of the membrane on actinoporin activity

Lipids, particularly SM, are the only membrane elements that actinoporins require to interact with a membrane. This is clearly shown by their interaction with model membranes that lack non-lipid components and the fact that incubation with SM, or the removal of SM from erythrocytes with sphingomyelinase, inhibits their action [12,13,26,31,32,34,138]. Membrane phase coexistence in the presence of Chol, the fluidity and compactness of the bilayer, and the interfacial hydrogen bonding network of SM also affect the activity that actinoporins display when encountering a given membrane [32,35,76,77,79–83,139,140]. Since actinoporins present an array of basic amino acids (see Section 3.4), a preference toward anionic phospholipids (PLs) could be expected. However, net charge has not been observed to play a significant role in the interaction [109,130]. In fact, charged residues are essentially absent from the binding site, mostly unaffected by the overall charge distribution on the protein [130,141].

4.1. The role of sphingomyelin

The presence of SM in the membrane, however, does not guarantee actinoporin binding to the bilayer, which is largely influenced by the aforementioned phase state of the membrane [26,31,32,34,82,83,138,141,142]. If ceramide (Cer) and SM, both fully saturated, are used together, the membrane will contain a highly ordered phase [143–145]. SM and Cer will then partition together, rendering SM out of the actinoporins' reach [81]. Thus, it can be said that the requirement for membrane binding is not just SM presence in the membrane but SM availability.

The SM selectivity displayed by actinoporins happens despite of PC and SM sharing the same head group moiety, POC. Therefore, the capability of discriminating between PC and SM must be based on the parts of the molecules that differ between them under the head group [130]. The 2NH and 3OH groups of SM, which constitute the main structural difference between SM and PC, should thus participate in the interaction with the toxin. Using the analogs of SM that were methylated in either of the groups, it was shown that replacing palmitoyl-SM (PSM) with either of its modified analogs impaired sticholysin activity, as reported by calcein release assays, and membrane binding, as measured by ITC and surface plasmon resonance (SPR), just as if the membranes were made of pure POPC [134]. The residues that take part in this interaction, which have been highlighted previously (see Section 3.3), also interact with the phosphate moiety of the POC. Just as the 2NH and the 3OH of SM are essential for the interaction, so is the phosphate group, and to a similar level of detail. It has been shown that the basis for avoiding self-toxicity lies precisely at the head group of SM [146]. This was done studying the activity displayed by the cytolysin of the sea anemone *Phymactis clematis*, which also belongs to the actinoporin family when it faces *P. clematis* lipids or other exogenous lipids [146–148]. The membrane composition of the sea anemone *P. clematis* does not include SM. Instead, it presents phosphonosphingolipids (PnSL), which are structural analogs of SM. In these molecules, the oxygen atom between the choline moiety and the phosphorus atom is missing, resulting in a direct bond between the phosphorus and the corresponding carbon atom of the choline [146]. Moreover, the degree of methylation of the nitrogen group of the ethanolamine moiety is also different, being mostly unsubstituted or monomethylated, though it can also be found trimethylated [146,149,150]. Based on later research, it appears that, while the trimethylation of the nitrogen of choline is likely important in terms of occupancy, the missing oxygen, which never appears in PnSL, is the basis of the selectivity. In fact, the sidechain of Arg51 in StnII is predicted to interact, precisely, with this oxygen [134]. Going back to Section 3.3, the mutation of Arg51 resulted in a mutant unable to induce calcein release in all instances and in membrane binding in the absence of Chol [119]. The cause would most likely be that the interaction with that oxygen can no longer take place. PnSL have also been observed in other sea anemones, including *S. helianthus*, and, in general, in the Cnidaria phylum [151–154]. This reinforces the hypothesis that these lipids are responsible for the actinoporins' ineffectiveness on the membranes of sea anemones, which can contain 20 mol% of PnSL [154].

Still, some exceptions to the need for SM availability have been observed. In such cases, the vesicles employed were made of PC with significant amounts of Chol, which can induce phase separation [32,74,75]. It has also been observed that SM is not enough to ensure

pore formation on giant unilamellar vesicles (GUVs), requiring the coexistence of the L_o and L_d phases, with pores being formed at the interface [75]. Nevertheless, it should be pointed out that that last report was made using EqtII mutants labeled with a large, highly hydrophilic fluorophore placed at positions in the N-terminal α-helix that correspond to hydrophobic residues in the WT protein. Back to phase separation, it has been shown that StnII forms pores on COS-7 cells precisely at the regions of the membrane that are enriched in SM and Chol [139]. Although much more complex than model membranes with L_d and L_o phases, these parts of the cell membrane are also considered to be laterally segregated. This point of view, which highlights the importance of phase separation, is also supported by a recent molecular dynamics work which indicates that the structure of the FraC pore would not have preference for SM over PC [155]. In this regard, similar observations have been made for EqtII as well. Those results indicate that when phase separation occurs, EqtII binds to lipid domain boundaries [76,156]. However, it can also recognize SM in the absence of phase separation and form pores when SM is in the L_d phase [156,157].

4.2. The role of cholesterol

The role played by Chol in the actinoporin-membrane interaction is unclear. For example, pore formation by StnI and StnII is enhanced by the presence of different sterols, regardless of their domain-formation capability [80,83]. It was shown that the hydrogen-bond acceptor capability of the sterol 3β-OH and an increase in membrane fluidity were responsible for enhancing the sticholysin-induced release of contents from LUVs, without a concomitant ordering of the SM phase [80,82,83]. In a way, this is consistent with the observations that actinoporins preferentially bind at the domain boundaries. These regions and fluid, more disordered membranes are richer in imperfections than the homogeneous, ordered phases. Thus, domain boundaries could facilitate membrane penetration, increasing the local concentration of toxin and reducing the energy barrier of the penetration step itself [76]. SM head groups at domain boundaries would be further exposed to the solvent due to the membrane's imperfections, easing the recognition process. Chol would be responsible for promoting phase separation. Incidentally, Chol is also able to modify the orientation and dynamics of the SM head group, according to the "umbrella hypothesis" (Fig. 9) [158,159]. This can be helpful for actinoporins when it comes to SM recognition [76]. Using an SM analog derivatized with *trans*-parinaric acid (tPa) as its acyl chain (tPa-SM), it was shown that, while including 10 mol% of Chol significantly increased StnII-induced calcein release, the acyl chain order of SM only increased modestly, as shown by the fluorescence anisotropy of tPa, indicating that something else was affected in SM [81].

Chol increasing lipid packing is a consequence of its interaction with the acyl chains of co-lipids, consequently affecting membrane fluidity [160,161]. Due to Chol's preference for SM, it will mostly affect the acyl chain order of SM when included in a model membrane made of PC-SM as well as the SM hydrogen-bonding network and, overall, the properties of the SM-rich phase [162–164]. The N-terminal α-helix residue Phe16 of FraC (equivalent to Phe14 in StnII) has been proven essential when facing tightly packed membranes, with its substitution resulting in mutants whose ability to induce calcein release is nearly abolished [118].

A later study has shown that the overall structure of Chol, and not only its -OH headgroup, is responsible for facilitating SM-recognition by StnII [87]. Oleoyl-ceramide, which also interacts with SM and has an equivalent interfacial hydroxyl moiety, did not promote the permeabilizing capabilities of StnII, nor promoted SM-acyl chain separation as Chol did. Furthermore, it was also shown that StnII-binding to membranes containing both SM and Chol induced a rearrangement of these lipids in the bilayer, increasing the overall separation of the acyl-chains of SM while also effectively extracting Chol from the PC-rich phase of the membrane [87]. In fact, a fluorescent analog of Chol, cholestatrienol, was shown to be preferentially distributed near StnII when equilibrium in the system was reached [87].

4.3. Other membrane effects

One of the hypotheses regarding the final configuration of the pore consists of actinoporins bending the membrane so that a toroidal pore is created. The pore walls would then be lined by both the toxins' α-helices and the polar head groups of lipids [165]. The presence of lipids that induce non-lamellar phases could increase the efficiency of pore formation. However, no substantial difference was observed when StnII was assayed against model membranes made of SM:Chol:glycerophospholipid in a 50:35:15 ratio, where the glycerophospholipid was PC or phosphatidylethanolamine (PE) [166,167]. PE induces an inverted hexagonal phase (H_{II}), which is characterized by having a curvature opposite to the one needed [168]. Nevertheless, phosphatidic acid (PA),

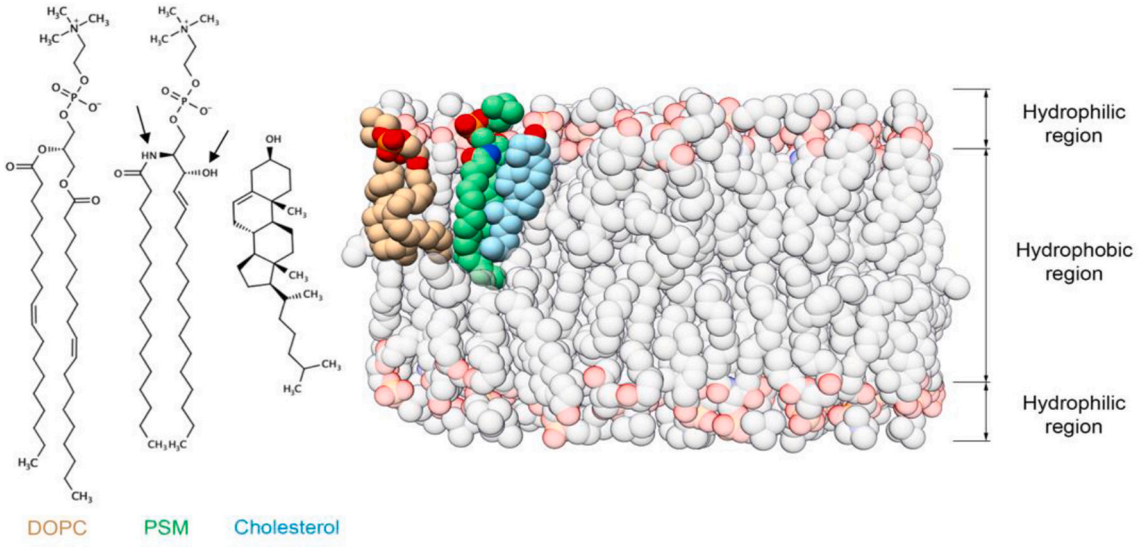

Fig. 9. Left: Schematic representation of the structures of DOPC, PSM and Chol. Arrows point at the 2NH and 3OH groups of PSM, referred in the main text. Schemes made with Marvin Sketch 19.23 (ChemAxon, 2019). Right: space-filling depiction of a lipid bilayer. A molecule of each DOPC, PSM, and Chol is highlighted in the color indicated on the left. Notice Chol's location beneath the head group of PSM.

which also induces the H_{II} phase, has been observed to increase the initial rate of calcein release [138,168]; perhaps the negative charge of this lipid also played a role in this observation. EqtII has been shown to induce non-lamellar phases, which are consistent with toroidal pores [169]. The formation of toroidal pores is also consistent with the observed increased rate of flip-flop transitions induced by sticholysins [138].

Bilayer thickness is another composition-dependent membrane characteristic that has been shown to affect the activity of sticholysins, both StnI and StnII [170]. The results presented indicated that, for an equivalent phase state, and SM and Chol content, these toxins preferred bilayer containing PC species whose acyl-chains consisted of 16 or 18 carbon atoms over those with shorter or longer acyl-chains [170]. The selectivity displayed appeared to correlate significantly with the length of the N-terminal α-helix responsible for membrane penetration. The fact that most abundant acyl-chains in the lipids of studied fish [171], marine crustaceans [172–174], mollusks [175,176], and insects [177] also contain 16 and 18 carbon atoms suggests that the length of the N-terminal α-helix of actinoporins is a result of evolutionary pressure since the mentioned organisms have been found in the gastrovascular cavity of *A. equina*, amongst other sea anemones, indicating that they represent a significant part of their diet [178].

Finally, some other potential membrane acceptors for actinoporins have been proposed on the grounds that erythrocytes can be lysed at comparatively smaller concentrations than those required to induce leakage on model vesicles [26,71]. Many actinoporins (11 out of the 20 aligned in Fig. 2) present an RGD-motif at position 141, which is located at one of the protein sides and, supposedly, close to the membrane in the pore structure. The RGD-motif is involved in the integrins' recognition of fibronectin [179]. It was proposed that actinoporins could use this motif to recognize integrins at the cellular surface, in spite of integrins being absent from the erythrocyte membrane [103]. However, the mutation of the Gly residue to Ala resulted in mutants whose oligomerization capability was severely restricted [180]. Moreover, it has been proposed that glycolipids could also facilitate actinoporin membrane binding since these toxins are eluted later than expected when put through a chromatography on a polysaccharide-based support using a buffer with low ionic strength. Nevertheless, this does not seem to be a specific interaction. Instead, it is probably a consequence of the negative charges displayed by the glycans and the overall positive charge of the protein [109,181].

5. Pore formation

As PFTs, the ultimate function of actinoporins is to form pores. Actinoporins are required to bind the target membrane and oligomerize. The roles played by different parts of these proteins in the process are understood to quite a high level of detail. Nevertheless, the sequence of events leading to pore formation is still unclear, being a source of controversy in the field.

5.1. *Membrane binding*

In the absence of membranes, actinoporins remain water-soluble. They retain their usual fold, consisting of a β-sandwich flanked by two α-helices. However, when they encounter a membrane with suitable characteristics, they attach themselves to it [182]. The process of membrane binding involves all protein regions mentioned previously (Sections 3.1 through 3.4) [182,183]. Kinetic measurements made with SPR using EqtII indicate that pore formation is a two-step process [109,130]. The first step would be membrane binding itself, with the second step comprising oligomerization and actual pore formation (Fig. 10).

Membrane binding would be driven mainly by the exposed cluster of aromatic residues, the array of basic amino acids, and the POC-binding site [73,182,183]. These elements would provide the required affinity for membrane binding and recognition. The array of basic amino acids and the aromatic cluster would likely be responsible for favoring protein

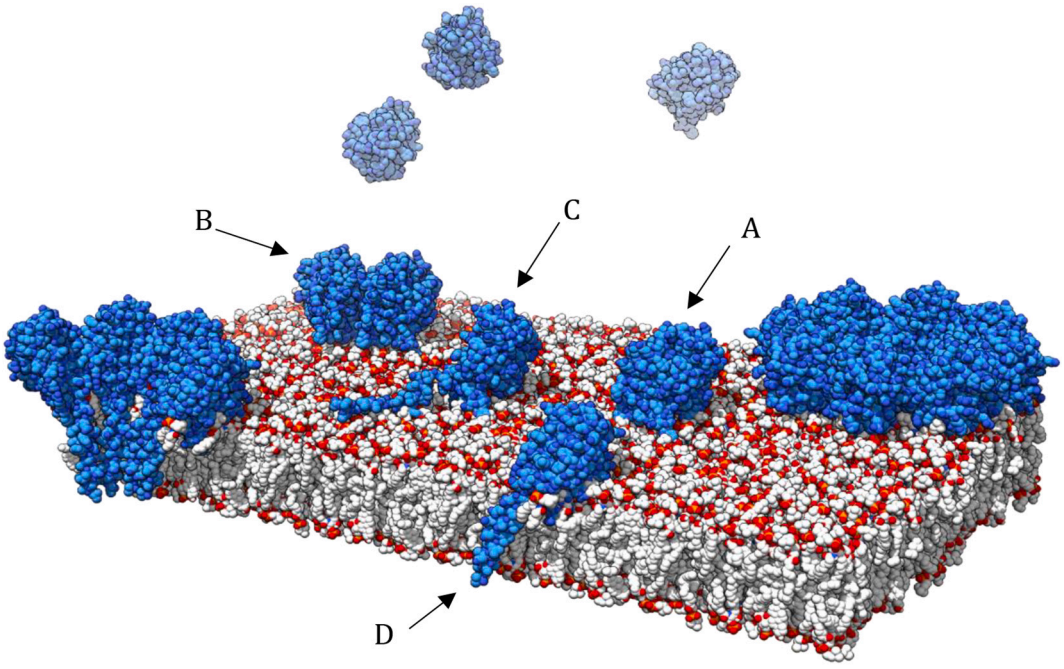

Fig. 10. Artistic all-atom representation of the process of pore formation by actinoporins, including some hypothetical intermediates. Lipids in white (carbon atoms), red (oxygen atoms), and orange (phosphorus atoms). Actinoporins in light blue (carbon atoms) and, for simplicity, dark blue (heteroatoms). Soluble monomers (top) would bind the membrane as monomers (A) or higher order oligomers (dimers in B). Then the helix would be deployed, lying on the surface of the membrane (C). Eventually, the helix would penetrate the bilayer (D) causing a perturbation on membrane continuity (not shown). Finally, those monomers would assemble into the final pores (left, showing the transitable channel, and right). Featured protein structures were made using the structure of StnII (PDB ID 1GWY) and the structure of the FraC pore (PDB ID 4TSY).

partition to the membrane surface [123]. However, this should not be a strong effect since no binding is observed when vesicles of pure PC are used [130,134]. The POC-binding site would then provide a firm attachment by recognition of and binding to SM. At this stage, the toxins are unlikely to have undergone significant structural changes other than at the sidechain level.

5.2. Oligomerization and membrane penetration

The oligomerization state is at this point of the process not clear, nor is whether several degrees of oligomerization are possible at this advanced stage of the mechanism. Though essentially monomeric in solution, there are reports showing that a part of the total population of StnII in solution consists of dimers, trimers, and tetramers, without the need for membrane binding [184]. Similar observations have been made regarding StnI. In that case, it was shown that StnI oligomerization was favored by the presence of StnII in the sample [185]. Thus, it is possible that the oligomerization process starts before encountering a bilayer. Nevertheless, this has not been observed in other actinoporins other than sticholysins. As to EqtII, for example, it has been proposed that this difference might lie in the distinct surface charge distribution of these two proteins [130].

Actinoporins undergo a significant structural change to transition from soluble, monomeric structures to oligomeric, transmembrane ensembles [66,73,113,182,183]. The biggest alteration of the soluble fold consists in the detachment of the N-terminal α-helix from the β-sandwich. This detachment of the N-terminal α-helix is associated with a conformational change in this segment, so that approximately all first 30 residues of the structure adopt an α-helical structure. How these events happen and how membrane penetration occurs is unclear. However, it has been shown that these events, and not the final, thermodynamically stable pore ensemble, are responsible for membrane permeabilization observed when performing calcein release or equivalent activity assays with StnII [186]. This observation is most likely applicable to all actinoporins.

Studies on FraC suggest that monomers would serve as the membrane-binding unit. In contrast, results obtained with StnI and StnII indicate that dimers, trimers, and tetramers could also be formed prior to membrane binding [66,184,185]. In any case, incorporation to the membrane surface results in a dramatic increase of the local concentration of toxin at the water-membrane interface (Fig. 10). The importance of this effect can be of several orders of magnitude, from nM in solution to mM locally at the membrane surface (assuming lipids are in the range of μM). After that, dimerization would occur (Fig. 10B) [66]. The sidechain of Phe16 of FraC (Phe14 in StnII; highly conserved among actinoporins) would be displaced by the sidechain of Val60 (Val62 of StnII; conserved in all sequences in Fig. 2) of the other subunit of the dimer, causing a partial unfolding of the N-terminal α-helix. In this case, it is proposed that further oligomerization would lead to the formation of a pre-pore ensemble, which would transition directly into the final pore [66,187]. According to this model, helix-detachment would be mediated and induced by oligomerization. The existence of a pre-pore is highly controversial, since some claim that it might not be large enough to allow the extension of the helices and their subsequent insertion in the bilayer.

However, it is also possible that helix deployment occurs before, being triggered, as presented in Sections 3.1 and 3.3, by SM binding. SM binding would be the only requirement in that scenario. After being released from the β-sandwich, the N-terminal α-helix would lie on the surface of the membrane due to its amphipathic character (Fig. 10C) [73]. Then, at some point, it would break the energy barrier and penetrate the bilayer (Fig. 10D). Some results indicate that the membrane insertion of the helix takes place just after membrane binding [188]. Since no other monomers would be required to detach the helix, it is possible that oligomerization takes place before helix detachment, while the helix lies on the membrane surface, once it has penetrated the membrane, or under any of the listed circumstances (Fig. 10B). Helix insertion, or simply deployment, prior to oligomerization implies that the pre-pore state would not be required [188]. EqtII single-molecule live-imaging and subsequent analysis indicated that, as proposed for FraC, the pores would be formed by the condensation of dimeric intermediates [189]. This is also supported by the observation that pre-assembled StnI dimers cross-linked by disulfide bridges at the N-terminal end appear to improve pore formation [190].

5.3. The pore

The final structure and stoichiometry of the pore, as with the oligomerization process, remains not agreed upon. Over the years, several different views and models of the structure have been obtained, with different degrees of acceptance in the scientific community.

The first direct observations of actinoporin pores were electron micrographs of ferritin-labeled sticholysin, obtained in 1977 [13]. Much later, StnII was crystallized on egg-PC and DOPC monolayers [61,191]. The volumes obtained could be used to fit the previously obtained soluble structures, including some modifications. The result was interpreted as a tetrameric ensemble in which the contacts between the monomers would be minor, between the N-terminal α-helix and the C-termini of the neighboring monomer. The monomers would induce the formation of toroidal pores. The height of the complex was 43 Å, with its outer diameter being 110 Å and the inner diameter being 50 Å. These dimensions could be considered surprising since functional assays performed previously indicated that the hydrodynamic diameter of the sticholysins pore ranged between 6 and 12 Å [32,89].

Years later, a crystal structure of FraC complexed with detergents was obtained [65]. This structure was that of a potential pre-pore ensemble, in which the sidechains of the aromatic residues are in contact with the molecules of detergent. Whether the pre-pore complex was or was not induced by the methodology can be considered controversial. In addition, some low-resolution volumes of FraC pores on LUVs were obtained. These two results were combined to obtain a structure of the pore consisting of nine subunits. In this structure, the N-terminal α-helices line the wall of the pore all by themselves. This time, the narrower constriction of the pore, at the tip of the α-helices, had a diameter of 15 Å, much closer to the aforementioned functionally predicted values for sticholysin. The outer vestibule of the complex was 50 Å wide, with the outer diameter of the whole structure being 130 Å.

In 2015, a new FraC complex was obtained [66]. This time, the structure of 3.2 Å resolution was composed of eight subunits, with the lumen of the pore being lined by both the N-terminal α-helices and lipids. The α-helices of the different subunits are in contact with those of the adjacent monomer, helping stabilize the structure. They are also in contact with the corresponding intercalated lipid. There are three lipid molecules per toxin monomer. One is bound at the POC-binding site, though with its head group adopting the inverse orientation to that of the POC shown in Fig. 7a. Another one is sitting on top of Trp112 (Trp110 in StnII), surrounding it with its acyl chains, while its head group is engaged in a cation-π interaction with Trp116 (Trp114 in StnII) in what could be a non-canonical binding site for SM. The last lipid is the one that is exposed to the lumen of the pore. Strikingly, the parts of the lipid that are exposed to the lumen of the pore and consequently to the solvent are acyl chains. In this case, the head group is sitting between two toxin subunits involved in a cation-π interaction with Tyr110 (Tyr108 of StnII) of one subunit and the phosphate moiety being stabilized by Arg79 (which in StnII is Thr77) of the other. In spite of being an octameric structure instead of nonameric, the diameters are very similar. The narrowest pass in the lumen is 16 Å, while the distance between the highest points in the structure (using the theoretical position of the membrane as reference) is 60 Å. The total width and height of the structure are 110 and 70 Å, respectively, with the part of the β-sandwich core being approximately 45 Å.

At this point, it is noticeable that the stoichiometry of the actinoporin

pore is not clear and, according to the bibliography, appears to vary among actinoporins. However, it is worth mentioning that a stoichiometry of eight subunits per pore has been described for FraC [66], for StnI, and the hybrid pores formed by StnI and StnII [185]. Furthermore, all obtained complexes have very similar dimensions. In fact, if the low-resolution volume used to fit sticholysin into a tetramer complex is inspected carefully, ignoring its four larger bumps, eight smaller, symmetrically-disposed bumps can be observed (see Fig. 5b of ref. [61]). It could be said that the FraC octamer fits in that volume except for the N-terminal α-helices. Altogether, the aforementioned results would suggest that the stoichiometry of the final thermodynamically stable pores would be the same for all actinoporins, regardless of the exact composition of the pore [185].

Before moving on, it is relevant to mention some interesting results regarding the stoichiometry of FraC pores. Calculations based on the conductance of FraC pores indicate that the stoichiometry of these pores would be pH-dependent [192]. While octamers would be predominant at pH 7.5 (~85 % of the pores), heptamers would also be present, with hexamers appearing at pH 4.5. Not only that, the proportion of these stoichiometries could be shifted to those smaller by the mutation of Trp112 and Trp116 (equivalent to Trp110 and Trp114 in StnII) [192].

Stoichiometry aside, the retention of the β-sandwich fold and the extension of the N-terminal α-helix are common features of all the models. These features are supported by the characterization of a wide variety of mutants. For example, using EqtII, it was shown that the N-terminal region was required to be flexible while the β-sandwich could not be substantially perturbed if the activity was to be maintained [112]. Similarly, covalently attaching the N-terminal region to the protein core or introducing a Pro residue in what would become the transmembrane α-helical segment significantly reduced the hemolytic activity of EqtII and StnII respectively [109,114].

A widely accepted model in the field is that of a toroidal pore in which the lumen is lined by both the proteins and the head groups of PLs, with the pores having a small range of stoichiometries [156,188,189,193,194]. Besides a hydrodynamic size of ~10 Å, functional experiments have also shown that, at least for EqtII and sticholysins, the pores are cation-selective [26,27,31,32,35]. This would be controlled by the character of the residues facing the lumen [26,31,111,195]. Interestingly, a version of FraC has been engineered to allow for the passage of anions by mutating some of the residues facing the inside of the pore-lumen [196]. This work has allowed for the development of actinoporin's nanopores-based sequencing and peptide-recognition technologies, which can be used to detect post-translational modifications [197,198], and for peptide identification [192,199,200]. Back to the selectivity of the pore, it has also been observed that the presence of anionic lipids such as PA enhances the selectivity for cations, supporting the exposure of the lipid head groups to the pore lumen [169]. There are many observations showing that the conductance of the pores varies over time, which is indicative of the small size and dynamic nature of the pores [35,195]. In spite of this, the average aperture of the pores appears to be concentration-independent [89]. In fact, experimental results indicate that a small number of monomers is enough to achieve the formation of functional pores [31,156,186].

6. Conclusions

Despite more than 40 years of intensive studies, there are still some holes in our understanding of the molecular details behind the mechanisms that dominate the transition of actinoporins from a water-soluble form to an integral membrane ensemble. Certainly, much progress has been made, not only solving the structural details of these proteins but also analyzing the features of the membranes they target, including the biophysical requirements of these lipid bilayers. Nevertheless, there is still much to be found out and determined. Some of the most relevant questions that remain to be definitely answered are the nature of the different intermediates leading to the formation of the final pore, the need (or not) for pre-pores, and the role of lipids in the stability and cation-selectivity of the final pores. Sticholysins constitute an optimum system to pursue solving these scientific puzzles.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This research was supported by the Sigrid Jusélius Foundation, the Jane and Aatos Erkko Foundation, and the Magnus Ehrnrooth Foundation (to J.P.S.) and by UCM-Banco Santander Grants PR75/18-21561, PR87/19-22556, and PR108/20-15 (to A.M.-d.-P.). A UCM-Banco Santander fellowship was granted to E.R.-d.-T. S.G.-L. is a Real Colegio Complutense-Harvard postdoctoral fellow. J.P.-O. has a funded doctoral student position from ISB/ÅA. We dedicate this article to our colleagues, co-authors and, above all, friends, Peter Slotte and Pepe Gavilanes, on the occasion of their professional retirement. We will miss them very much, but we will certainly do our best to maintain the high scientific standards they have always held.

References

[1] L. Eme, A. Spang, J. Lombard, C.W. Stairs, T.J. Ettema, Archaea and the origin of eukaryotes, Nat. Rev. Microbiol. 15 (12) (2017) 711.
[2] C. Lesieur, B. Vecseysemjen, L. Abrami, M. Fivaz, F.G. Vandergoot, Membrane insertion: the strategies of toxins, Mol. Membr. Biol. 14 (2) (1997) 45–64.
[3] M. Dal Peraro, F.G. Van Der Goot, Pore-forming toxins: ancient, but never really out of fashion, Nat. Rev. Microbiol. 14 (2) (2016) 77.
[4] P. Szczesny, I. Iacovache, A. Muszewska, K. Ginalski, F.G. Van Der Goot, M. Grynberg, Extending the aerolysin family: from bacteria to vertebrates, PLoS One 6 (6) (2011).
[5] R. Galinier, J. Portela, Y. Moné, J.F. Allienne, H. Henri, S. Delbecq, D. Duval, Biomphalysin, a new β pore-forming toxin involved in *Biomphalaria glabrata* immune defense against *Schistosoma mansoni*, PLoS Pathog. 9 (3) (2013).
[6] Y. Xiang, C. Yan, X. Guo, K. Zhou, Q. Gao, X. Wang, Y. Zhang, Host-derived, pore-forming toxin–like protein and trefoil factor complex protects the host against microbial infection, Proc. Natl. Acad. Sci. 111 (18) (2014) 6702–6707.
[7] H.J. Muller-Eberhard, The membrane attack complex of complement, Annu. Rev. Immunol. 4 (1) (1986) 503–528.
[8] E. Rivera-de-Torre, A. Martínez-del-Pozo, J.E. Garb, *Stichodactyla helianthus*' de novo transcriptome assembly: discovery of a new actinoporin isoform, Toxicon 150 (2018) 105–114.
[9] J.H. Welsh, Composition and mode of action of some invertebrate venoms, Annu. Rev. Pharmacol. 4 (1) (1964) 293–304.
[10] M.H. Baslow, Marine toxins, Annu. Rev. Pharmacol. 11 (1) (1971) 447–454.
[11] C. Lane, Toxins of marine origin, Annu. Rev. Pharmacol. 8 (1) (1968) 409–426.
[12] A.W. Bernheimer, L.S. Avigad, Properties of a toxin from the sea anemone *Stoichactis helianthus*, including specific binding to sphingomyelin, Proc. Natl. Acad. Sci. 73 (2) (1976) 467–471.
[13] R. Linder, A.W. Bernheimer, K.S. Kim, Interaction between sphingomyelin and a cytolysin from the sea anemone *Stoichactis helianthus*, Biochim. Biophys. Acta Biomembr. 467 (3) (1977) 290–300.
[14] R. Linder, A.W. Bernheimer, Effect on sphingomyelin-containing liposomes of phospholipase D from *Corynebacterium ovis* and the cytolysin from *Stoichactis helianthus*, Biochim. Biophys. Acta Biomembr. 530 (2) (1978) 236–246.
[15] J.P. Devlin, Isolation and partial purification of hemolytic toxin from sea anemone, *Stoichactis helianthus*, J. Pharm. Sci. 63 (9) (1974) 1478–1480.
[16] I. Ferlan, D. Lebez, Equinatoxin, a lethal protein from *Actinia equina*—I Purification and characterization, Toxicon 12 (1) (1974) 57–58.
[17] D. Sket, K. Drašlar, I. Ferlan, D. Lebez, Equinatoxin, a lethal protein from *Actinia equina*—II. Pathophysiological action, Toxicon 12 (1) (1974) 63–68.
[18] T. Giraldi, I. Ferlan, D. Romeo, Antitumour activity of equinatoxin, Chem. Biol. Interact. 13 (3-4) (1976) 199–203.
[19] P. Maček, D. Lebez, Kinetics of hemolysis induced by equinatoxin, a cytolytic toxin from the sea anemone *Actinia equina*. Effect of some ions and pH, Toxicon 19 (2) (1981) 233–240.
[20] W.M. Lafranconi, I. Ferlan, F.E. Russell, R.J. Huxtable, The action of equinatoxin, a peptide from the venom of the sea anemone, *Actinia equina*, on the isolated lung, Toxicon 22 (3) (1984) 347–352.
[21] C. Ho, J. Ko, H. Lue, C. Lee, I. Ferlan, Effects of equinatoxin on the guinea-pig atrium, Toxicon 25 (6) (1987) 659–664.
[22] C.-M. Teng, L.-G. Lee, C.-Y. Lee, I. Ferlan, Platelet aggregation induced by equinatoxin, Thromb. Res. 52 (5) (1988) 401–411.

[23] T. Turk, P. Maček, F. Gubensek, Chemical modification of equinatoxin II, a lethal and cytolytic toxin from the sea anemone *Actinia equina* L, Toxicon 27 (3) (1989) 375–384.

[24] U. Batista, P. Maček, B. Sedmak, The cytotoxic and cytolytic activity of equinatoxin II from the sea anemone *Actinia equina*, Cell Biol. Int. Rep. 14 (11) (1990) 1013–1024.

[25] R. Zorec, M. Tester, P. Maček, W.T. Mason, Cytotoxicity of equinatoxin II from the sea anemone *Actinia equina* involves ion channel formation and an increase in intracellular calcium activity, J. Membr. Biol. 118 (3) (1990) 243–249.

[26] G. Belmonte, C. Pederzolli, P. Maček, G. Menestrina, Pore formation by the sea anemone cytolysin equinatoxin-II in red blood cells and model lipid membranes, J. Membr. Biol. 131 (1993) 11–22.

[27] P. Maček, G. Belmonte, C. Pederzolli, G. Menestrina, Mechanism of action of equinatoxin II, a cytolysin from the sea anemone *Actinia equina* L. belonging to the family of actinoporins, Toxicology 87 (1-3) (1994) 205–227.

[28] G. Belmonte, G. Menestrina, C. Pederzolli, I. Krizaj, F. Gubensek, T. Turk, P. Maček, Primary and secondary structure of a pore-forming toxin from the sea anemone, *Actinia equina L.*, and its association with lipid vesicles, Biochim. Biophys. Acta Biomembr. 1192 (1994) 197–204.

[29] C. Alvarez, M. Tejuca, V. Morera, V. Besada, F. Pazos, R. Veitia, M. Lanio, Some characteristics of sticholysin: a novel cytolysin from *Stichodactyla helianthus*, Adv. Mod. Biotechnol. 2 (1994) 135–146.

[30] C. Alvarez, M. Tejuca, V. Morera, V. Besada, F. Pazos, M. Lanio, G. Padrón, Novel primary structure of sticholysin and its interaction with membranes, Toxicon 3 (34) (1996) 301.

[31] M. Tejuca, M. Dalla Serra, M. Ferreras, M.E. Lanio, G. Menestrina, Mechanism of membrane permeabilization by sticholysin I, a cytolysin isolated from the venom of the sea anemone *Stichodactyla helianthus*, Biochemistry 35 (47) (1996) 14947–14957.

[32] V. De los Ríos, J.M. Mancheño, M.E. Lanio, M. Oñaderra, J.G. Gavilanes, Mechanism of the leakage induced on lipid model membranes by the hemolytic protein sticholysin II from the sea anemone *Stichodactyla helianthus*, Eur. J. Biochem. 252 (2) (1998) 284–289.

[33] D.W. Michaels, Membrane damage by a toxin from the sea anemone *Stoichactis helianthus*. I. Formation of transmembrane channels in lipid bilayers, Biochim. Biophys. Acta Biomembr. 555 (1) (1979) 67–78.

[34] M.L. Shin, D.W. Michaels, M.M. Mayer, Membrane damage by a toxin from the sea anemone *Stoichactis helianthus*. II. Effect of membrane lipid composition in a liposome system, Biochim. Biophys. Acta Biomembr. 555 (1) (1979) 79–88.

[35] W. Varanda, A. Finkelstein, Ion and nonelectrolyte permeability properties of channels formed in planar lipid bilayer membranes by the cytolytic toxin from the sea anemone, *Stoichactis helianthus*, J. Membr. Biol. 55 (3) (1980) 203–211.

[36] W. Kem, J. Doyle, B. Dunn, K. Blumenthal, Purification and characterization of sea anemone (stoichactis) cytotoxins, in: Federation Proceedings 41, Federation Amer Soc Exp Biol, 9650 Rockville Pike, Bethesda, MD 20814-3998, 1982, p. 1643.

[37] K. Blumenthal, W. Kem, Primary structure of *Stoichactis helianthus* cytolysin III, J. Biol. Chem. 258 (9) (1983) 5574–5581.

[38] I. Ferlan, K.W. Jackson, Partial amino acid sequence of equinatoxin, Toxicon 21 (1983) 141–144.

[39] W.R. Kem, B.M. Dunn, Separation and characterization of four different amino acid sequence variants of a sea anemone (*Stichodactyla helianthus*) protein cytolysin, Toxicon 26 (11) (1988) 997–1008.

[40] J.W. Doyle, W.R. Kem, Binding of a radiolabeled sea anemone cytolysin to erythrocyte membranes, Biochim. Biophys. Acta Biomembr. 987 (2) (1989) 181–186.

[41] J.W. Doyle, W.R. Kem, F.A. Vilallonga, Interfacial activity of an ion channel-generating protein cytolysin from the sea anemone *Stichodactyla helianthus*, Toxicon 27 (4) (1989) 465–471.

[42] P. Maček, D. Lebez, Isolation and characterization of three lethal and hemolytic toxins from the sea anemone *Actinia equina* L, Toxicon 26 (5) (1988) 441–451.

[43] G. Anderluh, J. Pungerear, B. Strukelj, P. Maček, F. Gubensk, Cloning, sequencing and expression of equinatoxin II, Biochem. Biophys. Res. Commun. 220 (1996) 437–442.

[44] V. De los Ríos, M. Oñaderra, A. Martínez-Ruiz, J. Lacadena, J.M. Mancheño, A. Martínez-del-Pozo, J.G. Gavilanes, Overproduction in *Escherichia coli* and purification of the hemolytic protein sticholysin II from the sea anemone *Stichodactyla helianthus*, Protein Expr. Purif. 18 (1) (2000) 71–76.

[45] A. Ávila, C.M. De Acosta, A. Lage, A new immunotoxin built by linking a hemolytic toxin to a monoclonal antibody specific for immature T lymphocytes, Int. J. Cancer 42 (4) (1988) 568–571.

[46] A. Ávila, C.D. Mateo Acosta, A. Lage, A carcinoembryonic antigen-directed immunotoxin built by linking a monoclonal antibody to a hemolytic toxin, Int. J. Cancer 43 (5) (1989) 926–929.

[47] V. Morera, J. Gómez, V. Besada, R. Estrada, T. Pons, C. Álvarez, F. Pazos, Primary structure analysis of the haemolytic polypeptide sticholysin isolated from a sea anemone, 1994.

[48] A. Bellomio, K. Morante, A. Barlič, I. Gutiérrez-Aguirre, A.R. Viguera, J. M. González-Mañas, Purification, cloning and characterization of fragaceatoxin C, a novel actinoporin from the sea anemone *Actinia fragacea*, Toxicon 54 (6) (2009) 869–880.

[49] A.E. Mechaly, A. Bellomio, K. Morante, J.M. González-Mañas, D.M. Guerin, Crystallization and preliminary crystallographic analysis of fragaceatoxin C, a pore-forming toxin from the sea anemone *Actinia fragacea*, Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 65 (4) (2009) 357–360.

[50] J. Vincent, M. Balerna, J. Barhanin, M. Fosset, M. Lazdunski, Binding of sea anemone toxin to receptor sites associated with gating system of sodium channel in synaptic nerve endings in vitro, Proc. Natl. Acad. Sci. 77 (3) (1980) 1646–1650.

[51] J. Barhanin, M. Hugues, H. Schweitz, J.-P. Vincent, M. Lazdunski, Structure-function relationships of sea anemone toxin II from *Anemonia sulcata*, J. Biol. Chem. 256 (11) (1981) 5764–5769.

[52] A.W. Bernheimer, L.S. Avigad, C. Lai, Purification and properties of a toxin from the sea anemone *Condylactis gigantea*, Arch. Biochem. Biophys. 214 (2) (1982) 840–845.

[53] A.W. Bernheimer, L.S. Avigad, Toxins of the sea anemone *Epiactis prolifera*, Arch. Biochem. Biophys. 217 (1) (1982) 174–180.

[54] A.W. Bernheimer, L.S. Avigad, G. Branch, E. Dowdle, C.Y. Lai, Purification and properties of a toxin from the South African sea anemone, *Pseudactinia varia*, Toxicon 22 (2) (1984) 183–191.

[55] A.W. Bernheimer, C.Y. Lai, Properties of a cytolytic toxin from the sea anemone, *Stoichactis kenti*, Toxicon 23 (5) (1985) 791–799.

[56] N. El-Sherif, H.A. Fozzard, D.A. Hanck, Dose-dependent modulation of the cardiac sodium channel by sea anemone toxin ATXII, Circ. Res. 70 (2) (1992) 285–301.

[57] K.S. Khoo, W.K. Kam, H.E. Khoo, P. Gopalakrishnakone, M.C. Chung, Purification and partial characterization of two cytolysins from a tropical sea anemone, *Heteractis magnifica*, Toxicon 31 (12) (1993) 1567–1579.

[58] A. Aneiros, I. García, J.R. Martínez, A.L. Harvey, A.J. Anderson, D.L. Marshall, E. Karlsson, A potassium channel toxin from the secretion of the sea anemone *Bunodosoma granulifera*. Isolation, amino acid sequence and biological activity, Biochim. Biophys. Acta Gen. Subj. 1157 (1) (1993) 86–92.

[59] E. Tkacheva, E. Leychenko, M. Monastyrnaya, M. Issaeva, E. Zelepuga, S. Anastuk, E. Kozlovskaya, New actinoporins from sea anemone *Heteractis crispa*: cloning and functional expression, Biochemistry 76 (10) (2011) 1131.

[60] R.S. Norton, G. Bobek, J.O. Ivanov, M. Thomson, E. Fiala-Beer, R.L. Moritz, R. J. Simpson, Purification and characterisation of proteins with cardiac stimulatory and haemolytic activity from the anemone *Actinia tenebrosa*, Toxicon 28 (1) (1990) 29–41.

[61] J.M. Mancheño, J. Martín-Benito, M. Martínez-Ripoll, J.G. Gavilanes, J. A. Hermoso, Crystal and electron microscopy structures of sticholysin II actinoporin reveal insights into the mechanism of membrane pore formation, Structure 11 (11) (2003) 1319–1328.

[62] S. García-Linares, I. Castrillo, M. Bruix, M. Menéndez, J. Alegre-Cebollada, A. Martínez-del-Pozo, J.G. Gavilanes, Three-dimensional structure of the actinoporin sticholysin I. Influence of long-distance effects on protein function, Arch. Biochem. Biophys. 532 (1) (2013) 39–45.

[63] A. Athanasiadis, G. Anderluh, P. Maček, D. Turk, Crystal structure of the soluble form of equinatoxin II, a pore-forming toxin from the sea anemone *Actinia equina*, Structure 9 (4) (2001) 341–346.

[64] M.G. Hinds, W. Zhang, G. Anderluh, P.E. Hansen, R.S. Norton, Solution structure of the eukaryotic pore-forming cytolysin equinatoxin II: implications for pore formation, J. Mol. Biol. 315 (5) (2002) 1219–1229.

[65] A.E. Mechaly, A. Bellomio, D. Gil-Carton, K. Morante, M. Valle, J.M. González-Mañas, D.M. Guerin, Structural insights into the oligomerization and architecture of eukaryotic membrane pore-forming toxins, Structure 19 (2) (2011) 181–191.

[66] K. Tanaka, J.M. Caaveiro, K. Morante, J.M. González-Mañas, K. Tsumoto, Structural basis for self-assembly of a cytolytic pore lined by protein and lipid, Nat. Commun. 6 (1) (2015) 1–11.

[67] K. Morante, A. Bellomio, A.R. Viguera, J.M. Gonzalez-Manas, K. Tsumoto, J.M. M. Caaveiro, The isolation of new pore-forming toxins from the sea anemone *Actinia fragacea* provides insights into the mechanisms of actinoporin evolution, Toxins (Basel) 11 (7) (2019).

[68] M. Daly, D. Fautin, World list of actiniaria. Stichodactyla helianthus (Ellis, 1768), in: World Register of Marine Species, 2020. http://www.marinespecies.org/aphia.php?p=taxdetails&id=291135.

[69] M. Daly, D. Fautin, World list of actiniaria. Actinia fragacea (Tugwell, 1856), in: World Register of Marine Species, 2020. http://www.marinespecies.org/aphia.php?p=taxdetails&id=100805.

[70] M. Daly, D. Fautin, World list of actiniaria. Actinia equina (Linnaeus, 1758), in: World Register of Marine Species, 2020. http://www.marinespecies.org/aphia.php?p=taxdetails&id=100803.

[71] P. Maček, Polypeptide cytolytic toxins from sea anemones (Actiniaria), FEMS Microbiol. Immunol. 5 (1-3) (1992) 121–129.

[72] G. Anderluh, P. Maček, Cytolytic peptide and protein toxins from sea anemones (Anthozoa: Actiniaria), Toxicon 40 (2) (2002) 111–124.

[73] J. Alegre-Cebollada, M. Oñaderra, J.G. Gavilanes, A. Martínez-del-Pozo, Sea anemone actinoporins: the transition from a folded soluble state to a functionally active membrane-bound oligomeric pore, Curr. Protein Pept. Sci. 8 (6) (2007) 558–572.

[74] J.M. Caaveiro, I. Echabe, I. Gutiérrez-Aguirre, J.L. Nieva, J.L. Arrondo, J. M. González-Mañas, Differential interaction of equinatoxin II with model membranes in response to lipid composition, Biophys. J. 80 (3) (2001) 1343–1353.

[75] P. Schön, A.J. García-Saez, P. Malovrh, K. Bacia, G. Anderluh, P. Schwille, Equinatoxin II permeabilizing activity depends on the presence of sphingomyelin and lipid phase coexistence, Biophys. J. 95 (2) (2008) 691–698.

[76] A. Barlič, I. Gutiérrez-Aguirre, J.M. Caaveiro, A. Cruz, M.B. Ruiz-Argüello, J. Pérez-Gil, J.M. González-Mañas, Lipid phase coexistence favors membrane insertion of equinatoxin-II, a pore-forming toxin from *Actinia equina*, J. Biol. Chem. 279 (33) (2004) 34209–34216.

[77] D. Martínez, A. Otero, C. Álvarez, F. Pazos, M. Tejuca, M.E. Lanio, E. Lissi, Effect of sphingomyelin and cholesterol on the interaction of St II with lipidic interfaces, Toxicon 49 (1) (2007) 68–81.
[78] B. Bakrač, G. Anderluh, Molecular mechanism of sphingomyelin-specific membrane binding and pore formation by actinoporins, in: Proteins Membrane Binding and Pore Formation, Springer, 2010, pp. 106–115.
[79] L. Pedrera, M.L. Fanani, U. Ros, M.E. Lanio, B. Maggio, C. Álvarez, Sticholysin I-membrane interaction: an interplay between the presence of sphingomyelin and membrane fluidity, Biochim. Biophys. Acta Biomembr. 1838 (7) (2014) 1752–1759.
[80] L. Pedrera, A.B. Gomide, R.E. Sánchez, U. Ros, N. Wilke, F. Pazos, C. Álvarez, The presence of sterols favors sticholysin I-membrane association and pore formation regardless of their ability to form laterally segregated domains, Langmuir 31 (36) (2015) 9911–9923.
[81] I. Alm, S. García-Linares, J.G. Gavilanes, A. Martínez-del-Pozo, J.P. Slotte, Cholesterol stimulates and ceramide inhibits sticholysin II-induced pore formation in complex bilayer membranes, Biochim. Biophys. Acta Biomembr. 1848 (2015) 925–931.
[82] S. García-Linares, J. Palacios-Ortega, T. Yasuda, M. Astrand, J.G. Gavilanes, A. Martínez-del-Pozo, J.P. Slotte, Toxin-induced pore formation is hindered by intermolecular hydrogen bonding in sphingomyelin bilayers, Biochim. Biophys. Acta Biomembr. 1858 (6) (2016) 1189–1195.
[83] J. Palacios-Ortega, S. García-Linares, M. Astrand, M.A. Al Sazzad, J.G. Gavilanes, A. Martínez-del-Pozo, J.P. Slotte, Regulation of sticholysin II-induced pore formation by lipid bilayer composition, phase state, and interfacial properties, Langmuir 32 (14) (2016) 3476–3484.
[84] S. García-Linares, E. Rivera-de-Torre, K. Morante, K. Tsumoto, J.M. Caaveiro, J. G. Gavilanes, Á. Martínez-del-Pozo, Differential effect of membrane composition on the pore-forming ability of four different sea anemone actinoporins, Biochemistry 55 (48) (2016) 6630–6641.
[85] H.P. Wacklin, B.B. Bremec, M. Moulin, N. Rojko, M. Haertlein, T. Forsyth, R. S. Norton, Neutron reflection study of the interaction of the eukaryotic pore-forming actinoporin equinatoxin II with lipid membranes reveals intermediate states in pore formation, Biochim. Biophys. Acta Biomembr. 1858 (4) (2016) 640–652.
[86] M. Marchioretto, M. Podobnik, M. Dalla Serra, G. Anderluh, What planar lipid membranes tell us about the pore-forming activity of cholesterol-dependent cytolysins, Biophys. Chem. 182 (2013) 64–70.
[87] J. Palacios-Ortega, S. García-Linares, E. Rivera-de-Torre, J.G. Gavilanes, A. Martínez-del-Pozo, J.P. Slotte, Sticholysin, sphingomyelin, and cholesterol: a closer look at a tripartite interaction, Biophys. J. 116 (12) (2019) 2253–2265.
[88] X. Jiang, H. Chen, W. Yang, Y. Liu, W. Liu, J. Wei, A. Xu, Functional expression and characterization of an acidic actinoporin from sea anemone *Sagartia rosea*, Biochem. Biophys. Res. Commun. 312 (3) (2003) 562–570.
[89] M. Tejuca, M. Dalla Serra, C. Potrich, C. Álvarez, G. Menestrina, Sizing the radius of the pore formed in erythrocytes and lipid vesicles by the toxin sticholysin I from the sea Anemone *Stichodactyla helianthus*, J. Membr. Biol. 183 (2) (2001) 125–135.
[90] M.H. Saier Jr., V.S. Reddy, B.V. Tsu, M.S. Ahmed, C. Li, G. Moreno-Hagelsieb, The transporter classification database (TCDB): recent advances, Nucleic Acids Res. 44 (D1) (2016) D372–D379.
[91] E. Gouaux, Channel-forming toxins: tales of transformation, Curr. Opin. Struct. Biol. 7 (1997) 566–573.
[92] M.W. Parker, S.C. Feil, Pore-forming protein toxins: from structure to function, Prog. Biophys. Mol. Biol. 88 (1) (2005) 91–142.
[93] I. Iacovache, F.G. van der Goot, L. Pernot, Pore formation: an ancient yet complex form of attack, Biochim. Biophys. Acta Biomembr. 1778 (7-8) (2008) 1611–1623.
[94] M. Mueller, U. Grauschopf, T. Maier, R. Glockshuber, N. Ban, The structure of a cytolytic α-helical toxin pore reveals its assembly mechanism, Nature 459 (7247) (2009) 726–730.
[95] M. Mueller, N. Ban, Enhanced SnapShot: pore-forming toxins, Cell 142 (2) (2010), 334, 334 e331.
[96] B. Geny, M.R. Popoff, Bacterial protein toxins and lipids: pore formation or toxin entry into cells, Biol. Cell. 98 (11) (2006) 667–678.
[97] M.R. González, M. Bischofberger, L. Pernot, F.G. van der Goot, B. Freche, Bacterial pore-forming toxins: the (w)hole story? Cell. Mol. Life Sci. 65 (3) (2008) 493–507.
[98] G. Anderluh, J.H. Lakey, Disparate proteins use similar architectures to damage membranes, Trends Biochem. Sci. 33 (10) (2008) 482–490.
[99] R.H. Law, N. Lukoyanova, I. Voskoboinik, T.T. Caradoc-Davies, K. Baran, M. A. Dunstone, J.C. Whisstock, The structural basis for membrane binding and pore formation by lymphocyte perforin, Nature 468 (7322) (2010) 447–451.
[100] A. Basulto, V.M. Pérez, Y. Noa, C. Varela, A.J. Otero, M.C. Pico, Immunohistochemical targeting of sea anemone cytolysins on tentacles, mesenteric filaments and isolated nematocysts of *Stichodactyla helianthus*, J. Exp. Zool. A Comp. Exp. Biol. 305 (3) (2006) 253–258.
[101] E. Rivera-de-Torre, S. García-Linares, J. Alegre-Cebollada, J. Lacadena, J. G. Gavilanes, A. Martínez-del-Pozo, Synergistic action of actinoporin isoforms from the same sea anemone species assembled into functionally active heteropores, J. Biol. Chem. 291 (27) (2016) 14109–14119.
[102] Y. Wang, L.L. Yap, K.L. Chua, H.E. Khoo, A multigene family of *Heteractis* magnificalysins (HMgs), Toxicon 51 (8) (2008) 1374–1382.
[103] M. Monastyrnaya, E. Leychenko, M. Isaeva, G. Likhatskaya, E. Zelepuga, E. Kostina, E. Kozlovskaya, Actinoporins from the sea anemones, tropical *Radianthus macrodactylus* and northern *Oulactis orientalis*: comparative analysis of structure-function relationships, Toxicon 56 (8) (2010) 1299–1314.
[104] T. Turk, Cytolytic toxins from sea anemones, Toxin Rev. 10 (3) (1991) 223–262.
[105] The-UniProt-Consortium, UniProt: a worldwide hub of protein knowledge, Nucleic Acids Res. 47 (D1) (2018) D506–D515.
[106] Q.T. Hoang, S.H. Cho, S.F. McDaniel, S.H. Ok, R.S. Quatrano, J.S. Shin, An actinoporin plays a key role in water stress in the moss *Physcomitrella patens*, New Phytol. 184 (2) (2009) 502–510.
[107] I. Gutiérrez-Aguirre, A. Barlič, Z. Podlesek, P. Maček, G. Anderluh, J.M. González-Mañas, Membrane insertion of the N-terminal a-helix of equinatoxin II, a sea anemone cytolytic toxin, Biochem. J. 384 (Pt 2) (2004) 421–428.
[108] E.F. Pettersen, T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E. C. Meng, T.E. Ferrin, UCSF Chimera—a visualization system for exploratory research and analysis, J. Comput. Chem. 25 (13) (2004) 1605–1612.
[109] Q. Hong, I. Gutiérrez-Aguirre, A. Barlič, P. Malovrh, K. Kristan, Z. Podlesek, G. Anderluh, Two-step membrane binding by equinatoxin ii, a pore-forming toxin from the sea anemone, involves an exposed aromatic cluster and a flexible helix, J. Biol. Chem. 277 (44) (2002) 41916–41924.
[110] R.S. Norton, Structures of sea anemone toxins, Toxicon 54 (8) (2009) 1075–1088.
[111] P. Malovrh, G. Viero, M.D. Serra, Z. Podlesek, J.H. Lakey, P. Maček, G. Anderluh, A novel mechanism of pore formation: membrane penetration by the N-terminal amphipathic region of equinatoxin, J. Biol. Chem. 278 (25) (2003) 22678–22685.
[112] K. Kristan, Z. Podlesek, V. Hojnik, I. Gutiérrez-Aguirre, G. Guncar, D. Turk, G. Anderluh, Pore formation by equinatoxin, a eukaryotic pore-forming toxin, requires a flexible N-terminal region and a stable b-sandwich, J. Biol. Chem. 279 (45) (2004) 46509–46517.
[113] J. Alegre-Cebollada, A. Martínez-del-Pozo, J.G. Gavilanes, E. Goormaghtigh, Infrared spectroscopy study on the conformational changes leading to pore formation of the toxin sticholysin II, Biophys. J. 93 (9) (2007) 3191–3201.
[114] J. Alegre-Cebollada, M. Cunietti, E. Herrero-Galán, J.G. Gavilanes, A. Martínez-del-Pozo, Calorimetric scrutiny of lipid binding by sticholysin II toxin mutants, J. Mol. Biol. 382 (4) (2008) 920–930.
[115] F. Casallanovo, F.J. de Oliveira, F.C. de Souza, U. Ros, Y. Martínez, D. Penton, S. Schreier, Model peptides mimic the structure and function of the N-terminus of the pore-forming toxin sticholysin II, Biopolymers 84 (2) (2006) 169–180.
[116] A. Lima de Oliveira, E. Maffud Cilli, U. Ros, E. Crusca Jr., M.E. Lanio, C. Alvarez, A. Spisni, Insights on the structure-activity relationship of peptides derived from Sticholysin II, Pept. Sci. 110 (5) (2018), e23097.
[117] U. Ros, L. Pedrera, D. Diaz, J.C. Karam, T.P. Sudbrack, P.A. Valiente, C. Avarez, The membranotropic activity of N-terminal peptides from the pore-forming proteins sticholysin I and II is modulated by hydrophobic and electrostatic interactions as well as lipid composition, J. Biosci. 36 (5) (2012) 781–791.
[118] K. Morante, J.M. Caaveiro, K. Tanaka, J.M. González-Mañas, K. Tsumoto, A pore-forming toxin requires a specific residue for its activity in membranes with particular physicochemical properties, J. Biol. Chem. 290 (17) (2015) 10850–10861.
[119] S. García-Linares, I. Alm, T. Maula, J.G. Gavilanes, J.P. Slotte, A. Martínez-del-Pozo, The effect of cholesterol on the long-range network of interactions established among sea anemone Sticholysin II residues at the water-membrane interface, Mar. Drugs 13 (4) (2015) 1647–1665.
[120] E. Rivera-de-Torre, J. Palacios-Ortega, S. García-Linares, J.G. Gavilanes, A. Martínez-del-Pozo, One single salt bridge explains the different cytolytic activities shown by actinoporins sticholysin I and II from the venom of *Stichodactyla helianthus*, Arch. Biochem. Biophys. 636 (2017) 79–89.
[121] F. Casallanovo, F.J.F. de Oliveira, A.L.C.F. Souto, F.C. de Souza, E.M. Cilli, Y. Martínez, C. Álvarez, Peptides from the N-terminal domain of a pore-forming toxin, Sticholysin II. Conformation and activity, in: 48th Annual Meeting of the Biophysical Society, Biophys J, Baltimore, MD, 2004.
[122] U. Ros, A.L. Souto, F.J. de Oliveira, E. Crusca Jr., F. Pazos, E.M. Cilli, C. Alvarez, Functional and topological studies with Trp-containing analogs of the peptide StII1-30 derived from the N-terminus of the pore forming toxin sticholysin II: contribution to understand its orientation in membrane, Biopolymers 100 (4) (2013) 337–346.
[123] I. Castrillo, N.A. Araujo, J. Alegre-Cebollada, J.G. Gavilanes, A. Martínez-del-Pozo, M. Bruix, Specific interactions of sticholysin I with model membranes: an NMR study, Proteins 78 (8) (2010) 1959–1970.
[124] H. Mesa-Galloso, P.A. Valiente, M.E. Valdés-Tresanco, R.F. Epand, M.E. Lanio, R. M. Epand, U. Ros, Membrane remodeling by the lytic fragment of SticholysinII: implications for the toroidal pore model, Biophys. J. 117 (9) (2019) 1563–1576.
[125] A. Drechsler, C. Potrich, J.K. Sabo, M. Frisanco, G. Guella, M. Dalla Serra, R. S. Norton, Structure and activity of the N-terminal region of the eukaryotic cytolysin equinatoxin II, Biochemistry 45 (6) (2006) 1818–1828.
[126] M.R. De Planque, J.A. Kruijtzer, R.M. Liskamp, D. Marsh, D.V. Greathouse, R. E. Koeppe 2nd, J.A. Killian, Different membrane anchoring positions of tryptophan and lysine in synthetic transmembrane a-helical peptides, J. Biol. Chem. 274 (30) (1999) 20839–20846.
[127] J. Alegre-Cebollada, V. Lacadena, M. Oñaderra, J.M. Mancheño, J.G. Gavilanes, A. Martínez-del-Pozo, Phenotypic selection and characterization of randomly produced non-haemolytic mutants of the toxic sea anemone protein sticholysin II, FEBS Lett. 575 (1-3) (2004) 14–18.
[128] P. Malovrh, A. Barlič, Z. Podlesek, P. Maček, G. Menestrina, G. Anderluh, Structure-function studies of tryptophan mutants of equinatoxin II, a sea anemone pore-forming protein, Biochem. J. 346 (2000) 223–232.
[129] G. Anderluh, A. Razpotnik, Z. Podlesek, P. Maček, F. Separovic, R.S. Norton, Interaction of the eukaryotic pore-forming cytolysin equinatoxin II with model membranes: ^{19}F NMR studies, J. Mol. Biol. 347 (1) (2005) 27–39.

[130] B. Bakrač, I. Gutierrez-Aguirre, Z. Podlesek, A.F. Sonnen, R.J. Gilbert, P. Maček, G. Anderluh, Molecular determinants of sphingomyelin specificity of a eukaryotic pore-forming toxin, J. Biol. Chem. 283 (27) (2008) 18665–18677.
[131] S. García-Linares, T. Maula, E. Rivera-de-Torre, J.G. Gavilanes, J.P. Slotte, A. Martínez-del-Pozo, Role of the tryptophan residues in the specific interaction of the sea anemone *Stichodactyla helianthus*'s actinoporin Sticholysin II with biological membranes, Biochemistry 55 (46) (2016) 6406–6420.
[132] M.A. Pardo-Cea, I. Castrillo, J. Alegre-Cebollada, A. Martínez-del-Pozo, J. G. Gavilanes, M. Bruix, Intrinsic local disorder and a network of charge-charge interactions are key to actinoporin membrane disruption and cytotoxicity, FEBS J. 278 (12) (2011) 2080–2089.
[133] M.A. Pardo-Cea, J. Alegre-Cebollada, A. Martínez-del-Pozo, J.G. Gavilanes, M. Bruix, ^{1}H, ^{13}C, and ^{15}N NMR assignments of StnII-Y111N, a highly impaired mutant of the sea anemone actinoporin Sticholysin II, Biomol. NMR Assign. 4 (1) (2010) 69–72.
[134] T. Maula, Y.J. Isaksson, S. García-Linares, S. Niinivehmas, O.T. Pentikainen, M. Kurita, J.P. Slotte, 2NH and 3OH are crucial structural requirements in sphingomyelin for sticholysin II binding and pore formation in bilayer membranes, Biochim. Biophys. Acta Biomembr. 1828 (5) (2013) 1390–1395.
[135] B. Bakrač, A. Kladnik, P. Maček, G. McHaffie, A. Werner, J.H. Lakey, G. Anderluh, A toxin-based probe reveals cytoplasmic exposure of golgi sphingomyelin, J. Biol. Chem. 285 (29) (2010) 22186–22195.
[136] U. Ros, G.P. Carretero, J. Paulino, E. Crusca Jr., F. Pazos, E.M. Cilli, C. Alvarez, Self-association and folding in membrane determine the mode of action of peptides from the lytic segment of sticholysins, Biochimie 156 (2019) 109–117.
[137] U. Ros, W. Rodríguez-Vera, L. Pedrera, P.A. Valiente, S. Cabezas, M.E. Lanio, C. Álvarez, Differences in activity of actinoporins are related with the hydrophobicity of their N-terminus, Biochimie 116 (2015) 70–78.
[138] C.A. Valcarcel, M. Dalla Serra, C. Potrich, I. Bernhart, M. Tejuca, D. Martínez, G. Menestrina, Effects of lipid composition on membrane permeabilization by sticholysin I and II, two cytolysins of the sea anemone *Stichodactyla helianthus*, Biophys. J. 80 (6) (2001) 2761–2774.
[139] J. Alegre-Cebollada, I. Rodríguez-Crespo, J.G. Gavilanes, A. Martínez-del-Pozo, Detergent-resistant membranes are platforms for actinoporin pore-forming activity on intact cells, FEBS J. 273 (4) (2006) 863–871.
[140] B. Bakrač, G. Anderluh, Molecular mechanism of sphingomyelin-specific membrane binding and pore formation by actinoporins, Adv. Exp. Med. Biol. 677 (2009) 106–115.
[141] N. Rojko, M. Dalla Serra, P. Maček, G. Anderluh, Pore formation by actinoporins, cytolysins from sea anemones, Biochim. Biophys. Acta Biomembr. 1858 (3) (2016) 446–456.
[142] D. Martínez, A.M. Campos, F. Pazos, C. Álvarez, M.E. Lanio, F. Casallanovo, E. Lissi, Properties of St I and St II, two isotoxins isolated from *Stichodactyla helianthus*: a comparison, Toxicon 39 (10) (2001) 1547–1560.
[143] J.V. Busto, M.L. Fanani, L. De Tullio, J. Sot, B. Maggio, F.M. Goni, A. Alonso, Coexistence of immiscible mixtures of palmitoylsphingomyelin and palmitoylceramide in monolayers and bilayers, Biophys. J. 97 (10) (2009) 2717–2726.
[144] B.M. Castro, R.F. de Almeida, L.C. Silva, A. Fedorov, M. Prieto, Formation of ceramide/sphingomyelin gel domains in the presence of an unsaturated phospholipid: a quantitative multiprobe approach, Biophys. J. 93 (5) (2007) 1639–1650.
[145] S. Chiantia, N. Kahya, J. Ries, P. Schwille, Effects of ceramide on liquid-ordered domains investigated by simultaneous AFM and FCS, Biophys. J. 90 (12) (2006) 4500–4508.
[146] E. Meinardi, M. Florin-Christensen, G. Paratcha, J.M. Azcurra, J. Florin-Christensen, The molecular basis of the self/nonself selectivity of a coelenterate toxin, Biochem. Biophys. Res. Commun. 216 (1) (1995) 348–354.
[147] G. Anderluh, Z. Podlesek, P. Maček, A common motif in proparts of Cnidarian toxins and nematocyst collagens and its putative role, Biochim. Biophys. Acta Protein Struct. Mol. Enzymol. 1476 (2) (2000) 372–376.
[148] E. Meinardi, J.M. Azcurra, M. Florin-Christensen, J. Florin-Christensen, Coelenterolysin: a hemolytic polypeptide associated with the coelenteric fluid of sea anemones, Comp. Biochem. Physiol. Part B 109 (1) (1994) 153–161.
[149] T. Hori, Y. Nozawa, Phosphonolipids, in: New Comprehensive Biochemistry 4, Elsevier, 1982, pp. 95–128.
[150] R.L. Hilderbrand, T.O. Henderson, Phosphonic acids in nature, in: The Role of Phosphonates in Living Systems, 1983, pp. 5–30.
[151] G. Simon, G. Rouser, Phospholipids of the sea anemone: quantitative distribution; absence of carbon-phosphorus linkages in glycerol phospholipids; structural elucidation of ceramide aminoethylphosphonate, Lipids 2 (1) (1967) 55–59.
[152] J.D. Joseph, Lipid composition of marine and estuarine invertebrates: porifera and Cnidaria, Prog. Lipid Res. 18 (1) (1979) 1–30.
[153] M.M. Nelson, C.F. Phleger, B.D. Mooney, P.D. Nichols, Lipids of gelatinous Antarctic zooplankton: Cnidaria and Ctenophora, Lipids 35 (5) (2000) 551–559.
[154] P. Meneses, N. Navarro, ^{31}P NMR phospholipid profile study of seven sea anemone species, Comp. Biochem. Physiol. Part B 102 (2) (1992) 403–407.
[155] A. Sepehri, B. Nepal, T. Lazaridis, Lipid interactions of an actinoporin pore-forming oligomer, Biophys. J. 120 (8) (2021) 1357–1366.
[156] N. Rojko, B. Cronin, J.S. Danial, M.A. Baker, G. Anderluh, M.I. Wallace, Imaging the lipid-phase-dependent pore formation of equinatoxin II in droplet interface bilayers, Biophys. J. 106 (8) (2014) 1630–1637.
[157] A. Makino, M. Abe, M. Murate, T. Inaba, N. Yilmaz, F. Hullin-Matsuda, T. Kobayashi, Visualization of the heterogeneous membrane distribution of sphingomyelin associated with cytokinesis, cell polarity, and sphingolipidosis, FASEB J. 29 (2) (2015) 477–493.
[158] J. Huang, J.T. Buboltz, G.W. Feigenson, Maximum solubility of cholesterol in phosphatidylcholine and phosphatidylethanolamine bilayers, Biochim. Biophys. Acta Biomembr. 1417 (1) (1999) 89–100.
[159] A. Björkbom, T. Róg, K. Kaszuba, M. Kurita, S. Yamaguchi, M. Lönnfors, J. P. Slotte, Effect of sphingomyelin headgroup size on molecular properties and interactions with cholesterol, Biophys. J. 99 (10) (2010) 3300–3308.
[160] S. Jaikishan, A. Björkbom, J.P. Slotte, Sphingomyelin analogs with branched N-acyl chains: the position of branching dramatically affects acyl chain order and sterol interactions in bilayer membranes, Biochim. Biophys. Acta Biomembr. 1798 (10) (2010) 1987–1994.
[161] M. Feinstein, S. Fernandez, R. Sha'Afi, Fluidity of natural membranes and phosphatidylserine and ganglioside dispersions: effects of local anesthetics, cholesterol and protein, Biochim. Biophys. Acta Biomembr. 413 (3) (1975) 354–370.
[162] J. Aittoniemi, T. Rog, P. Niemelä, M. Pasenkiewicz-Gierula, M. Karttunen, I. Vattulainen, Tilt: major factor in sterols' ordering capability in membranes, J. Phys. Chem. B 110 (51) (2006) 25562–25564.
[163] T. Róg, M. Pasenkiewicz-Gierula, Cholesterol-sphingomyelin interactions: a molecular dynamics simulation study, Biophys. J. 91 (10) (2006) 3756–3767.
[164] K.S. Bruzik, B. Sobon, G.M. Salamonczyk, Nuclear magnetic resonance study of sphingomyelin bilayers, Biochemistry 29 (16) (1990) 4017–4021.
[165] K. Cosentino, U. Ros, A.J. García-Sáez, Assembling the puzzle: Oligomerization of a-pore forming proteins in membranes, Biochim. Biophys. Acta Biomembr. 1858 (3) (2016) 457–466.
[166] C. Álvarez, J.M. Mancheño, D. Martínez, M. Tejuca, F. Pazos, M.E. Lanio, Sticholysins, two pore-forming toxins produced by the Caribbean sea anemone *Stichodactyla helianthus*: their interaction with membranes, Toxicon 54 (8) (2009) 1135–1147.
[167] D. Martínez, C. Álvarez, M. Tejuca, F. Pazos, A. Valle, L. Calderón, M.E. Lanio, Los lípidos de la membrana actúan como moduladores de la actividad permeabilizante de Sticholisina II, una toxina formadora de poros con aplicaciones biomédicas, Biol. Aplicada 23 (3) (2006) 251–254.
[168] J. Jouhet, Importance of the hexagonal lipid phase in biological membrane organization, Front. Plant Sci. 4 (2013) 494.
[169] G. Anderluh, M. Dalla Serra, G. Viero, G. Guella, P. Maček, G. Menestrina, Pore formation by equinatoxin II, a eukaryotic protein toxin, occurs by induction of nonlamellar lipid structures, J. Biol. Chem. 278 (46) (2003) 45216–45223.
[170] J. Palacios-Ortega, S. García-Linares, E. Rivera-de-Torre, J.G. Gavilanes, A. Martínez-del-Pozo, J.P. Slotte, Differential effect of bilayer thickness on sticholysin activity, Langmuir 33 (41) (2017) 11018–11027.
[171] E. Prato, F. Biandolino, Total lipid content and fatty acid composition of commercially important fish species from the Mediterranean, Mar Grande Sea, Food Chem. 131 (4) (2012) 1233–1239.
[172] S. Chapelle, Lipid composition of tissues of marine crustaceans, Biochem. Syst. Ecol. 5 (3) (1977) 241–248.
[173] T. Farkas, S. Herodek, The effect of environmental temperature on the fatty acid composition of crustacean plankton, J. Lipid Res. 5 (3) (1964) 369–373.
[174] J.D. O'Connor, L.I. Gilbert, Aspects of lipid metabolism in crustaceans, Am. Zool. 8 (3) (1968) 529–539.
[175] D. Gardner, J. Riley, The component fatty acids of the lipids of some species of marine and freshwater molluscs, J. Mar. Biol. Assoc. UK 52 (4) (1972) 827–838.
[176] J.D. Joseph, Lipid composition of marine and estuarine invertebrates. Part II: mollusca, Prog. Lipid Res. 21 (2) (1982) 109–153.
[177] P.G. Fast, Insect lipids, in: Progress in the Chemistry of Fats and other Lipids 11, 1971, pp. 179–242.
[178] C. Chintiroglou, A. Koukouras, The feeding habits of three Mediterranean sea anemone species, *Anemonia viridis* (Forskål), *Actinia equina* (Linnaeus) and *Cereus pedunculatus* (Pennant), Helgoländer Meeresun. 46 (1) (1992) 53–68.
[179] E. Ruoslahti, Fibronectin and its receptors, Annu. Rev. Biochem. 57 (1) (1988) 375–413.
[180] S. García-Linares, R. Richmond, M.F. García-Mayoral, N. Bustamante, M. Bruix, J. G. Gavilanes, A. Martínez-del-Pozo, The sea anemone actinoporin (Arg-Gly-Asp) conserved motif is involved in maintaining the competent oligomerization state of these pore-forming toxins, FEBS J. 281 (5) (2014) 1465–1478.
[181] K. Tanaka, J.M.M. Caaveiro, K. Morante, K. Tsumoto, Haemolytic actinoporins interact with carbohydrates using their lipid-binding module, Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 372 (1726) (2017) 20162016.
[182] S. García-Linares, E. Rivera-de-Torre, J. Palacios-Ortega, J.G. Gavilanes, A. Martínez-del-Pozo, The metamorphic transformation of a water-soluble monomeric protein into an oligomeric transmembrane pore, in: A. Iglič, M. Rappolt, A.J. García-Sáez (Eds.), Advances in Biomembranes and Lipid Self-Assembly 26, 2017, pp. 51–97.
[183] L. García-Ortega, J. Alegre-Cebollada, S. García-Linares, M. Bruix, A. Martínez-del-Pozo, J.G. Gavilanes, The behavior of sea anemone actinoporins at the water-membrane interface, Biochim. Biophys. Acta Biomembr. 1808 (9) (2011) 2275–2288.
[184] V. De los Ríos, J.M. Mancheño, A. Martínez-del-Pozo, C. Alfonso, G. Rivas, M. Oñaderra, J.G. Gavilanes, Sticholysin II, a cytolysin from the sea anemone *Stichodactyla helianthus*, is a monomer-tetramer associating protein, FEBS Lett. 455 (1-2) (1999) 27–30.
[185] J. Palacios-Ortega, E. Rivera-de-Torre, S. García-Linares, J.G. Gavilanes, Á. Martínez-del-Pozo, J.P. Slotte, Oligomerization of Sticholysins from Förster Resonance Energy Transfer, Biochemistry 60 (4) (2021) 314–323.
[186] J. Palacios-Ortega, E. Rivera-de-Torre, J.G. Gavilanes, J.P. Slotte, Á. Martínez-del-Pozo, Evaluation of different approaches used to study membrane

permeabilization by actinoporins on model lipid vesicles, Biochim. Biophys. Acta Biomembr. (2020) 183311.
[187] K. Morante, A. Bellomio, D. Gil-Carton, L. Redondo-Morata, J. Sot, S. Scheuring, J.M.M. Caaveiro, Identification of a membrane-bound prepore species clarifies the lytic mechanism of actinoporins, J. Biol. Chem. 291 (37) (2016) 19210–19219.
[188] N. Rojko, K.C. Kristan, G. Viero, E. Zerovnik, P. Maček, M. Dalla Serra, G. Anderluh, Membrane damage by an a-helical pore-forming protein, Equinatoxin II, proceeds through a succession of ordered steps, J. Biol. Chem. 288 (33) (2013) 23704–23715.
[189] Y. Subburaj, U. Ros, E. Hermann, R. Tong, A.J. García-Sáez, Toxicity of an a-pore-forming toxin depends on the assembly mechanism on the target membrane as revealed by single-molecule imaging, J. Biol. Chem. 290 (8) (2015) 4856–4865.
[190] A. Valle, A. Lopez-Castilla, L. Pedrera, D. Martinez, M. Tejuca, J. Campos, S. Schreier, Cys mutants in functional regions of Sticholysin I clarify the participation of these residues in pore formation, Toxicon 58 (1) (2011) 8–17.
[191] J. Martín-Benito, F. Gavilanes, V. de Los Ríos, J.M. Mancheño, J.J. Fernández, J. G. Gavilanes, Two-dimensional crystallization on lipid monolayers and three-dimensional structure of sticholysin II, a cytolysin from the sea anemone *Stichodactyla helianthus*, Biophys. J. 78 (6) (2000) 3186–3194.
[192] G. Huang, A. Voet, G. Maglia, FraC nanopores with adjustable diameter identify the mass of opposite-charge peptides with 44 dalton resolution, Nat. Commun. 10 (1) (2019) 1–10.
[193] V. Antonini, V. Perez-Barzaga, S. Bampi, D. Penton, D. Martinez, M. Dalla Serra, M. Tejuca, Functional characterization of sticholysin I and W111C mutant reveals the sequence of the actinoporin's pore assembly, PLoS One 9 (10) (2014), e110824.
[194] M.A. Baker, N. Rojko, B. Cronin, G. Anderluh, M.I. Wallace, Photobleaching reveals heterogeneous stoichiometry for equinatoxin II oligomers, Chembiochem 15 (14) (2014) 2139–2145.
[195] K. Kristan, G. Viero, P. Maček, M. Dalla Serra, G. Anderluh, The equinatoxin N-terminus is transferred across planar lipid membranes and helps to stabilize the transmembrane pore, FEBS J. 274 (2) (2007) 539–550.
[196] C. Wloka, N.L. Mutter, M. Soskine, G. Maglia, a-Helical fragaceatoxin C nanopore engineered for double-stranded and single-stranded nucleic acid analysis, Angew. Chem. Int. Ed. Eng. 55 (40) (2016) 12494–12498.
[197] L. Restrepo-Pérez, C.H. Wong, G. Maglia, C. Dekker, C. Joo, Label-free detection of post-translational modifications with a nanopore, Nano Lett. 19 (11) (2019) 7957–7964.
[198] L. Restrepo-Pérez, G. Huang, P.R. Bohländer, N. Worp, R. Eelkema, G. Maglia, C. Dekker, Resolving chemical modifications to a single amino acid within a peptide using a biological nanopore, ACS Nano 13 (12) (2019) 13668–13676.
[199] F.L.R. Lucas, K. Sarthak, E.M. Lenting, D. Coltan, N.J. van der Heide, R.C. A. Versloot, G. Maglia, The manipulation of the internal hydrophobicity of FraC nanopores augments peptide capture and recognition, ACS Nano 15 (6) (2021) 9600–9613.
[200] G. Huang, K. Willems, M. Soskine, C. Wloka, G. Maglia, Electro-osmotic capture and ionic discrimination of peptide and protein biomarkers with FraC nanopores, Nat. Commun. 8 (1) (2017) 1–11.