



Sticholysins, two pore-forming toxins produced by the Caribbean Sea anemone *Stichodactyla helianthus*: Their interaction with membranes

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ABSTRACT

Sticholysins (Sts) I and II (StI/II) are pore-forming toxins (PFTs) produced by the Caribbean Sea anemone *Stichodactyla helianthus* belonging to the actinoporin family, a unique class of eukaryotic PFTs exclusively found in sea anemones. As for the rest of the members of this family, Sts are cysteine-less proteins, with molecular weights around 20 kDa, high isoelectric points (>9.5), and a preference for sphingomyelin-containing membranes. A three-dimensional structure of StII, solved by X-ray crystallography, showed that it is composed of a hydrophobic β -sandwich core flanked on the opposite sides by two α helices comprising residues 14–23 and 128–135. A variety of experimental results indicate that the first thirty N-terminal residues, which include one of the helices, are directly involved in pore formation. This region contains an amphipathic stretch, well conserved in all actinoporins, which is the only portion of the molecule that can change conformation without perturbing the general protein fold; in fact, binding to model membranes only produces a slight increase in the regular secondary structure content of Sts. Sts are produced in soluble form but they readily bind to different cell and model membrane systems such as lipidic monolayers, micelles, and lipid vesicles. Remarkably, both the binding and pore-formation steps are critically dependent on the physico-chemical nature of the membrane. In fact, a large population of toxin irreversibly binds with high affinity in membranes containing sphingomyelin whereas binding in membranes lacking this sphingolipid is relatively low and reversible. The joint presence of SM and cholesterol largely promotes binding and pore formation. Minor amounts of lipids favoring a non-lamellar organization also augment the efficiency of pore formation. The functional pore formed in cellular and model membranes has a diameter of ~ 2.0 nm and is presumably formed by the N-terminal α helices of four monomers tilted 31° in relation to the bilayer normal. Experimental evidence supports the hypothesis that sticholysins, as well as equinatoxin II, another actinoporin, form a toroidal pore in membranes in which the polypeptide chains as well as the polar head groups of phospholipids are involved.

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1. Introduction

Sea anemones (*Cnidaria*, *Anthozoa*, and *Actiniaria*) are relatively abundant along Cuban seacoasts, are relatively

easy to capture, and constitute prolific sources of bioactive compounds. Probably due to their mainly sedentary life, anemones are provided with an ensemble of molecules that acting together help these animals to capture prey or defend from predators. Members of the ensemble include neurotoxins (Mas et al., 1989; Loret et al., 1994), phospholipases (Pazos et al., 1993), K^+ channel blockers (Castañeda

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et al., 1995), protease inhibitors (Delfin et al., 1994), and pore-forming toxins, PFTs, (Bernheimer and Avigad, 1976; Kem and Dunn, 1988; Lanio et al., 2001). Proteins that form pores in cellular membranes have been identified in a plethora of species including bacteria, fungi, plants, and animals and in some cases exhibit mechanistic and structural similarities across large evolutionary distances (Gilbert, 2002). Pore-forming proteins from animals have been less studied. Therefore, the toxins' conformational changes, membrane-induced damage and the nature of the pore formed in cell barriers are not as well understood.

The Caribbean Sea anemone *Stichodactyla helianthus* (Fig. 1) produces two highly hemolytic proteins, sticholysins (Sts) I (StI) and II (StII) (Lanio et al., 2001) formerly described as CII and CIII (Kem and Dunn, 1988; Stevens et al., 2002).

StI and StII belong to the protein superfamily of PFTs and can be classified into the group of 20 kDa *actinoporins* inhibited by sphingomyelin according to the systematization proposed for cytolytic polypeptides isolated from anemones by Anderluh and Mačák (2002). StI and StII are cysteine lacking polypeptides containing a high content of nonpolar amino acids with highly basic isoelectric points (>9.5) and molecular weights of 19,392 (± 2) Da and 19,283 (± 2) Da, respectively (Huerta et al., 2001; Lanio et al., 2001); being isoforms, they exhibit high similarity (99%) and identity (93%). In spite of the homology existing between both sticholysins, they show different hemolytic activity (HA), i.e. StII is around six fold more active than StI against human red blood cells becoming natural tools to understand the structure–function relationship in actinoporins. Moreover, comparing the activity of StII with the homologous actinoporin equinatoxin II (EqII, 66% similarity), from *Actinia equina*, we have found a different ability to lyse human red blood cells when tested under the same experimental conditions. These results indicate how subtle differences in structure along this protein family can be reflected in their function. Interestingly, actinoporins

experience only small conformational changes upon binding to membranes (Menestrina et al., 1999; Alvarez et al., 2003; Alegre-Cebollada et al., 2007a) differently to other PFTs (Alouf, 2003). Perhaps a comparative analysis of the functional differences between these actinoporins could help to understand how these proteins, although water soluble, can interact with membranes and form a stable membrane pore. The soluble state structure of StII was resolved by X-ray crystallography (Mancheño et al., 2003) showing, as expected, analogies but also differences with EqII, the other actinoporin whose 3D structure has been resolved (Athanasiadis et al., 2001; Hinds et al., 2002).

The study of actinoporins is important not only to understand their biological roles in anemones' venom (Basulto et al., 2006) but also to investigate basic molecular mechanisms of protein insertion into membranes, protein–lipid interactions as well as modulation of protein conformation by lipid binding. Furthermore, these toxins could also be potential tools for the construction of antiparasite chimeric molecules (Tejuca et al., 1999, 2009), immunotoxins against tumor cells (Avila et al., 1988, 1989; Pederzoli et al., 1995; Potrich et al., 2000; Tejuca et al., 2004) or tumor protease-activated PFTs (Potrich et al., 2005). A detailed understanding of the basic molecular mechanisms involved in the toxin–cell interaction would facilitate the rational design of these nanocomplexes.

2. Sticholysins StI and StII: a multidisciplinary approach to the structure–function relationships

A precise structural characterization of Sts would require the determination of the high-resolution three-dimensional structure of both the water-soluble and the membrane-bound states, and also those of other potential intermediates functionally relevant. Certainly, the intrinsic experimental difficulties associated to these studies are the main responsible for the scarcity of structural information, not only within Sts, but also with other members of the

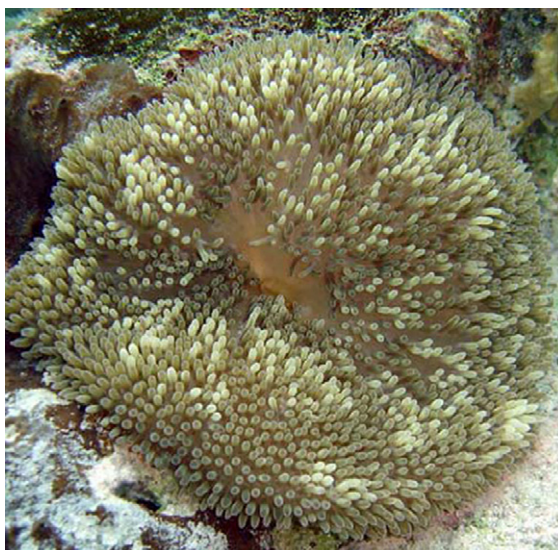


Fig. 1. *Stichodactyla helianthus* (Photo courtesy of Dr. J. Espinosa, Institute of Oceanology, Cuba).

actinoporins family, and in general, in PFTs, where only just one member, α -hemolysin from *Staphylococcus aureus*, has been characterized at high resolution in the oligomeric state (Song et al., 1996).

Some progress has been done in the structural characterization of actinoporins at high resolution in recent years. StI and StII belong to a highly conserved and definite group of proteins (Anderluh and Maček, 2002), as derived from multiple sequence alignment analyses (Fig. 2). This result is consistent with the presence of a common overall protein fold for all members of the family and consequently a similar mechanism of membrane pore formation. Particularly, StI and StII share a 93% sequence identity but exhibit

significant differences in HA (Martinez et al., 2001), which clearly suggests that the observed substitutions modulate the membrane pore-forming activity. In fact, all nonconservative changes are located close to the N-terminal end of the protein (Huerta et al., 2001), a region which is relevant in the formation of the pore. A direct consequence of these nonconservative replacements is a different charge distribution in both proteins, which would make StI almost equivalent to EqtII (Fig. 2).

A variety of low-resolution spectroscopic techniques such as UV-fluorescence, far- and near-UV circular dichroism (CD) and ATR-FTIR have been used to characterize the secondary and tertiary structure of Sts both in the

Or-A	-----ATFRVLAKVLAELGKVS	RKIAVGVDNESGGSWTALNAYFRSGTTDVI	47		
Or-G	----GAI IAGAALGFNVHQTVL	KALGQVSRKIAIGVDNESGGTWTALNAYFRSGTTDVI	55		
EqtII	SADVAGAVIDGASLSFDILKTV	LEALGNVVRKIAVGVDNESGKTWTALNTYFRSGTSDIV	60		
Ten-C	SADVAGAVIDGASLSFDILKTV	LEALGNVVRKIAVGVDNESGKTWTALNTYFRSGTSDIV	60		
EqtIV	SVAVAGAIKGAALTFFNVLQTV	LKALGDISRKIAVGVDNESGKTWTALNTYFRSGTSDIV	60		
EqtV	SVAVAGAVIEGATLTFNVLQTV	LKALGDISRKIAVGIDNESGMTWTAMNTYFRSGTSDVI	60		
StnI	--SELAGTIIIDGASLTFEVL	DKVLGELGKVS RKIAVGIDNESGGTWTALNAYFRSGTTDVI	59		
StnII	--ALAGTIIAGASLTFQVL	DKVLEELGKVS RKIAVGIDNESGGTWTALNAYFRSGTTDVI	58		
HMgIII	SAALAGTIIIEGASLGFQILD	DKVLGELGKVS RKIAVGVDNESGGSWTALNAYFRSGTTDVI	60		
RTX-A	--ALAGAI IAGASLTFQILD	DKVLAELGQVSRKIAIGIDNESGGSWTAMNAYFRSGTTDVI	58		
SrcI	-KISGGTVIAAGRLTDL	LLKTLGLTGLSISRKIAIGVDNETGGLITGNNVYFRSGTSDDI	59		
AvtI	SAAVAGAVIAGGELALKILT	KILDEIGKIDRKIAIGVDNESGLKWTALNTYYKSGASDVT	60		
Pstx20	SAAVAGAVIAGGELALKILT	KILDEIGKIDRKIAIGVDNESGLKWTALNTYYKSGASDVT	60		
Or-A	LPDLV	PNQKALLYRGGKDTGPVATGVVGVLAYAMS	DGNTLAILFSVPYDYNLYSNWNVK	107	
Or-G	LPEFV	PNQKALLYSGQKDTGPVATGAVGVLAYYMS	DGNTLGVMFSPVPFDYNLYSNWWDVK	115	
EqtII	LPHKV	PHGKALLYNGQKDRGPVATGAVGVLAYLMS	DGNTLAVLFSVPYDYNWYSNWWNVNR	120	
Ten-C	LPHKV	PHGKALLYNGQKDRGPVATGAVGVLAYLMS	DGNTLAVLFSVPYDYNWYSNWWNVNR	120	
EqtIV	LPHKV	PHGKALLYNGQKDRGPVATGAVGVLAYAMS	DGNTLAVLFSVPYDYNWYSNWWNVNR	120	
EqtV	LPHTV	PHGKALLYNGQKDRGPVATGVVGVLAYAMS	DGNTLAVLFSIPFDYNLYSNWNVK	120	
StnI	LPEFV	PNTKALLYSGRKSSGPVATGAVAAFAYYMS	NGNTLGVMFSPVPFDYNWYSNWWWDVK	119	
StnII	LPEFV	PNTKALLYSGRKDTGPVATGAVAAFAYYMS	SGNTLGVMFSPVPFDYNWYSNWWWDVK	118	
HMgIII	LPEFV	PNQKALLYSGRKDTGPVATGAVAAFAYYMS	NGHTLGVMFSPVPFDYNFYSNWWWDVK	120	
RTX-A	LPEFV	PNQKALLYSGRKNRGPD	TTGAVGALAYYMSNGNTLGVMFSPVPFDYNLYSNWWWDVK	118	
SrcI	LPHRV	ETGEALLYTARKTKGPVATGAVGVFTYYL	SDGNTLAVLFSVPFPDYNFYSNWWNVK	119	
AvtI	LPYEV	ENSKALLYTARKSKGPVARGAVGVLAYKMS	SGNTLAVMFSPVPFDYNLYSNWNVK	120	
Pstx20	LPYEV	ENSKALLYTARKSKGPVARGAVGVLAYKMS	SGNTLAVMFSPVPFDYNLYTNWNVK	120	
Or-A	VYSGKRRADQGMSE	DLSSYGN-PYGGDNGWHARKLA-YGLKERGF	MKSSAQSIETIHATKA	165	
Or-G	VYGRRRRADQAMY	EGLLYGI-PYGGDNGWHARKLG-YGLKGRGF	MKSSAQSIETIHVTKA	173	
EqtII	IYKGRRADQRM	YEELYYNLS-PFRGDNGWHTRNLG-YGLKSRGF	MNSSGHAILEIHVSKA	179	
Ten-C	IYKGRRADQRM	YEELYYNLS-PFRGDNGWHTRNLG-YGLKSRGF	MNSSGHAILEIHVSKA	179	
EqtIV	IFKGRRRADQRM	YEELYLYLS-PFRGDNGWHERHLG-YGLKSRGF	MNSGGQAILEIHVTKA	179	
EqtV	VYKGRRRADQRM	YEELYYNLS-PFRGDNGWHNRDLG-YGLKGRGF	MNSSGQSILEIHVTKA	179	
StnI	IYFGKRRADQGM	YEDYYGN-PYRGDNGWYQKNLG-YGLRMKG	IMTSAGEAKMQIKISR-	176	
StnII	IYSGKRRADQGM	YEDLYYGN-PYRGDNGWHEKNLG-YGLRMKG	IMTSAGEAKMQIKISR-	175	
HMgIII	VYSGKRRADQGM	YEDMYYGN-PYRGDNGWHQKNLG-YGLRMKG	IMTSAGEAILQIRISR-	177	
RTX-A	VYSGKRRADQAM	YEDLYYSN-PYRGDNGWHQKNLG-YGLKMKG	IMTSAGEAIMEIRISR-	175	
SrcI	IYSGKRNADYD	MYHELYYDANPFEGDDTWYRYLG-YGMRMEG	YMNSPGEAILKITVMPD	178	
AvtI	IYDGEKKADEK	MYNELYNNNNPIKP-STWEKRD	LQDGLKLRGFM	TNSGDAKLVIHIEKS	179
Pstx20	IYDGEKKADEK	MYNELYNNNNPIKP-SIWEKRD	LQDGLKLRGFM	TNSGDAKLVIHIEKS	179

Fig. 2. Multiple alignment of actinoporin amino acid sequences. Only complete sequences (excluding propeptide regions) are considered in the analysis. Sequences shown are: actinoporins Or-A and Or-G from *Oulactis orientalis* (Il'ina et al., 2005); equinatoxins EqtII (Anderluh et al., 1999), EqtIV, EqtV from *Actinia equina*; sticholysins StI and StII from *Stichodactyla helianthus* (Lanio et al., 2001); tenebrosin C (Ten-C) from *A. tenebrosa* (Simpson et al., 1990); magnificolysins HMgII and HMgIII from *Radianthus magnifica* (Wang et al., 2000); actinoporin RTX-A from *Radianthus macrodactylus* (Il'ina et al., 2006); actinoporin SrcI from *Sagartia rosea* (Jiang et al., 2002); actinoporins AvtI from *Actinaria villosa* (Uechi et al., 2005); actinoporin Pstx20 from *Phyllodiscus semoni* (Nagai et al., 2002). Color code: yellow, residues conserved in all actinoporins; green, non-conserved residues between sticholysins StI and StII. (For interpretation of colour in this figure, please refer to the web version of this article).

absence (Menestrina et al., 1999; Mancheño et al., 2001; Martinez et al., 2001; Alegre-Cebollada et al., 2007a) and presence of model membranes (Menestrina et al., 1999; Alvarez et al., 2003; Lanio et al., 2003; Pazos et al., 2006; Alegre-Cebollada et al., 2007a). These experimental approaches provided relevant information both on potential intermediates in the mechanism of StII membrane interaction (Mancheño et al., 2001) and also on the structure of the StII pore state (Alegre-Cebollada et al., 2007a). In the first case, it has been shown that StII adopts conformations with spectroscopic properties typical of partially folded states. FTIR (Menestrina et al., 1999; Alegre-Cebollada et al., 2007a) and circular dichroism (Alvarez et al., 2003) measurements have detected increments in the α helical content of sticholysins upon lipid binding that are compatible with the extension of the N-terminal helix. A slight increase in β -structure has also been detected by FTIR, though no clear interpretation can be currently given (Menestrina et al., 1999; Alegre-Cebollada et al., 2007a). Importantly, the N-terminal helix would have a 31° tilt with respect to the membrane normal (Alegre-Cebollada et al., 2007a), which fairly coincides with the structural model of the StII pore previously proposed (Mancheño et al., 2003).

The three-dimensional crystal structure of the water-soluble state of StII and the crystal structure of the complex formed with its water-soluble state and phosphocholine (POC) have been solved (Mancheño et al., 2003). The water-soluble states of EqtII (Athanasidis et al., 2001) and that of the double mutant EqtII Cys 8–69 (Kristan et al., 2004) have also been determined by X-ray crystallography; moreover, the structure of EqtII has also been solved by NMR (Hinds et al., 2002).

The structure of StII (Fig. 3A) has been solved by the molecular replacement method at 1.7 Å resolution (Mancheño et al., 2003). The water-soluble state of StII is a monomeric species with a globular shape with overall dimensions of $45 \times 32 \times 27$ Å. The overall protein fold is based on a β -sandwich composed of ten β strands, flanked on each side by two α helices comprising residues 14–23 and 128–135. The N-terminal α helix mainly interacts with strands $\beta 1$ and $\beta 2$, and the C-terminal one interacts with strands $\beta 7$ and $\beta 8$. The interactions are predominantly hydrophobic although salt bridges are also observed. Considering the membrane-interacting properties of actinoporins, the most remarkable characteristic of this structure is the presence of an exposed cluster of polar-aromatic residues: Trp 110, Tyr 111, Trp 114 (from the connecting loop $\beta 6$ – $\beta 7$), and Tyr 131, Tyr 135 and Tyr 136 (from the C-terminal α helix). It has been demonstrated that these aromatic residues have a specific affinity for a region near the lipid carbonyls (Killian and von Heijne, 2000). As expected for a fundamental feature in the mechanism of membrane interaction, the aromatic cluster would be present in all members of the actinoporins family (Fig. 2). Experimental evidences demonstrating the relevance of this region in the membrane binding step came from biochemical studies carried out for EqtII (Hong et al., 2002). Comparison of the crystal structures of StII (PDB code: 1GWY) and EqtII (PDB code: 1IAZ) reveals that they superpose almost perfectly, with some deviations being observed in the connecting loops.

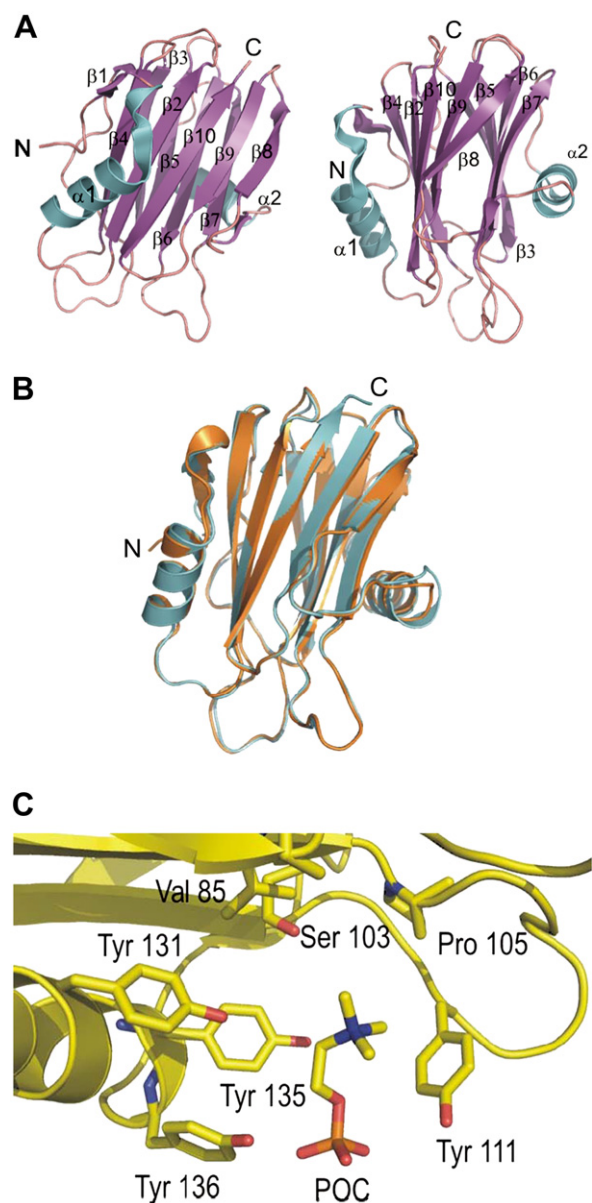


Fig. 3. Structural characterization of StII. **A.** Overall structure of StII; two views of the StII overall fold depicted as a ribbon diagram are shown. For clarity, α helix and β strand identifiers are used. **B.** Three dimensional superposition of StII (cyan) and EqtII (orange). The rms deviation for 172 C_α atoms was 0.6 Å. **C.** Phosphocholine binding-site of StII. Residues forming the POC binding site are shown as sticks. The figure was prepared with PyMol.

Further structural analyses on the water-soluble state of StII led to the cocrystallization of StII with phosphocholine (POC) which revealed for the first time the existence of a lipid-binding site in actinoporins (Mancheño et al., 2003) (Fig. 3C). POC binds to a cavity which exhibits a complex composition: Tyr 111, Tyr 131, Tyr 135, Tyr 136 (polar aromatic residues), Ser 52 and Ser 103 (polar residues), and Val 85 and Pro 105 (apolar residues). One of the most remarkable features of the interactions between StII and POC is the stabilization of the positive charge of the choline

moiety by cation– π interactions with the aromatic rings of Tyr 111 and Tyr 135. Interestingly, Tyr 111 and Tyr 135 are strictly conserved in all actinoporins, with the exception of actinoporins Or-A and Or-G from *Oulactis orientalis* which lack Tyr 135 (Fig. 2). As otherwise expected due to its small size, POC does not promote significant conformational changes in StII apart from minor rearrangements in local side chains and backbone modifications in the $\beta 6$ – $\beta 7$ loop (Mancheño et al., 2003).

Site-directed mutagenesis has also been applied to the study of the structure–function relationship in sticholysins. Elucidation of the functional role of specific protein residues involved in the lytic action will improve our understanding of how these proteins initially produced as water-soluble monomers, become membrane-embedded proteins, forming oligomeric pores within membranes. More than 30 actinoporins have been so far described (Maček, 1992; Anderluh and Maček, 2002), however, only six of them have been produced as recombinant proteins in *Escherichia coli*: EqtII (Anderluh et al., 1996) from *A. equina*, 6HisStnII (De los Ríos et al., 2000) and StI (Pazos et al., 2006) from *S. helianthus*, magnificallysin III (HMgIII) from *Heteractis magnifica* (Wang et al., 2000), Srcl from *Sagartia rosea* (Jiang et al., 2003a,b), and AvtI from *Actinergia villosa* (Uechi et al., 2005).

De los Ríos et al. (2000) and Alegre-Cebollada et al. (2007b) produced in *E. coli* recombinant variants of StI and StII that included a six-histidine tag at the N-terminus. The heterologously expressed StII showed reduced HA and decreased pore-formation ability in liposomes, in spite of an efficient binding to lipidic bilayers (Pazos et al., 2003) pointing to the relevance of the N-terminus on toxin function. However, the presence of the His-tag at the N-terminal precluded a clear interpretation of the differences between StII and its modified variant. Other recombinant actinoporin (HMgIII) with a His-tag on the N-terminal resulted a less hemolytic molecule than its corresponding wild-type protein, thus supporting the importance of the N-terminus for its cytolytic activity (Wang et al., 2000). Alegre-Cebollada et al. (2004) randomly produced mutants of 6HisStII in order to select those with reduced HA. Seven mutants were produced and purified to homogeneity having similar structural characteristics and thermostability but large differences in terms of their HA. Three single mutation protein variants of StII, at residues Lys 19, Phe 106 and Tyr 111, showed a significantly decreased HA probably due to the localization of mutations in or close to functionally important regions of the toxin, the N-terminal sequence (Lys 19), and the aromatic cluster involved in the initial binding of actinoporins to membranes (Tyr 111) (Mancheño et al., 2003).

StI has also been obtained by a recombinant procedure (rStI) lacking the His tag (Pazos et al., 2006). rStI primary structure shows one substitution of a single amino acid at the N-terminus (Glu 16 Gln). This change renders the charge of this segment intermediate between those of the two native Sts' isoforms. rStI behaves as StI in several model systems in terms of pore-formation in bilayers, interaction with lipidic monolayers and bilayers, and adsorption to the air/water interface. In spite of this, the pseudo-wild-type toxin is significantly more lytic than StI (Pazos et al., 2006)

reinforcing the importance of the N-terminal sequence in sticholysins' activity. Interestingly, Alegre-Cebollada et al. (2007b) also found in a recombinant variant of StI the amino acid change (Glu 16 Gln) reported by Pazos et al. (2006).

Work is in progress on engineered sticholysins in order to obtain site-directed mutants to understand the mechanisms of interaction of these proteins with membranes and pore-formation. The introduction of Cys in the primary sequence would allow forming spontaneous inactive dimers by disulphide bond and the introduction of different probes mediated by sulfhydryl-specific reagents.

3. Sticholysins and their interactions with cells: the functional pore

Hemolysis has been the most used procedure to assess and characterize the activity of actinoporins, being a very simple and sensitive assay for most membrane damaging toxins. StI and StII are extremely efficient in lysing red blood cells from different species, for instance, rat, sheep, rabbit, and human erythrocytes (Tejuca et al., 1994). The concentration at which 50% of a human red blood cells suspension (5×10^6 cells/ml) is lysed after 30 min was around 3×10^{-10} M for StI and 1×10^{-10} M for StII. The most noticeable difference between both toxins is the higher activity of StII, both measured in terms of erythrocyte internal K^+ exit or hemolysis (Martinez et al., 2001). The lysis is a consequence of the colloidal osmotic shock induced by the formation of pores in the membranes. As a result of an outward osmotic gradient of non-permeant molecules (such as hemoglobin), net influx of water increases cell volume until the cell membrane breaks (MacGregor II and Tobias, 1972).

For sizing the radius of the pore formed by sticholysins in erythrocytes and lipid vesicles we took advantage of the fact that osmotic shock can be prevented by addition to the incubation medium of an osmotic protectant of appropriate size. The pore radii estimated for StI were 1.09 nm and 0.96 nm when oligosaccharides and polyethylene glycols (PEGs) were used, respectively, while for StII the radius determined with PEGs was 1.05 nm. The osmometric behavior of large unilamellar vesicles (LUV) formed by equimolar mixtures of sphingomyelin (SM) with phosphatidylcholine (PC) was also used to estimate the toxin lesion radius in lipid vesicles. The StI channel radius was around 1.2 nm, both for sugars and for PEGs. Taken together these studies clearly demonstrate that the sticholysin pore has a constant size, independent of toxin concentration, and virtually the same in natural and artificial membranes, suggesting it has a fixed predominant structure (Tejuca et al., 2001).

Actinoporins are very potent toxins affecting almost all kind of eukaryotic cells on which they have been tried. Avila et al. reported in 1988 the cytotoxic activity of a hemolytic fraction isolated from the sea anemone *Stichodactyla* (formerly *Stoichactis*) *helianthus* on human myelocytic leukemia, lymphoblastic leukemia, peripheral mononuclear and chronic myelogenous leukemia cells. Later on, the cytotoxic effect of this fraction was evaluated on two lines of human breast carcinoma (Avila et al., 1989)

and more recently the effect of StI on a human colorectal cancer cells was assayed (Tejuca et al., 2004). The cytotoxic activities expressed as the toxin concentration necessary to kill half of the cells in the assay (C_{50}) are shown in Table 1. The concentration ranges from 10^{-10} to 10^{-7} M in agreement with the values obtained for the actinoporin family (10^{-11} to 10^{-7} M; Anderluh and Menestrina, 2001). The cytotoxic effects of the sticholysins on the unicellular parasite *Giardia duodenalis* have also been studied (Tejuca et al., 1999) and, curiously, and in contrast to the observed HA, StI is more efficient killing the parasite than StII (Table 1).

The high cytotoxic activity of these proteins prompted researchers to use sticholysins for the construction of Immunotoxins (ITs), chimeric molecules formed by cell binding ligands coupled to a killer toxin addressed to a specific cell. In the first approaches Sts' ITs were produced by chemical coupling of the hemolytic fraction isolated from *S. helianthus* to a monoclonal antibody (mAb) that recognizes a specific antigen expressed on immature T lymphocytes (Avila et al., 1988) and to an mAb directed against the carcinoembryonic antigen (Avila et al., 1989). More recently StI was linked to an mAb recognizing a colon tumor-associated antigen (Tejuca et al., 2004). The results obtained in these works support the feasibility of directing sticholysins to the surface of cells expressing tumor-associated antigens. However, these approaches were limited by the generation of unspecific linkages between the targeting molecule and the toxin and hence kept the unspecific activity associated with the toxic moiety, a common problem of most ITs. An approach designed to overcome this limitation is the production of ITs using PFTs activated by tumor-associated proteases. Promising results using this approach have already been obtained with engineered *Bacillus anthracis* lethal toxin containing either

matrix metalloproteinases (MMP) (Liu et al., 2008) or urokinase plasminogen activator (Su et al., 2007) cleavage sites.

Based on the understanding of the structure–function relationship in actinoporins, a mutant of StI (StI W111C) with a cysteine residue in the cytolysin-membrane binding region has been obtained to engineer a tumor proteinase-activated IT. The W111 residue is located at the Trp-rich region described for actinoporins as essential for protein anchoring to membrane (Anderluh et al., 1999). The mutant protein and the binding ligand will be attached through a protease-sensitive peptide by the StI 111 residue. As a result the binding region will be sterically blocked that in turn will reduce the IT unspecific killing activity. Currently, the construction of an MMP-activated IT based on StI W111C is in development.

4. Surfactants as model membrane systems. The effect of surfactants on sticholysin structure–function depends on the amphiphile nature

The activity of StI and StII is extremely dependent on those factors that change their conformation such as: amino acid group modification, ionic strength and pH (Campos et al., 1999; Pazos et al., 1998; Alvarez et al., 1998, 2001). Binding of surfactants to proteins induces changes in the secondary and/or tertiary structures strongly modifying the function of the macromolecule (Gebicka, 1999). The conformational changes of StI and StII upon interaction with micelles could mimic the transient conformations of these toxins in the early stages of the protein–membrane interactions leading to their insertion into lipidic bilayers.

Sodium dodecyl sulfate (SDS) is a widely used surfactant that forms well-characterized micelles at relatively high concentrations (8 mM in pure water). Its effect upon the conformation and HA of StI and StII strongly depends on its concentration (Lanio et al., 2003). At relatively low surfactant concentrations, SDS leads to the formation of aggregates; in contrast, at higher SDS concentrations the protein adducts disaggregate, as deduced from their intrinsic fluorescence properties, their quenching by acrylamide and simple observation of the solution turbidity. SDS effect was qualitatively similar for both toxins, as was inferred from Stern-Volmer constants, although with more significant changes for StI than for StII (Table 2). In agreement with an aggregated state of the proteins a lower quenching efficiency was observed for both toxins at maximal fluorescence emission (1 mM SDS). On the contrary, at higher SDS concentrations the quenching efficiency was significantly larger suggesting a higher exposure of Trp residues, probably due to a disaggregated and more relaxed structure. Concomitantly, StI and StII exhibited a loss of three-dimensional structure as followed by near-UV Circular Dichroism spectra and a small increase in α helix content, as deduced from far-UV CD spectra (Lanio et al., 2003). In spite of these noticeable changes, at higher SDS concentrations, the toxins partially recovered their HA, almost completely lost at 1 mM SDS. Although an explanation for these results is not completely evident, the authors assumed that the non-native α helix intermediates present in a more relaxed structure at higher SDS concentration

Table 1
Cytotoxic activity of sticholysins on different cell types.

Cells	Toxin	C_{50}	Reference
HL-60 (Human myelocytic leukemia)	Hemolytic fraction from <i>Stichodactyla helianthus</i>	1×10^{-7} M	Avila et al., 1988
Human peripheral mononuclear		5×10^{-8} M	
CEM (Lymphoblastic leukemia)		3×10^{-7} M	
K562 (Chronic myelogenous leukemia)		3×10^{-7} M	
MDA-MB-231 (Human breast carcinoma)		3×10^{-8a} M	Avila et al., 1989
MDA-MB-134 (Human breast carcinoma)		3×10^{-9a} M	
SW948 (Human colorectal cancer)	Sticholysin I	3×10^{-10} M	Tejuca et al., 2004
<i>Giardia duodenalis</i>	Sticholysin I	5×10^{-10} M	Tejuca et al., 1999
	Sticholysin II	2×10^{-9} M	

C_{50} : Cytotoxic activity, mean concentration required for 50% reduction of viability.

^a Value estimated from the original dose-dependence graphic.

Table 2

Effect of surfactant head group charge on StI and StII spectroscopic properties.

Surfactant	Toxin	F/F_0	Ksv (M^{-1})	Conformational changes ^a	HA ^b
SDS (Lanio et al., 2003)	StI	0.5	10.8	Large increase in α helical content, total loss of tertiary structure	Decreased, partially recovered at higher SDS concentrations
	StII	0.5	8.1		
HPS (Lanio et al., 2002)	StI	–	4.5	Negligible changes in secondary structure, progressive loss of tertiary structure	Unchanged
	StII	1.8	7.4		
CTAB (Lanio et al., 2007)	StI	2.0	6.5	Small changes in secondary structure and modification of tertiary structure	Increased
	StII	1.65	5.0		
None	StI	–	3.5 ± 0.3	–	–
	StII	–	4.8 ± 0.4		

 F/F_0 is the ratio of fluorescence intensities in the presence and absence of surfactant.

Ksv are the Stern–Volmer constants obtained for acrylamide quenching.

Surfactants were used at concentrations above their CMC.

Taken from Lanio et al. (2007), with permission.

^a Obtained by far- and near-UV CD measurements.^b HA: hemolytic activity.

could shift to the mainly β -sandwich native structure in the presence of membrane. This structure should contain both the very exposed cluster rich in hydrophobic amino acids and the flexible N-terminus involved in the first and last steps, respectively, of the pore formed by actinoporins (Malovrh et al., 2003).

Although zwitterionic and cationic surfactants have been considerably less studied, the available data indicate that their effects on proteins can be significantly different from those elicited by anionic surfactants (Henriquez et al., 1993; Moriyama et al., 1996; Gelamo and Tabak, 2000). N-hexadecyl-N'-9-dimethyl-3-ammonio-1-propane-sulfonate (HPS), a zwitterionic surfactant, readily binds to StI and StII (Lanio et al., 2002). After a slight decrease of StI and StII intrinsic fluorescence at low HPS concentrations, a significant increase in Trp fluorescence intensity occurs with the addition of surfactant. These changes indicate that HPS modifies the protein conformation, changing the Trp environment. The increase in fluorescence intensity upon HPS addition was sided by a shift of the Trp fluorescence band toward longer wavelengths, reflecting greater exposure of the average Trp residue toward the polar external solvent and apparently contradicting the fluorescence intensity increase observed for both toxins (Lanio et al., 2002). These results could be explained in terms of a lower quenching by internal groups as a consequence of partial unfolding of the protein (Gelamo and Tabak, 2000). Accordingly, StI and StII Trp emission exhibited an increase in the lifetimes associated with the main components with the addition of surfactant. Consequently and compatible with a higher exposure to the external solvent, HPS enhanced the efficiency of acrylamide quenching. Nevertheless, binding of HPS did not induce noticeable modification in the secondary structure of StI or StII as deduced from the far-UV CD spectra, but elicited a progressive loss of the protein three-dimensional structure as revealed by the near-UV CD studies (Lanio et al., 2002). In summary, all the spectroscopic information was compatible with a relatively more expanded tertiary structure of the proteins upon

addition of HPS. Interestingly, under these conditions the toxins fully retained their HA (Table 2). This result could suggest an irrelevant role of the conformational changes to the toxin function, and/or their reversible character in the presence of the red blood cell membrane. This last idea was rejected due to the irreversible character of the conformational changes upon addition of HPS (Lanio et al., 2002). In the hemolytic assays, the fast association of the toxins to the membranes (Alvarez et al., 2001) must take place with the protein conformation acquired during pre-incubation with HPS that resulted irrelevant to the function of the toxin. This was a rather unexpected result, because previous studies had shown that the HA was the parameter most sensitive to StI and StII conformational changes (Alvarez et al., 1998, 2001).

The effect of three cationic surfactants bearing the same polar head group and different chain length (CTAB, cetyltrimethyl ammonium bromide, C_{16} ; TTAB, tetradecyltrimethylammonium bromide, C_{14} ; DTAB, dodecyltrimethylammonium bromide, C_{12}) upon the conformation and function of StI and StII was also studied (Lanio et al., 2007). StI and StII fluorescence intensity increased upon addition of the cationic surfactants, shifting their maximum emission toward longer wavelengths. The increase of fluorescence intensity occurred in a cooperative manner at concentrations below the surfactant CMC and was more pronounced for StI than for StII. These differences increased with the alkyl chain length of surfactant, suggesting that hydrophobic interactions are more important for StI than for StII, a result compatible with the more hydrophobic character of the former toxin (Martinez et al., 2001). Lanio et al. (2007) suggested that the high cooperativity of the changes in the fluorescence intensity could mainly result from toxin–micelle hydrophobic interaction and not from a non-cooperative surfactant monomer–toxin interaction. The interaction with the cationic detergents provoked a red shift of protein emission λ_{max} , in contrast to the increase in intrinsic fluorescence, results that were explained in terms of a decreased intramolecular protein quenching associated

to changes in conformation (Lanio et al., 2007), as explained for HPS. StI and StII–cationic micelle interaction takes place without significant loss of the toxin native structure as assessed by UV CD spectroscopy that could correlate with the retention and/or increase of their pore-forming ability. The HA was strongly conditioned by the cationic detergent chain length. CTAB and TTAB increased StI and StII HA while DTAB did not show any apparent effect. The association of StI and StII with CTAB and TTAB micelles could induce changes in the toxins that, keeping their native structure, favor the acquisition of a more lytic competent structure. The invariant HA with DTAB could be explained by a reversible binding of sticholysins to this less hydrophobic surfactant when compared to CTAB and TTAB (Lanio et al., 2007).

Table 2 shows a summary of our results, regarding the interaction of StI and StII with surfactants of different nature. The high isoelectric points of StI and StII ($pI > 9.0$, Lanio et al., 2001) could favor, at the working pH, an electrostatic interaction with anionic surfactants, explaining the non-cooperative effects observed for SDS at low concentrations (Lanio et al., 2003). On the other hand, the association with cationic surfactants is highly cooperative and evoked only at concentrations near the surfactant CMC, a result compatible with a predominantly hydrophobic interaction.

5. Membrane lipid composition modulates the functional activity of sticholysins

Sticholysins are effective pore formers on model lipid membranes (Tejuca et al., 1996; Alvarez-Valcarcel et al., 2001), thus it follows that a protein receptor is not strictly required for their action. There are different interpretations of the role of lipid composition in the interaction of actinoporins with membranes. Some authors suggest that each consecutive step in the complex pore-forming process could be influenced by a single physico-chemical characteristic of the lipid molecule, a combination of several of their properties, or by physical parameters arising from the collective nature of lipids in membranes (Anderluh and Maček, 2002). Hence, fatty acyl-chain ordering parameter and membrane dielectric constant and thickness (Maček et al., 1997), gel-to-crystalline phase transition of lipid (Poklar et al., 1999), structural defects related to occurrence of lipid microdomains (De los Ríos et al., 1998; Barlic et al., 2004), and membrane curvature (Maček et al., 1994; Alvarez-Valcarcel et al., 2001) have all been suggested to modify toxin membrane binding, insertion and pore formation.

Next we will discuss how the main lipid components of mammal membranes, such as PC, SM, cholesterol (Cho), and other minor components modulate the interaction of sticholysins with model lipidic membranes.

5.1. Role of sphingomyelin

Vesicles comprised of an equimolar PC:SM mixture have been considered to be good targets for the permeabilizing activity of sticholysins (Tejuca et al., 1996; Alvarez-Valcarcel et al., 2001). The essential role of SM in the interaction of Sts with membranes has been discussed by several authors. In

their pioneer work, Bernheimer and Avigad (1976) purified the *S. helianthus* toxin reported by Devlin (1974) and demonstrated that the HA was specifically inhibited by sphingomyelin proposing that this phospholipid could function as its receptor in the erythrocyte membrane. Shin et al. (1979) in a systematic study of the effect of membrane lipid composition on toxin action found that SM was not essential for destabilizing liposomes as did De los Ríos et al. (1998). However, SM strongly promotes irreversible binding and pore formation in model membranes (Alvarez-Valcarcel et al., 2001; Martinez et al., 2007). It appears that actinoporins recognize SM both at the level of the head group, as indicated by the fact that SM phosphono-analogs are resistant to the permeabilizing activity of these toxins (Meinardi et al., 1995), and by the ceramide moiety, since the ganglioside GM1 with the same ceramide moiety as SM can mimic its action (Maček et al., 1994). The recent finding of a POC binding-site in the three-dimensional structure of StII by Mancheño et al. (2003) supports the role of the SM-head group in toxin binding to membranes. Nevertheless, considering the existence of a POC binding-site in actinoporins, a question that still remains open concerns to the nature of the structural elements that permit actinoporins to discriminate SM from PC.

Recently, Martinez et al. (2006) have demonstrated by surface pressure experiments, fluorescence assays and permeabilization studies that SM in model lipidic systems enhances sticholysin binding and pore-forming ability. In this regard, the presence of SM increased the critical pressure values (π_c) and intrinsic fluorescence (F_L/F_0) of StII as indicators of an enhanced lipid binding. Accordingly, C_{50} and t_{50} , the toxin concentration and time required to permeabilize half of the vesicles in the assay, respectively, indicated that the process is strongly favored in membranes containing SM (Table 3).

5.2. Role of cholesterol

The presence of Cho in membranes exclusively formed by PC leads to pore formation, even under circumstances where little toxin is associated with the lipids (De los Ríos

Table 3

Effect of lipid composition on StII–membrane interaction.

Lipid composition	π_c^a (mN·m ⁻¹)	F_L/F_0^b	C_{50}^c (nM)	t_{50}^d (s)
PC:SM (50:50)	44.9	1.24 ± 0.07	48	60
PC:Cho (70:30)	31.8	1.07 ± 0.01	82	240
SM:PC:Cho (50:15:35)	51.8	1.57 ± 0.09	25	20
PC:PE (70:30)	32.2	1.00	>1160	∞
SM:PC:PE (50:35:15)	43.6	1.25 ± 0.03	50	50
SM:Cho:PE (50:35:15)	51.9	1.53 ± 0.03	25	20

PC: phosphatidylcholine, SM: sphingomyelin, PE: phosphatidylethanolamine, and Cho: cholesterol.

Taken from Martinez et al. (2006), with permission.

^a Critical pressures determined by extrapolating regression lines from $\Delta\pi$ vs. π_0 plots. 35 mN m⁻¹ is the lateral pressure of a typical biological membrane (Brockman, 1999).

^b Ratio between fluorescence intensities of the bound (F_L) and free (F_0) protein.

^c Toxin concentrations required to permeate half of the vesicles in the assay (100 μ M lipid).

^d Time required to permeabilize 50% of the initial liposome ensemble.

et al., 1998 and Table 3). However, the joint presence of SM and Cho in membrane significantly favors binding and permeabilizing activity of StII (Table 3).

Mixtures of sphingomyelin, phosphatidylcholine, and cholesterol are characteristic of the so-called microdomains in which the concentration of membrane components (lipids and proteins) and their physicochemical properties are different from the surrounding environment. The existence and importance of lipid microdomains in cell and model membranes and their implication in many crucial biological processes has been extensively reviewed (Brown and London, 2000; Lommerse et al., 2004). SM and Cho are two of the main components of lipid rafts which are thought to form membrane microdomains in a liquid-ordered-like phase surrounded by lipids in a liquid-disordered-like phase (Simons and Vaz, 2004). The study of actinoporins using lipidic systems in order to model lipid rafts has demonstrated that binding and pore formation by the toxins in these membranes are specially favored (Barlic et al., 2004; Alegre-Cebollada et al., 2006; Martinez et al., 2007).

In fact, Barlic et al. (2004) showed that the coexistence of gel and liquid-crystal lipid phases in SM/PC mixtures as well as the coexistence of liquid-ordered and liquid-disordered lipid phases in PC/Cho or SM/PC/Cho mixtures favor membrane insertion and pore-forming activity of EqTII. It seems that lipid packing defects arising at the interfaces between coexisting lipid phases may function as preferential binding-sites for the toxin. They argued that association with the interfaces between domains is an efficient concentration strategy because it confines the toxin to a linear space where oligomerization and pore formation can take place at very low protein bulk concentrations.

In agreement with the previously mentioned results, Alegre-Cebollada et al. (2006) demonstrated the involvement of rafts in the StII permeabilization mechanism and suggested that binding is based on the recognition of the particular physical state represented by these microdomains in natural cell membranes. Depletion of cholesterol from the COS-7 cell plasma membrane using methyl β -cyclodextrin led to their protection against toxin permeabilization. Isolation of detergent-resistant cellular membranes showed that StII colocalizes with caveolin-1, one of the most common markers of cellular rafts, in fractions corresponding to raft-like domains. The interaction of StII with such domains is irreversible and only lipid dependent, as it also occurs in the absence of any other membrane-associated protein. The results suggest that sticholysin II promotes pore formation in COS-7 cells through interaction with membrane domains which behave like cellular rafts. The results suggest that actinoporin pore formation on COS-7 cells is mediated by binding to domains enriched in sphingomyelin and cholesterol known as cellular rafts.

Basically, the investigation by Martinez et al. (2007) shows that SM and large amounts of Cho lead to the most favorable interaction with liposomes, and the fastest rate of pore formation, in spite of the rigidity of the layers. The phase state of the membrane seems not to be the major determinant factor in the interaction of StII with the lipids, although, the ordered-liquid phase characteristic of Cho-

rich mixtures appears to favor both the interaction and function of the toxin. Particularly, it was suggested that there are two different bound states depending on membrane lipid composition; the first of them occurs in membranes containing SM and is characterized by a large population of toxin irreversibly bound to the membrane with high affinity. The second one appears in membranes lacking SM but containing cholesterol where toxin binding is relatively low and reversible. Finally, permeabilization rate is mostly dependent on the population of bound toxin where only a small fraction of the bound toxin molecules are involved in pore formation.

5.3. Role of other membrane phospholipids

In addition to the main cell membrane lipid components, the influence of other phospholipids in the functional activity of StI and StII has been thoroughly examined. Since both toxins are highly basic proteins (Lanio et al., 2001), Alvarez-Valcarcel et al. (2001) assessed the influence of introducing negatively charged phospholipids in membrane composition on the activity of sticholysins. The inclusion of even small proportions of phosphatidic acid (PA) into PC:SM vesicles increased pore formation by the toxins. Inclusion of other negatively charged lipids: phosphatidyl serine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), or cardiolipin (CL) also elicited an increase of the toxin activity, the potency being in the order $CL \approx PA \gg PG \approx PI \approx PS$. However, some boosting effects were obtained by including the zwitterionic lipid phosphatidylethanolamine (PE) or even, albeit to a lesser extent, the positively charged lipid stearylamine. This indicated that the effect was not mediated by electrostatic interactions between the cytolysin and the negative surface of the vesicles. On the other hand, the toxins strongly promoted the rate of transbilayer movement of lipid molecules indicating local disruption of the lamellar structure. The authors suggested that the insertion of the toxin channel could induce the formation in the bilayer of a toroidal lipid pore. Therefore, it is possible that the presence of minor amounts of lipids favoring this non-lamellar organization could also augment the efficiency of pore formation. Other indirect experimental evidence supporting the hypothesis of the toroidal pore was derived from electron paramagnetic resonance spectra of intercalated fatty acid spin probes carrying the nitroxide moiety at different carbons. Upon addition of the toxins, a component ascribed to boundary lipid was clearly detectable for the C-12-labeled probe but it was absent when the label was at C-16, indicating a lack of lipid-protein interaction close to the lipid terminal methyl group probably associated to a toroidal pore (Alvarez et al., 2003).

PE is a typical non-bilayer forming lipid, characterized by the induction of hexagonal phase when mixed with PC. The inclusion of PE in vesicles containing both PC and SM, did not substantially modify the insertion of StII, but increased the rate of pore formation (Table 3) probably associated to the reorganization of lipids in the membrane induced by the toxin, as has been previously discussed (Alvarez-Valcarcel et al., 2001). Similarly, EqTII induced a reorganization of the lipid bilayer even in the absence of

lipids with a known propensity to form non-bilayer phase (Anderluh et al., 2003). Furthermore, Malovrh et al. (2003) postulated that EqtII insertion could be associated with the redistribution of lipid molecules around the protein and might result in the formation of a lipidic pore whose walls might be lined with the hydrophilic face of its amphipathic N-terminal α helix and the polar head groups of the phospholipids, in a toroidal arrangement.

6. Integration of biochemistry, electron microscopy and X-ray diffraction results: a putative model of the StII pore

The amphipathic α helix located at the N-terminus of sticholysins (Huerta et al., 2001; Mancheño et al., 2003) is a conserved element of all actinoporins (Anderluh and Maček, 2002) forming a functional pore of approximately 2 nm diameter (Tejuca et al., 2001) which is composed of 3–4 monomers (Tejuca et al., 1996; Mancheño et al., 2003). The inner surface of the pore is probably formed by the combination of the hydrophilic faces of amphiphilic helices of the toxin and the polar head of the membrane lipids. The

toroidal pore model implies that each pore behaves as a point of fusion of the inner lipid monolayer with the outer one, so that the amphiphilic helices get stacked between the lipid polar heads. Fig. 4 shows a cartoon of the toroidal pore generated by the sticholysins. Lipids inducing positive curvature (conical shape with head group at the base) are favored in the section of the lipid torus perpendicular to the plane of the membrane while lipids inducing negative curvature (conical shape with head group at the tip) are favored in the central section parallel to the plane of the membrane.

A high-resolution model of the StII tetramer has been obtained by docking the atomic model of the soluble StII into the 3D envelope obtained by electron microscopy (EM) (Mancheño et al., 2003) allowing integration of the EM and X-ray diffraction results into the putative model of the StII pore. The initial docking revealed that only two regions the soluble StII atomic model, the N-terminal region (Ala 1 to Val 27) and the connecting loop $\beta 7$ – $\alpha 2$, did not fit into the EM envelope, but protruded from it. Furthermore, the model suggested that a pseudorigid body movement of the N-terminal region about the loop $\alpha 1$ – $\beta 2$ would be sufficient

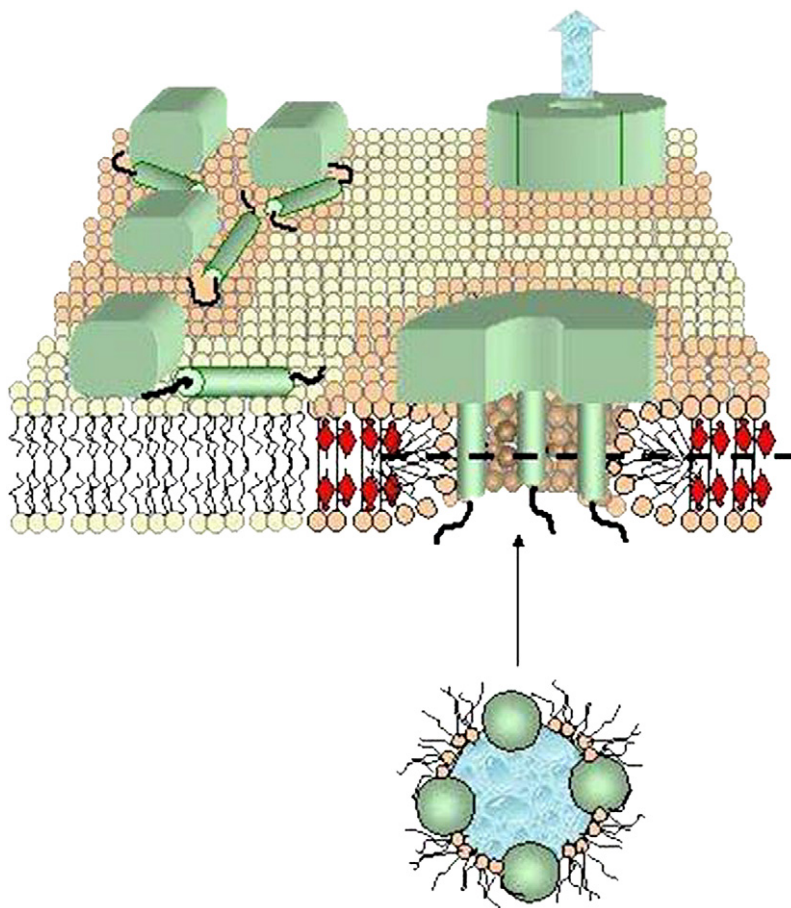


Fig. 4. Cartoon of the toroidal pore generated by sticholysins. The amphiphilic helix of the toxin is represented by a cylinder. Spheres represent phospholipids polar heads, and the lines the acyl chains. Cholesterol molecules are represented in red. The toxins are preferentially associated to SM and Cho enriched microdomains, oligomerizing and eventually forming the tetrameric pore. During this process, the lipid membrane is reorganized originating the toroidal lipid pore, where the non-bilayer lipids (as PE) are preferentially distributed between amphiphilic helices, forming the walls of the pore (lower view). Helices are arranged with hydrophilic faces toward the pore lumen and the hydrophobic faces in contact with acyl lipid chains.

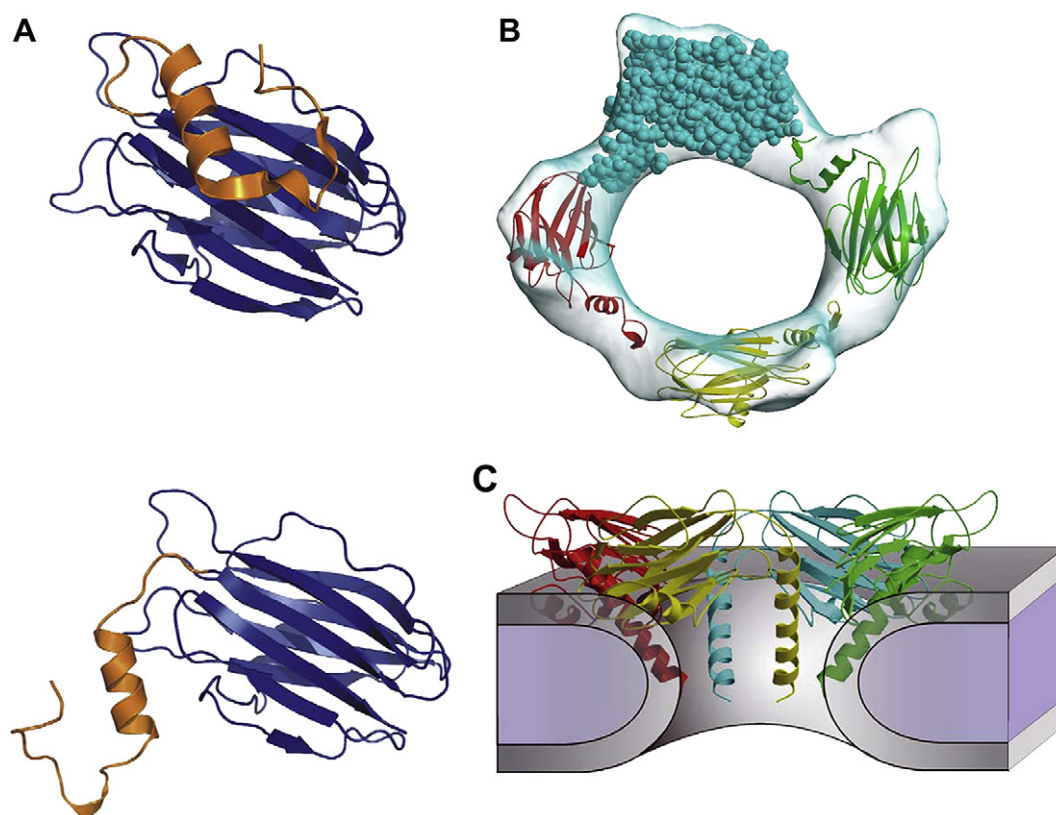


Fig. 5. Conformational changes of StII underlying formation of the tetrameric assembly in a lipid film and putative model for the functional pore. **A.** Pseudorigid body movement of the N-terminal region of StII explaining the electron microscopy envelope obtained with 2D crystals (Mancheño et al., 2003). **B.** Model for the tetrameric assembly of StII. A protomer is shown as cpk model to highlight the excellent fit of the atomic model into the EM envelope (light blue). **C.** Putative model for the functional pore of StII, in which the N-terminal region of the protomers would adopt a helical conformation. Lipid headgroup regions are shown as gray layers. The walls of the pore would be formed both by lipid molecules and four N-terminal helices.

to fill up the EM density in between monomers (Fig. 5A, Mancheño et al., 2003). A plausible hypothesis is that this movement constitutes the structural basis for the translocation of the N-terminal region from the β -sandwich to the membrane interface, as shown for EqtlI (Hong et al., 2002), and therefore, the tetrameric structure would correspond to the oligomerization of M2-states (Malovrh et al., 2003). Considering the above atomic model of the tetrameric complex, a putative model for the functional pore has been proposed in which the N-terminal regions of the four protomers would adopt a helical conformation (Fig. 5B). Alegre-Cebollada et al. (2007a) estimated a 31° tilt angle of the N-terminal helix with respect to the membrane normal, in excellent agreement with the proposed model (Fig. 5C).

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Conflict of interest

The authors declare that there are no conflicts of interest.

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