Biochimica et Biophysica Acta 1828 (2013) 1390–1395

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

2NH and 3OH are crucial structural requirements in sphingomyelin for sticholysin II binding and pore formation in bilayer membranes

Terhi Maula [a], Y. Jenny E. Isaksson [a], Sara García-Linares [b], Sanna Niinivehmas [c], Olli T. Pentikäinen [c], Mayuko Kurita [d], Shou Yamaguchi [d], Tetsuya Yamamoto [d], Shigeo Katsumura [d], José G. Gavilanes [b], Álvaro Martínez-del-Pozo [b], J. Peter Slotte [a,*]

[a] Biochemistry, Department of Biosciences, Åbo Akademi University, Turku, Finland
[b] Departamento de Bioquímica y Biología Molecular I, Universidad Complutense, Madrid, Spain
[c] Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland
[d] School of Science & Technology, Kwansei Gakuin University, 2-1 Gakuen, Sanda City, Hyogo 669-1337, Japan

ARTICLE INFO

Article history:
Received 27 November 2012
Received in revised form 9 January 2013
Accepted 22 January 2013
Available online 30 January 2013

Keywords:
Molecular docking
Membrane permeabilization
Isothermal titration calorimetry
Surface plasmon resonance

ABSTRACT

Sticholysin II (StnII) is a pore-forming toxin from the sea anemone *Stichodactyla heliantus* which belongs to the large actinoporin family. The toxin binds to sphingomyelin (SM) containing membranes, and shows high binding specificity for this lipid. In this study, we have examined the role of the hydrogen bonding groups of the SM long-chain base (i.e., the 2NH and the 3OH) for StnII recognition. We prepared methylated SM-analogs which had reduced hydrogen bonding capability from 2NH and 3OH. Both surface plasmon resonance experiments, and isothermal titration calorimetry measurements indicated that StnII failed to bind to bilayers containing methylated SM-analogs, whereas clear binding was seen to SM-containing bilayers. StnII also failed to induce calcein release (i.e., pore formation) from vesicles made to contain methylated SM-analogs, but readily induced calcein release from SM-containing vesicles. Molecular modeling of SM docked to the phosphocholine binding site of StnII indicated that the 2NH and 3OH groups were likely to form a hydrogen bond with Tyr135. In addition, it appeared that Tyr111 and Tyr136 could donate hydrogen bonds to phosphate oxygen, thus stabilizing SM binding to the toxin. We conclude that the interfacial hydrogen bonding properties of SM, in addition to the phosphocholine head group, are crucial for high-affinity SM/StnII-interaction.

1. Introduction

Sticholysin II (StnII) is a pore-forming single-peptide toxin isolated from the sea anemone *Stichodactyla heliantus* [1]. Structurally, it is highly related to other single peptide toxins in the actinoporin family, including equinatoxin II (EqtII) [1,2]. The StnII structure is based on a β-sandwich fold composed of 10 β-strands, with two α-helices interacting with both sides of the β-sandwich [3]. The N-terminal helix is amphiphilic and has been proposed to extend and be inserted into the bilayer, to form the pore walls [4,5]. Incorporation of the toxin into the bilayer depends largely on the composition and physicochemical state of the membrane [6–8]. A common and functionally important feature of actinoporins is their high specificity for sphingomyelin (SM) [9,10], but actinoporin/membrane association is also influenced by the physical properties of the bilayer membranes [7,11–13].

StnII binds to SM-containing phospholipid-membranes with high affinity ($K = 1.7 \times 10^8\ M^{-1}$) [11]. Mutation of a tyrosine residue (Y111N) in the phosphocholine (POC) binding site on StnII was shown by isothermal titration calorimetry (ITC) to reduce the bilayer binding constant of StnII by two orders of magnitude [11]. Since this residue was located in the POC-binding site, the results suggested that binding to SM was crucial for the high membrane affinity of StnII. However, the exact nature of SM/StnII interaction is not known, since no 3D structure of StnII is available with bound SM.

The specificity of actinoporins for SM has been convincingly demonstrated [10]. However, it appears that under specific conditions, some actinoporins may show low-affinity interaction with phosphatidylcholines and cause membrane permeabilization, but only in the presence of phase coexistence, most probably mediated by cholesterol [7,13,14]. In a dot-blot binding assay, phosphatidylcholine is not recognized by equinatoxin II, neither is ceramide nor sphingosylphosphorylcholine [10]. These observations together suggest that both the phosphocholine head group and the hydrogen bonding interfacial groups in SM (the 2NH and the 3OH) are important for toxin binding. Sphingomyelinase, which is specific for SM and does not degrade phosphatidylcholine,

Abbreviations: EqtII, equinatoxin II; ITC, isothermal titration calorimetry; LUV, large unilamellar vesicle; pbSM, procine brain sphingomyelin; POC, phosphocholine (binding site); POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PSM, palmitoyl SM; SM, sphingomyelin; pbSM, porcine brain SM; sSM, truncated SM analog used for docking optimization; SPR, surface plasmon resonance; StnII, sticholysin II

* Corresponding author. Tel.: +358 22154689.
E-mail address: jpslotte@abo.fi (J.P. Slotte).

0005-2736/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamem.2013.01.018

also uses the interfacial hydrogen bond donating functional groups (in addition to the phosphocholine head group) of SM to stabilize binding to the active site [15,16].

In this study, we examine the importance of the 2NH and 3OH in SM for pore formation by StnII. By chemical synthesis, we have prepared SM analogs in which either the 2NH or the 3OH is methylated [17], to yield 2NMeSM and 3OMeSM, respectively. We have prepared 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayers containing either palmitoyl SM (PSM), or one of the methylated PSM analogs (POPC:SM molar ratio 4:1). With such bilayers, we found that StnII caused extensive calcein leakage only in PSM-containing bilayers. With surface plasmon resonance (SPR) analysis, only PSM-containing vesicles caused StnII binding. Very similar results were shown using isothermal titration calorimetry (ITC) to study toxin binding to bilayer membranes. StnII was found to interact only with PSM-containing vesicles. Finally, using molecular docking of PSM to a 3D model of StnII, we suggest that Y135 in the POC-binding site stabilized SM binding by interactions with the 2NH and 3OH of PSM, whereas Y111 and Y136 formed hydrogen bonds to phosphate oxygen, possibly explaining the binding specificity for SM.

2. Materials and methods

2.1. Material

POPC, porcine brain SM (pbSM), and egg SM were obtained from Avanti Polar Lipids (Alabaster, AL, USA). PSM was purified from egg SM using preparative HPLC on a reverse phase (C18) column, as described previously [18]. The acyl chains of pbSM are mostly saturated (50% 18:0 and19% other saturated) while 24:1 is present at 21% (Avanti Polar Lipids). Methylated analogs of PSM (i.e., 2NMeSM and 3OMeSM) were prepared by chemical synthesis, as described previously [17]. Calcein and Sephacryl S200HR were obtained from Sigma/Aldrich (St. Louis, MO, USA). StnII was produced in an *E.coli* expression system, and purified, as described previously [19].

2.2. Calcein leakage assay

Calcein-entrapped large unilamellar vesicles (LUVs) were prepared from POPC and a SM-analog (4:1 molar ratio), by extrusion through 200 nm filters at 60 °C [20]. Briefly, the desired lipids were mixed and dried under a stream of nitrogen. The lipids were redissolved in chloroform and dried again before removal of any traces of remaining solvent in vacuum for 60 min. Prior to extrusion, the dry lipid films were hydrated for 30 min at 60 °C in Tris buffer containing calcein. The calcein concentration was 100 mM (10 mM Tris, 140 mM NaCl, pH 7.4), and the total lipid concentration was 1.25 mM. LUVs were separated from non-entrapped calcein by gel filtration on Sephacryl S200HR. The LUVs were used for permeabilization studies within 24 h. The concentration of LUV and StnII during calcein leakage experiments was 2.5 μM and 20 nM, respectively. Emission at 550 nm was followed at 23 °C as a function of time (Ex 480 nm). Fluorescence emission was measured on a PTI Quanta-Master spectrofluorimeter (Photon Technology International, Inc. NJ, USA). The released fraction of calcein was determined based on the maximum calcein release which was induced by Triton X-100 induced LUV disintegration. To ensure that no spontaneous leakage occurred, the emission was measured for each sample for 5 min before addition of toxin. A steady signal level, indicating intact vesicles, was observed for all samples.

2.3. Surface plasmon resonance spectroscopy

The association of StnII with vesicle-coated gold chips were performed as follows: LUVs were prepared from POPC or POPC/SM analog (4:1, molar ratio) in Tris buffer (10 mM Tris, 140 mM NaCl, pH 7.4) by extrusion through 100 nm polycarbonate filters at 60 °C. StnII binding to the vesicles was studied at 23 °C with a BioNavis SPR Navi 200 instrument (BioNavis Ltd, Tampere, Finland). The sensor gold chip was coated with a carboxymethylated dextran layer which was treated with N-hydroxysuccinimide and N-ethyl-N′ (dimethylaminopropyl) carbodiimide to activate the surface for capturing phospholipid membranes [21]. All solutions used for SPR were filtered through 0.2 μm membrane filters and degassed by bath sonication before use. The running buffer was 10 mM Tris, 140 mM NaCl, pH 7.4 and the flow rate was 5 μl/min. First, the chip surface was cleaned with two injections of 10 mM CHAPS. Then extruded LUVs (0.5 mM lipid concentration) were applied on the surface (12 min injection) and unbound vesicles were removed by one (3 min) injection of 50 mM NaOH. Bovine serum albumin (0.1 mg/ml, 3 min injection) was used to verify that the chip did not have uncovered areas. Finally StnII (1.0 μM) was applied for 22–24 min after which buffer alone was injected for 10 min to study toxin dissociation. The chip was regenerated with CHAPS as in the beginning of the experiment.

2.4. Isothermal titration calorimetry

The interaction between StnII and LUVs prepared from POPC and SM analogs (4:1 molar ratio, 100 nm diameter) was measured using a VP-ITC (MicroCal, Northampton, MA, USA), as described previously [11].

2.5. Computational studies

The structure of SM was sketched and energy minimized in SYBYL-X2.0 (Tripos, St. Louis, MO, USA) using the conjugate gradient method with MMFF94s charges [22] and a dielectric constant of 10. The termination gradient was set to 0.05 kcal/mol, and calculations were iterated until convergence was reached. PSM was drawn with two different configurations: PSM and a shortened version composed of the phosphocholine part, the long-chain base until C5, and the *N*-linked acyl chain until the Cα (sSM).

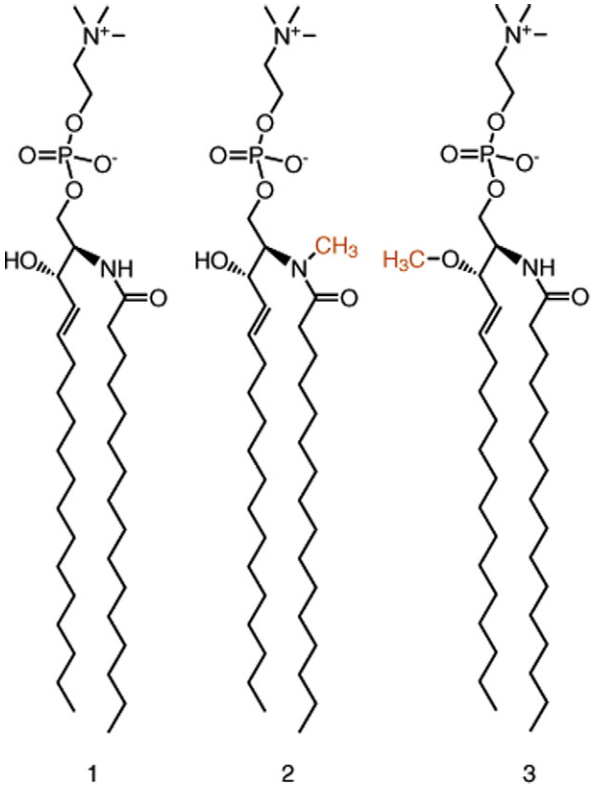
The 3D structure of StnII (PDB: 1O72 [3]) was acquired from the Protein Data Bank [23]. Hydrogen atoms were added into the StnII structure using TLEAP in ANTECHAMBER 1.27 [24]. The docking of PSM to the phosphocholine binding site of StnII was performed flexibly with GOLD 5.0 [25] using ChemScore scoring function, diverse solutions option, and the most accurate (slow) docking. Docking atom selection was not restricted entirely to the solvent-accessible surface. Both PSM and the sSM were used in docking experiments. However, due to the conformational space that the docking should cover for PSM, and since the main focus was on the exploring of the interfacial portion of the SM, the docking of sSM was studied more thoroughly. In addition, the stability of the ligand-protein complex was studied with molecular dynamics (MD) simulations. MD simulations were done as described previously [16]. Multiple starting conformations of PSM (obtained via molecular docking) were used in the MD.

3. Results

3.1. StnII pore formation and membrane binding

The importance of specific functional groups in SM structure for StnII binding to bilayers, and for subsequent pore formation, was examined using chemically synthesized SM analogs in which either the 2NH or the 3OH was methylated (see Scheme 1 for molecular structures). The methylated SM analogs have markedly reduced capability to form hydrogen bonds compared to unmodified SM [17].

StnII binding to SM-rich bilayers is known to lead to pore formation, and to membrane permeabilization for small solutes. We measured calcein leakage from LUVs which were prepared to contain POPC and one of the SM analogs (4:1 molar ratio; Fig. 1). While PSM-containing membranes were permeabilized efficiently by StnII, both



Scheme 1. Molecular structures of the three SM analogs used in the study: (1) palmitoyl SM, (2) 2NMe palmitoyl SM, and (3) 3OMe palmitoyl SM.

of the methylated PSM analogs failed to support StnII pore formation. The leakage from POPC LUVs was also very slow under the conditions used (Fig. 1).

Since calcein leakage only indicates pore formation, but gives no information about StnII binding to vesicles *per se*, we next used SPR to examine StnII binding to gold chips coated with different vesicles containing POPC and PSM or one of the methylated PSM analogs (Fig. 2). The SPR sensogram showed efficient binding of StnII to bilayers containing PSM, but not to bilayers containing methylated PSM, or POPC alone. The off-rate of StnII from PSM-containing bilayers was also negligible, indicating high affinity binding. The small initial deviations in the SPR response after injection of StnII could relate to mismatch in solvents (vesicle loading solvent versus StnII-containing solvent).

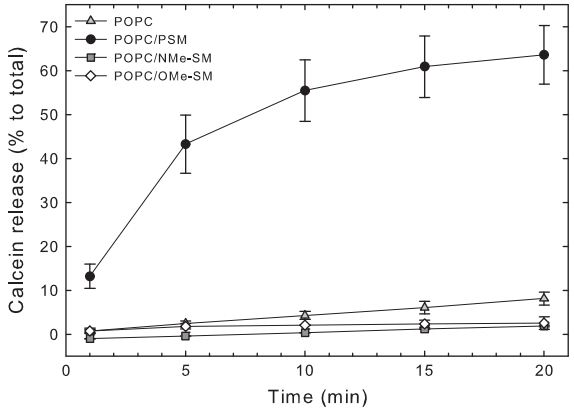


Fig. 1. The StnII-induced release of calcein from LUVs containing POPC and SM analogs was examined at 23 °C. The LUVs contained POPC and SM in a 4:1 molar ratio. StnII was added to the sample cell at time zero, and the RF (%) was calculated at time points indicated. Each value is the average from 3 to 5 separate experiments ± SD.

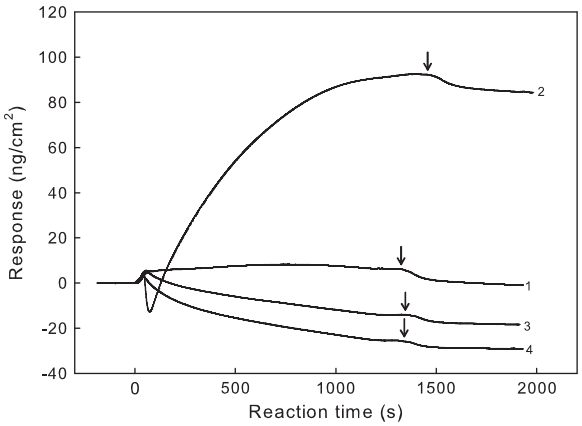


Fig. 2. SPR sensogram showing the binding of StnII to immobilized vesicles of different lipid composition. The vesicles consisted of 1: POPC, 2: POPC/PSM (4:1), 3: POPC/NMe-SM (4:1) or 4: POPC/OMe-SM (4:1). Vesicles (0.5 mM total lipid) were first immobilized on the chip surface for 12 min. Then StnII (1.0 μM) was allowed to bind for 22–24 min (starting at 0 s). The arrows indicate the injection of buffer (10 mM Tris, 140 mM NaCl, pH 7.4) to study toxin dissociation. The flow rate was 5 μl/min and the temperature was 23 °C. The figure shows representatives of at least 3 independent experiments.

In order to study direct toxin/bilayer binding with another method, we measured StnII/vesicle interaction by using ITC, which in addition also gives thermodynamic data for the binding process. The heat released after injection of POPC bilayers to StnII was close to zero (Fig. 3), indicating no substantial binding of StnII to POPC. Inclusion of PSM in the POPC bilayers resulted in clear exothermic signals when StnII was added. The calculated K for StnII binding to POPC/PSM (4:1 molar ratio) was 1.80×10^6 M^{-1} (Table 1). A very similar result ($K=1.44\times10^6$ M^{-1}, Table 1) was obtained when using pbSM containing vesicles. pbSM has a very different composition from PSM in terms of fatty acids composition (see Section 2). With methylated PSM analogs in the POPC vesicles, StnII failed to interact with the bilayers, since the interaction energy was close to zero (Fig. 3).

3.2. Computational studies of sphingomyelin binding to sticholysin II

To deduce the structural details that are important for SM recognition by StnII, and to outline the binding interactions of SM with StnII, SM was docked to the POC binding site of StnII. Overall, the docking pose of the phosphocholine moiety of SM is relatively similar to the pose appeared in the StnII crystal structure complexed with phosphocholine (PDB:1O72 [3]). sSM was used in the initial docking experiments, because interactions of the toxin with the phosphocholine moiety and with both 2NH and 3OH are likely to be important for SM recognition. Additionally, there are no clear grooves on the surface of the toxin for the hydrocarbon chains, and thus, the chains are not supposed to interact with the binding site of StnII, as suggested before [10].

The ligand binding cavity of StnII contains a cluster of aromatic amino acids, and is partly hydrophobic and partly hydrophilic. For example side chains of Val85, Pro105, and Trp114, and aromatic rings of several tyrosine residues (Tyr111, Tyr131, Tyr135, and Tyr136) are hydrophobic, whereas side chains of Ser52, and Ser103, and phenolic hydroxyl groups of the above-mentioned tyrosine residues add hydrophilic properties to the binding site [3]. Docking results suggested that the 2NH and 3OH groups of SM donate a hydrogen bond to the phenolic hydroxyl group of Tyr135 (Fig. 4). Furthermore, Tyr111 and Tyr136 could form hydrogen bonds to the oxygen atoms of the phosphate moiety through the phenolic hydroxyl groups (Fig. 4). Additionally, the phosphate moiety could be further stabilized by the cationic side chain of Arg51. Throughout the MD simulation, the phosphocholine moiety of PSM remains similarly bound into the ligand binding site. However, the long hydrocarbon chains do not find stable conformation but remain flexible as there are no grooves on the surface of the protein

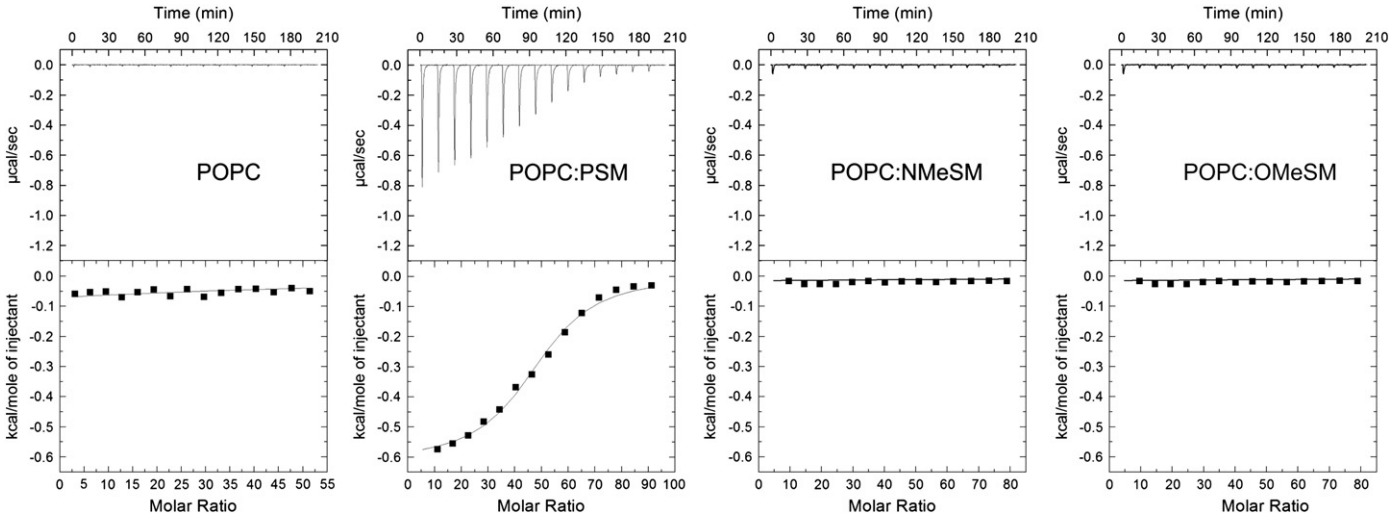


Fig. 3. Binding of StnII to POPC/SM analog vesicles at 25 °C. Protein solutions at 10 μM were titrated by injection of 20 μL aliquots of lipid suspensions (5 mM). Binding isotherms were adjusted to a model were the protein binds the membrane involving *n* lipid molecules [11].

for the hydrocarbon chains. This observation is also in good agreement with the pbSM ITC results which indicate that the fatty acid chain length is not important for binding (Table 1).

4. Discussion

Sphingomyelins are important phospholipids in animal cell membranes [26]. They often have saturated or monounsaturated acyl chains, and are thus ordered lipids [26,27]. Their ordered nature, and their extensive hydrogen bonding properties help to form SM-rich ordered domains in cell membranes [28]. Cholesterol associates with SM-rich domains and helps to keep them in a liquid ordered state [29]. In addition to maintaining membrane structure, and serving as precursors for many bioactive sphingolipids [30], SMs are also used by extracellular proteins as "receptors" to which they may bind [31]. This is also evidenced by the toxins from the actinoporin family, which are known to lyse membranes which contain SM [1,2,8,32,33].

The lipid specificity of equinatoxin II (EqtII) binding has been shown to be very high, since only SM is recognized by the toxin, from a range of other sphingolipids or glycerophospholipids [10]. However, EqtII is known to penetrate into monolayers from both SM and phosphatidylcholine, if the initial surface pressure is below 25–26 mN/m [14]. The toxin was also shown to cause bilayer permeabilization in vesicles made from cholesterol and phosphatidylcholine [7,14]. It appears that while binding of SM to StnII is of high affinity, phosphatidylcholine can bind to StnII but with much less affinity, and somehow cholesterol appears to facilitate this at least in *in vitro* studies.

All the results in this study clearly show that StnII failed to interact with or cause pore formation with bilayers which contained SM analogs in which the 2NH and 3OH functional properties were compromised. It therefore appears that these two functional groups are important for giving specificity and high affinity for StnII/membrane interaction and for pore formation. Methylation of the 2NH of SM was recently shown to reduce its gel-phase stability [17], suggesting that hydrogen-bonding involving the 2NH are important for SM–SM interlipid interactions. The 3OMe analog also showed slightly decreased gel-phase stability, but much less than for 2NMe. Sterol affinity for bilayers containing the methylated SM analogs was markedly lowered compared to PSM.

The 2NH and 3OH in SM have in many studies been shown to be of critical importance for the biophysical membrane properties of SM [17,34], and for the specificity of sphingomyelinase for SM [15,16]. The amino acid residues in the phospholipid binding site of StnII (or EqtII), which are most likely to give specificity for SM, are Tyr 111 (Tyr 113 in EqtII) and Trp 110 (Trp 112 in EqtII). Replacing Trp112 in EqtII with more polar residues led to loss of pore formation in DOPC:SM bilayers (as deduced from membrane permeabilization) [10,35]. Conversion of Tyr113 in EqtII to Ala113 also led to loss of pore formation in DOPC:SM bilayers, but Tyr113 could be converted

Table 1
Isothermal titration calorimetric analysis of the binding of StnII to POPC:PSM or POPC:pbSM (4:1) vesicles at 25 °C.

Vesicles	*n*	$K \times 10^{-6}$ (M^{-1})	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (cal mol^{-1} K^{-1})
POPC:PSM	48 ± 1	1.80 ± 0.32	−6.4 ± 0.8	−29.3 ± 1.5	−77 ± 7
POPC:SM	45 ± 1	1.44 ± 0.22	−6.3 ± 0.5	−25.4 ± 1.1	−64 ± 6

Values are the mean of at least two independent determinations (±SEM). Binding isotherms were adjusted to a model were the protein binds the membrane involving *n* lipid molecules [11].

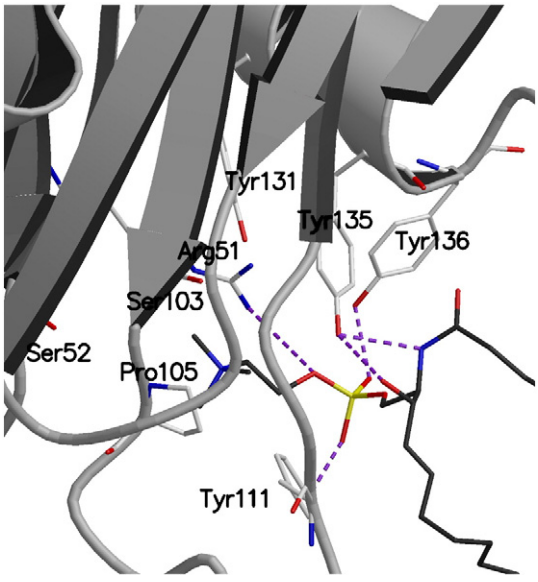


Fig. 4. Molecular docking of SM to the POC binding site of StnII. The protein structure (PDB: 1O72 [3]) is shown with secondary structure in gray whereas SM and key amino acids are shown in stick models. Hydrogen bonds are indicated with purple dashed lines. The 2NH and 3OH groups form a hydrogen bond with Tyr135. Additionally, Tyr111 and Tyr136 donate hydrogen bonds to the phosphate group. The figure was prepared using BODIL [36], MOLSCRIPT v 2.1.2 [37], and the RASTER3D package [38].

to Phe113 without significant loss of membrane association or pore formation [10]. In StnII, mutation of Tyr111 to Asn111 significantly reduced hemolytic activity of the toxin. This mutation also led to a 2 orders of magnitude less membrane affinity, compared to the wild type StnII [11].

Our SM docking model suggests that the phosphate oxygens interact with Tyr111 and Tyr136, while the 2NH and 3OH engage in electrostatic interactions with Tyr135. Additionally, the phosphate moiety could be further stabilized by the cationic side chain of Arg51. Methylation of 2NH and 3OH would prevent such interactions. Also MD simulations support the stable positioning of the phosphocholine head group of the SM into the ligand binding site.

It remains unclear what contribution, if any, the steric size (and hydrophobicity) of the methyl group would impose on SM/POC binding site interactions. However, in the case where these methylated SM analogs were allowed to interact with the binding site of sphingomyelinase, it was reasoned that attenuated hydrogen bonding was a more important factor than the steric size of the methyl, to eliminate their substrate capability [15,16]. This model does not contradict results from mutation studies involving Tyr111 (or Trp112 and Tyr113 in EqtII). It suggests, however, that mutation studies in which Tyr135 and Tyr136 would be changed, would be worthwhile to undertake, in order to better clarify the role of these residues for stabilizing SM/toxin interaction in the POC site.

The results of this study do not directly address questions related to the role of 2NH or 3OH in the SM structure for (i) membrane binding of StnII, and (ii) for the subsequent oligomerization and pore formation. However, the lack of apparent membrane binding of the methylated SM analogs (SPR and ITC data, Figs. 2 and 3), and the failure to cause significant membrane permeabilization (Fig. 1), suggest that the methylated SM analogs failed to support stable membrane association of StnII. This would confirm that high affinity interaction between StnII and SM is required for stable membrane association of StnII. The slow kinetics (relatively speaking) of both membrane permeabilization (calcein leakage) and membrane association (SPR sensogram) suggest that pore formation (i.e., oligomerization) is rate limiting relative to membrane association.

This study is the first to have used SM analogs with compromised hydrogen bonding properties, in the characterization of SM/StnII interaction. With the help of mutational studies with actinoporin toxins, the amino acid residues important for SM recognition have been identified. Our results with the methylated SM analogs complement the current understanding of SM/toxin interaction, and provide further evidence for the mechanisms involved in the high affinity binding of StnII to SMs.

Acknowledgments

The Turku group thanks Sofie Kvarnström and Josefin Halin for their help with some of the experiments. The work was funded by generous grants from the Sigrid Juselius Foundation (JPS), the Åbo Akademi Foundation (JPS), National Doctoral Programme in Nanoscience (SN), Grant-in-Aid for Science Research on Priority Areas 16073222 from the Ministry of Education, Culture, Sports, Science and Technology (SK) and also the Matching Fund Subsidy for a Private University, Japan (SK), and BFU2009-10185 from the Spanish Ministerio de Ciencia e Innovación (JGG and AMP).

References

[1] 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 1808 (2011) 2275–2288.
[2] G. Anderluh, P. Macek, Dissecting the actinoporin pore-forming mechanism, Structure 11 (2003) 1312–1313.
[3] J.M. Mancheno, J. Martin-Benito, M. Martinez-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 (2003) 1319–1328.
[4] A. Athanasiadis, G. Anderluh, P. Macek, D. Turk, Crystal structure of the soluble form of equinatoxin II, a pore-forming toxin from the sea anemone *Actinia equina*, Structure 9 (2001) 341–346.
[5] K. Kristan, Z. Podlesek, V. Hojnik, I. Gutierrez-Aguirre, G. Guncar, D. Turk, J.M. Gonzalez-Manas, J.H. Lakey, P. Macek, G. Anderluh, Pore formation by equinatoxin, a eukaryotic pore-forming toxin, requires a flexible N-terminal region and a stable beta-sandwich, J. Biol. Chem. 279 (2004) 46509–46517.
[6] C.A. Valcarcel, S.M. Dalla, C. Potrich, I. Bernhart, M. Tejuca, D. Martinez, F. Pazos, M.E. Lanio, 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 (2001) 2761–2774.
[7] V. de los Rios, J.M. Mancheno, M.E. Lanio, M. Onaderra, 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 (1998) 284–289.
[8] D. Martinez, A. Otero, C. Alvarez, F. Pazos, M. Tejuca, M.E. Lanio, I. Gutierrez-Aguirre, A. Barlic, I. Iloro, J.L. Arrondo, J.M. Gonzalez-Manas, E. Lissi, Effect of sphingomyelin and cholesterol on the interaction of St II with lipidic interfaces, Toxicon 49 (2007) 68–81.
[9] M. Tejuca, M.D. 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 (1996) 14947–14957.
[10] B. Bakrac, I. Gutierrez-Aguirre, Z. Podlesek, A.F. Sonnen, R.J. Gilbert, P. Macek, J.H. Lakey, G. Anderluh, Molecular determinants of sphingomyelin specificity of a eukaryotic pore-forming toxin, J. Biol. Chem. 283 (2008) 18665–18677.
[11] J. Alegre-Cebollada, M. Cunietti, E. Herrero-Galan, J.G. Gavilanes, A. Martinez-Del-Pozo, Calorimetric scrutiny of lipid binding by sticholysin II toxin mutants, J. Mol. Biol. 382 (2008) 920–930.
[12] B. Bakrac, G. Anderluh, Molecular mechanism of sphingomyelin-specific membrane binding and pore formation by actinoporins, Adv. Exp. Med. Biol. 677 (2010) 106–115.
[13] P. Schon, A.J. Garcia-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 (2008) 691–698.
[14] J.M. Caaveiro, I. Echabe, I. Gutierrez-Aguirre, J.L. Nieva, J.L. Arrondo, J.M. Gonzalez-Manas, Differential interaction of equinatoxin II with model membranes in response to lipid composition, Biophys. J. 80 (2001) 1343–1353.
[15] M.D. Lister, Z.S. Ruan, R. Bittman, Interaction of sphingomyelinase with sphingomyelin analogs modified at the C-1 and C-3 positions of the sphingosine backbone, Biochim. Biophys. Acta 1256 (1995) 25–30.
[16] C. Sergelius, S. Niinivehmas, T. Maula, M. Kurita, S. Yamaguchi, T. Yamamoto, S. Katsumura, O.T. Pentikainen, J.P. Slotte, Structure-activity relationship of sphingomyelin analogs with sphingomyelinase from *Bacillus cereus*, Biochim. Biophys. Acta 1818 (2012) 474–480.
[17] A. Bjorkbom, T. Rog, P. Kankaanpaa, D. Lindroos, K. Kaszuba, M. Kurita, S. Yamaguchi, T. Yamamoto, S. Jaikishan, L. Paavolainen, J. Paivarinne, T.K. Nyholm, S. Katsumura, I. Vattulainen, J.P. Slotte, N- and O-methylation of sphingomyelin markedly affects its membrane properties and interactions with cholesterol, Biochim. Biophys. Acta 1808 (2011) 1179–1186.
[18] S. Jaikishan, A. Bjorkbom, 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 1798 (2010) 1987–1994.
[19] J. Alegre-Cebollada, G. Clementi, M. Cunietti, C. Porres, M. Onaderra, J.G. Gavilanes, A.M. Pozo, Silent mutations at the 5′-end of the cDNA of actinoporins from the sea anemone *Stichodactyla helianthus* allow their heterologous overproduction in *Escherichia coli*, J. Biotechnol. 127 (2007) 211–221.
[20] T.K. Nyholm, P.M. Grandell, B. Westerlund, J.P. Slotte, Sterol affinity for bilayer membranes is affected by their ceramide content and the ceramide chain length, Biochim. Biophys. Acta 1798 (2010) 1008–1013.
[21] S. Löfås, B. Johnsson, A novel hydrogel matrix on gold surfaces in surface plasmon resonance sensors for fast and efficient covalent immobilization of ligands, J. Chem. Soc., Chem. Commun. (1990) 1524–1526.
[22] T. Halgren, Merck molecular force field. I. Basis, form, scope, parameterization, and performance of MMFF94, J. Comput. Chem. 17 (1996) 490–519.
[23] H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, P.E. Bourne, The Protein Data Bank, Nucleic Acids Res. 28 (2000) 235–242.
[24] J. Wang, W. Wang, P.A. Kollman, D.A. Case, Automatic atom type and bond type perception in molecular mechanical calculations, J. Mol. Graph. Model. 25 (2006) 247–260.
[25] G. Jones, P. Willett, R.C. Glen, Molecular recognition of receptor sites using a genetic algorithm with a description of desolvation, J. Mol. Biol. 245 (1995) 43–53.
[26] Y. Barenholz, Sphingomyelin–lecithin balance in membranes: composition, structure, and function relationships, in: M. Shinitzky (Ed.), Physiology of Membrane Fluidity, vol. 1, CRC Press, Boca Raton, 1984, pp. 131–174.
[27] Y. Barenholz, T.E. Thompson, Sphingomyelins in bilayers and biological membranes, Biochim. Biophys. Acta 604 (1980) 129–158.
[28] K. Simons, W.L. Vaz, Model systems, lipid rafts, and cell membranes, Annu. Rev. Biophys. Biomol. Struct. 33 (2004) 269–295.
[29] J.P. Slotte, Sphingomyelin–cholesterol interactions in biological and model membranes, Chem. Phys. Lipids 102 (1999) 13–27.
[30] Y.A. Hannun, The sphingomyelin cycle and the second messenger function of ceramide, J. Biol. Chem. 269 (1994) 3125–3128.
[31] V.R. Gupta, H.K. Patel, S.S. Kostolansky, R.A. Ballivian, J. Eichberg, S.R. Blanke, Sphingomyelin functions as a novel receptor for *Helicobacter pylori* VacA, PLoS Pathog. 4 (2008) e1000073.

[32] V. de los Rios, J.M. Mancheno, P.A. Martinez del, C. Alfonso, G. Rivas, M. Onaderra, J.G. Gavilanes, Sticholysin II, a cytolysin from the sea anemone *Stichodactyla helianthus*, is a monomer–tetramer associating protein, FEBS Lett. 455 (1999) 27–30.
[33] P.S. Garcia, G. Chieppa, A. Desideri, S. Cannata, E. Romano, P. Luly, S. Rufini, Sticholysin II: a pore-forming toxin as a probe to recognize sphingomyelin in artificial and cellular membranes, Toxicon 60 (2012) 724–733.
[34] R. Bittman, C.R. Kasireddy, P. Mattjus, J.P. Slotte, Interaction of cholesterol with sphingomyelin in monolayers and vesicles, Biochemistry 33 (1994) 11776–11781.
[35] Q. Hong, I. Gutierrez-Aguirre, A. Barlic, P. Malovrh, K. Kristan, Z. Podlesek, P. Macek, D. Turk, J.M. Gonzalez-Manas, J.H. Lakey, 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 (2002) 41916–41924.
[36] J.V. Lehtonen, D.J. Still, V.V. Rantanen, J. Ekholm, D. Bjorklund, Z. Iftikhar, M. Huhtala, S. Repo, A. Jussila, J. Jaakkola, O. Pentikainen, T. Nyronen, T. Salminen, M. Gyllenberg, M.S. Johnson, BODIL: a molecular modeling environment for structure-function analysis and drug design, J. Comput. Aided Mol. Des. 18 (2004) 401–419.
[37] P. Kraulis, MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures, J. Appl. Crystallogr. 24 (1991) 946–950.
[38] E.A. Merritt, D.J. Bacon, Raster3D: photorealistic molecular graphics, Methods Enzymol. 277 (1997) 505–524.