The behaviour of sea anemone actinoporins at the water-membrane interface.

Lucía García-Ortega[1], Jorge Alegre-Cebollada[1,2], Sara García-Linares[1], Marta Bruix[3], Álvaro Martínez-del-Pozo[1,*] and José G. Gavilanes[1, *]

[1]*Departamento de Bioquímica y Biología Molecular I, Facultad de Ciencias Químicas, Universidad Complutense, 28040 Madrid, Spain*.

[2]Present address: *Department of Biological Sciences, Columbia University, 1212 Amsterdam Ave., New York, NY 10027, USA.*

[3]*Instituto de Química-Física Rocasolano, CSIC, Serrano 119, 28006 Madrid, Spain.*

*To whom correspondence can be addressed: AMP (alvaro@bbm1.ucm.es) and JGG (jggf@bbm1.ucm.es)

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Abbreviations: Avt, actinoporins from *Actineria villosa*; ALP, actinoporin-like protein; ATR, attenuated total reflection; Bc2, actinoporin from *Bunodosoma caissarum*; CD, circular dichroism; Chol, cholesterol; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleylphosphatidylcholine; DPC, dodecylphosphocholine; DrI, ALP from *Danio rerio*; EM, electron microscopy; Ent, actinoporin from *Entacmea quadricolor;* Eqt, equinatoxin; FTIR, Fourier transform infrared spectroscopy, Fra C, actinoporin from *Actinia fragacea*; GUV, giant unilamellar vesicles; ITC, isothermal titration calorimetry; NLP, necrosis and ethylene-inducing peptide 1 (Nep1)-like protein; NMR, nuclear magnetic resonance; PE, phosphatidylethanolamine; PFT, pore forming toxin; PpBP, ALP from *Physcomitrella patens*; Pstx, actinoporins from *Phyllodiscus semoni*; SM, sphingomyelin; SPR, surface plasmon resonance; Stn, sticholysin; TFE, trifluoroethanol.

Abstract

Actinoporins constitute a group of small and basic α-pore forming toxins produced by sea anemones. They display high sequence identity and appear as multigene families. They show a singular behaviour at the water-membrane interface: In aqueous solution, actinoporins remain stably folded but, upon interaction with lipid bilayers, become integral membrane structures. These membranes contain sphingomyelin, display phase coexistence, or both. The water soluble structures of the actinoporins equinatoxin II (EqtII) and sticholysin II (StnII) are known in detail. The crystalline structure of a fragaceatoxin C (FraC) nonamer has been also determined. The three proteins fold as a β-sandwich motif flanked by two α-helices, one of them at the N-terminal end. Four regions seem to be especially important: A cluster of aromatic residues, a phosphocholine binding site, an array of basic amino acids, and the N-terminal α-helix. Initial binding of the soluble monomers to the membrane is accomplished by the cluster of aromatic amino acids, the array of basic residues, and the phosphocholine binding site. Then, the N-terminal α-helix detaches from the β-sandwich, extends, and lies parallel to the membrane. Simultaneously, oligomerization occurs. Finally, the extended N-terminal α-helix penetrates the membrane to build a toroidal pore. This model has been however recently challenged by the cryo-EM reconstruction of FraC bound to phospholipid vesicles. Actinoporins structural fold appears across all eukaryotic kingdoms in other functionally unrelated proteins. Many of these proteins neither bind to lipid membranes nor induce cell lysis. Finally, studies focusing on the therapeutic potential of actinoporins also abound.

"...the native conformation [of a protein] is determined by the totality of interatomic interactions and hence by the amino acid sequence, in a *given environment*"

Christian B. Anfinsen, 1972, Nobel Lecture

Christian B. Anfinsen was well aware of the importance of environment on the acquisition of the final native three-dimensional structure of a polypeptide chain. In fact, the statement shown above summarizes what could be considered as the *dogma of protein folding*. As it is well known, most proteins fold in an aqueous environment and remain soluble in this polar media while functionally active. Another smaller number, though not negligible, have evolved to fold into, or close to, the highly hydrophobic environment of the lipid bilayer. Even the most basic Biochemistry textbooks sustain the clear cut classification that distinguishes between soluble and membrane proteins. Surprisingly, actinoporins, the family of proteins which constitute the subject of this review, escape to this simple classification. They show what can be described as a dual behaviour at the water-membrane interface. In water solution, actinoporins remain mostly monomeric and stably folded but, upon interaction with lipid membranes of specific composition, they become oligomeric integral membrane structures. Thus, this family of proteins represent an optimal system to study the transition from a soluble monomeric folded conformation to an oligomeric transmembrane protein. Indeed, this transition to form a functionally active membrane-bound pore constitutes the core of their biological function. Actinoporins, in short, demonstrate how an identical amino acid sequence can fold into two different structures, showing the environmental influence on the energy landscape of a protein, as essentially stated by Anfinsen more than 35 years ago. They are a natural and beautiful example of protein plasticity.

Sea anemone actinoporins

Actinoporins constitute a widespread family of toxic proteins presumably stored within the nematocysts of sea anemones [1]. Contact with their tentacles triggers injection of these and other toxins into prey, leading to a variety of noxious effects [2].

Actinoporins are produced by different sea anemone species as single polypeptide chains of around 175 amino acids (Figure 1). They show basic isoelectric point values and are usually cysteinless [3-5].

Actinoporins belong to the much larger group of widely distributed pore forming toxins (PFTs). As it is quite obvious from their denomination, the toxic activity of these proteins relies on the formation of pores within biological membranes [6-13]. All PFTs show this same dual behaviour when encountering a membrane but, in order to study their ability to become integral membrane proteins, actinoporins show precisely the methodological advantage of being small and cysteinless.

The formation of pores by PFTs can be achieved by inserting either α-helices or β-hairpins within the cell membrane [6,9-13]. These structural motifs are employed to classify them into two well-defined groups. Thus, α-PFTs insert α-helices within the membrane, whereas β-PFTs use β-barrels to span it [14]. Actinoporins are α-PFTs since they seem to insert α-helices within the membrane core; a feature that they share with some of the most dangerous toxins known such as diphtheria or anthrax. They show haemolytic activity because they form cation-selective pores with a diameter of 1-2 nm within the membranes of erythrocytes [15-17]. Equinatoxin II (EqtII) from *Actinia equina* and sticholysins I and II (StnI and StnII) from *Stichodactyla helianthus* are the most thoroughly studied actinoporins [5,18].

Biological function

Many carnivorous sea animals possess toxins which function as chemical weapons to paralyze prey such as fish, crabs, worms, or bivalves [2,19,20]. This is particularly important if the considered animal lives a benthic life, spending most of its biological cycle attached to a fixed location. Maybe that is why these animals secrete a wide variety of different toxins including neurotoxins, cytotoxins, cardiotoxins, and hemolysins [2,21-23]. Within this idea, sea anemone actinoporins are believed to participate in functions like predation, defence, and digestion, and have been shown to be lethal to small crustaceans, molluscs, and fish [1], or even parasites such as the unicellular flagellated protozoan *Giardia duodenalis* [24]. The oligomeric cation-selective pores formed by them result in a colloid-osmotic shock that leads to cell death [16,25,26].

So far, actinoporins have been isolated from at least 20 different sea anemone species [3,5,27,28], showing their ubiquitous distribution within the *Actinaria* order. Moreover, they display high sequence identity (between 60 and 80%) (Figure 1) and appear as multigene families [29], giving rise to many protein isoforms within the same individual [3-5,30-32]. The small number of changes observed, however, result in large functional differences in terms of solubility and haemolytic activity [28,32-34] as exemplified by StnI and StnII. They are 91% identical but show quite different haemolytic activities. The presence of this large number of similar isotoxins in the venom probably helps to broad the range of prey for a given species [35]. Analogous to immunoglobulins, which require a plethora of highly diverse genes to counteract foreign antigens, sea anemone tentacles would produce many actinoporins isoforms. This feature has led some authors to propose that actinoporins could represent the embryo of a rudimentary immune system [32].

Water-soluble three-dimensional structure

The water-soluble structures of EqtII and StnII are known in detail [23,36-38]. The crystal structure of a FraC nonamer, obtained in the presence of detergent, has been also reported [39]. The three proteins fold as a β-sandwich motif composed of 10-12 β-strands flanked by two α-helices which interact with both sides of the β-sandwich (Figure 2). One of these helices is located near the N-terminal end (Figure 3) and is preceded by a short 3_{10} helix [23,36-39]. In fact, the first 30 residues appear to be the largest part of the protein that can adopt alternative conformations without disrupting the fold of the β-sandwich [36]. This feature and the amphipathic character of this α-helix seem to be extremely important for the final functionality of the pore since it has been proposed to extend and insert into the membrane to form the pore walls [40]. In addition to the N-terminal α-helix, three more regions of the structure seem to be especially important from a functional point of view: A phosphocholine (POC) binding site, a cluster of aromatic residues, and an array of basic amino acids (Figure 3). Determination of these crystal and soluble three-dimensional structures of actinoporins, including a StnII-POC complex, altogether with different biochemical and biophysical characterization of a high number of actinoporins mutants and natural variants, has led to putative models of their topology and mode of action.

The POC binding site

The existence of a specific POC binding site in StnII was determined after solving the X-ray structure of the corresponding complex [38]. This site is partly hydrophobic (side chains of Val-85 and Pro-105, and aromatic rings of Tyr-111 and Tyr-135) and partly hydrophilic (side chains of Ser-52 and Ser-103 and hydroxyl groups of Tyr-131, Tyr-135 and Tyr-136) (Figure 4). Some of the residues participating in this POC recognition site are also part of the conserved cluster of aromatic residues (Figures 3 and 5). Altogether, both overlapping regions seem to constitute the main recognition and binding sites to the membrane, as explained below. The positive charge of the choline moiety is stabilized by cation-π interactions with the aromatic rings of Tyr-111 and Tyr-135, whereas the phosphate group interacts with the phenolic hydroxyl groups of again Tyr-111 and Tyr-136, and is probably further stabilized by the cationic side chain of Arg-51 (Figure 4). Unexpectedly, POC binding does not promote significant conformational changes in StnII apart from minor rearrangements in local side chains and backbone modifications in the β6–β7 loop [38], at least when studied in the absence of lipidic media.

The importance of Tyr-111 is highlighted by the fact that it is 100% conserved among the actinoporins family (Figure 1). It is located in the very flexible loop linking the β5 and β6 strands (Figure 5) which appears highly disordered in EqtII [36,37] and StnII [38], both in the free and POC bound forms. However, in the crystal structure of POC-bound Stn II, the aromatic ring of Tyr-111 is pointing toward the phosphocholine moiety after a probable conformational change from its exposed free state [38], a fact that seems confirmed by the behaviour of the corresponding StnI Tyr-112 in the presence of dodecylphosphocholine (DPC) micelles [41]. Also in agreement, mutation of the equivalent Tyr-113 of EqtII diminished specific binding of SM, and prevented insertion of and binding into SM containing lipid monolayers and liposomes [42], suggesting that this residue is strictly required, at least for EqtII SM recognition. Accordingly, mutation of StnII Tyr-111 to Asn (StnII Y111N) resulted in a significantly decreased haemolytic activity and supports the interaction with the positively charged choline [43-46]. Finally, in the recent nonameric FraC structure, detergent molecules cocrystalized in contact with its equivalent Tyr-113 [39]. Surprisingly, Stn I Tyr-112 [41,47] is not affected by the micellar environment when studied by NMR in the

presence of DPC micelles [41] since it displays identical properties as in the lipid free protein. Thus, either Tyr-112 in StnI does not really interact with the micelle or the mobility of the lipid molecules is larger than in a real membrane, hampering the formation of a pore structure. The higher curvature of the micelle, in comparison to a real membrane, might be another reason to explain the observed absence of effect.

Altogether, all these data suggest the existence of a high conformational versatility around this Tyr residue when free in solution or in the presence of lipids. These properties may facilitate the fast conformational arrangements needed upon the environmental change produced when encountering the membrane and could be also responsible, in part, for specific recognition and pore formation.

The exposed cluster of aromatic residues

Most known actinoporins display an exposed and conserved cluster of aromatic amino acids (Figures 3 and 5), composed mainly of Tyr and Trp residues (Phe-106, Trp-110, Tyr-111, Trp-114, Tyr-131, Tyr-135 and Tyr-136 for StnII). Their implication in the interaction with membranes has been clearly established. In fact, mutations affecting this region result in less haemolytic variants with reduced affinity for lipids [43,44]. More recently, NMR studies of the aromatic resonances of the residues from this cluster, after the interaction of StnI with DPC micelles, have revealed that the motional flexibility of most of them was perturbed when compared with the protein in water [41]. Some of these residues (StnII Tyr-131, 135, and 136, for example) appear along the second α-helix (Figure 3, α_2 helix). Assuming that the nonameric FraC C crystal structure represents a membrane bound state [39], this α_2 helix would lie on the plane of the bilayer providing anchoring sites for attachment and stabilization of the β sandwich [39]. These observations are in good accordance with the very well known fact that aromatic chains preferentially appear at the membrane/water interfaces [48]. Consequently, the cluster seems to play an important role during the first steps of pore formation, most probably during membrane attachment [49, 50].

Apart from the already described importance of Tyr-111 (StnII numbering) in POC binding and SM recognition, another important residue for the establishment of the actinoporins-membrane interaction is StnII Trp-110 (Figures 3 and 5). Its implication in SM recognition has also been proven for the equivalent EqtII Trp-112 [42,51].

However, it can be substituted by any other bulky and hydrophobic amino acid such as Phe or Leu, while still maintaining a wild-type phenotype [42]. Within this same idea, attachment of a short peptide to a Cys residue substituting this Trp in StnI disturbs but does not prevent pore formation [52].

The determination of the soluble three-dimensional structure of a much less haemolytic StnII mutant (R29Q) has also revealed that things are far more complex. This Arg-29 is located at the hinge connecting the N-terminal helix to the β-sandwich core (Figure 3) and its substitution by Gln has revealed not only a perturbation of this region, but also long range effects affecting most of the residues located in the protein surface that presumably faces the membrane [46]. This perturbation includes the aromatic cluster residues, for example. Indeed, the distribution of the electrostatic potential along this surface changes dramatically, with a significant loss of the positive potential. This could affect the efficient targeting of the protein to the membrane and then perturb the specific interactions with the negative phosphate groups of the membrane phospholipids.

The array of basic amino acids

A region rich in basic residues has been proposed to play a role in the initial steps of EqtII membrane recognition via interaction with negatively charged regions of the lipid head groups [37]. Such a region in StnII would be comprised of Lys-118, Lys-149, Arg-156, and Arg-175, together with the contiguous sequence Lys-123-Arg-124-Arg-125 appearing along the loop linking the strand β7 with the second α-helix (Figures 3 and 6). Interestingly, most of the Arg in this StnII stretch appear as Lys in EqtII, and vice versa (Lys-118, Lys-149, Arg-156, and Arg-175 in StnII against Arg-120, Arg-152, Lys159, and Lys-178 in EqtII) (Figure 1). An additional EqtII Lys residue (Lys-123) appears as a Ser in the equivalent StnII position (Ser-121). Maybe these small changes correlate with the apparent different lipid affinities of these two proteins [31,42,44,53,54].

The N-terminal α-helix

Actinoporins N-terminal end, comprising about their first 30 amino acid residues (Figure 3), is an essential region to achieve the correct formation of the pore and, therefore, to exert their haemolytic activity, although it does not seem to be so important

for membrane attachment [49,55]. For example, it is well established how removal of this N-terminus of EqtII precludes pore formation, but does not prevent membrane binding [56]. A more recent characterization of StnII variants where single mutations had been introduced within these first 30 amino acid residues also supported the idea that the N-terminus is not needed for membrane recognition [44]. Furthermore, the use of disulfide mutants that locked the N-terminal helix to the β-sandwich elegantly showed that helix detachment was necessary for pore formation [49,55]. On the other hand, taking into consideration this crucial role of the N-terminal region in the formation of the pore, it is somehow striking that it is precisely the most variable region in the sequence of actinoporins (Figure 1). However, this variability is located mainly at the hydrophilic residues, whereas a higher degree of conservation is observed for hydrophobic ones, preserving its amphipathic nature. Thus, it seems feasible that distinct actinoporins may form pores with slightly different conductivity properties, resulting in differences in their toxicity. If that is the case, it could help to explain why one specific anemone produces several different isoforms in terms of a strategy of broadening of its venomous condition. In fact, N-terminal sequence differences found between StnI and StnII have been used to explain the observed different haemolytic activity of these two proteins against human erythrocytes [57,58].

The conformation of peptides mimicking the N-terminal of EqtII, StnI, and StnII has been studied in water and in the presence of lipid-like environments such as liposomes, bicelles, micelles, or even trifluoroethanol (TFE) [41,59-62]. All these compounds and materials are generally accepted as a first approximation to mimic the membrane environment of peptides and proteins; and they are thoroughly used with this purpose. Caution must be taken, however, when extrapolating the results obtained to a real membrane bilayer. With this precaution in mind, it still seems clear that this kind of approach has produced a good amount of useful data regarding the conformational possibilities of the N-terminal end of actinoporins. For example, the synthetic peptide corresponding to residues 1–30 of StnI displays a reasonably high tendency to form a helix in water but it is significantly more helical in either TFE or DPC micelles (Figure 7). In these lipid-mimicking environments it forms a helix-turn-helix motif with the last α-helical segment matching the native N-terminal α-helix (residues 14–24) present in the complete protein, while the first helix (residues 4–9) is less populated and is not

present in the water-soluble protein structure [41]. On the other hand, the amino-terminal peptides of EqtII and StnII are essentially unstructured and extended in water solution, respectively, although can also acquire large helical contents in hydrophobic environments like micelles or TFE [59-61]. Simulation of the behaviour in water of a peptide containing the first 32 residues of EqtII [62] revealed the adoption of a flexible loop and turn structures, in contrast to the helical structure seen in DPC micelles [60]. NMR studies confirmed that this peptide in aqueous solution did not adopt an ordered conformation [60] while they ~~also~~ showed that it becomes partly helical in the presence of lipids. This helical content is greater when SM is present, in agreement with the increased efficiency of actinoporins in the presence of this lipid [62]. Thus, producing more than one type of protein, such as StnI and StnII, with distinct structural tendencies along an important segment for pore formation, again may endow the anemone with a diverse arsenal suitable for attacking a wider variety of prey, including the possibility of a concerted and more efficient synergic cytotoxic action [41]. Indeed, all the studied peptides seem to display an environmental conformational plasticity which may be highly important for exerting their function when included in the complete protein.

All these NMR studies about the structure of different synthetic peptides and actinoporins in the presence of lipidic environments have rendered clues about their pore-forming mechanism [41,62,63]. For example, NMR analysis of StnI in water and in the presence of membrane-mimicking micelles has shown that the N-terminal α-helix is maintained in its native structure and that this micellar environment does not provoke its detachment from the protein core [41]. This direct observation that the dissociation of the StnI N-terminus does not occur in the presence of micelles [41] is in agreement with previous proposals for the homologous EqtII [51,64] and ~~also~~ with the assumption that this region does not participate in the recognition of the membrane [44].

It is suggested that actinoporins do not insert deeply in the membrane except for its N-terminus [49,53]. Within this idea, cysteine-scanning mutagenesis was used to propose that EqtII residues 10-28 are organized as an α-helix in the pore structure [40]. This proposal was later extended to suggest that the adoption of an N-terminal helical conformation would also take place along the first ten amino acids [65]. In fact, addition of an N-terminal 6xHis tag does not preclude pore formation but the channels formed are of lower conductance [65]. Indeed, these tagged proteins show haemolytic activity

significantly diminished [33,65,66]. The presence of 3_{10} helices within this protein segment may be indicative of the existence of intermediate structures prone to change their conformation to become elongated α-helices [39]. Such an extended helix would still be amphipathic, forming angle with the membrane normal of about 31º [53]. Accordingly, FTIR [53,67] and CD [68] measurements detected increments in the α-helical content of actinoporins upon lipid binding that were compatible with these observations. Within this same idea, as already mentioned, truncation of the first EqtII residues caused a decrease of its haemolytic activity and pore stability [56,65]. Finally, mutations that confer conformational stiffness, such as the introduction of Pro residues or a disulphide bridge, hamper pore formation most probably by diminishing the propensity to extend and detach the N-terminal helix once the protein is already bound to the membrane [44,49,55]. The already mentioned StnII R29Q mutant shows the existence of an increased dynamic flexibility at the hinge region between the N-terminal α-helix and the protein core [46] which would explain why this impaired variant is however more haemolytic than expected from its membrane binding affinity [46]. On the contrary, the structure of a StnII Y111N mutant [46] suggests not only a rigidification of the POC-binding site but also of the loop comprising residues 25 to 29. This conformational stiffness would hamper detachment of the N-terminal α-helix. Overall there would be a loss of the necessary plasticity to interact with the membrane. Both the local and the long-range effects in this mutant could explain why it is less haemolytic than predicted from its binding affinity [44].

Other protein regions

Regions around the N-terminal and the aromatic and positively charged residues are highly flexible and contribute to membrane binding as well as undergoing changes in secondary structure during interaction with bilayers [62]. Most actinoporins contain an Arg–Gly–Asp (RGD) motif that might be involved in promoting attachment to integrin receptors on the surface of cells [4,28,69-71], a possibility which does not seem to have been studied yet. The absence of this motif in the sequence of some members of this family, such as Avt-I from *Actineria villosa* or Pstx20 from *Phyllodiscus semoni* [34] (Figure 1), might be another structural feature to be taken into account when explaining the variety of prey killed by different sea anemones.

Finally, the detailed analysis of the soluble three-dimensional structure of StnII R29Q [46] has revealed the existence of other residues that are affected by conformational exchange processes. These residues are arranged around the β-hairpin composed by residues 145-150 and 156-161. Interestingly, these stretches comprise the RGD motif (StnII Arg-141, Gly-142, and Asp-143) as well as some of the components of the already discussed array of basic amino acids (Figure 3). It is speculated that these residues could be involved in other type of interactions apart from those directly related to lipid binding and pore formation such as oligomerization, for example. On the basis of the NMR results obtained so far it seems that the atomic determinants for membrane recognition and pore formation by actinoporins rely not only on a structure that provides the correct geometry but also on a finely tuned network arrangement and the adequate dynamic properties of the regions involved [5,46]. This geometric and dynamic arrangements are most probably modified by some of the membrane components when establishing the interactions needed to form the pore. These considerations would help to explain the different nature of the interactions occurring depending on the nature and composition of the bilayer studied.

The nature of the target lipid membrane

Actinoporins seem to be able to form pores within model membranes in the absence of any other protein, showing that they do not need a protein receptor [16,26,72,73]. In fact, the incorporation of the toxin into the bilayer, a crucial step for the final pore formation, depends largely on the composition and physicochemical state of the membrane [73-75]. Both factors influence the actinoporins conformational changes occurring upon the transition from the water media to the inserted state [44,67]. Thus, high affinity recognition of SM by the protein seems to be very important for its specific attachment to a particular membrane but the following effects observed probably depend also on the physical properties derived from its particular composition and not only from its SM content.

The importance of the presence of SM within the membrane has been already highlighted in the previous sections of this review. Many experiments have been performed during the last 15 years to prove the essential role of this lipid in actinoporins function [18,42,51,73-78]. Specific binding of EqtII to SM has been elegantly shown by

lipid dot blot assays and surface plasmon resonance (SPR) experiments [41]. This binding is so specific that it has been postulated that SM functions for EqtII as a real membrane receptor but of lipidic nature. The structural basis for this specificity seems to be based on the fact that EqtII could recognize a lipid moiety different from the headgroup, since PC and SM have the same one and POC does not bind with great affinity to actinoporins. A more recent ITC characterization of StnII binding to SM/DOPC/Chol (1:1:1) vesicles has ~~also~~ revealed a reversible interaction characterized by a high affinity constant [44], a fact that is contradictory with some previous results [15,76], but compatible with the observation that EqtII can slowly dissociate from DOPC/SM (1:1) membranes [42,49]. Overall, the results so far obtained support the belief that SM is the preferable lipid for EqtII, although permeabilization experiments with other actinoporins may not follow this strict requisite, at least when pore formation is being evaluated [73]. Definitively, this is an aspect that still remains to be completely solved.

Coexistence of different phases on the membrane seems to be another important factor, if not for attachment, at least for the final formation of the pore [44,67,73]. For example, studies performed with giant unilamellar vesicles (GUV) have shown that SM strongly enhanced binding of EqtII, but was not sufficient for membrane permeabilization. Pores were formed only when, in addition to the presence of SM, liquid ordered and disordered phases coexisted [78]. Moreover, there was permeabilization if these GUV were made only of DOPC/DPPC/Chol (1:1:1) mixtures which do not contain SM but do exhibit phase coexistence. Although these experiments were made at far higher concentrations of EqtII compared to the haemolysis assays, the results showed that this actinoporin can also interact with lipid membranes in the absence of SM. In this case, very similar results were obtained for StnII with other membrane systems. According to the results published so far for this particular actinoporin, the presence of SM does not seem to be as crucial as it is for EqtII [44,53,73,79].

Within this same idea, the increased permeabilizing potency of actinoporins observed against PC liposomes in the presence of Chol could be explained by the formation of microdomains, which may alter the accessibility of the phosphorylcholine group for toxin binding [80]. The presence of Chol in membranes exclusively formed

by PC leads to pore formation, even under circumstances where little toxin is associated with the lipids [73,81]. Moreover, SM and Chol coexistence in membranes significantly favours binding and permeabilizing activity of StnII. Consequently, solid-state NMR results also indicate that Chol destabilizes the DMPC bilayer in the presence of EqtII but leads to greater disruption when SM is in the bilayer. This supports the proposal that actinoporins are more lytic when both SM and Chol are present as a consequence of the formation of domain boundaries between liquid ordered and disordered phases in lipid bilayers [63,73,75,78]. It seems that lipid packing defects arising at the interfaces between coexisting phases may function as preferential binding-sites for the toxin. Association with these interfaces between domains would function as an efficient concentration strategy confining the toxins to a space where oligomerization and pore formation could take place at very low bulk protein concentrations [80]. If this is correct, minor amounts of lipids favouring non-lamellar organizations should augment the efficiency of pore formation. Accordingly, inclusion of PE in vesicles containing both PC and SM, did not substantially modify the insertion of StnII, but increased the rate of pore formation [81]. Within this same idea, the presence of small quantities of anionic lipids ~~also~~ rendered membranes more sensitive to actinoporin-induced permeabilization [74]. FTIR, ^{31}P NMR, and electron paramagnetic resonance (EPR) experiments have also proven how EqtII induces non-lamellar lipid structures which would be consistent with the formation of a toroidal lipid pore [53,74,82,83]. In addition, actinoporins are able to induce lipid flip-flop between internal and external leaflets of liposome membranes and to permeabilize liposomes in the presence of phosphatidic acid, a strong inducer of negative membrane curvature [74]. As mentioned above, synchrotron radiation CD spectroscopic studies have revealed that the peptide corresponding to residues 1–32 of EqtII adopts very different conformations when examined in water, DPC micelles, or small unilamellar vesicles composed of DOPC or DMPC [61]. These conformations were highly influenced by the presence of SM and Chol, confirming the importance of the lipid properties arising from the coexistence of liquid ordered and disordered phases and the dual role of SM as a specific attachment and ~~also~~ as a promoter of the bilayer organization necessary for membrane lysis [61]. In agreement with all these results, different experiments have showed that StnII is able to promote pore formation in COS-7 cells via its interaction with the domains enriched in SM and Chol known as cellular rafts [79].

It seems clear then that actinoporins permeabilizing activity depends on membrane lipid composition but ionic concentration and variety of the medium can be also a determinant factor. Results have shown that StnII haemolytic activity is promoted by Ca^{2+} and Mg^{2+} and inhibited by Co^{2+} and Mn^{2+} [84]. In particular, results recently reported suggest that StnII pore-forming ability is improved by an increase in intracellular calcium associated to membrane phospholipids translocation elicited by scramblases [85]. The oxidative status of the red blood cells, regarding membrane lipid composition, seems to be another feature to take into account when studying actinoporins effect on cells [86]. All these aspects of their function have not been deeply studied yet and deserve future attention and development.

From another point of view, it is also well known how StnII is about six fold more active than StnI against human red blood cells. Moreover, StnII and EqtII show a very different ability to lyse these cells when tested under the same experimental conditions [81]. Therefore, different actinoporins could display distinct lipid requirements to accomplish the formation of the pore, including different efficiencies for SM binding. This is a possibility that still remains as an open question to be answered in the near future.

Finally, sea anemones possess within their membranes analogues of SM to which actinoporins do not bind [87]. This would explain, at least in part, the molecular basis of their self/nonself selectivity. For example, the haemolytic activity of actinoporin from *Phymactis clematis* was inhibited by SM but not by sphingolipids purified from the same sea anemone. This specific recognition is probably based on the existence of a different phospholipid headgroup in the SM analogues.

The current model of pore formation

Actinoporins, as any other PFT, must suffer a transformation from a soluble monomeric protein to an oligomeric transmembrane channel to exert their biological function [6]. The accepted steps of the mechanism of actinoporins pore formation have been described in high detail before [5]. Therefore, only a brief summary of this mechanism (Figure 8) will be considered in the following lines, with special emphasis on the new results obtained along the last four years.

It is generally accepted that these proteins initially bind to the target membrane as monomers guided by their affinity to some membrane component, being SM the preferred candidate. Such an interaction would result in an increase of toxin concentration at the lipid-water interface that would lead to its oligomerization on the membrane surface. Interestingly, the presence of preassembled StnI dimers, via a disulfide bridge at the N-terminal end, seems to facilitate pore formation [88]. Kinetic measurements using SPR suggest this binding to membranes as a two-step process [42,49]. The first one would be driven by the POC-binding site, the aromatic cluster of amino acids, and the array of basic residues. The synergic or cooperative action of these domains seems to be possible, or even probable, but it has not been explicitly measured yet. During the second step, the N-terminal α-helix would detach from the β-sandwich, extend, and lie parallel to the membrane. Simultaneously, oligomerization would occur. A non-active oligomer, often referred as a "prepore" state, would then be formed (represented as T_1 or T_2 in Figure 8). Different intermediates and structures which could potentially represent this non-active state have been determined by two-dimensional crystallization on lipid mono-layers and electron and atomic force microscopies, combined with computer assisted docking [38, 89-91]. Interestingly, in all cases tetrameric ensembles were detected where the high resolution water soluble structure could be nicely fitted with only minimal modifications [38]. However, the recent determination of the FraC crystalline structure has challenged these conclusions. FraC was crystallized in the presence of a detergent [39] resulting in an ensemble consisting of two concentric and identical crown-shaped nonamers. The overall external diameter of each crown was almost double in size that the internal one of approximately 5.2 nm. The detergent molecules appear located at the basal crown rim and the nine N-termini line its inner wall. These N-terminal stretches still retain the conformation of the water soluble structures of actinoporins. Most of the residues belonging to the conserved cluster of aromatic amino acids constitute part of the crown base and their side chains enter into contact with the detergent molecules [39]. The authors of this work state that all these results are compatible with the assumption that this nonameric structure represents a high-resolution model of an actinoporin in its prepore state. Apart from the evident beauty and appeal of this proposal, direct evidence about the existence of this "prepore" as a definite structural intermediate en route to the formation of the pore is

still lacking. In contrast, this type of structure seems to have been perfectly identified in some β-PFTs [92].

On the basis of the NMR results obtained for StnI in the presence of DPC micelles [41], during the early steps of the interaction with the lipid bilayer most of the aromatic rings from the cluster would be in intimate contact with the membrane. The loop comprising Lys-27 to Lys-31 would be also close to it, and the N-terminal α-helix would remain in its native conformation. Experiments made with extensive double cysteine scanning mutagenesis [55] confirmed that changes of the β-sandwich structure are not necessary for formation of the pore. Instead, the core of the protein should remain compactly folded in order for the protein to be fully active. The recent determination of the structures of StnII R29Q and Y111N mutants [46] has revealed, however, the higher complexity of this process, as well as the crucial importance of long-range interactions along the protein and the overall distribution of the electrostatic potential of the surface facing the membrane. Within this picture, the β-sandwich would be crucial for lipid specificity, membrane targeting, and formation of the final pore structure, because it would constitute the solid scaffold where the distant parts responsible for the different and specific interactions would be positioned precisely to selectively act in the presence of the target. From this state, membrane permeabilization would be achieved by conformational changes within the toxin, which would expose hydrophobic patches of amino acids required for membrane insertion. Such lipid-protein interactions may be related to the ability of the protein to adopt a molten-globule state, a conformational intermediate that has been shown to exist for actinoporins under some experimental conditions [37,93-96]. One of these conformational changes would involve the detachment and extension of the N-terminal α-helix. This was shown, for example, by the behaviour of a double cysteine mutant where its N-terminus had been fixed to the protein core by a disulphide bond [49], impairing pore formation. Finally, this extended amino-terminal α-helix would penetrate the membrane to build a toroidal pore [5,97] where not only the protein but also the phospholipid headgroups would linen its walls. The existence of such a structure has been shown by different means, such as ATR-FTIR of isolated protein-membrane complexes [53] and would explain why these pores are noisy and less stable than those known to be made by β-PFT. Usually, pores formed by actinoporins do not seem to require more than 3–4 monomers

[16,26,98], while β-PFTs need instead a higher number of units [6] just because the lipid moieties are excluded from the pore lumen. Again, it has to be emphasized how the crystallization of FraC nonameric structure [39] has allowed these authors to build a model, based on cryo-EM results, of the protein bound to phospholipid vesicles, with the nine N-termini assembling as a compact α-helical structure where lipids would not be part of the pore wall. This new model challenges the consensus view in the field of actinoporins, and will probably be subject to experimental validation in the following years.

Furthermore, pore formation by StnII is an enthalpy-driven process [44], which indicates that the strength of interactions between the lipids and the protein is higher than those existing with the solvent [99]. Taking into consideration that ΔS is unfavourable, a higher binding constant is predicted when lowering temperature, which would agree with the higher activity of actinoporins at cooler temperatures [80].

It is clear that EqtII and StnII form cation selective pores with a hydrodynamic diameter of about 1-2 nm, according to the results of different experiments, including the use of osmotic protectants of different size and conductance measurements in planar bilayers [15-17,25,26,38,73]. This size appears to be independent of toxin concentration [17]. Thus, pores seem to have a fixed common predominant structure but may adopt slightly different conformations while keeping constant their size, depending on the protein and the nature of the membrane studied. EqtII pores, for example, have a broad conductance distribution [65]. The positively charged residues on the extended amphipatic N-terminal helices of the oligomer forming the pore would control binding of this protein segment by interacting with the negative phosphate groups of the membrane lipids, while negative charges within the pore lumen would contribute to cation selectivity as well as pore-forming activity [28]. Moreover, negatively charged lipids are able to increase the cationic selectivity of the pore supporting the proposition that lipids are part of the pore lumen [82]. The significant differences in actinoporins N-terminal segments [5,100] (Figure 1) could account for differences in activity, size, stability and maybe cation specificity of the resulting pore. Much work however still needs to be done in order to fully understand the complex mechanism underlying actinoporins functionality.

Actinoporin-like proteins

Actinoporins constitute a good example of how Nature preserves a common structural fold with different purposes [10]. Until very recently, actinoporins were thought to be restricted to sea anemones, but now actinoporin-like proteins (ALPs) have been identified in other cnidarians and a wide variety of vertebrates, in addition to a small number of plants, mosses, and fungi [101,102]. Surprisingly, many of these proteins, which show high levels of sequence and/or structural similarity with actinoporins, neither bind to lipid membranes nor induce cell lysis.

One of the most remarkable examples of this structural conservation is constituted by a family of fungal lectins, with insecticidal and anti-proliferative properties [103,104], such as XCL from *Xerocomus chrysenteron* or ABL from *Agaricus bisporus*. Although these proteins display less than 15% sequence identity to actinoporins [105,106] they exhibit a protein fold which is almost identical to the three-dimensional structures of EqtII and StnII, except for the absence of the N-terminal α-helix (Figure 9), in good agreement with their lack of permeabilizing activity. Furthermore, the specific structural features and sequence signatures in this protein family suggest a potential sugar binding site in these lectins, which would be evolutionary related to the actinoporins POC-binding site [105,107].

Very similar structures to actinoporins appear as well in other completely unrelated toxins, such as the necrosis and ethylene-inducing peptide 1 (Nep-1)-like protein (NLP) secreted by the phytopathogenic oomycete *Pythium aphanidermatum* [108]. This toxin belongs to the NLPs superfamily of proteins, produced by various prokaryotic and eukaryotic phytopathogenic microorganisms [109-112]. They trigger leaf necrosis and stimulate immunity associated defences in all dicotyledonous plants tested, but not in monocotyledonous ones [110,111]. Hence, NLPs have been proposed to have dual functions in plant pathogen interactions, acting both as triggers of immune responses and as toxin-like virulence factors [113]. Superimposition of actinoporins and *P. aphanidermatum* NLP three-dimensional structures suggests a significant degree of structural similarity but they are only distantly related proteins [108]. In addition, this ethylene-inducing peptide retains the N-terminal helical segment which in this case seems to be required for inducing necrosis and plant defence activation [114]. This and

other membrane permeabilization results suggest that NLPs and actinoporins may share a cytolytic, membrane-disintegrating mode of action [108].

Echotoxins are also actinoporin-related toxins that have been found in the salivary gland of the marine gastropod *Monoplex echo* [115]. They exhibit both haemolytic and mouse-lethal activities that are inhibitable by gangliosides. They share low sequence identity with actinoporins (12–16%), but some of the amino acid residues important for their biological activity are well conserved in both protein families [115]. Therefore, it has been hypothesized that echotoxins may have evolved from actinoporins or, at least, that both toxins originated from a common ancestor.

Bryoporin (PpBP), an ALP produced by the moss *Physcomitrella patens*, must be also mentioned. It shares 50% amino acid identity with EqtII. This protein is upregulated under water stress conditions, and overexpressing PpBP heightens drought resistance in this moss. Therefore, some ALPs might be involved in functions so important to terrestrial plants such as withstanding dehydration [102].

Search in public databases looking for sequences similar to EqtII yielded proteins from three animal and two plant phyla. Sequence conservation located to a region of the β-sandwich involved in membrane binding, suggesting that these homologues should be membrane-binding proteins. Interestingly, most of these sequences were retrieved from fish. One of them, corresponding to DrI, a protein of unknown function from zebra fish (*Danio rerio*), was cloned, isolated, and characterized. Surprisingly, DrI lacked SM specificity, was non-haemolytic and had only weak membrane binding capacity *in vitro* [101]. Clamlysin, another novel SM-binding protein, has been purified from the foot muscle of the clam *Corbicula japonica* [116]. This protein shows haemolytic activity which can be inhibited by SM, but not other phospholipids or glycosphingolipids. It displays high sequence similarity and a size compatible with actinoporins. However, the reported characterization [116] is still preliminary and much work has to be done yet to include it within the actinoporins family.

Noticeably, actinoporins show weak structural similarity with domain 4 of perfringolysin O (Figure 10), a Chol-dependent PFT from *Clostridium perfringens*. This domain, which is responsible for the initial membrane recognition and binding of this

much larger toxin, lacks the region equivalent to the N-terminal structure of actinoporins, and thus pore-formation requires the intervention of some of its other additional domains [117]. It can be concluded then that the archetypical actinoporin β-sandwich fold is not simply a lipid-recognition motif but it can be also used by other proteins to bind ligands of very different nature and exert a variety of other biological functions apart from pore formation [10].

Eventually some other toxins have been considered ALPs but their classification into this group of proteins is not really established yet. A good example of this situation would be a pore-forming cytolytic protein isolated from the Northern red sea anemone, *Urticina crassicornis* [118]. This cytolysin, named UcI, is able to cause haemolysis as a result of a colloid-osmotic shock caused by the opening of toxin-induced ionic pores. It binds to lipid monolayers and efficiently permeabilizes small lipid vesicles composed of SM and Chol. However, its cytolytic activity is not prevented by preincubation with either any of these two lipids. Sequencing of its N-terminal revealed no sequence similarity with actinoporins but to UpI, another cytolytic protein isolated from a related sea anemone species, *Urticina piscivora*. Both proteins most probably belong to a separate family of sea anemone cytolysins that is pending of being characterized [118]. As a final example, toxins from sea anemones *Gyrostoma helianthus* and *Radianthus koseirensis* have been sometimes classified as actinoporins because they are inhibited by SM [119]. However, this inclusion is rather controversial due to their much smaller size (10 kDa), and should wait for a more detailed inspection of their molecular properties.

In summary, these observations altogether suggest that actinoporins structural motif displays ubiquity and some kind of structural conservation which may be useful to understand protein evolution. Indeed, it seems clear that the archetypal actinoporin fold is widespread and used for specific binding of different proteins to other molecules or surfaces apart from plasmatic membranes.

Therapeutic potential

Finally, similarly to many other toxins, studies focusing on the therapeutic potential of actinoporins abound. These include the characterization of their different pharmacological effects, their presumable anticancer activities, and their use in the

development of highly specific immunotoxins. Cardiostimulatory, anti-tumour, and anti-parasite activity have been reported for some of these toxins from sea anemones [2,24,120-123].

Some actinoporins have been shown to induce concentration dependent inhibition of gamma amino butyric acid and choline uptake into rat brain synaptosomes [124]. Ionotropic, myocardial contractility, hypotension, arrhythmia, and vascular permeability effects have been reported for rats and guinea pigs treated with different doses of actinoporins [125-128], mostly EqtII. Although sometimes transient favourable effects were observed, most of the animals finally died due to a yet unexplained cardio-respiratory arrest [121,127]. StnI and StnII have been shown to display different activities on molluscan nervous system and mammalian cardiac tissues [129]. The effect of the release of different ions such as K^+ or Ca^{2+} due to the administration of actinoporins has been studied without arriving to definitive conclusions about the effect of these toxins on the mammal's cardiovascular system [2,128,130-134].

Cytolysins show anticancer activity but by a mechanism still poorly understood [135-137]. For example, both Bc2, from *Bunodosoma caissarum*, and EqtII decrease U87 glioblastoma cells viability in a concentration dependent manner. Cell death is concomitant with results showing a highly altered membrane permeability leading to cell swelling, activation of different intracellular signalling pathways, and necrosis [136,137]. Actinoporin RTX-A from *H. crispa* has been shown to exhibit cancer preventive and anticancer cytotoxic properties, at low non-cytotoxic concentrations [135], through the induction of p53-independent apoptosis and inhibition of the oncogenic AP-1 and NF-κB nuclear factors activity.

Many viruses, including HIV-1, are suggested to bud from raft-like membrane microdomains [138,139]. Consequently, HIV-1 membrane is highly enriched in Chol, SM, and other raft lipids. Interestingly, treatment of infectious HIV-1 with EqtII showed differential inhibition of infectivity in a SM concentration-dependent manner [140]. However, pores were not being formed into the viral membrane despite complete inactivation of viral infectivity suggesting that inactivation was primarily being caused by the toxin binding to the viral membrane. While pore forming toxins certainly cannot serve in this case as drug candidates, the authors of this work [140] propose testing of

other lipophilic compounds which could specifically bind and alter HIV-1 membrane structure.

Actinoporins, or some fragments such as their N-terminal peptide, have also been used in the construction of immunotoxins or tumour protease-activated PFTs, targeted to different cell lines or the parasite *G. duodenalis* [24,521,141-147]. *Plasmodium falciparum* is another parasite which has been targeted by actinoporins. EqtII has been employed to lyse the limiting membrane of infected and uninfected erythrocytes [148], permeabilizing both cellular populations with similar efficiency but without disrupting the parasitophorus vacuole where the microorganism resides. This study opened the door to designing new approaches and tools aimed to the analysis of the behaviour of this human pathogen [148].

On a very different register, a GFP-modified version of EqtII has been shown to be an important probe for membrane SM [54] that can be used to monitor the subcellular distribution of this phospholipid. This is a new and important use of actinoporins taking into account that SM is the precursor of many important lipid signalling molecules, such as ceramide and sphingosine or their corresponding phosphorylated versions.

Perspectives

Much work has been done during the last decade in order to explain the behaviour of actinoporins at the water-membrane interface, but many more experiments are still required to fully understand the mechanisms underlying their biological function.

A very important question to be answered is why phase coexistence in the target membrane promotes pore formation and what the precise role of SM in this mechanism is. The use of many more lipid mixtures, containing different amounts of SM and Chol, and their lipid analogues, seems to be a wise line of research to be pursued. In fact, it would be also important to extend many of the experiments made with EqtII and StnII to other actinoporins to confirm the generality of the actual assumptions made about their mechanism. Thermodynamic studies can be of great help in elucidating the influence of temperature. Taking into account that sea anemones live in a rather cool

environment, answering this question would be of great importance to understand actinoporins biological function.

Studies targeting the regions of actinoporins whose role has not been assigned yet must be another path to be followed. For example: what are the residues involved in oligomerization? Is there synergy and/or cooperativity among the different regions involved in membrane attachment and oligomerization? Or, what are the determinants of the specific SM recognition? Many more detailed studies regarding the biophysical and structural properties of these proteins are still needed. Characterization of mutants affecting different stages of pore formation will be of the highest interest, as well as, again, the employment of different actinoporins. The wide variety of known proteins from this family, altogether with their high sequence conservation can be considered as a wide natural palette of variants to help in this study of their functionality.

However, the most important and still unanswered question refers to the detailed mechanism of pore formation, as well as the structure of the final functional pore. In this regard, the isolation of a stable pore assembly would be of great help in order to understand the precise role of lipids in the pore, or what is the exact length of the N-terminal helix in the pore. The isolation of water-soluble pore structures seems to be an obliged step in order to solve this problem. Eventually, the discovery of more potential non-conducive "prepore" intermediates would be of great utility to dissect the mechanism of pore formation at the molecular level and establish the final pore structure. Employment of water-soluble lipidic platforms, such as nanodiscs [149], with this purpose seems to be a promising approach. Micelles and bicelles of different nature and composition, although have to be used with caution, represent convenient platforms to mimic the lipidic environment of the final pre and pore structures. Solid-state NMR and cryo-EM stand out as additional useful tools to help unveiling the mechanism of pore formation by actinoporins. Such structural studies may be hindered by the possibility that the pore structure is not a unique entity, as suggested by the broad conductance distribution of actinoporins pores. Again, mutagenesis can be used as a method to "freeze" or isolate some of these intermediate states.

Many more studies must be aimed at searching the distribution of the actinoporins motif within the protein universe and the different roles played in the

protein families where it appears. Data mining of genomic and structural data bases must be performed in order to identify the presence of this motif. More experiments should be done in order to clarify if there is a general mechanism, based on protein-lipid interactions, underlying the toxicity of those ALPs with cytotoxic properties. Finally, it must not be forgotten either the potential use of actinoporins, or different modified versions, as diagnostic or therapeutic tools. Their inclusion as part of different immunoconjugates seems to be one of the most promising alternatives in this regard.

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References

[1] 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. Zoolog. A Comp. Exp. Biol. 305 (2006) 253-258.

[2] D. Šuput, *In vivo* effects of cnidarian toxins and venoms, Toxicon 54 (2009) 1190-1200.

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

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

[5] 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 (2007) 558-572.

[6] M.W. Parker, S.C. Feil, Pore-forming protein toxins: from structure to function, Prog. Biophys. Mol. Biol. 88 (2005) 91-142.

[7] B. Geny, M.R. Popoff, Bacterial protein toxins and lipids: Pore formation or toxin entry into cells, Biol. Cell. (2006) 667-678.

[8] M.R. González, M. Bischofberger, L. Pernot, F.G. van der Goot, B. Frêche, Bacterial pore-forming toxins: the (w)hole story?, Cell Mol. Life Sci. 65 (2008) 493-507.

[9] I. Iacovache, F.G. van der Goot, L. Pernot, Pore formation: an ancient yet complex form of attack, Biochim. Biophys. Acta 1778 (2008) 1611-1623.

[10] G. Anderluh, J.H. Lakey, Disparate proteins use similar architectures to damage membranes, Trends Biochem. Sci. 33 (2008) 482-490.

[11] 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 (2009) 726-730.

[12] M. Mueller, N. Ban, Enhanced SnapShot: Pore-forming toxins, Cell 142 (2010) 334.e1.

[13] R.H. Law, N. Lukoyanova, I. Voskoboinik, T.T. Caradoc-Davies, K. Baran, M.A. Dunstone, M.E. D'Angelo, E.V. Orlova, F. Coulibaly, S. Verschoor, K.A. Browne, A.

Ciccone, M.J. Kuiper, P.I. Bird, J.A. Trapani, H.R. Saibil, J.C. Whisstock, The structural basis for membrane binding and pore formation by lymphocyte perforin, Nature 468 (2010) 447-451.
[14] E. Gouaux, Channel-forming toxins: tales of transformation, Curr. Opin. Struct. Biol., 7 (1997) 566-573.
[15] 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 (1980) 203–211.
[16] 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.
[17] 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 (2001) 125–135.
[18] B. Bakrač, G. Anderluh, Molecular mechanism of sphingomyelin-specific membrane binding and pore formation by actinoporins, Adv. Exp. Med. Biol. 677 (2010) 106-115.
[19] P. Favreau, R. Stöcklin, Marine snail venoms: use and trends in receptor and channel neuropharmacology, Curr. Opin. Pharmacol. 9 (2009) 594-601.
[20] D. Butzke, A. Luch, High-molecular weight protein toxins of marine invertebrates and their elaborate modes of action, EXS 100 (2010) 213-232.
[21] 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 (1990) 1013–1024.
[22] 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 (1990) 243–249.
[23] R.S. Norton, Structures of sea anemone toxins, Toxicon 54 (2009) 1075-1088.
[24] M. Tejuca, G. Anderluh, P. Maček, R. Marcet, D. Torres, J. Sarracent, C. Alvarez, M.E. Lanio, M. Dalla Serra, G. Menestrina, Antiparasite activity of sea-anemone cytolysins on *Giardia duodenalis* and specific targeting with anti-*Giardia* antibodies, Int. J. Parasitol. 29 (1999) 489–498.

[25] 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 (1994) 205-227.
[26] 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 (1996) 14947–14957.
[27] 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 (2009) 869-880.
[28] M. Monastyrnaya, E. Leychenko, M. Isaeva, G. Likhatskaya, E. Zelepuga, E. Kostina, E. Trifonov, E. Nurminski, E. Kozlovskaya, Actinoporins from the sea anemones, tropical *Radianthus macrodactylus* and northern *Oulactis orientalis*: Comparative analysis of structure-function relationships, Toxicon 56 (2010) 1299-1314.
[29] G. Anderluh, I. Križaj, B. Štrukelj, F. Gubenšek, P. Maček, J. Pungerčar, Equinatoxins, pore-forming proteins from the sea anemone *Actinia equina*, belong to a multigene family, Toxicon 37(1999) 1391-1401.
[30] T. Turk, Cytolytic toxins from sea anemones,. J. Toxicol.-Toxin Rev. 10 (1991) 223–262.
[31] 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 haemolytic protein sticholysin II from the sea anemone *Stichodactyla helianthus*, Protein Expr. Purif. 18 (2000) 71-76.
[32] Y. Wang, L.L. Yap, K.L. Chua, H.E. Khoo, A multigene family of *Heteractis* magnificalysins (HMgs), Toxicon 51 (2008) 1374-1382.
[33] J. Alegre-Cebollada, G. Clementi, M. Cunietti, C. Porres, M. Oñaderra, J.G. Gavilanes, A. Martínez-del-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.
[34] G. Uechi, H. Toma, T. Arakawa, Y. Sato, Molecular characterization on the genome structure of hemolysin toxin isoforms isolated from sea anemone *Actineria villosa* and *Phyllodiscus semoni*, Toxicon 56 (2010), 1470-1476.

[35] B.M. Olivera, J. Rivier, C. Clark, C.A. Ramilo, G.P. Corpuz, F.C. Abogadie, E.E. Mena, S.R. Woodward, D.R. Hillyard, L.J. Cruz, Diversity of *Conus* neuropeptides, Science 249 (1990) 257–263.

[36] 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 (2001) 341-346.

[37] 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 (2002) 1219–1229.

[38] 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 (2003) 1319-1328.

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

[40] P. Malovrh, G. Viero, M. Della Serra, Z. Podlesek, J.H. Lakey, P. Maček, G. Menestrina, G. Anderluh, A novel mechanism of pore formation: membrane penetration by the N-terminal amphipathic region of equinatoxin, J. Biol. Chem. 278 (2003) 22678-22685.

[41] 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 (2010) 1959-1970.

[42] B. Bakrač, I. Gutiérrez-Aguirre, Z. Podlesek, A.F. Sonnen, R.J. Gilbert, P. Maček, J.H. Lakey, G. Anderluh, Molecular determinants of sphingomyelin specificity of a eukaryotic pore-forming toxin, J. Biol. Chem. 283 (2008) 18665-18677.

[43] 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 (2004) 14-18.

[44] 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 (2008) 920-930.

[45] 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 (2010) 69-72.
[46] 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. (2011), *in press: doi: 10.1111/j.1742-4658.2011.08123.x.*
[47] I. Castrillo, J. Alegre-Cebollada, A. Martínez-del-Pozo, J.G. Gavilanes, J. Santoro, M. Bruix, ^{1}H, ^{13}C, and ^{15}N NMR assignments of the actinoporin Sticholysin I, Biomol. NMR. Assign. 3 (2009) 5–7.
[48] M.R. de Planque, J.A. Kruijtzer, R.M. Liskamp, D. Marsh, D.V. Greathouse, R.E. Koeppe II, B. de Kruijff, J.A. Killian, Different membrane anchoring positions of tryptophan and lysine in synthetic transmembrane α-helical peptides, J. Biol. Chem. 274 (1999) 20839–20846.
[49] Q. Hong, I. Gutiérrez-Aguirre, A. Barlič, P. Malovrh, K. Kristan, Z. Podlesek, P. Maček, D. Turk, J.M. González-Mañas, 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.
[50] 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.
[51] G. Anderluh, A. Razpotnik, Z. Podlesek, P. Maček, F. Separovic, RS. Norton, Interaction of the eukaryotic pore-forming cytolysin equinatoxin II with model membranes: ^{19}F NMR studies, J. Mol. Biol. 347 (2005) 27–39.
[52] D. Pentón, V. Pérez-Barzaga, I. Díaz, M.L. Reytor, J. Campos, R. Fando, L. Calvo, E.M. Cilli, V. Morera, L.R. Castellanos-Serra, F. Pazos, M.E. Lanio, C. Alvarez, T. Pons, M. Tejuca, Validation of a mutant of the pore-forming toxin sticholysin-I for the construction of proteinase-activated immunotoxins, Protein Eng. Des. Sel. (2011) *in press*.
[53] 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 (2007) 3191-3201.

[54] 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 (2010) 22186-22195.
[55] K. Kristan, Z. Podlesek, V. Hojnik, I. Gutiérrez-Aguirre, G. Gunčar, D. Turk, J.M. González-Mañas, J.H. Lakey, P. Maček, G. Anderluh, Pore formation by equinatoxin, a eukaryotic pore-forming toxin, requires a flexible N-terminal region and a stable β-sandwich, J. Biol. Chem. 279 (2004) 46509–46517.
[56] G. Anderluh, J. Pungerčar, I. Križaj, B. Strukelj, F. Gubenšek, P. Maček, N-Terminal truncation mutagenesis of equinatoxin II, a pore-forming protein from the sea anemone *Actinia equina*, Protein Eng. 10 (1997) 751–755.
[57] F. Pazos, A. Valle, D. Martínez, A. Ramírez, L. Calderón, A. Pupo, M. Tejuca, V. Morera, J. Campos, R. Fando, F. Dyszy, S. Schreier, E. Horjales, C. Álvarez, M.E. Lanio, E. Lissi, Structural and functional characterization of a recombinant sticholysin I (rSt I) from the sea anemone *Stichodactyla helianthus*, Toxicon 48 (2006) 1083-1094.
[58] E.M. Cilli, F.T. Pigossi, E. Crusca Jr., U. Ros, D. Martínez, M.E. Lanio, C. Álvarez, S. Schreier, Correlations between differences in amino-terminal sequences and different haemolytic activity of sticholysins, Toxicon 50 (2007) 1201-1204.
[59] F. Casallanovo, F.J. de Oliveira, F.C. de Souza, U. Ros, Y. Martínez, D. Pentón, M. Tejuca, D. Martínez, F. Pazos, T.A. Pertinhez, A. Spisni, E.M. Cilli, M.E. Lanio, C. Alvarez, S. Schreier, Model peptides mimic the structure and function of the N-terminus of the pore-forming toxin sticholysin II, Biopolymers 84 (2006) 169–180.
[60] A. Drechsler, C. Potrich, J.K. Sabo, M. Frisanco, G. Guella, M. Dalla Serra, G. Anderluh, F. Separovic, R.S. Norton, Structure and activity of the N-terminal region of the eukaryotic cytolysin equinatoxin II, Biochemistry 45 (2006) 1818–1828.
[61] A. Drechsler, A.J. Miles, R.S. Norton, B.A. Wallace, F. Separovic, Effect of lipid on the conformation of the N-terminal region of equinatoxin II: a synchrotron radiation circular dichroism spectroscopic study, Eur. Biophys. J. 39 (2009) 121–127.
[62] Y.H. Lam, A. Hung, R.S. Norton, F. Separovic, A. Watts, Solid-state NMR and simulation studies of equinatoxin II N-terminus interaction with lipid bilayers, Proteins 78 (2010) 858-872.
[63] A. Drechsler, G. Anderluh, R.S. Norton, F. Separovic, Solid-state NMR study of membrane interactions of the pore-forming cytolysin, equinatoxin II, Biochim. Biophys. Acta 1798 (2010) 244-251.

[64] A.J. Miles, A. Drechsler, K. Kristan, G. Anderluh, R.S. Norton, B.A. Wallace, F. Separovic, The effects of lipids on the structure of the eukaryotic cytolysin equinatoxin II: a synchrotron radiation circular dichroism spectroscopic study, Biochim. Biophys. Acta 1778 (2008) 2091–2096.

[65] 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 (2007) 539-550.

[66] I.F. Pazos, D. Martínez, M. Tejuca, A. Valle, A. Martínez-del-Pozo, C. Alvarez, M.E. Lanio, E.A. Lissi, Comparison of pore-forming ability in membranes of a native and a recombinant variant of sticholysin II from *Stichodactyla helianthus*, Toxicon 42 (2003) 571–578.

[67] G. Menestrina, V. Cabiaux, M. Tejuca, Secondary structure of sea anemone cytolysins in soluble and membrane bound form by infrared spectroscopy, Biochem. Biophys. Res. Commun. 254 (1999) 174–180.

[68] N. Poklar, J. Fritz, P. Maček, G. Vesnaver, T.V. Chalikian, Interaction of the pore-forming protein equinatoxin II with model lipid membranes: A calorimetric and spectroscopic study, Biochemistry 38 (1999) 14999-5008.

[69] E. Leininger, M. Roberts, J.G. Kenimer, I.G. Charles, N. Fairweather, P. Novotny, M.J. Brennan, Pertactin, an Arg–Gly–Asp-containing *Bordetella pertussis* surface protein that promotes adherence of mammalian cells, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 345–349.

[70] E. Ruoslahti, RGD and other recognition sequences for integrins, Annu. Rev. Cell Dev. Biol. 12 (1996) 697–715.

[71] G. Anderluh, P. Maček, Dissecting the actinoporin pore-forming mechanism, Structure 11 (2003) 1312-1313.

[72] 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 555 (1979) 79-88.

[73] 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 haemolytic protein sticholysin II from the sea anemone *Stichodactyla helianthus*, Eur. J. Biochem. 252 (1998) 284–289.

[74] C. Álvarez-Valcarcel, M. Serra, C. Patrich, I. Bernhart, M. Tejuca, D. Martínez, 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.

[75] D. Martínez, A. Otero, C. Alvarez, F. Pazos, M. Tejuca, M.E. Lanio, I. Gutiérrez-Aguirre, A. Barlič, I. Iloro, J.L. Arrondo, J.M. González-Mañas, E. Lissi, Effect of sphingomyelin and cholesterol on the interaction of St II with lipidic interfaces, Toxicon 49 (2007) 68–81.

[76] 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 (2001)1343–1353.

[77] B.B. Bonev, Y.H. Lam, G. Anderluh, A. Watts, R.S. Norton, F. Separovic, Effects of the eukaryotic pore-forming cytolysin equinatoxin II on lipid membranes and the role of sphingomyelin, Biophys. J. 84 (2003) 2382–2392.

[78] P. Schön, A.J. García-Sáez, 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.

[79] 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 (2006) 863-871.

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

[81] 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 (2009) 1135-1147.

[82] 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 (2003) 45216–45223.

[83] C. Álvarez, F. Casallanovo, C.S. Shida, L.V. Nogueira, D. Martínez, M. Tejuca, I.F. Pazos, M.E. Lanio, G. Menestrina, E. Lissi, S. Schreier, Binding of sea anemone

pore-forming toxins sticholysins I and II to interfaces--modulation of conformation and activity, and lipid-protein interaction, Chem. Phys. Lipids 122 (2003) 97-105.
[84] R. Veitia, M. Tejuca, C. Alvarez, M.E. Lanio, F. Pazos, Kinetic of a haemolysis induced by a cytolysin from *Stichodactyla helianthus*: Effect of temperature and divalent cations,. Biología 9 (2005) 15–22.
[85] G. Celedón, G. González, E. Lissi, T. Cerda, D. Martinez, C. Soto, M. Pupo, F. Pazos, M.E. Lanio, C. Alvarez, Effect of calcium on the haemolytic activity of *Stichodactyla helianthus* toxin sticholysin II on human erythrocytes, Toxicon 54 (2009) 845-850.
[86] G. Celedón, G. González, E. Lissi, T. Cerda, D. Bascuñant, M. Lepeley, F. Pazos, M.E. Lanio, C. Álvarez, Effect of pre-exposure of human erythrocytes to oxidants on the haemolytic activity of Sticholysin II. A comparison between peroxynitrite and hypochlorous acid, Free Radic. Res. 45 (2011) 400-408.
[87] 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 (1995) 348-354.
[88] A. Valle, A. López-Castilla, L. Pedrera, D. Martínez, M. Tejuca, J. Campos, R. Fando, E. Lissi, C. Álvarez, M.E. Lanio, F. Pazos, S. Schreier, Cys mutants in functional regions of Sticholysin I clarify the participation of these residues in pore formation, Toxicon (2011) *in press.*
[89] 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 (2000) 3186-3194.
[90] J.M. Mancheño, J. Martín-Benito, J.G. Gavilanes, L. Vázquez, A complementary microscopy analysis of Sticholysin II crystals on lipid films: Atomic force and transmission electron characterizations, Biophys. Chem. 119 (2006) 219-223.
[91] I. Gutiérrez-Aguirre, A. Barli, Z. Podlesek, P. Maček, G. Anderluh, J.M. González-Mañas, Membrane insertion of the N-terminal α-helix of equinatoxin II, a sea anemone cytolytic toxin, Biochem. J. 384 (2004) 421-428.
[92] A.P. Heuck, R.K. Tweten, A.E. Johnson, β-Barrel pore-forming toxins: Intriguing dimorphic proteins, Biochemistry 40 (2001) 9065–9073.

[93] V.E. Bychkova, R.H. Pain, O.B. Ptitsyn, The 'molten globule' state is involved in the translocation of proteins across membranes?, FEBS Lett. 238 (1988) 231-234.
[94] N. Poklar, F. Lah, M. Salobir, P. Maček, G. Vesnaver, pH and temperature-induced molten globule-like denatured states of equinatoxin II: A study by UV-melting, DSC, far- and near-UV CD spectroscopy, and ANS fluorescence, Biochemistry 36 (1997) 14345-14352.
[95] J.M. Mancheño, V. de los Ríos, A. Martínez-del-Pozo, M.E. Lanio, M. Oñaderra, J.G Gavilanes, Partially folded states of the cytolytic protein sticholysin II, Biochim. Biophys. Acta, 1545 (2001) 122-131.
[96] N.P. Ulrih, G. Anderluh, P. Maček, T.V. Chalikian, Salt-induced oligomerization of partially folded intermediates of equinatoxin II, Biochemistry 43 (2004) 9536-9545.
[97] K.C. Kristan, G. Viero, M. Dalla Serra, P. Maček, G. Anderluh, Molecular mechanism of pore formation by actinoporins, Toxicon 54 (2009) 1125-1134.
[98] 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 (1999) 27-30.
[99] S. Leavitt, E. Freire, Direct measurement of protein binding energetics by isothermal titration calorimetry, Curr. Opin. Struct. Biol. 11 (2001) 560–566.
[100] V. Huerta, V. Morera, Y. Guanche, G. Chinea, L.J. González, L. Betancourt, D. Martínez, C. Álvarez, M.E. Lanio, V. Besada, Primary structure of two cytolysin isoforms from *Stichodactyla helianthus* differing in their haemolytic activity, Toxicon 39 (2001) 1253–1256.
[101] I. Gutiérrez-Aguirre, P. Trontelj, P. Maček, J.H. Lakey, G. Anderluh, Membrane binding of zebrafish actinoporin-like protein: AF domains, a novel superfamily of cell membrane binding domains, Biochem. J. 398 (2006) 381-392.
[102] 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 (2009) 502-510.
[103] V. Trigueros, A. Lougarre, D. Ali-Ahmed, Y. Rahbe, J. Guillot, L. Chavant, D. Fournier, L. Paquereau, *Xerocomus chrysenteron* lectin: identification of a new pesticidal protein, Biochim. Biophys. Acta 1621 (2003) 292–298.

[104] C. Marty-Detraves, F. Francis, L. Baricault, D. Fournier, L. Paquereau, Inhibitory action of a new lectin from *Xerocomus chrysenteron* on cell-substrate adhesion, Mol. Cell Biochem. 258 (2004) 49–55.

[105] C. Birck, L. Damian, C. Marty-Detraves, A. Lougarre, C. Schulze-Briese, P. Koehl, D. Fournier, L. Paquereau, J.P. Samama, A new lectin family with structure similarity to actinoporins revealed by the crystal structure of *Xerocomus chrysenteron* lectin XCL, J. Mol. Biol. 344 (2004) 1409-1420.

[106] M.E. Carrizo, S. Capaldi, M. Perduca, F.J. Irazoqui, G.A. Nores, H.L. Monaco, The antineoplastic lectin of the common edible mushroom (*Agaricus bisporus*) has two binding sites, each specific for a different configuration at a single epimeric hydroxyl, J. Biol. Chem. 280 (2005), 10614-10623.

[107] L. Damian, D. Fournier, M. Winterhalter, L. Paquereau, Determination of thermodynamic parameters of *Xerocomus chrysenteron* lectin interactions with N-acetylgalactosamine and Thomsen-Friedenreich antigen by isothermal titration calorimetry. BMC Biochem. 6 (2005) 11.

[108] C. Ottmann, B. Luberacki, I. Küfner, W. Koch, F. Brunner, M. Weyand, L. Mattinen, M. Pirhonen, G. Anderluh, H.U. Seitz, T. Nürnberger, C. Oecking, A common toxin fold mediates microbial attack and plant defence, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 10359-10364.

[109] C.L. Pemberton, G.P.C. Salmond, The Nep1-like proteins: A growing family of microbial elicitors of plant necrosis. Mol. Plant Pathol. 5 (2004) 353–359.

[110] S. Kamoun, A catalogue of the effector secretome of plant pathogenic oomycetes, Annu. Rev. Phytopathol. 44 (2006) 41–60.

[111] M. Gijzen, T. Nürnberger, Nep1-like proteins from plant pathogens: Recruitment and diversification of the NPP1 domain across taxa, Phytochemistry 67 (2006) 1800–1807.

[112] H. Bae, M. Kim, R. Sicher, H.J. Bae, B. Bailey, Necrosis- and ethylene-inducing peptide from *Fusarium oxysporum* induces a complex cascade of transcripts associated with signal transduction and cell death in *Arabidopsis*, Plant Physiol. 141 (2006)1056–1067.

[113] D. Qutob, B. Kemmerling, F. Brunner, I. Küfner, S. Engelhardt, A.A. Gust, B. Luberacki, H.U. Seitz, D. Stahl, T. Rauhut, E. Glawischnig, G. Schween, B. Lacombe, N. Watanabe, E. Lam, R. Schlichting, D. Scheel, K. Nau, G. Dodt, D. Hubert, M.

Gijzen, T. Nürnberger, Phytotoxicity and innate immune responses induced by Nep1-like proteins,. Plant Cell 18 (2006) 3721-3744.
[114] G. Fellbrich, A. Romanski, A. Varet, B. Blume, F. Brunner, S. Engelhardt, G. Felix, B. Kemmerling, M. Krzymowska, T. Nürnberger, NPP1, a *Phytophthora*-associated trigger of plant defence in parsley and *Arabidopsis*,. Plant J. 32 (2002) 375-390.
[115] K. Gunji, S. Ishizaki, K. Shiomi, Cloning of complementary and genomic DNAs encoding echotoxins, proteinaceous toxins from the salivary gland of marine gastropod *Monoplex echo*, Protein J. 29 (2010) 487-492.
[116] T. Takara, T. Nakagawa, M. Isobe, N. Okino, S. Ichinose, A. Omori, M. Ito, Purification, molecular cloning, and application of a novel sphingomyelin-binding protein (clamlysin) from the brackishwater clam, *Corbicula japonica*, Biochim. Biophys. Acta. 1811 (2011) 323-332.
[117] R. Ramachandran, R.K. Tweten, A.E. Johnson, Membrane-dependent conformational changes initiate cholesterol-dependent cytolysin oligomerization and intersubunit β-strand alignment, Nat. Struct. Mol. Biol. 11 (2004) 697-705.
[118] A. Razpotnik, I. Krizaj, W.R. Kem, P. Maček, T. Turk, A new cytolytic protein from the sea anemone *Urticina crassicornis* that binds to cholesterol- and sphingomyelin-rich membranes, Toxicon 53 (2009) 762-769.
[119] D. Mebs, M. Liebrich, A. Reul, Y. Samejima, Hemolysins and proteinase inhibitors from sea anemones of the Gulf of Aqaba, Toxicon 21 (1983) 257-264.
[120] M. Thomson, R.L. Moritz, R.J. Simpson, R.S. Norton, Tenebrosin-A, a new cardiostimulant protein from the Australian sea anemone *Actinia tenebrosa*, Biochem. Int. 15 (1987) 711–718.
[121] R.S. Norton, G. Bobek, J.O. Ivanov, M. Thomson, E. Fiala-Beer, R.L. Moritz, R.J. Simpson, Purification and characterization of proteins with cardiac stimulatory and haemolytic activity from the anemone *Actinia tenebrosa*, Toxicon 28 (1990) 29–41.
[122] R.J. Simpson, G.E. Reid, R.L. Moritz, C. Morton, R.S. Norton, Complete amino acid sequence of tenebrosin-C, a cardiac stimulatory and haemolytic protein from the sea anemone *Actinia tenebrosa*, Eur. J. Biochem. 190 (1990) 319–328.
[123] M.M. Monastyrnaya, T.A. Zykova, O.V. Apalikova, T.V. Shwets, E.P. Kozlovskaya, Biologically active polypeptides from the tropical sea anemone *Radianthus macrodactylus*, Toxicon 40 (2002) 1197–1217.

[124] H.E. Khoo, J.P. Lim, C.H. Tan, Effects of sea anemone (*Heteractis magnifica* and *Actinia equina*) cytolysins on synaptosomal uptake of GABA and choline, Toxicon, 33 (1995) 1365-1371.

[125] 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 (1984) 347–352.

[126] M. Budihna, P. Maček, D. Šuput, Effects of equinatoxin II on the isolated guinea-pig heart, Eur. J. Pharmacol. 5 (1990) 2051–2052.

[127] R.S. Norton, P. Maček, G.E. Reid, R.J. Simpson, Relationship between the cytolysins tenebrosin-C from *Actinia tenebrosa* and equinatoxin II from *Actinia equina*, Toxicon 30 (1992) 13–23.

[128] M. Bunc, G. Drevensek, M. Budihna, D. Šuput, Effects of equinatoxin II from *Actinia equina* (L.) on isolated rat heart: the role of direct cardiotoxic effects in equinatoxin II lethality, Toxicon 37 (1999a) 109–123.

[129] T. García, D. Martinez, A. Palmero, C. Soto, M. Tejuca, F. Pazos, R. Menéndez, C. Alvarez, A. Garateix, Pharmacological effects of two cytolysins isolated from the sea anemone Stichodactyla helianthus, J. Biosci. 34 (2009) 891-898.

[130] G. Drevensek, M. Bunc, M.V. Budihna, D. Šuput, Lowering of the coronary flow in isolated rat heart by equinatoxin II depends upon extracellular Ca^{2+} concentration, Pflugers. Arch. 439 (2000) R150–R151.

[131] G. Drevensek, S. Kirbis, M. Bunc, M. Zitko, M.V. Budihna, D. Šuput, Tezosentan inhibits both equinatoxin II and endotelin-1 induced contractions of isolated porcine coronary artery in a similar way, J. Nat. Toxins 11 (2002) 231–244.

[132] F.A. Meunier, R. Frangez, E. Benoit, G. Ouanounou, B. Rouzaire-Dubois, D. Šuput, J. Molgo, Ca^{2+} and Na^{+} contribute to the swelling of differentiated neuroblastoma cells induced by equinatoxin-II, Toxicon 38 (2000), 1547–1560.

[133] M. Šentjurc, A. Štalc, D. Šuput, Influence of equinatoxin II on coronary smooth muscle membrane fluidity, Pflugers Arch. 431 (1996) R317–318.

[134] D. Šuput, R. Frangez, M. Bunc, Cardiovascular effects of equinatoxin III from the sea anemone *Actinia equina* (L.), Toxicon 39 (2001), 1421–1427.

[135] S. Fedorov, S. Dyshlovoy, M. Monastyrnaya, L. Shubina, E. Leychenko, E. Kozlovskaya, J.O. Jin, J.Y. Kwak, A.M. Bode, Z. Dong, V. Stonik, The anticancer

effects of actinoporin RTX-A from the sea anemone *Heteractis crispa* (=*Radianthus macrodactylus*), Toxicon 55 (2010) 811-817.
[136] R.C. Soletti, G.P. de Faria, J. Vernal, H. Terenzi, G. Anderluh, H.L. Borges, V. Moura-Neto, N.H. Gabilan, Potentiation of anticancer-drug cytotoxicity by sea anemone pore-forming proteins in human glioblastoma cells, Anticancer Drugs 19 (2008) 517-525.
[137] R.C. Soletti, T. Alves, J. Vernal, H. Terenzi, G. Anderluh, H.L. Borges, N.H. Gabilan, V. Moura-Neto, Inhibition of MAPK/ERK, PKC and CaMKII signalling blocks cytolysin-induced human glioma cell death, Anticancer Res. 30 (2010) 1209-1215.
[138] D.H. Nguyen, J.E. Hildreth, Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts, J. Virol. 74 (2000) 3264-3272.
[139] A. Ono, E.O. Freed, Plasma membrane rafts play a critical role in HIV-1 assembly and release, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 13925-13930.
[140] M. Lorizate, B. Brügger, H. Akiyama, B. Glass, B. Müller, G. Anderluh, F.T. Wieland, H.G. Kräusslich, Probing HIV-1 membrane liquid order by Laurdan staining reveals producer cell-dependent differences, J. Biol. Chem. 284 (2009) 22238-22247.
[141] A.D. Avila, M.C. de Acosta, A. Lage, A new immunotoxin built by linking a haemolytic toxin to a monoclonal antibody specific for immature T lymphocytes, Int. J. Cancer 42 (1988) 568–571.
[142] A.D. Avila, M.C. de Acosta, A. Lage, A carcinoembryonic antigen directed immunotoxin built by linking a monoclonal antibody to a haemolytic toxin, Int. J. Cancer 43 (1989) 926–929.
[143] C. Pederzolli, G. Belmonte, M. Dalla Serra, P. Maček, G. Menestrina, Biochemical and cytotoxic properties of conjugates of transferrin with equinatoxin II, a cytolysin from a sea anemone, Bioconjug. Chem. 6 (1995) 166–173.
[144] M. Tejuca, I. Díaz, R. Figueredo, L. Roque, F. Pazos, D. Martínez, N. Iznaga-Escobar, R. Pérez, C. Álvarez, M.E. Lanio, Construction of an immunotoxin with the pore forming protein StI and ior C5, a monoclonal antibody against a colon cancer cell line,. Int. Immunopharmacol. 6 (2004) 731–744.
[145] M. Tejuca, G. Anderluh, M. Dalla Serra, Sea anemone cytolysins as toxic components of immunotoxins, Toxicon 54 (2009) 1206-1214.

[146] C. Potrich, G. Viero, M. Tejuca, G. Anderluh, P. Maček, G. Menestrina, Construction of new immunotoxins by linking equinatoxin II to monoclonal antibodies via the biotin–avidin interaction. Cytotoxic effects on human tumour cells, Acta Biol. Slov. 43 (2000) 47–51.
[147] C. Potrich, R. Tomazzolli, M. Dalla Serra, G. Anderluh, P. Malovrh, P. Maček, G. Menestrina, M. Tejuca, Cytotoxic activity of a tumor protease-activated pore-forming toxin,. Bioconjug. Chem. 2 (2005) 369–376.
[148] K.E. Jackson, T. Spielmann, E. Hanssen, A. Adisa, F. Separovic, M.W. Dixon, K.R. Trenholme, P.L. Hawthorne, D.L. Gardiner, T. Gilberger, L. Tilley, Selective permeabilization of the host cell membrane of *Plasmodium falciparum*-infected red blood cells with streptolysin O and equinatoxin II, Biochem. J. 403 (2007) 167-175.
[149] T.H. Bayburt, S.G. Sligar, Membrane protein assembly into Nanodiscs, FEBS Lett. 3 (2010) 1721-1727.
[150] R. Koradi, M. Billeter, K. Wüthrich, MOLMOL: a program for display and analysis of macromolecular structures, J. Mol. Graph. 14 (1996) 29-32.
[151] W. Humphrey, A. Dalke, K. Schulten, VMD: visual molecular dynamics, J. Mol. Graph. 14 (1996) 33-38, 27-28.).

Figures

Figure 1.- Sequence alignment of all known primary structures from different sea anemone actinoporins. Eqt, equinatoxins from *Actinia equina*; Stn, sticholysins from *Stichodactyla helianthus*; Ten, tenebrosins from *Actinia tenebrosa*; Hmg and magnificalysin, magnificalysins from *Heteractis magnifica*; RTX, actinoporins from *Radianthus macrodactylus*; SrcI, acidic cytolysin from *Sagartia rosea*, AvtI, actinoporin from *Actineria villosa*; PSTX-20A, actinoporin from *Phyllodiscus semoni*; Caritoxin, actinoporin from *Actinia cari*; Ent, actinoporin from *Entacmea quadricolor*; SmT, actinoporins from *Stichodactyla mertensii*; Or, actinoporins from *Oulactis orientalis*; FraC, fragaceatoxin C from *Actinia fragacea*. It must be noted that EqtII and TenC have the same sequence. Black, grey, or white boxes indicate the different degree of conservation, in descending order, along all the reported actinoporins-sequences.

Figure 2.- Ribbon representation of the X-ray structures of EqtII (pdb 1IAZ) and StnII (pdb: 1GWY). The N- and C-ends are labelled. A) EqtII is shown in yellow and StnII in blue. B) Two views of the backbone superposition of EqtII and StnII rotated 180º are represented too. Figure prepared with MolMol [150].

Figure 3.- Diagram showing the distribution of the regular secondary structure elements along the StnII amino acid sequence (grey, α-helix; green, β-strand). The N-terminal 30 first residues are also indicated (red background) as well as those ones conforming the cluster of aromatic amino acids (blue letters with black background and white letters with blue background), the POC-binding site (black background), and the array of basic amino acids (white letters with green background).

Figure 4.- Ribbon representation showing the POC-binding site within the three-dimensional structure of StnII (pdb: 1GWY 1O72). This site is composed by the side-chains of residues Arg-51, Ser-52, Val-85, Ser-103, Pro-105, Tyr-111 (shown in green), Tyr-131, Tyr-135, and Tyr-136. A molecule of POC is also shown. Figure produced by VMD [151].

Figure 5.- Diagram showing the exposed cluster of aromatic amino acids within the three-dimensional structure of StnII (pdb: 1GWY). This cluster is composed by residues Phe-106, Trp-110, Tyr-111, Trp-114, Tyr-131, Tyr-135, and Tyr-136. The backbone is represented by a ribbon. The side-chains of selected amino acids are shown in blue

(Phe-106, Trp-114, Tyr-131, Tyr-135, and Tyr-136) and gold (Trp 110 and Tyr-111). Figure generated by MolMol [150].

Figure 6.- Diagram showing the protein surface (grey) with the relative location of the array of basic amino acids within the three-dimensional structure of StnII (pdb: 1GWY). This array is made of StnII residues Lys-118, Ser-121, Lys-123, Arg-124, Arg-125, Lys-149, Arg-156, and Arg-175. The side-chains corresponding to these residues are shown in blue. The backbone is represented by a ribbon with the α-helices in red and the β-strands in cyan. Figure produced by PyMol (DeLano Scientific LLC).

Figure 7.- Representation of the solution structure of a synthetic peptide corresponding to the first 30 amino acids of StnI [40]. Superposition of the backbone atoms of the 25 structures obtained by NMR in 30% TFE (A) or DPC micelles (B). One conformer of each family is represented by a ribbon.

Figure 8.- Scheme showing different steps of the most accepted mechanism of pore formation by actinoporins, exemplified by the proposal that StnII assembles into a tetrameric toroidal pore [5]. M_S: water soluble monomer. T_S: water soluble tetramer. The amount of monomers forming the pore, as well as the particular step where oligomerization occurs, are still matter of study. M_m: monomer bound to the membrane. T_1 and T_2: tetrameric non-conductive lipid-bound forms. P: tetrameric pore. The conformational changes involving detachment and extension of the N-terminal α-helix would take place along the $M_m \leftrightarrow T_1 \leftrightarrow T_2$ transitions.

Figure 9.- Diagram showing the three-dimensional structures of XCL (pdb: 1XIO), ABL (pdb: 1Y2T), and StnII (pdb: 1GWY). Images were generated with MolMol [150].

Figure 10.- Diagram showing the three-dimensional structures of Perfringolysin O (PFO; pdb: 1M3J) and its domain 4 (PFO d4;) and StnII. Images were generated with MolMol [150].

Figure 1

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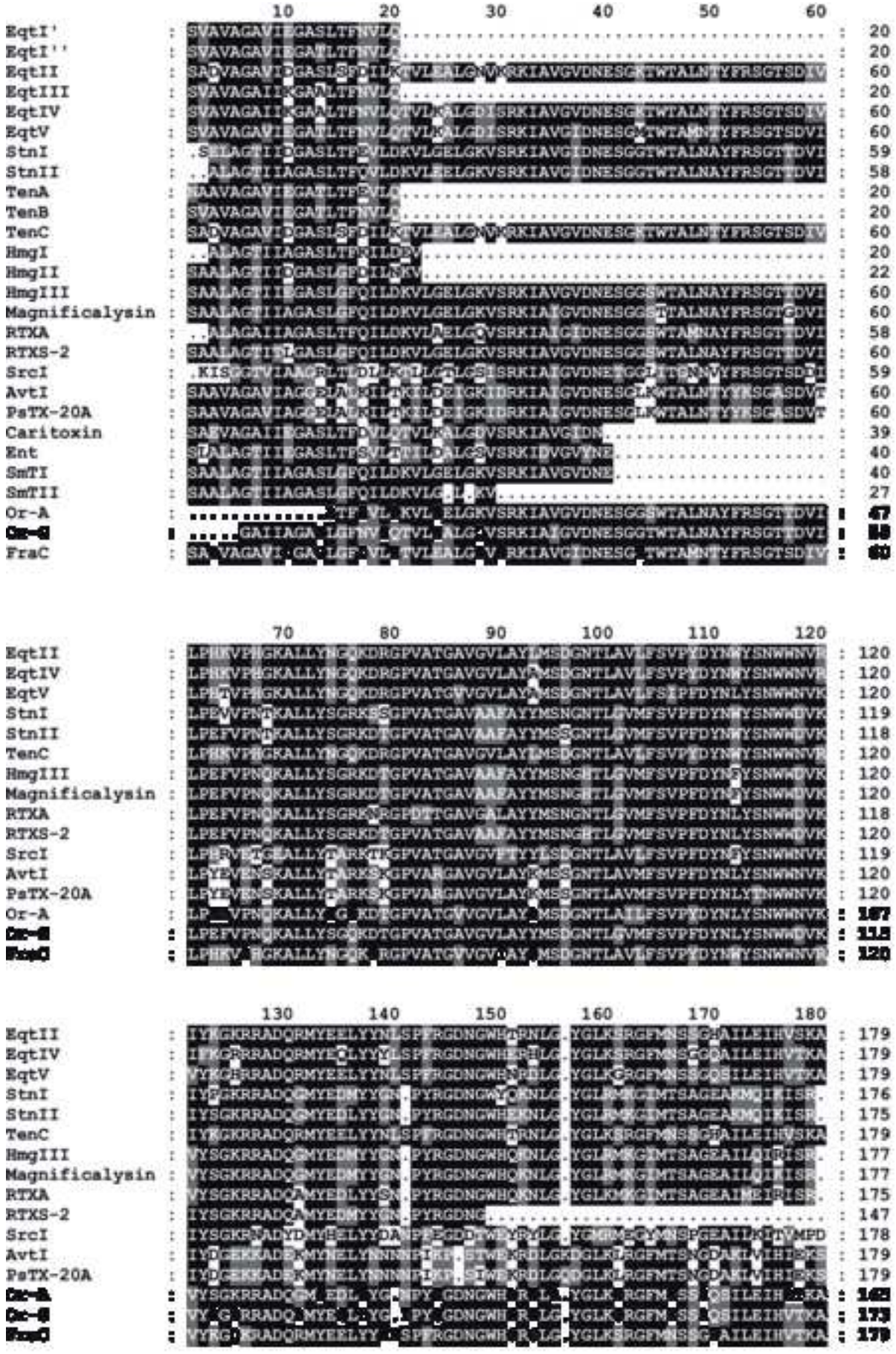

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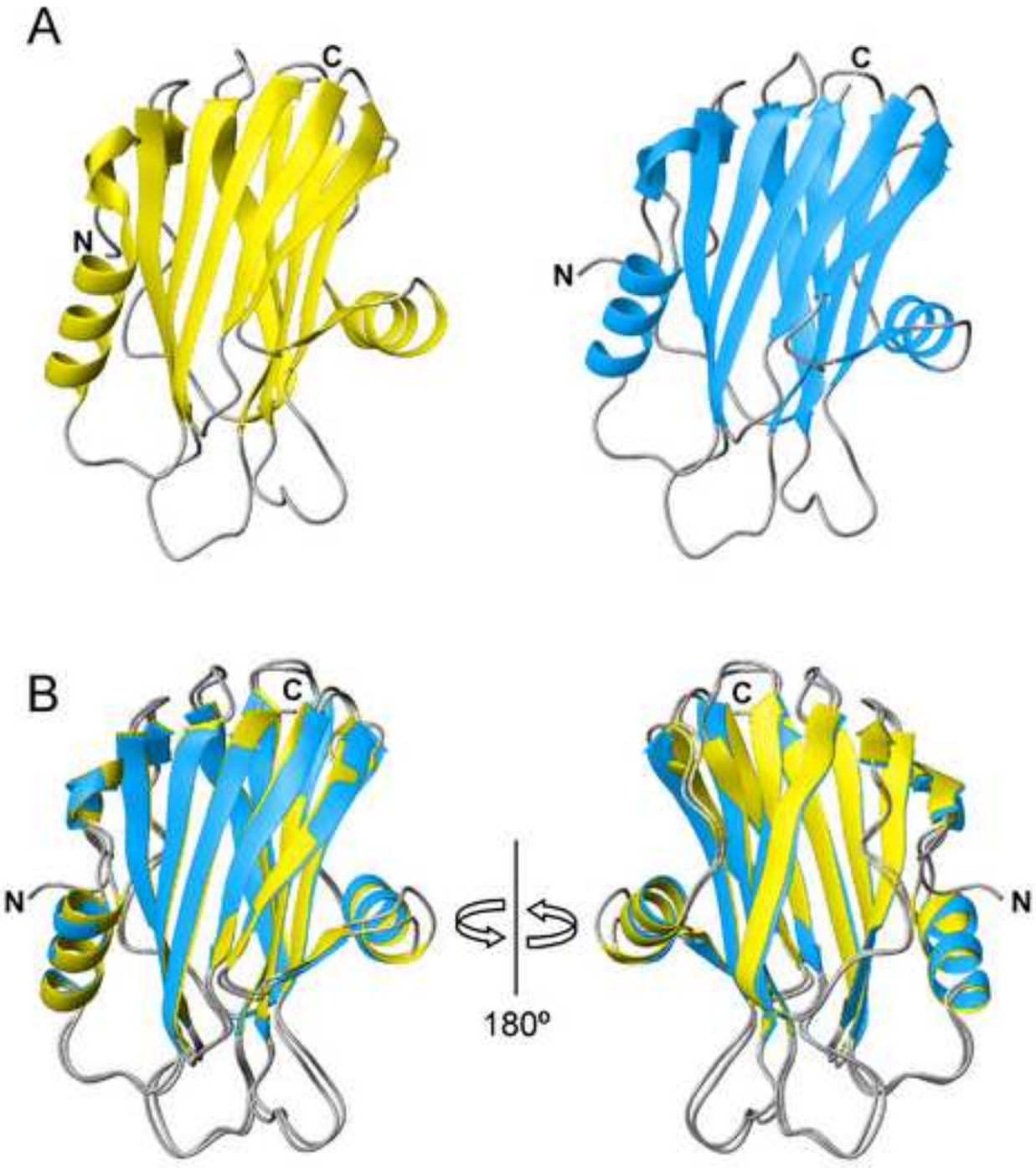

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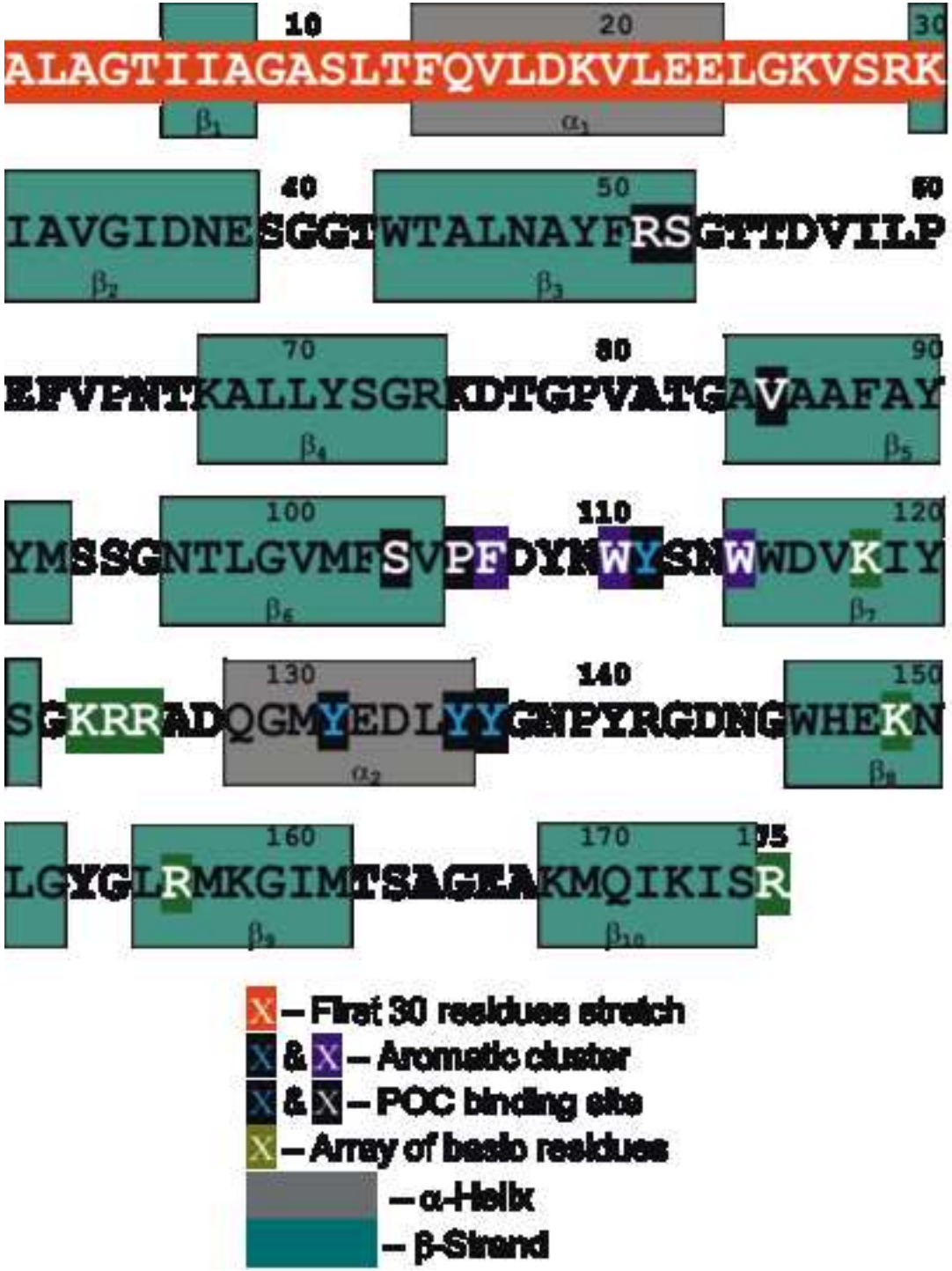

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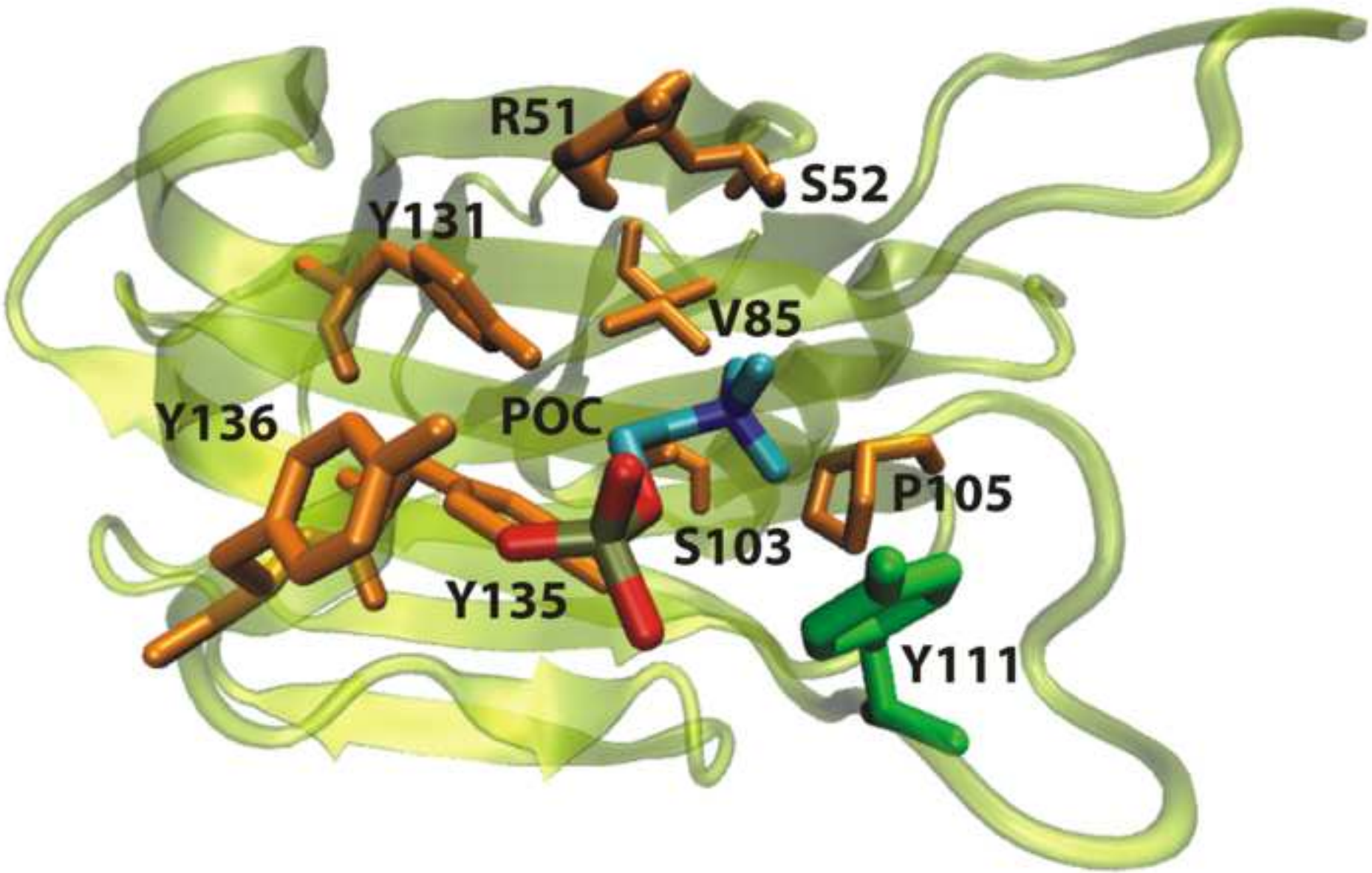

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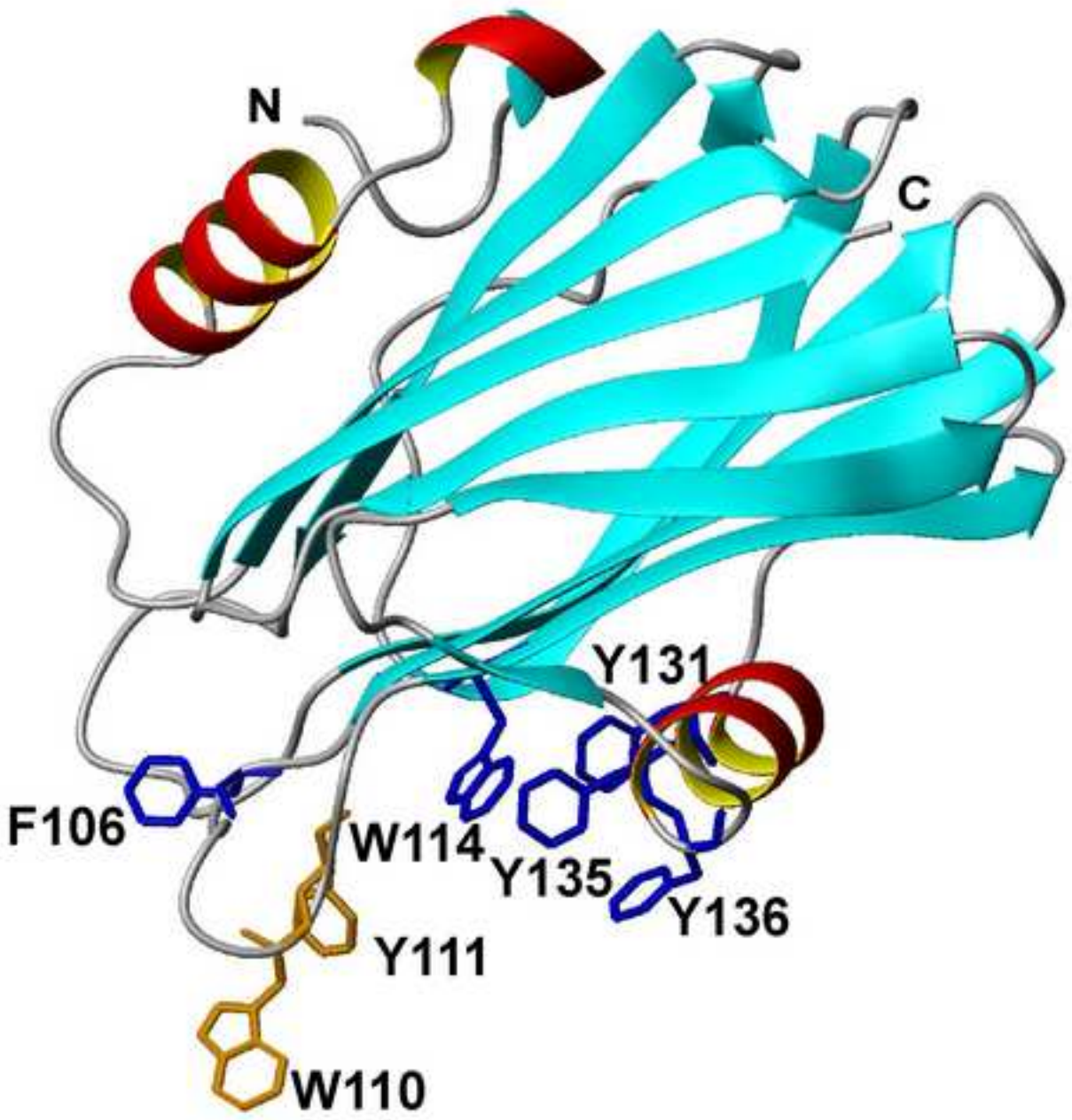

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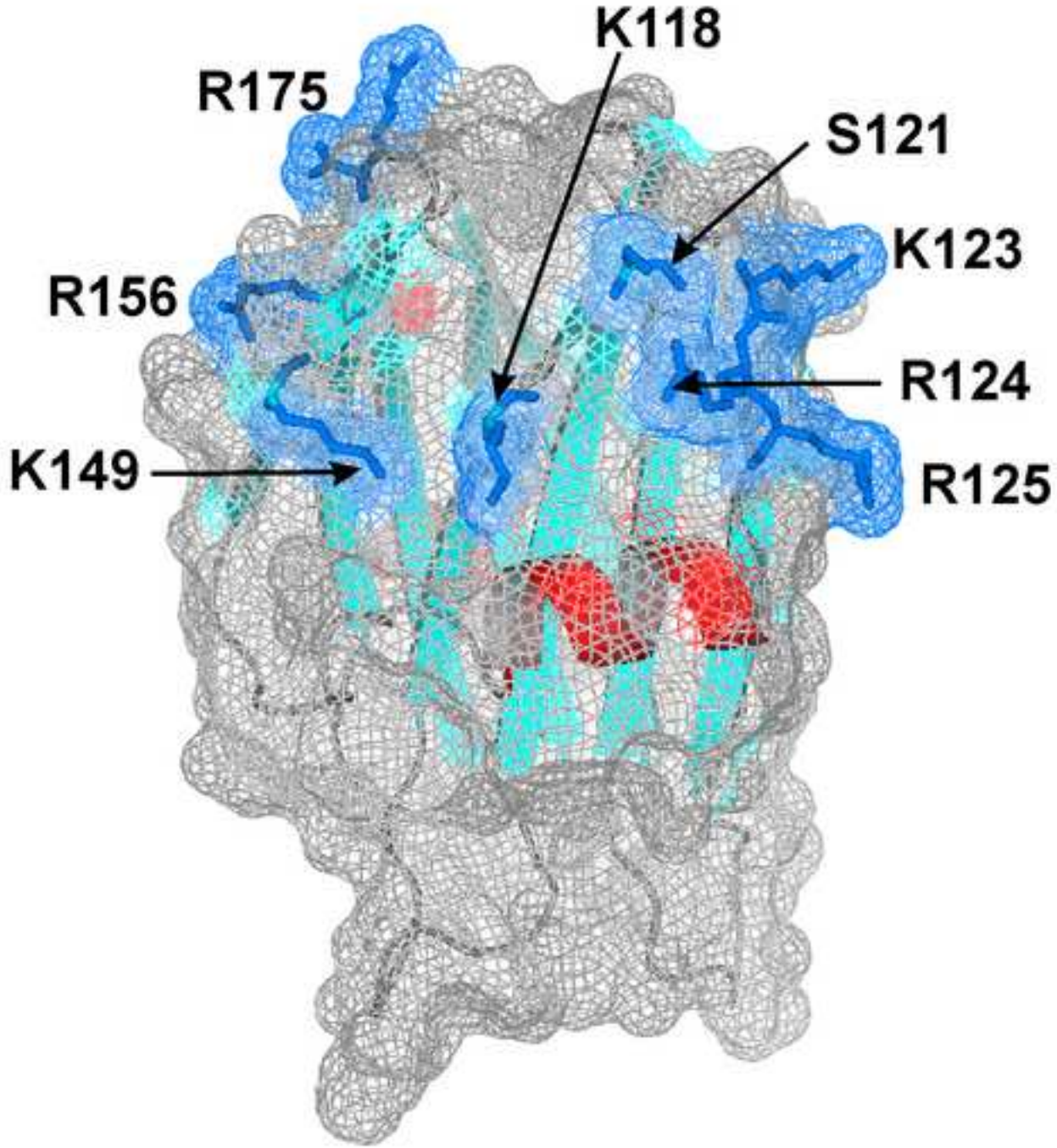

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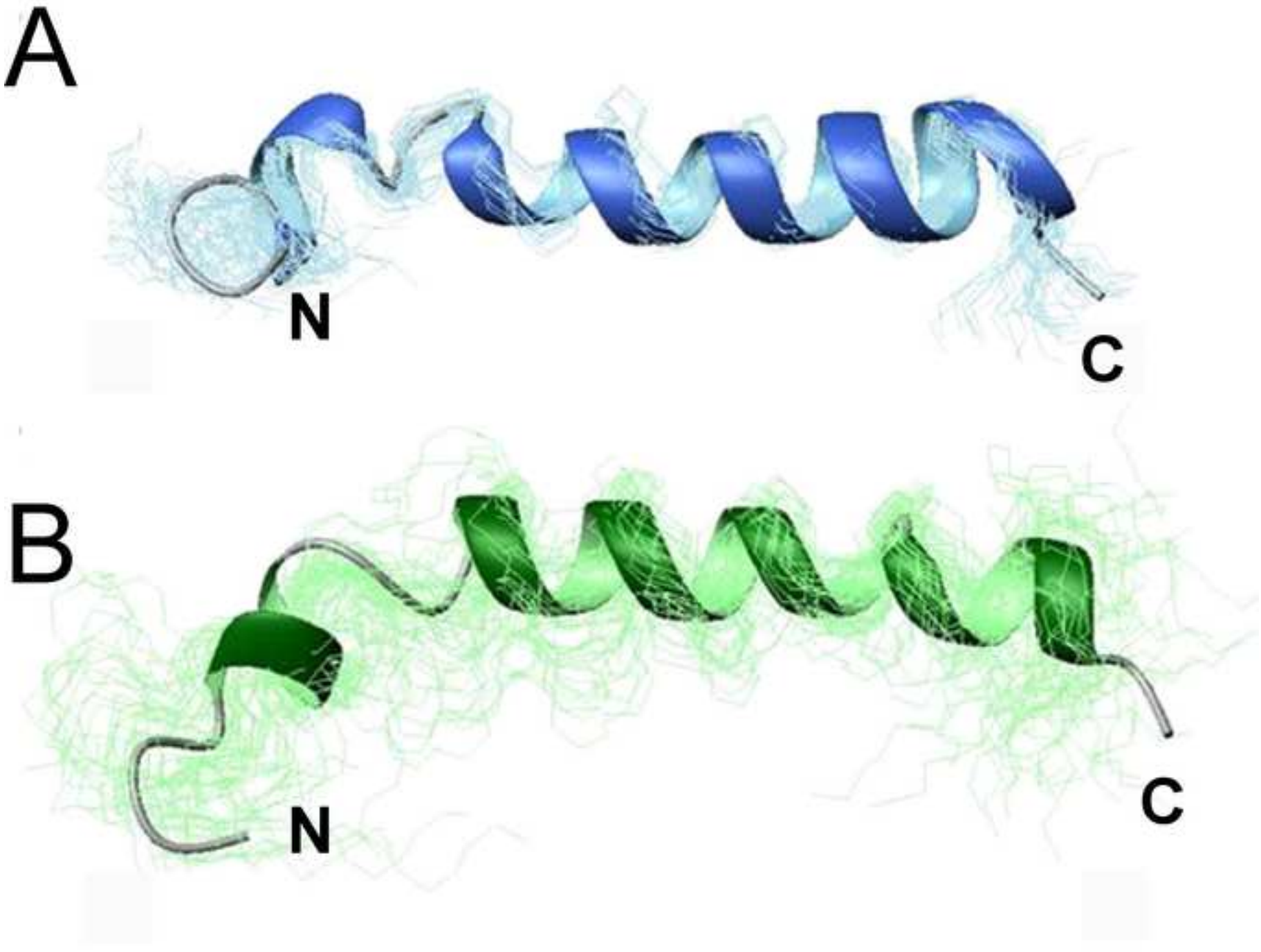

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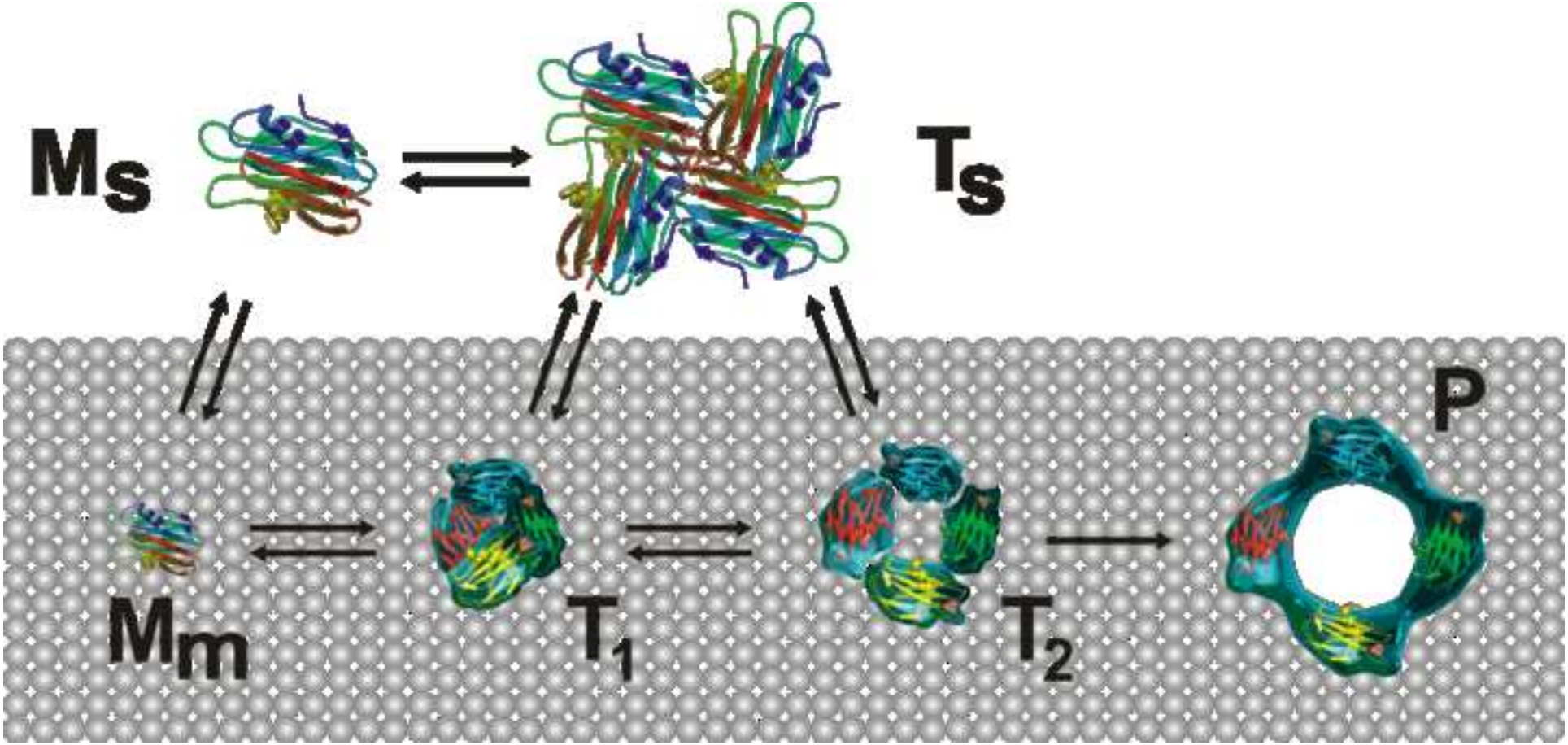

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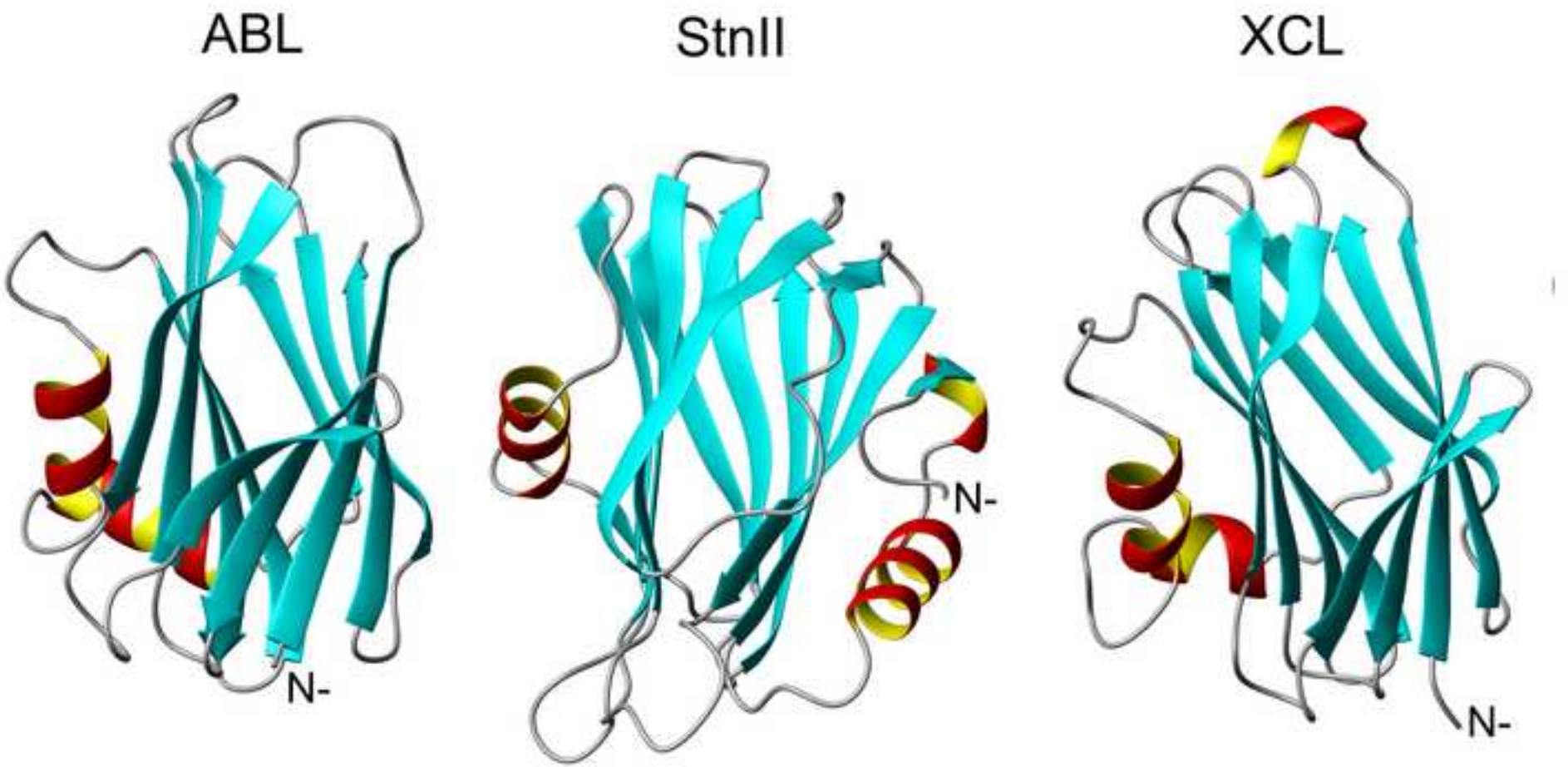

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