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Three-dimensional structure of the actinoporin sticholysin I. Influence of long-distance effects on protein function

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ABSTRACT

Actinoporins are water-soluble proteins with the ability to form pores upon insertion into biological membranes. They constitute a family of proteins with high degree of sequence identities but different hemolytic activities, suggesting that minor conformational arrangements result in major functional changes. A good example of this situation is the sea anemone *Stichodactyla helianthus* which produces two very similar actinoporins, sticholysins I (StnI) and II (StnII), but of very different hemolytic efficiency. Within this idea, given that the high resolution three-dimensional structure of StnII is already known, we have now solved that one corresponding to StnI in order to analyze the influence of particular residues on the conformation and activity of these proteins. In addition, random mutagenesis has been also used to produce five less hemolytic variants of StnI. All these mutations map to functionally relevant regions because they are probably involved in conformational changes associated with pore formation, which take place after membrane binding, and involve long-distance rearrangements of the polypeptide chain of actinoporins.

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Introduction

Actinoporins are an intriguing family of unique proteins produced by different sea anemone species from the *Actinaria* order [1–7]. They are highly basic, made of a single polypeptide chain of around 175 amino acid residues and remain stable as monomeric water-soluble proteins, yet they can oligomerize and integrate into lipid membranes [1–7]. Actinoporins are members of the larger family of pore-forming toxins [2](PFTs) [8] and are classified as α-PFTs because their mechanism of pore formation involves the insertion of an α-helix within the cell membrane [8–12]. These pore-forming properties explain their fast and efficient hemolytic activity [12–15].

Actinoporins from each anemone species appear as multigene families displaying high sequence similarity (between 60% and 80% of identity) [1–3,7,16–19]. Unexpectedly, a small number of sequence changes results in large differences in terms of aqueous solubility and hemolytic activity [7,19–21]. This observation has been functionally explained assuming that the presence of a large number of similar isotoxins in a single venomous secretion probably increases the prey range for a particular species [22]. A good representative example of this situation can be found in the tentacles of the sea anemone *Stichodactyla helianthus* which produce sticholysins I (StnI) and II (StnII), two actinoporins displaying 91.0% sequence identity but showing quite different hemolytic activities [4,20].

The three-dimensional monomeric structure of several actinoporins is already known [23–27], including that one corresponding to StnII [25]. All of them display a common fold characterized by a β-sandwich of 10–12 β-strands flanked by two α-helices which interact with both sides of the β-sandwich. This can be seen in Fig. 1, where the new structure reported now (StnI, in red) is compared with those ones of StnII (Fig. 1B, StnII blue) and EqtII (Fig. 1C, EqtII blue), which were already known [23–27]. One of the helices is always located near the N-terminal end. This N-terminal portion, about 30 residues long, can adopt alternative conformations without disrupting the β-sandwich motif [23], and has been proposed to form the pore walls [27–30]. Three additional regions have been also shown to participate in membrane recognition: an exposed cluster of

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[2] *Abbreviations used:* Chol, Cholesterol; CD, circular dichroism; DOPC, dioleylphosphatidylcholine; Fra, fragaceatoxins produced by *Actinia fragacea*; Eqt, equinatoxins produced by *Actinia equina*; MOPS, 3-(N-morpholino)propanesulfonic acid; PFT, pore-forming toxin; POC, phosphocholine; SM, sphingomyelin; Stn, sticholysins produced by *Stichodactyla helianthus*; wt, wild-type.

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aromatic residues, an array of basic amino acids, and a phosphocholine (POC) binding site [24,25,27,30–32]. Thus, the conservation of amino acid sequences is not only reflected but rather supported by the high degree of conformational identity. From this point of view, the observations made allow drawing two main conclusions. First, the general overall fold of actinoporins is absolutely required for their unique function and, therefore, it has been conserved along evolution [31]. Second, minor changes produce subtle conformational modifications with deep impact on the function of these proteins. However, very little is known regarding these conformational transitions and the residues that participate in the intramolecular communication between the membrane-binding regions and the N-terminal α-helix that irreversibly inserts into the membrane.

Within this idea, we have solved the monomeric three-dimensional structure of StnI in aqueous solution by NMR and studied its dynamical properties. Random mutagenesis has been used to select five StnI mutants that display decreased hemolytic activity. Altogether, the results obtained are discussed in terms of the involvement of particular actinoporins regions in the changes that occur after membrane binding.

Experimental procedures

Proteins production and purification

The cDNA coding for StnI mutants G26D, V28E, S53T, D57A, and E62G were obtained by random mutagenesis as described elsewhere [32] and selected after screening for a less hemolytic phenotype using blood-agar plates [32]. The unlabeled versions of the wild-type and the different mutants of StnI, as well as the double uniformly labeled $^{13}C/^{15}N$ wild-type StnI samples, were produced using an *Escherichia coli* expression system following a protocol previously described [20]. For the labeled form, cells were grown in a M9 minimal medium with $^{15}NH_4Cl$ (1 g/l) and $^{13}C_6$-glucose (4 g/l) as the sole nitrogen and carbon sources. Protein purification was achieved by ion exchange chromatography on CM52 cellulose equilibrated in 50 mM Tris–HCl at different pH values, depending on the estimated pI value of the protein purified (pH 6.0 for V28E and G26D; pH 6.8 for S53T; pH 7.8 for D57A and E62G). The homogeneity of all protein samples used was analyzed by SDS–PAGE and amino acid analysis after acid hydrolysis of the proteins (5.7 M HCl, 24 h, 110 °C). These amino acid analyses were

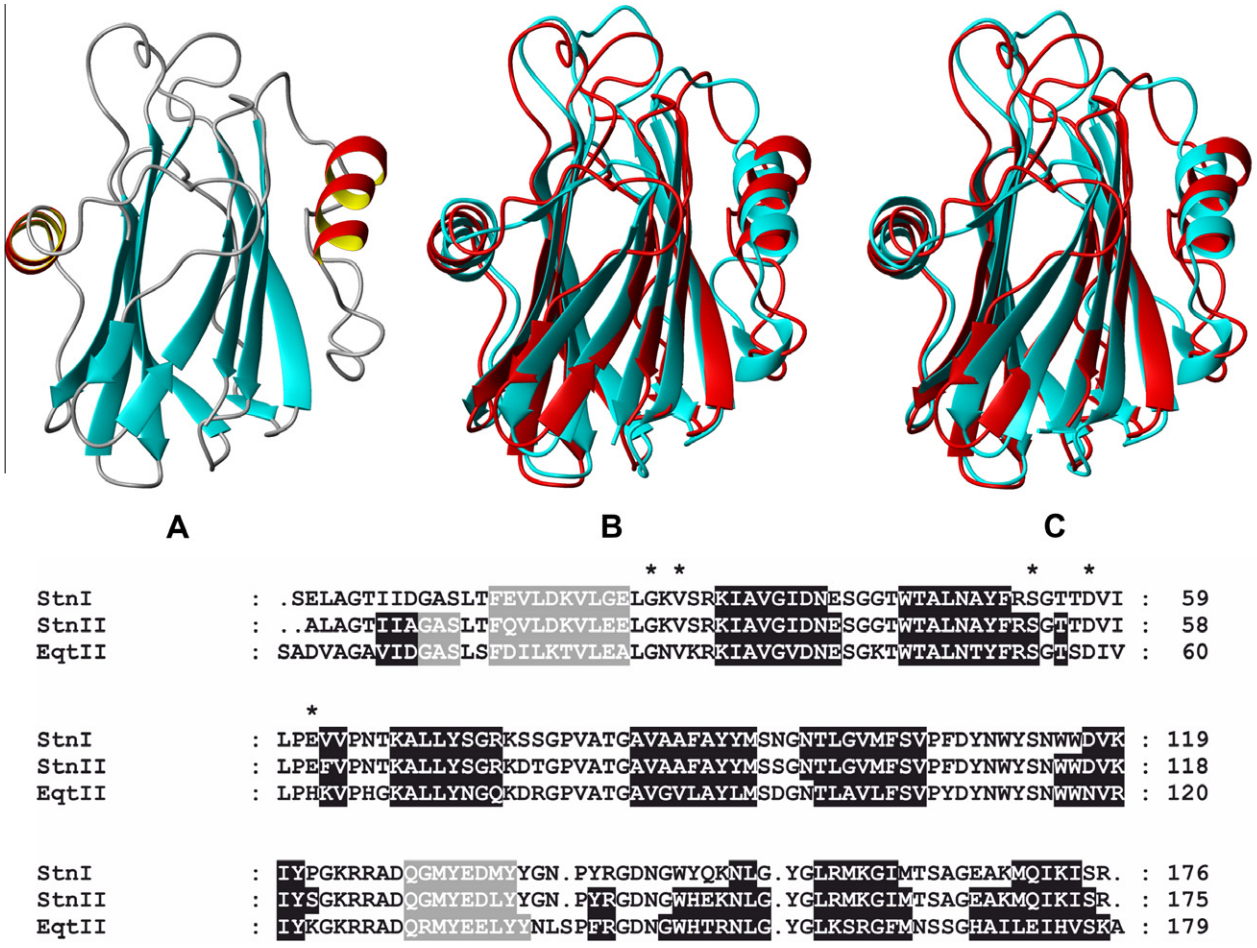

Fig. 1. *Upper panel*: Diagrams corresponding to the 3D structure of StnI (A) as well as its comparison (StnI in red) with those ones of StnII (B, StnII blue) [25] and EqtII (C, EqtII blue) [23]. Diagrams were constructed from the atomic coordinates deposited in PDB (Protein Data Bank, references 2KS4 for StnI, 1GWY for StnII, 1IAZ for EqtII). Structures were fitted by considering the atoms of the peptide bonds of residues 15–24, 68–75, 85–93, 97–105 and 129–136 (StnI), 14–23, 67–74, 84–92, 96–104 and 128–135 (StnII), and 16–25, 69–76, 86–94, 99–106 and 130–137 (EqtII). These peptide segments correspond to the two helical segments of the three proteins as well as three of their longer β strands. The resulting RMSD values were 1.355 Å for the pair StnI/StnII and 1.194 Å for StnI/EqtII. Images and fittings were generated by the MOLMOL program [41]. *Lower panel*: Sequence alignment of the three considered actinoporins, indicating the stretches corresponding to their different elements of ordered secondary structure (helical segments in grey, and β strands in black). The residues mutated in the StnI variants studied appear labeled with an asterisk. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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