RESEARCH LETTER

Sticholysin I–II oligomerization in the absence of membranes

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Actinoporins are pore-forming toxins found in the venom of many species of sea anemones [1–3]. The most studied actinoporins are sticholysins (Stns), produced by *Stichodactyla helianthus* [4–6], equinatoxins, from *Actinia equina* [7,8], and fragaceatoxins, from *Actinia fragacea* [9–11]. The sequences of actinoporins are usually very similar, sharing in most cases > 60% sequence identity [3,12,13]. This is especially remarkable in the case of isotoxins because many sea anemones, including the ones listed above, produced these isotoxins simultaneously [1,2,10,14–16]. The variation of the amino acid sequence among isotoxins from the same sea anemone species is much smaller than the average value obtained when considering other actinoporins. For example, StnI and StnII present a ~ 93% sequence identity (~ 94% similarity), EqtII and EqtIV, an ~ 87% (~ 94% similarity), and FraC and FraE, a ~ 98% (all but two residues).

It is striking that these small differences have, most often, a great impact on the activity of each of these proteins when assayed against both model membranes and erythrocytes [17]. These functional disparities have given rise to a variety of hypotheses regarding the existence of actinoporin isotoxins within single sea anemone individuals. One of the most popular is that this diversity could help broaden the range of accessible targets, both offensively and defensively [18–20]. Accordingly, one could expect the isotoxins to act separately. However, they are co-purified when the sea anemones are used directly as the toxin source [8,21–

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Taking this into account, it is feasible to speculate if some combined action between them may occur during the process of pore formation. Independent recombinant expression of the individual isotoxins allows to approach this question without interference of cross-contamination.

This is precisely what was observed for sticholysins [18]. A given concentration of a StnI–StnII mixture was shown to produce an effect not only greater than that of the same concentration of StnI alone, but also greater than that sum of the effect of the corresponding fractional concentrations of StnI and StnII present in those samples. Results of that study [18] also indicated that the interaction between the isotoxins took place, at least, during the membrane-binding steps of the pore-formation process. Hence, oligomerization in solution could be expected. In fact, StnII is known to form dimeric and tetrameric ensembles in absence of membranes [24]. In a much recent study [25], we also detected oligomerization of StnI and StnI–StnII mixtures in solution. However, the signal was very small, preventing the determination and quantification of the different oligomeric species.

To delve deeper into this phenomenon, we have now turned to analytical ultracentrifugation, using sedimentation velocity experiments, a hydrodynamic method that allows the determination of size, shape and interactions of the studied molecules [26–29]. Dynamic light scattering (DLS) was used to measure the Stokes radii of the monomeric species of StnI and StnII. Our results indicate that, separately, StnI is more prone to oligomerize than StnII. However, small amounts of StnII can increase the fraction of oligomeric species in StnI–StnII mixtures, in accordance with our previous activity results.

**Materials and methods**

Recombinant StnI and StnII were produced in *Escherichia coli*, strain RB791, and purified to homogeneity as described in Ref. [30]. Sedimentation velocity experiments were performed at 48 000 rpm (170 000 g) in an XL-I analytical ultracentrifuge (Beckman-Coulter Inc., Brea, CA, USA) equipped with both UV–VIS absorbance and Raleigh interference detection systems, using an An-50Ti rotor (Beckman-Coulter Inc.) at 20 °C. Samples in 15 mM MOPS, 100 mM NaCl buffer, pH 7.5, were loaded (320 µL) into 12-mm Epon-charcoal standard double-sector centerpieces, except for highly concentrated samples, where 3-mm centerpieces were used. The partial specific volume was calculated from the amino acid sequences by SEDNTERP [31], being 0.7324 and 0.7338 mL·g⁻¹ for StnI and StnII respectively. This allows to approximate the $M_w$.

Concentration scans were performed for both isotoxins separately, beginning at ~6 µm, and ending at ~390 µm. Sedimentation was followed using both interference and absorbance at 250 nm. At the highest concentration, interference data were used. StnI–StnII mixtures at various ratios were assayed at a total protein concentration of 150 µM. In this case, sedimentation was followed using absorbance at 300 nm. Differential sedimentation coefficient distributions were calculated by least-squares boundary-approximation modelling of sedimentation velocity data using the continuous distribution $c(s)$ Lamm equation model as implemented by SEDFIT [32]. These experimental $s$ values were corrected to standard conditions using the program SEDNTERP [31] to get the corresponding standard $s$ values ($s_{20,w}$).

For the separate isotoxins, it was possible to use the weight-averaged sedimentation coefficients results to fit the following isotherm

$$s_w(c_{tot}) = \sum_i \frac{s_{0,i}}{1 + K_i c_i^{c_i}} \approx \frac{1}{1 + K_i c_{tot}} \sum_i \frac{s_{0,i}}{c_i^{c_i}}$$

where $s_{0,i}$ is the species sedimentation coefficients, $K_i$ is their hydrodynamic nonideality coefficients, $c_i$ is the concentration of monomers in the samples regardless of their oligomerization state and $c_{tot}$ is the sum of the concentrations of all oligomeric species. In our case, we used the second part of Eqn (1), where the hydrodynamic nonideality coefficient is assumed to be the same for all species in the sample. In all cases, the value of the parameter was $< 5 \times 10^{-4}$, indicating that the systems behave ideally. The calculated $M_w$ of the oligomers observed in the sedimentation coefficient distribution $[c(s)]$ was used to estimate the stoichiometry of the oligomers. Accordingly, Eqn (1) was used so that it described a monomer–dimer–tetramer system [33,34].

The association constants obtained were used to calculate the oligomer fractions as function of total toxin concentration in the sample, which were compared to the fractions of each species calculated according to the $c(s)$ obtained.

This approach was also used to study the formation of heteromers in the mixtures of StnI and StnII. However, no satisfactory fits were obtained in this case.

The buffer for DLS (same composition as above) was filtered through 0.2-µm filters. Final toxin concentrations were 16 and 8.3 µM of StnI and StnII respectively.

**Results and Discussion**

**StnI**

Results from the concentration scan using StnI revealed the existence, in most cases, of three peaks in the $c(s)$ distribution, whose relative areas were
dependent on the loading concentration of StnI. Given that the peaks obtained are well clearly resolved from one another (Fig. 1A), the equilibrium between the oligomeric ensembles of StnI in solution does not seem to change much in the time scale of these experiments (~ 5.5 h) [27,28].

The values of $s_{w,20}$ and the approximates for $M_w$ obtained from the analysis are presented in Table 1. The estimated $M_w$ for the ensembles of the population with the smallest sedimentation coefficient was $24.6 \pm 1.6$ kDa, compatible with the $M_w$ of StnI monomers ($M_w = 19,391$ Da, according to the sequence). The upward deviation of the value is a consequence of the analysis and the significant population of larger oligomers detected which, during the fitting process, results in an average frictional coefficient that is overestimated for this population. The estimate for the Stokes radius ($r_{\text{Stokes}}$) of the monomers was $27.5 \pm 2.0$ Å. This value is an approximation and shows distortion from the analysis. The value obtained using DLS is $22.0 \pm 4.5$ Å. The latter value also agrees with the dimensions expected for a StnI monomer according to the available three-dimensional structures [35]. The estimated $M_w$ of the second population was $44.4 \pm 4.2$ kDa, indicating that it was most likely composed of StnI dimers. This value is again larger than the theoretical value, but follows the deviation explained above. The last detectable population had an estimated $M_w$ of $73.4 \pm 4.3$ kDa, compatible with that of a slightly elongated StnI tetramer.

The fact that no population compatible with the $M_w$ of StnI trimers was detected suggests that the likeliest pathway to tetramers is the condensation of dimers, with monomer–dimer interactions thus being somehow less favourable. This could be consequence of some dimerization-induced conformational change that, affecting both monomers involved, would alter their structure just enough so that a dimer–dimer interaction would be more favourable in water solution than monomer–dimer associations. This could also contribute to trimer scarcity by reducing the population of monomers, which are required to form trimers.

The isotherm fit to the weight-averaged sedimentation coefficients (Fig. 2A) allowed to estimate the association constants of the oligomers, obtaining values of $3 \times 10^3$ M$^{-1}$ and $2 \times 10^{10}$ M$^{-3}$ for the dimerization and tetramerization equilibria respectively. These constants were then used to calculate the fractional population of each oligomeric species as a function of StnI concentration (Fig. 2B).
assay (Fig. 1B). However, the analysis revealed some differences with StnI. First, only two major peaks were detected in most cases, with the second one becoming prominent only at the higher toxin concentrations. However, while in the case of StnI only three populations were required to adequately describe the sedimentation data, StnII showed residual populations (< 0.7% total) of higher sedimentation coefficients (> 6.5 S). These observations were more evident at the highest StnII concentration assayed (Fig. 3).

The values of $s_{20,w}$ and the approximates for $M_w$ obtained from the sedimentation velocity analysis are presented in Table 1. The estimate for the Stokes radius ($r_{\text{Stokes}}$) of the monomers was $22.7 \pm 0.8$ Å, an identical value to the DLS result of $22.5 \pm 4.5$ Å (DLS results are the same for StnI and StnII). As for StnI, no peaks compatible with trimers were observed, again suggesting that the formation of tetramers would occur via dimer condensation.

Isotherm fitting was now performed with these results (Fig. 2C), yielding association constants of $2 \times 10^3$ M$^{-1}$ and $3 \times 10^9$ M$^{-3}$ for the dimerization and tetramerization respectively. These results are reassuring, since the monomer–tetramer association constant for StnII was already reported to be in the range of $10^9$ M$^{-3}$ [24]. As for StnI, the association constants

### Table 1. Values of $s_{20,w}$ (S) derived from the sedimentation velocity experiments of StnI and StnII independently, and from those in which StnI–StnII mixtures were used. Values for $M_w$ and stoichiometry are estimates from the analysis. In each case, except for StnI–StnII mixtures at 150 µM, three populations could be detected in some of the samples. The monomer $M_w$ based on the sequence of StnI and StnII is 19 391 and 19 283 Da respectively. Indicated values are average ± SD of $n = 2$–6.

|         | $s_{20,w}$ (S) | $M_w$ estimates (kDa) | Stoichiometry (x monomer $M_w$) |
|---------|---------------|-----------------------|---------------------------------|
| StnI    | 2.13 ± 0.04   | 24.6 ± 1.6            | 1.27 ± 0.09                     |
|         | 3.15 ± 0.09   | 44.4 ± 4.2            | 2.29 ± 0.22                     |
|         | 4.28 ± 0.07   | 73.4 ± 4.3            | 3.79 ± 0.22                     |
| StnII   | 2.13 ± 0.03   | 20.6 ± 0.8            | 1.07 ± 0.04                     |
|         | 3.11 ± 0.04   | 36.1 ± 2.4            | 1.86 ± 0.13                     |
|         | 5.37 ± 0.47   | 81.2 ± 12.5           | 4.19 ± 0.65                     |
| StnI + StnII | 2.26 ± 0.04 | 17.7 ± 0.5            | 0.91 ± 0.02                     |
|         | 3.26 ± 0.14   | 31.4 ± 3.2            | 1.62 ± 0.17                     |

**StnII**

When identical experiments were performed with StnII, the results also suggested that the oligomerization equilibrium between StnII’s protomers in solution can also be considered static in the time scale of the

![Fig. 2.](image-url) (A) Isotherm of the weight-averaged sedimentation coefficient as a function of StnI concentration. The lower panel shows the residuals of the fitting. (B) Fraction of each detected StnI oligomeric species as a function of toxin concentration. Lines were drawn using the association constants obtained with the fitting shown in A. (C) Isotherm of the weight-averaged sedimentation coefficient as a function of StnII concentration. The lower panel shows the residuals of the fitting. (D) Fraction of each detected StnII oligomeric species as a function of toxin concentration. Lines were drawn using the association constants obtained with the fitting shown in C.
were used to calculate the fraction of each oligomeric species as a function of StnII concentration (Fig. 2D).

**StnI–StnII mixtures**

Sedimentation velocity experiments were next performed using StnI–StnII mixtures to see how their relative concentrations affected the oligomerization equilibrium in absence of membranes. These experiments were performed using total toxin concentration of 150 $\mu$M.

The values of $s_{w,20}$ and the approximates for $M_w$ obtained for all detected oligomeric populations are shown in Table 1. As for StnI and StnII separately, the estimates for the $M_w$ of most of the detected ensembles were consistent with those of monomers and dimers.

The fraction of monomers for all StnI–StnII mixtures assayed is shown in Fig. 4. It is interesting to notice that the fraction of monomers reaches a minimum, that is, a maximum in dimers, when the fraction of StnII is $\leq 10\%$, despite StnII been less prone to oligomerize than StnI. In those experiments, the effective StnI concentration has been reduced compared to when the fraction of StnI was 100%. For this reason, if the oligomerization equilibria were independent, the total monomer fraction in the sample should be increased, as the equilibria would be displaced towards monomers. However, this is not the case, indicating that hetero-oligomers are being formed. This observation would support the hypothesis that StnII, despite being less likely to oligomerize with itself, can promote the oligomerization of StnI [18]. In fact, possible residue interactions underlying this preference have already been proposed [25]. Based on FRET results using fluorescently labelled StnI, it was discussed how, apparently, StnII monomer–monomer associations would be more favourable than those of only StnI. Briefly, this would be so because, of the only 12 residues that differ between StnI and StnII, two of them are located on the monomer–monomer interface, according to the available pore structures of actinoporins [11]. The residue pairs that these amino acids would form with their complementary counterparts would establish stronger interactions for StnII than for StnI. However, given that both differences are on the same side of the toxin, a StnI–StnII dimer in the appropriate order, though not on the contrary, would, in principle, preserve the interactions that are presumed to occur on a StnII dimer (Fig. 5). Given that the population of sticholysin trimers is not detectable (at least in absence of membranes), further suggest that StnII monomers would present a preference to interact with StnI monomers but only using the most favourable monomer–monomer interface of the two available possibilities.
The role of these soluble oligomers in the process of pore formation, regardless of their isotoxin composition, remains unclear. Recent results suggest that these ensembles could be better at sphingomyelin recognition in membranes that lack Chol [25]. It is also likely that they may act as protomers, speeding up toxin oligomerization once on the membrane surface. However, further experiments are required to solve these questions.

Conclusions
In this study, the oligomerization of StnI and StnII in absence of lipid membranes has been studied using analytical ultracentrifugation and DLS, in particular sedimentation velocity experiments. These experiments have allowed us to determine the association constants for dimerization and tetramerization for StnI and StnII in solution, revealing that StnI is more prone to form oligomers, especially tetramers, than StnII. Not only that, but we have also observed that StnII is able to promote dimer formation in StnI–StnII mixtures beyond the oligomerization observed for StnI at ratios in which StnII is the minority component. Finally, we have proposed a hypothesis that might explain this behaviour based on the structure of both toxins and their potential monomer–monomer interactions. The role played by these oligomers in the process of pore formation is still an open question in the field and will certainly be the subject of future studies.

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Author contributions
SG-L, RA-S and CG-M prepared the samples for analysis. CA and JRL-O performed the ultracentrifugation experiments and the analysis to obtain the c(s) distributions. DH-M and JP-O prepared the samples for DLS. JP-O prepared the figures, performed the oligomerization analysis and wrote the first draft of the manuscript. All authors revised the final version of the manuscript, providing comments and corrections.

Data accessibility
The data supporting the findings of this study are available from the corresponding author (juan.palaciosb1a@gmail.com) upon reasonable request.

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