Structure-function relationship for the highly toxic crotoxin from *Crotalus durissus terrificus*

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Abstract. The three-dimensional structure of the highly toxic crotoxin from *Crotalus durissus terrificus* was modelled based on sequence analysis and the refined structure of calcium-free phospholipase of *Crotalus atrox* venom. Small-angle x-ray scattering experiments were performed on aqueous solutions of crotoxin. The radial distribution function derived from these scattering experiments and the one calculated from the model structure are in good agreement. Crotoxin consists of a basic and an acidic subunit. The model strongly suggests that the overall folding motif of phospholipases has been preserved in both subunits. The basic domain has an intact active site. The residues that are expected to contact the lipid tails of the phospholipid are different from other phospholipases, but they are all hydrophobic. The acidic domain consists of three independent chains interconnected by disulfide bonds. Compared to other phospholipases the active site for the greater part has been preserved in this domain, but it is not very well shielded from solvent. Most residues normally in contact with the lipid tails of the phospholipid are missing, which might explain the acidic subunit’s lack of phospholipase activity. A homology between the third chain of the acidic domain and neurophysins suggests that the acidic domain may act as a chaperone for the basic domain.

Key words: Crotoxin – Structure-function relationship – Modelling by homology – X-ray scattering

Introduction

In 1938 Slotta and Fraenkel-Conrat (1938) isolated a toxic protein from the venom of the South American rattlesnake *Crotalus durissus terrificus* that represented about 70% of the total venom protein. Crotoxin, as it is called, proved to be a highly toxic presynaptic neurotoxin. It causes paralysis and respiratory failure. These effects are of peripheral origin (Vital-Brazil 1966). The protein consists of two subunits, a larger, basic subunit with molecular weight 14 300 and pI = 8.9, and a smaller, acidic subunit with a total molecular weight of 9 200 and pI = 3.8. The acidic subunit, called crotapotin, consists of three independent chains (hereafter referred to as A, B and C) which are interconnected by disulfide bonds (Aird et al. 1985). The basic subunit exhibits phospholipase A₂ activity but no serious toxicity as a monomer. Upon dimerization of the basic and acidic subunits, the toxicity is increased strongly, but the phospholipase activity is diminished (Breithaupt et al. 1975; Chang and Su 1978).

Although phospholipases are among the structures solved with the highest resolution, not much is known about their mode of action. The main problem is that they act at a heterogeneous (water-membrane) interface, a chemical environment that is hard to probe or mimic. Several phospholipases show increased activity (up to a factor of 200) upon dimerization (Tomasselli et al. 1989). Sometimes acylation of lysines is needed for dimerization (Cho et al. 1988).

There is much controversy in the literature about the mode of action and the kinetics of phospholipases (for a review see Waite 1987). Neurotoxins like crotoxin are known to bind to proteins at the cholinergic nerve terminal membrane which are functionally related with the transmitter release mechanism (Chang 1985). The observed reduction in non-specific binding of the basic subunit after dimerization with crotapotin has led to the suggestion that crotapotin is not acting as an affinity probe to increase the affinity of crotoxin for the target site on the nerve terminal, but rather as a chaperone to minimize distraction and destruction en route to the site of action by curtailing non-specific affinity to muscle and other tissues (Chang 1985). The observed homology between the crotapotin C-chain and mammalian neurophysins supports this hypothesis (Aird et al. 1985).

The aim of the present study is to answer the following questions:
1) Does the tentative sequence for the 10 N-terminal amino acids of the crotapotin B-chain seem plausible?
2) Does it seem likely that crotapotin is a chaperone for crotoxin?
3) Can the available spectroscopic data be explained?
4) Could (auto-)acylation be needed for crotoxin complex formation?
5) Are the crotoxin subunits likely to contain calcium?
6) Does the structure suggest experiments that allow us to shed further light on the crotoxin structure-function relation?

In a general case one would be able to answer these questions on the basis of crystallographic or NMR data. Detailed study of crotoxin, however, is hampered by a lack of direct atomic data. Crystals of crotoxin were reported in 1985 (Achari et al. 1985), but attempts to determine its structure have not been successful yet. Therefore, we resorted to building a computer model of the crotoxin structure. In general it is possible to draw reliable conclusions with respect to the basic features of a protein on the basis of a model when the homology with the underlying real structure is high (Sander and Schneider 1991; Chothia and Lesk 1986). In order to assess the quality of our model we carried out small-angle x-ray scattering (SAXS) experiments on crotoxin solutions, and determined how well the model fits the experimental data.

Sequence comparisons have shown a high degree of homology with phospholipases and with several other toxins. Of those proteins for which a three-dimensional structure is available in the Protein Data Base (Bernstein et al. 1977), calcium-free phospholipase from *Crotalus atrox* venom (Brunie et al. 1985; 1PP2 in the Protein Data Base) shows the highest sequence homology with crotoxin. We therefore built the crotoxin model using the structure of this phospholipase and its sequence alignment with crotoxin.

## 2 Methods

### 2.1 X-ray scattering

Crotoxin was purified as described by Hendon and Fraenkel-Conrat (1971). Solutions of 60, 50, 40 and 20 mg/ml were prepared by adding appropriate amounts of crotoxin to an aqueous solution of formic acid at pH = 3.0. Scattering curves were obtained as described by Beltran et al. (1990) for scattering vector moduli ranging from 0.01840 to 0.478 Å⁻¹. The intensity curves I(h) were corrected for beam linear cross section shape and smoothed to facilitate extrapolation to zero concentration. The first 9 points (Ah = 0.00368 Å⁻¹) of the curve were eliminated prior to the calculation of the p(r) function because this region was still affected by inter-particle scattering effects. The radial distribution function p(r), defined as

\[ p(r) = \frac{1}{2\pi^2} \int_0^\infty I(h) h r \sin h r \, dh \]

describes the intra-molecular distance distribution. It is characteristic for the particles in dilute solution and can be determined unambiguously. From p(r) the maximal distance within one particle, D_max, can be obtained directly from the abscissa value where p(r) vanishes. The radius of gyration \( R \) can be calculated from

\[ 2R^2 = \int_0^\infty p(r) r^2 \, dr \]

The ITP program (Glatter 1982) was used for desmearing the SAXS curves and for the calculation of the experimental radial distribution function and radius of gyration. The Multibody program (Glatter 1980) was used to calculate the model radial distribution function and radius of gyration from the atomic coordinates. The same form factor was used for all atoms in the model calculation.

### 2.2 Modelling

Multi-sequence alignments were performed using HSSP (Sander and Schneider 1991). Figure 1 shows the multi-sequence alignment of crotoxin and the two phospholipases whose structure is known: bovine pancreatic phospholipase A₂ and the phospholipase from *Crotalus atrox* venom. Model building was carried out using the automatic model-by-homology option of the WHAT IF program (Vriend 1990). Torsion angles of the model were taken from the original structure whenever possible. Otherwise they were taken from a standard residue library. Where necessary the model was regularized (Vriend 1990; Dodson et al. 1976). Reasonable positions for side chains that showed considerable Van der Waals overlap were obtained in an iterative process of flipping through all \( \phi \)-angle rotamers. The resulting crude structure was further optimized by means of energy minimization and Molecular Dynamics simulations with the GROMOS package (Van Gunsteren and Berendsen 1987). The MD simulations were carried out merely to generate better geometries in regions near deletions where energy minimization proved ineffective. The “active sites” of both subunits were modelled on calcium-free phospholipase, although the basic subunit may contain calcium. In the basic subunit one water molecule, which in 1PP2 (Brunie et al. 1985) is an integral part of the active site, was retained during the simulations.

## 3 Results and discussion

Figure 2 shows the observed and the calculated radial distribution functions. The overall correspondence supports the proposed model. In addition, the maximum distance and radius of gyration derived from the model (52.0 Å and 16.6 Å, respectively) are in very good agreement with the values calculated from the experimental data (52.0 Å and 16.5 Å, respectively).

Modelling the basic subunit was rather straightforward because of the high homology with 1PP2 (see Fig. 1). In modelling the acidic subunit several problems