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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 14300 and pI = 8.9, and a smaller, acidic subunit with a total molecular weight of 9200 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 A_2 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:

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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 ($\Delta h = 0.00368\ Å^{-1}$) 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

$$p(r) = 1/2\,\pi^2 \int_0^\infty I(h)\, hr \sin hr\, dh$$

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 form the abscissa value where $p(r)$ vanishes. The radius of gyration R can be calculated from

$$2R^2 = \frac{\int_0^\infty p(r)\, r^2\, dr}{\int_0^\infty p(r)\, dr}.$$

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 A_2 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 χ-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

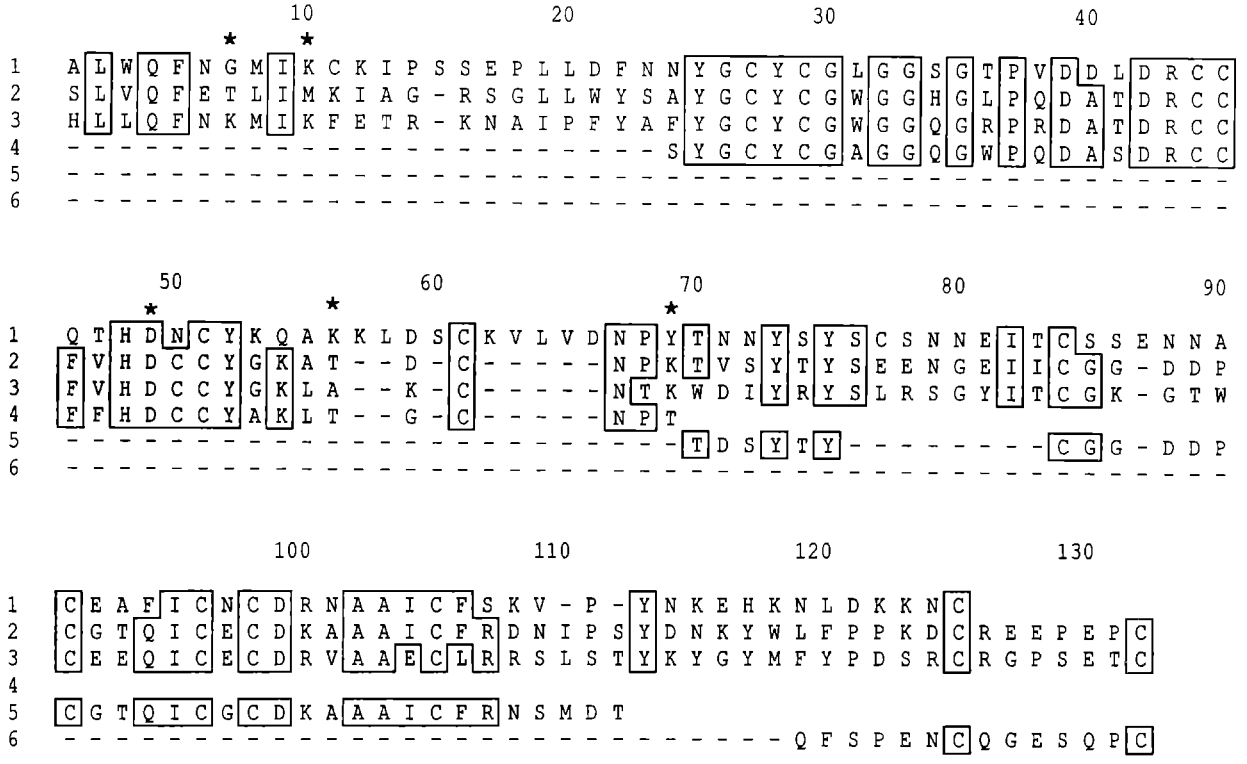

Fig. 1. Multi-sequence alignment of crotoxin, bovine pancreatic phospholipase A_2 (1BP2) and the phospholipase from *Crotalus atrox* venom (1PP2). 1) 1BP2, 2) 1PP2, 3) Crotoxin basic subunit, 4) Crotapotin A-chain, 5) Crotapotin B-chain, 6) Crotapotin C-chain. The alignment of 1BP2 and 1PP2 and the residue numbering is as in Renetseder et al. (1985). Asterixes indicate residues explicitly mentioned in the text

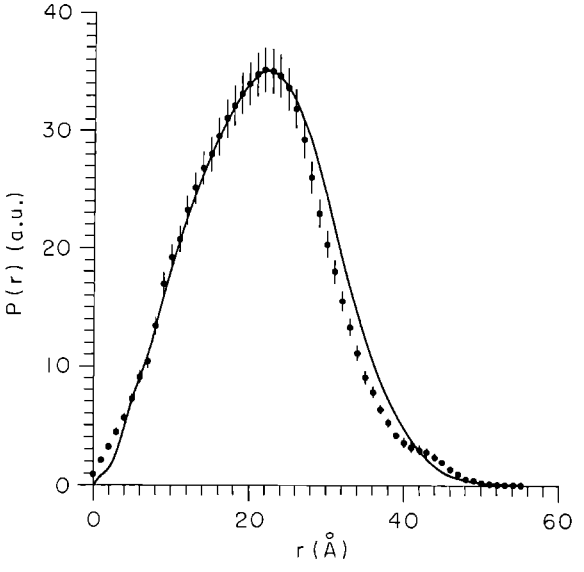

Fig. 2. Experimental and simulated radial distribution functions. The drawn curve is based on the model. Circles represent experimental data

had to be dealt with. The sequence of the first 10 amino acids of the B-chain of the acidic subunit, crotapotin, is only tentatively known (Aird et al. 1985; Laure 1988). Since most of these residues are part of a surface loop there are not many constraints on the allowed residues in these positions. Compared to 1PP2 crotapotin lacks 8 residues in the beginning of the B-chain. The sequence alignment and consequently the structure prediction may therefore not be fully reliable. Both tentative sequences give a cysteine in position 84, which is most likely correct because it would preserve the disulfide bond with Cys96. This fixes 4 residues in space, leaving only the spatial arrangement of the first 6 amino acids of the crotapotin B-chain to be predicted. These residues would most likely correspond in structure to residues 78–83 or to 70–75 in 1PP2. In 1PP2 residues 78–83 are part of a surface loop which covers several hydrophobic residues. Since these hydrophobic residues are absent in crotapotin and do not leave any other hydrophobic areas exposed, there is no equivalent role that the first six crotapotin B-chain residues could play. They would only be a protrusion into the solvent. However, in 1PP2 residues 70–75 contain one active-site residue (Tyr73) and in addition shield several hydrophobic residues near the active site. Although in crotapotin the "active site" lacks several residues compared to 1PP2 the first six B-chain residues of crotapotin could serve a similar purpose, and we therefore assume that they indeed occupy the same position in space as residues 70–75 in 1PP2. Tentative assignments give for residue 75 (in sequence equivalent with 83 since 8 residues are missing) either tyrosine (Laure 1988) or valine (Aird et al. 1985). In all sequences homologous to 1PP2 large aromatic residues are found at position 75. However, the disulfide bond between residues 84 and 96 makes accommodation of a tyrosine at position 75 very difficult. In our model we therefore use a valine at position 75.

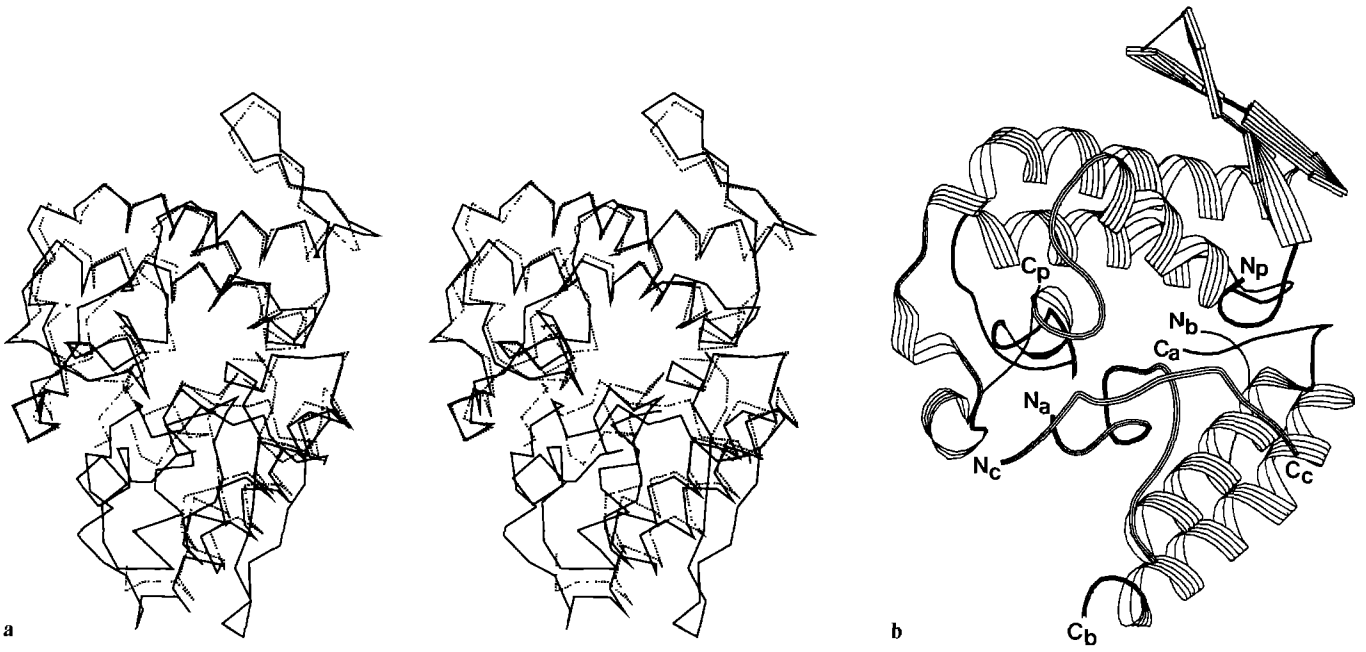

Fig. 3a, b. Two representations of the final crotoxin model. **a** Cα trace superimposed on the 1PP2 structure. 1PP2 is drawn solid, the crotoxin model dashed. The lower crotoxin subunit in this figure is crotapotin. **b** Ribbon plot generated with the program RIBBON (Priestle 1988). The N and C-terminal ends of the phospholipase subunit are labeled N_p and C_p, and the N- and C-termini of the crotapotin A, B, and C chains are labeled N_a, C_a, N_b, C_b, N_c and C_c

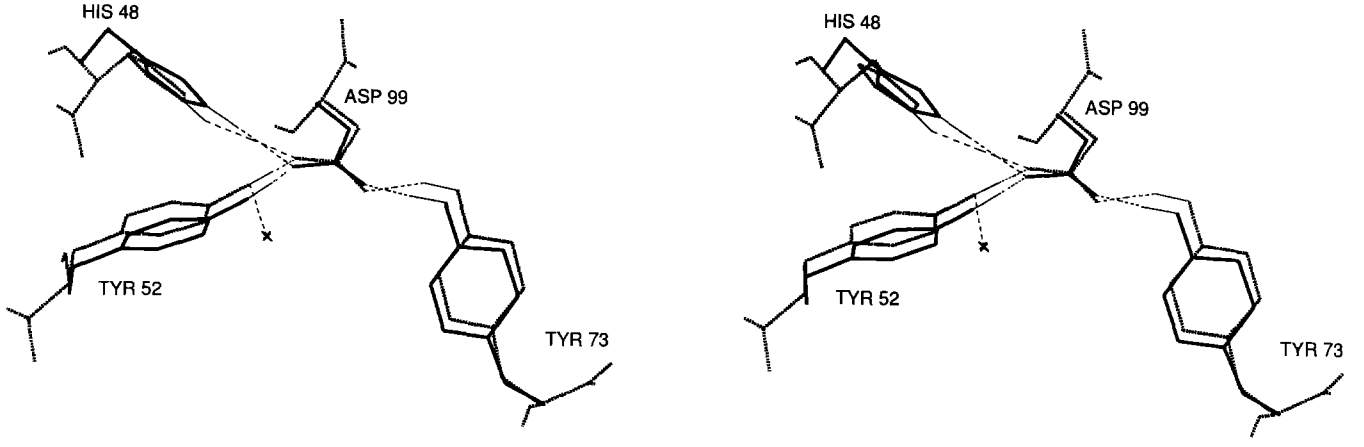

Fig. 4. The active sites of the basic subunit superimposed on the equivalent residues in 1PP2. 1PP2 side chains are drawn solid. The equivalent residues in the basic crotoxin subunit are dashed. The small cross indicates the water molecule explicitly included in the energy minimization and molecular dynamics. Thin solid and dashed lines indicate hydrogens and hydrogen bonds respectively

Figure 3 shows two representations of the crotoxin model: a $C\alpha$ trace superimposed on calcium-free phospholipase from *Crotalus atrox* and a ribbon plot. From Fig. 3 a it is clear that the phospholipase (basic) subunit of crotoxin corresponds well with 1PP2. Compared to 1PP2 the crotapotin subunit lacks a large number of residues but the part that is still present superimposes well on 1PP2. Evaluation of the complete model reveals that it has all characteristics of a "normal" protein. Packing quality (Vriend and Sander 1992), surface polarity (Baumann et al. 1989), side-chain torsion angles (McGregor et al. 1987; Ponder and Richards 1987), and energies (Van Gunsteren and Berendsen 1987), all fall in the ranges observed for normal proteins.

Figure 4 shows the details of the active site of the basic crotoxin subunit superimposed on the equivalent part of the 1PP2 structure (Brunie et al. 1985). This subunit clearly has a completely intact active site. Its residues occupy identical positions in space as 1PP2 and the greater part of the individual atomic contacts are preserved as well. Crotapotin's active site (not shown) lacks one residue (Gln4) and several residues that partly screen the active

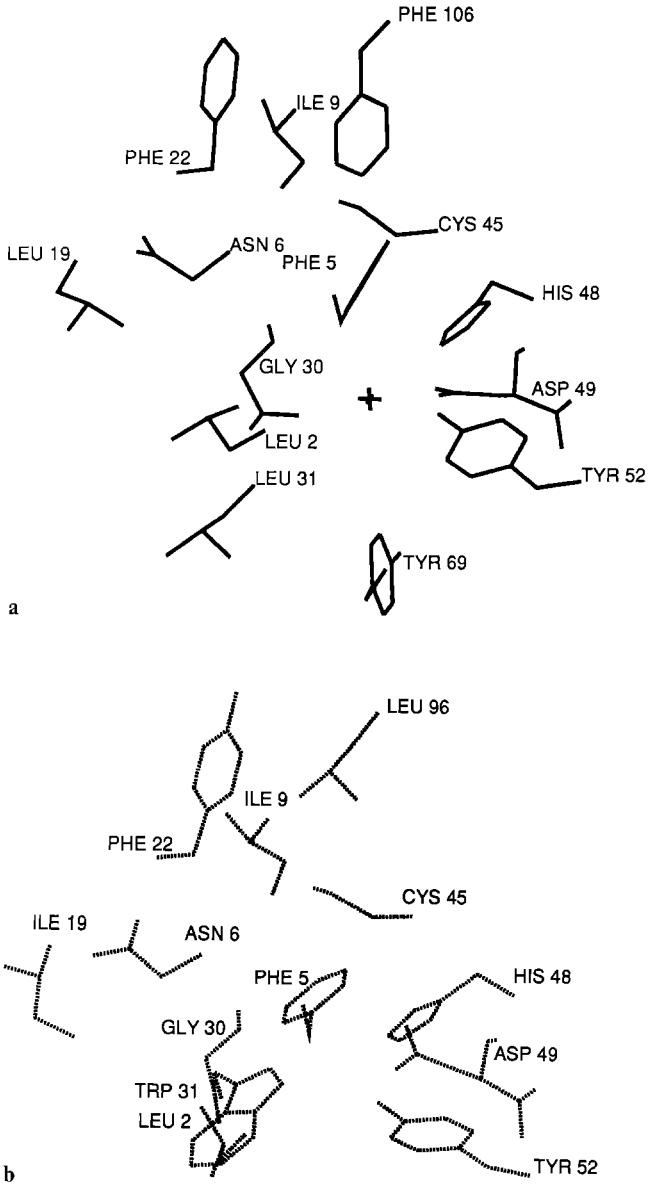

Fig. 5a, b. The lipid-binding residues in crotoxin and in 1BP2. **a** 1BP2 side chains involved in binding a competitively inhibiting substrate analog. Backbone atoms that contact the inhibitor (Gly30 and Asp49) are shown as well. The cross indicates the calcium ion. **b** The equivalent residues in crotoxin. Most residues are identical or of the same character (hydrophobic versus hydrophobic etc.). Tyr69 contacts a phosphate oxygen in 1BP2. This tyrosine is a lysine in crotoxin. Our modelling indicates that this lysine could perform the same function. However, without adequate substrate coordinates it will not be possible to model this lysine properly

site from solvent in 1PP2 are absent too, but the remainder are all found in the same positions as in 1PP2. This does imply however, that the two active sites are in a fully different electrostatic environment.

Figure 5 shows the residues involved in binding the hydrophobic lipid tails of the phospholipid (Thunnissen et al. 1990) in bovine pancreatic phospholipase A_2 (Dijkstra et al. 1978; 1BP2 in the Protein Data Base) and the equivalent residues in our model. We compare here with 1BP2 instead of 1PP2 since the mode of lipid binding has been established for 1BP2 (Thunnissen et al. 1990). All residues that are involved in binding the tails of the lipid moiety are functionally conserved between 1BP2, 1PP2 and the basic crotoxin subunit. They are lacking in crotapotin and thus prevent selective binding of phospholipids. These two clear differences (concerning the active-site and the lipid-binding regions) between crotapotin on the one hand and 1BP2, 1PP2 and the basic crotoxin subunit on the other lead to the conclusion that the acidic subunit cannot exert phospholipase activity and must serve a different purpose.

Crotapotin has been suggested to act as a chaperone for the basic crotoxin subunit (Aird et al. 1985). This suggestion was based on the strong sequence similarity of the crotapotin C-chain and neurophysins. Crotapotin may fool the defense systems of the host organism long enough to reach the membrane where the lethal action is to take place. The fact that this C-chain is rather prominent at the surface of the molecule (see Fig. 3) supports this idea.

Hanley (1979) examined the complex formation of crotoxin with fluorescence and circular dichroism techniques. He concluded that upon complex formation there was an increased amount of secondary, particularly β-sheet, structure. Our model clearly indicates that the amount of β-structure is rather limited, and those few β-strands that are present are at considerable distance from the subunit interface. It is therefore unlikely that complex formation brings about an increase in the amount of β-structure. Hanley also concluded that two of the four tryptophan residues (both on the basic subunit) get buried upon complex formation. Indeed, our model shows that two initially exposed tryptophans on the basic subunit become buried in the complex. They are not buried in a hydrophobic core, though, but only partly inaccessible to solvent. Most likely these tryptophans are shielded enough to prevent a probe like N-bromosuccinimide from binding to them.

Acylation of lysine residues has been shown to be important for dimerization and hence for potentiation of the presynaptic effect of phospholipases (Tomasselli et al. 1989; Cho et al. 1988). The residues indicated are lysine 56 in pancreatic phospholipase A_2, and lysines 7 and 10 in the venom of the American water moccasin *Agkistrodon piscivorus piscivorus*. There is no experimental evidence for acylation of lysine residues in crotoxin. Evaluation of all lysine residues in our model indicates that only Lys69 in the basic domain gets buried at the subunit interface upon complex formation, but it is involved in a salt bridge to Asp49 in crotapotin. Our model does not suggest that acylation of lysines in crotoxin plays a role in the dimerization process.

One of the differences between *Crotalus atrox* venom phospholipase (1PP2) and phospholipase A_2 from bovine pancreas (1BP2) is the presence of a calcium ion in the latter. Renetseder et al. (1985) conclude that the backbone conformation of the residues 30–34 in the structure of these two proteins has to be different because of the dimerization. However, a closer evaluation reveals that there is another important difference. In the *Crotalus atrox* venom phospholipase dimer Lys69 in one subunit interacts with Asp49 in the other. This aspartate is equiv-

alent to the one that binds calcium in 1BP2. In the crotoxin model the basic subunit has a lysine in the same position (69) as the *Crotalus atrox* venom phospholipase but the acidic subunit lacks it, while both subunits have the potentially calcium-binding aspartate at position 49. We therefore believe that the basic subunit of crotoxin binds a calcium ion, whereas the acidic subunit does not. It would be interesting to carry out dimerization studies as a function of calcium concentration in order to verify this hypothesis. Renetseder et al. (1985) suggest that upon dimerization steric hindrance of two bulky residues in the 30–34 region *Crotalus atrox* venom phospholipase is the main reason for the conformational differences in the backbone of the two phospholipase structures. In crotoxin the equivalent residues in the basic subunit are also bulky, but crotapotin lacks the residues that are responsible for steric hindrance in *Crotalus atrox* venom phospholipase.

The validity of our deductions depends heavily on the quality of our model. Fortunately, phospholipases are a highly homologous family of proteins. All structures known to date have the same overall fold, the same active site, and in case of dimerization, a highly homologous dimer interface (Renetseder et al. 1985). Using the laws that govern the correspondence between sequence homology and structural homology (Sander and Schneider 1991; Chothia and Lesk 1986), it is obvious that the model of crotoxin described in this study must have the correct fold. Most core residues are probably correctly located, and the high similarity of its active-site regions with equivalent sites in the structures of known, related proteins leads to confidence in that aspect of our model, too. It is difficult to assess the correctness of the surface loops, especially the one formed by the first ten residues of the crotapotin B-chain which are only tentatively known. Since these loops differ substantially between the homologous proteins with known structure (1BP2 and 1PP2) and have little effect on the function of the protein, we can safely conclude that the essence of our model, whose coordinates are available upon request, is correct.

The model presented here may prove useful for solving the x-ray crystal structure of crotoxin without having to resort to heavy atom derivatives. An attempt to solve the structure will be made shortly.

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