A Comparison of the Crystal Structures of Phospholipase A2 from Bovine Pancreas and Crotalus atrox Venom*

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The refined high resolution crystal structure of the bovine phospholipase A2 was compared with its counterpart from the venom of *Crotalus atrox*, the western diamondbacked rattlesnake. The strong similarity in their backbone conformations forms the basis of a common numbering system for the amino acid sequence. The three common major helices and much of the extended chain form a nearly identical "homologous core" structure. The variations in conformation usually arise from deletions/insertions or *en bloc* shifts of structural units. The exception to this is part of the highly conserved calcium-binding loop; however, this is not to be expected as 1) there is no calcium ion sequestered in the venom dimer as there is in the case of the bovine enzyme and 2) two side chains in that segment form dimer-stabilizing interactions between the subunits of the *C. atrox* enzyme.

The absolutely conserved catalytic network of hydrogen-bonded side chains formed by His 48, Tyr 52, Tyr 73, and Asp 99, as well as the hydrophobic wall that shields it, are virtually superimposable in the two structures. However, the details of the structural relationship between the amino terminus and the catalytic network differ in the two species and the ordered water molecules thought to be either functionally or structurally important in the pancreatic enzymes are not found in the crystal structure of the phospholipase A2 from *C. atrox*.

The most striking difference from a functional standpoint is the fact that the surface depression in the region of the catalytic network that has been commonly considered the active site is shielded substantially in forming the intersubunit contact surface of the dimeric venom enzyme.

Over the past several years the crystal structures of several phospholipases A2 and the pancreatic proenzyme have been determined and refined (1-5). Allowing for slight variations in their exact substrate preferences, it is clear that the members of this large family of homologous proteins share certain functional characteristics that presumably reflect common structural features (see reviews, Refs. 6 and 7). Most notable are the following functional similarities. (a) All of the enzymes require certain structural features of the phospholipid substrate; that is, they will hydrolyze only esters and not amides at the C2 of the naturally occurring enantiomer of phosphatic acid derivatives. (b) There is a marked enhancement of activity—sometimes by a factor as high as 105—when the phospholipid is condensed in a micellar aggregate. Whereas both the proenzyme and enzyme hydrolyze monomeric soluble substrates at about the same slow rate, only the enzyme's activity is enhanced by substrate aggregation. (c) Calcium ions, although weakly bound ($K_C > 10^{-4}$ M), are an obligatory requirement for activity and in most cases it binds to the enzyme before the phospholipid substrate. (d) Alkylation of His 48 destroys activity (5). (e) In the pancreatic enzymes at least, the terminal amino group is required for binding to aggregated substrate.

Brunie et al. (5) describe the refined crystal structure of a dimeric phospholipase A2 typical of rattlesnake venoms (5). Unlike the monomeric bovine and porcine enzymes, whose refined crystal structures have been reported earlier, this venom enzyme and certain close homologues function as dimers whose subunits dissociate with an equilibrium constant ranging from $10^{-9}$ to $10^{-11}$ M. In this paper we contrast this dimeric enzyme with its monomeric bovine counterpart to sharpen our focus on the common structural features underlying the above-mentioned functional characteristics of all phospholipases A2.

It should be borne in mind that crystallization conditions for these two crystal structures differ. Specifically the bovine enzyme has been crystallized from organic solvent at neutral pH, in the presence of 5 mM Ca$^{2+}$, and has a calcium ion bound to the protein; the venom dimer was crystallized from distilled water at pH 4.2. These chemical differences in the molecular environment and the fact that only the bovine enzyme bears a calcium ion makes the comparison of structure/function relationships less direct; nevertheless, there are striking similarities and significant differences that should help us understand phospholipase A2 function. Ultimately, to complete the picture comparisons will need to be made where the crystals of both the monomer and dimeric enzymes are at a comparable pH and contain (or are devoid of) the same functionally important ligands, such as calcium and substrate analogues.

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Comparison of Monomeric and Dimeric Phospholipase A<sub>2</sub>

**FIG. 1.** A common numbering system for phospholipase A<sub>2</sub>. Sequences were aligned primarily on the basis of the conformational similarity of the "homologous segments" in Table I and indicated here by a continuous heavy line (sinusoidal indicates helical structure). The broken lines indicate segments that are also clearly homologous but whose conformation deviates either locally (31 to 36) or globally (74 to 84) in order to make obvious functional or structural adjustments. Residues 59 and 61 of the <i>C. atrox</i> enzymes have been assigned their sequence numbers arbitrarily and deviate in position considerably from their bovine counterparts. The numbers in parentheses indicate the disulfide partner of the Cys residue below it. Position 123 contains an insertion in the equine phospholipase A<sub>2</sub>.

**MATERIALS AND METHODS**

The atomic coordinates of the molecular models used to compare the structures were determined for the monomeric bovine phospholipase (1.7 Å resolution) by Dijkstra et al. (1) and for the dimeric venom phospholipase (2.5 Å resolution) by Brunie et al. (5). Both sets have been deposited in the Brookhaven Protein Data Bank. Visual comparisons were carried out mainly by the program system GUIDE (8) and the displays photographed from an Evans and Sutherland Picture System 2 at the University of Groningen. Quantitative
comparisons were carried out in Groningen by programs written by Dr. Wim Hol and in Cambridge by programs written by Dr. Arthur Lesk.

**RESULTS AND DISCUSSION**

**A Common Numbering System Based on Structural Similarities**

To facilitate comparative studies of the structure, function, and evolution of the various members of this large family of homologous phospholipase A₂ molecules (reviewed in Refs. 7 and 9), it is necessary to have a common numbering system for the amino acid sequences. In Fig. 1 we propose a numbering system which optimizes the structural similarity in three dimensions between numerically corresponding residues in the bovine and the C. atrox enzymes. As these two sequences each represent a subclass near the extremes of the homologous family (9) they are ideally suited for this purpose. Moreover, the venom enzyme has two subunits with identical sequences but different environments in the crystal lattice. These subunits are crystallographically independent structures and were refined without imposing subunit symmetry; therefore, contrasting the subunits of the C. atrox enzyme (Table I and Fig. 2) provides a measure of the largest expected differences due to lattice effects or experimental errors.

Fig. 1 is based on structural similarity in a segmental sense. The sequences were divided into segments whose individual structures could be superimposed by a least squares minimization of which the rms² deviation of the corresponding Cα atoms was less than 0.60 Å (range 0.28 to 0.59 Å) and no pair of Cα atoms deviated by more than 1.0 Å. These segments and the quality of their fit are tabulated in Table I. These criteria of structural homology were chosen somewhat arbitrarily to find those segments that were unequivocally similar in conformation. As discussed below, the substructure formed by these homologous segments provides an excellent basis for superimposing the subunits of the C. atrox enzyme on the bovine enzyme. The segments range in length from 7 to 20 residues and together comprise 82 residues or 62% of the positions in the common numbering system. Of the 82 residues in the structurally homologous segments, 39 or 48% are identicalically conserved compared to 13 identical residues or 41% of the 32 common sequence positions regions outside these structurally homologous segments.

In addition to the structural differences caused by obvious deletions or insertions, part of the “calcium-binding loop” from residues 31 to 36 is not structurally similar. Despite the high degree of sequence homology, a different conformation would be expected for residues 31 to 36 since the bovine structure contains a calcium ion sequestered, in part, by the backbone carbonyl oxygens of residues 28, 30, and 32 and the calcium ion. Moreover, the side chains of Trp 31 and His 34 are involved in interactions between the subunits of the venom dimer that have no counterparts in the monomeric bovine enzyme. The relationship between calcium binding and intersubunit contacts of the calcium-binding loop has been discussed recently by Brunie et al. (5) and will be discussed further below. The “β-wing,” comprising residues 74 to 84, does not quite meet the criteria of segmental similarity stated above. Although shifted *en bloc* as a structural unit (Fig. 3), it is clearly similar in conformation and presents no difficulty in the sequence alignment.

### Table I

| Sequence segments | Bovine vs C. atrox left subunit | Bovine vs C. atrox right subunit | C. atrox left subunit vs C. atrox right subunit |
|-------------------|--------------------------------|---------------------------------|-----------------------------------------------|
| Homologous segments |                                  |                                 |                                               |
| 1-12              | 0.39                            | 0.42                            | 0.17                                          |
| 19-30             | 0.57                            | 0.54                            | 0.22                                          |
| 37-54             | 0.37                            | 0.34                            | 0.31                                          |
| 67-73             | 0.55                            | 0.50                            | 0.23                                          |
| 90-109            | 0.43                            | 0.28                            | 0.36                                          |
| 113-126           | 0.44                            | 0.41                            | 0.27                                          |
| β-wing (74-84)    | 0.36                            | 0.69                            | 0.54                                          |
| Homologous core   | 0.66                            | 0.64                            | 0.31                                          |
| Entire sequence   | 1.79                            | 1.65                            | 0.61                                          |

* A comparison of the left and right subunits of the C. atrox dimer is included as a point of reference (“left” and “right” are defined in Brunie et al. (5)).

* A segment is considered homologous if, following optimal superimposition by least squares, the rms difference between all Cα atoms in the segment is less than 0.6 Å and no Cα is more than 1.0 Å from its counterpart. These segments are indicated by a continuous heavy line in Fig. 1.

* This segment meets the criteria for a homologous segment when considering only the left subunit of C. atrox.

* The backbone structure composed of the six homologous segments taken as a single rigid unit.

* The 114 common sequence positions for which there is a residue in both the bovine and C. atrox structures.

* This segment, although homologous in sequence, and bounded by structurally homologous segments has large conformational differences probably related to calcium binding and/or subunit interactions (see text).

The “Homologous Core” Structure Has a Nearly Superimposable Backbone

Taken as a unit, the six homologous segments of Table I (designated by a continuous heavy line in Fig. 1) represent a backbone conformation that is globally preserved in both the pancreatic and venom enzymes. When combined and superimposed as a rigid structural unit, the Cα atoms of this homologous core structure in the bovine enzyme deviate from their counterparts in the dimeric venom enzyme by an rms distance of 0.66 and 0.64 Å for the left and right subunits, respectively. In both comparisons only 9 sequence positions of the 82 homologous residues have disparities between corresponding Cα atoms that are greater than 1.0 Å, the largest being 1.56 Å.

The structural similarity between the bovine and C. atrox structures is shown schematically in Fig. 2 which graphs the disparity in Cα positions and in Fig. 3 which shows a stereo-diagram of the Cα backbone superimposed by a least squares fit of the homologous core. A similar picture emerges if one attempts to superimpose the entire common backbone of the bovine and venom enzyme and improves the fit iteratively by rejecting those pairs that deviate badly. It will not be surprising to see this core structure preserved in all phospholipase A₂ structures of this homologous family, including the phospholipase subunits of the hetero-oligomeric presynaptic neuromuscles.
The bovine enzyme by a much longer connection in the C. atrox enzyme in which Lys 11 forms a long noncovalent link to the side chains of Glu 77 and Glu 78 through several water molecules.

The absence of the disulfide bridge provides a potential for a hinge-like movement of the β-wing in this and homologous crotaid enzymes, as suggested by the fact that the "thermal parameters" that characterize the motional freedom of the backbone atoms are 30 to 100% higher in the distal part of the wing than in the molecule in general.2 Table I and Fig. 3 show that the structure of the β-wing in the bovine phospholipase A2 is similar to its counterpart in each of the subunits of the C. atrox enzyme. The similarity of the backbone conformations is remarkable in view of the fact that in the two different molecular species the wings occupy different positions relative to the main body of the protein and, therefore, despite the high degree of sequence homology (55%) cannot make comparable tertiary structural interactions. This segmental similarity indicates that the conformation of this loop of β-structure is intrinsically well defined.

The "Missing" Loop—In the segment 57 to 66 all but Asp 59 and Cys 61 are deleted in the C. atrox sequence; that is, the mammalian and elapid phospholipases A2 have an "extra" loop. The difference in conformation starts at residue 55 which prematurely terminates a helix in the C. atrox enzyme to accommodate the deletion and ends at residue 67 where the structures become similar again. In both species Cys 61 holds this nonhomologous segment to the homologous core through a disulfide bridge with Cys 91. Not surprisingly the least squares superimposition leaves the Cα of Cys 61 4.0 and 4.2 Å from its counterparts in the C. atrox dimer; nevertheless, a common sequence position was assigned because of the disulfide's homologous structural role. (The preceding Asp residue of the venom enzyme has no obvious structural counterpart and was assigned to position 59 arbitrarily.) A structural comparison of the Cys 61 to 91 disulfide bridge and its neighbors suggests that the common practice of using conserved disulfide bridges to indicate regions of high structural homology carries some risk.

The extra loop in the bovine enzyme would appear to prevent dimerization similar to that of C. atrox in two ways. As shown in Fig. 3 the extra loop encroaches on the dyad axis of the C. atrox dimer and would thereby sterically block dimer formation. In addition to causing steric hindrance, the extra loop would also prohibit a dimer-stabilizing interaction found in the C. atrox enzyme (5). As shown in Fig. 4 the severe shortening of this segment in the C. atrox structure disrupts a helical hydrogen bond at residue 52 and exposes the carbonyls of Tyr 52 and Ala 55 to dimer-stabilizing hydrogen bonds with the Nδ2 of Asn 67 in the opposing subunit. This type of interaction would not occur in the bovine enzyme since the insertion of eight additional residues in the bovine sequence extends the helix to include the carbonyl oxygen of residues Tyr 52 and Ala 55 in helical hydrogen bonds. It should be kept in mind, however, that this extra loop of the pancreatic enzyme can undergo considerable conformation adjustments. A comparison of crystalline bovine and porcine phospholipases A2 (10) showed that in the segment 59 to 70 the bovine and porcine enzymes have a significantly different backbone conformation even though there is only one difference in the amino acid sequence (bovine Val 63 ↔ porcine Phe 63).

The Carboxyl-terminal Extension—The sequences of crotaid venom phospholipase A2 possess a characteristic extension of 6 to 8 residues beyond the carboxyl terminus of the

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5. S. Brunie and P. B. Sigler, unpublished observation.
mammalian and elapid sequences. Brunie et al. (5) point out in an earlier paper that this unusual sequence, which starts and ends with a disulfide bridge and contains three charged residues and two prolines, maintains a position that is virtually outside the globular fold as if it were a bannister or railing. Despite the fact that the carboxyl-terminal Cys 132 is covalently linked, via Cys 50, to a sequence segment containing the functionally important residues His 48, Asp 49, and Tyr 52 and even though the carboxyl-terminal extension is situated near the dimer interface; this segment does not interact directly with either the catalytic network or the calcium-binding region, nor does it contribute to the interaction between the dimer subunits. If this extension were added to the bovine enzyme, it would appear remote to any of the structural features considered essential to the enzyme's function.

The Catalytic Networks Are Identical

The side chains of the absolutely conserved residues—His 48, Tyr 52, Tyr 73, and Asp 99—form an identical hydrogen-bonded network in both the pancreatic and venom enzymes (1–3, 5). This conserved network is almost certainly involved in the catalytic function of phospholipase A2 as alkylation of the N61 of His 48 by p-bromophenacyl bromide completely inhibits the enzyme without interfering with binding to micelles and vesicles (for a cogent review of this point see Ref. 7). Fig. 5 shows that when the structural cores of both phospholipases are aligned, the hydrogen-bonded catalytic networks of the two enzymes are almost superimposed. Indeed, the three-dimensional structure of the catalytic network is almost as well conserved when comparing the bovine enzyme with a venom dimer subunit (rms difference for all atoms is 0.56 Å (left), 0.47 Å (right)) as when comparing the venom subunits with one another (rms difference for all atoms is 0.36 Å). Fig. 5 also shows the similarity in three-dimensional structure of a wall of almost invariant nonpolar side chains (Phe, Ile, Phe(Tyr), Cys, Ala, and Ala) that partially encloses this catalytic network.

In both the bovine and C. atrox structures there are several water molecules close to the functional groups of the catalytic network. The water molecule in the region of His 48 of the bovine enzyme is hydrogen-bonded to the N61 of the imidazole ring and has been implicated as the attacking nucleophile in the catalytic mechanism (11). Unlike the almost exact struc-
would undoubtedly shift the position of their side chains the orientation of the backbone carbonyl functions of Trp 31 and His 34 would be altered. There is an additional and perhaps related loop.

The Calcium-binding Region

One of the best conserved regions in the amino acid sequences of the phospholipase A₂ family is the segment from Tyr 25 to Gly 35. In virtually all sequences only two positions, 31 and 34, are not absolutely identical. The presence of 2 half-cysteine and 4 glycine residues suggested a well-defined and specially conformed backbone underlying an important function. Dijkstra et al. (1) established the functional role by showing that three of the carbonyl groups—Tyr 28, Gly 30, and Gly 32—formed part of the oxygen cage that sequesters a calcium ion—presumably the calcium ion critical for enzymatic activity. This conserved sequence was called the "calcium-binding loop."

The C. atrox structure contains no Ca²⁺ (5) and not unexpectedly, the carbonyl groups of Gly 30 and Gly 32 are oriented differently to their calcium-sequestering counterparts in the bovine enzyme. There is an additional and perhaps related reason for the dissimilarity between the conformation of the calcium binding loops. Fig. 6 shows that the two residues of the calcium-binding loop in the C. atrox dimer that are not universally conserved (Trp 31 replacing Leu 31 and His 34 replacing Ser 34) form intersubunit interactions with Leu 2 and Glu 6 of the opposing subunit. Fig. 6 shows that these interactions confer a different conformation on the calcium-loop. As pointed out by Brunie et al. (5) it would seem that if the C. atrox enzyme were to bind a calcium ion with the same stereochemistry as the bovine crystal structure, the reorientation of the backbone carbonyl functions of Trp 31 and His 34 would undoubtedly shift the position of their side chains and the stereochemistry of their intersubunit interactions would be altered.

The Amino Terminus

Proteolytic removal of the amino-terminal seven residues converts the pancreatic phospholipase A₂ to the enzyme. The newly created terminal amino group confers on the enzyme the ability to rapidly hydrolyze phospholipids in micellar aggregates at rates which can be several orders of magnitude higher than the rate at which soluble monomeric phospholipids are hydrolyzed. Since the hydrolysis of monomeric substrate is catalyzed at about the same slow rate by both proenzyme and enzyme, the enzyme's amino terminus appears to be the key element in creating a structure that binds aggregated substrates in a productive mode. This has been borne out by experiments with the pancreatic enzymes where modifying or removing the terminal amino group or changing its optical configuration destroys the special capacity of enzyme to attack aggregated substrates (6, 12). A comparison of the various enzyme structures (1-3, 5) with that of bovine proenzyme (4) suggests that the enzyme's unique capacity to attack aggregated substrates results—at least in part—from a structural transition that converts residues 1 through 4 and 67 through 73 from a disordered state in the proenzyme (4) into a well-defined state in the enzyme.

In the crystal structures of both bovine and porcine pancreatic phospholipase A₂, a water molecule has been implicated as the structural connection between the amino terminus and the catalytic site (1, 3). Brunie et al. (5) point out that a corresponding water molecule is not present in the C. atrox crystal structure and, since its catalytic network is superimposable on that of the pancreatic enzyme, the structure of the catalytic network in the C. atrox enzyme obviously does not depend on this water linkage. Instead they suggest (5) an "activation network" of hydrogen-bonds involving the terminal amino group, the side chain of Glu 4 and the backbone segment 67 to 73 that stabilize the conformation of the enzyme necessary for productive-mode aggregated substrate. Fig. 7 shows the differences in the conformation of the amino terminus and its hydrogen-bonded interactions.

Solvent Structure

As noted above, neither of the two water molecules considered to have functional significance in the pancreatic enzymes have counterparts in the C. atrox crystal structure; that is, the water molecule near His 48 that has been implicated as the attacking nucleophile and the water molecule that links the amino-terminal region to the functional groups of the
Fig. 6. Comparison of the calcium-binding loops. Bovine (continuous line) and C. atrox (broken line) segments superimposed by least squares fit of C, atoms in the homologous core. Calcium ion seen only in the bovine. In the C. atrox structures of N61 of Trp 31 and N82 of His 34 form hydrogen bonds with the side chain carboxyl oxygen of Glu 6 on the opposing subunit.

Fig. 7. Comparison of the amino-terminal regions. Bovine (continuous line) and C. atrox (broken line) segments superimposed by least squares fit of C, atoms in the homologous core. The filled and open circles are the terminal amino group of the bovine and venom enzymes, respectively. W’s indicate water molecules previously implicated (1) in linking the amino group to the catalytic network.

catalytic network. It is difficult to ascribe functional significance to this discrepancy since the crystals of the C. atrox enzyme contain no calcium ion and have a much lower pH. However, there is one striking similarity in solvent structure which occurs at the surface of a highly homologous and well-ordered helical region (residues 44 to 51 and 98 to 105). Here a cluster of solvent peaks was so well defined in the electron-density map of the bovine enzyme’s crystal structure that it was considered a molecule of the precipitating agent, methyl pentanediol, and was refined as such, albeit not very satisfactorily. A similar density on the corresponding surface of both subunits of the dimeric C. atrox enzyme was initially interpreted as a citrate ion until binding studies with radioactive citrate showed that no citrate ion was bound to the crystals and, therefore, these sites must be firmly bound water molecules (5). Since these water molecules in the C. atrox crystal structure coincide with atomic centers of the putative methyl pentanediol bound to the crystalline bovine enzyme, it is likely that this surface of the molecule, which is the same in both the bovine and venom enzymes, forms the same high affinity binding site for a water cluster. It is noteworthy that in both enzymes this surface is on the opposite side of the helices that contribute the side chains of His 48, Tyr 52, and Asp 99 to the catalytic network and Asp 49 to the liganding case of the calcium-binding site. It is not clear if these nearly isostructural clusters of water molecules have any functional significance.

The Catalytic Surface

In an earlier paper, Brunie et al. (5) pointed out that the dimeric organization of the C. atrox phospholipase A2 appears to prevent access to much of the catalytic network. In particular the catalytic interactions with the head group and interfacial recognition surface suggested by Dijkstra et al. (1) do not easily apply to a functional C. atrox dimer whose structure is similar to that seen in the crystal structure. We assume that both the bovine pancreatic enzyme and its C. atrox
counterpart have similar general mechanisms of substrate binding and catalysis. This structural comparison suggests, however, that mechanistic inferences must be drawn with care from any one particular crystallographically determined molecular model. Further studies of structural variants of phospholipase A₂, either alone or in complexes with appropriate functional ligands, will be required to provide a confident understanding of phospholipase A₂ function.

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