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The X-ray structure of a snake venom Gln48 phospholipase A_2 at 1.9 Å resolution reveals anion-binding sites☆

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Abstract

Phospholipase A_2 is an "interfacial" enzyme and its binding to negatively charged surfaces is an important step during catalysis. The Gln48 phospholipase A_2 from the venom of *Vipera ammodytes meridionalis* plays the role of chaperone and directs a toxic His48 PLA_2 onto its acceptor. In the venom the two phospholipases A_2 exist as a postsynaptic neurotoxic complex, Vipoxin. The X-ray structure of Gln48 PLA_2, complexed to sulphate ions, which mimic the negatively charged groups of anionic membranes, has been determined by the molecular replacement method and refined to 1.9 Å resolution. The protein forms a homodimer stabilized by ionic, hydrophobic, and hydrogen-bond interactions. The structure reveals two anion-binding sites per subunit. These sites are probably involved in interactions with the negatively charged membrane surface and, in this way, in the "targeting" of the toxic component to the receptors of the postsynaptic membranes. In the absence of the chaperone subunit the toxin changes the target of the physiological attack. A comparison of the homodimeric Gln48 PLA_2 structure with that of the heterodimeric Vipoxin reveals differences in regions involved in the pharmacological activity of the toxin. This fact, except the active site histidine substitution, can explain the absence of toxicity in the Gln48 protein in comparison to the His48 phospholipase A_2.

Keywords: X-ray structure; Phospholipase A_2; Neurotoxin; Snake venom

Phospholipase A_2 (phosphatide *sn*-2 acylhydrolase, PLA_2, EC 3.1.1.4) enzymes hydrolyse specifically the *sn*-2 ester bond of natural long fatty acid chain phospholipids [1]. The reaction products contain lysophospholipids, which can induce tissue damage, and arachidonic acid, a precursor of eicosanoid mediators of inflammation. PLA_2s participate in the digestion, remodelling, and metabolism of phospholipids, host defence, and signal transduction [2]. However, secreted phospholipolytic enzymes are involved in inflammatory and other human diseases such as rheumatoid arthritis, asthma, allergy, brain injury, cancer development, and cardiovascular disorders [3 and references therein]. Snake venom PLA_2s exhibit a wide variety of pharmacological activities: neurotoxicity, cardiotoxicity, myotoxicity, hypotensive, antiplatelet, haemolytic, hemorrhagic, edema-inducing, coagulant or anticoagulant effects [4].

The postsynaptic neurotoxin Vipoxin is the major lethal component in the venom of *Vipera ammodytes meridionalis*, the most toxic snake in Europe. It is a complex between a highly toxic His48 PLA_2 and a non-toxic component, Gln48 PLA_2, in which the active site histidine is substituted by a glutamine [6,7]. Both PLA_2s are closely related proteins with 62% sequence identity and the same length of the polypeptide chains, 122 residues [7]. Probably, the Gln48 protein is a product of evolution of the toxic enzyme. In the complex it reduces the activity and toxicity of His48 PLA_2 and Vipoxin is the first reported

☆ *Abbreviations:* PLA_2, phospholipase A_2.

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example of modulation of the toxic function generated by molecular evolution [8]. Gln48 PLA_2 plays the role of chaperone and directs its toxic counterpart to the receptors of neuromuscular junctions avoiding a non-specific binding. The toxin prevents the binding of acetylcholine to its receptor and in this way exerts its lethal effect. In the absence of chaperone subunit the toxic PLA_2 changes the binding site on the target membrane [9].

PLA_2s are "interfacial" enzymes and catalysis by these hydrolases involves initial binding to the lipid–water interface, followed by the catalytic step [10]. For this reason anion-binding sites on the surface of the enzyme molecule play an important role for the complex formation with the negatively charged natural substrates, phospholipids. It was shown that such sites in the porcine pancreatic PLA_2 are located in the interface region of the enzyme which makes contact with the substrate interface [11].

Here, we describe the X-ray structure of the *V. a. meridionalis* Gln48 PLA_2 complexed to sulphate ions, which mimic negatively charged groups on the membrane surface. Anion-binding sites have not been identified so far in the chaperone subunits of snake venom heterodimeric neurotoxic complexes. The structure reveals sites which can participate in the PLA_2 binding to anion interfaces during the interfacial catalysis. A comparison of the present structure with that of Vipoxin shows differences in the region of the toxicity site. Most probably, this is one of the reasons for the absence of toxicity in the Gln48 PLA_2.

Materials and methods

Crude venom was collected from *V. a. meridionalis* and the neurotoxin Vipoxin was isolated as described in [12]. The dissociation of the complex and separation of Gln48 PLA_2 from His48 PLA_2 were performed according to the procedure given in [7]. The lyophilized chaperone subunit was dissolved in distilled water at a concentration of 10 mg/ml. The crystallization was performed by the hanging-drop vapour diffusion method using 2% PEG-8000, 0.5 M Li_2SO_4 at 298 K. The hanging drops were 4 μl in volume and contained 2 μl of the protein solution and 2 μl of the reservoir solution. After 3 days crystals suitable for high resolution X-ray data collection were obtained. Diffraction data to 1.9 Å resolution were collected from a flash frozen crystal at 100 K with synchrotron radiation ($\lambda = 0.802$ Å) at the consortium beamline X13 at HASYLAB/DESY—Hamburg. The images were processed using the *DENZO* program package [13]. The initial phase problem was solved by the molecular-replacement techniques applying the program *AMoRe* [14] and using our coordinates of chain A of Vipoxin refined to 1.4 Å resolution (PDB code: 1jlt). The refinement was performed by molecular-dynamic techniques using the program *CNS* [15]. The *CCP*4 program suite [16] and the program *TURBO-FRODO* [17] were used for calculations and model building, respectively. The solvent molecules were added during refinement at chemically reasonable positions where $(F_0 - F_c)$ difference density exceeded 3σ. The final model has been analysed using the program *PROCHECK* [18] which indicated that 85.6% of all residues fall into the most favoured region of the φ, ψ plane. The average isotropic *B* factor for all protein atoms is 22.9%. The R_{value}/R_{free} ratio was 18.5% for the resolution range 25–1.9 Å. The model of the dimeric Gln48 PLA_2 contains 370 water molecules and four sulphate ions. The final stereochemical parameters, the data-collection, and refinement parameters are summarized in Table 1. The surface accessibility was calculated using the program *DSSP* [19].

Table 1
Data-collection and refinement statistics

Space group	$P4_122$
Unit-cell parameters (Å)	$a = b = 58.95$, $c = 139.12$
Crystal volume per Dalton (V_M) (Å^3 Da^{-1})	2.04/2 molecules per AU
Wavelength used (Å)	0.802
Data collection and refinement	
Resolution (Å)	25–1.9
No. of unique reflections	19,560
R_{merge} (%)	5.0 (13.0)
Completeness (%)	94.5 (97.9)
Refinement statistics	
R_{value}/R_{free} (%)	18.5/25.3
No. of amino acids	244
No. of solvent molecules	370
Average *B* factors (Å^2)	
Protein	22.9
Solvent molecule	33.7
R.m.s. deviations from ideal values	
Bond distances (Å)	0.021
Bond angle (°)	2.09
Torsion angles (°)	6.254
Ramachandran plot: non-Gly residues in	
Most favoured regions (%)	85.6
Additionally allowed regions (%)	13.4
Generously allowed regions (%)	1.0

Values in parentheses are for the last resolution shell.

Atomic coordinates. Coordinates of the refined model of the Gln48 PLA_2, containing bound sulphate ions, have been deposited in the Protein Data Bank with the entry code 1Q5T.

Results and discussion

Overall structure

The crystal of Gln48 PLA_2 diffracted to 1.9 Å resolution and belonged to the space group $P4_122$ with unit cell dimensions $a = b = 58.95$ and $c = 139.12$ Å. The structure contains two molecules forming a homodimer in the asymmetric unit. The homodimer has an ellipsoidal shape and dimensions of $58 \times 38 \times 40$ Å (Fig. 1).

The overall secondary structure of each polypeptide chain consists of two long and antiparallel α-helices (residues 40–54 and 89–109), a shorter N-terminal α-helix (residues 2–14), and the so-called "β-wing," a section of antiparallel β-sheet (residues 74–85) which extends outward from the globule. The structure is stabilized by seven disulphide bonds, Cys27–Cys126, Cys29–Cys45, Cys44–Cys105, Cys51–Cys98, Cys61–

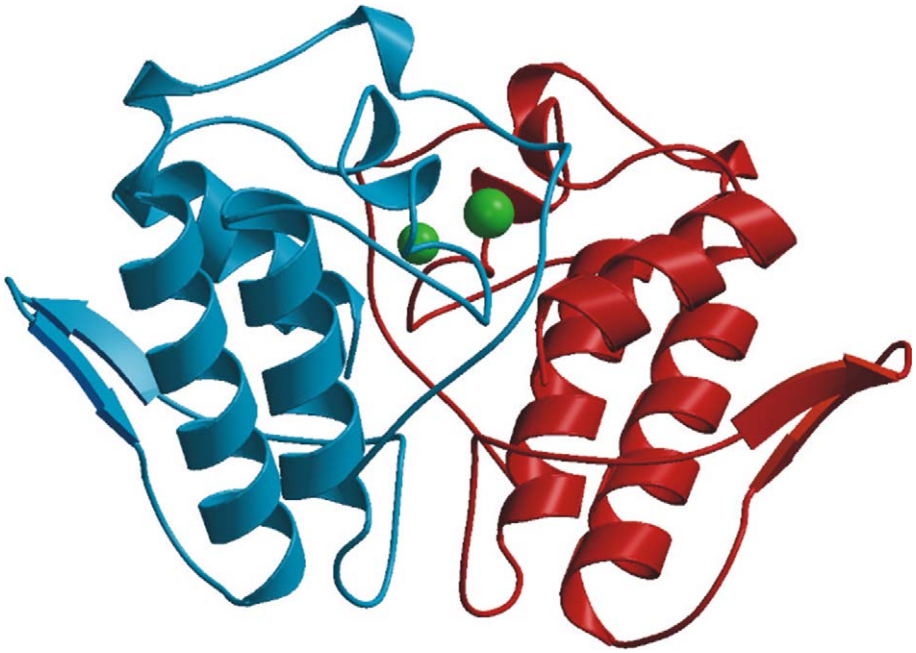

Fig. 1. Ribbon representation of the Gln48 PLA_2 dimer. The anion-binding site which is located in the same position in both monomers is shown as a circle.

Cys91, Cys96–Cys84, and Cys133–Cys50. The mutual orientation of the two long α-helices is fixed by two S–S bridges, Cys44–Cys105 and Cys51–Cys98. It is impressive how the relatively small PLA_2 molecule is "saturated" by seven disulphide bonds which add a considerable rigidity to the structure. S–S bonds in the protein polypeptide chains impose distance and angle constraints between the C^{β} and S^{γ} of the joined cysteine residues. Devedjiev et al. [20] published the crystal structure of the non-toxic subunit of the *V. a. meridionalis* toxin. Unfortunately, this structure is not deposited in the Protein Data Bank and we could not compare it directly with our three-dimensional structure. From the description of the model in [20] it seems that the general organization of the subunits in the two structures is similar. We have also observed similar hydrophobic interactions at the dimer interface. The major differences are in the localization and description of anion-binding sites, which have not been observed by the authors mentioned above. Another significant aspect of our work is the comparison of the Gln48 homodimeric structure with that of the heterodimeric Vipoxin, which revealed conformational differences between the non-toxic and toxic subunits in regions involved in the pharmacological activity.

Anion-binding sites

PLA_2 is an "interfacial" enzyme which is activated after binding to the anionic membrane surface. The structure determination of a PLA_2 complexed to an interface is hindered by experimental difficulties. For this reason we tried to model the binding of the enzyme to a negatively charged membrane surface by complexing with sulphate ions which mimic the negatively charged groups on the surface of the target membrane. Gln48 PLA_2 was crystallized in the presence of 0.5 M Li_2SO_4 and the structure revealed two anion-binding sites per subunit, one of them being the same for both components of the dimer. These regions can be considered as sites for interactions between the protein and negatively charged surface of aggregated substrates. A clear non-protein electron density was observed during the model refinement and interpreted as sulphate anions. These anions are bound through direct contacts with the positively charged side chains of Arg36 and Arg43 and by H-bonding interactions. The site 1 is located in both subunits in a cavity at the protein surface, in the C-terminal part of the polypeptide chain (Fig. 2A). The same region in His48 PLA_2 is involved in the neurotoxicity [21]. Fig. 2B shows a surface representation of the anion-binding site 1. The ligands are the side chains of Arg36 and Tyr120, as well as the main chain N-atoms of Arg36, Cys126, Thr127, and Glu128 (Table 2). The third sulphate anion is bound to chain A by the side chain of Arg43. This anion is coordinated by seven ligands including four water molecules (Table 2). The site is approximately 14 Å from the C-terminus of the molecule and is completely exposed on the protein surface. The fourth sulphate anion is liganded by the main chain N-atoms of Leu90 and Cys91, the side chain of Asp89, and five water molecules (Table 2).

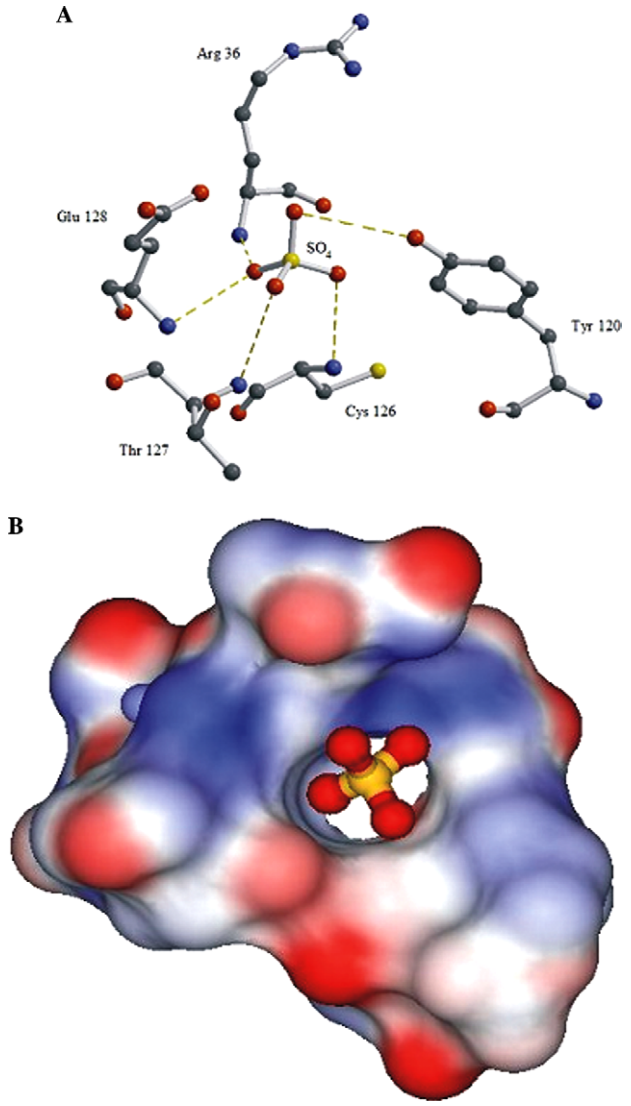

Fig. 2. (A) The anion-binding site in the Gln48 PLA_2 dimer, conserved in the two subunits. Interactions are shown by dotted lines; (B) surface representation of the same site. The program GRASP was used.

All anion-binding sites in the Gln48 PLA_2 dimer are exposed on the protein surface and can interact with substrate molecules. The anionic interface binding is important for the phospholipase A_2 catalysis and for the interfacial activation [22]. During the interfacial catalysis PLA_2 associates with the negatively charged phospholipids at the membrane surface. The observed anion-binding sites can be considered as potential sites for enzyme–substrate interactions. Studies in solution on the binding of crotoxin from the *Crotalus d. terrificus* venom to membranes showed that most probably the chaperone subunit of the toxin participates in the formation of a transient ternary complex, chaperone-toxic PLA_2-acceptor, contributing directly to the targeting of the toxin [5]. This can be valid also for the *V. a. meridionalis* Gln48 PLA_2.

Table 2
Coordination of sulphate ions at the anion-binding sites of each subunit of the homodimeric Gln48 PLA_2

Anion	Residue/atom	Subunit	Distance (Å)
SO4 (1,2)		A,B	
O1	Tyr120, OH		3.56
O2	Arg36, N		2.81
O2	Glu128, N		3.15
O2	Thr127, N		3.44
O3	Arg36, NH1		2.57
O3	Arg36, NE		3.05
O3	Thr127, N		3.48
O3	Cys126, N		3.55
O4	Cys126, N		2.82
SO4 (3)		A	
O2	Arg43, NH2		3.12
O2	Arg43, NE		2.39
O2	OW332		2.87
O3	Arg43, NH2		2.59
O3	OW154		2.59
O4	OW154		3.45
O4	OW332		3.30
SO4 (4)		B	
O1	OW193		2.88
O1	OW198		2.90
O2	OW201		2.58
O3	Cys91, N		3.14
O3	OW201		2.79
O4	Leu90, N		2.69
O4	Cys91, N		3.46
O4	Asp89, OD2		3.11
O4	OW193		2.71

Comparison of the homodimeric Gln48 PLA_2 with the heterodimeric (Gln48 PLA_2/His48 PLA_2) Vipoxin

The superposition of the Gln48 PLA_2 structure with that of the neurotoxin Vipoxin shows that several regions of the non-toxic subunit differ significantly (r.m.s. deviation > 1.0 Å) in their backbone conformations in the homo- and heterodimer (Fig. 3A). The major differences are in the region between residues 50 and 67, which includes part of an α-helix, the β-wing (residues 75–82), the segment between residues 87 and 91 (the beginning of α-helix), and the C-terminal part of the polypeptide chain (residues 126–131). The maximal deviation of approximately 3 Å demonstrates the flexibility of the β-wing region which has different orientation in the non-toxic component of hetero- and homodimer. The model shows that the C-terminus of the molecule is also flexible.

The three-dimensional structures of the toxic and non-toxic subunits of Vipoxin are very similar. They contain the same secondary structure elements: three α-helices, calcium binding loop, and β-wing. Moreover, the substrate-binding channel is also preserved in Gln48 PLA_2. One important difference is the substitution of the active site histidine by glutamine in the chaperone protein. However, it is known that the pharmacological

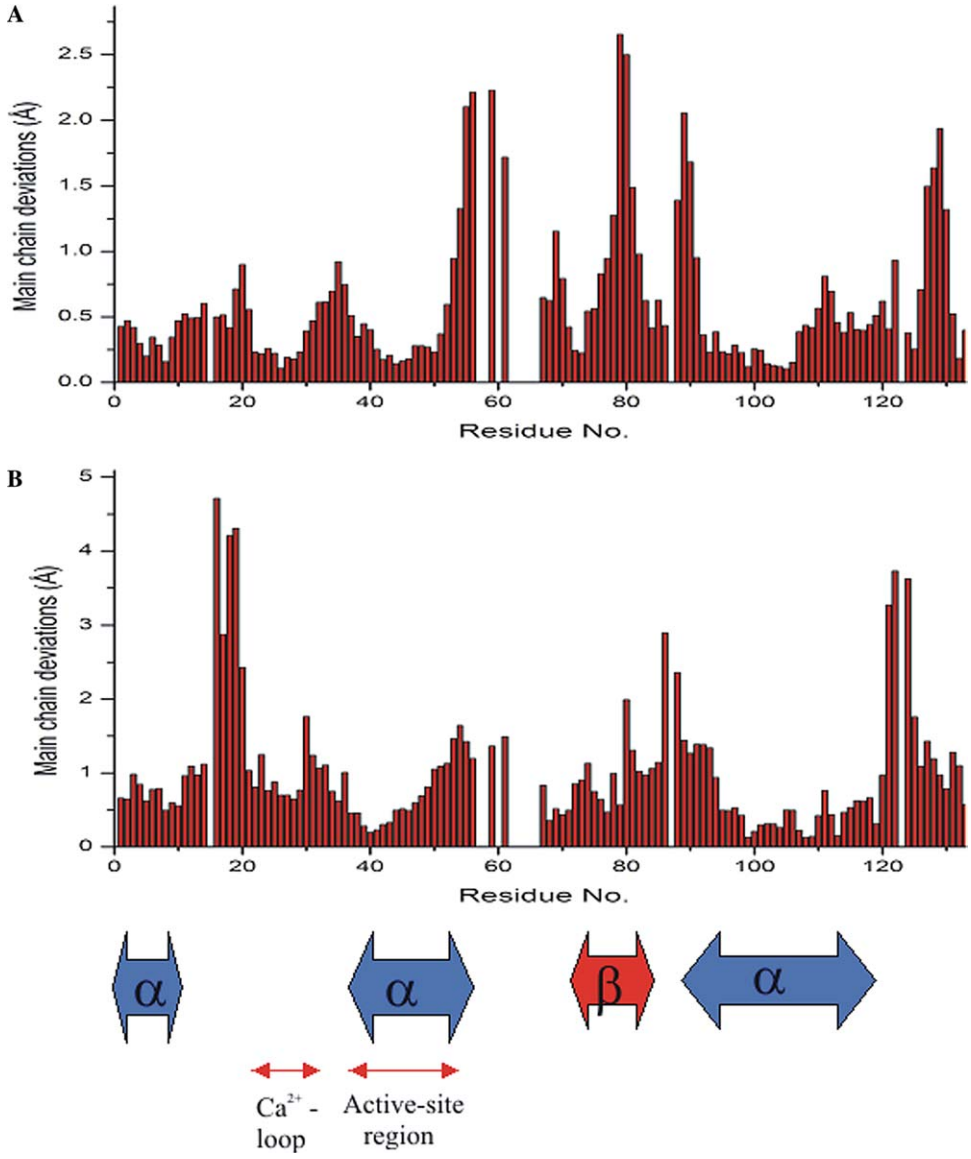

Fig. 3. (A) Main chain deviations plot between Gln48 PLA_2 in the homodimer and its counterpart in the heterodimer Vipoxin. (B) Main chain deviations plot between Gln48 PLA_2 in the homodimer and His48 PLA_2 of Vipoxin.

sites are independent of but sometimes overlap with the active site of PLA_2 [1]. There are examples in which no quantitative relationship between PLA_2 activity and toxicity has been observed [23]. This suggests that the difference in the pharmacological activities could be also due to structural differences in the regions involved in toxicity. The toxic function of PLA_2 has not been explained satisfactorily yet in terms of three-dimensional structure–function relationships. The regions where the Gln48 PLA_2 differs from the toxic His48 PLA_2 structure are displayed in Fig. 3B. They include the segment between residues 12–20 (the end of the N-terminal α-helix which contributes to the formation of the "lip" of the substrate-binding channel), the end of an α-helix (residues 53–61), the end of the β-wing, and the beginning of the longest α-helix (residues 86–89), and the toxicity site at the C-terminus of the molecule (residues 121–125) with a maximal deviation of approximately 5 Å. The region 121–125, residue 12, and the β-wing are involved in the PLA_2 neurotoxicity [24]. These structural differences, except the active site His48 substitution, may explain, at least in part, the difference in the pharmacological activity of the two subunits.

The interfacial region between the two subunits

The homodimeric complex is stabilized through ionic, hydrophobic, and hydrogen-bond interactions. The salt bridge between the carboxylic O atoms of Asp49 from one subunit and Lys69 NZ, from the other, is of special

interest because it includes the major Ca^{2+} ligand, Asp49. No bound metal ion was found at the calcium-binding loop. We have observed the same interaction also in heterodimeric PLA_2 complexes [21,25]. Probably, this is a common mechanism for stabilization of PLA_2s in the absence of added calcium not only for oppositely charged chains but also for identical subunits. The interface includes the side chains of Trp31 from each monomer. The bulky side chains of tryptophyl residues are involved in hydrophobic interactions which are important for the stabilization of the protein complexes. The complex formation decreases the accessible surface area of the two monomers by $\sim 1500\,\text{Å}^2$. It can be concluded that one important reason for the complex formation between the two Gln48 PLA_2 molecules is the free-energy minimization by excluding hydrophobic side chains from the water solvent.

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