# Crystal structure of vipoxin at 2.0 Å: an example of regulation of a toxic function generated by molecular evolution

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Abstract Vipoxin is the main toxic component in the venom of the Bulgarian snake *Vipera ammodytes meridionalis*, the most toxic snake in Europe. Vipoxin is a complex between a toxic phospholipase  $A_2$  (PLA<sub>2</sub>) and a non-toxic protein inhibitor. The structure is of genetic interest due to the high degree of sequence homology (62%) between the two functionally different components. The structure shows that the formation of the complex in vipoxin is significantly different to that seen in many known structures of phospholipases and contradicts the assumptions made in earlier studies. The modulation of PLA<sub>2</sub> activity is of great pharmacological interest, and the present structure will be a model for structure-based drug design.

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*Key words:* X-ray structure; Synchrotron radiation; Vipoxin; PLA<sub>2</sub>-complex

## 1. Introduction

Phospholipases represent a class of enzymes that catalyse the hydrolysis of membrane phospholipids to release free fatty acids. In particular,  $PLA_2$  (EC 3.1.1.4) hydrolyses the sn-2acyl bond of phospholipids producing equimolar amounts of lysophospholipids and free fatty acids. In addition to this activity,  $PLA_{2S}$  from snake venom include a wide variety of pharmacological activities such as neurotoxic effects and septic shock [1]. The ability to produce substrates for the generation of inflammatory lipid mediators in the process of tissue injury and rheumatoid arthritis [2] makes this specific class of phospholipases of medical and pharmaceutical interest. Structure-based drug design for the development of potent and specific inhibitors for these enzymes has led to the characterization and structural analysis of several  $PLA_{2S}$  and their complexes with substrate analogues [3–11].

The vipoxin complex consists of an alkaline PLA<sub>2</sub> and an acidic protein inhibitor. Both, the PLA<sub>2</sub>-subunit and the inhibitor consist of a polypeptide chain with 122 amino acids. The inhibitor-subunit of vipoxin reduces phospholipase activity in vitro by up to 60% [12]. The separated PLA<sub>2</sub>-subunit is so far the most toxic phospholipase [13] characterized to date  $(LD_{100} = 1-3 \ \mu g \text{ per } 20 \ g \text{ mouse})$ , and is the only PLA<sub>2</sub> known to form a complex with a highly homologous natural inhibitor. The high degree of sequence homology between the

PLA<sub>2</sub>-subunit and the inhibitor makes the complex of great genetic interest [14,15]. The vipoxin complex is also a neurotoxin with postsynaptic action, whereas all other known PLA<sub>2</sub>s exhibit a presynaptic action [13]. However, when the PLA<sub>2</sub>-subunit is separated as an isolated component from the vipoxin complex, it also exhibits presynaptic action [16].

### 2. Materials and methods

Details of purification are available from the authors. Crystals with high quality were grown within two weeks at 20°C by sitting drop technique from a solution with a final composition of 14% PEG 3350, 15% PEG 400, 10 mM CaCl<sub>2</sub>, 100 mM Na acetate at pH 4.8 containing 12 mg/ml Protein. One crystal was used to collect data up to 2.0 Å. All diffraction measurements were performed at -172°C on a flash frozen crystal of approximate dimensions of 0.2×0.1×1.5 mm<sup>3</sup>. Diffraction data were collected at EMBL-Hamburg, synchrotron beam line BW7B using a MAR Image Plate Scanner. The space group was assigned as  $P2_12_12$  with one molecule in the asymmetric unit. The cell dimensions are pseudotetragonal with a = 67.64, b = 67.69 and c = 46.82 Å, giving a packing parameter  $V_{\rm M}$  of 2.0. The images were processed using the DENZO program package [19] with a resulting  $R_{\rm symm} = 8.6\%$ . The phase problem was solved by molecular replacement techniques applying the program AmoRe [20] and one monomer of the dimeric phospholipase  $A_2$  from Crotalus atrox (pdb entry=1 PP2) as search model. The initial structure was refined by the maximum likelihood method using the program refmac [21]. The present crystallographic R factor is 0.16 ( $R_{\text{free}}$  is 0.23) using all data between 20.0 and 2.0 Å including 285 water molecules and one acetate. On average, bond lengths, interbond angle distances and planarities deviate from ideality by 0.013, 0.034 and 0.025 Å, respectively.

## 3. Results and discussion

We can show, that as expected from the extensive sequence homology (Fig. 1) the overall fold and disulphide network are similar to other known PLA<sub>2</sub>s. As shown in Fig. 2, the structures of vipoxin-PLA<sub>2</sub>-subunit and the inhibitor can both be closely superimposed on a PLA<sub>2</sub> from *Crotalus atrox* [11] with an r.m.s. difference for the  $C_{\alpha}$  positions of 1.48 and 1.46 Å, respectively.

One of the most striking features of the vipoxin complex is the manner in which the complex is formed. The reported structures of  $PLA_2$  from *Crotalus atrox*, and recently the structure of the isolated inhibitor of vipoxin [17] show that both form dimers with an almost exact two-fold rotational symmetry. Based on this arrangement Devedjiew et al. [17] have proposed a model for the vipoxin complex with a similar complex conformation. However the structure presented here shows that this is not the case and that the relative positions

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|                           |     |     |                           |     |                 |      |      |     |     | 10  |     |     |          |     |     |     |     |     |           | 20  |
|---------------------------|-----|-----|---------------------------|-----|-----------------|------|------|-----|-----|-----|-----|-----|----------|-----|-----|-----|-----|-----|-----------|-----|
|                           |     | α   | α                         | α   | α               | α    | α    | α   | α   | α   | α   | α   | α        | α   |     |     |     |     |           |     |
| PLA <sub>2</sub> Vipoxin  | Asn | Leu | Phe                       | Gln | Phe             | Ala  | Lys  | Met | Ile | Asn | Gly | Lys | Leu      | Gly | -   | Ala | Phe | Ser | Val       | Trp |
| Inh. Vipoxin              | Asn | Leu | Phe                       | Gln | Phe             | Gly  | Åsp  | Met | Ile | Leu | Gln | Lys | Thr      | Gly | -   | Lys | Glu | Ala | Val       | His |
| PLA <sub>2</sub> C. Atrox | Ser | Leu | Val                       | Gln | Phe             | Glu  | Thr  | Leu | Ile | Met | Lys | Ile | Ala      | Gly | -   | Arg | Ser | Gly | Leu       | Leu |
|                           |     |     |                           |     |                 |      |      |     |     | 30  | •   |     |          | 5   |     | U   |     | 5   |           | 40  |
|                           |     |     |                           |     |                 |      |      |     |     |     |     |     |          |     |     |     |     |     |           | α   |
| PLA <sub>2</sub> Vipoxin  | Asn | Tyr | Ile                       | Ser | Tyr             | Gly  | Cys  | Tvr | Cys | Glv | Tro | Glv | Glv      | Gln | Glv | Thr | Pro | Lvs | Asp       | Ala |
| Inh. Vipoxin              | Ser | Tyr | Ala                       | Ile | Tyr             | Gly  | Cys  | Tyr | Cys | Gly | Trp | Glv | Gly      | Gln | Gly | Arg | Ala | Gln | Asp       | Ala |
| PLA <sub>2</sub> C. Atrox | Trp | Tyr | Ser                       | Ala | Ťyr             | Gly  | Cys  | Tyr | Cys | Gly | Trp | Glv | Gly      | His | Gly | Leu | Pro | Gln | Asp       | Ala |
|                           | -   | •   |                           |     | •               | •    | •    | •   | •   | 50  | -   | •   | 2        |     | 5   |     |     |     | · · · · F | 60  |
|                           | α   | α   | α                         | α,  | α               | α    | α    | •   |     | α   | α   | •   | α        | α.  |     |     |     |     |           |     |
| PLA <sub>2</sub> Vipoxin  | Thr | Asp | Arg                       | Cys | Cys             | Phe  | Val  | His | Asp | Cys | Cys | Tvr | Glv      | Arg | Val | Arg | -   | -   | Glv       | -   |
| Inh. Vipoxin              | Thr | Asp | Arg                       | Ċys | Ċys             | Phe  | Ala  | Gln | Asp | Cys | Cys | Tyr | Gly      | Arg | Val | Asn | -   | _   | Asp       | -   |
| PLA <sub>2</sub> C. Atrox | Thr | Asp | Arg                       | Cys | Cys             | Phe  | Val  | His | Asp | Cys | Ċys | Tyr | Gly      | Lys | Ala | Thr | -   | -   | Asp       | -   |
|                           |     |     |                           | -   | •               |      |      |     | -   | 70  | •   | •   | -        | -   |     |     |     |     | 1         | 80  |
|                           |     |     |                           |     |                 |      |      |     |     |     |     |     |          | β   | β   | β   | β   | β   | β         | ß   |
| PLA <sub>2</sub> Vipoxin  | Cys | -   | -                         | -   | -               | -    | Asn  | Pro | Lys | Leu | Ala | Ile | Tyr      | Ser | Tyr | Ser | Phe | Lys | Lys       | Gly |
| Inh. Vipoxin              | Cys | -   | -                         | -   | -               | -    | Asn  | Pro | Lys | Thr | Ala | Thr | Tyr      | Thr | Tyr | Ser | Phe | Glu | Asn       | Gly |
| PLA <sub>2</sub> C. Atrox | Cys | -   | -                         | -   | -               | -    | Asn  | Pro | Lys | Thr | Val | Ser | Tyr      | Thr | Tyr | Ser | Glu | Glu | Asn       | Gly |
|                           |     |     |                           |     | Na 2201270antes |      |      |     |     | 90  |     |     |          |     | -   |     |     |     |           | 100 |
|                           | β   | β   | β                         | β   | β               |      |      |     | a   | α   | α   | α   | α        | α   | α   | α   | α   | a   | •         | a   |
| PLA <sub>2</sub> Vipoxin  | Asn | Ile | Val                       | Cys | Gly             | Lys  | -    | Asn | Asn | Gly | Cys | Leu | Arg      | Asp | Ile | Cys | Glu | Cys | Asp       | Arg |
| Inh. Vipoxin              | Asp | Ile | Val                       | Cys | Gly             | Asp  | -    | Asp | Asp | Leu | Ċys | Leu | Arg      | Ala | Val | Cys | Glu | Ċys | Asp       | Arg |
| PLA <sub>2</sub> C. Atrox | Glu | Ile | Ile                       | Cys | Gly             | Gly  | -    | Asp | Asp | Pro | Cys | Gly | Thr      | Gln | Ile | Cys | Glu | Cys | Asp       | Lys |
|                           |     |     | 1017 - 101 alia - 104 - 1 |     |                 |      |      |     |     | 110 |     |     |          |     |     |     |     |     | -         | 120 |
|                           | α   | α   | α                         | α   | α               | α    | α    | α   | α   |     |     |     |          |     |     |     |     |     |           |     |
| PLA <sub>2</sub> Vipoxin  | Val | Ala | Ala                       | Asn | Cys             | Phe  | His  | Gln | Asn | Lys | Asn | Thr | Tyr      | Asn | Arg | Asn | Tyr | Lys | Phe       | Leu |
| Inh. Vipoxin              | Ala | Ala | Ala                       | Ile | Cys             | Leu  | Gly  | Glu | Asn | Val | Asn | Thr | Tyr      | Asp | Lys | Asn | Ťyr | Ġlu | Tyr       | Tyr |
| PLA <sub>2</sub> C. Atrox | Ala | Ala | Ala                       | Ile | Cys             | Phe  | Arg  | Asp | Asn | Ile | Pro | Ser | Tyr      | Asp | Asn | Lys | Tyr | Тгр | Leu       | Phe |
|                           |     |     |                           |     |                 |      |      | -   |     | 130 |     |     | •        | -   |     | -   | •   | •   |           |     |
| PLA, Vinovin              | Sar | Ser |                           | Sar | Ara             | Cure | ٨٠٠  | C1- | Th- | Sar | Chr | Cl- | <b>C</b> |     |     |     |     |     |           |     |
| Inh Vinovin               | Ser | Ser | -                         | Ser | Hie             | Cys  | The  | Gh  | Glu | Sor |     | Gin | Cys      |     |     |     |     |     |           |     |
| PLA. C Atrov              | Pro | Dro | -                         | Lvc | Acr             | Cys  | 1.11 | Glu | Ch  | Dro | Ch  | Dro | Cys      |     |     |     |     |     |           |     |
|                           | 110 | 110 | -                         | Lys | Loh             | Cys  | nig  | Olu | Ju  | E10 | Old | riu | Cys      |     |     |     |     |     |           |     |

Fig. 1. Sequence comparison showing the sequences of the vipoxin-PLA<sub>2</sub>, the vipoxin inhibitor and the *Crotalus atrox* PLA<sub>2</sub> [11]. The sequence numbering refers to the bovine pancreatic PLA<sub>2</sub> [10] accordance to the numbering scheme commonly adopted in literature. Catalytic site residues (ovals), the residues supposed to be involved in a potential calcium binding (squares), and the regions of secondary structure are indicated accordingly.

of the two subunits are significantly different to other dimeric PLA<sub>2</sub>s and most interestingly also to the isolated inhibitor. In the vipoxin complex one subunit is rotated by about 180° to give a pseudo mirror symmetry (Fig. 3) and the complex is stabilized by twelve intermolecular contacts which obviously differ from those in the dimeric PLA<sub>2</sub> from *Crotalus atrox* (Table 1). Furthermore there is no evidence for the complex 'recognition site' involving residues Phe<sup>3</sup>, Trp<sup>31</sup> and Tyr<sup>119</sup> of the inhibitor as proposed by Devedjiew. Table 1 summarises the contacts found in the vipoxin complex in comparison to those found in the dimer of *Crotalos atrox* PLA<sub>2</sub>.

The lack of activity for the inhibitor is explained by the replacement of the catalytically essential His<sup>48</sup> by glutamin (Fig. 1) whereas in the PLA<sub>2</sub>-subunit the side chains of His<sup>48</sup>, Asp<sup>99</sup> and Tyr<sup>52</sup> (Fig. 4) form an active site which is conserved in all phospholipases [3–11]. However, in the vipox-in-PLA<sub>2</sub>-subunit, the region referred to be the calcium-bind-ing loop is structurally different from other PLA<sub>2</sub>s. The Ca<sup>2+</sup>-

binding region found in the refined crystal structure of bovine PLA<sub>2</sub> [10] and of human PLA<sub>2</sub> [8] the three backbone carbonyl oxygens of Tyr<sup>28</sup>, Gly<sup>30</sup> and Gly<sup>32</sup> and the carboxylate group of Asp<sup>49</sup> form a Ca<sup>2+</sup> coordination site. This conformation is not possible in the PLA2-subunit, because the backbone carbonyl oxygens of Tyr28 and Gly32 (Table 1) are already involved in intermolecular contacts with the inhibitor. Moreover, the carbonyl oxygen of  $Gly^{32}$  (PLA<sub>2</sub>) is about 5.5 Å apart from the potential calcium-binding site. Based on the  $Ca^{2+}$ -free structure of PLA<sub>2</sub> from *Crotalus atrox*, Keith et al. [11] have suggested that the positively charged alkylammonium side chain of Lys<sup>69</sup> may satisfy the need for partial charge neutralisation in the region of the structurally shielded Asp<sup>49</sup> carboxylate. Also in vipoxin there is an ionic interaction between Lys<sup>69</sup> (Inhibitor) and Asp<sup>49</sup> (PLA<sub>2</sub>). The binding of calcium by the PLA2-subunit would require a change in the conformation of the complex, which would weaken the stability of the complex. The requirement of Ca<sup>2+</sup> for activity needs



Fig. 2. Stereo-plot showing the superimposed  $C_{\alpha}$ -backbone structures of the phospholipases  $A_2$  of vipoxin (red), and from *Crotalus atrox* (green) and the Vipoxin inhibitor (blue). The three  $\alpha$ -helices fit very close. Only in the region 65–75 of the flexible  $\beta$ -sheet and the area around the potential  $Ca^{2+}$  binding loop (residues 27–33) higher deviations are visible. The r.m.s. deviations for the  $C_{\alpha}$  positions using Vipoxin-PLA<sub>2</sub> as target are 1.48 Å for the inhibitor and 1.50 Å for the PLA<sub>2</sub> from *Crotalus atrox*.

further discussion as, although greatly reduced, the PLA<sub>2</sub>-subunit in complex with the inhibitor still shows enzyme activity in the absence of calcium.

White et al. [7] have reported the crystal structure of Cobra-Venom PLA<sub>2</sub> in complex with a phosphonate transition-stateanalogue which shows the interactions in the active site. For vipoxin the electron density maps have clearly shown the presence of an acetate, originating from the crystallization medium, in the active site. Acetate is the terminal part of a fatty acid and therefore it is interesting to note that this acetate is recognized in the position of the free fatty acid after cleavage of the 2-sn-acyl bond [7]. Fig. 4 shows that the acetate forms hydrogen bonds with His<sup>48</sup> and Gly<sup>30</sup> as well towards a water molecule in the active site. Considering the absence of calcium and the remaining reduced activity, this is an indication for an active site coordinated substrate-analogue. No acetate is found in the corresponding position of the inhibitor as might be expected due to the missing histidine in position 48.

The vipoxin complex displays bifunctional behaviour. Whilst the phospholipase activity is reduced by up to 60% if complexed with the inhibitor, it has also been shown that, when separated from the inhibitor, the phospholipase soon

irreversibly loses its enzymatic activity [16]. Similarly, the neurotoxic activity of vipoxin in complex persists for at least four years, whereas it is lost within some days after separation of the complex [12]. Snake venom contains several phospholipases and in the case of vipoxin, importance is placed on preservation of toxicity. Thus, the inhibitor provides stability to sustain the toxicity for long periods at the expense of phospholipase activity. Nature has found a compromise between these two biological functions. The crystal structure of the vipoxin complex reveals the molecular interactions, which effect these modifications of biological functions.

To elucidate the new and upcoming aspects in the structure function relationship of vipoxin further X-ray studies to high resolution, including studies of complexes with bound substrate analogues are intended.

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Table 1

Intermolecular contacts stabilizing the Vipoxin-complex in comparison to contacts in the dimeric PLA<sub>2</sub> complex of Crotalus atrox

| Vipoxin  |   |   |   |  | Dimer of PLA <sub>2</sub> from Crotalus atrox   |  |  |   |  |  |  |  |  |
|--|---|---|---|--|---|--|--|---|--|--|--|--|--|
| Atom of PLA <sub>2</sub>   |   | Atom of   | inhibitor                                 | Distance<br>(Å)                                      | Atom of<br>Chain R  | PLA <sub>2</sub>                                 | Atom of<br>Chain L   | PLA <sub>2</sub>                                  | Distance<br>(Å)                                      |  |  |  |  |
|  | Νδ2<br>Νδ2<br>Ν<br>Ο<br>Νε<br>Ο<br>Οδ1<br>Οδ2 | $\begin{array}{c} {\rm Gly^{33}}\\ {\rm Gln^{34}}\\ {\rm Gly^{32}}\\ {\rm Lys^{69}}\\ {\rm Lys^{12}}\\ {\rm Leu^2}\\ {\rm Lys^{69}}\\ {\rm Lys^{69}}\\ {\rm Lys^{69}}\end{array}$ | Ο<br>Οε1<br>Ο<br>Νζ<br>Ο<br>Ν<br>Νζ<br>Νζ | 3.1<br>2.9<br>2.8<br>2.7<br>3.1<br>3.0<br>2.9<br>3.0 | $\begin{array}{c} {\rm Glu}^6 \\ {\rm Glu}^6 \\ {\rm Trp}^{31} \\ {\rm His}^{34} \\ {\rm Asp}^{49} \\ {\rm Tyr}^{52} \\ {\rm Ala}^{55} \\ {\rm Asn}^{67} \end{array}$ | ΟεΙ<br>ΟεΙ<br>Νε2<br>Νε2<br>ΟδΙ<br>Ο<br>Ο<br>Νδ2 | His <sup>34</sup><br>Trp <sup>31</sup><br>Glu <sup>6</sup><br>Glu <sup>6</sup><br>Lys <sup>69</sup><br>Asn <sup>67</sup><br>Asn <sup>67</sup><br>Ala <sup>55</sup> | Νε2<br>Νε1<br>Οε1<br>Οε1<br>Νζ<br>Νδ2<br>Νδ2<br>Ο | 2.6<br>2.8<br>3.0<br>2.9<br>3.3<br>2.5<br>3.3<br>3.0 |  |  |  |  |
| Val <sup>55</sup><br>Gly <sup>59</sup><br>Cys <sup>61</sup><br>Lys <sup>69</sup> | Ο<br>Ο<br>Οδ1<br>Νζ                           | Asn <sup>56</sup><br>Asn <sup>56</sup><br>Asp <sup>49</sup><br>Cys <sup>29</sup>  | Νδ2<br>Νδ2<br>Ο<br>Ο                      | 2.6<br>3.3<br>2.8<br>3.2                             | Asn <sup>67</sup><br>Lys <sup>69</sup>  | Νδ2<br>Νζ  | Tyr <sup>52</sup><br>Asp <sup>49</sup>   | Ο<br>Οδ1  | 2.7<br>3.3   |  |  |  |  |



Fig. 3. A cartoon plot of the overall structure of the vipoxin complex (PLA<sub>2</sub> in blue and inhibitor in red). Based on the orientation of the vipoxin-PLA<sub>2</sub>, the dimeric PLA<sub>2</sub> from *Crotalus atrox* is superimposed and only the part of the dimer corresponding to the inhibitor is shown (in white and dashed lines). The relative position of the inhibitor to the corresponding PLA<sub>2</sub> from *Crotalus atrox* is obviously different. The drawing was created by the program MOLSCRIPT [18].

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Fig. 4. A view towards the active site of the Vipoxin showing also the coordination of the acetate. The supposed calcium binding region close to  $Asp^{49}$  is blocked by the side chain of  $Lys^{69}$  of the inhibitor, which forms a salt bridge with  $Asp^{49}$ . Hydrogen bonds are shown in dashed lines.

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