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Asp49 phospholipase A_2–elaidoylamide complex: a new mode of inhibition☆

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Abstract

The inhibition of phospholipase A_2s (PLA_2s) is of pharmacological and therapeutic interest because these enzymes are involved in several inflammatory diseases. Elaidoylamide is a powerful inhibitor of a neurotoxic PLA_2 from the *Vipera ammodytes meridionalis* venom. The X-ray structure of the enzyme–inhibitor complex reveals a new mode of Asp49 PLA_2 inhibition by a fatty acid hydrocarbon chain. The structure contains two identical homodimers in the asymmetric unit. In each dimer one subunit is rotated by 180° with respect to the other and the two molecules are oriented head-to-tail. One molecule of elaidoylamide is bound simultaneously to the substrate binding sites of two associated neurotoxic phospholipase A_2 molecules. The inhibitor binds symmetrically to the hydrophobic channels of the two monomers. The structure can be used to design anti-inflammatory drugs.

Keywords: Phospholipase A_2; Neurotoxin; Elaidoylamide; X-ray structure

Phospholipase A_2s (phosphatide *sn*-2 acylhydrolases, PLA_2s; EC 3.1.1.4) are a rapidly growing superfamily of enzymes which hydrolyze stereospecifically the *sn*-2 ester bond of natural phospholipids. They catalyze reactions at lipid-aqueous interfaces. PLA_2s play an important role in a number of biological processes such as phospholipid metabolism and remodelling, mediator production, homeostasis of cellular membranes, host defense, and signal transduction [1]. The liberated lysophospholipids are important in cell signalling, membrane perturbation, and can induce tissue damage [2,3]. One of the reaction products is arachidonic acid which is a precursor of eicosanoids, potent mediators of inflammation and signal transduction. In this way, PLA_2 is involved in chronic inflammatory diseases such as rheumatoid arthritis, asthma, platelet aggregation, acute hypersensitivity and pancreatitis, sepsis and septic shock, respiratory distress syndrome, etc. [4–8]. Snake venom PLA_2s exert additionally a wide variety of pharmacological activities: neurotoxicity, cardiotoxicity, myotoxicity, hypotensive, antiplatelet, hemolytic, hemorrhagic, edema-inducing, coagulant or anticoagulant effects [9]. For this reason it is of pharmacological and medical interest to develop specific inhibitors for PLA_2.

The venomous snake *Vipera ammodytes meridionalis* is of public health significance in Europe. Envenomation by this viper causes very often rapid death due to the effect of the venom on the neuromuscular junctions, which prevents the binding of acetylcholine to its receptor. The major toxic factor has been identified as a basic PLA_2 associated with an acidic non-toxic PLA_2-like protein in a neurotoxic complex [10,11]. The acidic component modulates the enzymatic and pharmacological activities of the toxic subunit [11].

☆ *Abbreviations:* PLA_2, phospholipase A_2.

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Here, we report the crystal structure of PLA_2 isolated from the neurotoxic complex of the *V. a. meridionalis* venom and complexed to elaidoylamide (the amide of *trans*-9-octadecenoic acid). Fatty acids are important components of the biological membranes where they are bound to phospholipids and cholesterol esters or exist as free compounds. Fatty acid derivatives modulate the lipid membrane properties which, on the other side, regulate membrane protein functions such as enzyme activity, protein–membrane interactions, etc. [12]. We have found that elaidoylamide is a powerful inhibitor of the *V. a. meridionalis* venom PLA_2 [13]. The present structure reveals a new mode of PLA_2 inhibition: the hydrocarbon chain of one molecule of fatty acid is bound simultaneously to the substrate binding hydrophobic channels of two PLA_2 molecules. To our knowledge, this is the first crystal structure of Asp49 PLA_2 inhibited by an added fatty acid.

Materials and methods

Crude venom was collected from *V. a. meridionalis* inhabiting southeastern Europe. The neurotoxin Vipoxin was isolated as described in [14]. The isolation of His48 PLA_2 was performed according to the procedure given in [15]. Elaidoylamide is a generous gift of Prof. M. Jain from the University of Delaware, USA. The crystallization conditions are described in [13]. Diffraction data were collected from a flash-frozen crystal at 100 K with a synchrotron radiation ($\lambda = 0.802$ Å) at the consortium's beamline X13 at HASYLAB/DESY—Hamburg. The images were processed using the *DENZO* (SCALEPACK) suite program [16]. The initial phase problem was solved by the molecular replacement techniques using the program *AMoRe* [17] and using our coordinates of Vipoxin refined to 1.4 Å resolution (PDB code: 1jlt). The refinement was performed by molecular-dynamic techniques using the program *CNS* [18]. The *CCP*4 program suite [19] and the program *TURBO-FRODO* [20] were used for calculations and model building, respectively. The final model has been analyzed using the program *PROCHECK* [21].

Atomic coordinates. Coordinates of the refined model of the PLA_2–elaidoylamide complex have been deposited in the Protein Data Bank with the entry code 1RGB.

Results and discussion

Overall structure

The crystal of elaidoylamide–PLA_2 complex belonged to the space group $P2_12_12_1$ with unit cell dimensions of $a = 46.6$ Å, $b = 82.7$ Å, $c = 199.5$ Å. The structure contains two identical homodimers in the asymmetric unit. One of the dimers is rotated by approximately 90° with respect to the other. In the dimer AB the molecular dyad is approximately parallel to the *C*-axis and in the dimer CD the molecular dyad is approximately parallel to the *B*-axis. In each dimer one molecule of the inhibitor is bound simultaneously to both protomers which are related by a non-crystallographic 2-fold symmetry. The shape of the homodimer, which is composed of two covalently identical subunits, is similar to that of oblate ellipsoid. The relative orientation of the partners can be described as head-to-tail (Fig. 1). Data-collection and refinement parameters are summarized in Table 1.

The "catalytic network" formed by His48, Tyr52, Tyr73, and Asp99, as well as secondary structure elements characteristic for the Group II PLA_2s, is conserved also in the enzyme isolated from the *V. a. meridionalis* venom. In the present structure the ε-NH_2 of Lys69 of one subunit forms a salt bridge with the carboxylic group of Asp49 and vice versa which stabilizes the local conformation. The structure is dominated by 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 the disulfide bonds Cys27–Cys126, Cys29–Cys45, Cys44–Cys105, Cys51–Cys98, Cys61–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.

The interfacial region between the two subunits. Stabilization of the dimer

The homodimeric complex is stabilized through ionic, hydrophobic, and hydrogen-bond interactions summa-

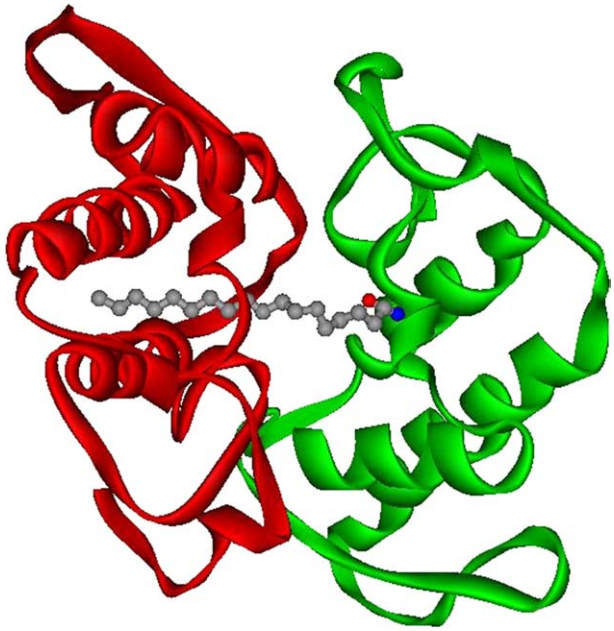

Fig. 1. Three-dimensional structure of the complex between the inhibitor elaidoylamide and the dimer of *V. a. meridionalis* neurotoxic phospholipase A_2, ribbon representation. Molecule A is colored in red, molecule B—in green, and the hydrocarbon chain of the inhibitor—in grey. The monomers are related by a non-crystallographic 2-fold symmetry. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Table 1
Data-collection and refinement statistics

Space group	$P2_12_{11}$
Unit-cell parameters (Å)	
a	46.57
b	82.68
c	119.47
Crystal volume per Dalton (V_M Å^3 Da^{-1})	2.13
Data collection and refinement	
Wavelength used (Å)	0.802
Resolution range (Å)	20.0–3.3
Total number of reflections used	69.923
No. of unique reflections	7251
Average I/σ	13 (3)
R_{merge}[a]	0.117 (0.573)
Completeness (%)	99.1 (98.6)
Refinement statistics	
R_{value}/R_{free}	0.234/0.286
No. of amino acids	488
Modelled water molecules	0
Average atomic *B* factor (Å^2)	34
R.m.s. deviations from ideal values	
Bond distances (Å)	0.009
Bond angle (°)	1.6
Ramachandran plot: non-Gly residues in (%)	
Most favored regions	64.7
Additionally allowed regions	31.7
Generously allowed regions	3.6
Number of diffraction images	127

Values in parentheses are for the high resolution bin.
[a] $R_{merge} = \sum |I - \langle I \rangle| \sum I$, where $\langle I \rangle$ is the average intensity and the summation is done for all reflections.

Table 2
Intermolecular contacts stabilizing the complex between the potent inhibitor elaidoylamide and the dimer of the *V. a. meridionalis* phospholipase A_2

PLA_2 molecule A	Distance (Å)	Elaidoy-lamide	Distance (Å)	PLA_2 molecule B
His48 ND1	3.0	N1		
Gly30 N	3.0	O2		
His48 CE1	4.2	C2		
Phe5 CE2	4.4	C2		
Gly30 CA	3.7	C3		
		C4	4.4	Trp31 CZ2
		C5	3.7	Trp31 CE2
		C6	4.0	Trp31 CD1
Leu2 CD1	4.0	C7	4.2	Trp31 CD1
		C8	3.5	Leu2 CD1
Lys69 NZ	3.7	C9		
Trp31 CE2	4.5	C9		
Trp31 CZ2	3.7	C10		
Trp31 CZ2	3.7	C11		
		C11	3.4	Gly30 CA
Trp31 CH2	3.4	C12		
		C13	4.1	Cys29 CA
		C14	3.6	Phe5 CE2
		C14	3.8	Phe106 CZ
		C15	3.5	Cys45 CB
		C15	4.0	Phe5 CZ
		C16	3.4	Phe5 CZ
		C16	3.7	Cys45 CA
		C16	3.8	His48 CB
		C16	3.5	Phe106 CZ
		C17	3.2	Phe106 CZ
		C18	3.6	Phe106 CG
		C18	4.0	Ala102 CB
		C18	3.4	Cys44 CB

rized in Table 2. The hydrophobic substrate binding channels are occupied by the hydrocarbon chain of the inhibitor. The interface between the monomers includes mainly non-polar side chains. The bulky indole groups of Trp31 and Trp20 from both subunits are involved in hydrophobic interactions which play an important role for the stabilization of the complex. The salt bridge between the carboxylic O atoms of Asp49 from one subunit and Lys69 NZ, from the other, contributes significantly to the structural integrity of the bound polypeptide chains. We have observed the same interaction also in the cases of heterodimeric PLA_2 complexes [22,23]. Finally, the long hydrocarbon chain of elaidoylamide, which occupies the substrate-binding channels of the monomers and participates in a number of interactions with non-polar groups, serves as a hydrophobic anchor stabilizing the dimer (Fig. 1). The inhibitor additionally supports the mutual orientation of the two monomers.

Binding of elaidoylamide to the dimer of Asp49 PLA_2

Fig. 2 shows $F_o - F_c$ omit electron density of elaidoylamide bound simultaneously to the substrate binding channels leading to the catalytic sites of the two PLA_2 molecules associated in a dimer. In this way the inhibitor blocks the access of the substrate to the enzyme active site. This is a new type of inhibition of Asp49 PLA_2 by added fatty acid. Dynamic light scattering measurements confirmed that the *V. a. meridionalis* PLA_2 molecule, at the concentration used for the crystallization experiments, forms stable dimers in solution [24]. The dimers are catalytically and pharmacologically active. The structure shows that the binding of elaidoylamide is stabilized by two hydrogen bonds between the inhibitor's amide group, on one side, and the ND1 atom of the catalytic site His48, and the main chain nitrogen atom of Gly30, on the other. The length of these bonds was calculated to be 3.0 Å (Table 2). Theoretical ab initio fitting of an amide ligand (formamide) at the PLA_2 active site allowed one to predict the existence of a hydrogen bond between ND1 of His48 and the nitrogen atom of the amide [25]. The "mouths" of the two hydrophobic channels are arranged in a manner suitable for simultaneous binding of a fatty acid chain. The inhibitor binds to the monomers in symmetrical manner. The hydrocarbon chain of elaidoylamide participates in a number of hydrophobic interactions with residues from the substrate binding

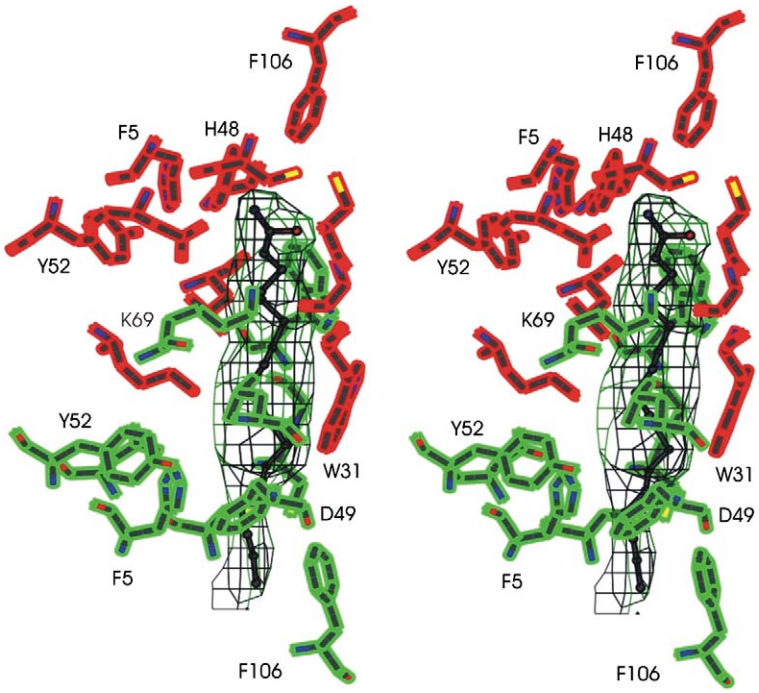

Fig. 2. $F_o - F_c$ omit electron density of elaidoylamide bound simultaneously at the hydrophobic pockets of two monomers in the PLA_2 dimer. The carbohydrate chain of the inhibitor is shown in black and the residues of the substrate binding site of each monomer in red and green, respectively. The figure is prepared by the program *MOLSCRIPT* [36]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

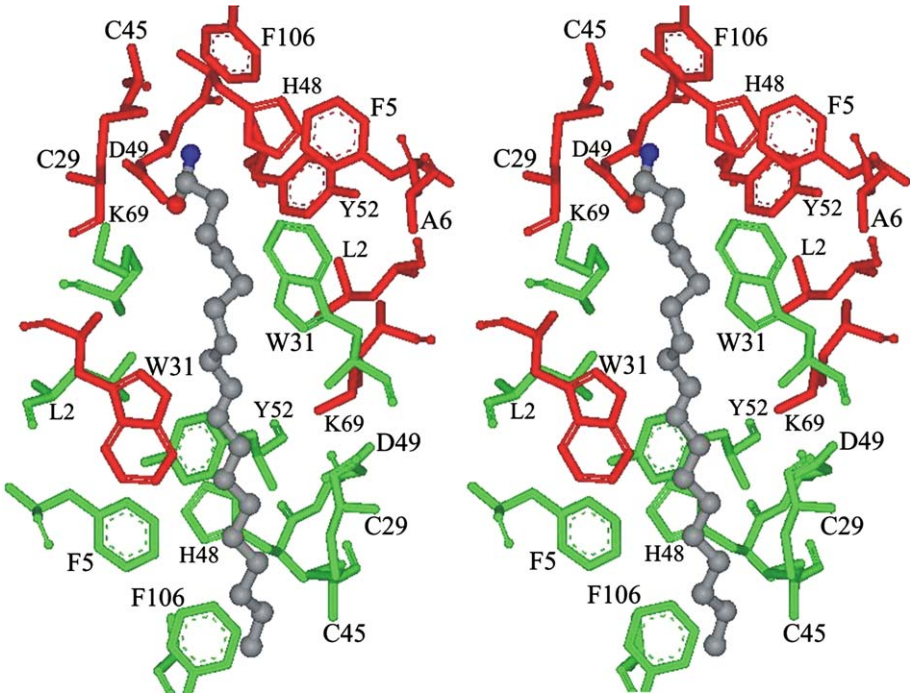

Fig. 3. Stereofigure of the binding of elaidoylamide at the active sites of two *V. a. meridionalis* phospholipase A_2 molecules associated in a dimer. The figure demonstrates symmetrical interactions between the hydrocarbon chain of the inhibitor and the side chains of residues in identical positions in the two polypeptide chains.

channel, such as Leu2, Phe5, Cys29, Trp31, Lys69, and Phe106. The interacting residues are symmetrically located on opposite sides of the inhibitor (Fig. 3). The conformation of Trp 31, which is located at the "mouth" of the channel, is suitable for hydrophobic interactions with elaidoylamide. The respective van der

Waals interactions are described in Table 2. All these interactions provide the major stabilizing effect for the enzyme–inhibitor complex and are schematically present in Fig. 4.

Here, we present the observed mode of binding of elaidoylamide at the substrate binding sites of the monomers in the dimer. As the two monomers are identical and the chemical environment of the binding pocket for elaidoylamide is symmetrical, theoretically there is an equal probability of the N-atom of the inhibitor NH_2 group to be bound to His48 of each monomer.

Pedersen et al. [26] showed fatty acid binding exclusively to Lys49 mutants of PLA_2. The substitution of Asp49 by lysine results in greatly reduced phospholipolytic activity or in its absence. Bound fatty acids (in protein:fatty acid ratio 1:1) have thus so far only been

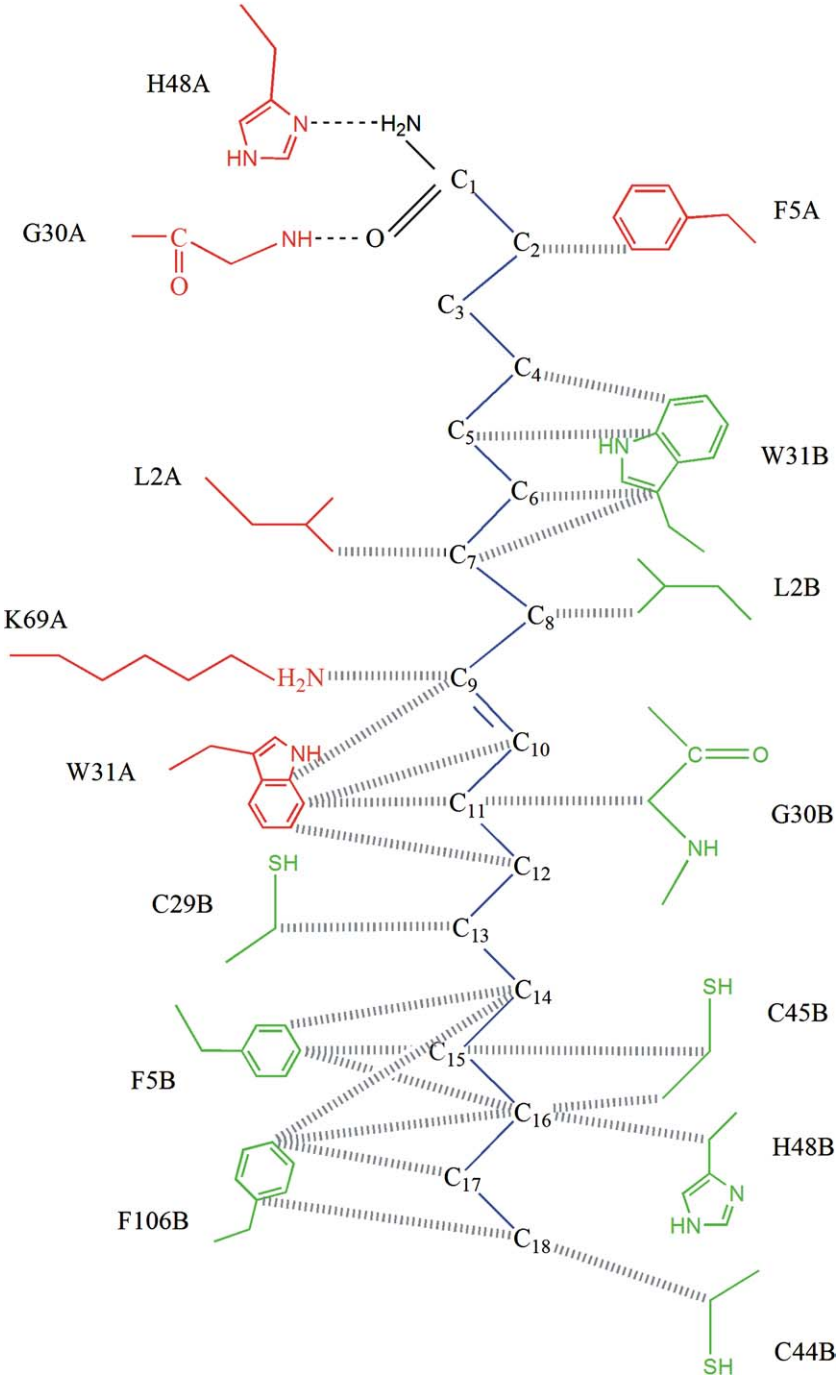

Fig. 4. A scheme of the enzyme–inhibitor interactions in the complex between elaidoylamide and the dimer of the *V. a. meridionalis* neurotoxic phospholipase A_2. Residues from the molecule A are colored in red, those from molecule B—in green, and the hydrocarbon chain of the inhibitor—in blue. Hydrogen bond (- - -) and van der Waals (|||) interactions are labelled. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

observed in two crystal structures of Lys49 homologues of PLA_2 [27]. The fatty acids have not been added to the crystallization solution but probably result from an incomplete catalysis, with a failure of product release [27].

The present crystal structure is therefore different to those of phospholipid analogues complexed to PLA_2s from different sources [28–32]. Thus, the crystal structure of the complex between a dimeric PLA_2 from the *Naja naja atra* venom and L-1-*O*-octyl-2-heptylphosphonyl-*sn*-glycero-3-phosphoethanolamine shows that one molecule of this compound is bound to each enzyme molecule. From the orientation of the *sn*-1 and *sn*-2 substituents of the inhibitor it is highly unlikely that both active sites of the enzyme dimer can simultaneously interact with the same substrate [28]. The crystal structure of the anion-assisted dimer of porcine pancreatic PLA_2, complexed to the products of hydrolysis of the platelet activating factor, shows that a molecule of the

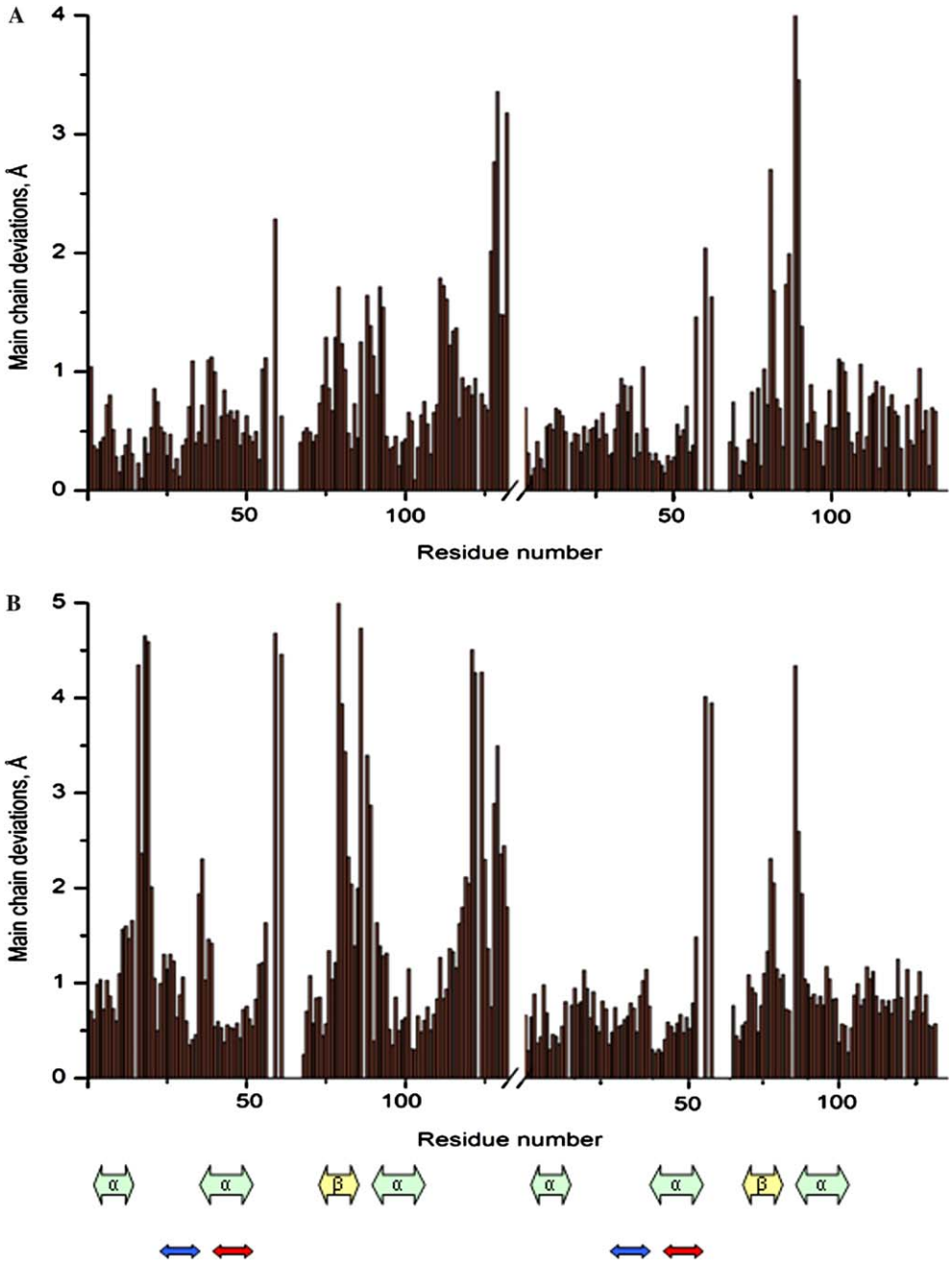

Fig. 5. Main chain r.m.s. plots between: (A) the two PLA_2 homodimers in the asymmetric unit and (B) the PLA_2 homodimer and the heterodimer of Vipoxin. The α-helices are indicated by green arrows and the β-wing by yellow arrow. The calcium binding loop and the active site are labelled by blue and red arrows, respectively. In the region of the β-wing, which is a part of the toxicity site, r.m.s. differences between 1 and 6 Å were calculated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

product acetate is bound to subunit A and the other product, 1-octadecyl-*sn*-glycero-3-phosphocholine, to subunit B [33]. In the complex between α-tocopherol and dimeric PLA_2 from the *Vipera russelli* venom one molecule of vitamin E is bound specifically to one of the subunits [34].

Comparison of the neurotoxic homodimeric PLA_2 structure with that of the heterodimeric toxin Vipoxin

The *V. a. meridionalis* neurotoxic complex Vipoxin binds to the postsynaptic membranes of the neuromuscular junctions preventing the binding of acetylcholine to its receptor [35]. In this way it blocks the neuromuscular transmission of skeletal muscles and exerts its lethal effect. The separation of the toxic PLA_2 from the chaperone subunit changes the target of the physiological attack from the post- to presynaptic membranes affecting the neurotransmitter release. To correlate the change of the pharmacological activity with possible structural changes in the separated neurotoxin, we have compared the toxic dimeric PLA_2 structure with that of Vipoxin using the PDB code 1jlt. The overall conformation of the two homodimers in the asymmetric unit is highly similar, with an average r.m.s. displacement of 0.9 Å for the main chains (Fig. 5A). Comparison of each of the two PLA_2 homodimers with the hetorodimer Vipoxin showed an average main chain r.m.s. of 1.4 Å (Fig. 5B). However, more differences were observed in the region of the β-wing (residues 76–81), which is a part of the toxicity site, with r.m.s. deviations of 1–5 Å. This suggests that the change of the pharmacological activity is connected with conformational changes in the toxic PLA_2.

Conclusions

The present investigations reveal a new mode of PLA_2 inhibition by an amide of fatty acid. Probably, the proposed inhibitor will be tolerant to living organisms. Additional studies in this direction will be performed. The design of specific PLA_2 inhibitors is of great pharmacological interest and requires a detailed knowledge of the mode of binding of the inhibitor to the enzyme active site. The three-dimensional structure of the complex elaidoylamide–PLA_2 can be used to design anti-inflammatory drugs. The simple hydrocarbon chain of the inhibitor can be in principle well adjusted in the very homologous (conserved) substrate binding channels leading to the catalytic sites of Group I/II/III PLA_2s. In this respect the inhibitor can have more universal applications.

The change of the target of the physiological attack of the separated from Vipoxin neurotoxic PLA_2 from the post- to presynaptic membranes of the neuromuscular junctions is connected with a conformational change of its polypeptide chain in the region of the toxicity site.

Acknowledgments

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