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Non-steroidal anti-inflammatory drugs as potent inhibitors of phospholipase A_2: structure of the complex of phospholipase A_2 with niflumic acid at 2.5 Å resolution

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Phospholipase A_2 (PLA_2; EC 3.1.3.4) catalyzes the first step of the production of proinflammatory compounds collectively known as eicosanoids. The binding of phospholipid substrates to PLA_2 occurs through a well formed hydrophobic channel. Surface plasmon resonance studies have shown that niflumic acid binds to *Naja naja sagittifera* PLA_2 with an affinity that corresponds to a dissociation constant (K_d) of 4.3×10^{-5} *M*. Binding studies of PLA_2 with niflumic acid were also carried out using a standard PLA_2 kit that gave an approximate binding constant, K_i, of $1.26 \pm 0.05 \times 10^{-6}$ *M*. Therefore, in order to establish the viability of PLA_2 as a potential target molecule for drug design against inflammation, arthritis and rheumatism, the three-dimensional structure of the complex of PLA_2 with the known anti-inflammatory agent niflumic acid {2-[3-(trifluoromethyl)anilino]nicotinic acid} has been determined at 2.5 Å resolution. The structure of the complex has been refined to an *R* factor of 0.187. The structure determination reveals the presence of one niflumic acid molecule at the substrate-binding site of PLA_2. It shows that niflumic acid interacts with the important active-site residues His48 and Asp49 through two water molecules. It is observed that the niflumic acid molecule is completely buried in the substrate-binding hydrophobic channel. The conformations of the binding site in PLA_2 as well as that of niflumic acid are not altered upon binding. However, the orientation of the side chain of Trp19, which is located at the entry of the substrate-binding site, has changed from that found in the native PLA_2, indicating its familiar role.

1. Introduction

Phospholipase A_2 (PLA_2; EC 3.1.1.4) catalyzes the hydrolysis of the ester linkage at the *sn*-2 position of phospholipids, leading to the production of free arachidonic acid and lysophospholipids (Smith, 1992). It displays enhanced activity towards lipids in lamellar and micellar aggregates both in membranes and at other lipid–water interfaces (Pieterson *et al.*, 1974; Verger, 1976; Jain & Berg, 1989; Gelb *et al.*, 1995, 1999). The release of free arachidonic acid from the membrane pool is responsible for the biosynthesis of eicosanoids. The eicosanoids are implicated in the triggering of inflammation. The role of inhibitors of PLA_2 in controlling inflammatory effects is not as yet clearly understood. On the other hand, non-steroidal anti-inflammatory drugs (NSAIDs), with their well known anti-inflammatory effects, have not been correlated with the inhibition of PLA_2 enzymes. Since high levels of PLA_2 have been detected in inflammatory conditions, the relief caused by NSAIDs in inflammatory disorders is

indicative of a unique relationship between PLA_2 and inflammatory disorders. In order to determine the binding of NSAIDs with PLA_2 enzymes and the nature of their interactions, we report here the results of binding studies of niflumic acid {2-[3-(trifluoromethyl)anilino]nicotinic acid} with PLA_2 and the crystal structure of the complex formed between PLA_2 and niflumic acid at 2.5 Å resolution. The PLA_2 used here is from cobra (*Naja naja sagittifera*) venom, which belongs to the group I family of enzymes and is hence similar to other group I PLA_2s from human pancreas (Grataroli *et al.*, 1981, 1982; Nishijima *et al.*, 1983) and spleen (Nakaguchi *et al.*, 1986; Kanda *et al.*, 1989). Therefore, the mechanism of binding of substrates to these enzymes is also expected to be similar. It has been shown that the mode of inhibitor binding is similar in both snake and human PLA_2 enzymes (Schevitz *et al.*, 1995; Singh, Ethayathulla *et al.*, 2005).

2. Materials and methods

2.1. Purification of the protein

Crude venom of the south Indian/Andaman cobra subspecies *N. naja sagittifera* was obtained from Irula snake farm in Tamil Nadu (India). 250 mg venom was dissolved in 25 ml deionized water. This was centrifuged at 16 000 rev min^{-1} for 20 min to remove insoluble material. The supernatant was collected and diluted twice with 50 m*M* ammonium acetate buffer pH 6.0. The diluted sample was loaded onto a Cibacron blue affinity column (30.0 × 2.5 cm) pre-equilibrated with the same buffer. The column was washed with 20 m*M* ammonium acetate buffer pH 6.0 to remove unbound fractions. In the next step, 50 m*M* ammonium bicarbonate buffer pH 8.0 was passed through the column to remove weakly bound proteins. Finally, 20 m*M* ammonium carbonate buffer pH 10.5 was used to elute the bound PLA_2. These fractions were dialyzed against ammonium acetate buffer pH 7.5 and then loaded onto a diethylaminoethyl (DEAE) Sephacel anion-exchange column (10 × 2.5 cm) at the same pH. In order to elute the acidic PLA_2, a salt gradient of 0.0–0.5 *M* NaCl in 50 m*M* ammonium acetate buffer pH 7.5 was used. Fractions with PLA_2 activity were eluted at 0.25 *M* NaCl and dialyzed against ammonium acetate buffer pH 6.0. Minor contaminants were removed by passage through a sulfopropyl (SP) Sephadex cation-exchanger column (10 × 2.5 cm) equilibrated with ammonium acetate buffer pH 6.0. The unbound fractions were collected and dialyzed against water to remove salt. The purified samples were lyophilized.

2.2. Enzymatic assay

The purified samples of PLA_2 were used in the kinetic studies. All assays were carried out using Correlate-Enzyme Assay PLA_2 kit (Assay Design Inc., catalogue No. 907-002) at 298 K. The assay was performed using the specific PLA_2 substrate hexadecylphosphocholine (HePC) from the kit, which is converted into sulfhydryl product by PLA_2. The presence of sulfhydryl product is detected colorimetrically using Ellman's reagent [DTNB; 5,5′-dithio-bis(2-nitrobenzoic acid)], which forms a yellow-coloured product owing to the presence of sulfhydryl. Niflumic acid (Sigma–Aldrich, USA) was dissolved to 10 µ*M* in 10 m*M* sodium phosphate buffer pH 7.0 containing 2 m*M* $CaCl_2$ and 10% methanol. Stock solutions of PLA_2 (0.196 mg ml^{-1}) and of the substrate (Assay Design Inc., catalogue No. 907-002; 10 m*M*) were prepared in the same buffer. The enzyme concentration was fixed at 1.4×10^{-7} *M*. Four different concentrations of niflumic acid, 1.4×10^{-7}, 2.8×10^{-7}, 4.2×10^{-7} and 14×10^{-7} *M*, were used for kinetic studies. The enzyme was incubated with the above concentrations of niflumic acid for 3 h. The substrate was dissolved at 0.1–2.1 m*M* at steps of 0.4 m*M* in the reaction-mixture solution. The initial velocity of the reaction was calculated from the changes in absorbance recorded at 405 nm with a Perkin–Elmer spectrophotometer (model Lambda 25). The data were analyzed using the Lineweaver–Burk method (Lineweaver & Burk, 1938).

2.3. Binding studies of niflumic acid using surface plasmon resonance (SPR)

Binding studies were carried out using the BIACore 2000 apparatus (Pharmacia BIACore AB, Upsala, Sweden). The BIACore apparatus is a biosensor-based system for real-time specific interaction analysis (Szabo *et al.*, 1995). The CH5 sensor chips, surfactant 120 and the amine-coupling kit containing *N*-hydroxysuccinimide (NHS), *N*-methyl-*N*′-3-(diethylaminopropyl) carbadiimide (EDC) and ethanolamine hydrochloride were used. The running buffer used was 5 m*M* sodium phosphate pH 6.0, 0.005% surfactant P20. The immobilization of PLA_2 was performed at a flow rate of 10 µl min^{-1} at 298 K. The dextran on the chip was equilibrated with running buffer and carbomethylated matrix was activated using an EDC/NHS mixture. The immobilized PLA_2 was studied by injecting niflumic acid at concentrations of 50, 75, 100, 125 and 150 µg ml^{-1} in 50 m*M* sodium phosphate pH 6.0 with 5 m*M* $CaCl_2$. The samples of niflumic acid were injected separately at different cycles at a flow rate of 10 µg ml^{-1}. The dissociation of niflumic acid was also studied in 50 m*M* sodium phosphate buffer pH 6.0. Regeneration of the immobilized protein was performed with 10 m*M* glycine–HCl buffer pH 2.5. Analysis of the data was carried out using the *BIA* evaluation software (Pharmacia BIACore; Nieba *et al.*, 1996). 210 µl PLA_2 from *N. naja sagittifera* (50 µg ml^{-1}) in 10 m*M* sodium phosphate pH 4.8 was injected and unreacted groups were blocked by injection of ethanolamine pH 8.5. The SPR signal for immobilized PLA_2 was found to be 5049 RVs.

2.4. Crystallization

The niflumic acid was dissolved in 10 m*M* sodium phosphate buffer pH 6.0 containing 2 m*M* $CaCl_2$ and 30% methanol. Purified protein was dissolved in the above solution to a final protein concentration of 10 mg ml^{-1}. Crystallization was performed using the hanging-drop vapour-diffusion method at 293 K in 24-well plates. 10 µl protein drops were equilibrated against reservoir solution containing 10 m*M* sodium phosphate buffer with 2 m*M* $CaCl_2$ and 30% ethanol.

Table 1
Data-collection statistics.

Values in parentheses correspond to the highest resolution shell (2.59–2.50 Å).

Space group	$P4_1$
Unit-cell parameters (Å)	$a = b = 42.3$, $c = 65.9$
V_M (Å^3 Da^{-1})	2.1
Solvent content (%)	41
No. of molecules in the unit cell	4
Resolution range (Å)	20.0–2.5
Total No. of measured reflections	55852
No. of unique reflections	4011
Completeness (%)	99.7 (99.5)
R_{sym} (%)	6.3 (23.8)
$I/\sigma(I)$	20.6 (7.6)

Diamond-shaped crystals of dimensions up to 0.3 × 0.3 × 0.3 mm were obtained after a week.

2.5. X-ray intensity data collection

The X-ray intensity data were collected at 285 K using a MAR Research 345 mm imaging-plate scanner mounted on a RU-300 rotating-anode X-ray generator (Rigaku, Japan) equipped with Osmic mirrors. The data were processed using *DENZO* and *SCALEPACK* from the *HKL* package (Otwinowski & Minor, 1997). This gave a data set with 99.7% completeness to 2.5 Å resolution (Table 1). The space group was $P4_1$, with unit-cell parameters $a = b = 42.3$, $c = 65.9$ Å. The packing density for one PLA_2–niflumic acid complex molecule in the asymmetric unit of these crystals was 2.1 Å^3 Da^{-1}, corresponding to a solvent content of approximately 41%, a reasonable value for globular proteins (Matthews, 1968).

2.6. Structure determination and refinement

The crystal structure determination of the complex formed between PLA_2 and niflumic acid took place using the molecular-replacement method with the program *AMoRe* (Navaza, 1994). The coordinates of native PLA_2 from *N. naja sagittifera* (PDB code 1sz8; Singh, Jabeen *et al.*, 2005) were used to build the initial search model. The top peak in both the rotation and translation functions gave the best solution in the correct space group $P4_1$. The solution was further improved with rigid-body refinement, which gave a correlation coefficient of 68% and an R factor of 42%. Manual fitting of the model to the electron density was carried out using the program *O* (Jones *et al.*, 1991). 10% of the reflections were randomly selected to create an independent data set of test reflections for cross-validation of the refinement procedure. The structure was refined in the resolution range 20.0–2.5 Å, initially with the program *X-PLOR* (Brünger, 1992) using protocols for rigid-body refinement, slow cooling, simulated annealing and minimization. The final steps of refinement were carried out with *CNS* (Brünger *et al.*, 1998) using torsion molecular dynamics, slow cooling, simulated annealing and maximum-likelihood target function, energy minimization and individual B-factor refinements. After these steps, the R and R_{free} factors fell to 0.238 and 0.261, respectively. At this stage, characteristic non-protein electron density in the $|F_o - F_c|$ at 2.5σ was observed in the hydrophobic substrate-binding site of PLA_2 into which the molecule of niflumic acid (Krishna Murthy & Vijayan, 1979) was modelled (Fig. 1). The coordinates of niflumic acid thus fitted were included in the further steps of refinement. The spherical electron densities for the Ca^{2+} atom in both Fourier $|2F_o - F_c|$ and difference Fourier $|F_o - F_c|$ maps were observed at greater than 6σ. The positions of 101 water molecules were also identified from the difference Fourier $|F_o - F_c|$ maps and were checked manually for their interactions with protein atoms. The R factor for the final model was 0.187 and the R_{free} factor was 0.227. The crystallographic refinement statistics are given in Table 2.

Table 2
Refinement statistics.

PDB code	1td7
Resolution limits (Å)	20.0–2.5
No. of reflections	4011
R_{cryst}† (%)	18.7
R_{free}‡ (%)	22.7
Protein atoms	904
Niflumic acid atoms	20
Calcium ions	1
Water molecules	101
R.m.s.d. bond lengths (Å)	0.01
R.m.s.d. bond angles (°)	1.8
R.m.s.d. dihedral angles (°)	15.9
Overall G factor	0.07
Mean B factor (Å^2)	
Main-chain atoms	31.3
Side-chain atoms and waters	32.8
All atoms	32.0
Niflumic acid	78.2
Ramachandran plot	
Residues in most favoured regions (%)	90.4
Residues in additionally allowed regions (%)	9.6

† $R_{cryst} = \sum ||F_{obs}| - |F_{calc}|| / \sum |F_{obs}|$. ‡ R_{free} is based on 10% of the total reflections excluded from refinement.

The final refined coordinates of the structure have been deposited in the Protein Data Bank (PDB) with code 1td7. The figures were drawn using the programs *SwissPDBViewer* (Guex & Peitsch, 1997) and *PyMOL* (DeLano, 2002).

3. Results and discussion

3.1. Inhibition of PLA_2 by niflumic acid and determination of its binding constants

The kinetic data obtained from the measurements using a commercial kit and purified PLA_2 indicated that the binding of niflumic acid was competitive in nature. The approximate value of the inhibition constant was calculated to be $1.26 \pm 0.05 \times 10^{-6}$ M. Although conventional kinetic assays do not provide accurate estimates of K_i for PLA_2 enzymes, they do allow an approximate determination. Even the visual observations indicated the inhibitory effects of niflumic acid. The accurate value of the binding constant was obtained with SPR. The dissociation constant (K_d) from the SPR data was calculated to be 4.3×10^{-5} M. This interaction strength is expected to allow association of niflumic acid *in vivo*.

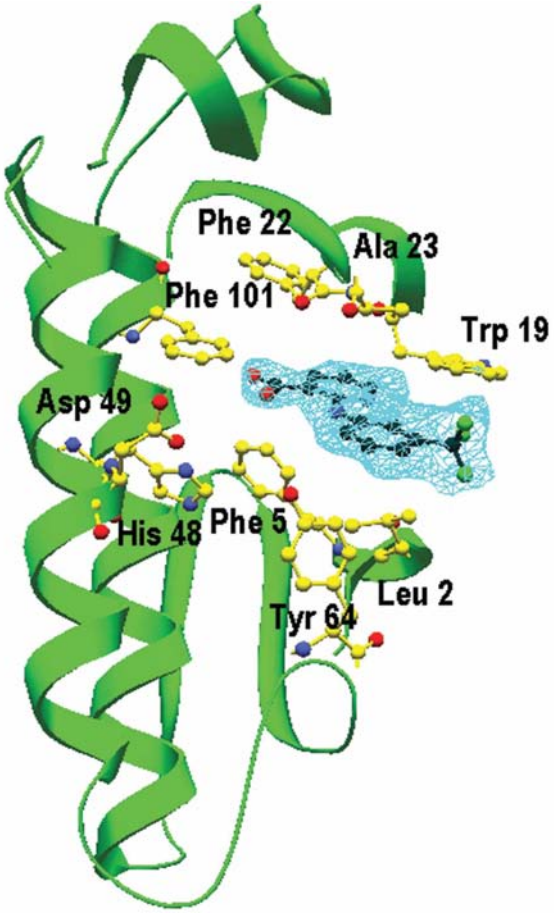

Figure 1
Final $|2F_o - F_c|$ electron density for the niflumic acid molecule at 2.0σ in the substrate-binding site.

3.2. Quality of the model

The final model consists of one molecule of PLA_2 complexed with niflumic acid, one calcium ion and 101 water molecules. The refinement of the final model converged to R_{cryst} = 18.7% and R_{free} = 22.7% for 4011 reflections in the resolution range 20.0–2.5 Å. The final model has a good geometry with r.m.s. deviations of 0.01 Å and 1.8° for bond lengths and bond angles, respectively. The Ramachandran plot (Ramachandran & Sasisekharan, 1968) obtained with the program *PROCHECK* (Laskowski *et al.*, 1993) showed that 90.4% of the dihedral angles were present in the most favoured regions, while the remaining 9.6% of residues were found in the additionally allowed regions. The average *B* value for all the atoms was 31.3 $Å^2$.

3.3. Overall PLA_2 structure

The molecular topology of PLA_2 conserves all the main features of the PLA_2 type of folding, with an N-terminal helix (H1) from residue 2 to residue 12, helix 2 (H2) from residue 40 to residue 55 and helix 3 (H3) from residue 86 to residue 103. There are two short helical turns involving residues 19–22 (SH4) and 108–110 (SH5) (Fig. 2). The overall structure of PLA_2 in the complex is essentially similar to its native structure, showing an r.m.s. deviation of 0.34 Å for C^α chains (Singh, Jabeen *et al.*, 2005). Superposition of the C^α chain of PLA_2 in the present complex with other PLA_2 structures from *N. naja* subclasses such as *N. naja atra* (PDB code 1poa; Scott *et al.*, 1990) and *N. naja naja* (PDB code 1psh; Fremont *et al.*, 1993) (Fig. 3) show r.m.s. deviations of 0.69 and 0.78 Å, respectively, indicating the similarity in the overall folding of their main chains. However, significant variations were

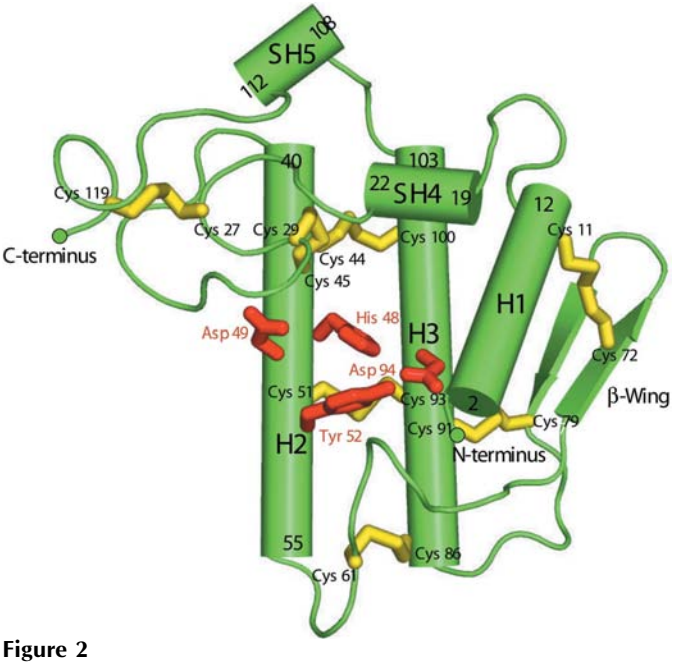

Figure 2
Schematic diagram showing the overall structure of the present PLA_2. Helices are shown as cylinders. The three main helices (H1, H2 and H3), two short helices (SH4 and SH5) and β-wing are indicated. Active-site residues are shown in red and disulfide linkages in yellow.

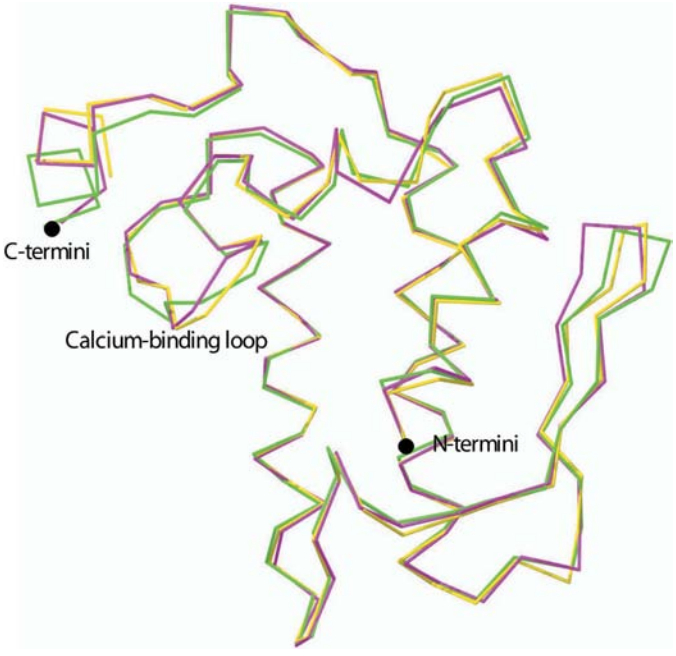

Figure 3
Superposition of PLA_2 from the present complex (green) on PLA_2s from *N. naja atra* (yellow; Scott *et al.*, 1990) and *N. naja naja* (magenta; Fremont *et al.*, 1993).

Table 3
Hydrogen bonds formed between PLA_2 and niflumic acid (NFA).

NFA atom	Residue/atom	Distance (Å)	Residue/atom	Distance (Å)
O8	Gly30 N	2.94		
O8	OW62	2.96	His48 $N^{\delta1}$	3.10
			Asp49 $O^{\delta1}$	3.12
O8	OW97	3.19	Gly30 O	2.70
			Asp49 $O^{\delta1}$	3.27
			Tyr64 OH	3.26
F1	OW99	2.96		
F2	OW98	2.87		
F3	Lys N^{ζ}	2.82		

observed in the conformations of various loops in these structures (Scott *et al.*, 1990; Fremont *et al.*, 1993). The most notable differences were observed in the conformations of their calcium-binding loops (Fig. 3). As in other structures, the calcium ion was found to be coordinated by the two carboxylate O atoms of Asp49 and three main-chain O atoms of Tyr28, Gly30 and Gly32. Two structurally conserved solvent water molecules complete the coordination sphere of the Ca^{2+} ion, forming a distorted pentagonal bipyramid. The present PLA_2 structure was also compared with group I mammalian PLA_2 structures (PDB codes 1bp2 and 1p2p; Dijkstra *et al.*, 1981, 1983). Superposition of C^{α} atoms showed r.m.s. deviations of 0.89 and 0.93 Å, respectively. The side chains of important residues in the substrate-binding cleft of these structures also correspond well (Fig. 4).

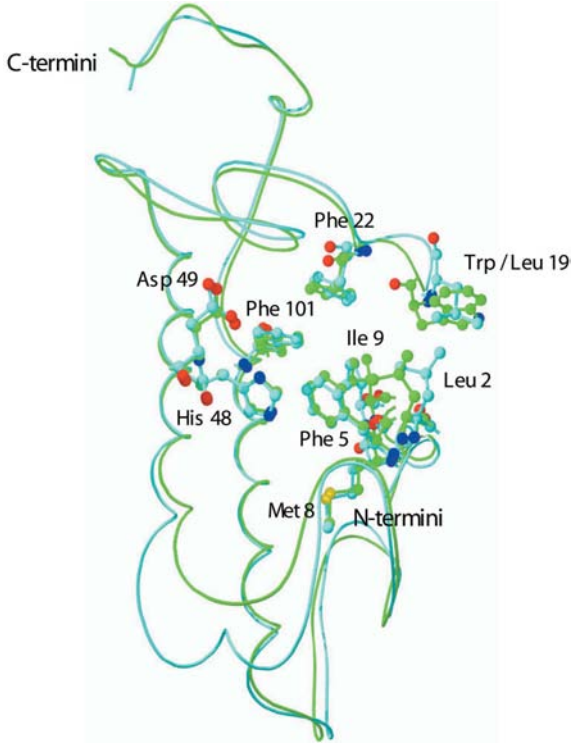

Figure 4
Superposition of PLA_2 from the present complex (green) on bovine pancreatic PLA_2 (blue; Dijkstra *et al.*, 1981). The side chains of important residues of the hydrophobic substrate-binding site that are involved in recognition have also been indicated.

3.4. Binding of niflumic acid to PLA_2

The high quality of the electron density for niflumic acid in the $|F_o - F_c|$ map allowed an accurate determination of its atomic coordinates. The niflumic acid molecule was located almost in the centre of the hydrophobic channel of PLA_2 (Fig. 5). It is completely buried in the substrate-recognition site and is only visible through the opening of the substrate-binding channel (Fig. 6). The niflumic acid molecule was also found to be favourably oriented to interact with important residues in the substrate-binding site of the PLA_2 (Fig. 7). The O8 atom of the carboxylic group of niflumic acid forms three hydrogen bonds with Gly N, OW62 and OW97. In turn, OW62 and OW97 form several interactions, including those with His48 and Asp49, although the interactions of Asp49 $O^{\delta1}$ and Tyr64 OH with OW97 are weak hydrogen bonds (Table 3, Fig. 7). On the opposite side of the molecule, the F atoms interact with Lys6 N^{ζ}, OW98 and OW99. The aromatic bulk of the molecule also contributes to the overall strength of interactions with PLA_2 through several hydrophobic interactions. The notable residues that are responsible for the hydrophobic interactions are Leu2, Phe5, Ile9, Trp19, Phe22, Ala23, Gly30 and Tyr64. The initial complementarity between niflumic acid and the substrate-binding site of PLA_2 appears

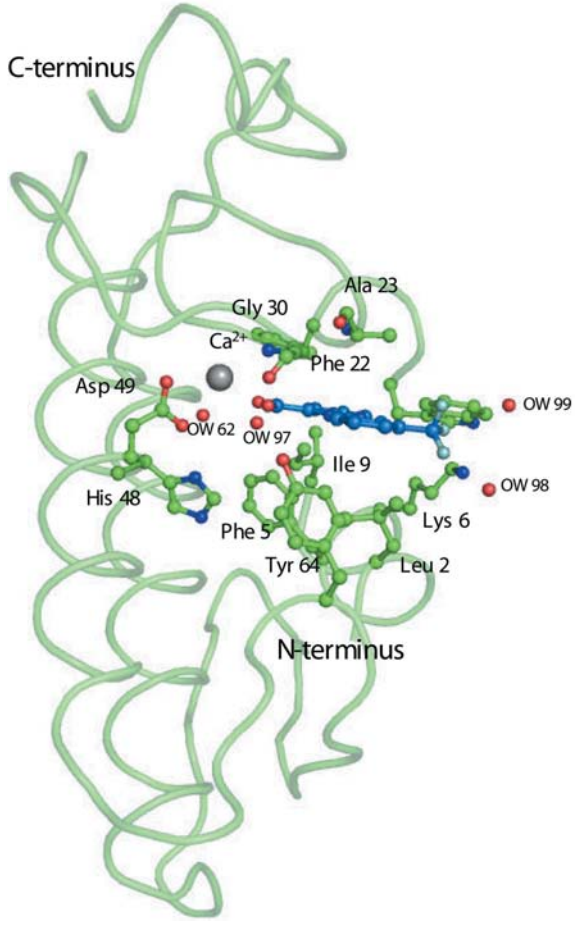

Figure 5
The structure of PLA_2 (green) with niflumic acid (blue) showing the binding site.

to be very high. Therefore, upon binding only small conformational changes occur in both PLA_2 and niflumic acid. The superposition of the coordinates of niflumic acid from the present complex on those in the free state (Krishna Murthy & Vijayan, 1979) shows a good agreement (Fig. 8). The values of various dihedral angles in the niflumic acid indicate that the structure is essentially planar, as observed in the crystal structure of niflumic acid. The phenyl ring and the pyridine ring in the complex are inclined at a nearly the same angle as those in the free state. The orientation of the carboxylic group with respect to the plane of the pyridine ring has changed only slightly and shows a departure from coplanarity by 7.3 (5)° compared with the values observed in the free state. Overall, the molecule of niflumic acid fit well into the substrate-binding cavity of PLA_2 ($K_i = 1.26 \times 10^{-6}$ *M*). So far, only eight other complexes of group I PLA_2s with various ligands have been studied. Out of these complexes, one is with the natural compound anisic acid (PDB code 1o2e; Sekar *et al.*, 2003), five are with substrate/transition-state analogues (PDB code 5p2p, Thunnissen *et al.*, 1990, $K_m = 0.8 \times 10^{-3}$ *M*; PDB code 1pob, White *et al.*, 1990; PDB code 1fdk, Sekar *et al.*, 1997; PDB code 1mkv, Sekar *et al.*, 1998; PDB code 1fx9, Pan *et al.*, 2001), one with a designed peptide Val-Ala-Phe-Arg-Ser (VAFRS; PDB code 1mf4; Singh *et al.*, 2003; $K_i \simeq 1.02 \times 10^{-8}$ *M*) and one with the non-steroidal anti-inflammatory drug aspirin (PDB code 1oxr; Singh, Ethayathulla *et al.*, 2005; $K_i \simeq 6.4 \times 10^{-5}$ *M*). Superimposition of the C^{α} traces of PLA_2 from the present complex on the other complexes mentioned above shows r.m.s. displacements for the C^{α} atoms ranging from 0.82 to 1.21 Å. The location of niflumic acid in the substrate-binding site of PLA_2 is almost identical to those observed in other complexes of group I PLA_2s (Figs. 9*a* and 9*b*). Comparison of the interactions observed between niflumic acid and PLA_2 with the interactions reported for other complexes show that the majority of key interactions are identical and involve residues Tyr28, Gly30, His48 and Asp49.

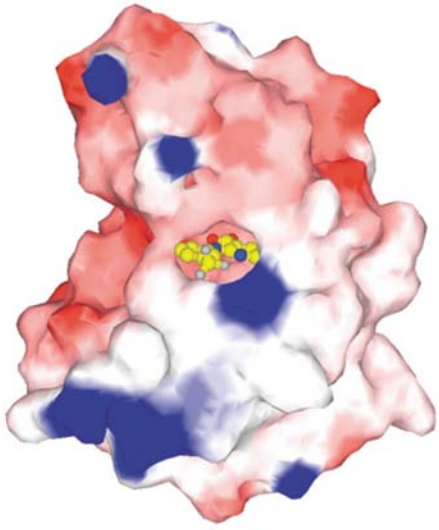

Figure 6
Electrostatic surface potential of the PLA_2 complex with niflumic acid. The niflumic acid molecule is seen completely buried in the substrate-binding pocket.

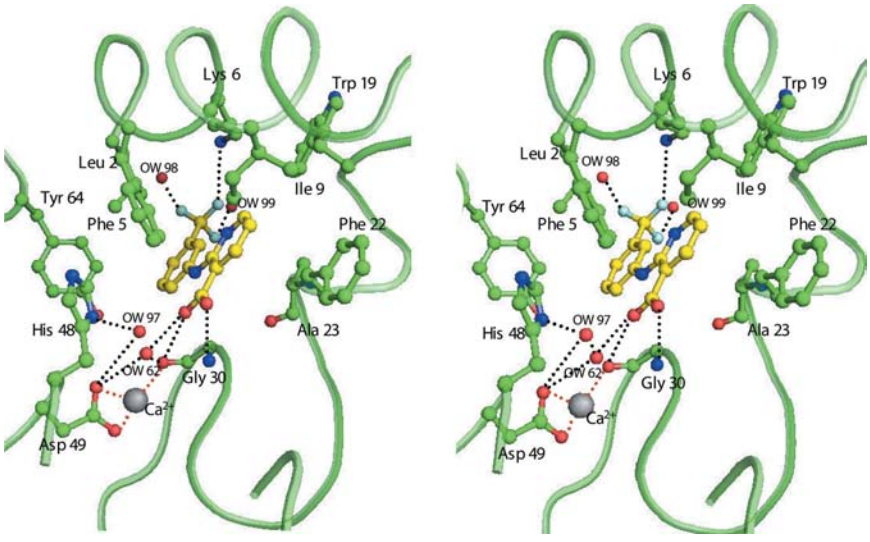

Figure 7
Stereoview of the interactions of niflumic acid in the substrate-binding site of PLA_2. Hydrogen bonds are shown as black dotted lines, while calcium coordinations are represented by dotted lines in red.

4. Conclusions

The structure of the complex formed between PLA_2 and niflumic acid demonstrates that the substrate-binding site of PLA_2 is stereochemically compatible with the binding of niflumic acid. The interactions involving Asp49, His48 and Gly30 that are generally important in the recognition of substrate binding in PLA_2 are also observed in the present complex. The structure shows that niflumic acid is nearly 100% buried in the substrate-binding cleft of the enzyme and is only visible through the opening of the substrate-binding channel. Thus, these results clearly show that the native structures of PLA_2 and NSAIDs such as niflumic acid remain unaltered upon binding to each other, indicating a high complementarity between the substrate-binding site of PLA_2 and the structure of niflumic acid. The value of the binding constant (K_i) as determined using kinetic data is of the order of 10^{-6} *M*. The value of the dissociation constant (K_d) as determined using SPR is 4.3×10^{-5} *M*. These values also indicate that the association of niflumic acid with PLA_2 can occur *in vivo*. Thus, it is likely that the resulting anti-inflammatory effects of niflumic acid in particular and NSAIDs in general might have partly been induced by their inhibition of PLA_2. These observations support the usefulness of PLA_2 as a potential target for structure-based drug design against various inflammatory disorders.

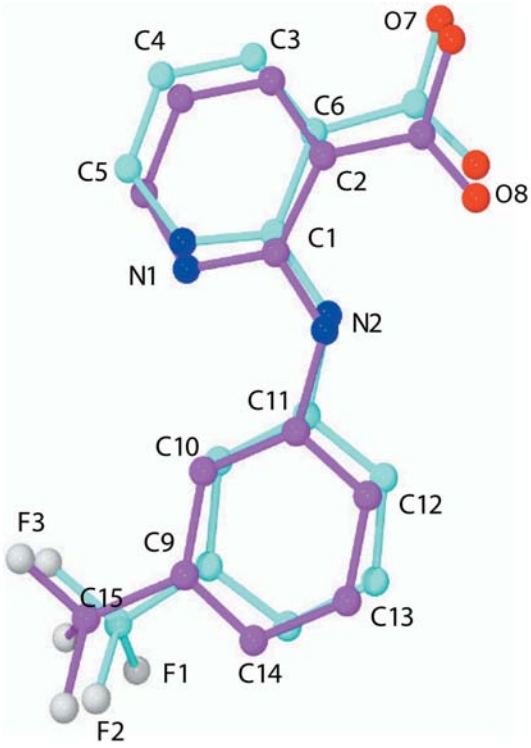

Figure 8
Least-squares superposition of the niflumic acid molecule in the complex (blue) on its structure in the free state (magenta).

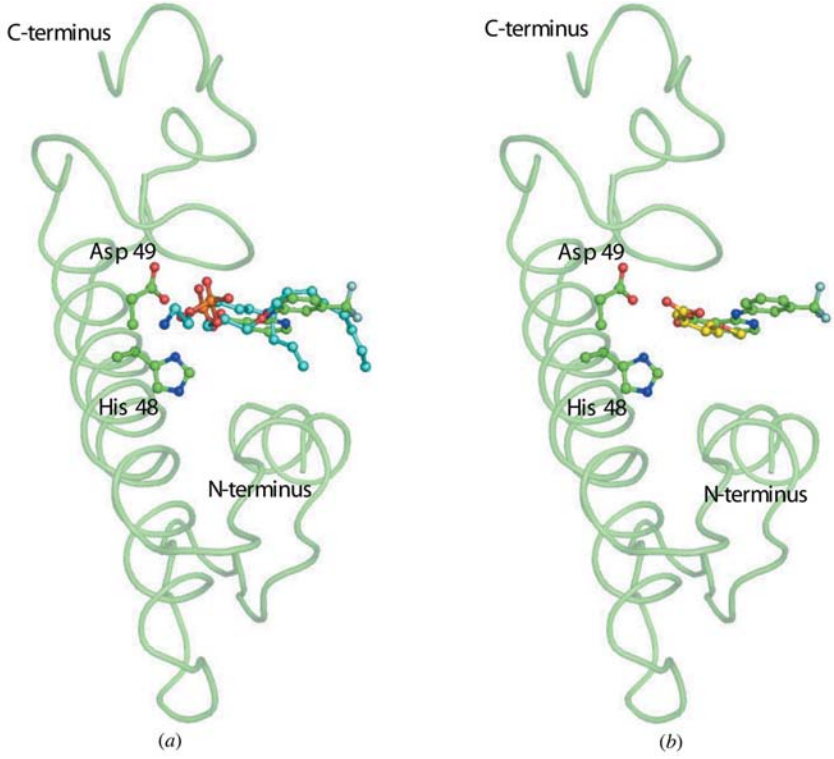

Figure 9
(*a*) Superimpositions of niflumic acid (green) and a transition-state analogue (1-*O*-octyl-2-heptylphosphonyl-*sn*-glycero-3-phosphoethanolamine; blue) in complexes with *N. naja sagittifera* PLA_2 and *N. naja atra* PLA_2 (White *et al.*, 1990). Only the backbone of *N. naja sagittifera* is drawn for the sake of clarity. (*b*) Superimpositions of niflumic acid (green) and anisic acid (yellow) in complexes with *N. naja sagittifera* PLA_2 and bovine pancreatic PLA_2 (Dijkstra *et al.*, 1981). Only the backbone of *N. naja sagittifera* is drawn for the sake of clarity.

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