Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Specific binding of non-steroidal anti-inflammatory drugs (NSAIDs) to phospholipase A_2: structure of the complex formed between phospholipase A_2 and diclofenac at 2.7 Å resolution

Nagendra Singh, Talat Jabeen, Sujata Sharma, R. K. Somvanshi, Sharmistha Dey, A. Srinivasan and T. P. Singh*

Department of Biophysics, All India Institute of Medical Sciences, New Delhi 110029, India

Correspondence e-mail: tps@aiims.aiims.ac.in

Received 20 September 2005
Accepted 30 January 2006

PDB Reference: phospholipase A_2–diclofenac complex, 2b17.

Type IIA secretory phospholipase A_2 (PLA_2) enzymes catalyze the hydrolysis of the *sn*-2 ester bond of glycerophospholipids to release fatty acids and lysophospholipids. In order to elucidate the role of PLA_2 in inflammatory disorders and to determine the mode of binding of non-steroidal anti-inflammatory drugs (NSAIDs) to PLA_2, the detailed three-dimensional structure of a complex formed between a group IIA PLA_2 from *Daboia russelli pulchella* and 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid (diclofenac) has been determined. The preformed complex was crystallized by equilibrating the protein solution against a mixture of 0.20 *M* ammonium sulfate and 30% PEG 4000. The crystals belong to space group $P4_3$, with unit-cell parameters $a = b = 53.0$, c = 48.4 Å. The structure was solved by the molecular-replacement method and refined to $R_{\rm cryst}$ and $R_{\rm free}$ factors of 0.192 and 0.211, respectively, using reflections to 2.7 Å resolution. The structure showed that diclofenac occupies a very favourable position in the centre of the substrate-binding hydrophobic channel that allows a number of intermolecular interactions. The binding mode of diclofenac involved crucial interactions with important residues for substrate recognition such as Asp49, His48 and Gly30. In addition, it included three new interactions involving its Cl atoms with Phe5, Ala18 and Tyr22. It also showed an extensive network of hydrophobic interactions involving almost all of the residues of the substrate-binding hydrophobic channel. The binding affinity of diclofenac was determined using surface plasmon resonance, which gave an equilibrium constant of $4.8 \pm 0.2 \times 10^{-8}$ *M*.

1. Introduction

Phospholipase A_2 (PLA_2, EC 3.1.1.4, phosphatide *sn*-2 acylhydrolase) stereospecifically hydrolyzes the ester bond at the *sn*-2 position of phospholipids (Dennis, 1994, 1997) and also displays enhanced activity towards lipids in lamellar and micellar aggregates both in membranes and at other lipid–water interfaces (Pieterson *et al.*, 1974; Verger & de Hass, 1976; Jain & Berg, 1989; Scott *et al.*, 1990; Wery *et al.*, 1991; Gelb *et al.*, 1995, 1999). There have been several reports suggesting PLA_2 to be a viable target for the design of anti-inflammatory agents because of its critical involvement in physiological and pathological events such as phospholipid turnover and production of pro-inflammatory lipid mediators (Smith, 1992; Balsinde *et al.*, 1995; Chilton *et al.*, 1996; Dennis, 2000). Furthermore, it plays an important role in modulating both the cyclooxygenase and 5′-lipooxygenase pathways which catalyze the simultaneous reactions of inflammatory processes. It is well established that the production of PLA_2-catalyzed arachidonic acid leads to the biosynthesis of pro-inflammatory mediators such as prostaglandins, leukotrienes, thromboxanes

and platelet-activating factors that are commonly known as eicosanoids (Smith, 1992). It has been shown that there is more than one form of PLA_2 that might be involved in the production of pro-inflammatory eicosanoids (Dennis, 1994). In this regard, the involvement of extracellular group II and intracellular group IV PLA_2s have been suggested, although the roles of PLA_2s from other groups cannot be ruled out. Thus, the use of group II PLA_2s from various sources as targets for the design of potent anti-inflammatory agents is recommended. The modes of inhibitor binding to group II PLA_2s from both human and snake-venom sources were also found to be very similar (Thunnissen *et al.*, 1990).

The use of non-steroidal anti-inflammatory drugs (NSAIDs) has been widespread since the 19th century and the notion held until now was that NSAIDs inhibit the biosynthesis of prostaglandins from arachidonic acid, thus targeting cyclo-oxygenases. NSAIDs have proved to be valuable tools in the elucidation of prostaglandin-biosynthesis pathways and have provided a starting point for the synthesis of new anti-inflammatory agents. To date, the role of PLA_2s as the target for NSAIDs has neither been well investigated nor sufficiently understood. In order to generate much needed information about the role of PLA_2s in inflammatory disorders and the overall basis of the anti-inflammatory effects of NSAIDs, we have been carrying out systematic studies on the structures and functions of PLA_2s (Chandra *et al.*, 2001; Singh *et al.*, 2001; Jasti *et al.*, 2004; Jabeen, Sharma, Singh, Singh, Kaur *et al.*, 2005; Jabeen, Sharma, Singh, Singh, Verma *et al.*, 2005) and their complexes with natural compounds (Chandra, Jasti, Kaur, Betzel *et al.*, 2002; Chandra, Jasti, Kaur, Srinivasan *et al.,* 2002; Singh, Jasti *et al.*, 2005), NSAIDs (Singh *et al.*, 2004; Jabeen, Singh *et al.*, 2005; Singh, Ethayathulla *et al.*, 2005) and designed peptides (Chandra, Jasti, Kaur, Dey, Perbandt *et al.*, 2002; Chandra, Jasti, Kaur, Dey, Srinivasan *et al.*, 2002; Singh *et al.*, 2003; Singh, Singh *et al.*, 2005). We report here the results of binding studies of 2-[(2,6-dichlorophenyl)amino]-benzeneacetic acid (diclofenac) to a group IIA PLA_2 from *Daboia russelli pulchella* and the detailed three-dimensional structure of the complex formed between the PLA_2 and diclofenac.

2. Experimental

2.1. Purification of PLA_2

The PLA_2 enzyme, belonging to group IIA, was used for detailed binding studies with diclofenac as well as for three-dimensional structure determination. It was purified as reported previously (Singh *et al.*, 2004) from crude *D. russelli pulchella* venom. The venom was obtained from the Irula Cooperative Snake Farm in Tamil Nadu, India. 250 mg lyophilized venom was dissolved in 50 m*M* ammonium acetate buffer pH 6.0 to a final concentration of 10 mg ml^{-1}. After centrifugation, the solution was loaded onto a Cibacron Blue affinity column and the fractions eluted with ammonium carbonate at pH 10.5 were pooled and concentrated and loaded onto a CM-cellulose C-25 cation-exchange column. The bound fractions eluted using a 0.0–0.5 *M* gradient of NaCl in 50 m*M* ammonium acetate buffer pH 6.0 were collected and desalted. Analysis took place using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI–TOF; Kratos, Shimadzu, Japan). A single band was observed on SDS–PAGE and a molecular weight of 13 612.8 Da was determined using MALDI–TOF.

2.2. Kinetic studies

In order to evaluate the nature of the binding of diclofenac to PLA_2 in the presence of substrate, kinetic studies were carried out using a chromogenic assay based on the catalysis of hexadecylphosphocholine (HePC) by PLA_2 (Ikeda *et al.*, 1984). The presence of the released product is detected colorimetrically using Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)]. The assay was performed using a PLA_2 Detection Kit. Various concentrations of HePC ranging from 4.6 to 34 m*M* were used for PLA_2 kinetic studies. 5 µl of colouring agent (DTNB) was added to each assay reaction. The assay included a 30 min incubation of enzyme with diclofenac prior to the addition of substrate to the reaction vial. 1 µ*M* PLA_2 was added to the reaction mixtures, which contained four different concentrations of substrate, and the absorbance was measured at a wavelength of 405 nm using 0.62 µ*M* (I_1), 1.15 µ*M* (I_2) and 2.25 µ*M* (I_3) concentrations of diclofenac on a UV spectrometer (Lambda 25, Perkin–Elmer, USA). All measurements were repeated three times in order to estimate the mean error. To determine the inhibition constant (K_i), the observed reaction rates were plotted against substrate concentrations using the Lineweaver–Burk method (Lineweaver & Burk, 1934). The Lineweaver–Burk plot is shown in Fig. 1.

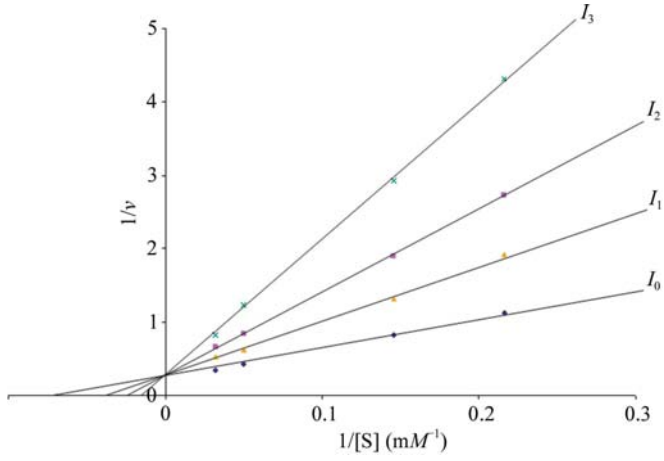

Figure 1
Analysis of kinetic parameters of the PLA_2-driven hydrolysis of HePC in the presence of diclofenac. Diclofenac was added to each reaction at concentrations of I_1 (0.65 µ*M*), I_2 (1.12 µ*M*) and I_3 (2.25 µ*M*). Diclofenac is seen to be a competitive inhibitor of PLA_2 activity. The K_i value for the hydrolysis of HePC was estimated to be $6.2 \pm 0.6 \times 10^{-7}$ *M*.

2.3. Surface plasmon resonance (SPR) analysis of the interaction of PLA_2 with diclofenac

The binding properties of diclofenac were also investigated by SPR. All SPR measurements were performed at 298 K using a BIACore-2000 apparatus (Pharmacia Biosensor AB, Uppsala, Sweden). The BIACore-2000 apparatus used is a biosensor-based system for real-time specific interaction analysis (Szabo *et al.*, 1995). CM5 sensor chips, surfactant P20 and an amine-coupling kit containing *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-3-(diethylaminopropyl)carbodiimide (EDC) and ethanolamine hydrochloride (Pharmacia Biosensor AB, Uppsala, Sweden) were used. The running buffer used was 5 m*M* HEPES pH 7.4 containing 0.005% surfactant P20. The sensor chip CM5 (a disposable sensor chip, the surface of which was covered with a thin gold layer coated with carboxymethyl dextran residue for covalent protein immobilization) was purchased from Pharmacia Biosensor AB (Uppsala, Sweden). The coated CM5 chip was used for protein immobilization. The immobilization of PLA_2 was performed at a flow rate of 10 µl min^{-1} at 298 K using the amine-coupling kit. The dextran on the chip was equilibrated with running buffer and carbomethylated matrix was activated with an EDC–NHS mixture. 210 µl PLA_2 (50 µg ml^{-1}) in 10 m*M* sodium acetate pH 4.8 was injected and unreacted groups were blocked by injecting ethanolamine pH 8.5. The SPR signal for immobilized PLA_2 was found to be 5049 RUs. The association of diclofenac with PLA_2 was determined by injecting 10, 20, 30, 40 and 50 µg ml^{-1} diclofenac separately at different cycles in 5 m*M* HEPES pH 7.0 with 5 m*M* $CaCl_2$ at a flow rate of 10 µl min^{-1}. The dissociation of diclofenac was initiated by 5 m*M* HEPES pH 7.0. The kinetic parameters were obtained using the *BIAevaluation* software package (Nieba *et al.*, 1996). The association (k_{on}) and dissociation (k_{off}) rate constants for diclofenac binding to PLA_2 were calculated and the equilibrium (K_d) value was determined by the mass-action relation $K_d = k_{off}/k_{on}$.

2.4. Crystallization

The purified samples of PLA_2 were dissolved in deionized water containing 10% methanol and 5 m*M* $CaCl_2$ to attain a final protein concentration of 15 mg ml^{-1}. Diclofenac was dissolved in the above solution at a ten times higher molar concentration than that of protein. Droplets containing a mixture of 10 µl of the above solution and 10 µl reservoir solution were equilibrated against reservoir solution containing 0.20 *M* ammonium sulfate and 30% PEG 4000 in the sitting-drop vapour-diffusion experiment at 298 K. Crystals of dimensions up to 0.3 × 0.3 × 0.3 mm appeared after a week.

2.5. X-ray intensity data collection and processing

Freshly grown crystals of the PLA_2 complex with diclofenac were used for data collection at 285 K. X-ray intensity data were obtained to 2.7 Å resolution using a 345 mm MAR Research imaging-plate scanner mounted on an RU-300 Rigaku rotating-anode X-ray generator equipped with Osmic mirrors. The data were processed and scaled using the programs *DENZO* and *SCALEPACK* from the *HKL*2000 package (Otwinowski & Minor, 1997). The results of data collection and processing are given in Table 1.

Table 1
Data-collection and refinement statistics of the PLA_2 complex with diclofenac.

Values in parentheses are for the highest resolution shell.

Data collection	
Space group	$P4_3$
Unit-cell parameters (Å)	
$a = b$	53.0
c	48.4
Z	4
V_M (Å^3 Da^{-1})	2.5
Solvent content (%)	50
Resolution range (Å)	20.0–2.7 (2.76–2.70)
Total no. of measured reflections	42951
No. of unique reflections	3288
Completeness of data (%)	96.8 (95.9)
R_{sym} (%)	11.6 (20.6)
$I/\sigma(I)$	7.3 (2.1)
Refinement	
PDB code	2b17
R_{cryst}/R_{free} (%)	19.2/21.1
No. of protein atoms	944
No. of diclofenac atoms	19
No. of water molecules	86
No. of sulfate ions	2
R.m.s. deviations from ideal values	
Bond lengths (Å)	0.006
Bond angles (°)	1.4
Dihedral angles (°)	22.4
Ramachandran plot	
Residues in most favourable regions (%)	89.3
Residues in additionally allowed regions (%)	10.7
Mean B factors (Å^2)	
For main-chain atoms	24.4
For side-chain atoms and waters	31.4
For all atoms	28.2

2.6. Structure determination and refinement

The crystal structure was determined using the molecular-replacement method with the program *AMoRe* (Navaza. 1994). The coordinates of the native PLA_2 from *D. russelli pulchella* (PDB code 1fb2; Chandra *et al.*, 2001) were used as a model for the structure determination. The refinement was carried out with the program *REFMAC* 4.01 from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994). The progress of refinement was monitored by the use of R_{free} and 5% of the data were set aside for cross-validation before refinement. At each refinement step, $|2F_o - F_c|$ and $|F_o - F_c|$ maps were calculated for manual model building using the program *O* (Jones *et al.*, 1991) on a Silicon Graphics O2 workstation. This resulted in a decrease of the R_{cryst} and R_{free} factors to 0.243 and 0.264, respectively. Refinement of individual B factors reduced the R factor further to 0.228. Extra non-protein electron density was observed at the substrate-binding site of the enzyme in the difference Fourier $|F_o - F_c|$ map at 2.5σ cutoff. A molecule of diclofenac (Castellari & Ottani, 1997) was fitted into the observed

electron density. The positions of two sulfate ions and 85 water molecules were also determined from the subsequent difference Fourier maps. The final cycles of refinement using all the reflections in the resolution range 20.0–2.7 Å caused the R_{cryst} and R_{free} factors to converge to 0.192 and 0.211, respectively. The final statistics of refinement are given in Table 1.

3. Results and discussion

3.1. Inhibition of PLA_2 by diclofenac

A purified sample of the PLA_2 monomer from *D. russelli pulchella* venom was used in the kinetic studies. The maximum inhibition of PLA_2 attained with diclofenac was 90%. Analysis of the data obtained from the kinetic experiments (Fig. 1) indicated that diclofenac inhibited PLA_2 competitively and its binding constant (K_i) was estimated to be $6.2 \pm 0.6 \times 10^{-7}$ *M*.

The binding of diclofenac by PLA_2 was also determined by the SPR method. The data obtained were analyzed using *BIAevaluation* software (Nieba *et al.*, 1996) provided by Pharmacia Biosensor AB. The sensograms obtained are shown in Fig. 2. The association (k_{on}) and dissociation (k_{off}) rate constants for diclofenac binding to PLA_2 were calculated to be 2.7×10^4 M^{-1} s^- and 1.3×10^{-3} s^{-1}, respectively. From these values, the equilibrium constant (K_d) was calculated to be $4.8 \pm 0.2 \times 10^{-8}$ *M*. This interaction strength is sufficient to allow specific binding of diclofenac to PLA_2 under *in vivo* conditions.

3.2. Quality of the model

The final model consists of 944 protein atoms, 19 atoms of diclofenac, two sulfate ions and 85 water O atoms. Calcium ions are not present in the structure. Instead, a water molecule is found at the calcium-ion site. Protein atoms occupy approximately 50% of the unit-cell volume. The final $|2F_o - F_c|$ electron-density map shows an excellent electron density at 1.2σ cutoff for diclofenac (Fig. 3). The refined model has good geometry, with r.m.s. deviations of 0.006 Å and 1.4° for the bond lengths and angles, respectively. The stereochemistry of the final model was checked using the program *PROCHECK* (Laskowski, Macarthur *et al.*, 1993; Laskowski, Moss *et al.*, 1993), which showed that 89.3% of the backbone torsion angles were in the most favoured regions and 10.7% in the additionally allowed regions of the Ramachandran φ,ψ plot (Ramachandran & Sasisekharan, 1968). The average *B* factor for this structure is 28.2 $Å^2$, which is slightly higher than the commonly observed *B* factor for PLA_2 structures ($B_{av} \simeq 25.8$ $Å^2$). Other refinement details are listed in Table 1.

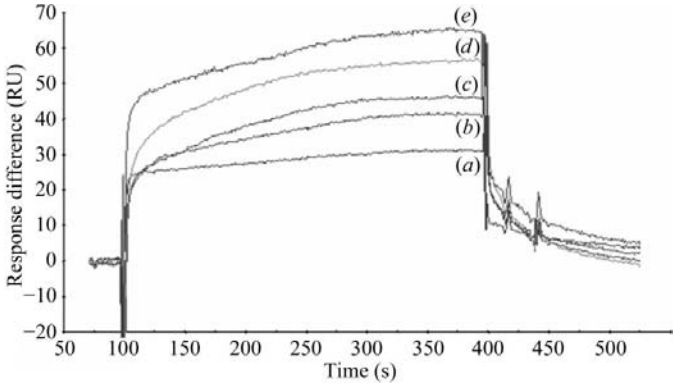

Figure 2
Observed BIAcore sensograms for binding of diclofenac to PLA_2 are shown. The curves represent different concentrations of diclofenac: (*a*) 10, (*b*) 20, (*c*) 30, (*d*) 40 and (*e*) 50 μg ml^{-1} diclofenac separately at different cycles in 5 m*M* HEPES pH 7.0 with 5 m*M* $CaCl_2$ at a flow rate of 10 μl min^{-1}. The K_d value for diclofenac binding to PLA_2 was determined to be $4.8 \pm 0.2 \times 10^{-8}$ *M*.

3.3. Overall molecular structure

The crystal structure contains one PLA_2 molecule with a diclofenac molecule bound to it at the substrate-binding site. The molecular topology of the present group IIA PLA_2 conserves all the main features of the group II PLA_2 type of folding (Fig. 3). It contains an N-terminal helix 1 (H1) that runs from residues 2 to 12. Helix 2 (H2) extends from residues 40 to 55, while helix 3 (H3) spans residues 90–108. The structure also contains a double-stranded antiparallel β-sheet designated as a β-wing (residues 75–78 and 81–84). There are two short helical turns involving residues 115–117 (SH4) and residues 122–125 (SH5). Although 5 m*M* $CaCl_2$ was present in the protein droplets used for crystallization, the structure did not show the presence of Ca^{2+} ion in the so-called calcium-binding loop. The Ca^{2+} ion is generally considered to be essential for the catalytic activity of secretory PLA_2s (Berg *et al.*, 1991; Yu *et al.*, 1993), but in various active isoforms of venom PLA_2s the presence of Ca^{2+} ion has not been reported (Perbandt *et al.*, 1997; Chandra *et al.*, 2001). In the present structure, as in some others (Perbandt *et al.*, 1997; Chandra *et al.*, 2001), instead of the Ca^{2+} ion a water molecule is observed that stabilizes the conformation of the calcium-binding loop. The residues of the short N-terminal amphiphilic helix H1 form part of the highly conserved protein core and its residues interact extensively with other parts of the molecule *via* hydrogen bonds. In addition, this helix forms several contacts with the β-wing. The hydrophobic residues on the inner surface of the helix H1 are highly conserved and form one wall of the hydrophobic channel that provides access to the catalytic site. Additional contributions to the hydrophobic channel include amino acid 19 located in the short turn following the helix H1, amino acid 31 located within the calcium-binding loop and amino acid 69 located before the first strand of the β-wing. Two sulfate ions were observed in the structure: one in the proximity of loop 13–22 that forms a salt bridge with Lys16 and the other at the C-terminus which interacts with Lys131. The two sulfate ions are also involved in interactions with two symmetry-related molecules on both sides of PLA_2.

3.4. Binding of diclofenac to PLA_2

The electron density for diclofenac as indicated by the final difference $|2F_o - F_c|$ Fourier map was of good quality, indicating high occupancy. Diclofenac occupied a very favourable

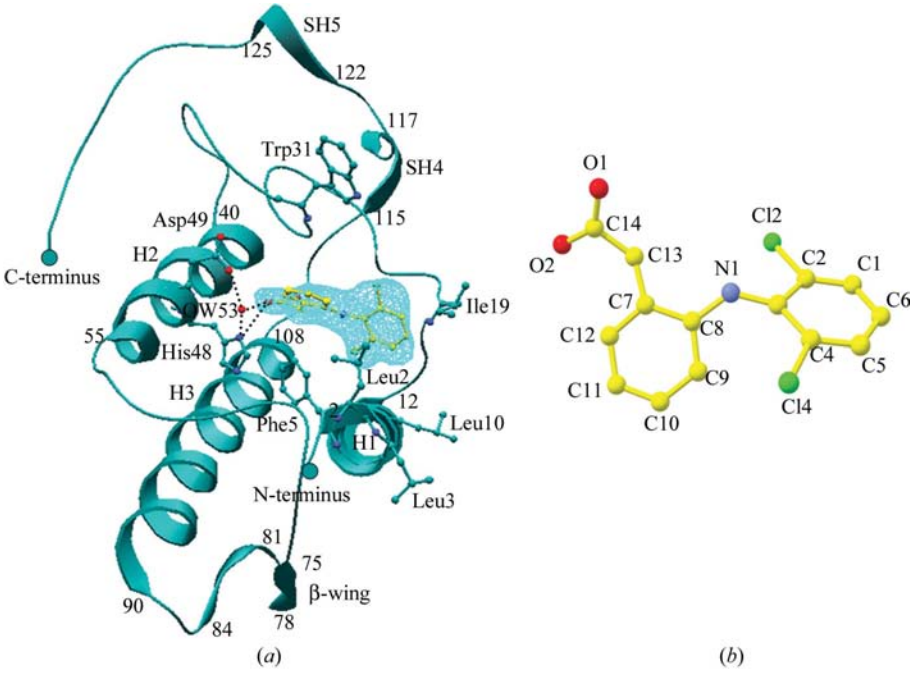

Figure 3
(*a*) Final ($2F_o - F_c$) map showing electron density for diclofenac at a 1.2σ cutoff. A diclofenac molecule (yellow) is overlaid on the density. The overall molecular structure of PLA_2 is shown along with some of the important residues of the substrate-binding channel. (*b*) The numbering scheme in the diclofenac molecule is indicated. The figure was drawn with the program *SwissPDBViewer* (Guex & Peitsch, 1997).

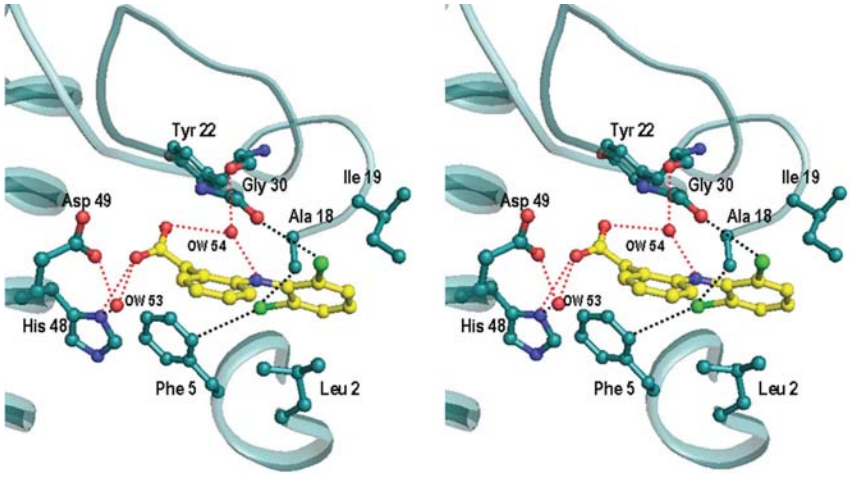

Figure 4
A stereoview of showing the interactions of diclofenac with PLA_2. Hydrogen bonds are shown in red, while van der Waals interactions with Cl atoms are indicated in black. The figure was drawn with *PyMOL* (DeLano, 2002).

space in the substrate-binding channel of PLA_2 that resulted in a number of interactions with the enzyme (Fig. 4). The bulk of the hydrophobic interactions involve the residues of the N-terminal helix (residues 2–12), the residues of the external loop 17–22 and Gly30 of the so-called calcium-binding loop (Fig. 4). In addition, five hydrogen bonds, one direct and four *via* water molecules, have been observed. The O1 atom from the carboxylic group of diclofenac forms two hydrogen bonds, one with His48 $N^{\delta1}$ and another with conserved water molecule OW53. In turn, OW53 forms a bridge between His48 and Asp49. The O2 and N1 atoms of diclofenac interact with OW54, which forms a bridging interaction with Tyr22 O. The two Cl atoms Cl2 and Cl4 form van der Waals contacts with Gly30 O and with Phe5 $C^{\delta2}$ and Ala18 C^{β}, respectively. The molecule of diclofenac is completely inside the substrate-binding cleft of PLA_2. Furthermore, a superimposition of the present complex of PLA_2 with an previously reported structure of the complex formed between PLA_2 and the well known anti-inflammatory drug 4-butyl-1-(4-hydroxyphenyl)-2-phenyl-3,5-pyrazolidinedione (oxyphenbutazone) shows that diclofenac and oxyphenbutazone occupy a nearly identical space in the substrate-binding site, thus indicating the viability of the substrate-binding site in PLA_2 for interacting with NSAIDs.

Diclofenac is a well known NSAID. The structure of the complex formed between another important enzyme of the pathway, cyclooxygenase (COX-2), and diclofenac (Rowlinson *et al.*, 2003) has shown that both the carboxylic O atoms are involved in interactions with the residues in the active site of COX-2, forming three direct hydrogen bonds and several van der Waals interactions. A comparison of the nature and strength of binding of diclofenac to COX-2 show it to be quite similar to its binding with PLA_2. The interactions involving the atoms of diclofenac with COX-2 and PLA_2 clearly indicate that binding of diclofenac may occur to both these enzymes with comparable affinity (the equilibrium constant K_d with PLA_2 is $4.8 \pm 0.2 \times 10^{-8}\,M$ and IC_{50} with COX-2 is $7.7 \times 10^{-8}\,M$; Rowlinson *et al.*, 2003). These values indicate that the overall anti-inflammatory effects of diclofenac *in vivo* may be the result of its binding to both these enzymes.

As seen from Fig. 5, the observed conformation of diclofenac in the complex with COX-2 is very similar to the conformation observed in the crystal structure of diclofenac (Castellari & Ottani, 1997), whereas its carboxylic group has undergone a rotation of −163.7° about the C7—C13 bond in the complex with PLA_2. Similarly, the dichlorophenyl ring of diclofenac in the latter has rotated by 17° about the N1—C3 bond with respect to its orientation in the crystal structure of diclofenac (Castellari & Ottani, 1997). These conformational changes of diclofenac in the complex with PLA_2 clearly reveal an induced adaptability as a result of its

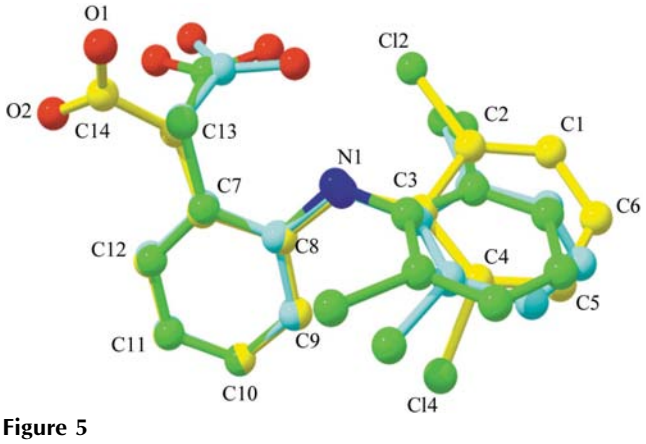

Figure 5
The superimposition of diclofenac from the present complex with PLA_2 (yellow) on diclofenac from the complex with COX-2 (blue) and on uncomplexed diclofenac (green).

interactions with PLA_2. It may also be noted that in diclofenac an intramolecular hydrogen bond between its imino nitrogen and one of its carboxylic O atoms was observed in the structure of uncomplexed diclofenac as well as in the complex with COX-2, but was absent in the complex with PLA_2. Loss of an intramolecular hydrogen bond in a ligand improves its capability for intermolecular interactions with other molecules.

4. Conclusions

Kinetic, SPR and structural studies show that the NSAID diclofenac binds to PLA_2 enzyme with an affinity comparable to that of its affinity for COX-2. The placement of diclofenac in the substrate-binding site and the observed interactions between PLA_2 and diclofenac indicate that its binding to PLA_2 is specific. It binds to PLA_2 at the substrate-binding site and physically blocks the entry of the substrate. These observations suggest clearly that diclofenac will most likely also bind to PLA_2 under *in vivo* conditions. The structure of the complex also indicates the potential sites in diclofenac that can be replaced by new groups to further enhance its affinity. For example, Cl2 may be replaced by NH_2 or OH and CH_3 can be substituted in place of Cl4. Similarly, O2 may be replaced by $NHCH_3$. Overall, these studies have (i) suggested that PLA_2 is potentially an important target for drug development against inflammation, (ii) suggested that diclofenac occupies a favourable site in the substrate-binding region of PLA_2 and (iii) indicated sites in diclofenac for new substitutions/modifications to the ligand to enhance its potency.

The authors greatfully acknowledge financial support from the Department of Science and Technology (DST), New Delhi under the FIST program. NS and TJ thank the DST for grants under the Fast Track Scheme for Young Scientists in Life Sciences. TPS thanks the Alexander von Humboldt Foundation for financial support.

References

Balsinde, J., Bianco, I. D., Ackermann, E. J., Conde-Frieboes, K. & Dennis, E. A. (1995). *Proc. Natl Acad. Sci. USA*, **92**, 8527–8531.
Berg, O. G., Yu, B. Z., Rogers, J. & Jain, M. K. (1991). *Biochemistry*, **30**, 7283–7297.
Castellari, C. & Ottani, S. (1997). *Acta Cryst.* C**53**, 794–797.
Chandra, V., Jasti, J., Kaur, P., Betzel, C. & Singh, T. P. (2002). *Biochemistry*, **41**, 10914–10919.
Chandra, V., Jasti, J., Kaur, P., Dey, S., Perbandt, M., Srinivasan, A., Betzel, C. & Singh, T. P. (2002). *J. Biol. Chem.* **277**, 41079–41085.
Chandra, V., Jasti, J., Kaur, P., Dey, S., Srinivasan, A., Betzel, C. & Singh, T. P. (2002). *Acta Cryst.* D**58**, 1813–1819.
Chandra, V., Jasti, J., Kaur, P., Srinivasan, A., Betzel, C. & Singh, T. P. (2002). *J. Mol. Biol.* **320**, 215–222.
Chandra, V., Kaur, P., Jasti, J., Betzel, C. & Singh, T. P. (2001). *Acta Cryst.* D**57**, 1793–1798.
Chilton, F. H., Surette, M. E. & Winkler, J. D. (1996). *Adv. Exp. Med. Biol.* **416**, 169–172.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
DeLano, W. L. (2002). *The PyMol Molecular Graphics System.* DeLano Scientific, San Carlos, CA, USA. http://www.pymol.org.
Dennis, E. A. (1994). *J. Biol. Chem.* **269**, 13057–13060.
Dennis, E. A. (1997). *Nature Biotechnol.* **5**, 11060–11064.
Dennis, E. A. (2000). *Am. J. Respir. Crit. Care Med.* **161**, S32–S35.
Gelb, M. H., Cho, W. & Wilton, D. C. (1999). *Curr. Opin. Struct. Biol.* **9**, 428–432.
Gelb, M. H., Jain, M. K., Hanel, A. M. & Berg, O. G. (1995). *Annu. Rev. Biochem.* **64**, 653–658.
Guex, N. & Peitsch, M. C. (1997). *Electrophoresis*, **18**, 2714–2723.
Ikeda, K., Sano, S., Teshima, K. & Samejima, Y. (1984). *J. Biochem. (Tokyo)*, **96**, 1427–1436.
Jabeen, T., Sharma, S., Singh, N., Singh, R. K., Kaur, P., Perbandt, M., Betzel, C., Srinivasan, A. & Singh, T. P. (2005). *Proteins*, **59**, 856–863.
Jabeen, T., Sharma, S., Singh, N., Singh, R. K., Verma, A. K., Paramasivam, M., Srinivasan, A. & Singh, T. P. (2005). *Acta Cryst.* D**61**, 302–308.
Jabeen, T., Singh, N., Singh, R. K., Sharma, S., Somvanshi, R. K., Dey, S. & Singh, T. P. (2005). *Acta Cryst.* D**61**, 1579–1586.
Jain, M. K. & Berg, O. G. (1989). *Biochim. Biophys. Acta*, **1002**, 127–156.
Jasti, J., Paramasivam, M., Srinivasan, A. & Singh, T. P. (2004). *Acta Cryst.* D**60**, 66–72.
Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. (1991). *Acta Cryst.* A**47**, 110–119.
Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993). *J. Appl. Cryst.* **26**, 283–290.
Laskowski, R. A., Moss, D. S. & Thornton, J. M. (1993). *J. Mol. Biol.* **231**, 1049–1067.
Lineweaver, H. & Burk, D. (1934). *J. Am. Chem. Soc.* **56**, 658–662.
Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163
Nieba, L., Krebber, A. & Plückthun, A. (1996). *Anal. Biochem.* **234**, 155–165.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Perbandt, M., Wilson, J. C., Eschenburg, S., Mancheva, I., Aleksiev, B., Genov, N., Willingmann, P., Weber, W., Singh, T. P. & Betzel, C. (1997). *FEBS Lett.* **412**, 573–577.
Pieterson, W. A., Vidal, J. C., Volwerk, J. J. & de Haas, G. H. (1974). *Biochemistry*, **13**, 1455–1460.
Ramachandran, G. N. & Sasisekharan, V. (1968). *Adv. Protein Chem.* **23**, 283–438.
Rowlinson, S. W., Kiefer, J. R., Prusakiewicz, J. J., Pawlitz, J. L., Kozak, K. R., Kalgutkar, A. S., Stallings, W. C., Kurumbail, R. G. & Marnett, L. J. (2003). *J. Biol. Chem.* **278**, 45763–45769.
Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gelb, M. H. & Sigler, P. B. (1990). *Science*, **250**, 1541–1546.

Singh, G., Gourinath, S., Sharma, S., Paramasivam, M., Srinivasan, A. & Singh, T. P. (2001). *J. Mol. Biol.* **307**, 1049–1059.
Singh, G., Jasti, J., Saravanan, K., Sharma, S., Kaur, P., Srinivasan, A. & Singh, T. P. (2005). *Protein Sci.* **2**, 395–400.
Singh, N., Jabeen, T., Sharma, S., Somvanshi, R. K., Dey, S. & Singh, T. P. (2004). *Biochemistry*, **43**, 14577–14583.
Singh, R. K., Ethayathulla, A. S., Jabeen, T., Sharma, S., Kaur, P. & Singh, T. P. (2005). *J. Drug Target.* **2**, 113–119.
Singh, R. K., Singh, N., Jabeen, T., Sharma, S., Dey, S. & Singh, T. P. (2005). *J. Drug Target.* **13**, 367–374.
Singh, R. K., Vikram, P., Makker, J., Jabeen, T., Sharma, S., Dey, S., Kaur, P., Srinivasan, A. & Singh, T. P. (2003). *Biochemistry*, **42**, 11701–11706.
Smith, W. L. (1992). *Am. J. Physiol.* **263**, F181–F191.
Szabo, A., Stolz, L. & Granzow, R. (1995). *Curr. Opin. Struct. Biol.* **5**, 699–705.
Thunnissen, M. M., Ab, E., Kalk, K. H., Drenth, J., Dijkstra, B. W., Kuipers, O. P., Dijkman, R., de Haas, G. H. & Verheij, H. M. (1990). *Nature (London)*, **347**, 689–691.
Verger, R. & de Haas, G. H. (1976). *Annu. Rev. Biophys. Bioeng.* **5**, 77–117.
Wery, J. P., Schevitz, R. W., Clawson, D. K., Bobbitt, J. L., Dow, E. R., Gamboa, G., Goodson, T. Jr, Hermann, R. B., Kramer, R. M., McClure, D. B., Mihelich, E. D., Putnam, J. E., Sharp, J. D., Stark, D. H., Teater, C., Warrick, M. W. & Jones, N. D. (1991). *Nature (London)*, **352**, 79–82.
Yu, B. Z., Berg, O. G. & Jain, M. K. (1993). *Biochemistry*, **32**, 6485–6492.