J. Biochem. 123, 619-623 (1998)

Crystal Structure of Human Secretory Phospholipase A_2-IIA Complex with the Potent Indolizine Inhibitor 120-1032[1]

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Received for publication, October 13, 1997

Phospholipase A_2 is a key enzyme in a number of physiologically important cellular processes including inflammation and transmembrane signaling. Human secretory phospholipase A_2-IIA is present at high concentrations in synovial fluid of patients with rheumatoid arthritis and in the plasma of patients with septic shock. Inhibitors of this enzyme have been suggested to be therapeutically useful non-steroidal anti-inflammatory drugs. The crystal structure of human secretory phospholipase A_2-IIA bound to a novel potent indolizine inhibitor (120-1032) has been determined. The complex crystallizes in the space group $P3_121$, with cell dimensions of $a=b=75.8$ Å and $c=51.3$ Å. The model was refined to an R-factor of 0.183 for the intensity data collected to a resolution of 2.2 Å. It was revealed that the inhibitor is located near the active site and bound to the calcium ion. Although the binding mode of the 120-1032 inhibitor to human secretory phospholipase A_2-IIA is similar to that previously determined for an indole inhibitor LY311299, the specific interactions between the enzyme and the inhibitor in the present complex include the oxycarboxylate group which was introduced in this inhibitor. The oxycarboxylate group in 120-1032 is coordinated to the calcium ion and included in the water-mediated hydrogen bonding to the catalytic Asp49. In addition, the ethyl group in 120-1032 gains hydrophobic contacts with the cavity wall of the hydrophobic channel of the enzyme.

Key words: indolizine inhibitor, non-steroidal anti-inflammatory drug, phospholipase A_2, structure-based drug design, X-ray crystallography.

Phospholipase A_2 (PLA_2) [EC 3.1.1.4] stereospecifically hydrolyzes acyl esters at the *sn*-2 position of 3-*sn*-phosphoglycerides. Pharmacological interest in this reaction stems from the belief that PLA_2 may catalyze the release of arachidonate and thereby precipitate the inflammatory cascade involving prostaglandins, thromboxanes and leukotrienes (*1*). An imbalance in the production of these compounds can lead to chronic inflammatory diseases such as rheumatoid arthritis and asthma. Inhibitors of PLA_2 might, therefore, act to reduce the effects of inflammation.

Human secretory phospholipase A_2-IIA (hs-PLA_2-IIA) exists in high levels in synovial fluid from inflamed joints of arthritic patients as well as in the blood of patients with acute pancreatitis, adult respiratory distress syndrome, bacterial peritonitis and septic shock (*2*). It has been suggested that potent inhibitors of hs-PLA_2-IIA can be therapeutically useful drugs in the treatment of septic shock, acute respiratory distress syndrome, pancreatitis, trauma, bronchial asthma, allergic rhinitis, rheumatoid arthritis, gout, and other diseases (*2*).

Hs-PLA_2-IIA has been isolated from the synovial fluid of patients with rheumatoid arthritis (*3*), and its gene has been cloned and overexpressed (*4*). The protein has a 14,000 molecular weight and consists of 124 amino acid residues with seven disulfide bonds. It is a Ca^{2+}-dependent enzyme with a proton relay catalytic system involving His48, Asp99, and water.

The X-ray crystal structure of hs-PLA_2-IIA at 2.2 Å resolution was reported for the enzyme without the Ca^{2+} ion essential for substrate binding and catalysis (*5*). The crystal structures of its calcium-bound form were also determined at physiological pH in both the presence (at 2.1 Å resolution) and absence (at 2.2 Å resolution) of a phosphonate transition state analogue (*6*).

Recently, a series of increasingly potent indole inhibitors of hs-PLA_2-IIA, which display 1,500-fold selectivity when assayed against pancreatic PLA_2, were developed by researchers at Lilly Research Laboratories. Four crystal structures of hs-PLA_2-IIA complexed with these inhibitors were determined, which afforded the structural basis for understanding the binding mode and provided valuable insights for further development (*7*).

These results prompted us to study the structural effects of closely related indolizine compounds on the hs-PLA_2-IIA

[1] This work was partly supported by the "Research for the Future" Program (JSPS-RFTF 97L00501) from the Japan Society for the Promotion of Science to K.M.

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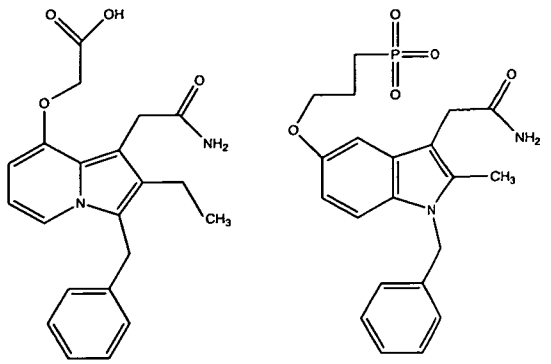

Fig. 1. **Chemical structures of the indolizine compound, 120-1032 (left), and the indole compound, LY311299 (right).**

activity. Having examined a variety of related compounds which were expected to indicate the inhibitor activity as indole, we found that indolizine is an effective candidate. The indolizine compounds have a similar skeleton to indole, including a delocalized 10 π-electron system to which aromaticity is attributed. Owing to its electronic and stereochemical similarity to indole, the indolizine system is considered to show a highly potent inhibitory activity for hs-PLA$_2$-IIA. We have already reported the synthesis of four types of indolizine derivatives and the evaluation of their hs-PLA$_2$-IIA inhibitory activity (*8*).

Here we report the crystal structure at 2.2 Å resolution of the hs-PLA$_2$-IIA complex with one of these indolizine derivatives, designated as 120-1032, to elucidate the interaction between hs-PLA$_2$-IIA and this inhibitor. The chemical formula of 120-1032 is shown in Fig. 1. Furthermore, the comparison of the present crystal structure with that of the hs-PLA$_2$-IIA complexed with a potent indole inhibitor, LY311299 (Fig. 1), previously determined (*7*), enabled us to discuss the structural differences of both complexes in detail.

EXPERIMENTAL PROCEDURES

Co-Crystallization and Data Collection—The protein sample of hs-PLA$_2$-IIA was overexpressed and purified as described (*5*). The inhibitor compound, 120-1032, was synthesized as reported previously (*8*). Crystals of the complex were prepared by co-crystallization of the native protein with 120-1032 in the mother liquor. The crystals were grown by the hanging-drop vapor diffusion technique from a solution containing 10.0 mg/ml of protein in 50 mM MOPS buffer at pH 7.2, 80% saturated sodium chloride and 1% dimethyl sulfoxide. The concentration of the inhibitor was adjusted to 3 molar equivalents. X-ray diffraction data were collected on a Rigaku R-AXIS IIc imaging plate system. The graphite monochromatized Cu$K\alpha$ radiation was generated by a Rigaku R-200 generator operated at 40 kV and 100 mA with a fine focus filament. Data collection and processing details are summarized in Table I.

Structure Solution and Refinement—The crystal structure was solved by the molecular replacement method using the *X-PLOR* program (*9*). The structure of native hs-PLA$_2$-IIA was employed as a starting model (*7*). Crystallographic refinement was also carried out by the *X-PLOR* program (*9*) using the energy minimization and simulated annealing with molecular dynamics. Cycles of refinement were followed by manual corrections of the model using the program *O* (*10*). The refined atomic model has an *R*-factor of 0.183 (R_{free} = 0.268) for 6,886 reflections between 8.0 and 2.2 Å resolution. Analysis of stereochemistry by the *PROCHECK* program (*11*) showed that all of the main chain atoms fall within the allowed regions of the Ramachandran plot and that the side chain geometry is inside expected regions at this resolution for χ^{-1} and χ^{-2} stereochemical parameters.

TABLE I. **Details in intensity data collection and refinement.**

Data collection	
Space group	$P3_121$
Cell dimension	$a=b=75.8$ Å, $c=51.3$ Å
Data collection detector	R-AXIS IIc
Resolution	2.2 Å
Rotation axis	b^*
Rotation range	0°-90°
Rotation step	3°/image
R_{merge}[a]	0.091
Total no. of reflections ($\|F\|>1\sigma$)	29,485
No. of independent reflections	7,076
Completeness	92.4%
Refinement	
Resolution (Å)	8.0-2.2
R_{cryst}[b]	0.183
R_{free}[c]	0.268
Number of reflections ($\|F\|>2\sigma$)	6,886
Total number of atoms	1,357
Water molecules	62
rms deviation of bond lengths (Å)	0.009
rms deviation of bond angles (degree)	1.50

[a] $R_{merge}=\Sigma h\Sigma i|I(h,i)-\langle I(h)\rangle|/\Sigma h\Sigma i I(h,i)$, where $I(h,i)$ is the intensity value of the i-th measurement of h and $\langle I(h)\rangle$ is the corresponding mean value of $I(h)$ for all i measurements. [b] $R_{cryst}=\Sigma||F_{obs}|-|F_{calc}||/\Sigma|F_{obs}|$, where $|F_{obs}|$ and $|F_{calc}|$ are observed and calculated structure factor amplitude, respectively. [c] R_{free} is the same as R_{cryst}, for a 5% subset of all reflections that was never used in the crystallographic refinement.

RESULTS AND DISCUSSION

Co-Crystals of hs-PLA$_2$-IIA and 120-1032—The native crystal of hs-PLA$_2$-IIA and its cocrystals with indole inhibitors are typical hexagonal-rod-shaped and belong to the hexagonal space group $P6_122$ with the similar cell dimensions of around $a=b=76$ Å and $c=90$ Å (*6, 7*). On the other hand, the crystals of the hs-PLA$_2$-IIA/120-1032 complex grown as hexagonal plates belong to the trigonal space group $P3_121$ with cell dimensions of $a=b=75.8$ Å and $c=51.3$ Å. The slightly different structures of these inhibitors caused changes in crystal symmetry and cell dimensions of the hs-PLA$_2$-IIA complex. Interestingly, the present crystal symmetry and cell dimensions are very similar to those of *Notechis scutatus* notexin, a snake venom neurotoxin, which has a closely homologous primary structure with hs-PLA$_2$-IIA (*12*).

Inhibitor Binding—The electron density of the 120-1032 inhibitor was readily interpretable in the difference Fourier map, the structural model of 120-1032 being located unambiguously (Fig. 2). It was found that the 120-1032

inhibitor binds at a similar site to those observed for the other indole inhibitors in the hs-PLA$_2$-IIA complexes (7) and that is directly interacts with the calcium ion (Fig. 3).

The active site of hs-PLA$_2$-IIA possesses a hydrophobic channel (5), which is formed by Leu2, Val3, Phe5, His6, and Ile9 on the N-terminal α1 helix (2–13) and Ala17 and Ala18 on the short α2 helix (16–24) (Fig. 3). The benzyl group of 120-1032 has van der Waals contacts with this hydrophobic channel.

Figure 4 highlights the interactions of the inhibitor with the active site of hs-PLA$_2$-IIA. The oxygen atom of the amide group of 120-1032 is coordinated to the calcium ion, while its nitrogen atom is hydrogen-bonded both to the imidazole nitrogen atom of His48 at the active site and to the side chain carboxylate oxygen atom of Asp49. One oxygen atom of the oxycarboxylate group of 120-1032 binds to the calcium ion. The other oxygen atom forms a water-mediated hydrogen bond to the backbone carbonyl oxygen of Asp49. The distances from this ordered water molecule to the oxygen atoms of the 120-1032 oxycarboxylate group and of the Asp49 carbonyl group are 3.48 and 3.00 Å, respectively. The ethyl group of 120-1032 is buried in a hydrophobic environment composed of Phe5, Ile9, Tyr22, and Phe106.

The calcium ion, which is essential for the hs-PLA$_2$-IIA activity, is also bound to the main chain carbonyl oxygen atoms of residues His28, Gly30, and Gly32 and to the side chain oxygen atoms of Asp49 (Fig. 4). Thus, the calcium ion is seven-coordinated with a pentagonal bipyramidical geometry.

Comparison of Binding Mode—We compared the binding modes of the inhibitors 120-1032 and LY311299 (7) in the crystal structures of both hs-PLA$_2$-IIA complexes. The inhibitors 120-1032 and LY311299 differ chemically at the following specific sites (Fig. 1) (8). In 120-1032, the nitrogen atom in the indole ring of LY311299 (7) is shifted to the neighbor ring position. Two of four substituents attached to the ring are different in both compounds, where the ethyl group in 120-1032 is specifically introduced to

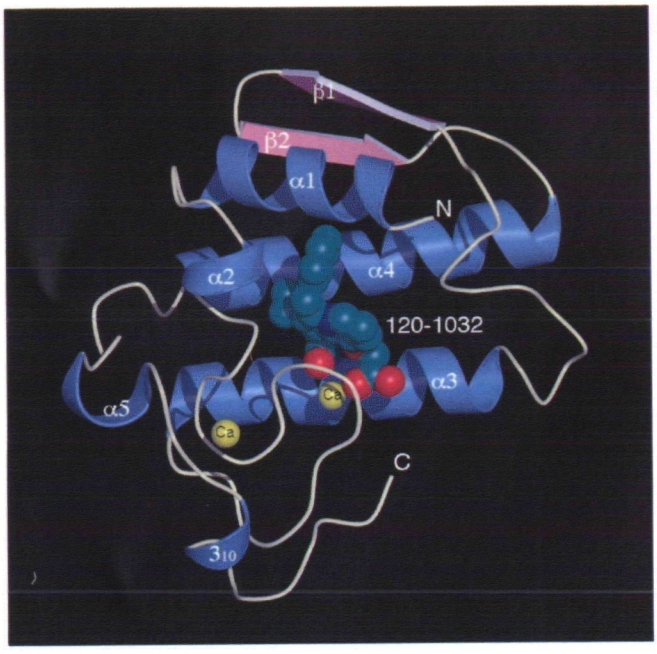

Fig. 3. **Ribbon and space filling representation of 120-1032 inhibitor bound to hs-PLA$_2$-IIA.** The figure is drawn with the program *MOLSCRIPT* (*14*) and rendered with *RASTER3D* (*15*). The β-strands are colored in pink, and the α- and 3_{10} helices are in light blue. The calcium ions are shown by large yellow spheres. The atoms of the inhibitor are color-coded by atom types: green-carbon, blue-nitrogen, and red-oxygen.

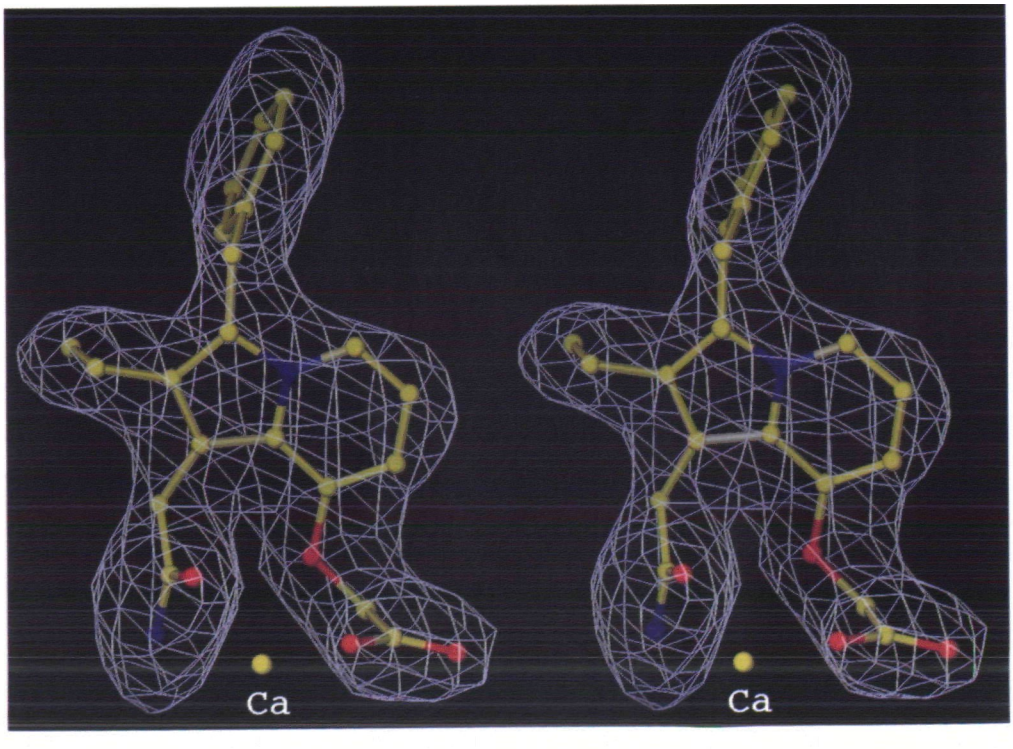

Fig. 2. **Stereo view of the electron density of the 120-1032 inhibitor.** An omit $|F_{obs}| - |F_{calc}|$ electron density map contoured at 2σ shows well the defined density of the 120-1032 inhibitor and the calcium ion in the active site of hs-PLA$_2$-IIA, where the inhibitor was not included in the structure factor calculation.

gain more hydrophobic contacts with hs-PLA_2-IIA. The oxycarboxylate group is employed instead of the phosphonate group of LY311299 in the present case because it is more commonly used for drugs when the medical use is considered. The values for the half-maximal inhibition (IC_{50}) measured by the chromogenic assay were 0.013 and 0.045 μM for 120-1032 and LY311299, respectively (*13*). LY311299 was successfully designed as a result of the modification of the indomethacin-like compounds by comparing the crystal structures of hs-PLA_2-IIA complexed with the phosphonate transition state analogue (*6*). In that analogue compound, one of the oxygen atoms of the substrate phosphate group is coordinated to the calcium ion and interacts with the side chain nitrogen atom of Lys69 *via* a water molecule (*6*). In the crystal structure of the hs-PLA_2-IIA/LY311299 complex (*7*), the phosphonate of LY311299 provides the same motif where the oxygen atoms function as a ligand to the active site calcium and as a hydrogen bond acceptor to Lys69 mediated by a water molecule. However, introduction of the phosphonate in LY311299 resulted in a

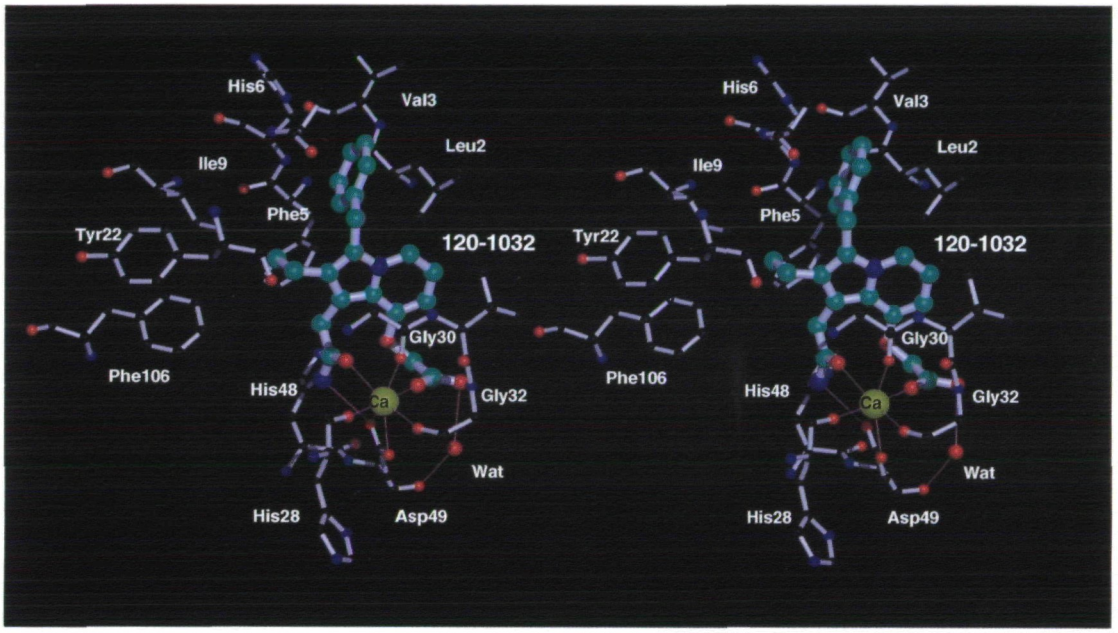

Fig. 4. **Stereo view of the binding of the 120-1032 inhibitor to hs-PLA_2-IIA in the active site.** Calcium, oxygen, nitrogen, and carbon atoms are indicated by yellow, red, blue, and cyan, respectively. The atoms of the inhibitor are color-coded by atom type: green-carbon, blue-nitrogen, and red-oxygen. Amino acid residues important for the inhibitor binding are indicated by three letter code. Specific protein-inhibitor interactions are indicated by thin line.

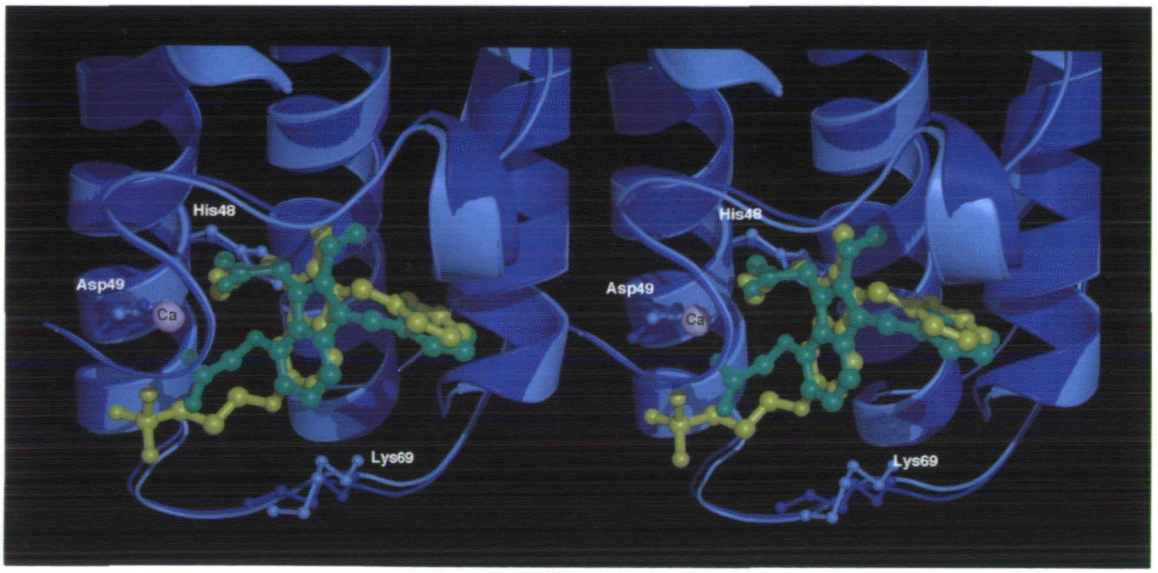

Fig. 5. **Stereo view of the superposition of the active site in the hs-PLA_2-IIA/120-1032 complex (light blue) and hs-PLA_2-IIA/LY311299 complex (blue).** The superposition is based only on the protein atoms in each complex. The inhibitors are shown by the colored ball-and-stick representation: 120-1032 (green) and LY-311299 (yellow). Calcium ion is represented by a large white sphere.

threefold increase both in binding to hs-PLA$_2$-IIA and in activity measured by the tissue-based assays against the indomethacin-like compounds (*7*).

Figure 5 shows the superposition of the active sites of the hs-PLA$_2$-IIA/120-1032 and hs-PLA$_2$-IIA/LY311299 complexes. The planes of indole and indolizine rings of the inhibitors are inclined at an angle 30° to each other. The overall hs-PLA$_2$-IIA structure in the hs-PLA$_2$-IIA/120-1032 complex is similar to that of the hs-PLA$_2$-IIA/LY311299 complex. Superposition of all α-carbon atoms of hs-PLA$_2$-IIA in both complexes resulted in an r.m.s. deviation of 0.46 Å. The hs-PLA$_2$-IIA enzyme has the flexibility to accommodate several inhibitors with different structures and binding modes. The remarkable structural differences in both complexes were found in the catalytic His48 and Asp49 residues. In the hs-PLA$_2$-IIA/120-1032 complex, the side chain of His48 moves 0.5 Å, whereas the side chain of Asp49 rotates 30° with a translational shift of 1.25 Å. This structural movement induced the closer contact between hs-PLA$_2$-IIA and 120-1032. Furthermore, there is no water molecule bound to Lys69 in the hs-PLA$_2$-IIA/120-1032 complex, and the side chain of this residue takes a different orientation from that in the hs-PLA$_2$-IIA/LY311299 complex. The terminal nitrogen atom of the Lys69 side chain in the 120-1032 inhibitor is shifted by 1.21 Å away from that in the LY311299 inhibitor.

As expected, the substitution of the ethyl group increased hydrophobic contacts with the cavity wall of the hydrophobic channel of the enzyme in the hs-PLA$_2$-IIA/120-1032 complex. This fact coincides with the data obtained by the chromogenic assay, where the ethyl substituent increased the ability to inhibit hs-PLA$_2$-IIA (*8*). As mentioned before, the substituents which occupy the positions of the transition state analogue (*6*), the phosphonate group in LY311299 and the oxycarboxylate group in 120-1032 (Fig. 4), are bound to the calcium ion and to the hs-PLA$_2$-IIA enzyme in different modes in the two complexes. These differences in binding interactions are probably connected with the fact that the binding affinity of the 120-1032 inhibitor is 3.5-fold higher than that of LY311299. This is a case in which the effects of the inhibitor on the binding ability predicted from the molecular modeling study are confirmed by the established three-dimensional structures. In addition, the X-ray structure revealed the unexpected existence of the water molecule which mediated the hydrogen bonding between the hs-PLA$_2$-IIA enzyme and the inhibitor. This indicates the significant contribution that X-ray crystallography can make to structure-based drug design.

We would like to thank our colleagues at the Lilly Research Laboratories, Indianapolis, USA, who have given the samples and coordinates of hs-PLA$_2$-IIA and collaborated with us during the course of this project. We also would like to acknowledge Dr. H. Arita's support as the director of this project. We are grateful to Dr. Y. Katsuya, Hyogo Prefectural Institute Industrial Research, for his help in data collection and processing.

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