Journal of
Medicinal Chemistry
J. Med. Chem., 1996, 39(20), 3878-3881, DOI:10.1021/jm960502g

Enzyme Purification:

All chromatography, concentration, and dialysis steps were carried out at 4 °C. All other steps were carried out at room temperature. CHO cell culture media was adjusted to pH 2.0 with HCl, filtered through 0.3 micrometer depth filters (Millipore Polyguard CR) and concentrated using a tangential flow plate and frame device fitted with a 3 kDa molecular weight cut-off cassettes (Millipore Prostak). The pH of the concentrate was adjusted to 4.1 with NaOH and the conductivity was adjusted to 35 mS/cm with NaCl. The adjusted concentrate was loaded on a 25.2 x 5 cm SP-C25 column (Pharmacia) equilibrated with 10 mM acetate buffer pH 4.5 containing 0.5 M NaCl. Bound proteins were eluted with a 0.5 M to 2.0 M NaCl gradient in the same buffer. Eluted fractions having PLA_2 activity were pooled and concentrated with a tangential flow apparatus using a 3 kDa molecular weight cut-off cassettes (Millipore Prostak). The concentrate was chromatographed on a 5 x 100 cm Superdex 75 column (Pharmacia) equilibrated with the same acetate buffer. The active fractions were dialyzed extensively against 100 mM ammonium bicarbonate, lyophilized to dryness and stored at -80 °C. The lyophilized material was resuspended in 100 mM TrisCl buffer pH 7.5 containing 35% NaCl and 5 mM $CaCl_2$ and chromatographed on a 1 x 30 cm FPLC analytical Superose 12 column equilibrated with resuspension buffer. This step separated misfolded enzyme from correctly folded enzyme (main peak). The purified enzyme had a specific activity of 400 units/mg using ^3H-arachidonate labeled *E. coli* membrane and appeared as a single band at ~14 kDa on SDS-PAGE. Typically, 1L of CHO cell culture yielded 1 mg of pure enzyme.

Crystallization and X-ray Data Collection:

The crystals of the complex were obtained by vapor diffusion against the precipitant containing 70% saturated NaCl, 0.1M TrisCl buffer (pH 7.6), and 0.05 M $CaCl_2$. **I** was added as a solid onto a droplet containing 2 µL of hnps-PLA_2 solution (30 mg/ml, TrisCl buffer, pH 7.6) and 2 µL of the precipitant, immediately before equilibrating the droplet with 1.0 mL of the same precipitant solution in a hanging drop setting at 7 °C. It was critical to add the inhibitor immediately before equilibrating the droplet with the precipitant. Other variations such as cocrystallization failed to yield crystals. Best crystals grew to a size of about 0.5x0.3x0.2 mm in about two weeks. Initially, diffraction intensities to 2.2 Å resolution were collected on a DIP2000 area detector on a Mac Science M18XHF X-ray generator operated at 90 mA and 50 kV at room temperature. The cumulative completeness of the data was 85% and 74% ($> 1\sigma$) up to 2.5 and 2.2 Å, respectively. Later, during the structure refinement, diffraction

intensities to 2.0 Å resolution were collected on the same instrument with a larger crystal. The internal *R*merge on I_{hkl} is 7.1% for a total of 81,302 reflections (47,102 unique reflections). The cumulative completeness of the data is 95% and 78% ($> 1\sigma$) up to 2.5 and 2.0 Å, respectively. All data sets were processed with DENZO (Otwinowski, Z. in *Data Collection and Processing*, Sawyer, L., Isaacs, N., Bailey, S., Eds. CCP4 Study Weekend, SERC Daresbury Laboratory, Warrington, UK, 1993, pp 56-62). The inhibited enzyme crystallized in space group of $P2_1$ with cell parameters *a*=64.62, *b*=114.44, *c*=64.71 Å, β=119.9° (six molecules in the asymmetric unit). Figure 1 shows the hexameric packing of the complex.

Structure Determination:

X-PLOR program package (*X-PLOR Version 3.0.*,Yale University, New Haven, Connecticut, 1992) was used for the structure determination by molecular replacement. Reflections in the resolution shell 10-4 Å of the 2.2 Å data were used throughout the molecular replacement procedures. A self-rotation search (using maximum Patterson vector length of 30 Å) resulted in a strong local 2-fold peak (psi=90.0°, phi=30.0°, kappa=180°) which was 5.13 σ higher than the highest non-related peak. A cross-rotation search (using maximum Patterson vector length of 45 Å) were carried out on a 1° grid in modified Eulerian angles $\theta+,\theta_2,\theta-$ which was restricted to 0-720°, 0-90°, 0-360°, respectively according to rotation space group (Rao *et al. Acta Crystallogr.* **1980**, A36, 878-884). The top 50 rotation function (RF) peaks from the search were further refined by the Patterson Correlation (PC) refinement which consisted of 15 steps of rigid-body conjugate gradient minimization (Powell, M. J. D. *Math. Programming* **1977**, 12, 273-278). The PC refinement resulted in four different refined rotation solutions distinguishable from the noise level, all of which were not related by the non-crystallographic 2-fold symmetry defined earlier. The two-dimensional (*ac* plane) translation searches for three of the four PC refined RF peaks on a 1 Å sampling interval yielded a top translation function peaks only 1.85 σ (molecule A), 2.33 σ (molecule B), and 1.75 σ (molecule C) higher than the next highest peak, respectively. The local 2-fold symmetry information was used to check whether the position of molecule A is actually the correct translation solution. After generating a molecule (molecule $A_{2\text{-fold}}$) by applying the non-crystallographic 2-fold symmetry to molecule A, two translation searches (along the *ac* plane and along *b*) were carried out to determine the position of molecule $A_{2\text{-fold}}$ with respect to molecule A. The second translation search (along *b*) for origin definition resulted in a top translation peak 3.1 σ higher than the next highest peak. The two molecules showed favorable contacts with each other with no overlap

between symmetry-related molecules, confirming that the positions of molecule $A_{2\text{-fold}}$ as well as molecule A were correct. Correct positions of the remaining four molecules in the asymmetric unit were determined by the same procedure with the other two top translation function peaks (molecule B and C), and origin definition with respect to molecule A. Subsequent rigid body refinement (20 steps of conjugate gradient minimization against reflections at 8-2.5 Å resolution) of the six translation solutions lowered the R-factor from 43.1 to 41.9%. At this stage, in the 2Fo-Fc map, the strong electron density was found for the bound inhibitors as well as the calcium ions which were not included in the map calculation. Multiple rounds of electron density calculation and manual refitting of many side chains plus the backbone atoms of C-terminus and residues 78-81 were necessary due to the completely different crystal packing, and probably due to a minor error in tracing electron density of uninhibited enzyme. The retraced residues 78-81 fall into the region of energetically favorable main-chain conformation on the Ramachandran plot while Ala78, Lys79, and Asp81 in the model of uninhibited enzyme do not. Thereafter, further structure refinement was carried out with the 2.0 Å data. Final atomic position refinement by the simulated annealing protocol including built-in inhibitor molecules, calcium ions, and 476 water molecules lowered standard R-factor to 20.1% and free R-factor to 27.3 (10% of the total reflections) for reflections at 8-2.0 Å resolution ($> 1\sigma$). Non-crystallographic restraints were not employed during the refinement because of the differences in the local molecular environment. The final model of the complex, with rms deviations from ideality of 0.018 Å and 1.56^{o} in bond distance and bond angles, contains no residue deviating from the energetically allowed region on the Ramachandran plot except for glycines.

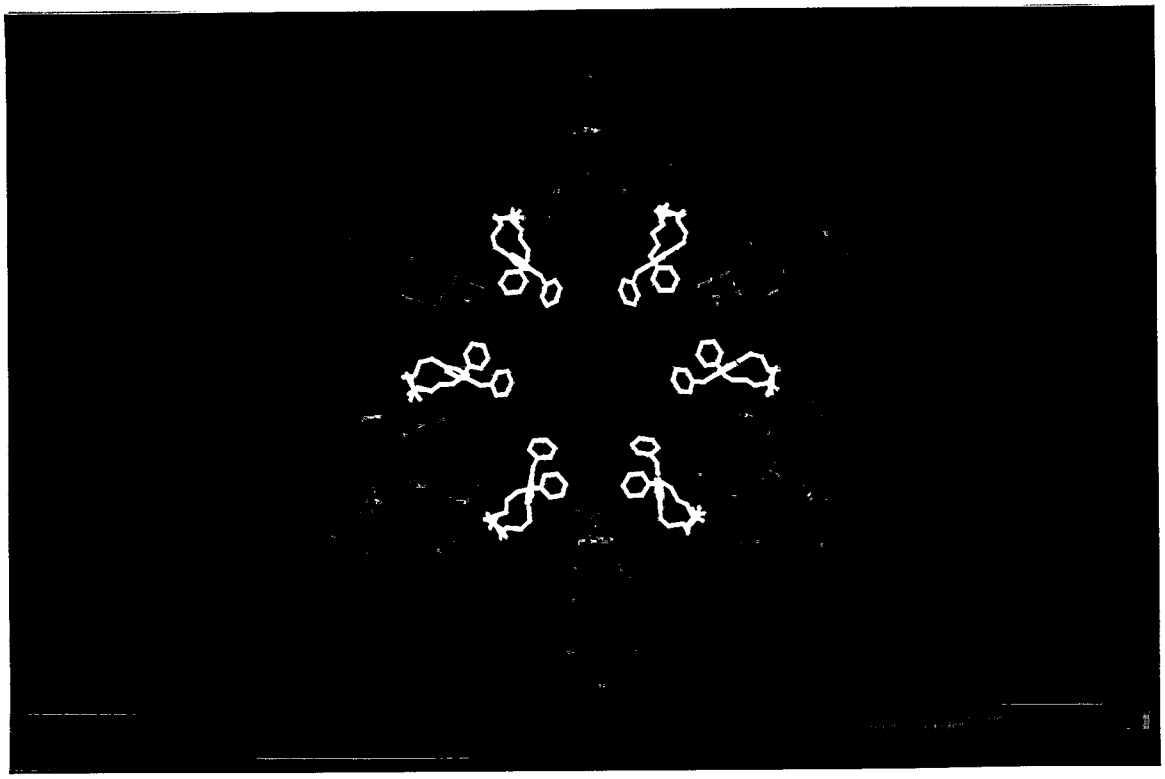

Figure 1. The hexameric crystal packing of hnps-PLA_2 complexed with **I** (white thick lines) viewed roughly along a local three fold axis. Three protomers at the front are in green, and other three protomers at the back are in purple. Only backbone atoms of the molecules are shown for clarity.