Fluoroketone Inhibition of Ca\(^{2+}\)-Independent Phospholipase A\(_{2}\) through Binding Pocket Association Defined by Hydrogen/Deuterium Exchange and Molecular Dynamics

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ABSTRACT: The mechanism of inhibition of group VIA Ca\(^{2+}\)-independent phospholipase A\(_{2}\) (iPLA\(_{2}\)) by fluoroketone (FK) ligands is examined by a combination of deuteration exchange mass spectrometry (DXMS) and molecular dynamics (MD) simulations. Empty pockets were identified during the simulations and studied for their ability to accommodate FK inhibitors. Ligand docking techniques showed that the potent inhibitor 1,1,1,3-tetrafluoro-7-phenylheptan-2-one (PHFK) forms favorable interactions inside an active-site pocket, where it blocks the entrance of phospholipid substrates. The polar fluoroketone headgroup is stabilized by hydrogen bonds with residues Gly486, Gly487, and Ser519. The nonpolar aliphatic chain and aromatic group are stabilized by hydrophobic contacts with Met544, Val548, Phe549, Leu560, and Ala560. The binding mode is supported by DXMS experiments showing an important decrease of deuteration in the contact regions in the presence of the inhibitor. The discovery of the precise binding mode of FK ligands to the iPLA\(_{2}\) should greatly improve our ability to design new inhibitors with higher potency and selectivity.

INTRODUCTION

Group VI phospholipase A\(_{2}\) (GVI PLA\(_{2}\)), also known as Ca\(^{2+}\)-independent phospholipase A\(_{2}\) (iPLA\(_{2}\)), constitutes one group of the superfamily of phospholipase A\(_{2}\) enzymes that hydrolyzes the sn-2 ester bond of phospholipids to release free fatty acids. These fatty acids can be metabolized into pro-inflammatory eicosanoids, including prostaglandins and leukotrienes.\(^3\)

Ca\(^{2+}\)-independent phospholipases were so named after the discovery that their activity is not increased in the presence of Ca\(^{2+}\), in contrast to the other then-known PLA\(_{2}\)s. The first discovered and best-studied iPLA\(_{2}\) is the 85-kDa GVI iPLA\(_{2}\) (also known as iPLA\(_{2}\beta\)).\(^4\) Different variants of iPLA\(_{2}\)s were later discovered, leading to their classification into various subgroups. To date, six subgroups have been identified, including GVI (iPLA\(_{2}\beta\), PNPLA9),\(^5,8\) GVIC (iPLA\(_{2}\gamma\), PNPLA8),\(^9,10\) GVIE (iPLA\(_{2}\delta\), PNPLA6),\(^11\) GVID (iPLA\(_{2}\epsilon\), PNPLA3),\(^12\) GVIF (iPLA\(_{2}\zeta\), PNPLA2),\(^13,15\) and GVIF P A\(_{2}\) (iPLA\(_{2}\eta\), PNPLA4).\(^16-18\) These GVI iPLA\(_{2}\)s all contain a serine active site in a patatin-like catalytic domain. GVI iPLA\(_{2}\) has been reported to be activated by oligomerization, ATP binding, caspase cleavage, and membrane interaction\(^1\) and to localize in mitochondria in various types of cells.\(^19-21\)

The important roles of iPLA\(_{2}\) in regulation of the inflammatory response and in mitochondrial maintenance suggest that it could be implicated in a large number of genetic and age-related diseases. GVI iPLA\(_{2}\) has been suggested to be responsible for the deacylation of cardiolipin and monolyso cardiolipin accumulation in Barth syndrome and hypertensive heart failure.\(^22,23\) Clinical studies have demonstrated that mutations of GVI iPLA\(_{2}\) are associated with neurodegeneration with brain iron accumulation (NBIA) disorders and infantile neuroaxonal dystrophy.\(^24-27\) Recently, it was demonstrated that GVI iPLA\(_{2}\) plays a key role in the onset and progression of experimental autoimmune encephalomyelitis (EAE) indicating that this enzyme may be a target for the development of novel therapies for multiple sclerosis.\(^28\) Recent cellular studies have also suggested that iPLA\(_{2}\) may interfere with the protein p53 pathway. In particular, inhibition of iPLA\(_{2}\) was shown to decrease prostate cancer cell growth, suggesting iPLA\(_{2}\) could be a new anticancer target.\(^29,30\)

Due to the considerable pharmaceutical interest in iPLA\(_{2}\), a current challenge is the design of a safe inhibitor that is also highly selective for this class. Several ligands have already been shown to inhibit the phospholipase, lysophospholipase, and transacylase activities of GVI iPLA\(_{2}\).\(^31-34\) The most commonly used iPLA\(_{2}\) inhibitor is bromoenol lactone (BEL),...
and the S enantiomer (S-BEL), has been shown to preferentially inhibit iPLA₂.33–37 Although BEL inhibits iPLA₂ through acylation of a critical cysteine,38 it is also highly toxic, as it reacts with cysteines in other proteins. Methyl arachidonyl fluorophosphonates, known as MAFPs, are potent inhibitors of iPLA₂;39 but their action is irreversible, which typically translates into increased toxicity in vivo. In addition, 2-oxamides have been shown to inhibit GIVA iPLA₂ but are also active against GIVA cPLA₂.39 Finally, fatty acyl trifluoromethyl ketones, including arachidonyl and palmitoyl trifluoromethyl ketones, have been identified as promising inhibitors of GIVA iPLA₂.33 Fluoroketone (FK) inhibitors were designed to target serine active sites and consequently are also active against cPLA₂. However, FK inhibitors were shown to become selective for iPLA₂ versus cPLA₂ and sPLA₂ after a modification of the fluoroketone group, and the addition of a hydrophobic terminus connected by a medium-length carbon chain to mimic the fatty acid chain.40 One pentafluoroketone, FKGK11, has been shown to be a potent and selective GIVA iPLA₂ inhibitor demonstrating in vivo activity against EAE.27 Another FK ligand, 1,1,1,3-tetrafluoro-7-phenylheptan-2-one (PHFK),40 contains a phenyl ring, a five-carbon linker, and an additional fluorine on the carbon adjacent to the ketone of the trifluoromethylketone. It shows great promise as a lead compound for iPLA₂-associated diseases; hence, we have focused in this paper on resolving its binding mode.

Previous computational studies on the inhibition of PLA₂ have mainly focused on group IVA (GIVA, cPLA₂) proteins,41,42 where high-resolution structural data exist. The task of determining the binding mode of FK inhibitors for GIVA iPLA₂ is complicated by the lack of high-resolution X-ray structures. GVI iPLA₂ enzymes are known to be composed of an N-terminal regulatory domain containing seven ankyrin repeats and a C-terminal catalytic domain containing the active-site dyad (Ser519/Asp652) (Figure 1A). The catalytic domain has low sequence homology to other lipases, such as cPLA₂, and is distinct from the highly disulfide-bonded sPLA₂s. Previously, we proposed a rudimentary model of the catalytic domain based on the structure of the patatin protein,43 a lipid acyl hydrolase found in potato with sequence similarity with the catalytic domain of iPLA₂. The model could explain why some regions of the catalytic domain appeared particularly accessible to the solvent in deuterium exchange mass spectrometry (DXMS) experiments.43 However, the binding of inhibitors and the stability of the model in molecular dynamics (MD) simulations were not studied.

In this paper, computational methods are coupled with experimental DXMS techniques to study the atomic-level details of the iPLA₂–PHFK complex. A new model for the catalytic domain of iPLA₂ is proposed and shown to be stable by extensive MD simulations under aqueous conditions. Ligand docking techniques were able to uncover a favorable binding mode for FK inhibitors, and H/D exchange experiments provided experimental information about the effect of PHFK binding on solvent accessibility, giving strong support to the proposed binding mode. The details of protein–inhibitor interactions are discussed, as well as implications for the catalytic mechanism.

### MATERIALS AND METHODS

#### Materials

1-Palmitoyl-2-arachidonoyl-sn-phosphatidylcholine (PAPC) was obtained from Avanti Polar Lipids, and D₂O (99.96%) was obtained from Cambridge Isotope Laboratories. The PHFK ligand was synthesized as previously described.40 All other reagents were analytical reagent grade or better.

#### Protein Expression, Purification, and Activity Assay

GVIA iPLA₂ containing an N-terminal His₆-tagged insertion at the third residue was expressed by use of recombinant baculovirus in suspension cultures of Sf9 insect cells.43 For the specific activity of iPLA₂, assays (Figure 3) were performed in buffer composed of 100 mM N-(2-hydroxyethyl)piperazine-N'-ethanesulfonic acid (HEPES) at pH 7.5, 2 mM ATP, and 5 mM dithiothreitol (DTT). The mixed micelles were composed of 0.1 mM PAPC (80 000 cpm) and 0.4 mM Triton X-100 in a final volume of 500 μL. The reaction was initiated by adding 0.2 μg of iPLA₂ to mixed micelles and incubated at 40 °C for 30 min. After incubation, the reaction was quenched, and the fatty acids were extracted by a modified Dole protocol as previously described.46

#### Preparation of Deuterated Samples

Before the DXMS experiments were carried out, 5 mM PHFK inhibitor (100X stock in 100% dimethyl sulfoxide, DMSO) was added to iPLA₂ to a final 50 μM PHFK in 1% DMSO and incubated at 23 °C for 10 min. Control experiments contain 1% DMSO. Hydrogen/deuterium exchange experiments were initiated by mixing 40 μL of iPLA₂ (containing 50 μg of iPLA₂) in protein buffer with 80 μL of D₂O buffer to a final concentration of 63.3% D₂O at pH 7.0. D₂O buffer was prepared by mixing 99.99% D₂O with 20X buffer solution in H₂O and generated 95% D₂O buffer. Final D₂O buffer contained 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) (pH 6.9), 100 mM NaCl, 2 mM DTT, and 2 mM ATP. The samples were incubated at 23 °C for an additional 10, 30, 100, 300, 1000, or 3000 s. The deuteration experiment was quenched by adding 80 μL of ice-cold quench solution (2.5% formic acid, 2.5 M guanidine hydrochloride) that acidified the sample to a final pH = 2.5. The samples were then immediately frozen on dry ice. Back exchange levels were calculated as previously reported.45,46 Vials with frozen samples were stored at −80 °C until analysis, usually within 3 days.

#### Proteolysis–Liquid Chromatography–Mass Spectrometry Analysis of Samples

All steps were performed at 0 °C as previously described.43–45 The quenched sample was digested by porcine pepsin-imobilized column and the proteolyzed peptides were separated on a C18 column. The peptides were analyzed on an LCQ Classic (Thermo Finningan, Inc.) electrospray ion trap–type mass spectrometer and an

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**Figure 1.** (A) Schematic representation of GIVA iPLA₂ sequence information with the location of the active-site dyad, Ser519/Asp652. (B) Initial guess for the structure of the catalytic domain based on patatin. (C) Equilibrated structure after extensive (~200 ns) MD simulation. (D) Superposition between initial guessed structure and equilibrated structure, showing a high degree of consistency.
Peptides were identified by tandem mass spectrometry (MS/MS) data through SEQUEST (Thermo Finnigan, Inc.). DXMS Explorer (Sierra Analytics Inc., Modesto CA) was used to analyze the mass spectra and calculate the average of the mass envelope. The procedure for further data processing was described previously.43,44

iPLA₂ Coverage Map of Pepsin Fragmentation and Deuterium On-Exchange. The recombinant iPLA₂ after purification was confirmed to be active in the mixed-micelle assay. The procedure of protein digestion by pepsin was previously described.43 Among them, 153 peptides had a sufficient signal-to-noise ratio for analysis, which covered 88% of the total protein sequence. From these 110 peptides, 46 different peptides with the least number of overlapped residues were selected to determine the deuteration level of iPLA₂ (Figure 2). Overall, the representative 46 peptides selected account for 71% coverage of the iPLA₂.

Deuterium exchange experiments were carried out by incubation of 40 μL of iPLA₂ with 80 μL of 95% D₂O buffer for six different time points (10, 30, 100, 300, 1000, and 3000 s) at 23 °C. The sample was quenched and injected onto the HPLC online digestion system.43−45 The triplicate experiments were carried out with three different batches of enzyme on different days. The deuteration level increase over time due to the amide H/D exchange. The exchange results in various regions were calculated as the ratio of incorporated deuterons to the maximum deuteration in a particular peptide (Figure 4). Because of the fast off-exchange rate of N-terminal residues, these residues are unable to retain any deuterons under the experimental conditions employed; hence, these residues were not shown in the results. Some peptides that showed a poor signal-to-noise ratio after deuteration were also excluded from analysis.

Structural Models and MD Simulation. The Prime protein structure prediction suite46 was used to build a homology model for the catalytic domain of iPLA₂, using as a template the X-ray structure of patatin (40% sequence homology), solved at a resolution of 2.2 Å (PDB code 1OXW).47 The 329 amino acid model for the catalytic domain was solvated in VMD,48 in a cubic periodic box containing a physiological concentration of Na⁺ and Cl⁻ ions of 0.10 mol/L. Ionizable side chains were kept in their default charge states for pH 7, and the His residues were kept uncharged. All simulations were conducted in NAMD 2.949 with the CHARMM27 force field.50 A time step of 2.0 fs was used in combination with the SHAKE algorithm.51 The temperature was regulated with a Langevin thermostat.52
Energy minimization was conducted for 25 000 steps with the protein backbone atoms fixed, followed by two consecutive 20 ns MD runs in the NPT and NVT ensembles. Subsequently, two production runs were conducted for 200 ns in the NVT ensemble. The overall structural stability of the model was indicated by a stable root-mean-square-deviation (RMSD) for the backbone atom positions during the simulations. After only ∼3 ns of MD, the potential energy had reached a plateau and the RMSD of backbone atoms stabilized below 3 Å. Inspection of the trajectories revealed that the integrity of the secondary structure was maintained (Figure 1C). In the spirit of the relaxed complex scheme, trajectories were clustered into a reduced ensemble of 50 representative structures for ligand docking. Empty pockets inside the protein were monitored by computing the solvent-accessible surface in VMD. The ligand was docked with Glide software, using the OPLS-2005 force field for its parametrization and an inner docking box of length 10 Å.

### RESULTS AND DISCUSSION

**DXMS Characterization of iPLA₂−PHFK Interaction.** The significant deuteration changes upon PHFK binding are located in the catalytic domain of iPLA₂ (Figure 5). In particular, five regions surrounding the active-site dyad, residues 483−493, 516−525, 544−549, 631−655, and 773−778, showed the most significant decrease of deuteration (Figure 6), while none of the regions showed an increase in H/D exchange.

Region 483−493 contains the DXMS information from three peptide fragments: 483−490, 483−493, and 486−493. In the absence of inhibitor, the deuteration level was observed to increase, from 10% to 30%, from 10 s to 50 min. The same experiment repeated in the presence of PHFK led to a deuteration level below 10% during the whole 50 min time course. Region 493−498 is located in a stable helix next to the C-terminus of region 483−493. It showed a very slow H/D exchange, of only 2%, after 50 min. Region 516−525, which contains the active-site Ser519, showed a clear decrease in deuteration upon PHFK binding. The largest decrease in this region was 18% at 50 min of exchange. Similarly, region 544−549 showed a significant decrease of deuteration after 16 min of H/D exchange. Region 631−655 contains fragments 631−650, 631−655, 632−655, and 632−656 from four individual peptides. Among these regions, the 631−650 fragment seems to be responsible for most of the changes. Region 773−778 showed a 12% decrease of H/D exchange at the 50 min time point, and region 710−730 showed 12% and 9% decrease of exchange after 10 and 30 s, respectively. Regions 404−442 in the linker region and 238−269 in the ankyrin repeats also showed greater than 10% decrease in H/D exchange.

**Computational Characterization of the iPLA₂−PHFK Interaction.** MD simulations showed that iPLA₂ residues, including active-site residues, are flexible (RMS fluctuation = ~0.6−0.8 Å). An analysis of the solvent-accessible surface...
during 2 × 200 ns MD simulations was carried out to discover transient pockets that could potentially accommodate the PHFK inhibitor. Fifty representative protein structures were extracted from the simulations. An analysis of the solvent accessibility surface revealed that several empty pockets were large enough to accommodate the inhibitor, including an empty pocket near the active-site Ser519. Ligand docking was performed on all the pockets and indicated that the best docking scores were systematically obtained when the box was centered on the active site, suggesting a very favorable binding mode.

In Figure 7, the docked iPLA₂−PHFK complex is shown. The inhibitor is found to sit snugly in the active-site with its FK group stabilized by polar residues, and its alkyl chain and benzene ring stabilized by nonpolar side-chains (Figure 8). More specifically, the carbonyl group of PHFK forms favorable interactions with the amide groups of Gly486 and Gly487. A "fluorophilic site" is created by Lys489, Asn658, and Lys729, while the aliphatic carbon chain is stabilized by a narrow hydrophobic tunnel formed by residues Thr520, Phe549, and Leu770. The benzene ring forms hydrophobic contacts with Met544, Val548, Phe549, and Leu560. DXMS experiments indicate that these residues display decreased H/D exchange in the presence of the PHFK ligand (Table 1), in agreement with the proposed binding mode. Such a noncovalent binding model is in accordance with previous results demonstrating reversible inhibition of iPLA₂ by trifluoromethyl ketones. Various experimental results demonstrated that the mechanism of inhibition of iPLA₂ by FKs is quite different than that observed by cPLA₂. In the case of iPLA₂, the reaction progress curve was linear and the binding was fast, indicating a reversible inhibition not involving slow or tight binding.
the catalytic domain through the linker. Whether and how ankyrin repeats regulate the catalytic activity of iPLA₂ remains unclear. DXMS experiments suggest that inhibitor binding to iPLA₂ decreases the rates of H/D exchange, with most changes appearing in the loops surrounding the active site in the catalytic domain. The decrease in H/D exchange in these regions appears to be fully explained by direct contact with the inhibitor, blocking access to phospholipid substrates and reducing solvent accessibility. However, a minor decrease in H/D exchange was also observed in the linker region and the ankyrin repeats, indicating that the perturbation caused by inhibitor can extend to the whole enzyme. Because only one linker region and one ankyrin region showed a small 10% decrease of deuteration after 30 min of H/D exchange, overall, the structure of the ankyrin repeats and the linker is unlikely to change dramatically, and only minor shifts in the relative positions between the ankyrin repeats and the catalytic domain may occur upon ligand binding. One cannot exclude that phospholipid substrates may have different properties when compared to the PHFK inhibitor studied here; however, we

Figure 7. Location of PHFK binding to iPLA₂ according to ligand docking and DXMS experiments. The color code indicates regions of decreased H/D exchange upon ligand binding. The membrane penetration domain (loop 695–730) is believed to anchor the protein at the membrane surface by entering the membrane. The left and right images correspond to the protein viewed from a direction parallel or perpendicular to the membrane surface.

Figure 8. Characterization of the inhibitor binding pocket in the catalytic domain of iPLA₂. Residues within a 5 Å radius of PHFK are shown.
expect the perturbation caused by ligand binding to remain localized to the active site in both cases.

The active-site dyad, Ser519 and Asp652, is conserved in both patatin and iPLA2. Asp652 is believed to accept a hydrogen from Ser519 during the initial stages of the catalytic reaction. Interestingly, no hydrogen bond is present between Ser519 H and Asp652 O, from 3.7 Å to 1.6 Å, creating a hydrogen bond between the two residues. This hydrogen bond suggests that the binding of PHFK helps shorten the distance −ected by PHFK binding. Ser519 is located in the €exible loops that could provide some form of regulation. The regulation of active-site accessibility by the surrounding environment is strongly affected by ligand binding. Ser519 is located in the region 516–525, showing a H/D exchange decrease of 18%, and Asp652 is in the region 632–655, which shows a decrease of 11%. MD simulations of the protein−PHFK complex suggest that the binding of PHFK helps shorten the distance between Ser519 H and Asp652 O, from 3.7 Å to 1.6 Å, creating a hydrogen bond between the two residues. This hydrogen bond was also observed in MD simulations of the apoprotein, where it was seen to typically break and re-form within ~1 ns.

The regulation of active-site accessibility by the surrounding flexible loops is another interesting possibility. The structure of the catalytic domain of iPLA2 is similar to the core structure of cPLA2, which is one of the well-studied PLA2s. Unlike the buried active sites of cPLA2, iPLA2 has a more exposed catalytic domain. However, the active-site serine is surrounded by several flexible loops that could provide some form of regulation, including residues 485−487 (glycine loop), 517−520 (catalytic loop), 540−545, 549−560, 631−653, 695−730 (membrane penetration arm), and 761−775. MD simulations confirmed a high degree of flexibility in the loop regions, in particular in the membrane penetration arm domains, (loop 695−730), which could play a role similar to the cap structure of cPLA2. DXMS experiments show that the effect of PHFK binding propagates to the loop regions, suggesting some degree of conformational flexibility in the unstructured loops. In particular, regions 710−722 and 710−730 in the membrane penetration arm showed a very slight drop of 7% and 12% in the deuteration level at 10 s of H/D exchange. This domain was previously found to undergo a dramatic decrease of H/D exchange upon binding to membranes. However, it appears to be only slightly affected by inhibitor binding, with a small degree of change relative to residues in direct contact with the inhibitor. Thus, we do not expect the binding of PHFK to lead to complete collapse of the membrane penetration arm to cover the active site, although the loop could still help create some form of regulation.

**CONCLUDING REMARKS**

Ligands containing a trifluoromethyl or pentafluoroethyl ketone and a hydrophobic tail have been identied as potent and selective inhibitors of GVIA iPLA2, encouraging their utility in developing new pharmaceutical agents. Computer modeling and DXMS experiments show that the binding of FG lidands occurs in the active site, with the inhibitors mimicking the binding of natural substrates. In the natural enzyme, hydrophobic interactions with the phospholipid tail are believed to help increase the binding affinity of the reactant with respect to the lysophospholipid product. Similarly, in the iPLA2−PHFK complex, favorable hydrophobic interactions can explain the high afinity of the inhibitor. In addition, the perfluorooalkyl headgroup of FK lidands can substitute for the headgroup of the phospholipids and enter a hydrophobic site, while the oxygen hole in the triglycine region is able to stabilize the FK carbonyl group through strong hydrogen bonds. The abundant empty space on both sides of the docked PHFK inhibitor suggests why FK lidands with larger head or tail groups are also able to bind to iPLA2. Discovery of the binding mode for PHFK and the available space for further decoration of the primary inhibitor represents an important rst step toward the rational design of new selective inhibitors. In future work, chemical alterations of current inhibitors will be pursued, based on the binding mode and structural model presented here.

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**Notes**
The authors declare no competing financial interest.

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