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Using Hydrogen/Deuterium Exchange Mass Spectrometry to Define the Specific Interactions of the Phospholipase A_2 Superfamily with Lipid Substrates, Inhibitors, and Membranes*[S]

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Jian Cao, John E. Burke, and Edward A. Dennis[1]

From the Department of Chemistry and Biochemistry and Department of Pharmacology, University of California, San Diego, La Jolla, California 92093-0601

The phospholipase A_2 (PLA_2) superfamily consists of 16 groups and many subgroups and constitutes a diverse set of enzymes that have a common catalytic activity due to convergent evolution. However, different PLA_2 types have unique three-dimensional structures and catalytic residues as well as specific tissue localization and distinct biological functions. Understanding how the different PLA_2 enzymes associate with phospholipid membranes, specific phospholipid substrate molecules, and inhibitors on a molecular basis has advanced in recent years due to the introduction of hydrogen/deuterium exchange mass spectrometry. Its theory, practical considerations, and application to understanding PLA_2/membrane interactions are addressed.

The phospholipase A_2 (PLA_2)[2] superfamily consists of a diverse set of enzymes that catalyze hydrolysis of the *sn*-2 ester bond of phospholipids, thereby producing lysophospholipids and free fatty acids with important downstream signaling roles (1). Although the PLA_2s have been formally classified into some 16 groups and numerous subgroups, each is more commonly thought of as being part of one of six main types based on a variety of sequence, structural, and other characteristics. The six main types are secreted ($sPLA_2$), cytosolic ($cPLA_2$), Ca^{2+}-independent ($iPLA_2$), platelet-activating factor acetylhydrolase (PAF-AH), lysosomal ($LPLA_2$), and adipose (AdPLA) (2). It is well known that PLA_2 enzymes are involved in the inflammatory process and various diseases, including atherosclerosis, diabetes, arthritis, and cancer (1, 3). A large body of evidence suggests that inhibiting the enzymatic activity of PLA_2s has potential benefits for the treatment of specific diseases (4–6). Therefore, there has been considerable interest in understanding how the PLA_2s interact with the lipid substrate, inhibitors, and membranes with the goal of developing effective new pharmaceutical agents.

Many PLA_2s display a dramatic increase in activity when the phospholipid substrate is in an aggregated form (micelles, vesicles, liposomes, etc.) rather than in monomeric form. Most PLA_2 enzymes have to bind to lipid/water interfaces and access the substrate from the lipid phase to complete a catalytic turnover. The concept of "surface-dilution kinetics" was successfully applied to explain these enzymes' kinetic characteristics (7). As part of surface-dilution considerations, the enzyme may undergo surface binding to membranes, whereby the enzyme either associates nonspecifically with the surface of the lipid aggregate or associates specially with a phospholipid(s) in the aggregate's surface (7). It is believed that PLA_2s contain an interfacial surface distinct from their specific active site in terms of topology and function, which associates with phospholipid membranes.

Precisely how PLA_2 enzymes associate with lipid membranes has long been a question in membrane protein enzymology. Many techniques have been applied to explore the interfacial surface, including some high resolution methods such as solution NMR and x-ray crystallography. Although these methods could provide structural information at an atomic level, the insolubility of the phospholipid substrate and its large aggregate form make it extremely difficult to study the interfacial surface. Moreover, some PLA_2 enzymes exhibit oligomeric and/or allosteric properties when they are associated with a substrate or phospholipid membranes in solution. Success in studying membrane association has been achieved only using protein lipid FRET (8) and electron paramagnetic resonance spectroscopy (9). Certain limitations restrict the type of enzymes that can be studied using these methods because paramagnetic resonance spectroscopy requires the insertion of non-wild type amino acids, and protein/membrane FRET does not allow for localization of changes within the enzyme upon membrane association. A newer method, hydrogen/deuterium exchange mass spectrometry (DXMS), has been used successfully to investigate the dynamics of proteins in solution, as well as protein/ligand and protein/protein interactions, despite the size of the proteins and the complexity of their binding partners (10–15). DXMS works by monitoring the exchange of a protein's backbone amide hydrogen atoms with deuterium atoms, provided by incubating the protein in D_2O. In recent years, DXMS has been used to study the structural dynamics and interactions of membrane proteins (16, 17) and membrane-associated proteins (18) such as various PLA_2s (19–24). Although DXMS provides only medium to semi-high resolution, when combined with methods like x-ray crystallography, fluorescence spectrometry, mutagenesis, and quantum mechanics/

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We dedicate this minireview to our longtime collaborator, the late Professor Virgil L. Woods, Jr., who contributed so much to the development of the DXMS technique over the years.

[S] This article contains supplemental material, Figs. S1 and S2, Equations 1–6, and additional references.

[1] To whom correspondence should be addressed. E-mail: edennis@ucsd.edu.

[2] The abbreviations used are: PLA_2, phospholipase A_2; $sPLA_2$, secreted PLA_2; $cPLA_2$, cytosolic PLA_2; $iPLA_2$, Ca^{2+}-independent PLA_2; PAF-AH, platelet-activating factor acetylhydrolase; DXMS, hydrogen/deuterium exchange mass spectrometry; H/D, hydrogen/deuterium; Lp-PLA_2, lipoprotein-associated PLA_2; G, Group; DMPC, dimyristoylphosphatidylcholine; MAFP, methyl arachidonyl fluorophosphonate; C2, Ca^{2+} binding domain.

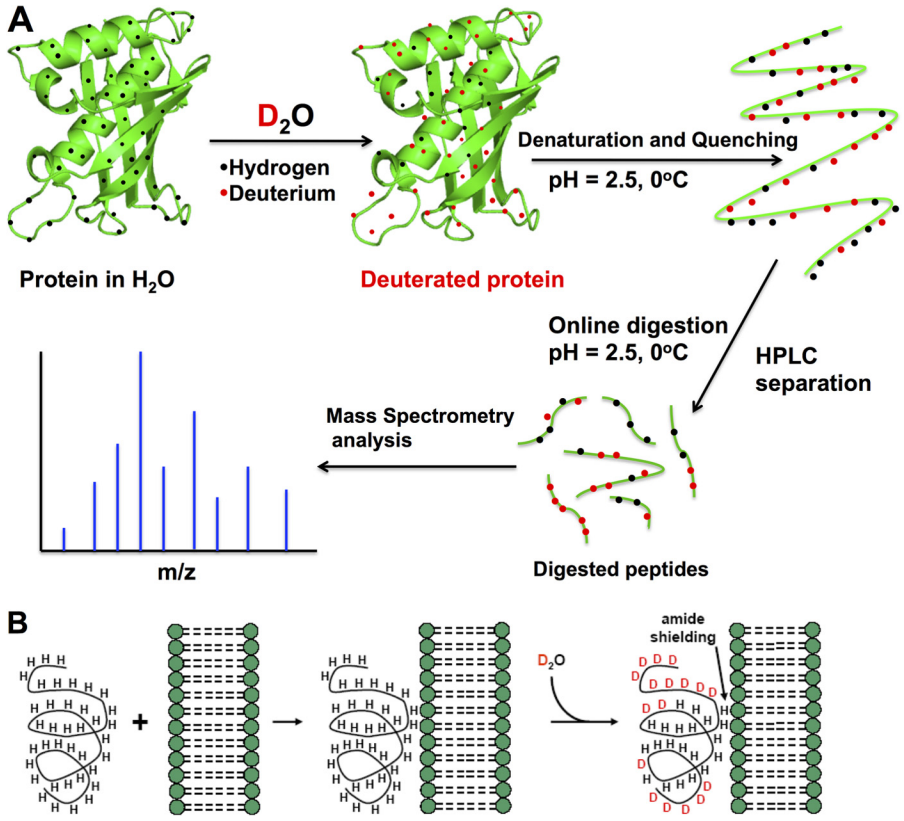

FIGURE 1. **Basis of H/D exchange.** *A*, the protein of interest is incubated with deuterated water and labeled via exchangeable hydrogens. The exchange is then quenched to lock the deuterium atoms in position by lowering the pH of the protein-containing solution (which also denatures the protein) and lowering the solution temperature. The protein is then loaded onto an HPLC column that contains an immobilized protease that digests the protein into peptide segments. The reversed-phase column also separates these peptide segments according to their hydrophobicity. The HPLC eluent is directly connected to the mass spectrometer, where the mass analysis is carried out (the peptide segments may be further fragmented in the mass spectrometer for analysis). *B*, PLA_2/lipid membrane interactions by DXMS. The peptide region of a protein associated with a membrane is less prone to H/D exchange (amide shielding), and this information can be used to infer this peptide region's interaction with a lipid membrane.

molecular mechanics, it can provide useful information on protein/membrane, protein/inhibitor, and protein/substrate interactions.

In this minireview, we focus on the relatively recent application of DXMS in investigating membrane-associated PLA_2 enzymes. We provide both a general description of how DXMS can be used to investigate protein/phospholipid membrane and protein/inhibitor interactions and examples of interfacial binding sites and/or interactions with specific inhibitors of specific PLA_2 enzymes that have been identified.

Hydrogen/Deuterium Exchange Mass Spectrometry

Hydrogen atoms in amino acid side chains and backbones are labile and are continuously exchanged with hydrogen atoms in the surrounding water. However, only the exchange rate of amide hydrogens can be conveniently monitored. The exchange rate of amide hydrogens in a protein varies greatly depending on their involvement in secondary structures as well as their access to the solvent. However, recent work has demonstrated that the involvement in hydrogen bonding networks, but not solvent accessibility, is the predominant factor in controlling hydrogen/deuterium (H/D) exchange rates (25). The H/D exchange rate of amide hydrogens is an acid-base catalyzed process with a calculated global minimum in the 2.5–4 pH range (26, 27), which is also highly dependent on temperature. When the protein is exposed to a D_2O environment, deuterons incorporated into the protein can be detected by mass spectrometry, which means that the rate of H/D exchange can be measured. A generalized scheme for a DXMS experiment is outlined in Fig. 1*A*.

For a typical PLA_2/membrane interaction experiment, DXMS experiments are carried out on the pure PLA_2 enzyme alone and on the PLA_2 enzyme in the presence of phospholipid membranes (lipid/protein ratio of 60 or higher) (Fig. 1*B*). It is important to note that H/D changes induced upon membrane binding can be caused by a variety of factors (oligomerization, allosteric conformational changes distant from the membrane-binding site, and direct membrane interactions). These H/D exchange differences must be interpreted in the context of previous biophysical and structural experiments. Detailed methodology is provided in the supplemental material, including typical data (supplemental Fig. S1), fragmentation schemes (supplemental Fig. S2), and a description of the kinetic parameters governing amide exchange. Further detail is provided in several recent excellent reviews (28–30).

Identifying the Interfacial Surfaces of PLA_2 Enzymes

The crucial first step for a PLA_2-catalyzed reaction is the association of the enzyme with membranes via its interfacial binding surface containing one or more "membrane interaction site(s)." Each PLA_2 enzyme also contains a unique "catalytic

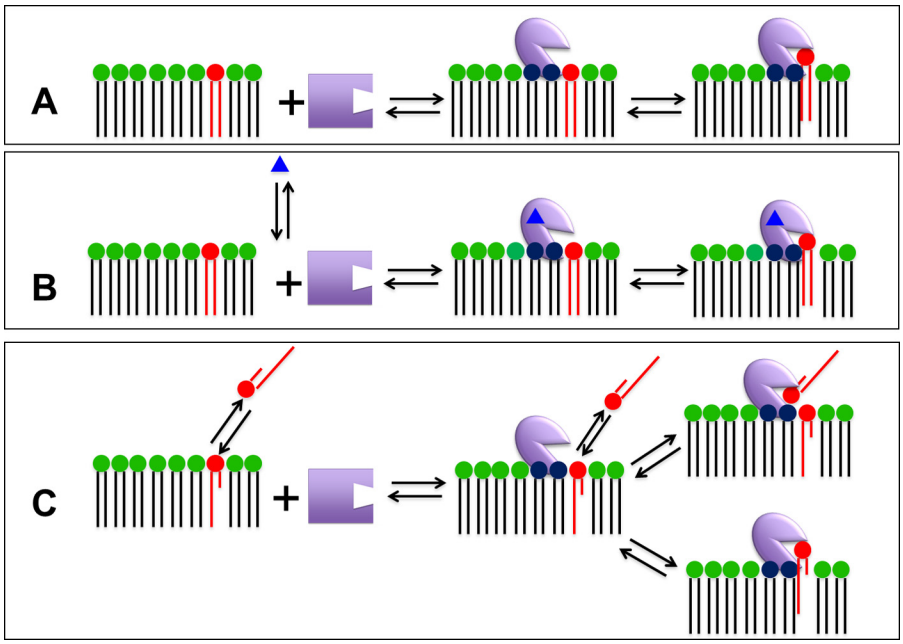

FIGURE 2. **Possible binding modes for PLA_2 via its membrane interaction site, another allosteric site, and its catalytic site.** *A*, the enzyme associates with the membrane interface via its membrane interaction site, which is distinct from its catalytic site. Once associated with the membrane, a phospholipid substrate molecule is bound by the catalytic site. *B*, regulated enzymes bind traditional small molecule activators or regulators, referred to as "allosteric ligands" (*blue triangles*), which are bound to an allosteric site on the enzyme to facilitate optimal interfacial binding via the membrane interaction site. *C*, for PLA_2s that act on water-soluble monomeric (or small aggregated) substrates, the enzyme associated with the interface through the membrane interaction site may, in principle, access a substrate phospholipid residing in either the aqueous or membrane phase.

site" that binds a substrate phospholipid for hydrolysis. Here, we consider membrane interaction sites to be typical allosteric sites such that when the PLA_2 enzyme is associated with ligand (in this case, the membrane), the enzyme exists in a different conformational state as described by Changeux and co-workers (31, 32) in terms of R-to-T transitions as illustrated in Fig. 2*A*. In addition to membrane interaction site(s), PLA_2s may contain one or more traditional "allosteric site(s)" that bind small molecules that regulate or activate the enzyme. We include the possibility that additional small molecule allosteric site(s) can affect the conformation of the membrane interaction sites(s) as illustrated in Fig. 2*B*. PLA_2s are further complicated by the fact that the substrate molecules are aggregated to compose the membrane (or micelle, lipoprotein, or other aggregated form). However, for some phospholipids, the *sn*-2 fatty acid chain is an acetate or oxidized, and these phospholipids have a critical micelle concentration such that the phospholipid substrate molecules may exist as monomers or small aggregates in aqueous solution under physiological conditions, so there is the possibility that the membrane-associated enzyme binds substrate in the aqueous solution rather than in the membrane surface as illustrated in Fig. 2*C*. Note that although allosteric enzymes have generally been associated with oligomerization and sigmoid kinetics, we do not distinguish here whether the active enzyme is monomeric or oligomeric or if association with membranes changes the oligomerization state of the enzyme, although these are distinct possibilities.

For example, $cPLA_2$ is generally considered to be a monomeric protein that binds membranes via more than one membrane interaction site and whose association and/or activity is regulated through one or more allosteric sites, including binding of Ca^{2+}, ceramide 1-phosphate, and/or phosphatidylinositol 4,5-bisphosphate (33, 34), as typified by Fig. 2*B*. For the $sPLA_2$s, if one considers the required Ca^{2+} as a regulator of membrane association or binding, then Ca^{2+} also constitutes an allosteric regulator, but in this case, the Ca^{2+} is located in the catalytic site, so it is hard to distinguish between the models depicted in Fig. 2 (*A* and *B*). $iPLA_2$, which is regulated to some extent by ATP and/or indirectly by Ca^{2+} (35), is also depicted in Fig. 2*B*. PAF-AH/lipoprotein-associated PLA_2 (Lp-PLA_2) is depicted in Fig. 2*C* with high critical micelle concentration substrates.

Although numerous PLA_2 crystal structures have been determined in the past decades, none of them have contained lipid substrates with normal fatty acyl chains or lipid membranes in the crystal lattice. DXMS is an ideal approach for studying PLA_2/substrate/lipid membrane interactions because DXMS results can be obtained in the presence of large lipid aggregates and monomeric lipid substrates. DXMS has been applied to the study of the PLA_2 superfamily of enzymes and has now been successfully used to identify the structural basis of four different types of PLA_2/membrane interaction sites.

Using DXMS to study membrane interactions does have inherent limitations, and many of the technical hurdles have been summarized in an excellent review (36). One of these limitations is that DXMS experiments do not directly measure perturbations caused solely by side chain interactions and allow only for the determination of dynamic changes that affect the rate of H/D exchange of amide hydrogens. Many changes that are dominated by side chain interactions may not be detected. However, side chain interactions may rigidify the secondary structure, causing decreased amide H/D exchange (37).

Also, the different H/D exchange rates in the presence or absence of lipid vesicles may not be caused by a direct lipid

interaction of the amides in that region, but by conformational changes induced by lipid binding or a combination. This includes both allosteric and oligomeric changes that may occur at the lipid surface. However, carefully controlled experiments on Group (G) IVA PLA$_2$ were able to discriminate conformational changes upon substrate binding *versus* the interfacial surface (23). Care should be taken when examining the differences upon lipid binding to determine the role of direct binding *versus* conformational changes/aggregation distant from the lipid-binding surface.

GIA sPLA$_2$

The interfacial enzymology of small, secreted, and highly disulfide-bonded sPLA$_2$s has been widely studied. Several kinetic models, including surface dilution, scooting mode, and quasi-scooting mode, have been developed to explain the interfacial mechanism of sPLA$_2$s (7, 38). Many sPLA$_2$ structures, either from NMR or x-ray crystallography, have become available in the past decades, and these structures have provided us with useful information for understanding the interfacial binding surfaces. Unfortunately, none of the structures were solved in the presence of a normal lipid substrate. However, DXMS has allowed for the study of the protein dynamics of GIA sPLA$_2$ in solution in the presence of divalent cations as well as lipid substrate (22). GIA sPLA$_2$ is a 13-kDa protein with seven disulfide bonds, four α-helices, and three β-sheets. Interestingly, two of the core helices barely exhibit H/D exchange, but the other two helices exchange very rapidly (22). Four disulfide bonds are involved in the two core helical regions, which exhibit extremely slow H/D exchange rates. These disulfide bonds keep these helical regions extremely rigid, preventing the small fluctuations in secondary structure required for H/D exchange.

GIA sPLA$_2$ requires Ca^{2+} to carry out its enzymatic activity. Structures of GIA PLA$_2$ have been solved with either two Ca^{2+} ions present (39) or only one present (40) The DXMS experiments showed decreased H/D exchange in the second Ca^{2+}-binding site upon exposure to either Ca^{2+} or Ba^{2+}. This finding supports previous results showing two Ca^{2+}-binding sites in the *Naja naja atra* GIA sPLA$_2$ structure (41). When using dimyristoylphosphatidylcholine (DMPC) lipid vesicles to mimic lipid bilayers in DXMS experiments, the membrane interaction site residues were identified. The H/D levels at amide hydrogens in regions containing Tyr-3, Trp-61, Tyr-63, Phe-64, and Lys-6 decreased dramatically in the presence of DMPC lipid vesicles. The aromatic residues on the surface are believed to insert into the lipid phase, which benefits the hydrophobic interaction between the protein and lipid acyl tails. Lys-6 should contribute to electrostatic interactions with the polar headgroup of a lipid membrane surface. Regions containing several other hydrophobic residues Phe-5, Ile-9, and Trp-19 also demonstrated a decreased H/D level. These residues may also be involved in the interfacial binding to mediate the hydrophobic interactions.

By mapping these decreases in the exchange rate onto the x-ray crystallographic structure, along with accounting for previous biochemical data, it was possible to generate a model for how this enzyme binds to the lipid surface (Fig. 3*A*). The aromatic amino acids, especially tryptophan, are thought to be the most potent contributors to interfacial binding. Indeed, tryptophan mutants have shown dramatically decreased binding affinity in human GV and GX sPLA$_2$s (42, 43). Electrostatic interactions were also shown to contribute to the interfacial binding of other sPLA$_2$s such as GIIA and GIB (44, 45). The decreases in H/D exchange are caused either by direct hydrogen bonding of the amide hydrogens to the lipid substrate or by side chain interactions rigidifying the secondary structure. Overall, the GIA sPLA$_2$ DXMS study is consistent with the results obtained by other methods and with other sPLA$_2$s but reveals more directly the lipid interface of the enzyme.

GIVA cPLA$_2$

GIVA cPLA$_2$ (21, 23) is a 85-kDa protein containing two domains linked by a flexible tether: a Ca^{2+}-binding domain (C2) and a catalytic domain (15). GIVA cPLA$_2$ has a distinct preference for substrates containing arachidonic acid at the *sn*-2 position (46). The crystal structure of GIVA cPLA$_2$ shows that it contains a lid region in the catalytic domain that could block the direct insertion of a substrate molecule in the active site. From this structure, it was postulated that binding to the lipid surface might induce a conformational shift in this lid region, allowing phospholipid substrates to access the active site (15). DXMS on this enzyme was focused on determining differences between a single lipid substrate binding in the active site *versus* a bulk lipid surface binding.

DXMS experiments showed an increase in H/D exchange in regions directly underneath the lid region of GIVA PLA$_2$ in the presence of both substrate vesicles and methyl arachidonyl fluorophosphonate (MAFP) alone. However, there was no further change in this region when comparing the MAFP-treated enzyme and the MAFP-treated enzyme plus vesicles. These experiments show that this conformational change can be caused by a single lipid molecule binding in the active site and does not require association with the lipid interface.

Comparing the DXMS data for the MAFP-treated enzyme with that for the MAFP-treated enzyme plus vesicles allowed the identification of regions that are responsible only for lipid surface binding. This allowed mapping of the interfacial binding surface of the enzyme, and by combining this work with previous biophysical studies (47–50) carried out on the C2 domain alone and full-length cPLA2 enzyme, this led to the creation of the model for membrane binding shown in Fig. 3*B*. Both the C2 and catalytic domains bind to phospholipid surfaces. Regions 35–39 and 96–98 on the C2 domain, which are very hydrophobic, can penetrate into the lipid phase. In contrast, regions 268–279 and 466–470 on the catalytic domain can mediate cPLA$_2$ interfacial binding through electrostatic interactions with phospholipid headgroups.

GVIA iPLA$_2$

The membrane interaction site of human GVIA-2 iPLA$_2\beta$ was also investigated using DXMS (19). GVIA-2 PLA$_2$ (iPLA$_2\beta$) contains seven ankyrin repeats and a catalytic domain connected by a linker region. There is no structural information available for this enzyme. The Protein Data Bank (PDB) contains only two homolog structures: human ankyrin-R, with 51% similarity to iPLA$_2$ ankyrin repeat residues 88–474, and the

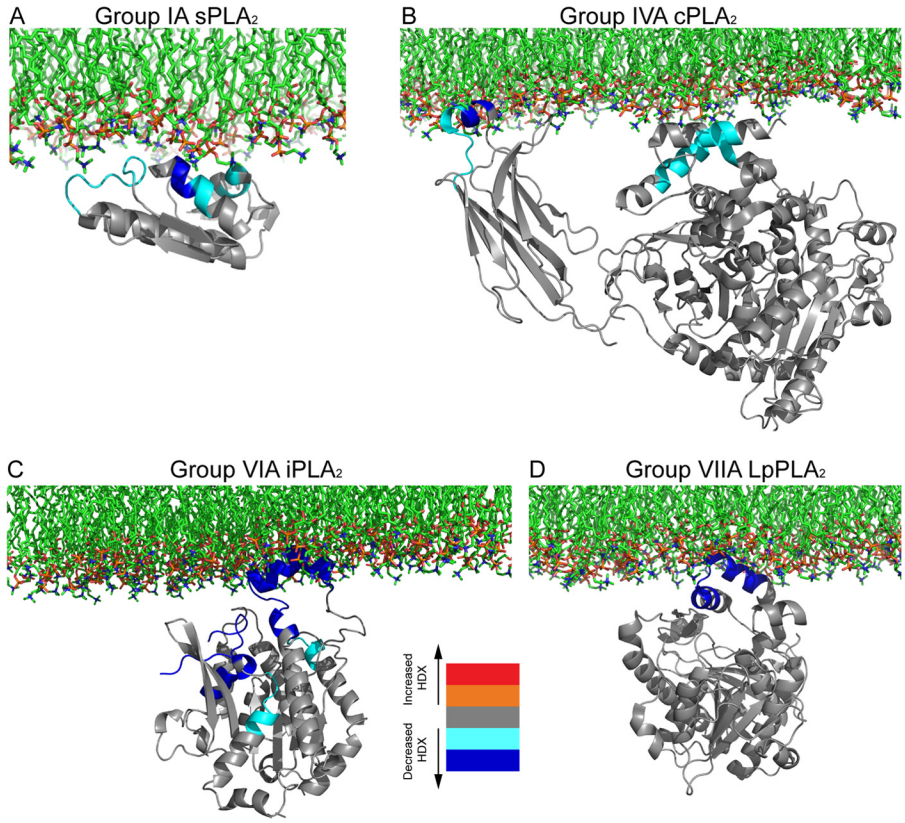

FIGURE 3. **Proposed schematic models of the interfacial binding surface of four different members of the PLA$_2$ superfamily.** Differences in H/D exchange (*HDX*) combined with biophysical experiments were employed to generate models of the monomers of the different PLA$_2$ enzymes bound to membranes. *A*, GIA sPLA$_2$ (Protein Data Bank code 1PSH), adapted from Ref. 22. *B*, GIVA cPLA$_2$ (code 1CJY), adapted from Ref. 23. *C*, GVIA iPLA$_2$ (homology model based on patatin crystal structure 1OXW), adapted from Ref. 19. *D*, GVIIA PAF-AH/Lp-PLA$_2$ (code 3D5E), adapted from Ref. 24. Note that the images of structures are not to the same scale.

lipase patatin, with 40% sequence similarity to iPLA$_2$ catalytic domain residues 475–806. Based on this structural data, two homology models were developed, one for the iPLA$_2$ ankyrin repeats and one for the catalytic domain (19). DXMS proved to be an effective approach to validate these homology models by allowing examination of the correlation between the H/D exchange rates and the predicted structure (51). GVIA-2 iPLA$_2$ H/D data provided a good fit with the homology model (19). This homology model also showed a high correlation when validated by a recently developed program, DXCOREX, by which H/D exchange results were used to quantitatively assess the accuracy of tertiary protein structure models (52). The membrane interaction site was determined in an iPLA$_2$/1-palmitoyl-2-arachidonoyl-*sn*-phosphatidylcholine lipid vesicle DXMS experiment in the presence of the inhibitor MAFP. The iPLA$_2$/MAFP DXMS experiment was also performed to exclude the possibility that any H/D changes in the membrane interaction site residue(s) were induced by the MAFP inhibitor. H/D results indicated that only the catalytic domain residues interacted with the lipid membrane and that ankyrin repeat regions were not involved in the iPLA$_2$/lipid association. Specifically, the hydrophobic region in the catalytic domain, including residues 708–730, was most likely to penetrate the membrane surface, and other regions, including residues 631–655, 658–664, and 773–778, were most likely to assist the iPLA$_2$/membrane binding by electrostatic interactions with the charged headgroup of the phospholipids (19). These effects were mapped onto the catalytic domain homology model as shown in Fig. 3*C*. The catalytic residue Ser-519 was underneath the membrane penetration region 708–730, and the active site was relatively open to the solvent, which may explain why iPLA$_2$ does not discriminate between substrates containing differing *sn*-2 acyl chains.

GVIIA PAF-AH PLA$_2$

Although most PLA$_2$ enzymes must first bind to the membrane and extract substrate phospholipid from the lipid phase, another type of PLA$_2$ enzyme, PAF-AH, seems to utilize a different mechanism because the substrate PAF and PAF analogues are partially water-soluble as monomers or small aggregates. PAF-AH might bind the interface only through its membrane interaction site and take substrate from the aqueous phase, but it is not necessarily activated in the interface (Fig. 2*C*) (53). GVIIA PLA$_2$ is a plasma PAF-AH, also known as Lp-PLA$_2$ because it is found associated mainly with LDL and HDL in human plasma (54) and hydrolyzes oxidized phospholipids (55, 56).

Considering that both LDL and HDL have >30% surface monolayer phospholipids, it is reasonable that GVIIA PLA$_2$ will associate with the lipid surface when it associates with lipoproteins and could bind substrate molecules from either the aqueous or lipid phase. Because GVIIA PLA$_2$ has been shown to bind DMPC lipid vesicles tightly (53), DMPC lipid vesicles were used in GVIIA PLA$_2$/membrane binding DXMS studies. Only one region in the enzyme, residues 113–120, demon-

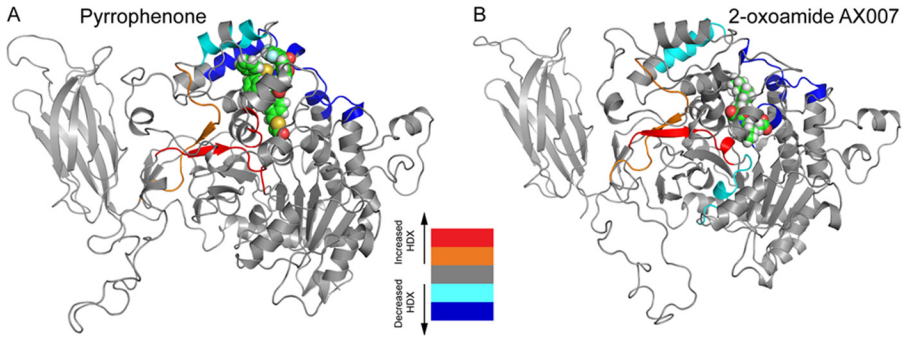

FIGURE 4. **Differences in deuterium exchange rates for GIVA cPLA$_2$ upon binding to two lipophilic inhibitors.** Time-dependent H/D exchange rates (*HDX*) in the presence and absence of the inhibitors pyrrophenone (*A*) and 2-oxoamide AX007 (*B*) are shown mapped onto the resulting molecular dynamics simulation of the structural model of GIVA cPLA$_2$ (Protein Data Bank code 1CJY). The decreases/increases in the exchange rates are color-coded as shown in the *legend* (from Ref. 20).

strated decreases in H/D exchange in the presence of DMPC lipid vesicles (24). Residues 113–120 are part of one surface of the α-helix, which is believed to be important for GVIIA PLA$_2$/LDL association (57). This helix contains numerous hydrophobic residues, and within this helix, Trp-115 and Leu-116 are considered the most hydrophobic residues. When Trp-115 and Leu-116 are replaced with alanine residues, the mutant protein loses its membrane binding capacity, as detected by both fluorescence assay and DXMS (24). This result indicates that the GVIIA PLA$_2$/lipid membrane interaction is due predominantly to hydrophobic interactions and that Trp-115 and Leu-116 are the major contributors. The proposed model is shown in Fig. 3*D*. Although GVIIA PLA$_2$ may not be interfacially activated, it binds to the lipid membrane using a surface helix binding, which leaves its active site open to solvent and, in principle, could also allow access to substrates such as oxidized PC from the lipid phase. It is also important to note that regions that cannot be crystallized or are disordered using x-ray crystallography were all detected by DXMS (24, 58) and showed very rapid H/D exchange rates, indicating the structural flexibility of the residues within these regions. DXMS has also been used to provide some useful information on the structural basis of Lp-PLA$_2$/HDL interactions (59).

DXMS Combined with Molecular Dynamics: Example of GIVA cPLA$_2$ Inhibitors

Many inhibitors of GIVA cPLA$_2$ have been developed, and although some of the inhibitors showed positive effects both *in vitro* and *in vivo*, none of them is currently in an advanced clinical trial due to side effects or bioavailability (1, 60). Understanding the location of inhibitor binding, as well as induced allosteric change, is important to design the next generation of inhibitors for therapeutic purposes. Membrane proteins, including the PLA$_2$s, are inhibited by extremely lipophilic compounds, which can cause numerous solubility issues, making the use of high resolution methods such as x-ray extremely challenging. DXMS has been used alongside site-directed mutagenesis to locate the exact binding site of certain inhibitors (61, 62). The ability to locate the binding sites of inhibitors that cannot be co-crystallized with a protein of interest is an important advantage of the DXMS method.

Two representative inhibitors from markedly different classes of molecules, pyrrophenone and a 2-oxoamide-derived inhibitor (AX007), were selected for GIVA cPLA$_2$/inhibitor DXMS experiments (20). In the presence of pyrrophenone, DXMS in three regions of GIVA cPLA$_2$ showed increased on-exchange rates (Fig. 4*A*). In the same regions, increased H/D exchange rates were also found in GIVA cPLA$_2$/lipid substrate binding DXMS experiments, which is caused by the opening of the lid region upon substrate binding to the active site (23). It seems that inhibitor pyrrophenone binding also induced the same lid opening and increased the solvent accessibility in these regions. Five other regions demonstrated decreased H/D exchange rates in the presence of pyrrophenone. Molecular dynamics studies have confirmed that residues in these regions can form a hydrophobic pocket to accommodate the inhibitor. The combination of results from DXMS, molecular docking, and molecular dynamics has resulted in a model that elucidates the molecular basis of the pyrrophenone inhibition mechanism (20).

The 2-oxoamide-derived inhibitor AX007 shared some binding sites with pyrrophenone, but not others. Molecular dynamics results indicated that both inhibitors have similar contacts in the shared regions (20). Not surprisingly, DXMS also exhibited some differences in the presence of AX007. Molecular dynamics indicated that interactions between Arg-200 and the carboxylic acid of the oxoamide and between the carbonyl of the 2-oxoamide and the oxyanion hole stabilize protein/inhibitor binding. Overall, the results suggest that the oxoamide inhibitor AX007 binds mainly to the active site, whereas pyrrophenone connects with the cap region near the interfacial binding surface.

Conclusions

DXMS has been an important tool for studying protein dynamics, conformation, and solvent accessibility. Recent advances in resolution, automation, and mass analysis capabilities have made this technique even more powerful and accessible to the scientific community. DXMS has been used to successfully define the interfacial binding surfaces of the four main types of PLA$_2$s. Another type of PLA$_2$ enzyme, adipose PLA, which has a transmembrane domain, undoubtedly interacts with membranes in still another unique way (63). Although the PLA$_2$ superfamily has common catalytic activity due to convergent evolution, different structures and catalytic residues (including His-Asp, Ser-Asp, Ser-His-Asp, and Cys-His combi-

nations) are engaged in distinct biological functions in the organism. Data obtained using DXMS are consistent and complementary to the data obtained with other biophysical and biochemical techniques. DXMS has also been shown to be an excellent method for identifying PLA_2/inhibitor interaction mechanisms and is helpful in building a precise inhibitor-binding model.

DXMS could also be a powerful tool for studying other peripheral and integral membrane protein interactions with membranes and inhibitors and for elucidating conformational changes. However, no method is perfect. Little information can be obtained if the region of interest is located in extremely protected or disordered regions because it is extremely difficult to see H/D differences between very slow or very fast exchange rates. It is also hard to understand changes in the exchange rates without the availability of a three-dimensional structure. However, homology models coupled with DXMS data and computational modeling may be very useful to allow interpretation of H/D dynamic information (52). DXMS has to be used as a tool alongside other methods such as x-ray, NMR, mutagenesis, fluorescence, and molecular modeling. Together, these techniques allow for a much greater understanding of the dynamic nature of the proteins being studied.

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