Title
Deuterium exchange mass spectrometry studies of the phospholipase A₂ superfamily

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Publication Date
2008

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Deuterium Exchange Mass Spectrometry Studies of the Phospholipase A₂ Superfamily

A Dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Chemistry

by

John Edmund Burke

Committee in Charge:

Professor Edward A. Dennis, Chair
Professor Michael Burkart
Professor Alexander Hoffmann
Professor Yitzhak Tor
Professor Virgil Woods, Jr.

2008
The Dissertation of John Edmund Burke is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego
2008
For my family and friends who have always been supportive on this long crazy ride.
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<tr>
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<th>Full Form</th>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>BEL</td>
<td>Bromo-enol lactone</td>
</tr>
<tr>
<td>C1P</td>
<td>Ceramide 1 phosphate</td>
</tr>
<tr>
<td>CamKII</td>
<td>Calmodulin kinase II</td>
</tr>
<tr>
<td>CID</td>
<td>Collision induced dissociation</td>
</tr>
<tr>
<td>cPLA2</td>
<td>Group IVA cytosolic PLA2</td>
</tr>
<tr>
<td>DMPC</td>
<td>Dimyristoyl phosphatidylcholine</td>
</tr>
<tr>
<td>DPPC</td>
<td>Dipalmitoyl phosphatidylcholine</td>
</tr>
<tr>
<td>DXMS</td>
<td>Deuterium exchange mass spectrometry</td>
</tr>
<tr>
<td>ECD</td>
<td>Electron capture dissociation</td>
</tr>
<tr>
<td>GdHCl</td>
<td>Guanidine Hydrochloride</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>iPLA2</td>
<td>Group VIA Ca(^{2+}) independent PLA2</td>
</tr>
<tr>
<td>LA</td>
<td>Linoleic acid</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>Lp PLA2</td>
<td>Lipoprotein associated Phospholipase A2</td>
</tr>
<tr>
<td>LUV</td>
<td>Large unilamellar vesicle</td>
</tr>
<tr>
<td>MAFP</td>
<td>Methyl arachidonyl flouro phosphonate</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>MD</td>
<td>Molecular dynamics</td>
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ix
<table>
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<tr>
<th>Acronym</th>
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<tr>
<td>MNK1</td>
<td>Mitogen activated protein kinase interacting kinase</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOE</td>
<td>Nuclear overhause effect</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PAPC</td>
<td>Palmitoyl arachidonyl phosphatidylcholine</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidyl choline</td>
</tr>
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<td>PE</td>
<td>Phosphatidyl ethanolamine</td>
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<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
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<td>PLA2</td>
<td>Phospholipase A2</td>
</tr>
<tr>
<td>PME</td>
<td>Particle mesh ewald</td>
</tr>
<tr>
<td>RMSD</td>
<td>Root mean square distance</td>
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<tr>
<td>SUV</td>
<td>Small unilamellar vesicle</td>
</tr>
<tr>
<td>sPLA2</td>
<td>Secreted PLA2</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris-carboxy ethyl phosphine</td>
</tr>
<tr>
<td>TFA</td>
<td>Tri-flouro acetic acid</td>
</tr>
<tr>
<td>VMD</td>
<td>Visual molecular dynamics</td>
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To my mom and dad and sister, who have supported me in all my endeavors. Being the son of two professors has really taught me to love academic science. (Of course there were a few rebellions). I cannot thank them enough for getting me into science, and always respecting me decision to choose my own path, ie wander through 7 different majors in undergard. Also I have to thank my girlfriend Carolyn who has stood by me the last two and a half years as I have finished my thesis. I finally have to start washing my dishes now that I no longer have the PhD excuse.
Last, but not least, thank you Ed for giving me all these opportunities and experiences. You demonstrated an incredible amount of support and patience, and always believed my projects would work. I hope someday I can be that kind of mentor for my own students.
# CURRICULUM VITA

## EDUCATION

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<td></td>
<td>BA in Molecular and Cellular Biology</td>
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## RESEARCH EXPERIENCE

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<tbody>
<tr>
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<td>Graduate Research Assistant</td>
<td>Edward A. Dennis</td>
<td>2004 – Present</td>
<td>Developed deuterium exchange methodology for both the Group IA and IVA phospholipase A2 enzymes. Characterized the lipid binding surface of each of these enzymes. Also determined structural changes upon binding various inhibitors of the GIVA enzyme. Developed skills in protein expression, purification, molecular biology, enzymology, and mass spectrometry. Also assisted in the preparation of a R01 grant as well as published two review articles on the phospholipase A2.</td>
</tr>
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<td>University of California, Berkeley</td>
<td>Undergraduate Research Assistant</td>
<td>Paul Bartlett</td>
<td>2001 – 2002</td>
<td>Synthesized unnatural amino acids that would induce beta sheet structure in a peptide.</td>
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## TEACHING EXPERIENCE

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<tr>
<td>University of California, San Diego – La Jolla, CA</td>
<td>Teaching Assistant, Department of Chemistry and Biochemistry</td>
<td>2003 – 2004</td>
<td>Organic Chemistry lab- (2 quarters), Biochemistry III (1 quarter), The Periodic Table (1 quarter), All 4 quarters received awards for excellence in teaching</td>
</tr>
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<td>Master TA for the chemistry department (3 quarters) 2007-2008</td>
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PUBLICATIONS


POSTER PRESENTATIONS


ORAL PRESENTATIONS

ABSTRACT OF THE DISSERTATION

Deuterium Exchange Mass Spectrometry Studies of the Phospholipase A₂ Superfamily

by

John Edmund Burke

Doctor of Philosophy in Chemistry

University of California, San Diego, 2008

Professor Edward A. Dennis, Chair
The phospholipase A₂ (PLA₂) superfamily consists of many different groups of enzymes that catalyze the hydrolysis of the sn-2 ester bond in a variety of different phospholipids. The products of this reaction, a free fatty acid, and lysophospholipid have many different important physiological roles. This reaction occurs on a two dimensional lipid surface. The mechanism of this reaction has important consequences for not only phospholipid hydrolysis, but all of lipid enzymology. To better understand the mechanism of different phospholipase A₂ enzymes we have employed deuterium exchange mass spectrometry (DXMS). This technique has the ability to determine solvent accessibility and conformational changes not seen with x-ray crystallography or other structural techniques. This methodology has proven particularly useful to determine lipid binding sites that would be impossible to characterize with standard NMR or x-ray methodologies.

This thesis first discusses the PLA₂ superfamily of enzymes and the different roles they have been shown to play in inflammatory diseases, as well as the deuterium exchange mass spectrometry methodology. With this background we were able to study the GIA cobra venom PLA₂ which has acted as a model lipid enzyme for over a hundred years, and determine the effects of binding both metal ions and phospholipid substrate. We also examined the GIVA human cPLA₂, which has been implicated in numerous diseases, and determined inter-domain contacts not seen in x-ray crystallography, as well as examining metal binding and phospholipid surface binding. The movement of the lid region was discovered using DXMS methods, that has until now only been a hypothesis. Finally we studied the binding of different inhibitors and used a combination of molecular modeling and DXMS towards the design of better GIVA PLA₂ inhibitors.
CHAPTER 1

Phospholipase A₂ Biochemistry, Structure/Function, Mechanism and Signaling
1.A Abstract

The phospholipase A₂ (PLA₂) superfamily consists of many different groups of enzymes that catalyze the hydrolysis of the sn-2 ester bond in a variety of different phospholipids. The products of this reaction, a free fatty acid, and lysophospholipid have many different important physiological roles. There are five main types of PLA₂: the secreted sPLA₂’s, the cytosolic cPLA₂’s, the Ca²⁺ independent iPLA₂’s, the PAF acetylhydrolases, and the lysosomal PLA₂’s. This chapter focuses on the superfamily of PLA₂ enzymes, and then uses three specific examples of these enzymes to examine the differing biochemistry of the three main types of these enzymes. These three examples are the GIA cobra venom PLA₂, the GIVA cytosolic cPLA₂, and the GVIA Ca²⁺-independent iPLA₂. We also review herein our current understanding of the structure and interaction with substrate phospholipid which resides in membranes for a representative of each of these main types of PLA₂. We will also briefly review the development of inhibitors of these enzymes and their roles in lipid signaling.

1.B Introduction

The last twenty-five years has witnessed a virtual explosion in our knowledge about the superfamily of phospholipase A₂ (PLA₂) enzymes. PLA₂ hydrolyzes the fatty acid from the sn-2 position of membrane phospholipids as shown in Fig 1-1. In vivo, the sn-2 position of phospholipids frequently contains polyunsaturated fatty acids and when released, these can be metabolized to form various eicosanoids and related bioactive lipid mediators (1).
Figure 1-1 Phospholipase A2 Reaction Mechanism. Reaction catalyzed by the PLA2 superfamily of enzymes. Phospholipid on the left is hydrolyzed at the sn-2 position to yield lysophospholipid and free fatty acid on the right.

From the end of the nineteenth and beginning of the twentieth century (2), PLA2 was known to be a major component of snake venoms and it was later recognized that PLA2 from old world snakes (Group I) differed in their disulfide bond pattern from new world snakes (Group II). Later it was discovered that the major mammalian digestive enzyme, pancreatic PLA2 was more similar to that from the old world snakes such as the Indian cobra (Group IA) and hence the pancreatic enzyme was named Group IB. With the isolation, sequencing, and cloning of the PLA2 from human synovial fluid in 1988 (Group IIA) (3, 4) which had a disulfide bond pattern more similar to the new world rattlesnakes (Group II), the more complicated PLA2 from bee venom (Group III) (5), and
in 1991 the human cytosolic calcium-dependent PLA2 from macrophages (Group IVA) (6, 7), the need for a more elaborate "Group Numbering System" became obvious (8). As the discovery of additional PLA2’s continued such as the macrophage secreted Group V PLA2 (9, 10) and the calcium-independent PLA2 (Group VI) (11), this system was expanded with fourteen distinct Groups and many subgroups appearing by 2000 (12). The latest review (13) lists fifteen distinct Groups of PLA2. They cluster in four main categories or types: secreted sPLA2’s, cytosolic cPLA2’s, calcium-independent iPLA2’s, and platelet activating factor (PAF) acetyl hydrolase/oxidized lipid lipoprotein associated (Lp) PLA2’s. Each of these types has been implicated in diverse kinds of lipid metabolism and disease progression so there has been a tremendous interest in the pharmaceutical and biotechnology industry in developing selective and potent inhibitors of each of these types.

1. C. The PLA2 Superfamily of Enzymes
1. C. 1 Secreted PLA2 Enzymes

PLA2 activity was first studied in phenomenological detail as early as the 1890’s using “poison” or venom from cobras (2, 14). The group numbering system was originally used to distinguish between different snake venoms, with the first use of the group numbering system seen in 1977 with Group I/II to distinguish between venoms from rattlesnakes and vipers from cobra and kraits (15), based on differences in disulfide bonding patterns. The secreted PLA2s are characterized by their requirement for histidine in the active site, low molecular weight, Ca\(^{2+}\) requirement for catalysis, and the presence of six conserved disulfide bonds, with one or two variable additional disulfide bonds (12,
13) The mechanism of action/structure of the GIA PLA₂ will follow in the following section, as well as mechanistic differences between the GIA sPLA₂ and other group members. The first non-venom PLA₂ named GIB was isolated from the pancreatic juices of cows, and was also found in many other animals (16). There is strong evidence that this enzyme plays a major role in the digestion of phospholipids in the stomach (17, 18). This was followed by the isolation of many other mammalian and other forms of secreted PLA₂'s shown in Table 1. The name sPLA₂ was coined from the high content of GIIA PLA₂ in the synovial fluid of patients with rheumatoid arthritis (4), but has come to stand for secreted.

<table>
<thead>
<tr>
<th>Group</th>
<th>Source</th>
<th>Molecular mass (kDa)</th>
<th>Disulfide bonds</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>Cobras and Kraits</td>
<td>13–15</td>
<td>7</td>
</tr>
<tr>
<td>IB</td>
<td>Human/porcine pancreas</td>
<td>13–15</td>
<td>7</td>
</tr>
<tr>
<td>IIA</td>
<td>Rattlesnake; human synovial</td>
<td>13–15</td>
<td>7</td>
</tr>
<tr>
<td>IIB</td>
<td>Gaboon viper</td>
<td>13–15</td>
<td>6</td>
</tr>
<tr>
<td>IIC</td>
<td>Rat/murine testis</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>IID</td>
<td>Human/murine pancreas/spleen</td>
<td>14–15</td>
<td>7</td>
</tr>
<tr>
<td>IIE</td>
<td>Human/murine brain/heart/uterus</td>
<td>14–15</td>
<td>7</td>
</tr>
<tr>
<td>IIF</td>
<td>Human/murine testis/embryo</td>
<td>16–17</td>
<td>6</td>
</tr>
<tr>
<td>III</td>
<td>Human/murine/lizard/bee</td>
<td>15–18</td>
<td>8</td>
</tr>
<tr>
<td>V</td>
<td>Human/murine heart/lung/macrophage</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>IX</td>
<td>Snail venom (conodipine-M)</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>X</td>
<td>Human spleen/thymus/lymphocyte</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>XIA</td>
<td>Green rice shoots (PLA₂-I)</td>
<td>12.4</td>
<td>6</td>
</tr>
<tr>
<td>XIB</td>
<td>Green rice shoots (PLA₂-II)</td>
<td>12.9</td>
<td>6</td>
</tr>
<tr>
<td>XII</td>
<td>Human/murine</td>
<td>19</td>
<td>7</td>
</tr>
<tr>
<td>XIII</td>
<td>Parovirus</td>
<td>&lt;10</td>
<td>0</td>
</tr>
<tr>
<td>XIV</td>
<td>Symbiotic fungus/bacteria</td>
<td>13–19</td>
<td>2</td>
</tr>
</tbody>
</table>

All of the sPLA₂ enzymes (except group III) (19) display a characteristic increase in activity when substrate is switched from monomeric to higher ordered lipid aggregates,
and this is known as interfacial activation (20). The mechanism of interfacial activation will be considered in the next section on the GIA PLA₂. Experiments conducted with the Group III bee venom have used electrostatic potential-modulated spin relaxation magnetic resonance to determine how that enzyme binds the lipid surface (21). The secreted enzymes show similar activity to phospholipids with different fatty acids in the sn-2 position (22).

The primary role of the mammalian secreted PLA₂ enzymes in eicosanoid signaling remains unclear and has been recently reviewed (23). The most well understood function of a mammalian sPLA₂ is Group IIA which has been shown to be a potent anti microbial agent. Many different studies have examined the role the secreted PLA₂’s play in eicosanoid release and these studies have been inconclusive. They show that the up regulation of Groups IIA, V, and X caused a cytosolic GIVA PLA₂ dependent increase in eicosanoids. However a specific inhibitor of the Group IIA inhibitor has been developed by Lily (24), with clinical trials of its efficacy against arthritis and allergens showing no therapeutic effects (23). The pro-inflammatory role of the secreted PLA₂ has been suggested to possibly be controlled by a protein binding event not dependent on PLA₂ activity. Receptors present in mouse tissues named the M-type receptors have been shown to bind different secreted phospholipases, but no M-type receptor in humans has been found that binds PLA₂ (25). Recent work however has shown that Group IIA PLA₂ binds to integrins, and this raises the interesting possibility that integrin-PLA₂ contacts may mediate pro-inflammatory activity (26).
1.C.2 Cytosolic PLA₂’s

The cytosolic PLA₂’s are larger than the sPLA₂ enzymes (61-114 kDa) and do not have the same disulfide bonding network as the sPLA₂ enzymes. The first cytosolic PLA₂ was isolated from neutrophils and platelets in 1986. The complete list of these enzymes is shown in Table 1-2. The detailed mechanism and biology of the GIVA PLA₂ will be explained in detail in Section 1.E. These enzymes all function through the action of a serine/aspartic acid dyad. All of the cytosolic PLA₂’s (except GIVC (27)) require Ca²⁺ for activity, due to the presence of C2 domains (28-31). The different GIV enzymes have different specificity for fatty acids in the sn-2 position. GIVA is specific for AA containing phospholipids (6), GIVB and GIVC have very little specificity (29, 30), GIVD appears to be specific for linoleic acid (LA) containing fatty acids (31), while GIVE and GIVF hydrolyze both AA and LA (28). For a more in-depth analysis of the biology of the other cytosolic enzymes see reviews (13, 32).

Table 1-2 Cytosolic Group IV PLA₂. Adapted from (12, 13)

<table>
<thead>
<tr>
<th>Group</th>
<th>Source</th>
<th>Molecular mass (kDa)</th>
<th>Features</th>
<th>Alternate names</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVA</td>
<td>Human/murine</td>
<td>85</td>
<td>C2 domain</td>
<td>cPLA₂α</td>
</tr>
<tr>
<td>IVB</td>
<td>Human</td>
<td>114</td>
<td>C2 domain</td>
<td>cPLA₂β</td>
</tr>
<tr>
<td>IVC</td>
<td>Human</td>
<td>61</td>
<td>acylated</td>
<td>cPLA₂γ</td>
</tr>
<tr>
<td>IVD</td>
<td>Human/murine</td>
<td>92–93</td>
<td>C2 domain</td>
<td>cPLA₂δ</td>
</tr>
<tr>
<td>IVE</td>
<td>Murine</td>
<td>100</td>
<td>C2 domain</td>
<td>cPLA₂ε</td>
</tr>
<tr>
<td>IVF</td>
<td>Murine</td>
<td>96</td>
<td>C2 domain</td>
<td>cPLA₂ζ</td>
</tr>
</tbody>
</table>

1.C.3 Ca²⁺ Independent PLA₂’s

The Ca²⁺ independent PLA₂ (iPLA₂s) includes six different types GVIA, GVIB, GVIC, GVID, GVIE, and GVIF PLA₂ as shown in Table 1-3. The term Ca²⁺ independent PLA₂ is misleading for the Group VIA enzymes. All of the enzymes in this group do not
require Ca\(^{2+}\) for activity, but the GIVC enzyme also does not require Ca\(^{2+}\) for activity, but was placed in the Group IV of PLA\(_2\) enzymes due to homology to other GIV enzymes (27). All of these enzymes function through a catalytic serine at the active site. A more in depth discussion of the mechanism of GVIA will be presented in section 1.F. There is no fatty acid chain specificity seen in any of the GVI enzymes.

The role of the GVIA PLA\(_2\) in different signaling pathways has been shown to be very complex. Initial reports of the functions of the GVIA PLA\(_2\) were determined using the inhibitor bromoenollactone (BEL) (33). Recent work has shown that this inhibitor is not specific for GVIA PLA\(_2\) and actually functions through activation of the inhibitor by GVIA PLA\(_2\) followed by non specific covalent modification of cysteine residues in all proximally located enzymes (34). Therefore it has been hard to evaluate early experiments using this inhibitor to determine the function of the GVIA PLA\(_2\). Experiments using the inhibitor BEL are reviewed elsewhere (35). Two major factors have allowed the determination of GVI PLA\(_2\)’s cellular functions. First the recent generation of GVIA PLA\(_2\) deficient mice has shown the importance of this enzyme in bone formation, apoptosis, insulin secretion, and sperm development (36-39). Secondly the recent development of specific fluoroketone inhibitors of GVIA PLA\(_2\) (40) have shown in mouse models that the GVIA PLA\(_2\) in combination with the GIVA PLA\(_2\) play an important role in Wallerian degeneration and axon regeneration in nerve injury (41). For reviews of the other GVI enzymes see (13).
### Table 1-3 GVI PLA2

<table>
<thead>
<tr>
<th>Group</th>
<th>Source</th>
<th>Molecular mass (kDa)</th>
<th>Features</th>
<th>Alternate names</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIA-1</td>
<td>Human/murine</td>
<td>84–85</td>
<td>8 ankyrin repeats</td>
<td>iPLA2</td>
</tr>
<tr>
<td>VIA-2</td>
<td>Human/murine</td>
<td>88–90</td>
<td>7 ankyrin repeats</td>
<td>iPLA3β</td>
</tr>
<tr>
<td>VIB</td>
<td>Human/murine</td>
<td>88–91</td>
<td>Membrane-bound</td>
<td>iPLAγ</td>
</tr>
<tr>
<td>VIC</td>
<td>Human/murine</td>
<td>146</td>
<td>Integral membrane protein</td>
<td>iPLA2δ, neuropathy target esterase (NTE)</td>
</tr>
<tr>
<td>VID</td>
<td>Human</td>
<td>53</td>
<td>Acylglycerol transacylase, triacylglycerol lipase</td>
<td>iPLA2ε, adiponutrin</td>
</tr>
<tr>
<td>VIE</td>
<td>Human</td>
<td>57</td>
<td>Acylglycerol transacylase, triacylglycerol lipase</td>
<td>iPLA2ζ, TTS-2.2</td>
</tr>
<tr>
<td>VIF</td>
<td>Human</td>
<td>28</td>
<td>Acylglycerol transacylase, triacylglycerol lipase</td>
<td>iPLA2θ, GS2</td>
</tr>
</tbody>
</table>

**1.C.4 PAF acetylhydrolases**

The PAF acetylhydrolases are composed of two PLA2 groups that both hydrolyze the acetyl group from the sn-2 position of platelet activating factor (PAF) as shown in Table 1-4. The function of this class of enzymes is of high interest due to the important roles played by PAF in the body. All of these enzymes function through the action of a catalytic serine. The PAF acetylhydrolases all have a Ser/His/Asp catalytic triad mediating hydrolysis (42, 43). These enzymes do not require Ca^{2+} for activity. The GVIIA PLA2 is a secreted enzyme that can also hydrolyze short chain fatty acids from the sn-2 position (44). This enzyme is not interfacially activated (44). This enzyme is also known as the lipoprotein associated PLA2. Recent work has identified regions in the catalytic domain important for binding to both HDL and LDL cholesterol molecules (45). The mechanisms that impart preferences for HDL and LDL are currently poorly understood.
understood. The GVIII PAF acetylhydrolases are also regulated through aggregation of
the catalytic and regulatory subunits (46).

**Table 1-4 GVII and GVIII PLA2.** Adapted from (12, 13)

<table>
<thead>
<tr>
<th>Group</th>
<th>Source</th>
<th>Molecular mass(kDa)</th>
<th>Features</th>
<th>Alternate names</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIIB</td>
<td>Human, murine, porcine, bovine</td>
<td>40</td>
<td>Intracellular, myristoylated, α/β hydrolase</td>
<td>PAF-AH II</td>
</tr>
<tr>
<td>VIIIA</td>
<td>Human</td>
<td>26</td>
<td>Intracellular, Ser/His/Asp triad, homodimer or heterodimer with GVIIIB associates with regulatory β-subunit</td>
<td>PAF-AH 1b (α1 subunit)</td>
</tr>
<tr>
<td>VIIIB</td>
<td>Human</td>
<td>26</td>
<td>Intracellular, Ser/His/Asp triad, homodimer or heterodimer with GVIIIA associates with regulatory β-subunit</td>
<td>PAF-AH 1b (α2 subunit)</td>
</tr>
</tbody>
</table>

This enzyme was cloned from human plasma in 1995, and was shown to have anti-inflammatory activity *in vivo* (43). These original studies led to the hypothesis that this enzyme might function in a protective role by stopping the pro-inflammatory roles of PAF; however several clinical studies of GVIIA PLA2 levels in patients have now established this enzyme as a definitive marker of coronary heart disease (47, 48).

With the classification of this enzyme as a positive risk factor in coronary heart disease, it has become a very attractive drug target. A specific inhibitor of this enzyme was developed in 2003 by GlaxoSmithKline (49), and recent clinical trials with this
inhibitor have showed a decrease in the complex atherosclerotic lesions that lead to unstable lesions, as well as other cardiovascular disease markers (50, 51).

1.C.5 Lysosomal PLA$_2$

The lysosomal PLA$_2$ is the newest type; it was purified from bovine brain and acylates ceramide using the acyl group from the sn-2 position of phospholipid as substrate (52, 53). This enzyme contains a conserved Ser-His-Asp triad and has four cysteine residues that are required for catalytic activity (54).

<table>
<thead>
<tr>
<th>Group</th>
<th>Source</th>
<th>Molecular mass (kDa)</th>
<th>Features</th>
<th>Alternate names</th>
</tr>
</thead>
<tbody>
<tr>
<td>XV</td>
<td>Human, murine, bovine</td>
<td>45 (deglycosylated)</td>
<td>Ser/His/Asp triad, glycosylated, N-terminal signal sequence</td>
<td>ACS, lysosomal PLA$_2$ (LPLA$_2$), LLPL</td>
</tr>
</tbody>
</table>

1.D Group IA PLA$_2$

One of the best studied PLA$_2$ enzymes is the cobra venom Group IA (GIA) PLA$_2$. This enzyme has acted as not only an important model of phospholipid metabolizing enzymes, but of all lipid enzymology. Many different crystal structures of this enzyme exist from different venom sources (55-58). These crystal structures all show some important traits as shown in Fig. 1-2A. The enzyme contains the six conserved disulfide bonds from 28-44, 26-118, 43-99, 50-92, 60-85, and 78-80, as well as the additional disulfide bridge from 11-71 (55). The active site dyad is composed of the conserved His-48, and Asp-99. The active site histidine is found to be conserved in all sPLA$_2$ enzymes (20, 59-61). The enzyme catalyzes hydrolysis through the activation of a water molecule
by extraction of a proton, and attack at the sn-2 ester bond (20, 62, 63). This mechanism explains the pH dependence of these enzymes at around 7-9. Recent work using unnatural phospholipid substrate with PC headgroups in the sn-2 position have shown that phospholipid hydrolysis is proportional to the ease of water accessibility to the active site (64, 65). The enzyme binds Ca$^{2+}$ through the conserved Asp-49 (66, 67), as well as the carbonyl oxygens of Tyr-28, Gly-30, and Gly-32 (55). The Ca$^{2+}$ ion is required for hydrolysis through orientation of the lipid substrate by coordination of the negative charge from the phosphate oxygen (56). Some structures have shown the presence of a secondary Ca$^{2+}$ ion that may act as a supplementary electrophile (56).

Experiments using NMR derived NOE results have been used to map the binding sites of a single phospholipid substrate in the cobra venom Group IA PLA$_2$ as shown in Fig. 1-2A (68). Recent work using deuterium exchange mass spectrometry with phospholipid surface present has generated a model of how this same enzyme binds to the lipid surface as shown in Fig. 1-2B (69). The Group IA enzyme appears to bind lipid substrate in the active site through the hydrophobic residues lining the active site channel, and binds neutral membrane substrate through interactions with a group of hydrophobic residues on the lipid binding surface of the molecule.

This enzyme is able to hydrolyze monomeric phospholipid substrates, but there is a substantial increase in activity when the enzyme acts on large lipid aggregates (70). This enzyme has also been shown to be activated by phospholipids containing phosphatidylycholine (PC) head groups (71), and two possible sites for this interaction have been suggested (57, 72). A combination of site-directed mutagenesis and
equilibrium dialysis has identified and confirmed there is an activator site distinct from the catalytic site (73, 74).

Figure 1-2 GIA Substrate Binding. (A) The Group IA PLA₂ with phospholipid substrate modeled in the active site. The active site residues His-48 and Asp-93 and the bound Ca²⁺ is shown in purple. Ca²⁺ is bound by Asp-49 as well as the carbonyl oxygens of Tyr-28, Gly-30, and Gly-32. Aromatic residues are shown in white. Adapted from Dennis (8). (B) Model of the lipid surface binding of the Group IA PLA₂ is shown with residues on the interfacial binding surface Tyr-3, Trp-19, Trp 61, and Phe 64 shown in stick form. Adapted from Burke et. al. (69).
The different sPLA\(_2\)'s all share different preferences for the charge state of the lipid membrane. For an excellent analysis of the different mouse and human sPLA\(_2\) membrane preferences see (22). The majority of sPLA\(_2\) enzymes preferentially hydrolyze anionic substrates (23). The GIA enzyme however is able to hydrolyze zwitterionic substrate equally as well as negatively charged lipid surfaces (71, 75). This is most likely due to the aromatic residues present on the interfacial binding surface of the GIA PLA\(_2\) as shown in Fig. 1-2A. Mutation of these residues significantly decreases the membrane binding of this enzyme (74, 75).

In comparison, the crystal structures of the GIB and GIIA enzymes demonstrate these enzymes have a cationic interfacial binding surface, and this may play a large role in their preference for anionic lipids (60, 76). It has been shown that a mutant form of the GIB enzyme with the pancreatic loop from 62-66 removed has increases in activity against zwitterionic substrate, and a decrease in activity against negatively charged substrate (77). Recent studies using a GIIA enzyme with a Trp residue mutated into the interfacial binding region dramatically increased zwitterionic phospholipid hydrolysis (78), as well as penetration (79, 80). The only other sPLA\(_2\) enzymes to have high affinity for zwitterionic vesicles are the GV, and GX enzymes (81-83) which also share the characteristic of having Trp residues in the interfacial binding region.

1.E Group IVA PLA\(_2\)

GIVA PLA\(_2\) is an 85 kDa enzyme that utilizes a catalytic serine for hydrolysis rather than histidine, as in the sPLA\(_2\) enzymes. This enzyme was initially isolated from human neutrophils (84), and platelets (85). This enzyme was sequenced in 1991, and
was shown to be specific for phospholipids containing arachidonic acid in the \textit{sn}-2 position (6). The crystal structure of the C2 domain was solved in 1998 (86) followed by the whole enzyme in 1999 and showed a Ca\textsuperscript{2+} binding C2 domain important for Ca\textsuperscript{2+} mediated membrane translocation, and a \(\alpha/\beta\) hydrolase domain that contains the catalytic site (87). The crystal structure is shown in Fig. 1-3. Of special note in this structure is the presence of a lid region that spans regions 415-432 that prevents the modeling of a phospholipid substrate in the active site. This structure confirmed previous work using mutant constructs showing the two independent functions of the C2, and catalytic domain (88). This structure showed the presence of a novel Ser/Asp dyad that mediated the hydrolysis of phospholipid substrate. The enzyme hydrolyzes substrate through the formation of a serine-acyl intermediate (89, 90). Previous work had suggested that the active site residues would consist of Ser-228, Asp-549, and Arg-200 due to inactivity of mutants containing mutations at any of these locations (91). This crystal structure shows that Ser-228, and Asp-549 are in the correct orientation to act as an active site dyad, but Arg-200 is too far away to form any contacts with either Ser-228, or Asp-549. This led to the proposal that Arg-200 may be important in binding the charged headgroups of phospholipid substrate (87).
Figure 1-3 Group IVA PLA₂ Crystal Structure. (as determined by Dessen et al (87)).

The C2 domain is shown in orange, with two bound Ca²⁺ ions shown in purple. The catalytic domain is shown on the right with the cap region colored yellow, and the lid region 415-432 colored magenta. The active site residues Ser-228, Asp-549 and Arg-200 are shown in stick form colored red. The PIP₂ binding site is shown in dark blue, and the C1P binding site is shown in cyan.
This enzyme also has both lysophospholipase, and transacylase activity (92, 93), however the lysophospholipase activity of this enzyme is insensitive to Ca\(^{2+}\) concentration. The PLA\(_2\) activity of the enzyme is active against monomeric substrate, but there is a substantial activation upon binding a membrane surface (88). For the enzyme to be active, it must be sequestered to a phospholipid interface. The binding of the GIVA PLA\(_2\) to the membrane is mediated through three mechanisms: Ca\(^{2+}\) mediated translocation, binding of secondary lipid messengers, and phosphorylation.

Ca\(^{2+}\) binding in the GIVA PLA\(_2\) is not required for catalysis as in the sPLA\(_2\) enzymes, but is required for translocation to the membrane surface (94-99). Ca\(^{2+}\) binding is mediated through the C2 domain of the enzyme. C2 domains are conserved domains present on many different lipid binding proteins (For an excellent review on membrane binding domains see (100)). The mechanism of Ca\(^{2+}\) binding to the C2 domain, and how this mediates phospholipid binding, has been studied through a variety of techniques, including x-ray reflectivity, site directed mutagenesis, NMR, EPR, and computational methods(101-107). These studies have shown that Ca\(^{2+}\) binding to this domain sequesters the protein to the lipid surface through penetration of Ca\(^{2+}\) binding loops one and three, composed of amino acids 35-39, and 96-98, into the interface. The C2 domain of the GIVA PLA\(_2\) is specific for membranes with PC headgroups (108, 109).

The GIVA PLA\(_2\) is also activated by binding many different lipid second messengers. It has been shown that phosphatidylinositol (4,5) bis phosphate (PIP\(_2\)) significantly activates the enzyme in a Ca\(^{2+}\) independent manner (110, 111). The location of the PIP\(_2\) binding site was identified through the use of site directed mutagenesis and is located at four lysines at position 485, 541, 543, and 544 (111, 112) as shown in Fig. 1-3.
We have also shown that this PIP$_2$ activation requires the presence of the C2 domain, even though the PIP$_2$ binding site is completely contained on the catalytic domain (111). Recently the lipid ceramide 1 kinase was discovered to also be an activator of GIVA PLA$_2$ (113-115). Ceramide 1-phosphate (C1P) binds to the enzyme at a specific site in the C2 domain consisting of Arg-57, Lys-58, and Arg-59 shown in Fig. 1-3. (116). Studies have also shown that the mechanism of C1P activation is Ca$^{2+}$ dependent, and decreases the kinetic dissociation constant from the membrane surface (117) while PIP$_2$ activation is caused by an increase in the catalytic efficiency, potentially through a conformational change (117).

The phosphorylation state of the GIVA PLA$_2$ also plays an important role in mediating lipid-enzyme interactions. Many different residues on the GIVA enzyme can be phosphorylated by a myriad of different kinases. The main residues found phosphorylated are Ser-505, Ser-515, and Ser-727. These residues are phosphorylated by mitogen activated protein kinases (MAPKs), mitogen activated protein kinase interacting kinase (MNK1), calmodulin kinase II (CamKII), and mitogen activated protein kinase interacting kinase (MNK1) respectively (118-121). Other phosphorylation sites have been reported at Ser-437, and Ser-454 in Sf9 cells (122), but there is currently no information on the effects of phosphorylation at these residues. Interestingly all of the residues that have been found to be phosphorylated are located in areas of the crystal structure with no traceable electron density (87). It has been shown that Ser-505, and Ser-727 are common phosphorylation sites in agonist-stimulated human platelets and HeLa cells (118), while Ser-505, and Ser-515 phosphorylation are found in vascular smooth muscle cells (123). Ser-505 phosphorylation has been shown to cause a very small increase in activity (124,
125), however recent work studying membrane binding found a 60 fold increase in membrane affinity at 2.5 μM Ca\(^{2+}\), and it is suggested that it induces a conformational change that causes tighter binding to the lipid surface (126). It has recently been shown that Ser-727 phosphorylation mediates GIVA PLA\(_2\) activity through disruption of the complex formed between annexin A2, p11, and GIVA PLA\(_2\) (127). Ser-515 phosphorylation has been shown to increase in-vitro activity of the enzyme 3 fold (120), and may also activate the enzyme through a conformational change.

The importance of GIVA PLA\(_2\) in many different inflammatory processes has been proven through the use of knockout mice deficient in GIVA PLA\(_2\). These mice showed significant decreases in allergic response, damage from acute lung injury, and postischaemic brain injury (128-130). For review see (131). Recognition of the importance of the GIVA PLA\(_2\) in inflammatory diseases, as well as important structural discoveries has made it a very attractive drug target, and many different laboratories have attempted to develop inhibitors. Two of the most promising drug candidates include the indole derivative inhibitors developed by Wyeth, and the 2-oxoamide inhibitors developed by Kokotos et. al. (132, 133). Both of these inhibitors have been used for in \textit{vivo} animal models of inflammation and have shown potency in reducing inflammatory effects (133, 134). Potential side effects of GIVA PLA\(_2\) inhibitors have been suggested by recent work examining a human patient with defects in GIVA PLA\(_2\) who showed decreases in PLA\(_2\) activity, eicosanoid biosynthesis, and the generation of many small intestinal ulcers (135).
The human Group VIA PLA₂ gene yields multiple splice variants, including GVIA-1, GVIA-2, GVIA-3 PLA₂, GVIA Ankyrin-1 and GVIA Ankyrin-2 (136, 137). At least two of these isoforms, GVIA-1 and GVIA-2 iPLA₂ are active. The human GVIA iPLA₂ contains 7-8 ankyrin repeats, a linker region, and a catalytic domain. The 85-kDa GVIA-2 iPLA₂ was first purified and isolated from the p388D1 cell line (11, 138), which possesses PLA₂ activity as well as lysophospholipase and transacylase activity (139). The active site serine of the GVIA iPLA₂ lies within a lipase consensus sequence (Gly-X-Ser⁵¹⁹-X-Gly) (140). The enzyme is not specific in what fatty acid is being released (11, 140). The activity of GVIA iPLA₂ has been reported to be regulated through many mechanisms. The enzyme possesses a caspase-3 cleavage site that is clipped in vitro (141-143). The caspase truncated enzyme was hyperactive and reduced cell viability when overexpressed in HEK293 cells (141). Caspase mediated activation has also been recently shown to be important in mediating cell migration in ovarian cancer cells (144). The enzyme is also regulated through ATP binding. ATP binding seems to protect the GVIA PLA₂ from losing activity (139). Fatty acyl-CoA was also shown to be a substrate for GVIA iPLA₂, showing a potential role for nucleotide binding (145).

The GVIA PLA₂ contains multiple ankyrin repeats which may be important in mediating protein-protein interactions. The enzyme when originally isolated was shown to be active as a tetramer (11). The splice variant GVI Ankyrin-1 was also suspected to be a negative regulator of GVIA PLA₂ through blocking potential formation of the active aggregate.(137). The importance of the ankyrin repeats is shown by studies done on the catalytic domain alone of GVIA PLA₂ showing no activity (137). The enzyme has also
been shown to be regulated by calmodulin which negatively regulates GVIA PLA₂ through direct binding on the residues 694-705 of GVIA-1 PLA₂ (146, 147). GVIA-2 iPLA₂ is membrane associated when overexpressed in COS-7 cells as well as rat vascular smooth muscle cells (136, 148). The other active splice variant, GVIA-1, is cytosolic and not specific in targeting membrane surfaces (136, 148).

1.G Conclusions

Experiments with members of the PLA₂ superfamily of enzymes have been carried out for over 100 years. Early kinetic and structural work established PLA₂ as a model of enzymes acting at the lipid-water interface applicable to all of lipid enzymology. With the discovery of multiple different family members of PLA₂ and their structural characterization, as well as the discovery of their cellular functions, the PLA₂ family has become a major drug target for many different diseases. The future of this field is very exciting as new knockout mouse models, along with specific inhibitors of these enzymes lead to further elucidation of PLA₂’s roles in cellular processes, along with new potential therapeutics.

1.H Acknowledgements

Edward A Dennis coauthored this chapter from work published and submitted in the Journal of Cardivascular disease and therapy and the Journal of lipid research respectively.
1.H References


CHAPTER 2

Deuterium Exchange Mass Spectrometry (DXMS) Methodology
2.A Abstract

Deuterium exchange mass spectrometry is a powerful tool for analyzing conformational changes and solvent accessibility of proteins. Amide hydrogens in solution freely exchange with solvent, and in the presence of D$_2$O, proteins can incorporate deuterium in the amide position. Amide hydrogen solvent exchange is an acid and base catalyzed reaction with a minimum of pH 2.5-4, so with the use of pH adjustment, deuterium exchange rates can be slowed. The use of an acid labile protease allows for fragmentation of the protein, and with mass spectrometry the isotope label can be followed by an increase in mass of a peptide fragment. This allows the localization of exchange and determination of the structural basis for this exchange. This technique has its beginnings in experiments started over fifty years ago examining amide hydrogen exchange, and has exploded in the last twenty through the use of mass spectrometers to analyze deuterium incorporation with the development of cheaper and better instruments. Amide hydrogen exchange had been studied using radioactivity and NMR measurements for many years, but mass spectrometry approaches have greatly expanded the power of the technique and simplified data analysis. In this chapter we review the beginning of amide hydrogen exchange, the methodology of deuterium exchange mass spectrometry (DXMS), and discuss applications of the method to biological problems, as well as future directions.
2.B Introduction

Proteins are not static structures in solution. Many of the structural studies performed in the last fifty years have focused on using x-ray crystallography to generate static structures of protein complexes. These studies have limitations due to viewing the protein in one state only, as well as not looking at the structure in solution. It is similar to deducing how an automobile’s engine works by taking a photo under the hood. The creation of techniques that can investigate the dynamics of proteins in solution is an important step in unifying the information from crystal structures with how the protein acts in solution.

To this end deuterium exchange mass spectrometry is an important tool in investigating the dynamics of proteins in solution. Examining the exchange of amide hydrogens in proteins has been used for over 50 years, with the first experiments performed in the lab of Linderstrom-Lang (1-3). It was predicted that amide hydrogens involvement in secondary structure of proteins would change the exchange rates, and from these rate changes important structural information could be inferred. From these initial experiments the fundamental equations defining amide hydrogen exchange were defined (4). From these studies the hydrogens in proteins could be divided into three classes based on their rates of exchange with solvent. Hydrogens attached to carbon almost never exchange with solvent (with the imidazole on the His side chain, being an exception), and the side chain hydrogens attached to oxygen, nitrogen, or sulfur on residues Cys, Ser, Arg, Lys, Asn, Asp, Glu, Gln, and His, exchange very rapidly, as well as the N or C terminus amine and carboxylic acid (Fig. 2-1). Amide hydrogens attached
to the peptide backbone however exchange with rates varying from seconds to months based on their involvement in secondary structure and solvent accessibility.

This method was made more powerful by the use of size exclusion chromatography combined with tritium labeling of amide hydrogens (5-8). The advent of HPLC technology along with acid labile proteolysis allowed for further refinement and use of amide exchange methodology (9-14). Advances in NMR technology allowed for the first uses of amide exchange methodology with NMR which allowed investigations into how different proteins fold and protein-protein interactions (15-20). The use of amide hydrogen isotope exchange with NMR have been recently reviewed (21). The discovery of ESI mass spectrometry led to the first uses of using mass spectrometry coupled with amide hydrogen exchange to determine structural information (22-26). In this chapter further refinements to deuterium exchange mass spectrometry methodology will be discussed as well as recent examples of experiments conducted using deuterium exchange mass spectrometry.
Figure 2-1 Hydrogens in Proteins. The three types of hydrogens in a protein can be divided into three different classes based on their rates of exchange with solvent. Hydrogens attached to carbon shown in green almost never exchange with solvent. Side group hydrogens, along with the N-terminal amine and the C terminal carboxylic acid, are shown in blue and they exchange rapidly with solvent. Amide hydrogens, shown in red however have exchange rates based on protein conformation and solvent accessibility that can vary from seconds to months.

2.C Methodology

2.C.1 Hydrogen Exchange

The exchange of amide hydrogens in a protein varies greatly depending on their involvement in secondary structure as well as their accessibility to solvent. This rate is also highly dependent on pH and temperature. The details of the mechanism for amide hydrogen exchange has been reviewed extensively (4, 13, 27). This section will present a
short summary of the kinetic parameters governing amide exchange in solvent. A recent review has also explained the derivations of all of these equations (28). Every amide hydrogen in an unfolded peptide has an intrinsic rate constant due to interactions with the local amino acids present. This rate constant is defined as $k_i$ and the values for this rate constant for unfolded peptides of varying sequences at varying pH and temperature values has been determined (29, 30). The rate of exchange of any given amide hydrogen in a folded protein is given by the following equation.

$\text{Equation 2-1}$

$$k_{ex} = k_f + k_u$$

Where $k_{ex}$ represents the experimental rate determined, $k_f$ and $k_u$ are the individual contributions to the rate from the folded and unfolded forms of the protein. This can be described by viewing figure 2-2 describing the mechanism by which a amide hydrogen can exchange. The rate of solvent exchange of an amide hydrogen in a totally folded protein that does not undergo any unfolding reaction is given by the equation.

$\text{Equation 2-2}$

$$k_f = \beta k_i$$

Where $k_i$ once again represents the intrinsic rate constant in an unfolded peptide, and $\beta$ represents the protection factor that can vary in value from 0 to 1, and is dependent on the involvement in secondary structure (intramolecular hydrogen bonding) as well as solvent accessibility, this is represented in the top line of figure 2-2. However any experimental rate constant also contains a potential contribution from exchange due to unfolding of the protein given by $k_u$. The equations governing this reaction are shown below.
Where $k_1$ and $k_{-1}$ are the rate constant for unfolding and folding respectively. $F_H$ represents the folded protein at that amide hydrogen and $U_H$ represents the unfolded state around that hydrogen. When $k_i \gg k_{-1}$, then the equation for $k_u$ becomes

(Equation 2-4)

$$k_u = k_1$$

This situation is known as EX1 kinetics and very few proteins undergo global EX1 kinetics under physiological conditions. EX1 kinetics can be thought of as a cooperative unfolding process where amides in the unfolded region exchange before refolding ($k_{-1}$). Much more likely under physiological conditions is when $k_{-1} \gg k_i$ the equation for $k_u$ becomes

(Equation 2-5)

$$k_u = \frac{k_1}{k_{-1}} k_i = K_{\text{unf}} * k_i$$

This situation is known as EX2 kinetics. EX2 kinetics can be thought of a case where there are very few large cooperative unfolding reactions and exchange with solvent occurs through micro fluctuations in secondary structure and solvent accessibility. Proteins can be forced into EX1 kinetics by use of denaturants (31), but certain regions in proteins may undergo both EX1 and EX2 kinetics depending on lid or hinge movements common in some proteins. Determining regions where either EX1 or EX2 deuterium exchange can be examined with mass spectrometry based on isotopic profiles (32, 33).

The exchange of amide hydrogens is an acid and base catalyzed process with a minimum around pH 2.5-4 (29, 30). The process is also temperature dependent, with the rate decreasing as the temperature decreases. This property allows amides to be locked
into place by manipulating pH and temperature, which allows for digestion and separation of peptides while locking amide hydrogens in place. Side group hydrogens exchange rapidly even at these temperatures and pH values allowing for removal of side chain hydrogens in the HPLC separation steps.

Figure 2-2 Amide Hydrogen Exchange in Proteins. Amide hydrogens can exchange through two different mechanisms. The amide hydrogen can exchange without protein unfolding occurring where the rate \( (k_f) \) will be based on solvent accessibility and conformation of the region where the amide is located. Exchange can also occur through a mechanism where the protein unfolds followed by amide exchange at a rate \( (k_i) \) based on the intrinsic characteristics of the peptide itself.
With the knowledge of hydrogen exchange rates and mechanisms, experiments can be designed to test important biological questions using deuterium exchange mass spectrometry. These methods have been recently reviewed (34). The simplest method for DXMS studies is continuous labeling experiments where the protein of interest is diluted into a fixed amount of D₂O for varying time points, and at the set time point the protein is quenched at pH 2.5 to 3 at 0°C followed by digestion, separation and mass spectrometry analysis. Pulse labeling with deuterium is another technique, where the protein is incubated with a denaturant or other stimuli and then pulsed with a very short burst of deuterium, ~0.5-10 seconds, followed by quench, so that only unfolded regions exchange. This technique has been used to follow protein unfolding. Other techniques include off exchange experiments, where the protein of interest is fully exchanged with deuterium and then incubated with an interacting protein/ligand/substrate, and then the protein is diluted into H₂O. The rate of amide off exchange can be followed and certain areas of protection can be observed.

2.C.2 Protein Digestion / Spatial Resolution

The usefulness of deuterium exchange methodologies is limited by the resolution of structural information. In mass spectrometry experiments this is determined by the number of peptide fragments that can be generated, along with overlapping peptides that can be used to isolate smaller regions of amide exchange. HPLC systems are generally used to separate the peptide fragments before addition to the mass spectrometer. Proteases used for deuterium exchange experiments need to be active under low pH conditions, as well as active under denaturant and reduction conditions. The first use of
acid labile proteases was used by Zhang and Smith (25). The majority of experiments performed have used pepsin as the acid protease, although recent work has used various fungal proteases to increase the resolving power of deuterium exchange experiments (35). Pepsin was shown to be the most effective in cleaving proteins into peptide fragments, but the fungal proteases can still increase the resolving power of deuterium exchange MS. Recent work using an automated HPLC attached pepsin column has allowed single amino acid resolution for small proteins (36, 37). The process of digestion is outlined in figure 2-3.

**Figure 2-3 Peptide Denaturation and Digestion.** The protein of interest is analyzed through denaturation using denaturants (and reductants if necessary), followed by digestion with a acid active protease, followed by HPLC separation and mass spectrometry analysis.
Resolution of the deuterium label can be obtained through mass spectrometry as well as proteolysis. In the mass spectrometer individual peptides can be separated into fragments though both collision induced dissociation (CID) as well as electron capture dissociation (ECD). The ions generated through fragmentation are shown in figure 2-4. Initial reports seemed to show that CID might be an effective way to determine high resolution data (38). However newer reports have shown that CID causes scrambling of the b and y ions, which invalidates this technique as a method of locating deuterium (39, 40).

The use of ECD however has been shown to not induce the scattering seen in CID methods (41-43). This allows use of mass spectrometric techniques to localize deuterium in peptides, but this method does require the use of fourier transform mass spectrometry methods that are beyond the funding and expertise levels of many laboratories performing deuterium exchange experiments.

2.C.3 Peptide Identification and Deuterium Quanitification

Once peptides have been digested and separated on HPLC systems they still need to be identified using the mass spectrometer. The identification of peptide fragments based on their fragmentation patterns has been an important tool of proteomics research, and database searches using this technique have been recently reviewed (44-46). This method uses collision induced dissociation to fragment peptides into different ion pairs as shown in figure 2-4. The parent mass of any peptide can be selected and then fragmented in a MS/MS dependent fashion. In CID the most abundant ion pairs created are usually b and y ion pairs. One of the main database search tools is the SEQUEST database search,
which uses a correlation function to compare a group of theoretical commonly found ion pairs to experimentally identified ions, and then ranks these comparisions with a quality score (47). An example of what SEQUEST identifies computationally is shown in figure 2-5. Peptide fragments separated on the HPLC are injected into the mass spectrometer, which cycles through a search of all parent mass ions, and selects the highest intensity parent masses, and fragments these with a collision gas. An example of a total ion count spectrum is shown in figure 2-5-A. For each parent ion that is fragmented there will be a MS/MS spectrum generated, an example of which is shown in figure 2-5-B. This allows for the identification of many of the different peptides generated through protease digestion.

Figure 2-4 MS/MS Fragmentation Patterns for Peptide Ions. Shown above are possible fragmentation patterns for a peptide fragment composed of 5 amino acids. The peptide can fragment into a-x pairs (shown in red), b-y pairs (shown in blue), and c-z (shown in green). These ions can be identified by the mass spectrometer.
Figure 2-5 Peptide Identification and Deuterium Quantification. (A) The total ion count chromatogram for the entire HPLC run (32 minutes) is shown for the GIVA PLA2. (B) The fragmentation of peptide 266-279 charge state 2 is focused on from the ion count shown in part A is shown. The different ion pair fragments identified are labeled on the figure. (C) The raw data for deuterium quantification for this peptide is shown with deuterium levels increasing from 0-10,000 seconds. The centroid values of these peaks are indicated by the black arrow.
The amount of deuterium in each peptide can be quantified as shown in figure 2-5-C. For each peptide there will be a certain amount of deuterium that exchanges on and off in the quench step, protein digestion step, as well as the HPLC separation. This can be corrected if both an on exchange control and back exchange control are prepared. The on exchange control is prepared by adding D$_2$O to a sample after the addition of quench buffer to control for deuterium addition during the processing steps. The back exchange control is prepared by generating a fully deuterated sample where all amides contain deuterium, and then that sample is exposed to the same processing. This controls for deuterium that exchanges off during processing steps. The equation that governs quantification is shown below.

\[
D = \left[ \frac{m_s - m_{0\%}}{m_{100\%} - m_{0\%}} \right] N
\]

Equation (2-6)

Where $m_i$ is the centroid of the peptide exposed to $s = \text{sample conditions}$, 0\% = On exchange quench control and 100\% is fully deuterated and $N = \text{maximum deuterium level of peptide}$ (25).

There are problems with this method for quantification with some proteins. This method requires the generation of a fully deuterated sample, and this can be technically challenging in certain situations such as dealing with large globular proteins. Instead of formal quantification, experiments can be performed where the relative amount of deuterium in each peptide is compared. This still allows the determination of differences in between different conditions, without possible complications from full deuteration sample preparation. This topic has been recently reviewed (34).
2.D Applications of Method

The application of deuterium exchange mass spectrometry towards numerous different applications is shown schematically in figure 2-6, and is discussed in the following sections.

2.D.1 Protein Structure

Numerous studies have been performed testing the solvent structure of different proteins, and how amide hydrogens may contribute to protein structure and dynamics (Fig. 2-6-A) (22, 23, 28, 36, 48-56). A few selected examples are highlighted. Deuterium exchange mass spectrometry has been used to study transmembrane proteins and determine the position of helices spanning the membrane through decreases in amide exchange (56). A recent study has also examined hydrogen bonding inside of membrane proteins and a combination of deuterium exchange and x-ray crystallography was used as a means to test the protein dynamics of a variety of H-bonding mutants for changes in protein dynamics (55). Work has also been undertaken examining the structure of amyloid fibrils that are important in alzheimer’s disease. These fibrils have resisted crystallography, but using deuterium exchange combined with mass spectrometry has allowed structural information to be determined (36). Finally deuterium exchange mass spectrometry has been proven to be an excellent technique to generate protein constructs that are more rapidly crystallized through the removal of highly disordered regions (52, 53).
Figure 2-6 Deuterium Exchange Applications. Examples of different experiments (A-C) where deuterium exchange experiments can give structural information about protein dynamics / conformational changes/ solvent accessibility.
2.D.2 Protein Folding

Deuterium exchange mass spectrometry has also been an excellent tool to test how different proteins fold, and to try and identify different folding intermediates (31, 32, 57-63). These experiments have used denaturants followed by pulse deuterium experiments to determine the folded and unfolded of a variety of proteins such as triosephosphate, tryptophan synthase, and aldolase and locate the specific regions and timing of these proteins during folding and unfolding (57-59, 61, 62).

2.D.3 Inhibitor and Ligand Binding

One of the main uses of deuterium exchange mass spectrometry has been to test the location of how inhibitors and various other ligands bind, as well as conformational changes that these inhibitors/ligands may induce (Fig. 2-5-B+C) (64-71). DXMS studies have allowed the study of how these inhibitors and ligand influence the conformation of different proteins, and has led to a deeper understanding of the structural aspects of how proteins change conformation in response to different stimuli.

2.D.4 Protein-Protein Interactions

The determination of different protein-protein interfaces has been an important area of research in deuterium exchange mass spectrometry studies. Numerous protein interfaces have been determined with this technique, including proteins lacking x-ray structural information (54, 72-83). These studies have also been combined with computational approaches determining protein-protein binding surfaces using algorithms integrating deuterium exchange information (77).
2.E Conclusions

Deuterium exchange has been an important tool for studying protein dynamics, conformation, and solvent accessibility. The incorporation of mass spectrometry into this technique, along with improvements in proteolysis, separation, and mass analysis makes deuterium exchange mass spectrometry an important tool for protein structure analysis.

2.F References


CHAPTER 3

Interaction of Group IA Phospholipase A₂ with Metal Ions and Phospholipid Vesicles Probed with Deuterium Exchange Mass Spectrometry
3.A ABSTRACT

Deuterium exchange mass spectrometric evaluation of the cobra venom (Naja naja naja) Group IA phospholipase A2 (GIA PLA2) was carried out in the presence of the metal ions Ca^{2+} and Ba^{2+} and phospholipid vesicles. Novel conditions for digesting highly disulfide bonded proteins and a methodology for studying protein-lipid interactions using deuterium exchange have been developed. The enzyme shows unexpectedly slow rates of exchange in the two large alpha helices at regions 43-53 and 89-101, which suggests that these alpha helices are highly rigidified by the four-disulfide bonds in this region. The binding of Ca^{2+} or Ba^{2+} ions decreased the deuterium exchange rates for five regions of the protein (24-27, 29-40, 43-53, 103-110, and 111-114). The magnitude of the changes was the same for both ions with the exception of regions 24-27 and 103-110 which showed greater changes for Ca^{2+}. The crystal structure of the N. naja naja GIA PLA2 contains a single Ca^{2+} bound in the catalytic site, but the crystal structures of related PLA2s contain a second Ca^{2+} binding site. The deuterium exchange studies reported here clearly show that in solution the GIA PLA2 does in fact bind two Ca^{2+} ions. With dimyristoyl phosphatidylcholine (DMPC) phospholipid vesicles with 100 µM Ca^{2+} present at 0 °C, significant areas on the i-face of the enzyme showed decreases in exchange. These areas included regions 3-8, 18-21, and 56-64 which include Tyr-3, Trp-61, Tyr-63, and Phe-64 proposed to penetrate the membrane surface. These regions also contained Phe-5, and Trp-19, proposed to bind the fatty acyl tails of substrate.
3.B Introduction

The phospholipase A$_2$ (PLA$_2$) superfamily consists of fifteen different groups and many subgroups that hydrolyze the ester bond of 2-acyl fatty acids from phospholipids (1, 2). The products of this reaction, free fatty acids and lysophospholipids, play many different roles as second messengers and precursors for important bioactive molecules (3). One of the best studied PLA$_2$ enzymes is the cobra (*Naja naja naja*) venom Group IA (GIA) PLA$_2$. This is one of the secreted PLA$_2$s that are characterized by their low molecular weight, Ca$^{2+}$ requirement for catalysis, and the presence of seven disulfide bonds. This enzyme is able to hydrolyze monomeric phospholipid substrates, but there is a substantial increase in activity when the enzyme acts on large lipid aggregates (4). This enzyme has also been shown to be activated by phospholipids containing phosphatidylcholine head groups (5), and two possible sites for this interaction have been suggested (6, 7). Site-directed mutagenesis identified an activator site distinct from the catalytic site (8). Extensive kinetic and biophysical studies have been done to understand how soluble enzymes interact with lipid interfaces and to define how the interface affects enzyme kinetics. Many of these studies were carried out on the GI and GII PLA$_2$s. Thus, the cobra venom GIA PLA$_2$ has been an important model of not only phospholipid metabolism, but for all of lipid enzymology as well (9).

Biophysical studies on lipid metabolizing enzymes acting on phospholipids are complicated because this system involves two large macromolecules, the enzyme and the aggregated phospholipid vesicle. The size of the lipid aggregates present limitations for many standard biophysical techniques such as solution NMR and X-ray crystallography. For example, many X-ray crystal studies of the secreted PLA$_2$s have been conducted
including two of the GIA *N. naja naja* enzyme (7, 10). These studies were carried out in the presence of metal ions, but not in the presence of large lipid aggregates. Crystallography reports only the state of the enzyme in a crystal not its state in solution, yet the solution dynamics of the interactions between the PLA₂ and the interface may affect enzyme activity. The *N. naja naja* enzyme has also been studied with NMR in the presence of a monomeric small lipid inhibitor, and a micellar phospholipid analogue (11, 12), but no studies have been reported with natural phospholipids. In an attempt to utilize a technique that can yield both structural information in solution and information about the dynamics of the enzyme-phospholipid surface interaction, we have employed deuterium exchange mass spectrometry to study PLA₂ lipid/surface interactions and used this technique to add valuable information about these processes.

Peptide amide hydrogen/deuterium exchange analyzed via liquid chromatography/mass spectrometry (DXMS) has been widely used to analyze protein-protein interactions (13, 14), protein conformational changes (15, 16), protein dynamics (17), and protein-lipid interactions (18) on proteins lacking disulfide bonds. Tris-carboxyethyl phosphine (TCEP) has regularly been used to reduce proteins containing one or two disulfide bonds, and occasionally more (19). However, denaturing and reducing as highly a disulfide bonded protein as the GIA PLA₂ which contains 14 cysteines, all in disulfide bonds, out of 119 amino acids is extremely challenging and not previously reported using the DXMS technique. We have now employed DXMS to examine the effects of metal ion binding on solvent accessibility and protein structure. We have probed the structural dynamics of the protein and found large changes in accessibility of the amide hydrogens in heavily disulfide bonded regions of the protein. We have also confirmed the presence
of the primary Ca\(^{2+}\) -binding site and a secondary Ca\(^{2+}\) -binding site. We have also studied the interactions of the soluble GIA PLA\(_2\) with a lipid surface and found a deuterium exchange difference on the interfacial side of the enzyme in the presence of phospholipid vesicles. This has advanced our understanding of the structure and dynamics of the secreted PLA\(_2\)’s in solution.

3.C MATERIALS AND METHODS

3.C.1 Materials

Cobra venom PLA\(_2\) was purified as described previously (20) and stored at –20 °C in 10 mM Tris pH 7.5. Phospholipids were purchased from Avanti Polar Lipids. All other reagents are analytical reagent grade or better.

3.C.2 On Exchange Experiments

D\(_2\)O buffer contains 10 mM Tris (pH 7.5), 50 mM NaCl, (+/-)1mM CaCl\(_2\) or BaCl\(_2\) in 98% D\(_2\)O. Hydrogen/Deuterium exchange experiments were initiated by mixing 10 µl of 10 mM Tris pH (7.5) containing 40 µg of GIA PLA\(_2\) with 30 µl of D\(_2\)O buffer producing a final D\(_2\)O concentration of 74% at pH 7.5. In the experiments examining metal ion binding, the GIA PLA\(_2\) was pre-incubated with 4 mM BaCl\(_2\) or CaCl\(_2\) at 22 °C for 5 min. The optimized temperature for separating both fast and slow exchanging regions was 22 °C.

The H/D exchange samples were incubated in 22°C for 15 s, 60 s, 600 s, 3600 s, and 14,400 s at pH 7.5. This gave the greatest distribution of deuterium incorporation over our time course. For the phospholipid experiments, the last time point was dropped. Each time point was repeated three times. The deuterium exchange was quenched by
adding 160 µl ice-cold quench solution (8 M GdHCl, 1 M TCEP) buffered with formic acid to a pH of 2.5. The samples were placed on ice for 15 min.

3.C.3 Full Exchange Sample Preparation

A fully deuterated sample was prepared by adding GIA PLA₂ to a solution of 8 M GdHCl and 1 M TCEP dissolved in 100% D₂O and allowing this to stand at room temperature for 24 hrs. The GdHCl and TCEP were removed by diluting the solution with 1 volume of 100% D₂O and concentrating the protein with an Amicon Ultra 5000 MW cutoff centrifuge filter (Millipore) at 3000g until the volume was half of the original volume. This was repeated 10 times to remove all TCEP and GdHCl. The protein was allowed to sit for 2 hrs after removal of all denaturants and was then digested under normal conditions.

3.C.4 Off Line Protein Digestion

To adequately digest the GIA PLA₂, an off line digestion step had to be added in front of the digestion that occurs during the automated process outlined below. The quenched protein solutions containing 40 µg of protein were added to a 50 µl slurry of ice cold immobilized pepsin (Pierce Biotechnology) and fungal XIII proteases immobilized on 6% cross linked agarose beads suspended in 0.8% formic acid and allowed to sit for 15 min. Immobilized fungal XIII protease was prepared as described previously (15). This solution was placed in a 5 Micron ultrafree-MC centrifugal filter (Millipore) and centrifuged at 700 G (eppendorf centrifuge 5415C) to remove the immobilized proteases and the remaining solution was frozen on dry ice to stop all further amide hydrogen exchange.
3.C.5 Automated Proteolysis-liquid-chromatography-mass spectrometry Analysis

Samples were then loaded onto the automated DXMS system (13, 15) that digested the proteins, separates the peptides and performs the mass spec analysis. All steps were performed at 0 °C as previously described (13, 15). The samples were hand-thawed on melting ice and injected onto a protease column (66 µl bed volume) containing porcine pepsin (Sigma; immobilized on Poros 2 AL medium at 30 mg/ml following the manufacturer’s instructions, Applied Biosystems), at a flow rate of 100 ul/min with 0.05% (v/v) trifluoracetic acid (TFA). The eluate from the pepsin column flowed directly onto a C18 column 50 mM length 1.0 mM ID (Vydac cat #218MS5150). The peptides were eluted with a linear gradient from 0.046% TFA, 6.4% (v/v) acetonitrile to 0.03% TFA, 38.4% acetonitrile at 50 ul/min. The C18 column eluate flowed directly into a Finnigan LCQ Classic mass spectrometer via its ESI probe operated with a capillary temperature of 200°C as previously described (13, 15).

The presence of large amounts of phospholipid in peptide eluates injected into the mass spectrometer would significantly degrade the peptide analysis. We found that the C-18 column employed in the peptide separation bound all of the phospholipids that were carried through the workup in the phospholipid binding experiments. Thus, no extra steps were required to remove it. Up to ten runs could be completed before the phospholipid bound to the columns began to degrade the peptide separation or yields. After every ten runs the HPLC column was flushed with 100% methanol for 30 minutes to remove the bound phospholipids.
3.C.6 Lipid preparation and Lipid Binding Experiments

Lipid vesicles were prepared by adding the lipid in chloroform to a small glass tube and evaporating the solvent under argon. The lipid was resuspended in 100 mM KCl at 40 mM and allowed to sit at 50 °C for 30 min. This solution was then bath sonicated for 10 min. This solution was then size excluded using 10 passes over a mini extruder (Avanti Polar Lipids) with a 0.03 µM polycarbonate membrane. For lipid binding studies, the enzyme was studied with two different lipid systems dipalmitoyl phosphatidylcholine (DPPC) and dimyristoyl phosphatidylcholine (DMPC) and they were used to study lipid binding at 22 °C and 0 °C, respectively. Experiments were performed on the gel state of the lipid to prevent excess activity and to prevent the lag phase in activity associated with lipids above the phase transition temperature (26). For DPPC experiments, the enzyme was pre-incubated with 4 mM DPPC SUVs at 22 °C for 5 min plus the addition of either 4 mM BaCl₂ or 4 mM EDTA. This gave a total concentration after addition of deuterium of 1 mM DPPC and 1 mM BaCl₂ or EDTA.

For DMPC experiments, the enzyme was preincubated with 4 mM DMPC SUVs at 0°C in the presence of 100 µM Ca^{2+} to minimally activate the enzyme. A modified version of the Dole assay (8) using the exact same conditions as used for deuterium exchange experiments was used to assay enzyme activity. Deuterium exchange results were only obtained for timepoints with less then 10% hydrolysis of lipid vesicles to prevent acidification of exchange buffer, and effects of non phospholipid lipid aggregates. The enzyme was pre-incubated with 4 mM DMPC lipid vesicles with 400 µM Ca^{2+} for 30 seconds at 0 °C. This gave a total concentration after dilution by the addition of deuterated buffer of 1 mM DMPC and 100 µM Ca^{2+}. The K_d of the enzyme for
lipid vesicles at 40 °C is 4.4 mM, but lipid concentrations over 1 mM were limited by the amount of protein required for mass spectrometry analysis. The mole ratio of lipid molecules to protein molecules in the samples was ~16:1.

3.C.7 Data processing

SEQUEST (Thermo Finnigan Inc.) was used to assign peptide sequences to the ions peaks eluting from the C-18 column from their ms/ms spectra. Peaks that SEQUEST identified where then analyzed with DXMS Explorer (Sierra Analytica Inc, Modesto CA) as previously described (13, 15). All peptides selected for analysis had to first pass the quality-control thresholds setup in the DXMS software and then were manually checked. If the same peptide was found with different charges (1, 2 or 3), the one with the best signal/noise ratio was selected for analysis. The mass of the peptide was determined by measuring the centroid of the isotopic envelope of the peptide. The level of deuterium on a given peptide is expressed as the incorporated deuteron number (Inc#). The incorporated deuteron number is the difference between the centroids of a given peptide in non-deuterated and deuterated samples. Back-exchange was ~35-50% due to the long digestion time but was corrected using the fully deuterated control experiments. The deuteration level (D) is the ratio of the Inc# of the sample to the Inc# of a fully deuterated sample given by the following equation (21).

$$D = \left[ \frac{m_x - m_{0\%}}{m_{100\%} - m_{0\%}} \right] N$$

Equation (3-1)
Where \( m_i \) is the centroid of the peptide exposed to \( s = \) sample conditions, \( 0\% = \) On exchange quench control and \( 100\% \) is fully deuterated and \( N = \) maximum deuterium level of peptide.

Deuterium that has been incorporated into the amides of an intact protein is rapidly lost from the very rapidly exchanging amino terminus proton and most N-terminal amide proton of peptides created under exchange quench conditions. Thus in our experiments, no hydrogen exchange information is available for the first two amino acids of a probe peptide. We use the term region to represent all amides where we can track deuterium incorporation. In some cases we specifically state the peptide identity as shown by mass spectrometry and label those as peptides (13, 15). Regions of exchange were calculated by subtracting overlapping peptide fragments as in previous work (22). Percent changes reported are calculated by the difference in exchange between conditions over the total number of deuterons in a region.

3.D RESULTS

3.D.1 Protein Digestion of GIA PLA₂

GIA PLA₂ is a very rigid protein due to its small size (13,500 kD) and the presence of seven disulfide bonds. Because of this, finding conditions for digesting the protein was a major hurdle to obtaining a good peptide map. TCEP has been employed to reduce disulfide bonds under the low pH conditions needed for deuterium exchange quenching (19). In our initial experiments, we added TCEP under a variety of conditions to the chromatographic solvents employed with the pepsin column in the automated DXMS system. This met with no success. We found that significant digestion could only
be achieved by predigesting the enzyme with pepsin and fungal XIII protease prior to loading the samples onto the automated DXMS system described in the methods. The predigestion was achieved by quenching protein samples in a solution containing 1M TCEP and 8M GdHCl for 15 min followed by dilution into a solution containing immobilized pepsin and fungal XIII proteases for 15 min. This solution was then loaded onto the automated DXMS system and run through another immobilized pepsin column before being loaded onto the C18 column. Back exchange levels of ~50% were found with this technique, but by using fully deuterated controls, the amount of back exchange could be measured and used to determine corrected deuterium levels.

Using these conditions 55 good quality peptides were identified. If peptides occurred in more than one charge state, we have shown only the one with the best signal to noise ratio. This reduced the number of useful ions with no redundant data to 45, which are shown in Figure 3-1. This map covers 96% of the protein and has a high degree of overlap allowing for the determination of deuterium levels for regions of the protein smaller than the corresponding peptide. The difference in deuterium levels of the two overlapping peptides is the number of deuterons associated with the unmatched amino acids. This allows us to determine the deuteration levels of regions of the protein smaller than either peptide.

This map was generated in the presence of both metal ions and lipid without any changes in the number and intensity of peptide fragments. The number of deuterons incorporated was measured for all 55 peptides including peptides with multiple charge states, but to simplify the graphics only 23 of the peptides, those shown in bold lines in figure 1, were chosen to generate the deuterium exchange data for all of the other figures.
However all 55 peptides were analyzed in each experiment to make sure that the exchange data agreed with that of the 21 selected peptides.

Figure 3-1 Pepsin digested peptide coverage map of the GIA PLA₂ sequence. Alpha helical (α) and beta sheet (β) regions are indicated by symbols above the sequence. Black lines represent peptides chosen for this study. Dotted lines represent peptides that were identified and analyzed but were only used as a comparison for the bold line peptides. There are 45 distinct peptides which were studied with only one selected for peptides that have multiple charge states.
3.D.2 Deuterium Exchange of GIA PLA$_2$

On exchange experiments were carried out on the native GIA PLA$_2$ employing the DXMS methods outlined above. Figure 3-2 show deuterium exchange percentages for these experiments. Figure 3-2 is divided into 17 different regions. The deuterium levels of each region were generated by analyzing several overlapping peptides that allowed us to measure the deuteration level of the smallest protein regions possible.

The data show that both the N and C terminal regions (1-23, 102-119) of the protein exchange very rapidly (Fig 2). The first 23 amino acids encompass the first $\alpha$ helix and show over 80% deuteration within the first minute of on exchange with the exception of amino acids 9-10. Region 9-10 exchanges much slower and does not reach 80% deuteration until 60 min. This region encompasses the last two residues of the first $\alpha$ helix and is next to the disulfide bond bridging residues 11 and 71 as shown in Figure 3-3. This may explain the decreased rate of exchange. The C terminal area stretching from amino acid 102-119 also shows over 80% deuteration within the first min of on exchange.
Figure 3-2 Amide hydrogen/deuterium exchange analysis of the calcium and barium effect on GIA PLA₂. The deuterium exchange map with and without Ba²⁺ and Ca²⁺ is shown with the amino acid sequence of the GIA PLA₂. Each condition is divided into rows corresponding to each time point, from 15s to 240 min from top to bottom. The colors code for the amount of H/D exchange in the given time period.
Figure 3-3 Deuteration level of GIA PLA$_2$ visualized on the crystallographic model of the *N. naja naja* enzyme (1PSH). The deuterium exchange map of GIVA PLA$_2$ after 3000 seconds of on-exchange is shown with the color indicating the exchange rates detected by DXMS. Disulfide bonds are shown in yellow.
The region encompassing amino acids 56-86, which contains the two antiparallel β sheets, also shows very rapid exchange with deuteration rates comparable to those seen in both the N and C termini. The region from residue 24-40 is less flexible than the N and C termini and takes 60 min to fully exchange. In contrast, the rates of exchange on α helices 39-55 and 84-101 are very slow. The region from 89-101 exchanges less than 10% even after 24 hrs of on exchange. The region from 43-53 does exchange but only exchanges 50% after 4 hrs.

3.D.3 Changes in Deuterium Exchange of GIA PLA2 when Ba<sup>2+</sup> or Ca<sup>2+</sup> is Bound

The GIA PLA<sub>2</sub> requires Ca<sup>2+</sup> for activity with one molecule binding in the catalytic site. A second Ca<sup>2+</sup> may also bind to the enzyme, but its role in activity has not been defined. Ba<sup>2+</sup> also binds to the primary Ca<sup>2+</sup> binding site in the catalytic site but it inhibits activity although it does produce similar structural changes in the enzyme as detected by UV-Vis spectroscopy (23). Thus, we measured deuterium on exchange of the GIA PLA<sub>2</sub> from 15 sec to 4 hrs in the presence of 1 mM BaCl<sub>2</sub> or CaCl<sub>2</sub> to ascertain if these metals produce different exchange patterns. Several areas in GIA PLA<sub>2</sub> show changes in deuterium exchange in the presence of Ca<sup>2+</sup>. Three of these, 24-27, 29-40, 43-53 (Fig 3-4) are part of the primary Ca<sup>2+</sup> -binding site of the GIA PLA<sub>2</sub> which consists of the backbone oxygens of Y27, G29, and G31 and the aspartic acid oxygens of D48 (10). Thus Ca<sup>2+</sup> binding appears to decrease the deuterium exchange seen in regions 24-27, 29-40, and 43-53. Interestingly the decrease in deuterium exchange in regions 29-40 and 43-50 appears to mainly affect slow exchanging amide hydrogens. This is shown by the
greatest difference in deuterium exchange being seen from 3,000-10,000 seconds (Fig. 3-4).

Figure 3-4 Deuterium exchange of the GIA PLA2 upon Metal Binding. The number of incorporated deuterons at 5 time points are plotted for regions that show changes upon either calcium or barium (or both) binding. The changes in deuterium exchange are shown at two different time points mapped on the N. naja naja GIA PLA2 crystal structure (1PSH) with the Ca$^{2+}$ ion shown in pink. The color represents the changes in deuterium levels.
One other region is 103-114 where the changes affect only fast exchanging amide hydrogens with no difference seen after 60 minutes of on-exchange. Since these residues are not part of the primary Ca\(^{2+}\) binding site, these results would be consistent with a second Ca\(^{2+}\) binding site. The crystal structures of the structurally related *N. naja atra* GIA PLA\(_2\) have shown such a secondary Ca\(^{2+}\)-binding site with contacts between Asn 112, and Asp 23 and a secondary Ca\(^{2+}\) ion (24). By comparing the structures of these two enzymes, which are superimposable in this region, and overlaying the exchange data, it is clear that a Ca\(^{2+}\) binding to this second site in the *N. naja naja* PLA\(_2\) would account for the exchange data (Fig 3-5).

The rates of exchange with Ba\(^{2+}\) are essentially the same for regions 29-40, 111-114, and 43-53. Ba\(^{2+}\) also causes changes in residues 103-110, and 24-27 although the magnitude of these changes is not as great as with Ca\(^{2+}\).

These changes in exchange obviously have important structural implications because Ba\(^{2+}\) seems to bind the same sites, but in such a way as to inactivate the enzyme. These results are consistent with a picture in which Ba\(^{2+}\) binds competitively for the Ca\(^{2+}\)-binding site. The affinity of Ba\(^{2+}\) and Ca\(^{2+}\) is different with \(K_d\) values of 0.15 mM and 0.4 mM, respectively (23), and these differences in affinity may explain the difference in exchange seen at the Ca\(^{2+}\) binding sites.
Figure 3-5 Crystal structure of the *N. naja atra* GIA PLA2 with a Secondary Ca\(^{2+}\) Site Present. The deuterium exchange data from *N. naja naja* PLA2 was overlaid onto the *N. naja atra* structure (1POA) for two time points. Ca\(^{2+}\) shown in pink. Amino acids involved in binding the calcium ions are labeled. The color represents the change in deuterium levels.
3.D.4 Deuterium Exchange of GIA PLA₂ in the Presence of Lipid

One of the main goals of this research was to determine if deuterium exchange could yield information about how the soluble GIA PLA₂ interacts with large substrate aggregates. To that end deuterium exchange of GIA PLA₂ at time points varying from 15 sec to 1 hr was analyzed in the presence of 1mM small unilamellar vesicles (SUVs) of DPPC. These experiments were carried out in the absence of Ca²⁺ to prevent hydrolysis of the phospholipid vesicles. EDTA was included to insure that no free Ca²⁺ was present. Previous work in our lab has shown very low levels of enzyme binding to phospholipid without metal present, but that either Ca²⁺ or Ba²⁺ increased this level of phospholipid binding (25). For this reason experiments were also carried out using Ba²⁺ (data not shown) as a replacement for Ca²⁺, which achieved the exact same exchange patterns as with lipid in the absence of metal ions. Studies were done at 22 °C because it has been shown that the GIA PLA₂ has a significant lag phase with lipid above the phase transition temperature (26), so we desired our lipid surface to be only in the gel state. Most regions of the GIA PLA₂ show no change in deuterium exchange in the presence of DPPC lipid vesicles, except for one small region of the protein. This area is amino acids Ile9, and Lys10 (Fig. 3-6). The deuteration level of region 9-10 was calculated by subtracting the deuterium levels of peptide 1-8 from 1-10; the same decrease in exchange with lipid is seen in 5 other peptides (1-17, 1-21, 1-27, 5-21, 5-27) that include region 9-10. This effect is not seen in peptides 9-21, 9-17, 11-21, and 9-27 which do not include region 9-10 (noting that the first two amino acids fully exchange with the technique and are not included in the analysis). This shows that the effect is strictly limited to the region 9-10. There is a 20-30% change between the lipid free and DPPC containing protein samples.
Figure 3-6 Deuterium exchange of the GIA PLA$_2$ with areas of decreased exchange upon phospholipid binding in the presence of 1 mM EDTA. The number of incorporated deuterons at four time points is plotted for region 9-10. Changes were mapped onto the *N. naja naja* GIA PLA$_2$ crystal structure (1A3D).
Further experiments were done using 100 µM Ca\(^{2+}\) with phospholipid vesicles to minimally activate the enzyme. Original experiments using DPPC vesicles with 100 µM Ca\(^{2+}\) at 22 °C caused high levels of phospholipid hydrolysis. Further experiments were done at 0 °C to further slow hydrolysis so total phospholipid hydrolysis stayed below 10% over the deuterium on exchange time course. Due to the change in temperature, the lipid used was shifted from DPPC to DMPC. The phase transition temperatures of DPPC and DMPC are 41 and 23 respectively, so we used DMPC at 0 °C to view a similar physical state of the lipid as DPPC studies at 22 °C. Activity assays performed showed that at 300 seconds the enzyme had not hydrolyzed more than 10% of the lipid surface. Four regions of the protein, namely regions 3-5, 6-8, 18-21, and 56-64, had significant decreases in exchange upon exposure to lipid in the presence of calcium (Fig. 3-7). Deuterium content for region 6-8 was calculated through subtraction of peptides 1-5 from 1-8, and deuterium content of region 18-21 was calculated through subtraction of peptides 1-17, from 1-21.
Figure 3-7 Deuterium exchange of the GIA PLA₂ upon DMPC vesicle binding at 0 °C in the presence of 100 µM Ca²⁺. The number of incorporated deuterons at 4 time points are plotted for regions that show changes upon DMPC binding. The changes in deuterium exchange are shown mapped on the *N. naja naja* GIA PLA₂ crystal structure (1PSH) with the calcium ion shown in pink. The color represents the changes in deuterium levels.
Regions 3-5, and 6-8 are part of the first alpha helix located on the i-face of the enzyme. Due to multiple overlapping peptides in this region we were able to localize changes in this helix. The largest change was seen from region 3-5 containing Tyr-3, and Phe-5 and had a 20-30% decrease in exchange over all timepoints. Interestingly from using peptide overlap data from peptide 1-4 and peptide 1-5, it seems that both amino acids 3-4 and amino acid 5 have independent decreases in exchange. Region 3-4 had a smaller decrease in exchange at 100 and 300 seconds, with residue 5 having a constant deuterium decrease. Region 6-8 also had a 10-20% decrease in exchange. The region 18-21 had a 5-10% decrease in exchange, and it contains Trp-18, and Trp-19. Region 56-64 had a 10-20% decrease in exchange until 100 seconds, with less than 10% at 300 seconds. The region 9-10 which had a decrease in exchange with lipid and no Ca\(^{2+}\) present at 22 °C (Fig 3-6) had zero on exchange at 0 °C up to 300 seconds, so no change could be detected with lipid in the presence of Ca\(^{2+}\) at these lower temperatures. Importantly, this experiment shows interaction of phospholipid with the i-face of the protein and demonstrates the potential of deuterium exchange mass spectrometry to study proteins that catalyze reactions at the lipid surface.

3.E Discussion

There are many crystal structures of GIA PLA\(_2\), but these structures only show a static picture of the enzyme under crystallization conditions. Using deuterium exchange mass spectrometry, we are able to study the protein in solution under varying conditions. This represents the first use of deuterium exchange mass spectrometry to probe the structure of a PLA\(_2\) and its interaction with a lipid surface. In the present study, we show
a region of the protein that is solvent accessible blocked from exchange by extensive disulfide bonds, the presence of a secondary Ca\(^{2+}\) binding site in the *N. naja naja* GIA PLA\(_2\) as well as showing the ability to probe lipid-lipase interactions with deuterium exchange mass spectrometry.

The on exchange experiments on the native GIA PLA\(_2\) showed that the three \(\alpha\)-helices exhibited dramatically different exchange rates. The N-terminal helix exchanges vary rapidly while the two core helices exhibit almost no exchange even though they are on the surface of the protein and at least some of the amide protons should be accessible to water. The difference is that helices 39 to 55 and 84-101 are involved in four disulfide bonds with other parts of the protein while the N-terminus is not. Other regions that are not involved in extensive disulfide bonding also show very fast deuterium exchange rates as would be expected for such a small solvent exposed protein. Studies performed on a recombinant macrophage colony stimulating factor-beta with nine disulfide bonds (6 intramolecular and 3 intermolecular), did not reveal this same lack of deuterium exchange in surface exposed disulfide bonding areas (19). That study concluded that exchange rates were mainly due to solvent accessibility. The exchange rates of \(\alpha\) helices in this protein were correlated to depth from solvent rather than conformational constraints from disulfide bonds. This protein is a dimer of two 221 amino acid subunits that is quite large and contains 9 disulfide bonds. The difference in the exchange rates between this protein and GIA PLA\(_2\) is because of the higher percentage of disulfide bonds in this protein and the localization of these bonds intertwined with the two \(\alpha\) helices. The amino acids participating in disulfide bonds are shown in figure 3 with six of the cysteine residues located on the two helices. This is a novel finding as it has not
been previously demonstrated that extensively disulfide bonded areas of proteins are held extremely rigidly by those bonds and that exchange with the solvent is restricted.

Because of this tight disulfide bond structure the protein is very resistant to both digestion and denaturation and needed extreme conditions to fragment the protein. This extremely tight disulfide bonded network is a possible evolutionary adjustment in toxin containing PLA_2s to protect against protease digestion in the venom or in their prey. Our use of a pre-digestion with TCEP was necessary to achieve these results and provides a procedure to now investigate other highly disulfide bonded proteins.

Two crystal structures of the *N. naja naja* GIA PLA_2 have been previously solved by our laboratory (7, 10). In both of these crystals, the enzyme was present as trimers. The *N. naja atra* GIA PLA_2 structure was solved as a dimer and contained two Ca^{2+}-binding sites per enzyme molecule (24). The structure of *N. naja naja* by Fremont et al. (10) contained Ca^{2+} in the primary binding site, but did not show this secondary Ca^{2+}. However the trimer structure is held together by an intermolecular salt bridge between Arg30 on one monomer and Asp23 on a second monomer. Asp23 is one of the essential residues in binding the secondary Ca^{2+} as shown in the *N. naja atra* crystal. This trimer interaction occurs at high enzyme concentrations and may block the incorporation of the secondary Ca^{2+} ion. We could not isolate Asp23 in the DXMS analysis because of the lack of overlap of peptides in this region.

The C terminal region from 103-114 also forms part of the secondary site. This region does show a decrease in exchange that would suggest the presence of Ca^{2+} in this site. This indicates that when the enzyme is in solution, Ca^{2+} is bound to the second site, and that the lack of the second Ca^{2+} in the original crystal structure was due to the trimer
contacts with Asp23. If a model is generated with the *N. naja naja* crystal structure containing a Ca\(^{2+}\) ion in the secondary Ca\(^{2+}\) binding site shown in the *N. naja atra* structure, the amino acids are in the correct position for Ca\(^{2+}\) binding.

Ba\(^{2+}\) showed decreases in deuterium exchange as did Ca\(^{2+}\), but the magnitude of the change was less. Decreases in deuterium exchange at the Ca\(^{2+}\) binding sites were lower in the presence of Ba\(^{2+}\) then at the same levels of Ca\(^{2+}\). This is most likely due to the different affinities of these ions for the enzyme of 0.15 mM for Ca\(^{2+}\) and 0.4 mM for Ba\(^{2+}\) (23). These results suggest that Ba\(^{2+}\) binds in exactly the same place as the Ca\(^{2+}\) ion, but there are differences in the affinity at the site of binding.

The interfacial activation of PLA\(_2\) has been an area of interest for many years. The use of DPPC vesicles, without Ca\(^{2+}\) present to probe lipid binding did not show changes in deuterium exchange in large areas of the protein. However there was a distinct effect in residues 9-10. This region contains isoleucine 9, which has been postulated to be one of the residues in the hydrophobic core in the active site of the protein based on the crystal structure (24, 27). This change in deuterium exchange may well be the interaction of the sn-2 fatty acid from the phospholipid with the hydrophobic residue.

Experiments carried out in the presence of low levels of Ca\(^{2+}\) with DMPC SUVs showed significant decreases in exchange in large areas of the enzyme proposed to bind the membrane interface. A hypothetical scheme of membrane binding of the GIA *N. naja naja* PLA\(_2\) is shown in Fig. 3-8.
Figure 3-8 Hypothetical model of GIA PLA₂ binding to a DMPC membrane. PLA₂ before binding the surface (left) and after associating with the membrane (right). Areas with decreases in exchange have been colored and amino acid residues Tyr-3, Phe-5, Lys-6, Trp-18, Trp-19, His-47, Trp-61, Tyr-63, Phe-64, and Asp-93 have been drawn in stick form. Figure was created in PyMol.
Our experiments showed decreases in exchange at regions containing Tyr-3, Trp-61, Tyr-63, and Phe-64. We propose that the aromatic residues here are inserting into the membrane bilayer. Mutations of the aromatic amino acids have been tested in the structurally similar N. naja atra GIA PLA₂ enzyme and found to significantly diminish interfacial activation (28). Similar experiments in the N. naja naja GIA enzyme showed mutations at Trp-61, Tyr-63, and Phe-64 also significantly reduced interfacial inactivation (8). Our experiments also show a decrease in region 6-8 containing Lys-6, which is in the correct orientation to interact with phosphate headgroups of the membrane surface. Experiments using the GIA PLA₂ inhibitor manoalide showed that reaction of manoalide with Lys-6 caused a large decrease in activity, maybe inducing an incorrect protein orientation at the surface (29). The deuterium exchange data suggests that the majority of the interactions between the enzyme and the interface are mediated by regions 3-8, and 56-64. The area 18-21 has a very small change in exchange compared to changes seen from 3-8 and 56-64. We propose that binding to a lipid surface is mediated by the aromatic and charged residues in these areas and blocks them from solvent exposure. Our experiments confirm that these areas of the enzyme are interacting with the membrane surface, but only upon exposure to Ca²⁺.

Our experiments also demonstrate a decrease at regions containing Phe-5, Ile-9, and Trp-19. We propose these amino acids are mediating binding of the fatty acyl tails of phospholipid substrate. Previous NMR studies carried out by our laboratory using an amide substrate analogue dispersed in micelles showed differences in Leu-2, Phe-5, Trp-19, Ala-22, and Phe 100 with GIA PLA₂ (11, 12). There also exists a crystal structure of the N. naja atra enzyme with an inhibitor bound showing hydrophobic interactions
between Leu-2, Phe-5, Ile-9, Trp-19, and Tyr-69 (27). Our deuterium exchange results very closely match the results seen in both inhibitor bound GIA PLA2’s. In our studies we were not able to isolate Leu-2, Ala-22, or Phe-100 due to very slow exchange in these regions, or lack of resolution. The changes seen at Ile-9, and Phe-5 have a constant level of decrease in exchange at all timepoints, where regions proposed to penetrate the membrane surface have smaller decreases in exchange at later timepoints. This suggests that substrate binds very tightly and blocks this area from on exchange at all timepoints, while the enzyme may hop on and off substrate, allowing surface penetrating residues to exchange at later timepoints.

3. F Conclusion

This study shows rigidification of the two extensively disulfide bonded helices, and the presence of a secondary Ca^{2+} binding site present in the N. naja naja group Group IA PLA2. Also we have shown that both of these sites show changes upon Ba^{2+} binding, but not at the same levels of exchange. This study also shows an interaction between the soluble enzyme and the lipid surface at both surface and substrate binding regions of the protein. This study marks a novel use of deuterium exchange mass spectrometry to study lipid-lipase interactions, as well as using novel digestion and denaturation methods to work with very highly disulfide bonded proteins.

3. G Acknowledgements

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3.H References


CHAPTER 4

Calcium Binding Rigidifies the C2 Domain and the Intra-Domain Interaction of GIVA Phospholipase A₂ as Revealed by Hydrogen/Deuterium Exchange Mass Spectrometry
4.A Abstract

The GIVA phospholipase A$_2$ (PLA$_2$) contains two domains: a calcium binding domain (C2) and a catalytic domain. These domains are linked via a flexible tether. GIVA PLA$_2$ activity is Ca$^{2+}$ dependent in that calcium binding promotes protein docking to the phospholipid membrane. In addition, the catalytic domain has a lid that covers the active site, presumably regulating GIVA PLA$_2$ activity. We now present studies that explore the dynamics and conformational changes of this enzyme in solution utilizing peptide amide hydrogen/deuterium exchange coupled with liquid chromatography-mass spectrometry (DXMS) to probe the solvent accessibility and backbone flexibility of the C2 domain, the catalytic domain and the intact GIVA PLA$_2$. We also analyzed the changes in H/D exchange of the intact GIVA PLA$_2$ upon Ca$^{2+}$ binding. The DXMS results showed a fast H/D exchanging lid and a slow exchanging central core. The C2 domain showed two distinct regions: a fast exchanging region facing away from the catalytic domain and a slow exchanging region present in the “cleft” region between the C2 and catalytic domains. The slow exchanging region of the C2 domain is in tight proximity to the catalytic domain. The effects of Ca$^{2+}$ binding on GIVA PLA$_2$ are localized in the C2 domain and suggest that binding of two distinct Ca$^{2+}$ ions causes tightening up of the regions that surround the anion hole at the tip of the C2 domain. This conformational change may be the initial step in GIVA PLA$_2$ activation.
4.B Introduction

The cytosolic GIVA phospholipase A2 (PLA2), also known as cPLA2, was purified and cloned in 1991 (1, 2). It is one of the few phospholipases in the phospholipase A2 superfamily shown to be important in lipid mediator biosynthesis (3, 4). GIVA PLA2 hydrolyzes membrane phospholipids at the \( sn-2 \) position to release free arachidonic acid, which is the precursor of numerous eicosanoids including the prostaglandins and leukotrienes (5, 6), involved in the inflammatory and pain response (6-8). Understanding the regulation of the catalytic activity of GIVA PLA2 is crucial for understanding eicosanoid metabolism.

The activity of the 85-kDa GIVA PLA2 has been suggested to be regulated by several factors including the intracellular \( Ca^{2+} \) concentration, its phosphorylation state and the binding of various activators. Since the GIVA PLA2 was discovered in 1986, the activity of this enzyme has been known to be \( Ca^{2+} \) dependent (9). The \( Ca^{2+} \)-dependent lipid-binding domain (C2 domain) at the N-terminus which is linked to a catalytic domain where phospholipid hydrolysis occurs, was later identified (10). Further studies of the GIVA PLA2 activity showed upregulation by p38 protein kinase mainly through Ser505 phosphorylation (11-13). Various membrane associated activators have been shown to bind to GIVA PLA2 and upregulate its activity. In particular, we have shown that the membrane associated phosphatidylinositol-(4,5)bis-phosphate (PtdIns(4,5)P_2) can bind to the “lysine pocket” of GIVA PLA2 with high affinity and specificity to activate the GIVA PLA2 independent of \( Ca^{2+} \) (14, 15). Another membrane associated activator, ceramide-1-phosphate (C1P), has been shown to bind to the cation groove of the C2 domain to activate GIVA PLA2 in a \( Ca^{2+} \) dependent manner (16, 17).
Calcium binding is crucial for GIVA PLA$_2$ activation. Two calcium ions coordinate with the calcium binding loops, CBL1, CBL2 and CBL3, in the C2 domain, which is illustrated in known C2 domain structures (18-20). These two Ca$^{2+}$ ions neutralize the negative charge in the anion hole to facilitate the C2 domain’s hydrophobic interaction with phospholipid membranes (19, 20). Various models of membrane penetration for the GIVA PLA$_2$ have been proposed to explain the hydrolysis activity of GIVA PLA$_2$ (21, 22). However, the X-ray crystal structure cannot fit the model perfectly, unless there is a slight twist of the catalytic domain. Intra-domain interactions may change the position of the C2 domain relative to the catalytic domain from that assumed in the crystal structure interpretation. Also, structural information regarding the calcium free form of GIVA PLA$_2$ is limited, because its NMR and X-ray crystal structure are not available. It is necessary to analyze the entire GIVA PLA$_2$ and especially its intra-domain interactions to fully understand the regulation of GIVA PLA$_2$.

Peptide amide hydrogen/deuterium exchange coupled with liquid chromatography mass spectrometry (DXMS) has been widely used to analyze the interface of protein-protein interactions (23, 24), protein conformational changes (25, 26), and protein dynamics (27, 28). The present study represents the first attempt to use DXMS to study the intra-domain interactions and the Ca$^{2+}$ binding effect on the GIVA PLA$_2$. This technique should complement what can be learned from NMR and X-ray crystallographic analysis. Our results describe the intra-domain interactions between the C2 domain and the catalytic domain and confirm that the major conformational responses to Ca$^{2+}$ are localized to the calcium binding loop of the C2 domain. A tightened conformation of the
C2 domain appears upon Ca\(^{2+}\) binding. Herein, we explore the resultant effects on the catalytic domain.

4.C Experimental Procedures

4.C.1 Materials

All reagents were analytical reagent grade or better.

4.C.2 Protein Expression and Purification

C-terminal His\(_{6}\)-tagged GIVA PLA\(_{2}\), the C2 domain and the catalytic domain were expressed using recombinant baculovirus in a suspension culture of Sf9 insect cells (14). The cell pellet was lysed in 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM β-mercaptoethanol, and 2 mM EGTA and then the insoluble portion was removed by centrifugation at 12,000\(\times\)g for 30 min. The supernatant was passed through a column comprised of 6 ml nickel-nitrilotriacetic acid agarose (Qiagen, Valencia, CA). The protein in the native state was eluted in the “protein buffer” (25 mM Tris-HCl pH 8.0, 100 mM NaCl, 125 mM imidazole and 2 mM dithiothreitol). The protein concentration was measured using the Bradford assay and the activity was assayed using mixed micelles in the modified Dole assay (29, 30). Purified GIVA PLA\(_{2}\) (2 mg/ml) was stored in the protein buffer on ice for DXMS experiments.
4.C.3 Preparation of Deuterated samples

D₂O buffer contained 50 mM HEPES (pH 6.9), 100 mM NaCl, 2 mM dithiothreitol (DTT), (+/-)200 µM CaCl₂. Hydrogen/deuterium exchange experiments were initiated by mixing 20 µl of GIVA PLA₂, the C2 domain or the catalytic domain (containing 40 µg) in protein buffer with 60 µl of D₂O buffer to a final concentration of 70.4% D₂O at pH 7.0. In calcium binding experiments, the GIVA PLA₂ in protein buffer was pre-incubated in the presence of 240 µM CaCl₂ in a 23 °C water bath for 5 min. The D₂O buffer was then added and the samples were incubated at 23°C for an additional 10 s, 30 s, 100 s, 300 s, 1000 s, 3000 s or 200 min. Each time point of the intact GIVA PLA₂ was repeated three independent times. The deuterium exchange was quenched by adding 120 µl of ice-cold quench solution (0.96% formic acid, 1.66 M guanidine hydrochloride) that acidified the sample to a final pH = 2.5. The samples were placed on ice at the quenched condition for 10 min to partially denature the protein for the purpose of better peptide map coverage and then frozen on dry ice. Fully deuterated samples were incubated in 2.5 M guanidine hydrochloride, 75% D₂O at 23°C for two days. Vials with frozen samples were stored at −80 °C until analysis, usually within three days.

4.C.4 Proteolysis-liquid-chromatography-mass spectrometry analysis of samples

All steps were performed at 0°C as previously described (24, 26). The samples were hand-thawed on melting ice and injected onto and passed through a protease column (66 µl bed volume) filled with porcine pepsin (Sigma; immobilized on Poros 20 AL medium at 30 mg/ml following the manufacturer's instructions, Applied Biosystems), at a flow rate of 100 µl/min with 0.05% trifluoroacetic acid (TFA). The eluate from the
pepsin column was directly loaded onto a C18 column (Vydac cat #218MS5150). The peptides were eluted at 50 µl/min with a linear gradient of 0.046% TFA, 6.4% (v/v) acetonitrile to 0.03% TFA, 38.4% acetonitrile for 30 min. The eluate from the C18 column was directed to a Finnigan Classic LCQ mass spectrometer via its ESI probe operated with a capillary temperature of 200 °C as previously described (24, 26).

4.C.5 Data processing

SEQUEST software (Thermo Finnigan Inc.) was used to identify the sequence of the peptide ions. DXMS Explorer (Sierra Analytics Inc, Modesto CA) was used for the analysis of the mass spectra as previously described (24, 26). All selected peptides had first passed the quality-control threshold of the software and were then manually checked for the mass envelope fitting with the calculated mass envelope for data reduction. The highest signal/noise ion was picked if multiple ionization charges (1, 2 or 3) of a peptide were detected. Normally, the peptide with lower charge gave a better signal. Incorporated deuteron number was the centroid shift between the non-deuterated and the partially deuterated mass envelope. The deuteration level of each peptide was calculated by the ratio of the incorporated deuteron number to the maximum possible deuteration number.

4.D RESULTS

4.D.1 GIVA PLA₂ Coverage Map of Pepsin Fragmentation

The protein digestion procedure was optimized to produce a peptide map that yielded the best coverage of GIVA PLA₂. These optimizations included testing various denaturants and denaturing conditions, denaturation times, amounts of protein, and flow
rates for the online pepsin digestion. The final method is described in the Experimental
Procedures. The optimized condition gave 92% coverage of the GIVA PLA₂ sequence
with 157 distinct peptic peptides being identified, see Figure 4-1. The C2 domain and the
catalytic domain alone generated the same digestion pattern as their respective parts did
in the intact GIVA PLA₂. There are 58 unresolved amino acid residues. These are due to
regions without identified peptides and the fact that the first two residues of each peptide
are rapidly exchanging and do not retain deuterons during the processing. Although we
analyzed all 157 peptides, some regions of the protein contained several overlapping
peptides. In some cases, comparing the deuterium levels of overlapping peptides will
yield information about the deuterium levels of a subsection of the peptide. Thus, one can
obtain information about regions of the protein smaller than the peptides covering it. In
many cases, however, the orientation of the overlapping peptides is such that no
additional information can be obtained. Also, in some cases, the error propagation
through these calculations yields large variances that prevent further analysis. Because of
these latter two reasons, peptides that could not provide additional information were
eliminated. In the end, 59 peptide fragments, which cover 82% of the protein, were
employed. In this article, when we use the term "peptide", we are referring to an actual
peptide that was identified in the pepsin digestion. When we use the term "region", we
are referring to a section of the protein for which the deuterium exchange has been
calculated but may or may not correspond to an actual pepsin peptide.
4.D.2 Deuterium on-exchange of GIVA PLA₂

Full deuteration was also tried under various conditions such as two days of exchange in 0.5 % formic acid, high temperature exchange for four hours (50°C), and partial denaturation in 2.5 M guanidine hydrochloride. The resulting low deuteration observed for the α/β hydrolase region indicated that the protein was not fully deuterated under all conditions. Peptide fragment 266-279 containing a solvent exposed loop-helix structure had the highest deuteration level (89%). Hence, this peptide was used as a system back-exchange control. Back-exchange (12%) according to the fully deuterated control and the deuteration level (70.4%) was corrected for the centroid shifts.

Deuterium on-exchange experiments of GIVA PLA₂ (+/-) Ca²⁺ were incubated at pH 7.0, 23 °C for seven different time points ranging from 10 s to 200 min, and were processed as described above. Under these conditions of temperature, pH and time, the deuteration levels of the peptides were well distributed between 3-90% and there was a 12% back-exchange. The deuteration level after deuterium on-exchange over the time course is shown in Figure 4-2. Figure 4-1 shows the actual physical peptides that were isolated, detected, and analyzed via DXMS. Figure 4-2 shows the deuterium exchange levels of various regions of the protein, not the peptides per se. The presence of overlapping peptides in Figure 4-1 allows one to define the deuteration level of regions of the protein smaller than the peptides by comparing the deuterium levels of several overlapping peptides. Note that the first two N-terminal residues of each pepsin-digested DXMS peptide do not retain deuterium after the pepsin and C18 columns, this fact is also taken into account when calculating the deuteration levels of the protein regions shown in Figure 4-2 (26, 31).
Figure 4-1 Pepsin-digested peptide coverage map of GIVA PLA₂. Identified and analyzed peptides resulting from pepsin-digestion are shown below the primary sequence of GIVA PLA₂. Only the peptides shown as solid lines were used in this study.
Figure 4-2 Deuterium exchange of the GIVA PLA2 in the presence and absence of Ca²⁺. There are two main sets of colored bars, one for exchange with Ca²⁺ and one without Ca²⁺. Each bar is divided into rows corresponding to each time point from 10s to 200 min (top to bottom). The color coding indicates the percent of H/D exchange in the given time period.
The deuteration level after 3000 s on-exchange was plotted onto the GIVA PLA$_2$ structure (1CYJ.PDB) using a color index to illustrate the H/D exchange results for the structure as shown in Figure 4-3. The C2 domain consists of 8 antiparallel $\beta$ strands that are roughly divided into two domains. The first contains the $\beta$6, $\beta$7 and part of the $\beta$8 strands (which includes residues 6-13, 28-39, 73-76 and 125-129) and exhibits fast deuterium exchange. The second domain contains $\beta$1, $\beta$2 and $\beta$5 strands (which include residues 16-25, 41-48 and 91-101) and exhibits slow H/D exchange. The fast exchanging regions of the C2 domain tend to face away from the catalytic domain.

The slow exchanging regions are on the opposite side of the C2 domain and face the cleft between the C2 and catalytic domains. The crystal structure indicates that there is a significant amount of open space between these two domains, but of course the crystallographic data reflects the crystal packing rather than the solution conformation. This would predict that there would be significant accessibility of water to the internal face of the C2 domain, see Figure 4-3. The slow exchange in this region of the C2 domain could be due to the rigidity of the protein structure here or it could be due to the fact that when free in solution the C2 domain is folded against the catalytic domain.

There are five regions in the X-ray crystal structure that could not be resolved because of the flexibility of the chains. These are indicated in Figure 4-3 as dashed lines. It should be noted, that the DXMS method was able to detect and measure H/D exchange in each of these regions. As expected these regions showed significant levels of exchange. Two of these regions are the hinges for the catalytic site lid (20).
Figure 4-3 Deuteration level of GIVA PLA₂. The deuterium exchange map of GIVA PLA₂ is shown after 3000 s of on-exchange with the color coding indicating the percentage of H/D exchange. There are five regions in the protein for which there is no crystal structure information and these regions are shown by a dashed line. The line color indicates the exchange rates detected by DXMS, but the position and length of these dashed lines are not based upon any structural information and were added simply to show the DXMS data.
The catalytic domain contains a cap region (residues 370-548), a central core (residues 139-369 and 549-719) and a c-terminal tail (residues 720-742). The cap region contains a high percentage of fast exchanging residues. They include residues 403-441, 458-478, 501-509, and 524-542. The lid (residues 413-457) in the cap region that regulates the active site also shows fast exchange. The central core contains two regions (335-342 and 622-632) that show fast exchange. Most of the α/β central core exhibits slow exchange (residues 207-211, 223-237, 294-298, 365-371, 572-576, 594-599, 633-642, 692-699 and 709-714). The C-terminus (residues 720-742) and N-terminus also exhibit fast exchange.

4.D.3 Deuterium on-exchange of the C2 domain and the catalytic domain of GIVA PLA₂

To test the hypothesis that there is a dynamic interaction between the C2 domain and the catalytic domain, we expressed and purified His₆-tagged C2 and His₆-tagged catalytic domains individually and conducted DXMS experiments on both under the same conditions as the intact GIVA PLA₂. The differences in the deuteration level between the C2 domain alone and the intact GIVA PLA₂ are shown in Figure 4-4. The C2 domain alone showed greater than a 20% increase of solvent accessibility (residues 41-48, 91-95 and 51-71) on β2, β3, β4 and β5 in the cleft. Most regions in β1, β6, β7 and β8 outside of the cleft were unchanged, except for region 125-129 which was the only one showing a greater than 10% increase, but it was not significant after 100 seconds. This result indicates that the catalytic domain either interacts with the C2 domain or it
changes the conformation of the C2 domain through the linker region, leading to an increase in its solvent accessibility (Figure 4-4).

To find out if the increase in the solvent accessibility of the C2 domain is through the contact with the catalytic domain, we carried out hydrogen/deuterium exchange on the isolated catalytic domain (Figure 4-5). The DXMS of the catalytic domain showed over a 20% increase in the region of residues 363-372, 373-388, 389-397 and 494-498 in the cleft. Also, several regions in the CAP (residues 254-265, 266-279, 280-291, 292-298, 479-495 and 541-553) showed a 10-20% increase. The effect of the C2 domain on the catalytic domain is much more than the linker region and the contact region next to the linker region shown in the crystal structure, which indicates a more extensive interaction between the two domains.

When we mixed the C2 domain and the catalytic domain in a one to one molar ratio for DXMS, the result was the same as running them individually (Data not shown.) No evidence for an interaction between the two domains was observed. Clearly, the linker region is necessary for the interaction between the C2 and catalytic domains. Comparison of the DXMS results of the C2 domain and the catalytic domain alone to the intact GIVA PLA2 allowed us to localize where the C2 domain interacts with the catalytic domain. This result supports the hypothesis that there is an interaction between the C2 and the catalytic domain and allowed us to map out the interaction regions between them.
Figure 4-4 Differences in the deuteration level of the C2 domain alone in comparison to the C2 domain in the intact GIVA PLA₂. The percentage of deuteration at 7 time points showed a maximum of over 20% increase in three regions in red (residues 41-48, 91-95 and 51-71) of the C2 domain alone (■) over the C2 domain in the intact GIVA PLA₂ (○). The regions in orange showed a maximum of 10-20% increases and are labeled with the residue number.
Figure 4-5 Differences in the deuteration level of the catalytic domain alone in comparison to the catalytic domain in the intact GIVA PLA₂. The percentage of deuteration at 7 time points showed a maximum of over 20% increases in four regions in red (residues 363-372, 373-388, 389-397 and 494-498) of the catalytic domain alone (■) over the catalytic domain in the intact GIVA PLA₂ (◇). The regions in orange showed a maximum of 10-20% increases and are labeled with the residue number. The area in black has no DXMS data.
4.D.4 Calcium binding effects on GIVA PLA$_2$

The Ca$^{2+}$ binding site on GIVA PLA$_2$ consists of the three distinct Ca$^{2+}$ binding loops CBL1, CBL2 and CBL3. This region has been shown to be important for lipid interface binding mediated by CBL1 and CBL3 (21, 32, 33). We carried out the on exchange in the presence and absence of 200 µM Ca$^{2+}$. The two Ca$^{2+}$ ions coordinate with D40, T41, and D43 in CBL1, with N65 in the CBL2 region, and D93, A94, and N95 in CBL3. T41 and D43 in CBL1 exhibit slow exchange in the absence of Ca$^{2+}$, see Figure 4-6. There was only an additional 5% decrease in H/D exchange at 200 min when Ca$^{2+}$ was bound. Residue D40 in CBL2 is in a gap between two resolved DXMS peptides, and thus no H/D exchange information for this residue. D93, A94, and N95 in CBL3 are located in the DXMS peptide 91-101 and this peptide exhibited only a 10% H/D exchange after 200 min without Ca$^{2+}$. Its exchange rate did not change with Ca$^{2+}$. N65 in CBL2 is the only residue that is located on a DXMS peptide that showed a 10% decrease in H/D exchange upon Ca$^{2+}$ binding, see Figure 4-6. However, the resolution in this region was not sufficient to assign the H/D exchange changes to N65.
Figure 4-6 Deuterium exchange in the Ca$^{2+}$ binding site of the C2 domain. The number of incorporated deuterons at 7 time points in two regions, 41-48 and 91-101 of the C2 domain of the intact GIVA PLA$_2$, are plotted in the presence (■) and absence of Ca$^{2+}$ (▲). The calcium binding residues are shown in stick representation. In the figure, the region in green corresponds to residues 91-101 and the region in orange corresponds to residues 41-48. The error bars are based on the standard deviation of triplicates.
There were, however, dramatic changes in other areas of the C2 domain that indicated a conformational change in the protein. Four regions in the C2 domain had a significantly decreased H/D exchange rate; these were residues 26-39, 51-71, 102-106 and 125-129. The incorporated deuterium number over the time course in these four regions is plotted in Figure 4-7. The average change in deuteration level of the 4 time points from 5-200 min were also mapped onto the GIVA PLA2 structure in the same figure.

Residues 28-35 on CBL1 exhibited a 22% decrease in deuteration levels upon Ca\(^{2+}\) binding. We have four DXMS peptides that cover the region from 26-39, including 26-35, 26-38, 26-39, and 26-48. All four peptides showed between a 1 and 2 deuteron decrease after Ca\(^{2+}\) binding. By comparing the H/D exchange of the peptides 26-35, 26-38 and 26-39, we determined that the protein region 36-39 is not affected by calcium binding and retained about 3 deuterons that accounts for 75%-80% deuteration. Interestingly, residues 28-35 decreased the incorporation of 2 deuterons caused by binding of Ca\(^{2+}\).

Two DXMS peptides, 49-71 and 49-74 containing CBL2, showed an 11% deuteration level decrease upon Ca\(^{2+}\) binding in this region. Residues 102-106, which are next to CBL3, showed a 22% deuteration level decrease upon Ca\(^{2+}\) binding. Comparing DXMS peptides 115-129 and 115-124 showed that protein residues 125-129 on the β6-β7 loop showed a 13% deuteration level decrease upon Ca\(^{2+}\) binding.

We also examined H/D exchange levels on the rest of the protein and found no significant change in the H/D exchange levels in the catalytic domain. It is worth noting that the linker region (residues 139-146) showed a 6% solvent accessibility decrease upon
calcium binding, which suggests minor interaction or orientation changes between the C2 and the catalytic domain.

**Figure 4-7 Ca\(^{2+}\) binding effects on deuterium exchange of the intact GIVA PLA\(_2\).**

The number of incorporated deuterons in the C2 domain of GIVA PLA\(_2\) in the presence (■) and absence of Ca\(^{2+}\) (▲) are shown for regions in which deuterium exchange decreased upon Ca\(^{2+}\) binding as indicated in green or blue on the structural diagram. The decreased percentage of deuteration mapped onto the GIVA PLA\(_2\) structure was calculated by taking the average of the last three time points shown in the plots. The two calcium ions are shown in pink. The error bars are based on the standard deviation of triplicates.
4. E DISCUSSION

The DXMS results provide a detailed H/D exchange rate profile of the catalytic domain. The crystal structure of the GIVA PLA$_2$ contains a lid in the cap region that is tethered to the rigid catalytic core by two very flexible peptides and that blocks the catalytic site in the crystal structure. The lid apparently must move out of the way so that substrate can bind to the catalytic site. It has also been suggested that the cap is involved in membrane association (22). Our deuterium exchange results are consistent with these hypotheses. The lid and the tethers on either side of it show fast exchange. Interestingly, the deuterium DXMS method was able to detect and measure the exchange of the two tethers which could not be seen via X-ray crystallography and have confirmed that they have fast exchange. These results are consistent with the hypothesis that the lid is a flexible regulatory structure that can move when the enzyme interacts with lipid interfaces to expose the active site in the rigid catalytic domain.

The GIVA PLA$_2$ has been shown to translocate to the Golgi and perinuclear regions of cells upon the release of Ca$^{2+}$ (34-36). This movement has been suggested to be one important way for the cell to control the action of this enzyme and eicosanoid production. The C2 domain is clearly responsible for this action. The Ca$^{2+}$-dependent membrane docking of the C2 domain alone of this and many other proteins has been extensively studied to understand how its structure affects membrane penetration and the sequestration of the enzyme at the lipid surface. It is believed that the C2 domain allows the GIVA PLA$_2$ to bind to the membrane and that it positions the active site in a way that facilitates the binding of the substrate. Two Ca$^{2+}$ ions coordinate with Asp and Asn residues in the anion hole on the tip of the C2 domain to neutralize negative charges in
this region. The hydrophobic residues on the tip of CBL1 (Phe35, Met38 and Leu39) and CBL3 (Tyr96, Val97 and Met98) interact with the hydrophobic phospholipid headgroup (19). After the binding to the phospholipid bilayer, the dissociation rate of Ca\(^{2+}\) decreases (37, 38). Ca\(^{2+}\) binding may cause a conformational change of the C2 domain which effectively hides the hydrophilic residues and exposes the hydrophobic residues.

One of the most dramatic differences that we observed occurred in the C2 domain. The surface of the C2 domain that faces away from the catalytic domain showed significant H/D exchange while the opposite surface that faces the catalytic domain showed slow exchange. H/D exchange rates are affected by accessibility to water and can reflect the rigidity of the protein secondary structure and the strength of the hydrogen bonds. Both of these regions are composed of antiparallel \(\beta\) sheets and one might expect that they would have the same structural characteristics and thus the H/D exchange rates would be similar. The decreased exchange on the surface facing the catalytic domain could be due to the fact that in solution the C2 domain rests up against the catalytic domain and thus water accessibility is decreased. The slow exchange regions in the cleft between the C2 domain and the catalytic domain had over a 20% increase of the solvent accessibility when we conducted the DXMS experiments on the C2 domain or the catalytic domain alone. This would imply that the cleft delineated in the x-ray structure is in a different orientation of the protein when there is no calcium binding. It should be noted that the C2 and catalytic domains are held together by a single short peptide linker that should be fairly flexible. The interaction or the contact regions between the C2 domain and the catalytic domain can be mapped out by the solvent accessibility difference of the two individual domains and the intact GIVA PLA\(_2\) (Figure 4-4, 4-5).
To compare the effects of calcium binding on structural perturbations and charge neutralization, the electrostatic potential and the Ca\textsuperscript{2+} binding effects measured by DXMS were plotted onto the molecular model of GIVA PLA\textsubscript{2} (Figure 4-8). The anion hole surrounded by CBL1, CBL2 and CBL3 on the top of the C2 domain is highly negatively charged. The cleft between the C2 and catalytic domains has a low to positive electrostatic potential (Figure 4-8a). We also showed low solvent accessibility of the β2 and β5 sheets (Figure 4-3). The linker region between the C2 domain and the catalytic domain is possibly a flexible loop, because the regions 132-136 and 147-156 may be over-digested by pepsin and were not covered in the peptide map (Figure 4-1). This evidence suggests that the C2 domain is probably in physical contact with the catalytic domain. Additionally, the two Ca\textsuperscript{2+} ions bind directly to the anion hole to neutralize the negative charge (Figure 4-8a).
Figure 4-8 The electrostatic potential and the decreased deuteration level of the C2 domain in the intact GIVA PLA2. (a) The electrostatic potential mapped to the molecular surface is calculated by Swiss-Pdb Viewer 3.7, based on the simple coulomb interaction. (b) The decreased deuteration level is mapped to the molecular surface with the same orientation as the electrostatic potential.
We observe the largest change in electrostatics in the anion hole; however, the overall decreased H/D exchange regions do not occur in this region (Figure 4-8b). The most significantly decreased regions are on either side of the anion hole. Figure 4-6 shows that the regions that directly bind Ca$^{2+}$ have very slow exchange that did not change upon calcium binding. This suggests that the anion hole has either a low D$_2$O accessible/exchanging location or is very rigid. On the other hand, the surfaces surrounding the anion hole showed more exchange without calcium and showed decreased H/D exchange rates upon calcium binding. Both the C2 surface facing away from the catalytic site and the surface facing it showed decreased H/D exchange upon Ca$^{2+}$ binding. Since both of these surfaces have a decreased exchange and there is nothing on the outside surface that would hinder access to water, we conclude that the observed effects are due to Ca$^{2+}$ tightening up the pleated sheet structure in these regions and thus stabilizing the hydrogen bonds. CBL1 and CBL3 are both components of the anion hole. These two loops show slow exchange on the side of the anion hole and fast exchange on the molecular surface. These results are consistent with the proposed membrane penetration model (22) and indicate that the conformational change and negative charge neutralization occurs immediately after Ca$^{2+}$ binding.

4.F Conclusion

DXMS has allowed us to observe the interaction between the C2 domain and the catalytic domain and the effects of Ca$^{2+}$ binding on the intact GIVA PLA$_2$ from a unique perspective. The C2 domain shows extensive interaction with the catalytic domain beyond the linker region. While the calcium binding effects are mainly on the C2 domain,
Ca\textsuperscript{2+} binding may extend its effects and have implications for the catalytic domain. Binding of two Ca\textsuperscript{2+} ions cause a conformational change on the C2 domain, tightening up the regions surrounding the anion hole. The rigidified conformation of the C2 domain constitutes an initial step leading to enzyme interfacial activation, but this work shows clearly that changes in the cap and lid of the catalytic domain also play a critical role in enzyme interactions and the full catalytic activity of the enzyme.

4.G Acknowledgements

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4.H References


CHAPTER 5

A Phospholipid Substrate Molecule Residing in the Membrane Surface Mediates
Opening of the Lid Region in Group IVA Cytosolic Phospholipase A_2
5.A Abstract

The Group IVA phospholipase A$_2$ (GIVA PLA$_2$) associates with natural membranes in response to an increase in intracellular Ca$^{2+}$ along with increases in certain lipid mediators. This enzyme associates with the membrane surface as well as binding a single phospholipid molecule in the active site for catalysis. Employing deuterium exchange mass spectrometry (DXMS), we have identified the regions of the protein binding the lipid surface, and conformational changes upon a single phospholipid binding in the absence of a lipid surface. Experiments were carried out using natural palmitoyl arachidonyl phosphatidylcholine vesicles with the intact GIVA enzyme as well as the isolated C2 and catalytic domains. Lipid binding produced changes in deuterium exchange in eight different regions of the protein. The regions with decreased exchange included Ca$^{2+}$ binding loop one, which has been proposed to penetrate the membrane surface, and a charged patch of residues which may be important in interacting with the polar head groups of phospholipids. The regions with an increase in exchange are all located in either the hydrophobic core underneath the lid region or near the lid and hinge regions from 403-457. Using the GIVA PLA$_2$ irreversible inhibitor methyl-arachidonyl fluorphosphonate (MAFP) we were able to isolate structural changes caused only by pseudo-substrate binding. This produced results that were very similar to natural lipid binding in the presence of a lipid interface with the exception of the C2 domain and region 466-470. This implies that most of the changes seen in the catalytic domain are due to a substrate mediated, not interface mediated, lid opening which exposes the active site to water. Finally experiments carried out with inhibitor plus phospholipid vesicles showed decreases at the C2 domain as well as charged residues on the putative membrane
binding surface of the catalytic domain revealing the binding sites of the enzyme to the lipid surface.

5.B Introduction

The 85 kDa GIVA phospholipase A2 (GIVA PLA2) is a member of the superfamily of phospholipase A2 enzymes (1, 2) that cleave fatty acids from the sn-2 position of membrane phospholipids. This enzyme was initially isolated from human platelets and it is specific for phospholipids containing arachidonic acid in the sn-2 position (3, 4). The release of arachidonic acid is the critical first step in the biosynthesis of eicosanoids, which are potent mediators of inflammation and pain (5). There are a number of enzymes and routes by which arachidonic acid can be released from phospholipids, but experiments with GIVA PLA2 knockout mice have demonstrated its importance in many inflammatory processes (6-8) thereby confirming the key role of the GIVA enzyme.

The enzyme is composed of two domains, a Ca\(^{2+}\) binding C2 domain and a \(\alpha/\beta\) hydrolase domain that contains the catalytic site (9). Crystal structures of both the intact enzyme (10) and the C2 domain alone (11) have been solved. For the enzyme to be active, it must be sequestered to a phospholipid interface. Binding Ca\(^{2+}\) to the C2 domain accomplishes this as does the binding of two different lipid mediators, e.g. ceramide 1 phosphate (C1P) (12, 13) and phosphatidylinositol-(4, 5) bis-phosphate (PtdIns (4, 5) P2) (14, 15).

The Ca\(^{2+}\) binding C2 domain is a conserved domain that is present on many different lipid binding proteins (16). Ca\(^{2+}\) binding to this domain sequesters the protein to
the lipid surface. Extensive studies have been carried out on the C2 domain using a variety of techniques to determine how Ca$^{2+}$ binding accomplishes lipid surface binding. These studies have included x-ray reflectivity, site directed mutagenesis, NMR, EPR, and computational methods (17). These studies have shown (18-23) that lipid binding entails the penetration of Ca$^{2+}$ binding loops one and three, composed of amino acids 35-39, and 96-98, into the interface. However these studies only deal with C2 binding to the membrane, not the intact cPLA$_2$ enzyme. How the presence of the α/β hydrolase domain affects surface binding has not been determined in detail due to the difficulties of working with such a large protein.

Unlike the C2 domain, the binding of the catalytic domain of the enzyme to the lipid surface has not been extensively studied. Numerous studies using site directed mutagenesis of the intact protein have localized amino acids that are important for lipid binding and activation by secondary lipid mediators, as well as activation by phosphorylation of specific residues, especially residue Ser-505 (24, 25). We have also shown that there is an increase in activity upon binding PtdIns (4, 5) P2 containing lipids in a specific lysine binding pocket (14, 15). The enzyme has been shown to be minimally active on monomeric lipid substrates, but has substantial activation upon binding an interfacial surface (9). The enzyme also contains a lid region spanning amino acids 415-432 that covers the active site. Many different lipase proteins exist in both a closed lid conformation and upon lipid binding the enzyme shifts to an open lid conformation causing interfacial activation (26-28). Many of these open lid conformations have been crystallized in the presence of an inhibitor binding in the active site (26). Other than the crystal structure of the native GIVA PLA$_2$ with the lid region obstructing the active site,
there has been no evidence for this mechanism in the GIVA enzyme. The technique of deuterium exchange mass spectrometry (DXMS) is well equipped to examine the issues of the catalytic domain binding the lipid surface, as well examining the conformational changes upon the lid region opening. We have recently demonstrated for the first time that DXMS can be used to characterize membrane lipid binding to a PLA₂ (29).

Peptide amide hydrogen deuterium exchange analyzed via liquid chromatography/mass spectrometry has been widely used to analyze protein-protein interactions (30, 31), protein conformational changes (32, 33) and protein dynamics (34). We have recently conducted deuterium exchange studies on the GIVA enzyme showing intra domain interactions of the enzyme as well as structural changes caused by Ca²⁺ binding (35). This technique is an excellent way to probe the effects of lipid binding across the entire enzyme. The present study represents a continuation of our studies of PLA₂ binding a lipid surface (29). We have now identified specific regions of the GIVA PLA₂ that bind the lipid surface, specifically regions 28-39, 268-279, and 466-470. We now have also demonstrated conformational changes in regions 391-397, 481-495, and 543-553 hypothesized to be due to the conversion from the closed to the open lid conformation of the enzyme induced by natural phospholipid as well as the specific inhibitor MAFP binding to the active site of the enzyme.
5.C Materials and Methods

5.C.1 Materials

All reagents were analytical reagent grade or better.

5.C.2 Protein Expression and Purification

C-terminal His\textsubscript{6}-tagged GIVA PLA\textsubscript{2}, and the C2 and catalytic domains were expressed using recombinant baculovirus in a suspension culture of Sf\textsubscript{9} insect cells. The cell pellet was lysed in 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM β-mercaptoethanol, and 2 mM EGTA and then the insoluble portion was removed by centrifugation at 12,000 \texttimes g for 30 min. The supernatant was passed through a column comprised of 6 ml nickel-nitrilotriacetic acid agarose (Qiagen, Valencia, CA). The protein in the native state was eluted in the “protein buffer” (25 mM Tris-HCl pH 8.0, 100 mM NaCl, 125 mM imidazole and 2 mM dithiothreitol). The protein concentration was measured using the Bradford assay (Bio-rad) to manufacturer’s standards, and the activity was assayed using mixed micelles in a modified Dole assay (36) using the same conditions employed for deuterium exchange experiments. Purified GIVA PLA\textsubscript{2} (2 mg/ml) was stored in the protein buffer on ice for DXMS experiments. Experiments were performed immediately after elution from the nickel column.

5.C.3 Preparation of lipid vesicles

Lipid vesicles were prepared by evaporating solutions of 1-palmitoyl 2–arachidonic phosphatidylcholine in chloroform under argon to dryness. The lipid film was resuspended in 100 mM KCl and allowed to sit in a 40°C water bath for 30 min.
The lipid solution was then probe sonicated 5 times for 30 sec each with 30 sec breaks in ice between sonications. This solution was then centrifuged at 5,000 g to remove large lipid aggregates and titanium particles released from the probe sonicator. This solution was then used immediately for deuterium exchange studies. SUVs were chosen for this study because kinetic studies performed with both SUVs and LUVs with the GIVA PLA₂ have shown similar kinetics (25, 37). We have previously used this technique with SUVs on the GIA PLA₂ to study lipid binding (29), and we chose to expand on this using GIVA PLA₂.

5.C.4 Preparation of Deuterated Samples for On Exchange Experiments

D₂O buffer contained 10 mM Tris (pD 7.5), 50 mM NaCl in 98% D₂O. Hydrogen/deuterium exchange experiments were initiated by mixing 20 µl of GIVA PLA₂, or the C2 domain or catalytic domain (containing 40 µg) in protein buffer with 60 µl of D₂O buffer to a final concentration of 73% D₂O at pH 7.5. In lipid vesicle and MAFP binding experiments, the GIVA PLA₂ in protein buffer was pre-incubated in the presence of 60 µM MAFP added from a 1 mM stock dissolved in ethanol and/or 2 mM PAPC vesicles in a 23 °C water bath for 1 min. The final concentration of ethanol was 0.25% for experiments with MAFP and MAFP controls. MAFP was allowed to incubate with the enzyme for 3 hrs on ice, followed by 10 min at room temperature pre-D₂O incubation. The D₂O buffer was then added and the samples were incubated at 23°C for an additional 10 sec, 30 sec, 100 sec, 300 sec, 1,000 sec, 3,000 sec or 10,000 sec. For experiments with lipid present, time points were only taken while the percent hydrolysis of the lipid vesicles was under 10%. The activity of the enzyme against these lipid
vesicles was measured using a modified Dole assay that exactly matched deuterium exchange conditions (33). The deuterium exchange was quenched by adding 120 µl of ice-cold quench solution (0.96% formic acid, 1.66 M guanidine hydrochloride (GdHCl)) that acidified the sample to a final pH = 2.5 and concentrations of formic acid of 0.58%, and 1 M GdHCl. The samples were placed on ice for 10 min to partially denature the protein and obtain optimal peptide maps. Vials with frozen samples were stored at −80 °C until analysis, usually within three days.

5.C.5 Proteolysis-liquid-chromatography-mass spectrometry analysis of samples

All steps were performed at 0°C as previously described (30, 32). The samples were hand-thawed on melting ice and injected onto and passed through a protease column (66 µl bed volume) filled with porcine pepsin (Sigma); immobilized on Poros 20 AL medium (Applied Biosystems) at 30 mg/ml following the manufacturer's instructions, at a flow rate of 100 µl/min with 0.05% trifluoroacetic acid (TFA). The eluate from the pepsin column was directly loaded onto a C18 column (Vydac cat #218MS5150). The peptides were eluted at 50 µl/min with a linear gradient of 0.046% TFA, 6.4% (v/v) acetonitrile to 0.03% TFA, 38.4% acetonitrile for 30 min. The eluate from the C18 column was directed to a Finnigan Classic LCQ mass spectrometer via its ESI probe operated with a capillary temperature of 200 °C as previously described (30, 32).

5.C.6 Data Processing

SEQUEST software (Thermo Finnigan Inc.) was used to identify the sequence of the peptide ions. DXMS Explorer (Sierra Analytics Inc, Modesto CA) was used for the
analysis of the mass spectra as previously described (30, 32). All selected peptides had first passed the quality-control threshold of the software and were then manually checked to insure that the observed mass envelope agreed with the calculated mass envelope. The highest signal/noise ion was picked if multiple ionization charges (1, 2 or 3) of a peptide were detected. Incorporated deuteron number was obtained by measuring the centroid shift between the non-deuterated and the partially deuterated mass envelope.

The deuteration level of each peptide was calculated by the ratio of the incorporated deuteron number to the maximum possible deuteration number. Peptide deuteration levels in replicate samples, measured by our DXMS methods, have been found to vary by less than 10%, and we therefore regard changes greater than 10% as significant (30). All experiments were performed at least twice, and representative data is shown. Trends in the data were similar from experiment to experiment, but total deuterium content varied by roughly 5-10% in similar experiments carried out weeks apart. For all regions that showed a greater then 10% increase or decrease, other peptides that include some or all of the regions of interest are provided in other figures.

5.D Results

5.D.1 GIVA PLA₂ Coverage Map of Pepsin Fragmentation

The protein digestion procedure was optimized to produce a peptide map that yielded the best coverage of GIVA PLA₂ as previously described (35). The optimized condition gave 157 distinct peptides that gave 92% coverage of the GIVA PLA₂ sequence. The same condition was used to digest the C2 domain, which generated 32 peptides covering 89% of the sequence (Fig 5-1). Sixty one peptide fragments are shown
that cover 82% of the protein and 17 peptide fragments for the C2 domain covering 89% of the sequence, were analyzed for the data shown in the figures. These peptides are shown as bold lines in figure 5-1. In this article, when we use the term "peptide", we are referring to an actual peptide that was identified in the pepsin digestion. When we use the term "region", we are referring to a section of the protein for which the deuterium exchange has been calculated, but it may or may not correspond to an actual pepsin peptide.
Figure 5-1 Pepsin-digested peptide coverage map of GIVA PLA2 and C2 domain.

Identified and analyzed pepsin-digested peptides shown underneath the primary sequence of GIVA PLA2. Only the peptides in bold lines are used in this study.
5.D.2 C2 lipid binding experiments

On exchange experiments were performed on the isolated C2 domain containing amino acids 12-140. C2 domain lipid binding has been extensively studied by other techniques and these studies served as an important control to compare this technique against previously published results obtained with other approaches. C2 on exchange experiments were performed under five different conditions (Fig. 5-2). The change in exchange upon binding to 500 µM lipid vesicles was tested in the presence of 0, 200 and 1000 µM Ca\(^{2+}\). Significant decreases upon exposure to the lipid surface were only achieved in the presence of 1 mM Ca\(^{2+}\) (Fig. 5-2) and not with 0 or 200 µM Ca\(^{2+}\) (data not shown). The changes in exchange upon Ca\(^{2+}\) binding were exactly the same as the changes observed with intact full length cPLA\(_2\) (35) and included regions 28-35, 51-71, 101-106, and 125-129 as well as a new region 41-48 (data not shown). This new region 41-48 is part of the C2 domain that shows very slow exchange rates in the full enzyme and faster exchange in the C2 only domain (35). A significant decrease in exchange due to binding of lipid vesicles was seen in the region 36-39, as well as a small change in the region 28-35 in the presence of 1 mM Ca\(^{2+}\) and PAPC lipid vesicles (Fig 5-2). These regions include the hydrophobic residues Phe-35, Met-38, and Leu-39 present on the first Ca\(^{2+}\) binding loop. Previous work has also implicated region 96-98 in lipid binding (17-23), but no peptides were identified that contained these amino acids. These results agree with the previous experiments demonstrating lipid binding of the C2 domain alone.
Figure 5-2 Deuterium exchange of the isolated C2 domain binding the phospholipid membrane in the presence of Ca\(^{2+}\). DXMS was performed on the C2 domain under the following three conditions: 0, and 1mM Ca\(^{2+}\) without PAPC, as well as 1 mM Ca\(^{2+}\) with PAPC. The number of incorporated deuterons for three regions are shown: 28-35, 36-39, and 51-71. Changes in deuteration between the 1mM Ca\(^{2+}\) and 1mM Ca\(^{2+}\) + 500 µM PAPC conditions greater then 10% are represented in color on the crystal structure (see legend).
5.D.3 cPLA₂ Lipid Binding Experiments

On exchange experiments were performed on the intact cPLA₂ enzyme in the presence of a phospholipid membrane to determine how the catalytic domain interacts with the surface and to determine if it changed membrane binding of the C2 domain. These experiments were carried out in the presence of 500 µM PAPC and 200 µM Ca²⁺ for time points varying from 10 seconds to 300 sec. The literature K_d values of this enzyme interacting with membranes varies from 1 µM to 90 µM depending on the presentation of the lipid substrate (25, 37). Generation of product interfering with membrane binding was a concern in these experiments. For this reason these experiments were carried out at shorter time points with low levels of Ca²⁺ to keep the hydrolysis of phospholipids at the lipid membrane below ten percent. Experiments were carried out testing lipid binding in the presence of 1mM EGTA to test binding without Ca²⁺. No changes greater then 10% were observed upon lipid binding without Ca²⁺ present.

Activity assays were done under the same experimental conditions as the deuterium exchange experiments to measure the levels of arachidonic acid release. In the presence of 200 µM Ca²⁺ the % hydrolysis at 10 sec was ~0.2%, and the % hydrolysis proceeded to 6% at 300 sec. Samples taken at 1000 seconds showed 16% hydrolysis, and we decided to limit our time course to only 300 seconds. The specific activity of the enzyme under our conditions was ~20 nmol min⁻¹ mg⁻¹ which is consistent with literature values under similar protein to lipid ratios (25). Four regions of the protein (28-39, 258-265, 268-279 and 466-470) exhibited decreased exchange in the presence of lipid vesicles (Fig 5-3). Multiple additional peptides covered portions of these regions, and peptide data for these regions is shown in Figure 5-4. Region 28-39 exhibited similar changes in
deuterium exchange as did the isolated C2 domain. Changes were seen due to membrane surface binding in the intact enzyme in the presence of 200 µM Ca\(^{2+}\) with 500 µM PAPC vesicles that required 1 mM Ca\(^{2+}\) to be seen in the C2 domain alone. It is important to note that there is a large change in exchange induced upon Ca\(^{2+}\) binding in region 28-35 and a much smaller change induced upon lipid binding. Once again no peptides spanning region 96-98 were identified, so no data on lipid interactions of that region was available. No other regions on the C2 domain showed changes upon lipid binding.

The region 268-279 showed a 10-15% decrease in exchange upon membrane binding but no change upon Ca\(^{2+}\) binding alone. This region contains a group of charged amino acids, Lys-273, Arg-274, and Glu-277 as well as the polar Gln-270 that are oriented to possibly interact with the zwitterionic head groups of a lipid surface. There are also multiple hydrophobic residues including Val-272, Tyr-275, and Leu-279 pointing towards the catalytic site of the enzyme that may interact with fatty acyl chains of the substrate. The region 466-470 is located on the same face as 268-279 and has a similar decrease in exchange, with a decrease of around 10-15% at all time points. This area contains a charged group at R-467 and one hydrophobic residue Ile-469.

There was also a smaller decrease going from 5-10% over the time course in the region from 256-265 that contains a group of four leucines at 262-265 that have extensive hydrophobic contacts with other non-polar residues on the interior of the protein. There are no specific residues that would obviously interact with the membrane surface, so this decrease could be due to a conformational shift upon the membrane binding to the lipid surface or interactions between the leucines and the fatty acyl chains of the substrate.
Figure 5-3 Deuterium exchange of the GIVA PLA₂ binding the phospholipid membrane in the presence of Ca²⁺. Ca²⁺ was present at 200 µM and vesicles were present at 500 µM. The number of incorporated deuterons at four time points in seven different regions, 28-35, 36-39, 256-265, 268-279, 391-397, 466-470, and 543-553 in GIVA PLA₂ are plotted. Decreases or increases in deuteration greater than 10% are represented by the color scheme in the legend.
Figure 5-4  Deuterium exchange of the GIVA PLA$_2$ binding the phospholipid membrane in the presence of Ca$^{2+}$. Ca$^{2+}$ was present at 200 $\mu$M and vesicles were present at 500 $\mu$M. The number of incorporated deuterons at four time points in GIVA PLA$_2$ are plotted for regions that overlap regions shown in Fig.3. Regions 240-253, 375-392, and 375-393 are included as examples of regions with no changes in exchange. CS1, 2, 3 stands for charge state of the peptide ion 1, 2, or 3.
There are two regions that show increases in exchange upon lipid binding, and these are 391-397, and 543-553. Region 391-397 has a >10% increase at early time points, with no change in exchange at 300 sec. Region 543-553 has a larger increase of ~25% at early time points with almost no change in exchange seen at 300 sec. These rates imply that these regions of the protein are becoming more accessible to solvent. These regions contain hydrophobic residues that are part of a channel that leads to the active site residues Ser-228 and Asp-549. These residues are all in contact with the lid region spanning 415-432. These increases may be caused by a lid opening caused by substrate binding or membrane surface binding. Region 543-553 also contains the active site Asp-549. Experiments were also performed using the isolated catalytic domain in the presence of lipid vesicles, but no changes in deuteration greater than 10% were observed (data not shown).

5.D.4 On exchange results using MAFP inhibited GIVA PLA$_2$

MAFP is a potent irreversible inhibitor of the GIVA PLA$_2$ enzyme (38). Inhibitor bound $\alpha/\beta$ hydrolases have been used to crystallize the open lid conformation of various lipases (26). MAFP was used to determine if substrate binding caused opening of the lid region, or if interfacial binding of the lipid surface caused this change. Using MAFP allowed us to separate substrate binding effects from membrane surface binding effects. MAFP was selected due to its similarity to natural substrate, because it contains an arachidonic acid in a similar position to the sn-2 fatty acid chain of the natural substrate, although there is no phospholipid head group, or sn-1 fatty acid tail in MAFP. This acts as an excellent mimic to the lysophospholipase and acyl transferase activity of cPLA$_2$. 
MAFP binding was tested at seven time points from 10 to 10,000 sec at a concentration of 15 µM. There were five regions that saw changes upon MAFP binding. Multiple peptides covered these regions, and additional peptide data for these regions is shown in 5-6.

Two regions of the enzyme 256-265 and 268-279 showed decreases in exchange upon MAFP binding (Fig. 5-5). The region from 268-279 showed a decrease in deuteration of >10%, which is less than observed with phospholipid binding experiments. The region from 258-265 had a >10% decrease in exchange similar to the phospholipid binding experiments. This indicates that these regions are either binding the arachidonic acid tail of the MAFP molecule, or that conformational change that occurs when the lid opens blocks access to these residues or rigidifies the protein structure in this region. Other hydrophobic residues that should bind MAFP in the active site funnel have very low levels of exchange even in the inhibitor free samples, so no changes could be recorded upon inhibitor binding.

All of the regions that had increases upon lipid binding had increases upon MAFP binding. These increases were of the same magnitude as with lipid binding. Region 391-397 has a constant 5-15% increase in exchange over all time points. Other peptides overlapping this region (Fig. 5-6) shows that the majority of this increase is localized to region 394-397. The region 543-553 has a 25% increase at early time points with less of an increase at later timepoints. The region 481-495 also had an increase in exchange upon exposure to MAFP. This region had a 10-20% increase in exchange across all time points. All of these regions are partially located under the lid region.
spanning 415-432. MAFP binding closely mimics natural phospholipid binding in the increases seen in the catalytic domain of the protein, even without a membrane surface.

Figure 5-5 Deuterium exchange upon binding of 15 µM MAFP. A. The number of incorporated deuterons at seven time points in five different regions, 258-265, 268-279, 391-397, 481-495, and 543-553 in GIVA PLA₂ are plotted. Decreases or increases in deuteration greater than 10% are represented by the color scheme in the legend.
Figure 5-6 Deuterium exchange upon binding of 15 µM MAFP. A. The number of incorporated deuterons at seven time points are plotted for regions overlapping regions shown in Fig. 4. CS1, 2, 3 stands for charge state of the peptide ion 1, 2, or 3.
5.D.5 On exchange results with MAFP inhibited GIVA PLA₂ binding phospholipid

On exchange experiments were performed with both MAFP and PAPC vesicles in an attempt to identify those changes due to the presence of an interface as opposed to those due to the binding of the pseudo substrate in the catalytic site. The presence of MAFP in the active site should block any binding of phospholipid in the active site, so the effects should only be from the enzyme binding to the phospholipid surface and not from conformational changes induced by substrate binding. This also acted as an important control to see effects of lipid binding without product generation. The interface experiments were performed for time courses from ranging from 10 sec up to 1,000 sec. These experiments were performed in the presence of Ca²⁺ to activate membrane binding. An experiment was also performed in the presence of EGTA with lipid vesicles to see if any changes were seen without Ca²⁺ present. Experiments with EGTA showed no significant changes in deuterium exchange upon exposure to lipid vesicles without Ca²⁺ present. (Data not shown).

There were four regions of the protein that showed decreases in exchange upon MAFP inhibited lipid binding (Fig 5-7). Multiple peptides covered these regions, and peptide data for these regions is shown in Figure 5-8.

The regions 28-35 and 36-39 are located on the C2 domain. Exchange at peptide 28-35 is decreased significantly by the presence of Ca²⁺ but even so the changes induced by lipid were greater than 10%. The greatest change was seen in region 36-39 located on the hydrophobic alpha helix proposed to penetrate the membrane surface. These peptides showed no changes in the presence of lipid without Ca²⁺ present demonstrating that these
changes caused by lipid binding are Ca\textsuperscript{2+} dependent. These changes were almost identical to those seen with uninhibited enzyme mixed with lipid.

The region from 268-279 on the catalytic domain also showed a >10% decrease in exchange. This area is also one of the regions that show a significant decrease upon MAFP binding. This region appears to both bind the interfacial surface, as well as bind the single phospholipid molecule in the active site. The region from 466-470 that had decreases upon non-inhibited lipid binding shows a similar exchange pattern upon inhibitor bound lipid binding. These results suggest that the C2 domain, as well as the region of the catalytic domain containing regions 466-470 and 268-279 are important in binding the lipid surface.

The regions that increased due to lipid binding in region 391-397, and 543-553 showed no changes in exchange between the MAFP inhibited enzyme and the inhibited enzyme incubated with lipid vesicles (Figure 5-8).
Figure 5-7 Deuterium exchange upon Ca²⁺ mediated PAPC vesicle binding in the presence of 15 µM MAFP. Ca²⁺ was present at 200 µM and vesicles were present at 500 µM. The number of incorporated deuterons at five time points in four different regions, 28-35, 36-39, 268-279, and 466-470 in GIVA PLA₂ are plotted. Possible amino acids that may interact with the lipid surface have been shown in stick form. Decreases or increases in deuteration greater than 10% are represented by the color scheme in the legend.
Figure 5-8 Deuterium exchange upon Ca\(^{2+}\) mediated PAPC vesicle binding in the presence of 15 µM MAFP. Ca\(^{2+}\) was present at 200 µM and vesicles were present at 500 µM. The number of incorporated deuterons at five time points are plotted. CS1, 2, 3 stands for charge state of the peptide ion 1, 2, or 3.
5.E Discussion

C2 domain binding to membrane of the GIVA PLA2 has been extensively studied (17-23). However, how the intact enzyme binds a lipid surface, and the allosteric changes caused by this binding has been much more difficult to determine. This is the first study to examine the structural basis of the intact enzyme binding to the lipid surface as well as conformational changes upon binding a pseudo substrate in the active site with deuterium exchange mass spectrometry. Ca\(^{2+}\) mediated C2 binding to the membrane surface has been shown to be mediated by hydrophobic residues in the Ca\(^{2+}\) binding loop one 35-39 and residues in the Ca\(^{2+}\) binding loop two 96-98 (17-23). No peptides existed that spanned region 96-98 so information about how Ca\(^{2+}\) binding loop two penetrates the surface was unavailable. Our results with deuterium exchange of the isolated C2 domain with lipid show decreases in regions 28-35 and 36-39, which are consistent with these regions penetrating the lipid interface. These experiments on the C2 domain alone were used as a control to demonstrate that we could examine membrane surface binding using the DXMS technique.

The results of the intact cPLA2 enzyme binding to the lipid surface showed similar changes to the C2 domain alone, which showed lipid binding at regions 28-35 and 36-39, but with less Ca\(^{2+}\) required to see effects with the intact enzyme in deuterium exchange. This corresponds well with previous work showing an increased residence time of the intact GIVA PLA2 on the membrane surface of the golgi compared to the C2 domain alone (41). This implies that the presence of the catalytic domain helps bind the enzyme to the lipid surface, or causes changes in the binding of Ca\(^{2+}\) in the intact enzyme. The hydrophobic core of the protein around the active site gave rise to significant increases in
exchange upon Ca\textsuperscript{2+} mediated lipid binding. This region contains many hydrophobic residues that are blocked from solvent exposure in the closed form of the enzyme. When lipid binds in the active site there must be a change in the structure that causes the lid to reorient in some way which increases solvent accessibility to the hydrophobic core. The other decreases in deuteration in the catalytic domain upon Ca\textsuperscript{2+} mediated lipid binding were in regions that could be caused by interactions with either the membrane surface or fatty acyl chains of the substrate.

We used the inhibitor MAFP to differentiate lipid surface binding effects from conformational changes induced by the enzyme binding substrate in the active site. Previous work has used inhibitors to force lipases into an open lid conformation (26). MAFP gave rise to the same increases in the hydrophobic core as seen with phospholipid vesicles (Fig. 5-8), except for a slightly greater increase in solvent accessibility in region 481-495 compared to lipid binding results. The decreases in exchange seen in regions 256-265 and 268-279 of the catalytic domain were very similar to those seen with only lipid binding. MAFP covalently modifies the active site Ser-228 with an arachidonic acid and is very similar to the sn-2 fatty acid of a natural phospholipid substrate. The decreases in exchange in regions 256-265 and 268-279 upon MAFP binding without a lipid surface present suggest that residues are interacting with the arachidonic acid fatty acid. Residues Leu-264, Leu-267, and Tyr-275 could be in the correct orientation to interact with the fatty acid tail of substrate. The other hydrophobic amino acids that would bind to substrate are in the hydrophobic core of the protein that has very low levels of exchange (35), and therefore no changes in exchange can be seen. The increases in
exchange seen upon inhibitor binding are almost certainly caused by a shift of the lid region from 415-432 as shown in figure 5-9.

**Figure 5-9 Opening of the lid induced by inhibitor binding.** A. Molecular surface of the enzyme with areas that show an increase in exchange upon inhibitor binding colored red or orange, and the lid region from 415-432 is colored green. B. The lid region surface is removed and shown as a ribbon with most areas in red underneath the lid. The deuterium exchange data implies that the lid region from 415-432 shifts position to expose the regions underneath. Molecular surface was generated using Swiss PDB viewer.
There have been very few studies that have looked at how the intact enzyme binds the surface. The enzyme is assumed to shift to an "open conformation" in which the lid region has moved out of the way of the catalytic site. This hypothesis was suggested due to the inability to otherwise model a phospholipid in the active site (10), but was not based on or verified by experiment. The DXMS results reported here with natural phospholipids and the inhibitor MAFP clearly demonstrates this conformational change.

The lid region of 415-432 has amphipathic character with amino acids involved in hydrophobic interactions with amino acids underneath, and many charged amino acids exposed to solvent. There are significant increases in exchange in areas of the protein directly covered by the lid region. There are significant hydrophobic contacts seen in the crystal structure between the lid region and areas with an increase in exchange upon MAFP binding. There are contacts between four amino hydrophobic amino acids on the lid, Met-417, Leu-421, Ile-424, and Ile-429, and amino acids in the regions with increases in exchange, Ala-396, Phe-397, Ala-486, Val-548, and Leu-552. The breaking of these hydrophobic contacts between the lid region and the regions underneath would explain the increase in deuterium exchange seen in this region. This increase in exchange could be due to the lipid substrate binding to the hydrophobic residues at Phe-397, Ile-399, Leu-400, Val-404, and Leu 405 and causing the lid region to undergo a conformational shift opening up the active site. Previous studies using mutants of the GIVA PLA₂, specifically mutants of Ile-399, and Leu-400 showed a vast decrease in activity, membrane affinity and membrane penetration (24). The proposal was that these amino acids were mediating penetration of the membrane surface. This is unlikely due to the many charged residues that would also need to penetrate the membrane surface to allow Ile-399, and Leu-400 to
penetrate. It is possible that these residues are mediating substrate binding, and this substrate binding helps mediate activity and surface binding by moving the lid region. These increases in exchange are seen with natural phospholipid substrate, and with the MAFP substrate analog. This implies that the presence of an inhibitor in the active site closely mimics the open form of the enzyme that works on natural phospholipid substrate.

Our results using MAFP inhibited enzyme binding to the lipid surface were used to show how the enzyme binds to the lipid surface without complicating effects caused by conformational changes upon substrate binding, since those changes have already occurred due to MAFP in the active site. Upon addition of phospholipid to the MAFP-inhibited enzyme the majority of decreases are seen in the region from 28-35 and 36-39 which contains the hydrophobic alpha helix thought to penetrate the membrane surface. However there is still a significant change in exchange in regions 268-279, and 466-470. These regions contain a group of charged amino acids Lys-271, Lys-273, Arg-274, Arg-467 as well as the polar Gln-270 that could all interact with zwitterionic PC head groups. Lys-27, Arg-274 and Arg-467 are the only residues from the crystal structure that are oriented in the correct way to interact with PC head groups. However region 268-279 had significant decreases with MAFP inhibitor bound without the lipid interface present, which implies it is binding substrate in the active site. This interaction may help to orient the other residues in this region to interact with the head groups of a membrane surface. Region 466-470 is near Trp-464 that could penetrate into the hydrophobic core of the membrane, as proposed in previous studies (41). Previous work has shown that mutations
to residues Arg-467 and Lys-273 decreases binding to negatively charged phospholipids (24).

Figure 5-10 Hypothetical model of initial membrane binding before lid opening. Ca$^{2+}$ binding at the C2 domain causes translocation of the enzyme to the surface and penetration of the hydrophobic core of the membrane. There seems to be no penetration of the catalytic domain and its interactions with the surface are mediated through electrostatic contacts from regions 268-279, 466-470 to PC head groups. Model was generated using PyMol.
From these results we have generated a model of the initial step in Ca\textsuperscript{2+} mediated membrane binding before opening of the lid shown in figure 5-10. This model corresponds to our deuterium exchange data, where region 35-39 and 96-98 along with Trp-464 penetrate the membrane surface, and electrostatic contacts between Lys-273, Arg-274, and Arg-467 help bind the zwitterionic head groups on the membrane surface. Interactions between the catalytic domain alone are not sufficient to bind membrane phospholipid, with Ca\textsuperscript{2+} binding causing the enzyme to translocate to the membrane surface through the C2 domain, and this binding is increased through electrostatic and hydrophobic interactions between the membrane surface and the catalytic domain. Large regions of the catalytic domain do not penetrate the membrane surface, based upon the observation that there are no decreases in deuterium exchange in areas of the i-face of the enzyme upon lipid surface binding. Upon this binding, the lid region is opened through hydrophobic contacts between the enzyme and a single phospholipid diffusing into the active site, substrate binds in the open form of the enzyme in the active site, and free arachidonic acid is liberated.

We plan to study the interactions of various other lipid activators of this enzyme using these same techniques, and to further characterize the structure of the open lid form of the enzyme. This work suggests interesting possibilities such as being able to isolate the open conformation of the enzyme by using inhibitor-enzyme complexes. Further study using deuterium exchange along with crystallography and molecular modeling techniques offers the potential to further define the interaction of the enzyme with membrane surfaces.
5.F Conclusion

This work represents the first DXMS study of an intracellular lipase binding a lipid surface. This study shows lipid binding effects of the entire enzyme instead of only the C2 domain as has been previously demonstrated with other techniques. It also uses inhibitor binding to probe the structure of the open conformation of the enzyme.

5.G Acknowledgements

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5.H References


CHAPTER 6

Inhibitor Binding of Group IVA Phospholipase A$_2$ Probed by Molecular Dynamics and Deuterium Exchange Mass Spectrometry
6.A Abstract

An analysis of Group IVA (GIVA) phospholipase A2 (PLA2) inhibitor binding was examined using a combination of deuterium exchange mass spectrometry (DXMS) and molecular dynamics (MD). Models of the GIVA PLA2 inhibitors pyrrophenone and the 2-oxoamide AX007 docked into the protein were designed based on deuterium exchange results, and extensive molecular dynamics simulations were run to determine protein-inhibitor contacts. The models show that both inhibitors interact with key residues that also exhibit changes in deuterium exchange upon inhibitor binding. Pyrrophenone is bound to the protein through numerous hydrophobic residues located distal from the active site, while the oxoamide is bound mainly through contacts near the active site. We also show changes in protein dynamics around the active site between the two inhibitor-bound complexes. This combination of computational and experimental methods is useful in defining more accurate inhibitor binding sites, and can be used in the generation of better inhibitors against GIVA PLA2.

6.B Introduction

The Group IVA phospholipase A2 (GIVA PLA2) is a member of the superfamily of phospholipase A2 enzymes that cleave a fatty acid from the sn-2 position of phospholipids (1, 2). The products of this reaction, a free fatty acid and a lysophospholipid play important roles as lipid second messengers. GIVA PLA2 was isolated in 1990 from U937 cells (3), and was discovered to be composed of a C2 domain, and a α/β hydrolase domain containing the active site (4). The GIVA PLA2 is specific for phospholipids with arachidonic acid in the sn-2 position, and the release of
arachidonic acid is the first step in the production of eicosanoids and leukotrienes which play important roles in many inflammatory diseases (5). Experiments performed using mice deficient in the GIVA PLA₂ enzyme have proven that GIVA PLA₂ is the critical PLA₂ enzyme for eicosanoid generation in many inflammatory disease models (6-8).

The enzyme was shown through site directed mutagenesis to contain an active site dyad composed of Ser-228 and Asp-549 (9), and this was later confirmed through x-ray crystallography of the enzyme (10). The enzyme contains an amphipathic lid region from 415-432 that prevents accession of phospholipid into the active site (11). The lid region has two disordered regions from 408-412, and 433-457 that may act as hinges that allow the lid region to open. It has been shown that this lid is in the open conformation when the enzyme is in the presence of lipid vesicles (its natural substrate) or when inhibitor is bound in the active site (11).

The knowledge that GIVA PLA₂ plays an important functional role in many inflammatory diseases has sparked an interest in the production of specific inhibitors against this enzyme. The first inhibitors of this enzyme were based around the specificity of the enzyme for phospholipids with arachidonic acid in the sn-2 position, and as such arachidonyl trifluoromethyl ketones (ATK) and methyl arachidonyl fluoro phosphonate (MAFP) (1) were synthesized and found to inhibit the enzyme in platelet models of eicosanoid generation (12-14). In recent years many different strategies have been pursued to create effective and specific GIVA PLA₂ inhibitors. These have included indole derivatives developed by Wyeth Pharmaceuticals (2) (15-18), pyrrolidine based inhibitors by Shinogi Pharmaceuticals (3) (19-22), substituted propan-2-ones by the Lehr group (23-25), as well as 2-oxoamide compounds by the Kokotos and Dennis groups (4)
as shown in Figure 6-1 (26-29). Of these inhibitors, there exist two docked structures in the GIVA PLA$_2$ active site, generated through computer modeling, (15, 30), but there are no in depth examinations of the binding pocket contacts between inhibitor and enzyme.

Figure 6-1 Inhibitors of GIVA PLA$_2$. 1. MAFP. 2. Efipladib. 3. Pyrrophenone. 4. AX007

The pyrrolidine derived inhibitor pyrrophenone displays some of the best inhibition but (due to chemical properties) is not useful as a drug (18). We have previously shown that the 2-oxoamide compounds show an antihyperalgesic effect in rat models (31). The invention of better 2-oxoamide inhibitors is a promising drug target, and to such end, we set out to model the 2-oxoamide inhibitor AX007, as well as the
pyrrolidine derived inhibitor pyrrophenone, bound in the active site. This required a technique to monitor changes in protein structure upon inhibitor binding.

Peptide amide hydrogen deuterium exchange analyzed via liquid chromatography/mass spectrometry has been widely used to analyze protein-protein interactions (32, 33), protein conformational changes (34, 35), and protein dynamics (36). We have previously used this technique to explore changes in lipid binding with the GIVA PLA₂ and discovered changes in exchange profiles in the presence of the irreversible inhibitor MAFP (11). Use of the DXMS technique, in conjunction with site-directed mutagenesis, has recently been used to identify regions interacting with different inhibitors (37, 38).

Coupled with these experimental techniques, computational methods can be employed to study the atomic-level details in the GIVA PLA₂-Inhibitor complex. Extensive simulations of the phospholipase A₂’s have been carried out. Most notably, Wee et al. recently conducted a coarse-grained simulation of the pancreatic phospholipase A₂, in which they demonstrate how the enzyme adheres to the lipid bilayer (39). Quantum mechanical methodologies have also been applied to the phospholipase system (40). This work has proven vital to the understanding of phospholipase A₂ chemistry and dynamics.

In turn, by running molecular dynamics (MD) simulations of GIVA PLA₂ with inhibitor, one can observe how the latter docks into and stabilizes itself in the enzyme. Contacts between the inhibitor and specific residues of GIVA PLA₂ can also be identified. This information augments the results from the deuterium exchange technique which at this time lacks the resolution to achieve single-residue data. In lieu of known
crystal structures depicting the enzyme-inhibitor complex, this computational work affords working models of the complexes and characterizes key enzyme-inhibitor interactions. The MD simulations and subsequent analysis aid in drawing comparisons between the oxoamide and pyrrophenone complexes.

The study of these two very different inhibitors provides an excellent model for generalized GIVA PLA₂ inhibition. The dual techniques of deuterium exchange mass spectrometry and MD simulation are excellent methods to probe the dynamical changes induced by binding of inhibitors to any enzyme. This study also represents a continuation of our deuterium exchange studies on the PLA₂ family of enzymes (11, 41, 42). We have identified specific regions of the protein that interact with the oxoamide and pyrrophenone inhibitors, and we have carried out extensive computer simulations to create a model of inhibitor binding in the active site. We have also identified significant differences in the way pyrrophenone and oxoamide bind GIVA PLA₂. This work leads to the possibilities of enhanced rational drug design through the powerful combination of experimental and computational work.

6.C MATERIALS AND METHODS

All reagents were analytical reagent grade or better. Pyrrophenone was the generous gift from Shinogi, and AX007 was synthesized as described previously (27).

6.C.1 Protein Expression and Purification.

C-terminal His6-tagged GIVA PLA₂, and the C2 and catalytic domains were expressed using recombinant baculovirus in a suspension culture of Sf9 insect cells. The
cell pellet was lysed in 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM β-mercaptoethanol, and 2 mM EGTA and than the insoluble portion was removed by centrifugation at 12,000 x g for 30 min. The supernatant was passed through a column comprised of 6 ml nickel-nitrilotriacetic acid agarose (Qiagen, Valencia, CA). The protein in the native state was eluted in the “protein buffer” (25 mM Tris-HCl pH 8.0, 100 mM NaCl, 125 mM imidazole and 2 mM dithiothreitol). The protein concentration was measured using the Bradford assay (Bio-rad) to manufacturer’s standards, and the activity was assayed using mixed micelles in a modified Dole assay (43). Purified GIVA PLA₂ (2 mg/ml) was stored in the protein buffer on ice for DXMS experiments. Experiments were performed immediately after elution from the nickel column.


D₂O buffer contained 10 mM Tris (pD 7.5), 50 mM NaCl in 98% D₂O. Hydrogen/deuterium exchange experiments were initiated by mixing 20 µl of GIVA PLA₂ (containing 40 µg) in protein buffer with 60 µl of D₂O buffer to a final concentration of 73% D₂O at pH 7.5. In inhibitor binding experiments, the GIVA PLA₂ in protein buffer was pre-incubated in the presence of 40 µM pyrrophenone, 40 µM o xoamide, or DMSO control. The inhibitors were added from 600 µM stock dissolved in DMSO. The final concentration of DMSO was 1.5% for all experiments. The inhibitors were allowed to preincubate with the enzyme for 10 minutes at 23°C before addition of D₂O buffer. The D₂O buffer was added and the samples were incubated at 23°C for an additional 10, 30, 100, 300, 1000, 3000 or 10000 seconds. The deuterium exchange was quenched by adding 120 µl of ice-cold quench solution (0.96% formic acid, 1.66 M
guanidine hydrochloride (GdHCl)) that acidified the sample to a final pH = 2.5 and concentrations of formic acid of 0.58%, and 1 M GdHCl. The samples were placed on ice for 10 min to partially denature the protein and obtain optimal peptide maps. Vials with frozen samples were stored at −80 °C until analysis, usually within three days.


All steps were performed at 0°C as previously described (32, 34). The samples were hand-thawed on melting ice and injected onto and passed through a protease column (66 µl bed volume) filled with porcine pepsin (Sigma); immobilized on Poros 20 AL medium (Applied Biosystems) at 30 mg/ml following the manufacturer's instructions, at a flow rate of 100 µl/min with 0.05% trifluoroacetic acid (TFA). The eluate from the pepsin column was directly loaded onto a C18 column (Vydac cat #218MS5150). The peptides were eluted at 50 µl/min with a linear gradient of 0.046% TFA, 6.4% (v/v) acetonitrile to 0.03% TFA, 38.4% acetonitrile for 30 min. The eluate from the C18 column was directed to a Finnigan Classic LCQ mass spectrometer via its ESI probe operated with a capillary temperature of 200 °C as previously described (32, 34).

6.C.4 Data processing.

SEQUEST software (Thermo Finnigan Inc.) was used to identify the sequence of the peptide ions. DXMS Explorer (Sierra Analytics Inc, Modesto CA) was used for the analysis of the mass spectra as previously described (32, 34). All selected peptides had first passed the quality-control threshold of the software and were then manually checked to insure that the observed mass envelope agreed with the calculated mass envelope. The
highest signal/noise ion was picked if multiple ionization charges (1, 2 or 3) of a peptide were detected. Incorporated deuteron number was obtained by measuring the centroid shift between the non-deuterated and the partially-deuterated mass envelope.

The deuteration level of each peptide was calculated by the ratio of the incorporated deuteron number to the maximum possible deuteration number. Peptide deuteration levels in replicate samples, measured by our DXMS methods, have been found to vary by less than 10%, and we therefore regard changes greater than 10% as significant (32). All experiments were performed at least twice, and representative data is shown. Trends in the data were similar from experiment to experiment, but total deuterium content varied by roughly 5-10% in similar experiments carried out weeks apart.

6.C.5 Molecular Dynamics Simulations.

In total, three systems were simulated: the apo form of GIVA PLA₂; GIVA PLA₂ with pyrrophenone bound; and GIVA PLA₂ with oxoamide bound. The structure of GIVA PLA₂ was obtained from the Protein Data Bank (code 1CJY) (10). In this reported structure, several segments were missing including residues 407-414, 431-462, 498-538, and 626-632. These apparently flexible regions were modeled into chain B of 1CJY using SWISS MODEL (Figure 6-2) (44). The resulting complete structure of GIVA PLA₂ was optimized by 500 steps each of steepest decent and conjugate gradient energy minimizations and used in all three systems.
Figure 6-2 Modeling uncrystalized residues. The residues without defined electron density in the crystallographic structure were modeled and are shown in purple.
The inhibitors pyrrophenone and oxoamide were constructed using the Accelrys Discovery Studio package, in which they were built and energy minimized to obtain their initial conformations. Each inhibitor was manually placed in the active site of GIVA PLA₂ using the aforementioned knowledge of the active site and residues implicated in binding. This was carried out using the Visual Molecular Dynamics (VMD) package (45), which was also used for further construction of all 3 systems. The protein (or the protein-inhibitor complex) was placed in a simulation box of dimensions 127 × 73 × 91 Å, solvated with approximately 22750 TIP3P water molecules, and neutralized with 28 sodium counterions (~80,000 atoms total for each system).

The minimization steps and all subsequent simulations were carried out using the NAMD molecular dynamics package (46). For the apo structure, an energy minimization was performed on the system first with the protein backbone atoms fixed—for 25000 steps, to allow the water/ions to conform to the shape of the protein—and then with no such fixation—for another 25000 steps, to relieve any unfavorable contacts in the entire system. For the inhibitor-bound systems, a similar energy minimization scheme was performed, with the addition of an extra minimization holding the inhibitor coordinates fixed, to relieve unfavorable contacts between the inhibitor and protein. For all three systems, a heating step was performed, in which the system was gradually heated (~50 ps) to a temperature of 300 K. During this heating and then the equilibration phase, the protein backbone and inhibitor atoms were restrained with a force constant of 5 kcal mol⁻¹ Å⁻². In the equilibration phase, pressure coupling was added and a series of restrained MD simulations were conducted. The restraints were gradually relieved and the free, un-
restrained system was equilibrated for 1 ns further. The production runs for each system commenced; each protein (or protein-inhibitor complex) was simulated for 50 ns.

For each of the production runs, the temperature was maintained at 300K using the Langevin thermostat, with a coupling constant of 2 ps\(^{-1}\). Pressure was maintained at 1.01325 kPa using the Langevin piston method, with the ‘GroupPressure’ and ‘FlexibleCell’ parameters turned on. Bonds to hydrogen atoms were held fixed while using a 2 fs timestep. Non-bonded and full electrostatics was calculated every 1 and 2 steps, respectively. Non-bonded interactions were smoothly switched off between 8.5 and 10 Å, while the cut-off for pairlist distance was set to 12 Å. Long-range electrostatic forces were evaluated using the particle mesh Ewald (PME) method (47). The CHARMM22/27 all-hydrogen parameter files were used for protein and inhibitors in all three simulations (48). The standard protein/amino acid parameters were used for GIVA PLA\(_2\) and pyrrophenone (by analogy to various amino acid side chain structures). The oxoamide was parameterized according to the lipid parameters provided in CHARMM27.

6.D Results and Discussion

6.D.1 GIVA PLA\(_2\) Digestion Map.

The protein digestion procedure was optimized to produce a peptide map that yielded the best coverage of GIVA PLA\(_2\) as described previously (11, 42). The optimized condition identified 185 distinct peptides that gave 94% coverage of the GIVA PLA\(_2\) sequence. From this group, the 71 peptides with the best signal to noise ratio with the least amount of redundant data were used to generate the Figures in the manuscript as
described previously (11, 42). All peptides were analyzed for deuterium content as a comparison, but only the ones with non-redundant data were used in the analysis.


The overall structure of the protein remained close to the crystal structure in all three simulations (Figure 6-3 RMSD). The regions missing in the X-ray structure, but model-built in the simulations, exhibited large fluctuations throughout the simulations, which is consistent with their being disordered in the crystals. However, the majority of the modeled regions remained solvated, without making significant contact with the rest of the protein. As a result, their motion did not affect the active site region of the enzyme. In the apo form, GIVA PLA₂ shows no significant conformational changes, as expected. In the inhibitor-bound forms, both pyrrophenone and the oxoamide show considerable movement in the first half of simulation but settle to a converged conformation and location in the last 25 ns, as judged by the root mean square (RMSD) of the inhibitors (see Figure 6-3). Thus, the last 25 ns of each simulation were used in all subsequent simulation analysis.
Figure 6-3 *Root mean square distance of protein and inhibitor*. For all C\(\alpha\) RMSD measurements the red represents the RMSD of all residues, with crystallized residues RMSD shown in blue (excluding 407-414, 431-462, 498-538, and 626-632). A. The RMSD of the C\(\alpha\) in the apoenzyme over the simulation time course (50 ns). B. The RMSD of the C\(\alpha\) in the pyrrophenone bound enzyme. C. The RMSD of the C\(\alpha\) in the oxoamide bound enzyme. D. The RMSD values of both the oxoamide (green) and pyrrophenone (red) over the simulation time course is plotted.

6.D.3 GIVA PLA\(_2\) Pyrrophenone Binding Experiments

We examined both the pyrrolidine derived inhibitor pyrrophenone as well as the 2-oxoamide derived inhibitor AX007. These compounds are structurally quite different and target different functionalities of the GIVA PLA\(_2\). Therefore, determining exactly
how these inhibitors bind is an important goal, because it allows the possibility of combining the best parts of each inhibitor to form new, more effective inhibitors to allow for further structure-activity studies. On-exchange experiments were performed on the intact GIVA PLA2 enzyme in the presence of both 10 μM pyrrophenone and 10 μM of the 2-oxoamide inhibitor AX007 to determine if inhibitor binding caused changes in deuterium exchange rates. The experiments were carried out at relatively low ratios of protein to inhibitor (1:2) in 1.5% DMSO to prevent possible complications from inhibitor aggregation and misleading deuterium exchange results. Experiments were carried out at seven time points varying from 10 to 10000 seconds. Both inhibitors showed multiple regions of the protein with greater than 10% change in the on-exchange rates between the inhibitor-bound and apo forms at all time points. These percent increases and decreases in on-exchange rates showed a strong correlation with computational data mapping percent chance of contact per residue number (Figure 6-4). The residues from 292-298 and 401-417 predicted to be in contact with the inhibitor (within 5 Å) from modeling that show no changes in deuterium exchange are all located in regions with either extremely slow or rapid exchange, and hence there is no significant difference in exchange between apo- and halo- forms (see Figure 6-5). The difference in on-exchange at the 100 second time point captured all of the major changes, and was used to generate the data shown in Figure 6-4.
Figure 6-4 Deuterium exchange information compared to computer simulation results. Panels A and B: The percent change in deuterium exchange between inhibitor free GIbA PLA2 and oxoamide-bound (panel A) or pyrrophenone-bound (panel B) GIbA PLA2 at 100 seconds of on-exchange is shown. Each bar represents a region that deuterium exchange was quantified. All changes greater than 10% are considered significant. Panels C and D: The percent chance of specific residues being within 5 Å of the docked inhibitor in the molecular dynamics simulation are plotted for oxoamide-bound (panel C) and pyrrophenone-bound (panel D).
Figure 6-5 Regions in contact with inhibitors with extremely fast or slow rates of exchange. Region 294-298 and 403-417 are plotted showing extremely fast rates of exchange (region 403-417), or extremely slow rates (region 294-298), with or without pyrrophenone or oxoamide present.

The inhibitor pyrrophenone was synthesized in 2001 (20); and contains a thiazoloidinedione ring postulated to target Arg-200 and a carbonyl group bridging the two benzoyl groups that is expected to target the active site Ser-228. This class of inhibitors was also shown, through structure-activity work, to have large increases in inhibitory potency with the addition of large bulky lipophilic substituents, suggesting the presence of a hydrophobic binding pocket in the enzyme (19). Using deuterium exchange and modeling we planned to test this hypothesis based on structure-activity work.

Eight regions of the GIVA PLA2 exhibited significant changes in deuterium exchange in the presence of pyrrophenone. Figure 6-6 shows these results both quantitatively and visually imposed on snapshots from the MD simulations. Three
regions of the protein, residues 393-397, 481-495, and 543-553 exhibited increased rates of exchange in the presence of pyrrophenone. Regions 393-397 and 543-553 had greater differences in on-exchange rates (between apo- and pyrrophenone-bound enzymes) at early time points of roughly 20-30%, with the difference going to zero at later time points. Region 393-397 contains Ala-396 and Phe-397 in contact with pyrrophenone. Region 481-495 had a constant 10-15% increase in exchange at all time points. These are the exact regions that we have previously shown had increases in on-exchange rates in the presence of the potent irreversible GIVA PLA2 inhibitor MAFP, as well as natural phospholipid substrate vesicles (11). We have hypothesized that these regions show an increase in exchange due to the opening of the lid region from 415-432, and that pyrrophenone also causes an opening of the lid region upon binding in the active site. In turn, this opening event induces an increase in the solvent accessibility and results in higher on-exchange rates. This lid opening was not seen in our simulations, and this is most likely a time dependent process that is too slow to view with molecular dynamics.
Figure 6-6 Deuterium exchange upon binding of 10 µM Pyrrophenone. A. The number of incorporated deuterons at seven time points in eight different regions, 256-265, 268-279, 393-397, 466-470, 473-478, 481-495, 543-553, and 684-689 in GIVA PLA2 are plotted onto the docked model of pyrrophenone binding at 50 ns of simulation time (the inhibitor is shown in space filled form). Decreases or increases in deuteration greater than 10% are represented by the color scheme in the legend.
Five regions of the protein, residues 256-265, 268-279, 466-470, 473-478, and 684-689 demonstrated decreases in exchange between apo- and pyrrophenone-bound enzymes (Figure 6-6). Region 268-279 exhibits greater than 30% decreases in exchange at all time points. Correlating with the MD simulation, this region harbors multiple residues that are in constant contact with the pyrrophenone. Regions 256-265 and 684-689 exhibit 20-30% decreases at early time points and drop to less than 10% at later time points. These regions also contain multiple hydrophobic residues demonstrated by simulation to be in contact with pyrrophenone; yet, this is to a lesser extent (and thus less of a decrease in exchange) as compared to region 268-279. Regions 466-470 and 473-478 showed 10-15% decreases in on-exchange rates from 30 to 300 seconds but no differences in exchange at earlier or later time points.

Our deuterium exchange results show decreases in exchange in numerous regions containing hydrophobic regions and this matches our modeling work as shown in Figure 6-7, where Pro-263, Leu-264, Leu -267, Val-272, Tyr-275, Trp-464, Ile-465, Ile-469, Met-470, and Phe-683 all make contact with the numerous phenyl groups in pyrrophenone. These residues most likely are acting as the hydrophobic pocket postulated through structure activity work with pyrrophenone, and our results correlate with recent data from Wyeth showing that GIVA PLA2 inhibitors become more potent when the steric bulk of the inhibitor is increased in functionally allowed regions (18). However we find that the thiazolidinedione functionality targets Ser-228, rather than targeting Arg-200, as originally suggested by shinogi (19, 20), with the carbonyl bridging the two phenyl groups being located at a large distant from the active site serine.
Figure 6-7 Residues involved in the binding pyrrophenone. The residues that have contact with pyrrophenone greater than 90% of the time in the molecular dynamics simulation are represented as red sticks and labeled on the figure. The inhibitor is shown in the licorice representation, with carbon, hydrogen, oxygen, nitrogen and phosphorus atoms colored cyan, white, red, blue and yellow, respectively.
6.D.4 GIVA PLA₂ Oxomaide Binding Experiments

The 2-oxoamide inhibitor AX007 was originally synthesized and shown to be an effective GIVA PLA₂ inhibitor in 2002 (26). It was postulated to target GIVA PLA₂ via an interaction between its 2-oxo amide functionality and the active site Ser-228. Also, the carboxylic acid moiety of the oxoamide was designed to target Arg-200, while the inhibitor’s long fatty acyl tail positions itself in the hydrophobic binding pocket (27). Using deuterium exchange and modeling we planned to test these hypothesis based on structure-activity work.

Seven regions of the GIVA PLA₂ exhibited significant changes in deuterium exchange in the presence of the oxoamide (Figure 6-8). Two regions of the protein, residues 481-495, and 543-553 exhibit an increase in exchange in the presence of the oxoamide. These regions show the exact same deuterium on-exchange profile as the pyrrophenone-bound enzyme. However, the region 393-397 does not show any difference in exchange in the presence of the oxoamide. This result allows for multiple interpretations; perhaps the lid region is opened in a different way (as compared to the enzyme-pyrrophenone complex), thus only increasing solvent accessibility for regions 481-495 and 543-553, but not for region 393-397. Or, the lid region may be opened in the same way, but increased contacts between region 393-397 and the oxoamide causes a comparative decrease in exchange rates between the two inhibitor-bound structures.
Figure 6-8 Deuterium exchange upon binding of 10 µM AX007. A. The number of incorporated deuterons at seven time points in seven different regions, 196-201, 256-265, 268-279, 555-564, 481-495, 543-553, and 684-689 in GIVA PLA2 are plotted onto the docked model of the oxoamide AX007 binding at 50 ns of simulation time (the inhibitor is shown in space filled form) Decreases or increases in deuteration greater than 10% are represented by the color scheme in the legend.
**Figure 6-9 Residues involved in the binding of the Oxoamide AX007.** The residues that have contact with the oxoamide greater than 90% of the time in the molecular dynamics simulation are represented as red sticks and labeled on the figure. The inhibitor is shown in the licorice representation, with carbon, hydrogen, oxygen, nitrogen and phosphorus atoms colored cyan, white, red, blue and yellow, respectively.
Five regions of the protein, residues 196-201, 256-265, 268-279, 555-564, and 684-689 exhibit decreases in exchange in the presence of the oxoamide. Regions 256-265 and 684-689 reveal the same on-exchange pattern in the presence of both the oxoamide and pyrrophenone. Simulation shows that along these regions, both inhibitors make similar hydrophobic contacts with Pro-263, Leu-264, and Phe-683 as shown in Figures 6-7 and 6-9. Region 268-279 has a 10-15% decrease in exchange at all time points, which is much lower than the corresponding differences in this region in the pyrrophenone-enzyme complex. This much smaller decrease in exchange in the the oxoamide-enzyme complex correlates well with the simulation, which shows no residues in 268-279 making contacts with the inhibitor. Region 555-564 displays a 10-20% decrease in exchange at all time points in the presence of the oxoamide. This region contains Asp-555 and neighbors Gly-551/Leu-552, all of which are in constant contact with the oxoamide during the simulation. Recent work by us has shown that the short, nonpolar, aliphatic R-group substituent on the oxoamide AX007 (residing on the linker between the 2-oxo and carboxylic acid) increased potency. We postulated that this is facilitated by a hydrophobic pocket in the enzyme that can accommodate this particular group (29). The residues in and around 555-564 appear to constitute this pocket, as shown here by both on-exchange results and simulation. Region 196-201 exhibits a 20-30% decrease in exchange at all time points in the presence of the oxoamide. This region contains the proposed oxy-anion hole—residues Gly-196, Gly-197, Gly-198, Gly-229, and Arg-200—required for catalytic activity, as well as Phe-199, which is part of the hydrophobic pocket for the substrate. The modeling data shows an interaction between the carboxylic
acid of the oxoamide and Arg-200, as well as the carbonyl of the 2-oxoamide in contact with the oxyanion hole composed of the numerous glycine residues.

6.D.5 Differences in Oxoamide and Pyrrophenone Binding

Numerous regions of the protein show the same decreases or increases in exchange with both pyrrophenone and the oxoamide AX007. These regions include 256-265, 481-495, 543-553, and 684-689, and they all show similar contacts in these regions between both AX007 and pyrrophenone as shown in Figures 6-7 and 6-9. There are also regions such as 466-470, 473-478, and 555-564 which show changes only in the presence of one or the other inhibitor, and this is explained by specific contacts only seen between pyrrophenone or the oxoamide and the protein.
Figure 6-10 Different inhibitors cause different rates of exchange of the active site residues of GIVA PLA2. The number of incorporated deuterons at seven time points in five different regions, 196-201, 225-232, 393-397, 577-591, and 670-682 in GIVA PLA2 are plotted onto the docked models. Areas that show less exchange with the oxoamide AX007 and more exchange with pyrrophenone are colored onto the respective structures.

However region 196-201 acts as an interesting example of the differences between pyrrophenone and oxoamide inhibitor binding. There are four different regions in the enzyme 196-201, 225-232, 577-591, and 670-682 that show an increase in
exchange with pyrrophenone and a decrease in exchange with the oxoamide (Figure 6-10). Many of these peptides do not show a change in deuterium exchange greater than 10% as compared to the apo structure. However, the comparison between pyrrophenone and the oxoamide does show a greater than 10% change in exchange. These peptide regions are all in or near the active site of the enzyme. Region 225-232, which contains the active site residue Ser-228, never exchanges greater than 25% at any time point. Yet, there is a greater than 10% change in exchange between the oxoamide and pyrrophenone-bound studies, with the oxoamide-enzyme complex showing less exchange than the pyrrophenone-bound sample. For regions 577-591 and 670-682, the main effects are most likely localized to 577-580 and 680-682, respectively, which are located within 5 Å of the active site. These results show that the 2-oxoamide inhibitor AX007 decreases the solvent accessibility of the active site while pyrrophenone has the opposite effect (an increase). These results help to explain why region 393-397 has an increase in exchange with pyrrophenone and not with the oxoamide. This region is located near the active site, and increases in exchange are seen with the presence of pyrrophenone, MAFP, and natural phospholipid substrate, (11) but not the oxoamide. The decreased solvent accessibility of the active site in the presence of the oxoamide would explain the lack of exchange increases in region 393-397. From viewing Figure 6-10, it is also apparent that the oxoamide mainly occupies the active site area, while pyrrophenone is mainly bound in the cap region near the interfacial binding surface of the enzyme.
6.E Conclusions

These results have greatly enhanced our knowledge of how these two different inhibitors bind GIVA PLA₂ and have allowed us to model all the residues contacting both inhibitors. This will allow us to create new inhibitors combining the 2-oxoamide functionality with a bulky lipophilic substituent in place of the acyl fatty acid tail to mimic how pyrrophenone binds GIVA PLA₂ through multiple hydrophobic contacts located on or near the cap region. This study is the first to combine deuterium exchange mass spectrometry with molecular dynamics simulations for the determination of inhibitor binding. This methodology is an exciting new tool in developing better inhibitors, and we plan to continue this work through the synthesis and testing of new GIVA PLA₂ inhibitors based on our results.

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6.G References


