Review

Pancreatic phospholipase A$_2$: new views on old issues

Chunhua Yuan $^a$, Ming-Daw Tsai $^{a,b,c,d,*}$

$^a$ Department of Chemistry, Ohio State University, 100 West 18th Avenue, Columbus, OH 43210-1173, USA
$^b$ Department of Biochemistry, Ohio State University, Columbus, OH 43210, USA
$^c$ Campus Chemical Instrument Center, Ohio State University, Columbus, OH 43210, USA
$^d$ Ohio State Biochemistry Program, Ohio State University, Columbus, OH 43210, USA

Received 17 February 1999; accepted 31 July 1999

Abstract

The recent development in the structure-function relationship of pancreatic phospholipase A$_2$ is reviewed. The results of extensive studies by a combination of site-directed mutagenesis, X-ray crystallography, and NMR have provided new insight into several old issues. In particular, we summarize current views on the active site, the interfacial binding site, the mechanism of interfacial activation, the roles of the hydrogen-bonding network and the catalytic dyad, and the conformational stability of the structure. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Pancreatic phospholipase A$_2$; Site-directed mutagenesis; Interfacial activation; Hydrogen-bonding network; Scooting mode kinetics; Nuclear magnetic resonance; X-Ray crystallography

Contents

1. Introduction .......................................................... 215
2. New views on old issues in pancreatic phospholipase A$_2$ .............................................. 216
   2.1. Interfacial catalysis: active site vs. interfacial binding site .................................. 217
   2.2. Interfacial activation: enzyme model vs. substrate model .................................. 218
   2.3. Hydrogen bonding network is more important for structure than for function .......... 218
   2.4. Catalytic dyad plays both functional and structural roles .................................. 219
   2.5. The structural paradox of PLA$_2$: rigid but fragile ........................................ 220
Acknowledgements .......................................................... 220
References ............................................................... 221

1. Introduction

Pancreatic phospholipase A$_2$ (PLA$_2$, EC 3.1.1.4), a sub-family of the growing PLA$_2$ super family (group IB) [1,2], hydrolyzes the sn-2 ester bond of phospho-
lipids. These Ca\(^{2+}\)-dependent and highly homologous enzymes are typically small (13–15 kDa); however, they possess several interesting structural features (Fig. 1). In a small globular fold there are a rich array of secondary elements (approx. 50% \(\alpha\)-helix and approx. 10% \(\beta\)-sheet), seven disulfide bonds, an Asp-His catalytic dyad, a ‘hydrophobic channel’, an ‘interfacial binding site’, and a hydrogen-bonding network. The function of PLA2 is also unique – it is a water-soluble protein working at a lipid-aqueous surface of micelles or bilayers. Furthermore, many homologous variants of PLA2 have entirely different biological functions. Some of them are potent toxins or allergens.

Pancreatic PLA2 is among the first studied and best characterized lipolytic enzymes, particularly with respect to enzymatic mechanism and structure-function relationship. Initially the information was obtained through various ‘classical’ chemical techniques, such as protein sequencing and chemical modification [3]. The crystal structure of a free enzyme (bovine PLA2) was first reported in 1978 [4–6], which together with the biochemical data led to a proposed catalytic mechanism [7]. Since then (particularly in the last decade), we have witnessed a great expansion of knowledge in this field due to three major approaches [8]. (1) Recombinant DNA techniques: the development followed by rapid expansion of these techniques set the stage for delineating structure-function relationships at the molecular level. The techniques permit high-level expression of proteins, site-directed mutagenesis, isotopic labeling, etc. Porcine [9] and bovine [10,11] pancreatic PLA2s are among the first PLA2 enzymes that were cloned and overexpressed in *Escherichia coli*. (2) Kinetic assays: catalysis at interfaces is complicated by the binding process of free enzyme (E) to interface-bound enzyme (E\(*\)). Jain and co-workers have developed both the scooting mode kinetics (the enzyme stays bound to the membrane during catalytic events) [12] and hopping mode kinetics (the enzyme hops through aqueous phase) [13] that simplify the kinetic model of data analyses. (3) Structural analyses: a significant number of PLA2 structures, including mutant enzymes as well as enzyme complexed with substrate and transition state analogs (EI), have been solved by crystallography in the past decade [14]. In the same time biomolecular nuclear magnetic resonance (NMR) has emerged as another powerful tool for structural analyses. To date, the solution structures of porcine PLA2 in the absence and presence of the micellar interface [15,16] and free bovine PLA2 [17] have been determined by multidimensional NMR.

There have been several comprehensive reviews on PLA2 in recent years, from different angles or with different focuses [1,2,14,18–21]. This review will update a recent one by Verheij [8] on the structure-function relationship of pancreatic PLA2 (bovine and porcine pancreas mainly), in which our laboratory has been interested for a number of years. In particular, we address the recent studies that have provided new views on several old issues.

### 2. New views on old issues in pancreatic phospholipase A\(_2\)

The vast majority of studies on pancreatic PLA2 have been performed on bovine and porcine proteins, which share approx. 85% sequence homology. Many residues that are highly conserved in the sub-family have been investigated by site-directed mutagenesis in conjunction with structural and functional analyses as summarized in Table 1. Due to space restraints the following sections will address only the most interesting points.
2.1. Interfacial catalysis: active site vs. interfacial binding site

Interfacial catalysis by PLA2 can be adequately described by the steps of $E \equiv E^* \equiv E*S$ (enzyme-substrate complex at the interface) $\equiv E*P$ (enzyme-product complex at the interface) $\equiv E^*+P$ (product). It is implicit in such a scheme that the interfacial binding site is topologically distinct from the active site. Binding of the enzyme to the interface and binding of the substrate to the active site are kinetically distinct events [22,23]. The interfacial binding surface (IBS) has been proposed to be the flat external surface that surrounds the active-site slot [18,23,24]. The substrate binding at the active site has been clearly revealed by crystal structures of several EI complexes [25–30]. For details the reader is referred to the recent review by Scott [14].

The active site residues have been thoroughly investigated as described in later sections. The obligatory role of Ca$^{2+}$ [31–33] and the contributions of residues in the hydrophobic channel [34] have also been confirmed by site-directed mutagenesis and scooting mode kinetics. The enzyme-lipid binding, by contrast, is relatively poorly understood. Attempts to determine the membrane-bound protein structures by crystallography or solution NMR are hampered by inherent difficulties. Much effort has been directed to mapping the putative IBS, which commonly contains a ring of cationic and hydrophobic residues. Bovine pancreatic PLA2 is the most thoroughly studied in this regard [35–42]. The IBS of this enzyme involves a cluster of N-terminal residues, a cluster of C-terminal residues, and several other residues (Fig. 2). It was found that the effects of modifying individual residues are usually modest because the IBS includes a large number of residues. The interfacial binding is likely governed by electrostatic as well as hydrophobic interactions.


The contributions of these residues to substrate binding at the active site and catalysis have been confirmed. In particular, substitutions of L2 showed an acyl chain length discrimination toward different substrates.

Calcium binding residues: D49(K, E)$^P$ [70], D49(N, E, Q, K, A)$^B$ [31,61], G305$^B$ [77]

Deletion of surface loop enhances activity and alters specificity in porcine PLA2, but causes a slight conformational change around the active site in bovine PLA2. V63 is responsible for the helix III conformation in bovine PLA2, which is absent in porcine PLA2.

The two proteins are indicated by superscripts ‘B’ and ‘P’, respectively.
roles. For example, K56 was revealed to be involved in the substrate binding at the active site as well as in the interfacial binding on IBS [36,39]. The binding of the enzyme to the interface is likely governed by electrostatic and hydrophobic interactions [43], but how PLA2 is positioned at the interface is still not entirely clear. Recently Lin et al. [44] have developed a method involving site-selective spin-labeling and electron paramagnetic resonance spectroscopy, with which it was demonstrated that PLA2 sits on the membrane surface rather than digging into the membrane.

2.2. Interfacial activation: enzyme model vs. substrate model

PLA2 is several orders of magnitude more active on aggregated substrates than on monomeric forms, a phenomenon termed ‘interfacial activation’. The molecular mechanism behind this intriguing phenomenon has been the subject of much study and debate for years [3,8,14,18,20]. The two prevailing hypotheses reflect the contribution from the physical forms of substrate (‘substrate model’) and conformational changes of enzyme (‘enzyme model’).

The substrate model attributes the activity enhancement to lipid orientation, lipid conformation, hydration of the polar headgroups, etc. This hypothesis culminated in the early 1990s when Sigler and co-workers proposed ‘facilitated substrate diffusion’ from the interfacial binding surface to the catalytic site [45]. The key evidence for their claim is that crystallography revealed little structural change between E and EI [25–27]. By further comparisons of several evolutionarily divergent PLA2s it was argued that optimal active-site architecture already exists in the free enzyme.

The enzyme model states that enzyme conformational change induced by lipid binding optimizes the active site and enhances catalysis. This model appears to be supported by several recent studies particularly on pancreatic PLA2. In contrast to the conclusion by Scott et al. based on their crystallographic work [45], a cation-binding kinetic study with UV difference spectroscopy has suggested a remarkable plasticity in the active site region [33]. More importantly, it was revealed by NMR [46–48], fluorescence [49], and even more recent crystallographic work [29] that some conformational changes do occur in the enzyme upon monomeric substrate analogue and/or lipid binding, which might provide a structural basis for the allosteric interfacial activation. Collectively, the regions that are flexible in the free enzyme, such as the N-terminus and surface loop, become more ordered in the complexed form. It appears that crystal structures of free enzyme resemble the E* forms [15,17], which may be due to ordering inherent in the crystallization process. The inability of proPLA2 (PLA2 with an additional seven residues at the N-terminus) to display interfacial activation could be rationalized in light of the fact that the enzyme could not form an ordered N-terminus and surface loop in either E [50,51] or EI [52] crystalline forms. However, it is important to note that the evidence for the enzyme model is suggestive rather than conclusive. Strictly speaking, it would be the conformational difference between ES (enzyme-substrate complex) and E*S that is significant. Moreover, it is possible that both types of activation mechanism act together since they are not mutually exclusive.

2.3. Hydrogen bonding network is more important for structure than for function

The water-mediated hydrogen-bonding network, involving the catalytic dyad (H49-D99), N-terminal region (A1 and Q4), and several other residues (Y52, Y73, etc.), is one of the salient features observed in the crystal structures of PLA2 (Fig. 3A) [4–6]. The tyrosine residues Y52 and Y73 are absolutely conserved in all known class I/II PLA2 sequences. The network, which links the active site to the interfacial
binding site, was suggested to be important for interfacial catalysis [3,7]. However, the fact that deletion of the phenolic OH group of Y73 did not affect the catalytic activity cast serious doubt on this proposition [53–56]. Moreover, single, double and even triple mutants have been constructed on the conserved residues (Y52, Y73 and D99) and they have been studied by scooting mode kinetics and crystallography [53–61]. In the Y52F/Y73F/D99N triple mutant, kinetic analysis demonstrated retention of considerable activity (with a $k_{cat}$ value of 5% compared to wild type), while X-ray crystal structure revealed a substantial disruption of the H-bonding network [57]. Thus the mounting evidence suggests that the so-called H-bonding network associated with residues Y52 and Y73 as well as the A1 ($\alpha$-NH$_3^+$) and Q4 [37] is not crucial for any form of interfacial activation and the effect on catalytic function is modest at best.

On the other hand, solution structures of free bovine and porcine PLA2 revealed a less defined H-bonding network [15,17]. It was reported that the network would be fully formed only when a substrate binds to the active site at the interface [16,46]. However, the portion of the H-bonding network away from the N-terminus is clearly defined in free bovine PLA2 (Fig. 3B) [17], as evidenced by direct observation of a strong H-bond between Y73 and D99 and an implicated interaction between D99 and H48 (see Section 2.4). The role of the H-bonding network is probably to support the His-Asp catalytic dyad as well as to contribute to the conformational stability. It has been shown that substitutions on the conserved residues destabilized the enzymes [54–58]. Analysis of a series of mutants constructed in bovine PLA2 indicated that the existence of the Y73-D99 H-bond correlates directly with the conformational stability of the mutant [17]. Loss of this H-bond results in a loss of 2–3 kcal/mole in the conformational stability.

2.4. Catalytic dyad plays both functional and structural roles

The D99-H48 dyad is the catalytic machinery in PLA2. The proposed catalytic mechanism involving the dyad and a catalytic water resembles that of the renowned ‘catalytic triad’ (Asp-His-Ser) for serine proteases [3,7,8,18]. It is thus within expectation that the protonation state of H48 is critical for the enzyme activity [60,62,63].

The essential features of this proposal have been confirmed by crystallographic studies as well as site-directed mutagenesis [25–30,64]. A striking finding is the structural importance of the N$^\alpha$ of H48 by functional and structural analyses on a series of mutants H48A, H48N and H48Q [64]. The amide nitrogen atoms of Asn and Gln mimic the N$^\alpha$ and N$^\beta$, respectively, of His. Kinetic analysis indicated that H48N retains $6 \times 10^{-5}$ of the original activity while
H48Q and H48A show no detectable activity. On the other hand, structural analyses revealed that the conformation and conformational stability are largely retained in H48Q but not in H48A and H48N [17,61,64,65]. In particular, the D99-Y73 H-bond was observed only in H48Q [17]. Based on these findings it was apparent that while the N\textsuperscript{\theta 1} atom of H48 is critical for the catalysis by PLA2, the N\textsuperscript{\theta 2} atom of the same residue plays an important structural role.

The N\textsuperscript{\theta 2} atom is hydrogen bonded to the carboxylate side chain of D99. While the strength of this H-bond has not been estimated at this point, the interaction between these two residues likely contributes to the structural integrity via several possible mechanisms [17]. First, it is evident that the interaction acts to orient the imidazole ring and fix its tautomeric form required for catalysis. Second, this H-bond anchors the D99 side chain to ensure correct formation of the Y73-D99 H-bond and others in the H-bonding network. The D99-H48 interaction could also aid in the stabilization of the two long anti-parallel disulfide-linked (C44-C105 and C51-C98) \alpha-helices, both of which are identified as the most stable secondary elements by hydrogen-deuterium exchange experiments [17,66]. Therefore, while D99-H48-H\textsubscript{2}O can be regarded as the catalytic triad in PLA2, our work has led to the significant finding of the ‘structural triad’ H48-D99-Y73, which is crucial for the enzyme’s conformation as well as conformational stability [17,54,55,60,64]. The dual role of the D99-H48 dyad illustrates an important point in enzyme evolution – the interplay between structure and function.

2.5. **The structural paradox of PLA2: rigid but fragile**

PLA2 proteins are well known to display a remarkable stability against denaturation (\(\Delta G_{d}^{H_{2}O} = 9.6\) kcal/mol for bovine PLA2). It is a general perception that a high number (six or seven) of disulfide bonds and a high content of secondary structure are stabilizing forces. Engineering on these disulfide bonds has been performed on bovine PLA2 [67] and the results are summarized in Table 1.

Surprisingly, the PLA2 structure appears to be very fragile to point mutations as revealed in the study of bovine pancreatic PLA2. Many mutants (> 20) designed at sites of presumed functional importance (e.g. catalytic dyad) display properties reminiscent of a molten globule state [38,54,55,57,64,67]. Further analyses of some of the mutants by NMR, fluorescence and circular dichroism spectroscopic methods indicated that the formation of such a ‘molten globule-like state’ is a pH-dependent phenomenon and that the tertiary structure is not completely lost [65]. The residual structure is generally located in the upper-half of the protein in Fig. 1, which could be attributed to the stabilization by some of the disulfide bonds. It is interesting to note that most of the functional residues (the active site, the hydrophobic channel, the interfacial binding site, and the calcium binding loop) reside in the remainder of the protein, which is well disrupted in tertiary interactions. The anti-parallel \(\beta\)-sheet was identified to be part of the residual structure [65]. Despite generally poor sequence conservation in this segment, all known class I/II PLA2s contain one well-developed \(\beta\)-wing. While this segment receives little attention in the structure-function relationship studies, its role is likely to maintain PLA2 structural integrity: partly through the Y73-D99 H-bond [17,55] and the C77-C11 disulfide bond [67].

The extreme sensitivity of the PLA2 structure to site-directed mutagenesis is unexpected, particularly in view of the extreme heat and acid stability of wild type PLA2. This unusual behavior is distinct from many other enzymes that are less stable against denaturation but demonstrate remarkable plasticity and tolerance to amino acid substitutions. One such example is adenylyl kinase (AK), a protein that has no disulfide bonds and is only marginally stable (\(\Delta G_{d}^{H_{2}O} = 3.8\) kcal/mol) [68]. Thus the behavior of PLA2 can be interpreted as ‘stable but fragile’, while AK can be said to be ‘unstable but plastic’. The biological significance of the fragility in PLA2 structure needs further investigation.

**Acknowledgements**

The work from our laboratory was supported by a grant from National Institutes of Health (GM 41788 to M.-D.T.). We sincerely thank M.K. Jain and M. Sundaralingam for long term collaboration with our group.