Binding of bee venom and human group IIa phospholipases A\textsubscript{2} to membranes: a minor role for electrostatics


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Introduction

Phospholipases A\textsubscript{2} (PLA\textsubscript{2}s) hydrolyse the sn-2 ester of phospholipids and have been studied extensively because of their role in liberating arachidonic acid from cellular membranes for the biosynthesis of eicosanoids [1,2]. There are now 10 different PLA\textsubscript{2} classes based on amino acid sequences [3,4]. Many of these are small (~14–18 kDa), secreted, calcium-dependent enzymes found in snake, insect, and lizard venoms, animal digestive fluids, and inflammatory exudates (sPLA\textsubscript{2}s). The pancreatic, cobra venom, and bee venom sPLA\textsubscript{2}s have been extensively studied from a structure–function point of view and as a paradigm for the action of water-soluble enzymes at the lipid–water interface (interfacial enzymology) [5–9]. The availability of X-ray structures of several sPLA\textsubscript{2}s [10] and the NMR structure of pancreatic sPLA\textsubscript{2}s [11,12] have provided the necessary components for studying the molecular basis for interfacial binding and catalysis [13–16]. In some cases, structure–function studies have been interpreted in terms of an analytical kinetic reaction scheme for interfacial catalysis [8,9].

sPLA\textsubscript{2}s bind tightly to anionic vesicles

Although it is certain that sPLA\textsubscript{2}s must transfer from the aqueous phase to the membrane interface to hydrolyse naturally occurring long-chain phospholipids that have virtually no solubility in water (i.e. lipolysis by phospholipase A\textsubscript{2} necessarily occurs at the interface [17]), until recently there was limited information on the molecular determinants of interfacial binding of these enzymes. The X-ray structures of sPLA\textsubscript{2}s reveal a deep (~15 Å) active site slot that accommodates about half of the length of a phospholipid molecule. The catalytic residues lie at the end of the slot away from the protein’s surface, and thus it is imagined that the substrate must leave the plane of the membrane and transfer down the length of the active site slot (Figure 1). It has been presumed that the surface of the enzyme that surrounds the opening to the active site slot makes direct contact with the membrane, although the precise orientation of the enzyme on the membrane has only recently been determined (see below).

To date there are no exceptions to the phenomenon that sPLA\textsubscript{2}s bind orders of magnitude tighter to anionic membranes than to neutral ones [18]. For example, pancreatic, bee venom, and human group IIa sPLA\textsubscript{2}s bind poorly to phosphatidylcholine vesicles (even with millimolar amounts of lipid present), but binding to anionic phosphatidylmethanol and phosphatidylserine vesicles is virtually irreversible such that lipolysis occurs without desorption of enzyme into the aqueous phase (scooting mode) [19–21]. Not surprisingly, it has been generally accepted that interfacial binding of sPLA\textsubscript{2}s to membranes is driven predominantly by electrostatic interactions between enzyme and negatively charged head groups of anionic phospholipids. In fact almost all sPLA\textsubscript{2}s have a net positive charge on their putative interfacial binding surfaces due to the presence of lysine and arginine residues and a deficit of anionic residues [22]. However, the role of these basic residues in supporting tight interfacial absorption has been explored only recently by using site-directed mutagenesis, and the results are startling as described below.

Electrostatics is not the major interfacial binding force for bee venom (bv)PLA\textsubscript{2} and human group IIa sPLA\textsubscript{2}

Extensive mutagenesis of the interfacial binding surfaces of bvPLA\textsubscript{2} and of human group IIa sPLA\textsubscript{2} has been carried out recently [23,24]. The X-ray structure of bvPLA\textsubscript{2} reveals a putative interfacial binding surface that contains six basic
Figure 1

Interfacial binding surfaces of bee venom (bv)PLA2 and gila monster venom sPLA2

The structures on the left and in the centre are a stereo pair of bvPLA2; the structure on the right is gila monster PLA2. The orientation of bvPLA2 is such that the membrane binding surface is facing the viewer (based on [25]). The structure of the gila monster enzyme has been predicted by sequence comparison with bvPLA2. A short-chain phospholipid analogue in the active site of bvPLA2 is shown as a dark ball-and-stick structure [30]. Basic residues are darkened and are labelled K (lysine) and R (arginine), hydrophilic uncharged residues are not darkened but are labelled, and hydrophobic residues are in grey but are not labelled.
residues (lysines and arginines), two histidines (which may be protonated), and only one anionic residue (aspartate-92) (Figure 1). Note that these basic residues form a collar around a large cluster of hydrophobic residues. The surface contains only a single non-charged hydrophilic residue (threonine). In fact, several sPLA2s (those for which an X-ray structure is available) have a similar disposition of basic and hydrophobic residues. An interesting exception is the sPLA2 from gila monster venom. Although a crystal structure of this enzyme is not available, the primary structure is very similar to that of bvPLA2, and it has been possible to build a homology model (Figure 1). The interfacial binding surface of the gila monster enzyme has a large number of lysines and arginines scattered over its entire surface. Three basic residues, R7, K10 and K16 lie closest to the interfacial binding surface, and so they are the residues responsible for the large effects of charge reversal mutation on interfacial binding observed [23]. This enzyme contains a large number of lysines and arginines scattered over its entire surface. Three basic residues, R7, K10 and K16 lie closest to the active site slot and are thus most probably on or close to the interfacial binding surface. The triple mutant R7E/K10E/K16E binds about 300-fold weaker to anionic vesicles than does wild-type protein. The specific activity of this enzyme is comparable with that of the wild-type enzyme in the presence of 2–200 μM anionic vesicles, which argues that the triple mutant is properly folded. It is estimated that only a small percentage of the total interfacial binding energy is due to this patch of basic residues. Charge reversal mutation of other basic residues further away from the centre of the putative interfacial recognition surface results in a smaller reduction of interfacial binding.

The effects of mutating the basic residues on the interfacial binding surface of bvPLA2 were studied [24]. Remarkably, mutating basic residues to glutamate (charge reversal) singly or collectively (five out of six lysines/arginines) on the putative interfacial binding surface of bvPLA2 (Figure 1) results in enzymes that display nearly normal catalytic properties. These mutants, like the wild-type bvPLA2, bind poorly to phosphatidylcholine vesicles, and binding to anionic vesicles (1,2-dimyristoyl phosphatidylmethanol or 1-palmitoyl-2-oleoyl phosphatidylserine) is sufficiently tight that the enzymes operate in the scooting mode. Tight binding to anionic vesicles was confirmed by direct binding studies using fluorescence resonance energy transfer from bvPLA2 tryptophan residues to a dansylated phospholipid probe present at low concentration in vesicles. Addition of excess anionic vesicles without dansylated probe to vesicles containing probe and bound bvPLS2 will result in a decrease in energy transfer as enzyme transfers between vesicles. Although this decrease was observed, it occurred very slowly for wild-type and charge reversal bvPLA2 mutants (half-life >1 h for both). It is estimated that of the >15 kcal/mol of binding energy for the wild-type bvPLA2-vesicle interaction, only 1–3 kcal/mol is due to these basic residues. These results and also the lack of salt-induced desorption of bvPLA2 from vesicles strongly argues that interfacial binding of bvPLA2 is not driven mainly by electrostatic interactions. Finally, gila monster sPLA2 also displays high activity on anionic vesicles compared with witterionic vesicles despite the fact that this enzyme has only a single basic residue (lysine-92).

Similar findings were found with mutants of human group Ila sPLA2, although somewhat larger effects of charge reversal mutation on interfacial binding were observed [23]. This enzyme contains a large number of lysines and arginines scattered over its entire surface. Three basic residues, R7, K10 and K16 lie closest to the active site slot and are thus most probably on or close to the interfacial binding surface. The triple mutant R7E/K10E/K16E binds about 300-fold weaker to anionic vesicles than does wild-type protein. The specific activity of this enzyme is comparable with that of the wild-type enzyme in the presence of 2–200 μM anionic vesicles, which argues that the triple mutant is properly folded. It is estimated that only a small percentage of the total interfacial binding energy is due to this patch of basic residues. Charge reversal mutation of other basic residues further away from the centre of the putative interfacial recognition surface results in a smaller reduction of interfacial binding.

### Locating the interfacial binding site on bvPLA2: a new structure technique

The modest effects of charge reversal mutagenesis on the interfacial binding of bvPLA2 and human group Ila sPLA2 to anionic vesicles is startling. To test the notion that the interfacial binding surface of sPLA2s is the surface that surrounds the active site slot, we developed a new technique based on electron paramagnetic resonance (EPR) for determining the orientation of interfacial enzymes at the membrane–water interface [25]. We used site-directed mutagenesis to introduce 13 cysteines, one per protein, at various points on the surface of bvPLA2 (mostly on or near the putative interfacial binding surface). The thiol groups of these cysteines were selectively modified with a nitroxide spin labeling reagent. EPR was used to detect the spin label for the enzyme in solution or bound to anionic vesicles (1,2-ditetradecyl phosphatidylmethanol, which cannot be hydrolysed by the enzyme) and in the presence and absence of 10 mM [Cr(Oxalate)]$_{1}^{3-}$ (Crox). Crox serves as a membrane-impermeant spin relaxant (its actual charge is $-2.3$), and its effect on the relaxation parameters of the spin probe depends on the concentration of Crox in the vicinity of the spin label. The latter depends on the Crox-
The mysterious role of hydrophobic residues in supporting interfacial binding

The clear message that emerges from the study of bvPLA2 and human group IIa sPLA2 is that interfacial tight binding to anionic vesicles is driven mainly by forces other than electrostatic effects. The EPR data show that the membrane binding surface contains a patch of several hydrophobic residues near the active site slot. This surface contains only a single basic residue, lysine-14. Thus, this EPR study is consistent with our mutagenesis results showing that the collection of six basic residues are not critical for interfacial binding.

Perhaps the bulky head groups of phosphatidylcholine provide a steric impediment to the formation of precise molecular interactions between enzyme and interface that drive interfacial binding. Finally, it is interesting to note that the binding of cellulases to the surface of microcrystalline cellulose is driven by hydrophobic residues, including tryptophans, on the face of their cellulose binding domains [29]. One would have thought that binding to cellulose surfaces is driven by hydrophilic interactions. There is much to be learned about how interfacial enzymes absorb on to their substrate-containing surfaces.

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Phospholipases catalyse the cleavage of cellular phospholipids leading to the liberation of lipid second messengers of signal transduction. In most cell types, phospholipase A2 is responsible for the liberation of arachidonic acid after cellular stimulation and thus initiates the production of biologically active eicosanoid metabolites. The majority of the phospholipase A2 activity in most cells is catalysed by a calcium-independent phospholipase A2 (iPLA2) which demonstrates selectivity for substrates containing a vinyl ether linkage at the sn-1 position (plasmalogens) and arachidonic acid at the sn-2 position. We first identified iPLA2 activity in myocardium in 1985 [1] and subsequent studies have demonstrated that iPLA2 is also the major phospholipase A2 in brain, smooth muscle, and pancreatic β cells [2-5]. In contrast, cPLA2 is the major phospholipase A2 in many circulating cells (e.g. platelets) [6]. Because of the apparent unique nature of myocardial phospholipase A2 activity (i.e. calcium independence) we undertook purification of this enzyme; this was facilitated by the demonstration that it possessed an ATP binding site [7]. Remarkably, ATP both activated and stabilized myocardial iPLA2 enzymic activity [8]. We purified a 40 kDa polypeptide by ATP affinity chromatography and demonstrated that the 40 kDa polypeptide was radiolabelled after treatment with the mechanism-based inhibitor [3H] (E)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one (BEL) [9, 10]. However, we were not able to obtain sequence data for the purified myocardial enzyme due to its low abundance. Subsequently, Jones and co-workers identified the sequence of an 80 kDa iPLA2 in Chinese hamster ovary cells [11] which possessed an identical active site to that of a previously purified iPLA2 from potatoes (i.e. 1985

Abbreviations used: iPLA2, calcium-independent phospholipase A2; BEL, (E)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one; AVP, arginine vasopressin; BAPTA-AM, 1,2-bis-(o-aminophenoxy)ethane-N,N',N''-tetraacetic acid tetra-(acetoxymethyl)ester; IP3, inositol 1,4,5-trisphosphate. Owing to exceptional unforeseen circumstances, Professor Gross was unable to attend the meeting to present this paper, but it is included here for the sake of completeness of the Colloquium.

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