Interfacial binding of secreted phospholipases A_2: more than electrostatics and a major role for tryptophan

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Secreted phospholipases A_2 have similar catalytic sites, but vastly different interfacial binding surfaces that modulate their binding affinity for different kinds of phospholipid vesicles by several orders of magnitude. The structure/function principles that dictate both the differential interfacial binding and the physiological function of these enzymes are beginning to be unraveled.

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Abbreviations

EPR	electron paramagnetic resonance
K_d	dissociation constant
sPLA2s	secreted phospholipases A_2

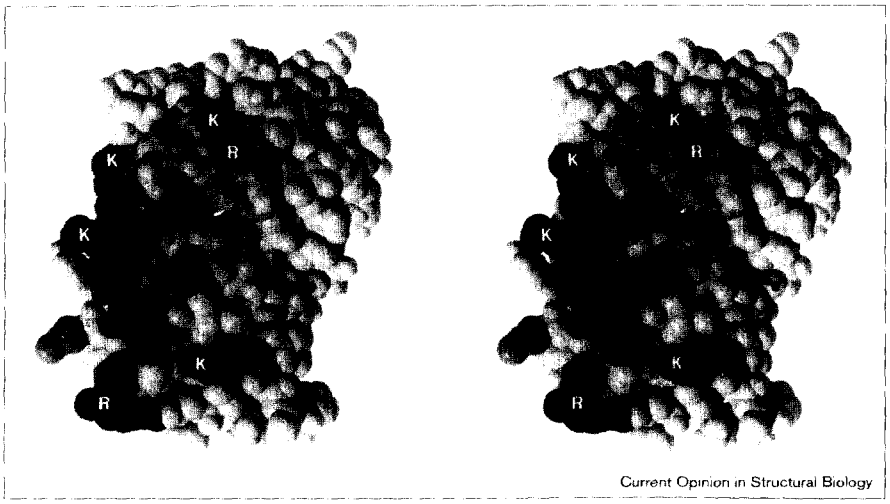
Introduction

Secreted phospholipases A_2 (sPLA2s) are small (~14 kDa), disulfide-rich, calcium-dependent enzymes that hydrolyze the *sn*-2 ester of glycerophospholipids to release a free fatty acid, such as arachidonic acid for eicosanoid biosynthesis, and a lysophospholipid [1]. According to amino acid sequences and disulfide number and positioning, nine groups of sPLA2s have been described [2,3]. As these enzymes act on naturally occurring phospholipids that have virtually no solubility in water, they must bind to the membrane interface to gain access to their substrates. The purpose of this review is to highlight some recent developments in our understanding of the molecular basis of the association of sPLA2s with the membrane interface.

Interfacial binding, catalytic site binding and crystal structures

The fact that sPLA2s can act in a highly processive scooting mode, in which the enzyme remains bound to the vesicle and undergoes many lipolysis reaction cycles, proves that the interfacial binding of the enzyme to the membrane ($E \rightarrow E^*$) and the binding of a substrate molecule to the catalytic site of the enzyme at the interface ($E^* + S^* \rightarrow E^* \bullet S^*$) are distinct steps [4,5]. The crystal structures of pancreatic, venom and human nonpancreatic sPLA2s demonstrate a high degree of structural homology [6]. All contain a catalytic site slot that extends from one surface of the enzyme to the opposite face (Figure 1). Recent structural studies using a novel, electron paramagnetic resonance (EPR)-based membrane docking technique have proven that the surface of the enzyme that surrounds the opening to the catalytic site slot constitutes the interfacial binding surface (i-face) [7••] (Figure 1). Interfacial binding is thought to seal the enzyme to the membrane, in order to facilitate phospholipid transfer into the catalytic site slot. Thus, the structural studies provide strong circumstantial evidence for separate the interfacial binding and catalytic site loading steps. The relative importance of electrostatic and

Figure 1



The i-face of bee venom sPLA2. Stereo pair depiction of the X-ray structure of bee venom sPLA2 [32]. A short-chain phospholipid analog bound in the catalytic site slot is shown as a black ball-and-stick structure. Residues that lie on the face that includes the opening to the catalytic site slot are basic (arginine and lysine; dark gray), hydrophobic (mid gray) and hydrophilic (histidine and threonine).

hydrophobic interactions in interfacial binding must ultimately depend both on the physicochemical composition of the membrane and on the structure of the i-face.

Quantification of interfacial binding

Measuring the affinity of sPLA2s for membrane interfaces can be quite difficult, especially when interfacial binding is high affinity. Furthermore, the products of phospholipid hydrolysis can greatly promote the binding of sPLA2s to zwitterionic phosphatidylcholine vesicles (see, for example, [5]). Conditions that prevent hydrolysis include the use of nonhydrolyzable phospholipid analogs, such as diether phospholipids, the absence of calcium, the use of catalytic site mutants that lack lipolysis activity and the use of polymerized phospholipid liposomes [8,9•,10–13]. Also, it must be remembered that the binding of the substrate within the catalytic site of the interfacial bound enzyme is a component of the apparent interfacial K_d (dissociation constant) value. The use of polymerized phospholipid liposomes or the exclusion of calcium prevents the E* + S* → E*•S* step, so that only the E → E* step is measured.

Most techniques for measuring interfacial binding require either physical separation of the bound and free enzyme or the use of a spectral change that is sensitive to binding. Physical separation under equilibrium conditions involves centrifugation to pellet the phospholipid aggregate. This separation is only possible with heavier phospholipid aggregates, such as sucrose-loaded large unilamellar vesicles or membrane fractions. These vesicles are fragile, however, and may not survive centrifugation; the use of polymerized phospholipid vesicles is helpful in this regard. Unfortunately, small unilamellar vesicles, which are commonly used for enzyme assays, do not readily sediment. Phospholipid-coated beads provide an alternative for presenting a stable phospholipid surface that can be readily sedimented [14], but it is difficult to know at the present time whether such artificial surfaces fully reproduce the interfacial protein-binding properties of bilayered vesicles.

Spectral methods for measuring interfacial binding do not require physical separation, but may require the use of nonphysiological reporter groups. The availability of a tryptophan residue (Trp3) on the interfacial surface of the pancreatic enzyme has been used, and the fluorescence change upon interfacial binding can be explained by desolvation, as the indole sidechain inserts into the interface [15]. An alternative approach involves the detection of resonance energy transfer between enzymic tryptophans and a fluorescent phospholipid, such as *N*-dansyl-phosphatidylethanolamine, that is present at a few mole percent in vesicles [15,16]. A major problem with these methods is that not only must the enzyme contain a tryptophan, but the efficiency of the energy transfer is highly variable, depending on the location of the fluorescent lipid probe in the vicinity of enzyme at the interface. For example, tryptophan-containing sPLA2s give strong spectral observables upon binding to anionic vesicles, but binding to phosphatidylcholine vesicles does not always give a response (MH Gelb, unpublished data).

There is a fundamental difficulty in quantifying interfacial binding if the value of K_d is very low ($< 10^{-7}$ M). For example, the sensitivity of fluorescent methods is not sufficient to detect the binding of subnanomolar concentrations of enzyme to $< 10^{-7}$ M lipid. This is a significant problem for the binding of sPLA2s to anionic vesicles, for which values of K_d are thought to be 10^{-8} M or smaller [14,15]. Improved methods for measuring tight interfacial binding are needed. The method of centrifuging polymerized phospholipid vesicles has been taken to its limit of measuring K_d values in the 1–10 nM range by very careful quantification of the free enzyme in the supernatant in the presence of low micromolar concentrations of phospholipid [17].

Amino acid determinants of sPLA2 interfacial binding as probed by site-directed mutagenesis

Extensive mutagenesis studies of catalytic residues in bovine and porcine pancreatic sPLA2s have been carried out (see, for example, [18,19]), but the following discussion will focus on studies that probe the contribution of i-face residues to interfacial binding. A hallmark of sPLA2s is their relatively high affinity for anionic versus neutral vesicles. Most sPLA2s bind weakly to phosphatidylcholine vesicles (K_d in the approximately millimolar range, except as noted below), and binding to anionic phosphatidylmethanol vesicles is high affinity ($K_d < 10^{-8}$ M) [14,15]. The i-faces of sPLA2s contain a collar of hydrophobic residues that surrounds the opening to the catalytic site slot and two or more cationic arginine or lysine residues (Figure 1). As a result of these basic residues, the i-faces of sPLA2s have a positive electrostatic potential [20], which could explain the high affinity of these enzymes for anionic interfaces.

Bovine pancreatic sPLA2 has 3–4 basic residues located on its putative i-face. Of these, K10, K56 and K116 were shown to make a contribution to interfacial binding, as mutating them individually to glutamates resulted in a 10- to 130-fold reduction in the affinity of binding to anionic phosphatidylglycerol vesicles [21]. A similar effect of charge-reversal mutagenesis was found for the sPLA2 from the venom of the water moccasin snake, in that a single, basic-residue mutation resulted in a 10- to 30-fold reduction in anionic vesicle binding [10]. The double mutant K7E/K10E bound 500-fold weaker than the wild-type to phosphatidylglycerol vesicles, corresponding to a 3.7 kcal/mol loss of interfacial binding energy. The location of these two basic residues matches the position of maximal positive electrostatic potential on the surface of the venom sPLA2. Like the venom enzyme, human group IIa sPLA2 contains basic residues at positions 7, 10 and 16, which are clearly on the i-face. The human enzyme

contains 11 additional basic residues that lie on the same side of the protein as the i-face, but that appear to be pushed back away from the planar surface of the enzyme that includes residues 7, 10 and 16 and the opening to the catalytic site slot. The group IIa triple mutant R7E/K10E/K16E binds 300-fold weaker than the wild-type to phosphatidylglycerol vesicles and mutation of groups of basic residues that make up the 11 additional positively charged sites results in a 10- to 200-fold reduction in interfacial binding [17]. The five-site mutant R7E/K10E/K16E/K124E/R127D binds 2200-fold weaker than the wild-type to anionic vesicles [22•].

All together, these studies show that the interfacial binding of these sPLA2s has a strong electrostatic component, but other factors, besides electrostatics, are at play. For example, the five-site group IIa sPLA2 mutant mentioned above has an overall negatively charged i-face and yet it still displays a respectable binding affinity for anionic vesicles (K_d ~2 μM). The sPLA2 from honey bee venom has the typical hydrophobic-residue collar (see above and Figure 1) and six basic residues on or near the i-face. The K_d for this enzyme bound to anionic phosphatidylmethanol vesicles is probably $< 10^{-9}$ M, because the enzyme operates on these vesicles in the scooting mode (processive catalysis) over several minutes, without leaving the surface [23]. Remarkably, a multisite mutant, in which five out of six basic residues were changed to glutamates, still operated on anionic vesicles in the scooting mode [9•]. Although it is possible that the interfacial K_d for bee venom sPLA2 was increased by charge-reversal mutation (values of K_d have not been measured to date because of their low magnitude), it is clear that the binding of bee venom sPLA2 to anionic vesicles is not driven by electrostatics. Thus, there must be other factors besides ionic interactions that govern the relatively high affinity of sPLA2s for anionic versus zwitterionic vesicles. Differences in the packing properties of phosphatidylcholine versus phosphatidylmethanol may alter the ability of enzyme residues to penetrate into the interfacial region of the bilayer, but such a working hypothesis is difficult to evaluate experimentally.

A new magnetic resonance technique has been recently developed to dock peripheral membrane proteins at the interface. Electron spin resonance, together with membrane impermeant and permeant spin-relaxing agents, is used to determine the distances between nitroxide spin labels, placed at specific points on the protein's surface, and the membrane surface [7••]. The docking of bee venom sPLA2 on the surface of anionic vesicles shows that the enzyme sits on the surface of the vesicle and that the six basic residues located on the face of the enzyme that includes the opening to the catalytic site slot do not directly contact the interface. This result provides a structural explanation for the modest effect of charge-reversal mutagenesis on interfacial binding (discussed above). The residues that are in direct contact with the interface are hydrophobic. Taken together, the results suggest that hydrophobic residues are better able to penetrate into the interfacial region of phosphatidylmethanol vesicles than into the interfacial region of phosphatidylcholine vesicles. For human group IIa, water moccasin and pancreatic sPLA2s, one might anticipate, according to the mutagenesis studies described above, that the basic residues are in direct contact with the interface, but there are no direct structural data that relate to this issue.

Not all sPLA2s bind weakly to phosphatidylcholine vesicles. A hallmark of the cobra venom sPLA2s is their relatively high affinity for zwitterionic vesicles (K_d for *Naja melanoleuca* sPLA2 of 8×10^{-7} M [24]). These enzymes have 1–3 tryptophans located on their putative i-faces, and numerous early studies have shown that chemical modification of tryptophan drastically lowers the activity of these enzymes on phosphatidylcholine vesicles (see, for example, [25,26]). There is mounting evidence that tryptophan is a key residue in supporting lipid–protein interactions. For example, spectroscopic studies have shown that simple indole analogs preferentially partition into the interfacial region of phospholipid bilayers (the region that includes the glycerol backbone, the esters that link fatty acyl chains to glycerol and the first 1–2 CH_2 groups of the fatty acyl chains) [27]. The powerful effect of tryptophan on interfacial binding is highlighted by studies with a mutant of human group IIa sPLA2 containing tryptophan replacing a valine located on the N-terminal helix that forms part of the i-face. The V3W mutant is 200- to 1000-fold more active than the wild-type on phosphatidylcholine vesicles [28••]. Although there are no direct interfacial binding data available for these proteins, the large kinetic effect of adding the i-face tryptophan is probably the result of enhanced vesicle affinity, because both the wild-type and the mutant show comparable activity on anionic vesicles. Also, whereas wild-type group IIa sPLA2 dissociates from phosphatidylmethanol vesicles with a half-time of a few minutes, the V3W mutant remains bound for more than 30 min and undergoes interfacial catalysis in the scooting mode (MK Jain, personal communication). Likewise, bovine pancreatic sPLA2 operates in the scooting mode on anionic vesicles, but the W3A mutant shows intervesicle exchange over minutes [29]. These results show that tryptophan also enhances interfacial binding to anionic vesicles. Recent studies, in which cobra venom tryptophans have been mutated, support the chemical modification studies and show that the indole ring makes a substantial contribution to interfacial binding (W Cho *et al.*, personal communication).

The recently discovered human group V sPLA2 behaves very differently than the group IIa enzyme with respect to interfacial binding. Whereas group IIa sPLA2 displays extremely poor activity on phosphatidylcholine vesicles, the group V enzyme is 7000-fold more active and both enzymes bind tightly to anionic vesicles [30•]. The i-face of group V sPLA2 contains a tryptophan and much fewer

basic residues than the i-face of group IIa sPLA2. Mutation of the i-face tryptophan to alanine renders group V sPLA2 40-fold less active on phosphatidylcholine vesicles [30•].

Conclusions and future directions

We are beginning to understand the molecular determinants of interfacial binding by sPLA2s. Clearly, for some enzymes, electrostatic interactions involving basic amino acids play an important role in allowing high-affinity binding to anionic vesicles. Further work is needed to understand the role of hydrophobic residues in supporting interfacial binding, especially in the case of bee venom sPLA2. Tryptophan emerges as a key residue in promoting interfacial binding to anionic and charge-neutral vesicles. This is most apparent with phosphatidylcholine vesicles, for which the interfacial binding of sPLA2s lacking i-face tryptophans is low affinity. Mammals contain multiple forms of sPLA2s. A reasonable working hypothesis is that these enzymes carry out different physiological functions that are governed by differences in their interfacial binding properties. For example, group IIa sPLA2 seems best fit to act as an antibacterial protein [31], as this enzyme displays a high affinity for anionic interfaces, such as bacterial membranes. Group V sPLA2 is much more active than the group IIa enzyme on the outer leaflet of mammalian cell membranes [30•], which are rich in zwitterionic lipids. Thus, group V sPLA2 may be a key player in releasing arachidonic acid for eicosanoid production.

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