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Interfacial Enzymology of Phospholipase A2

Michael H. Gelb*
Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195
Mahendra K. Jain*
Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716
Otto Berg*
Department of Molecular Biology, Uppsala University Biomedical Center, Uppsala, Sweden

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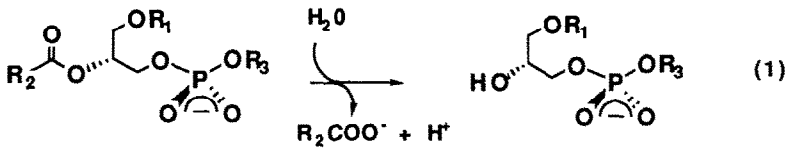
Abstract: Methods have been recently developed to analyze the phospholipase A_2-catalyzed hydrolysis of phospholipid aggregates in terms of standard kinetic theory. It is now possible to extract the Michaelis-Menten parameters, to determine the interfacial equilibrium constants for the binding of inhibitors and substrates to the active site of the enzyme bound to the interface, and to determine relative substrate preferences in the interface.

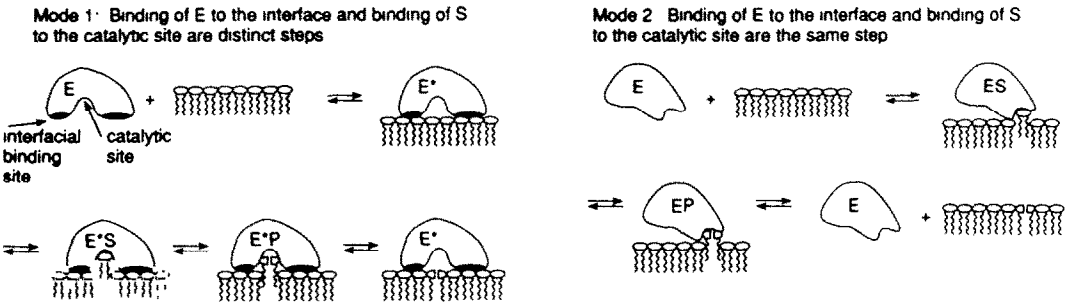
Phospholipase A_2 (PLA2) catalyzes the hydrolysis of glycero-phospholipids to release the fatty acid from the *sn*-2 position and to produce a lysophospholipid (Eq. 1).[1,2] There is considerable medicinal interest in these



(1)

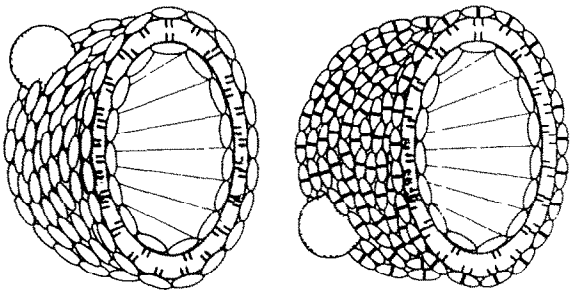
enzymes since they are likely to play a role in the inflammatory response.[3] For example, a 14 kDa, calcium-dependent PLA2 is released from stimulated platelets[4] and an 85 kDa cytosolic enzyme is probably responsible for the liberation of free arachidonic acid from the phospholipid pool for the biosynthesis of eicosanoids.[5,6,7] Calcium-independent PLA2s may be involved in tissue damage during acute myocardial ischemia.[8] Bee venom PLA2 is the major allergen present in bee venom.[9]

The quantitative analysis of PLA2 *in vitro* is not as straightforward as with most enzymes stemming from the fact that PLA2s must necessarily act on a phospholipid surface. This is because naturally-occurring phospholipids have no significant solubility in the aqueous phase. Thus, the enzyme must be capable of binding to the lipid/water interface. There are two modes of interfacial catalysis that can be envisioned. In mode 1, the binding of the enzyme to the interface and the loading of the active site with a single phospholipid molecule occur in sequence. In mode 2, there is only a single step in which both processes occur simultaneously. There is overwhelming evidence for mode 1. The X-ray crystal structures of four different 14 kDa PLA2s complexed with a short-chain phospholipid analogue inhibitor reveal that the phospholipid molecule must travel about 15 Å through a slot in the enzyme to reach the catalytic machinery.[10-13] Thus, external surfaces on the enzyme must contact the bilayer to gain access to the substrate. Kinetic proof for the sequential binding mode comes from the analysis of PLA2s on anionic vesicles such as phosphatidylmethanol[14] or on co-vesicles of phosphatidylcholine and phosphatidic acid.[15] A large number of secreted PLA2s bind much tighter to anionic vesicles than to neutral



vesicles and the enzyme catalyzes a *processive* reaction in which all of the phospholipids in the outer layer of the vesicles are hydrolyzed without desorption of the enzyme from the surface (termed "scooting mode catalysis"). If the binding of the enzyme to the interface occurs concomitantly with active site binding (mode 2), the release of products from the enzyme would necessarily involve enzyme desorption from the interface and the enzyme would have no tendency to return to the same vesicle.

Hydrolysis of Vesicles in the Scooting Mode



Scooting mode catalysis is important in the context of the kinetic analysis of PLA2. The fundamental problem associated with kinetic analysis of PLA2 is that the enzyme operates on substrate aggregates that contain relatively few substrates compared to the total amount of substrate in the reaction mixture. This is not the case for the action of water soluble enzymes on a homogeneous solution of substrate molecules. In the kinetic treatment of the latter, one assumes that each enzyme in the reaction "sees", in a global sense, the same concentration of substrates and products as any other enzyme in the reaction. This assumption is at the heart of kinetic theory of ensembles of molecules. Consider what would happen if portions of the solution phase reaction were sectioned off from other portions. Some portions would have more enzyme than others and for these, the build-up of product and the onset of product inhibition would occur earlier in time compared to portions that contain relatively few enzymes. Under these conditions, a set of *separate* reactions are occurring simultaneously and a mathematical description of the macroscopic observables, i.e. the total product formation and substrate depletion, requires knowledge of the events within each sub-compartment and also requires a

statistical method for the proper summation of such events. This situation can also be described as *time scrambling* in that those sub-reactions that contain more enzyme will appear to be further along in time compared to those that have fewer enzymes. Clearly, the kinetic theory that describes this situation is much more complex than that applied to homogeneous reactions under the fast exchange condition.

Often the action of PLA2s on detergent micelles that contain embedded phospholipids is studied and attempts are made to describe the quantitative aspects of this reaction using standard steady-state enzyme kinetic equations. This approach is likely to be fraught with difficulties. Since detergent micelles are relatively small (typically 100 monomers per micelle), only small numbers of phospholipid molecules, 10-20, can be incorporated into each micelle. Since a typical turnover time for PLA2 is 100 s^{-1}, it will take only milliseconds for an enzyme bound to a mixed-micelle to completely hydrolyze the substrate present in the particle. Thus, it is clear that reaction progress beyond the millisecond time-scale must necessarily involve either exchange of enzyme, or of phospholipids, or of both, among the ensemble of mixed-micelles. Recent studies have shown that inter-micelle exchange of phospholipids is relatively slow on the millisecond time-scale[16, 17] and is probably the rate-limiting process for enzymatic turnover, at least in some mixed-micelles.[18] Thus, observed rates of lipolysis of mixed-micelles may be more a reflection of the kinetics of inter-micelle exchange rather than of the catalytic efficiency of the PLA2. An inhibitor of interfacial catalysis on mixed-micelles by PLA2 could function in a pharmaceutically useful way by binding tightly to the active site of the PLA2, or it could function in an irrelevant way by decreasing the rate of inter-micelle phospholipid or enzyme exchange. Substrate preferences for PLA2 catalysis may also be influenced by exchange phenomenon.

The problems discussed above have been overcome by studying the kinetics of PLA2 acting on relatively large phospholipid aggregates, i.e. bilayered vesicles, under conditions in which neither the enzyme nor the phospholipids that make up the aggregate undergo inter-vesicle exchange. Since a typical vesicle contains greater than 10,000 phospholipid molecules, enzymatic turnover at 100 reaction cycles per second can be studied for several minutes without the need for the replenishment of substrate in the enzyme-containing vesicle. Furthermore, if the poly-dispersity in the size of the vesicles is small, if there are more vesicles than enzymes so that the vesicles contain at most one bound enzyme, and if the enzyme operates on these vesicles in the scooting mode then the kinetics of the system can be treated using classical kinetic theory. This is because each enzyme-containing vesicle will behave identically in time, on the average, and thus the experimentally observable product formation will simply be the summation of the contributions made by each enzyme-containing vesicle. It is clear that the chaotic time behavior associated with catalysis on small substrate aggregates, such as mixed-micelles, is no longer a problem. The processive nature of the catalysis on vesicles is important. If the enzymes were able to "hop" from one vesicle to another, the composition of vesicles would be a complex function of the reaction time and different enzymes would be bound to vesicles with different composition at any point in time. This problem is further complicated by the fact that the affinity of the PLA2 for the vesicle depends on the mole % of negatively charged fatty acid product in the vesicle. Thus, not only should the enzyme be bound tightly to the vesicle, but the time required for desorption from the vesicle should be longer than the time needed to complete the reaction, i.e. to hydrolyze all available substrate in the outer layer of the vesicle.

The action of PLA2s on anionic vesicles, such as phosphatidylmethanol, displays all of the features that allow the kinetics to be described by standard steady-state enzyme kinetic equations. Not only does the enzyme remain bound to the vesicle but conditions are selected so that the fusion of vesicles does not occur on the several minute time-scale needed for the hydrolysis of the entire outer layer of the phospholipid vesicle. For naturally-

occurring long-chain phospholipids, inter-vesicle exchange and exchange between the inner and outer vesicle monolayers is slow and need not be considered. The reaction progress curve is adequately described by the Michaelis-Menten equation adapted for interfacial catalysis (Eq. 2) and the integrated form (Eq. 3).[19] In Eq. 2, v_0 is the initial reaction velocity per enzyme and K_M^* is the interfacial Michaelis constant. The asterisk denotes

$$v_o = \frac{k_{cat}}{\left(1 + K_M^*\right)} \qquad (2)$$

$$k_i t = -\ln\left(1 - P_t / P_{max}\right) + \left(k_i N_S / v_o - 1\right) P_t / P_{max}$$

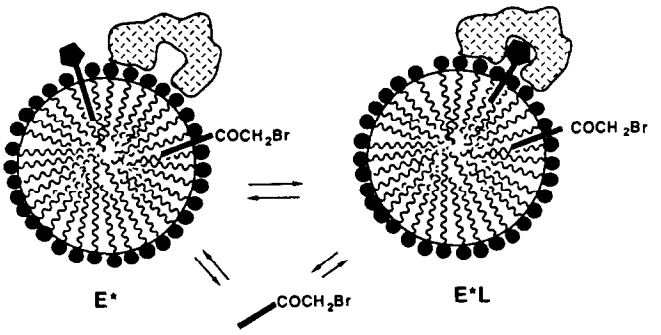
$$N_S k_i = k_{cat} / K_M^* \left(1 + 1 / K_P^*\right) \qquad (3)$$

that this constant is for the enzyme in the interface interacting with substrate in the interface. Thus, the unit of K_M^* is mole fraction which is related to the surface concentration of substrate (as opposed to bulk concentration, moles/volume). When the mole fraction of substrate in the vesicle equals its K_M^*, the value of v_0 will be $k_{cat}/2$. Eq. 2 is for vesicles made up only of substrate phospholipid so that the initial substrate concentration is 1 mole fraction and this appears in the denominator of Eq. 2. The reaction progress curve is described by the integrated Michaelis Menten equation (Eq. 3) which relates the amount of total product at time t (P_t) and the total product at the end of the reaction (P_{max}) to the kinetic parameters v_0 and k_i and the parameter N_S, which is the number of phospholipid molecules in the outer layer of a vesicle. The product $N_S k_i$ is the apparent second-order rate constant for the enzymatic reaction in the presence of product inhibition (K_P^* is the interfacial dissociation constant for the products; 1:1 mixture of fatty acid and lysophospholipid). With small vesicles, N_S is small (typically 5,000), and the ratio P_t/P_{max} will quickly approach 1 so that the initial linear velocity will be difficult to observe and the progess curve will be dominated by the logarithmic term and will take on a first-order appearance (i.e. the velocity will be continuously decreasing at each point in time). Conversely, with larger vesicles (N_S typically > 100,000), P_t/P_{max} remains very small for a relatively long time and so the logarithmic term will be linearized and one observes an initial constant velocity.

Fitting experimental reaction progress curves to Eq. 3 provides some of the rate constants that describe the interfacial catalysis. Other techniques are needed to evaluate all of the rate constants. For example, the observation of only a single oxygen-18 incorporated from solvent into the fatty acid product establishes that the enzyme-product complex does not return to the enzyme-substrate complex.[20] Carbon-14 carbonyl isotope effect studies establish that the enzyme-substrate complex decomposes mainly to give the enzyme-product complex rather than dissociation to give the free enzyme and free substrate.[20] A novel technique has been developed to measure equilibrium dissociation constants for enzyme-ligand complexes in the interface.[21] The approach relies on a *neutral diluent* which is defined as an amphiphilic molecule that forms an aggregate to which PLA2 can bind via its interfacial recognition surface but the affinity of a molecule of neutral diluent for the active site of the enzyme is low so that the active site remains ligand-free (filled with water). The binding of a ligand in the interface of the neutral diluent can protect the enzyme from alkylation of the active site histidine residue by a bromomethyl ketone. The dissociation constant for the enzyme-ligand complex in the interface (in units of mole

fraction) is simply the mole fraction of ligand in the interface that causes the half-time for enzyme alkylation to double.

PLA2 Bound to a Neutral Diluent in the Presence of an Active Site Ligand and an Alkylating Agent



Using these techniques, all of the rate and equilibrium constants for the interfacial catalysis of vesicles of 1,2-dimyristoylphosphatidylmethanol have been obtained.[19] The results are briefly summarized here. The interfacial dissociation for the reaction products, K_P^*, is 0.025 mole fraction, about 10-fold smaller than the K_M^* for the substrate of 0.3 mole fraction. With saturating concentrations of substrate (i.e. at time zero when the mole fraction of substrate is 1, which is above K_M^*), the k_{cat} value is 400 s^{-1} at ambient temperature. Under saturating substrate conditions, the reaction rate is fully limited by the lipolysis step and not by the product release step.[18] The fact that the enzyme can be saturated with substrate during steady-state turnover with experimentally obtained substrate concentrations (i.e. $K_M^* < 1$ mole fraction) establishes that the diffusion of the substrate to the active site of the enzyme is not rate-limiting. With substrate concentrations below K_M^*, the turnover number k_{cat}/K_M^* is limited by the rate constant for substrate binding to the active-site of the enzyme and not by the equilibrium dissociation constant for the enzyme-substrate, interaction, K_S^*.

The proper analysis of PLA2 inhibitors is important in light of the fact that numerous pharmaceutical companies are in the process of screening compound libraries with the hope of developing novel antiinflammatory agents. Again, the kinetic analysis is more problematic in the cases of interfacial catalysis. For the 14 kDa secreted PLA2s, many previously reported inhibition studies make use of substrate vesicles of pure phosphatidylcholine. The enzyme binds weakly to these zwitterionic vesicles. Thus, the observed inhibition may be due to the effective of the additive on the fraction of the enzyme bound to the interface. The additive may bind directly to the enzyme in the aqueous phase and prevent the interfacial binding or it could partition into the interface and alter the physical properties of the surface so that desorption of enzyme occurs. Finally, the inhibitor may bind specifically to the active site of the PLA2 in the interface and prevent substrate binding. It is now clear that most of the previously reported PLA2 inhibitors work by altering the physical nature of the interface. Such agents, which include alkanols, fatty acids, and cationic amphiphiles, and many others, are non-specific modulators of interfacial catalysis since they do not bind directly to the enzyme and inhibition is seen only when they are present at relatively high mole fraction in the bilayer. This is clear from the fact that studies

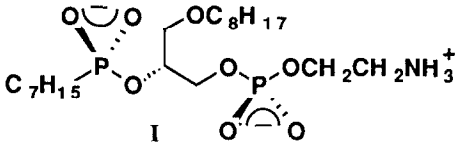
using fluorescent techniques to directly monitor the binding of the enzyme to the bilayer have shown that these agents cause enzyme desorption.[22] In addition, these agents do not inhibit the enzyme when it is tightly bound to the phospholipid vesicles (scooting mode).[21]

One approach to the problem of non-specific inhibition is to use the scooting mode analysis to evaluate true competitive inhibitors of PLA2. Studies using phospholipid-based transition state analogues have shown that these agents bind tightly to the enzyme in the interface.[23, 24] The equation that describes competitive inhibition for an enzyme operating in the scooting mode (Eq. 4) is analogous to the standard formalism. Here, the ratio of

$$\frac{v_o^o}{v_o^I} = 1 + \left[\left(1 + 1/K_I^*\right)/\left(1 + 1/K_M^*\right)\right]\left[X_I/\left(1 - X_I\right)\right] \qquad (4)$$

initial velocities in the absence to that in the presence of a competitive inhibitor depends on the affinity of the inhibitor for the enzyme (K_I^*) versus the substrate (K_M^*) and the mole fraction of inhibitor in the vesicle (X_I). The (1 - X_I) term that appears in the denominator of Eq. 4 is simply due to the fact that in a vesicle of constant size, the mole fraction of substrate in the vesicle will decrease as inhibitor molecules are added. For potent inhibitors this correction is negligible. It is often stated that PLA2 is difficult to inhibit since it exists in the presence of a "high" concentration of substrate, i.e. tightly packed phospholipid molecules. There is no basis for this statement since the K_M^* for the substrate is not significantly different in value than mole fraction 1.

Potent competitive inhibitors that have been described are phospholipid analogues in which the enzyme-susceptible ester has been replaced with a tetrahedral phosphonate (compound I),[23] a hydrated difluoro-ketone,[25] or an amide.[26, 27] Novel structures containing only a single phosphorous atom (compound II) are also potent PLA2 inhibitors.[24] All of these inhibitors produce a 50% reduction in the initial velocity of the enzymatic



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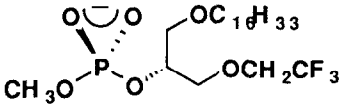

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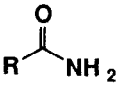

III

reaction when present in the vesicle at a concentration of 1 inhibitor per several hundred to several tens of thousands substrates. Fatty acid amides (Compounds III, R = alkyl chains of 14-20 carbons with 0-4 double bonds) were discovered in a screen for PLA2 inhibitors using a scooting mode-based strategy.[28] It is important to point out that in the course of the inhibitor screening of several thousand compounds, no false positives were detected. These simple amides are as potent as the phospholipid analogues. It is interesting to note that not all of the structural features of a phospholipid analogue are required for potent inhibition. Compound II lacks the *sn*-3 phosphate and Compounds III lacks the entire glycerol backbone. The X-ray crystal structures of Compound I bound to the active site of three different PLA2s clearly reveal that the *sn*-3 phosphate interacts in two ways with the enzyme. One of the non-bridging oxygens of the phosphate diester is a ligand for the calcium ion and the other oxygen accepts a H-bond from either a tyrosine or a threonine.[10-13] The inhibition data suggest that the energy of interaction of the *sn*-3 phosphate with the enzyme is not significantly different than the energy of

interaction of this group to other phospholipid molecules in the vesicle. A more detailed explanation must await a high resolution structure of the enzyme/inhibitor complexes. Recent studies show that interactions of the inhibitors with the phospholipids in the vesicle can modulate the K_I^* values.[29] A nice feature of Compounds III is that they are cell-permeable and should be useful as tools to establish the roles of secreted PLA2s in complex biological processes.

One limitation of a scooting mode-based inhibitor screen is that inhibitors that interact specifically with the enzyme in the aqueous phase and prevent the interfacial binding will probably not be detected owing to the fact that the dissociation constant for the PLA2 bound to negatively charged vesicles is very small (less than 10^{-12} M, based on the number of vesicles). These issues have been discussed in detail.[30] One could screen under conditions in which the enzyme binds weakly to the vesicle, but it is critical that the screen be run under conditions in which the mole fraction of test compound is small. The literature is flooded with reports of PLA2 inhibitors that work in the low μM range. These studies are almost always carried out with μM concentrations of substrate. For lipophylic test compounds, this is a problematic situation since a significant fraction of the substrate interface may contain the test compound. In aqueous phase enzymology, this is much less of a concern.

Studies of substrate preferences for PLA2 are also problematic unless the enzyme is operating in the scooting mode. Enzymatic velocities that are measured in separate experiments using different vesicles, each composed of a single phospholipid molecular species, will mainly reflect the relative affinities of the enzyme to the different vesicles. This has no connection to the intrinsic substrate specificity which is defined as the relative k_{cat}/K_M^* for substrates being hydrolyzed in a competitive manner with the enzyme in the scooting mode. These measurements are carried out by embedding small amounts of competing substrates (for example, each containing a different radioisotope) into an anionic vesicle that supports scooting mode catalysis.[15] These studies show that the 14 kDa secreted PLA2s do not significantly discriminate against phospholipids of differing head groups or acyl chains. A completely different picture emerges if one compares the relative velocities of the enzyme acting separately on vesicles composed of different pure phospholipids.

In summary, the scooting mode analysis allows the kinetics of interfacial catalysis by PLA2 to be analyzed using standard kinetic formalism. Assay systems that utilize relatively small substrate aggregates (such as micelles, mixed-micelles, or reverse micelles) can be treated theoretically only if the rates of inter-aggregate exchange are measured (a more accurate statement is that the rates of inter-aggregate exchange must be determined for *enzyme-containing* particles). This has not yet been accomplished. What relevance does the scooting condition have for the action of the enzyme *in vivo* ? This point is meaningless. The kinetic quantities that are measured in the scooting mode analysis of an ensemble of enzymes acting on vesicles are transferable to the action of a single enzyme molecule, or more precisely the average behavior of a single enzyme, acting on a vesicle. For example, the relative k_{cat}/K_M^* values measured in the scooting mode will dictate the substrate preferences of the enzyme even if it remains in the interface for a short time (i.e. non-processive catalysis) While the enzyme is in the bilayer, it will "choose" among the phospholipids that are present according to the relative k_{cat}/K_M^* values. The same is true for inhibitors (K_I^*). In thinking about the kinetics of interfacial catalysis, it is important to separate out what the enzyme "sees' locally in the vesicle to which it is bound from what is present in the entire reaction mixture. Although the *absolute* values of kinetic constants measured in a vesicle of, for example, 1,2-dimyristoylphosphatidylmethanol may be different than those measured in a vesicle of different composition, recent studies show that the *relative* interfacial parameters are similar in vesicles of vastly different

composition.[19, 21, 29] Of course other factors may alter the behavior of the enzyme *in vivo* such as specific PLA2/protein interactions, post-translational modification of the protein, etc. Even with these latter possibilities, it is critical to understand what fraction of the enzyme is bound the bilayer.

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