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REVIEW ARTICLE

Phospholipase A_2 at the Bilayer Interface

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ABSTRACT **Interfacial catalysis is a necessary consequence for all enzymes that act on amphipathic substrates with a strong tendency to form aggregates in aqueous dispersions. In such cases the catalytic event occurs at the interface of the aggregated substrate, the overall turnover at the interface is processive, and it is influenced the molecular organization and dynamics of the interface. Such enzymes can access the substrate only at the interface because the concentration of solitary monomers of the substrate in the aqueous phase is very low. Moreover, the microinterface between the bound enzyme and the organized substrate not only facilitates formation of the enzyme–substrate complex, but a longer residence time of the enzyme at the substrate interface also promotes high catalytic processivity.**

Binding of the enzyme to the substrate interface as an additional step in the overall catalytic turnover permits adaptation of the Michaelis–Menten formalism as a basis to account for the kinetics of interfacial catalysis. As shown for the action of phospholipase A_2 on bilayer vesicles, binding equilibrium has two extreme kinetic consequences. During catalysis in the scooting mode the enzyme does not leave the surface of the vesicle to which it is bound. On the other hand, in the hopping mode the absorption and desorption steps are a part of the catalytic turnover.

In this minireview we elaborate on the factors that control binding of pig pancreatic phospholipase A_2 to the bilayer interface. Binding of PLA2 to the interface occurs through ionic interactions and is further promoted by hydrophobic interactions which probably occur along a face of the enzyme, with a hydrophobic collar and a ring of cationic residues, through which the catalytic site is accessible to substrate molecules in the bilayer. An enzyme molecule binds to the surface occupied by about 35 lipid molecules with an apparent dissociation constant of less than 0.1 pM for the enzyme on anionic vesicles compared to 10 mM on zwitterionic vesicles. Results at hand also show that aggregation or acylation of the protein is not required for the high affinity binding or catalytic interaction at the interface.

Key words: phospholipase A_2, interfacial catalysis, interfacial activation, resonance energy transfer, lipid–protein interaction

INTRODUCTION

Interfacial catalysis on biomembranes is an intriguing biophysical phenomenon. Recently it has attracted considerable attention because such processes are believed to be responsible for cellular regulatory mechanisms in which soluble proteins act on the membrane localized substrates. Several classes of proteins are known to be functionally active at interfaces; examples include lipolytic enzymes, acyltransferases, protein kinases, and glycosidases. Interfacial catalysis by lipolytic enzymes is involved in such diverse processes as digestion of fats to tailoring of membrane lipids and modulation of signal transduction.[40] A resurgence of interest in phospholipase A_2 (PLA2) has been sparked by the possibility that the release of arachidonate and lysophospholipids from membrane phospholipids is the rate-limiting step in the biosynthesis of eicosanoids (prostaglandins, thromboxanes, leukotrienes, lipoxins) and platelet activating factor. These regulatory molecules have been implicated in the onset and control of a wide range of physiological and pathological states such as inflammation, asthma, ischaemia,

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Abbreviations: DMPC, dimyristoyl glycero-*sn*-3-phosphocholine; DTPC, ditetradecyl glycero-*sn*-3-phosphocholine; DMPM, dimyristoyl glycero-*sn*-3-phosphomethanol; DTPM, ditetradecyl glycero-*sn*-3-phosphomethanol; HDNS, dansyl-hexadecylphosphoethanolamine; NK-529, 1,3,3,1′,3′,3′-hexamethylindocarbocyananine; PLA2, phospholipase A_2 from pig pancreas; proPLA2, prophospholipase A_2 from pig pancreas.

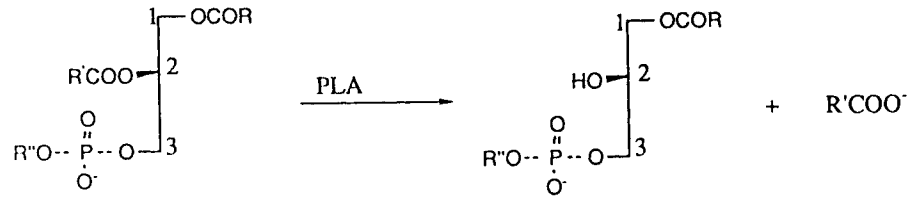


Fig. 1. The hydrolytic reaction catalyzed by phospholipase A_2. DMPM is R = R′ = $C_{13}H_{27}$; R″ = CH_3.

toxic shock, psoriasis, pancreatitis, burn trauma, and rheumatoid arthritis.

MOLECULAR CHARACTERISTICS OF PLA2

PLA2s are ubiquitous, and the enzymes from venoms and pancreas are abundant, stable, and biochemically well characterized. These water-soluble proteins have about 120 amino acids in a single peptide chain with a rigid three-dimensional structure stabilized by several disulfide bridges. All PLA2s have an absolute requirement for calcium, and His-48 is the catalytic site residue that participates in hydrolysis by a general base mechanism. The enzymes from venoms and pancreas have highly conserved regions and exhibit over 60% homology.[36] X-Ray crystallography[6,7,28,31] has revealed a common three-dimensional architecture in which 5 of the 7 disulfide bridges are also conserved. Certain intracellular PLA2s expressed from cDNA also exhibit a sequence homology with secretory PLA2s,[36] although a significant departure in their catalytic behavior is apparent.

CATALYTIC CHARACTERISTICS AT THE INTERFACE

In this review we focus on PLA2 from pig pancreas which has served as a prototype for the study of interfacial catalysis. It catalyzes the hydrolysis of the *sn*-2-ester bond in 1,2-diacyl-*sn*-3-phosphoglycerides (Fig. 1). Compared to a catalytic turnover number of less than 30 per min for PLA2 with solitary monomeric phospholipid molecules with short acyl chains, under optimum conditions the turnover number exceeds 25,000 per min for aqueous dispersions of long chain substrates. In order to appreciate the increase in the catalytic turnover at the interface it is necessary to invoke the binding of PLA2 to the interface as shown in Figure 2. In this review we focus on the factors that govern the binding step (E to E*), whereas a detailed analysis of the catalytic steps in the interface will be published elsewhere.

The molecular organization and dynamics of amphipathic molecules like phospholipids at an interface in aqueous dispersions depend on several factors,[2,22] and such factors ultimately control interfacial catalysis by PLA2. The hydrophobic effect provides the driving force for amphipathic molecules in water to form organized structures such as bilayers, monolayers, micelles, and emulsions. The cylindrial

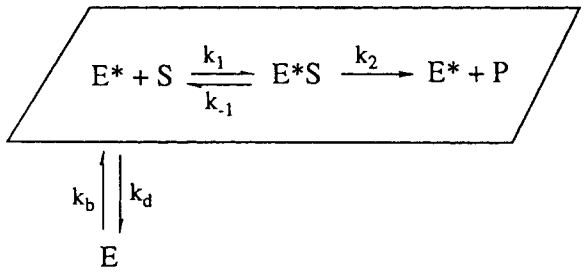


Fig. 2. The minimal Scheme I for interfacial catalysis by PLA2. Additional steps can be incorporated to account for binding of the bound enzyme (E*) to products or inhibitors. The species shown in the box plane are bound to the bilayer, where as the enzyme in the aqueous phase is shown as E. The values of the rate constants shown in the scheme are k_b = 4 sec^{-1}; k_d = <0.00002 sec^{-1}; k_1 = 1,350 sec^{-1} at mol fraction 1; k_{-1} = 35 sec^{-1} k_2 = 400 sec^{-1} for DMPM vesicles at 23C. If a product release step is postulated in the catalytic cycle the values of the kinetic parameters are K_{MS} = 0.3 mol fraction; k_{cat} = 400 sec^{-1}; K_S = 0.03 mol fraction; K_P = 0.025 mol fraction.

shape of naturally occurring phospholipids is mainly responsible for their propensity to organize as bilayers. The free energy for the removal of phospholipid molecules from a bilayer exceeds +20 kcal/mol, therefore the concentration of solitary monomers in the aqueous phase is below 0.1 nM. Due to such a low critical micelle concentration, the kinetics of removal of a phospholipid molecule from the interface is not favored and the rate of intervesicle exchange of phospholipids has a half-time of several hours.

Interfacial catalysis by PLA2 is a necessary consequence of the amphipathic character of phospholipids. How an enzyme molecule goes about optimizing the steps involved in the catalytic turnover cycle would ultimately depend on factors that control relative affinity of a substrate molecule for the enzyme and for the interface. Since the catalytic site of an enzyme is unlikely to be on its surface, it is necessary to insert or to localize the protein on the interface so as to dislodge the substrate without bringing in unfavorable hydrophobic effects. Thus an apparent activation of PLA2 at the interface can be viewed as a general regulatory process that is a necessary consequence of the availability of the substrate at the microinterface between the enzyme and the substrate interface.

The role of E to E* equilibrium leading to a higher overall catalytic turnover by PLA2 is reviewed in this article. In 1973, three different suggestions

were made to describe the contribution of binding of the enzyme to the interface on the overall catalytic turnover. Jain and Cordes[8,9] proposed that the equilibrium for the binding of the enzyme to the bilayer (E to E*) is perturbed by changing the organization and dynamics of the interface, while Verger et al.[37] proposed that the kinetic rate constant for the E to E* step is somehow regulated by the "quality of the interface." In contrast, Dennis[4,5] suggested that the "activation" occurs due to the aggregation of the enzyme in the interface. Over the intervening years these suggestions have been elaborated.[4,25,38,39] Here we review results of key experiments designed to evaluate the kinetic consequences and predictions of the E to E* equilibrium on interfacial catalysis on bilayers. We believe that these protocols and conclusions have a general validity for all biological interfacial catalytic processes if one takes into account the constraints of their organization and dynamics.

CONSTRAINTS OF INTERFACIAL CATALYSIS

The minimum kinetic model that accommodates unique features of interfacial catalysis is given as Scheme I in Figure 2. Scheme I is deceptively simple. It is, however, a remarkably versatile representation of interfacial catalysis. Reaction progress curves with virtually any shape can be generated within the constraints of Scheme I.[25] Here the enzyme in the aqueous phase (E) is in equilibrium with the enzyme in the interface (E*), where it undergoes catalytic turnover according to the steps shown in the box plane. Factors that regulate catalysis modulate the steps in the box, while a shift in the interfacial binding step E to E* is responsible for an apparently higher catalytic turnover number related to the changes in the molecular organization and dynamics of the interface.

When an enzyme in homogeneous solution acts on amphipathic substrate molecules organized at a lipid–water interface, a number of intriguing problems arise from the fact that the molecular organization and dynamics at the interface regulate the overall kinetic turnover. Since the catalysis occurs at the interface, the Michaelis–Menten complex of the substrate with the enzyme is no longer a solitary monomeric species in the aqueous phase; the concentration of the substrate lacks the usual meaning and it can not be readily manipulated; the transfer of the enzyme from the aqueous phase to the interface has to be introduced as an additional step for overall catalytic turnover; and for a detailed kinetic analysis it is often necessary to have an appreciation of the distribution and exchange dynamics of the enzyme, substrate, products, and other additives such as surface diluents, activators, and inhibitors.

Our approach for resolving such complexities is based on the premise that a reasonable quantitative description of the kinetics of interfacial catalysis requires not only the values of the equilibrium and the kinetic rate constants that are explicitly shown in the scheme, but it is also necessary to constrain implicit parallel kinetic processes arising from the organization and dynamics of phospholipids in the interface where catalysis occurs. As discussed elsewhere,[25] the uncontrolled variables in certain forms of aggregated substrates make it difficult to evaluate kinetics of interfacial catalysis even when the reaction progress curve has a linear steady-state region. For example, for emulsions and micelles the reaction progress curves have linear initial rates, however, their interpretation requires information about the size of the micellar complex of PLA2, an understanding of the rate of intermicellar fusion, as well as the rates of intermicellar exchange of the enzyme, substrate, and products. This is because such factors ultimately determine the rate of replenishment of the substrate on PLA2 containing micelles where catalytic turnover occurs. Similarly, for monolayer, there is considerable uncertainty about the role of the unstirred layer, as well as the levels of products and E*.

For the last 20 years, bilayers have been the focus of our kinetic and biophysical studies on interfacial catalysis. As outlined below, the molecular organization and dynamics of phospholipids in bilayers are reasonably well constrained, which permits an unequivocal description of interfacial catalysis under well-characterized experimental boundary conditions.[25] In bilayers and biomembranes the reaction sequence in the aqueous phase is neglected because the concentration of solitary substrate molecules (below 0.1 nM) is exceedingly low compared to the apparent affinity of the enzyme for the solitary substrate; the halftime for transbilayer movement (flip-flop) of phospholipids is of the order of several hours; the intervesicle exchange of the substrate and the products between the aqueous phase and the interface is negligible on the time scale usually employed for the kinetic studies;[22] under suitably chosen conditions, the rate of fusion of bilayer vesicles is negligibly small;[13,17] the products of hydrolysis of long chain phospholipids also form bilayers;[10,11] the integrity of the substrate bilayer is maintained even after all the substrate molecules in the outer monolayer of the target vesicles are hydrolyzed;[17,33,41] and the contents of the inner aqueous compartment of vesicles are not released even when the bilayer surface is substantially covered by PLA2. Within such constraints of organization and exchange dynamics of phospholipids in bilayer vesicles it is possible to rigorously describe the kinetic behavior of PLA2 on vesicles if the E to E* step is constrained, as is the case on anionic vesicles. Finally, it may be emphasized here that such constraints limit only the contribution of the parallel kinetic processes without compromising the underlying mechanism for catalytic turnover.

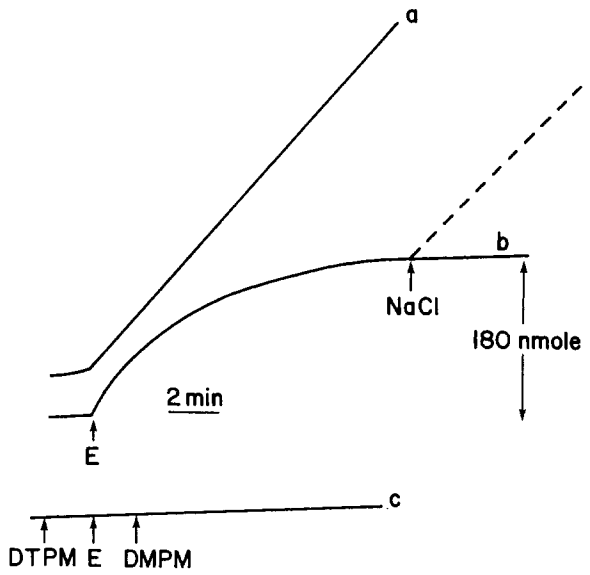


Fig. 3. Reaction progress curve for the hydrolysis of DMPM vesicles: **(a)** for large vesicles at 2.5 mM calcium; **(b)** for small sonicated vesicles at 0.5 mM calcium; **(c)** for small sonicated vesicles of DTPM to which PLA2 is added before the addition of small sonicated vesicles of DMPM at 0.5 mM calcium. For details see Jain et al.[17,18]

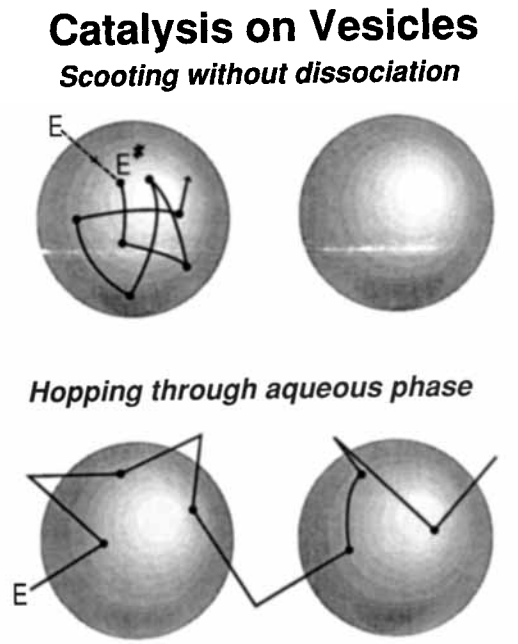


Fig. 4. A schematic drawing to illustrate key features of interfacial catalysis on phospholipid vesicles in the (top) scooting and (bottom) hopping mode. In the scooting mode, when the vesicle to enzyme ratio is more than 5, there is at most one enzyme per vesicle. With K_D < 0.1 pM for E* on DMPM vesicles, the bound enzyme does not leave the vesicle even when all of the substrate in the outer monolayer of the target vesicle is hydrolyzed. Therefore, excess vesicles are not hydrolyzed by the enzyme added initially unless the vesicles are allowed to fuse, or the bound enzyme undergoes intervesicle exchange, or the excess vesicles are hydrolyzed by adding excess enzyme so that there is at least one enzyme per vesicle. On the other hand during catalysis in the hopping mode, the enzyme desorbs from the vesicle surface between the catalytic cycles, and thus all vesicles are ultimately hydrolyzed even if the vesicle to enzyme ratio is >> 1.

CATALYSIS ON ANIONIC VESICLES

The E to E* equilibrium for PLA2 on vesicles of anionic phospholipids is >99.99% in favor of E*,[18] i.e., the dissociation constant for E* is <0.1 pM. For example, hydrolysis of 1,2-dimyristoyl glycero-*sn*-3-phosphomethanol (DMPM) vesicles by pig pancreatic PLA2 exhibits kinetic characteristics which show that the enzyme binds rapidly and essentially irreversibly. As shown in Figure 3, hydrolysis of DMPM vesicles starts immediately after the addition of PLA2. In the presence of excess enzyme 50 to 70% of the total substrate is hydrolyzed, corresponding to the fraction of the substrate present on the outer monolayer of the vesicles depending on their size. On the other hand, in the presence of an excess of small sonicated vesicles (more than five vesicles per enzyme to assure that there is at most one E per enzyme containing vesicle) the reaction stops when 4,400 substrate molecules have been hydrolyzed per enzyme. These observations are best interpreted in terms of the diagram shown in Figure 4. Here, only the substrate on the outer surface of the target vesicles is accessible to the enzyme, and the bound enzyme does not exchange with excess vesicles. Two kinds of experiments demonstrate that the enzyme is fully active at the end of the reaction progress curve. As shown in Figure 3, addition of salt promotes the desorption of the enzyme from vesicles so that the enzyme molecules hop from vesicle to vesicle, and ultimately hydrolyzes all substrate molecules in the outer monolayer of all vesicles in the reaction mixture. In other experiments, fusion of vesicles induced (e.g., by calcium, polymyxin B) at the end of the reaction progress curve (Fig. 3) promotes hydrolysis of excess vesicles by making them accessible to the bound enzyme. The fact that only the substrate in the outer monolayer of the target vesicles is hydrolyzed shows that the binding and catalysis by PLA2 does not require solubilization of phospholipids, or the formation of nonbilayer phases, e.g., hexagonal or micellar.

Other factors that influence the time course of interfacial catalysis on anionic vesicles also merit consideration because in this system the E to E* equilibrium is essentially completely in favor of E*: (1) The number of substrate molecules hydrolyzed by an enzyme molecule is the number of substrate molecules in the outer monolayer of the target vesicle, and the extent of hydrolysis per enzyme increases with the size of vesicles. (2) As expected, the polydispersity in the size of vesicles influences the shape of the reaction progress curve. (3) The reaction progress curves of type shown in Figure 3 have been observed with well over 50 PLA2s from differ-

ent sources, their isozymes, and mutant forms. (4) As expected, in such cases, the extent of hydrolysis per enzyme (in the presence of excess vesicles) remains essentially the same for enzymes from different sources, which shows that a single PLA2 molecule on a vesicle is catalytically active. (5) It may be emphasized here that the catalysis in the scooting mode does not exhibit any anomalous kinetic behavior at the gel-fluid thermotropic transition,[21,23] or during isothermal phase transition induced by lipophilic solutes.[27] Such observations show that the anomalous effects observed under comparable conditions with zwitterionic vesicles[1,12,34,35] (also see below) are due to a shift in the E to E* equilibrium.[16]

Characteristic features of interfacial catalysis are best observed in the scooting mode where an enzyme molecule bound to a vesicle "scoots" at the interface and hydrolyzes all the available substrate in the outer monolayer of the target vesicle (Fig. 4). As shown elsewhere[25] the reaction progress curves of type shown in Figure 3 are a direct consequence of the high affinity binding of PLA2 to vesicles where the enzyme, substrate, and products do not exhibit any intervesicle exchange even when all the substrate in the outer monolayer of the target vesicle has been completely hydrolyzed. We have obtained values of interfacial equilibrium and kinetic rate constants (Fig. 2), and through these it is possible to appreciate some of the otherwise inaccessible features of interfacial catalysis, such as the shape of the reaction progress curve, activation by calcium and other agents, substrate specificity,[26] specific competitive inhibition,[27] and the anomalous kinetics at thermotropic gel-fluid or isothermal phase transition in zwitterionic bilayers.[1,12,15,16,24,42]

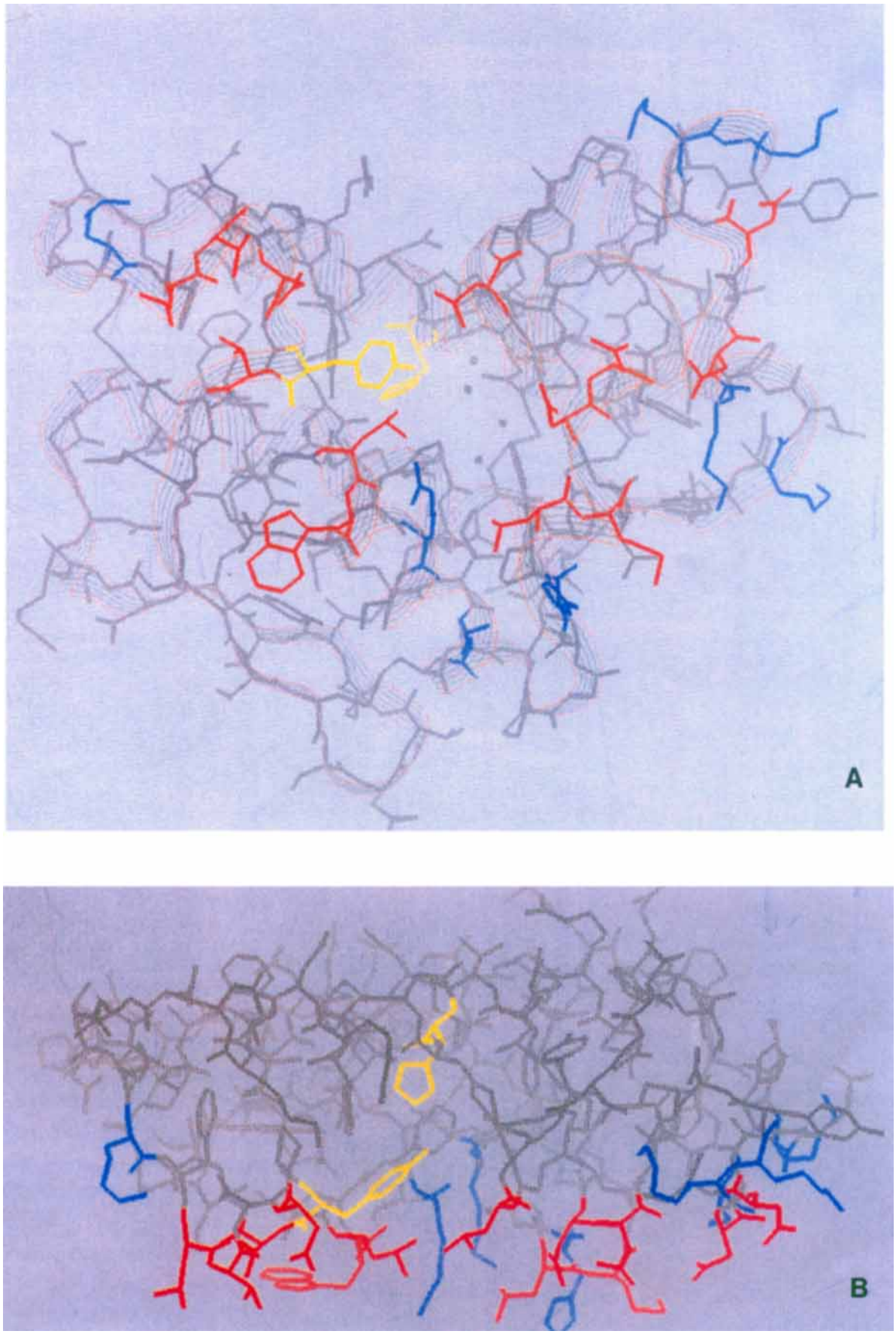
BINDING OF PLA2 TO BILAYER VESICLES

Binding of PLA2 to bilayers of nonhydrolyzable analogs of a phospholipid substrate can be monitored by several methods. The most convenient analog for the binding studies is 1,2-ditetradecyl-glycero-*sn*-3-phosphomethanol (DTPM), which forms bilayers with properties similar to those of the ester analog (DMPM). Direct evidence for high affinity binding of PLA2 to anionic vesicles is obtained from the experiments of type shown in Figure 3 (curve c), in which PLA2 on DTPM vesicles is inaccessible to DMPM vesicles added afterward. Other protocols to monitor binding of PLA2 to an interface include gel filtration, equilibrium dialysis, calorimetry, absorption and fluorescence spectroscopy of tryptophan and tyrosine, changes in anisotropy of Trp or a fluorescent label, and resonance energy transfer from tryptophan on the protein to a suitable acceptor group in the interface.[12,17–21]

Since proteins can bind nonspecifically to interfaces, it is necessary to develop criteria for evaluation of binding that is required for interfacial catalysis. A primary consideration is that binding be observed only under conditions that support catalysis, although catalysis is not necessary for the binding. Thus the catalytic significance of binding of PLA2 to vesicles of DTPM is demonstrated by observations such as binding requires calcium;[12] binding is observed only with anionic but not with zwitterionic bilayers unless the products of hydrolysis are also present;[18] the binding characteristics of pro-PLA2 are different;[21] the bilayer organization remains intact on binding of PLA2; only phospholipid molecules in the outer monolayer are accessible for binding and hydrolysis;[17,19] the rate of intervesicle exchange of the enzyme bound to DTPM or DMPM vesicles is very slow;[17,18] and the kinetic rate constants and the equilibrium dissociation constant for the E to E* step obtained from direct equilibrium binding, stopped-flow, and the kinetic measurements are comparable. As elaborated below such observations provide information about the enzyme–bilayer microinterface, i.e., the surface of PLA2 that interacts with the substrate interface (Fig. 5). Binding of PLA2 to DTPM vesicles causes a 2-fold increase in the quantum yield of the fluorescence emission from the only tryptophan residue (Trp-3) on the protein. The resonance energy transfer to a dansyl fluorophore (HDNS) in the polar head group region of the bilayer occurs with >90% efficiency, suggesting that the Trp-3 to dansyl distance is less than 10 Å.[21] As elaborated below, such an increase in the fluorescence emission from Trp-3 and dansyl fluorophores is useful for obtaining kinetic and thermodynamic information about the E to E* step.

Stopped-flow studies[23] show that the association rate constant, k_b, for the E to E* step has two components: a second-order diffusion limited step, followed by a first order rate constant of 4 sec^{-1}. The value of k_b for the micellar interface are the same as for anionic bilayers.[23] The upper limit for the dissociation rate constant, k_d, is estimated as <0.00002 sec^{-1}. These results corroborate with the fact that in the steady-state reaction progress curve the latency period is less than 3 sec; that the rate of intervesicle exchange of PLA2 is very slow during the hydrolysis of anionic vesicles; and that k_d and k_b are not a part of the catalytic turnover cycle in the steady state. The fact that the rate of catalytic turnover in the scooting mode is about 400 sec^{-1} also rules out the possibility that the catalytic turnover is limited by lateral diffusion, where the lower limit for the turnover rate would be 20,000 sec^{-1}.[25]

Binding isotherms of PLA2 to vesicles is quantitatively explained in terms of the E + nL to E* equilibrium,[12] where binding of one enzyme molecule to the interface requires n lipid molecules. The binding isotherms for PLA2 to anionic vesicles are steep hyperbola,[18] and the estimated upper limit of the apparent dissociation constant for E* is <100 nM. This is consistent with the kinetics of catalysis


A
B

in the scooting mode where the dissociation constant is estimated to be less than 0.1 pM.

The initial slope of the fluorescence intensity versus phospholipid concentration plots shows that 35 to 40 ($=n$) phospholipid molecules participate in the binding of each PLA2 to the interface.[12,17] One of the simplest interpretations of n is that the binding of one PLA2 molecule to a vesicle surface makes 35 phospholipid molecules inaccessible for the binding of other PLA2 molecules. If the cross-sectional area of a phospholipid monomer is 50 Å^2, the area occupied by PLA2 on a bilayer would be about 1,800 Å^2. Indeed, the face of PLA2 on which Trp-3 is located not only satisfies this constraint (Fig. 5a), but this remarkably flat face is the only face of PLA2 through which His-48 is accessible to a phospholipid head group of 6–8 Å diameter. At the edge of this face there is a ring of cationic residues which probably bind to the anionic interface. The middle collar around the entrance to the cavity consists largely of hydrophobic residues, and as discussed below it could be involved in the desolvation of the microinterface between the enzyme and the bilayer to promote binding of the substrate to the enzyme, i.e., in the E* + S to E*S step for the catalytic turnover.

NATURE OF THE MICROINTERFACE IN E*

There is no noticeable change in the circular dichroism spectrum of PLA2 on binding to anionic vesicles, which argues against a major conformational change in the E to E* step. The nature of the microinterface between the protein and the bilayer in E* has been probed by monitoring accessibility as well as the hydration and ionic characteristics of functional groups at the interface.[18,21] In free PLA2 Trp-3 is readily accessible to the bulk aqueous phase, and it is considerably less accessible in PLA2 bound to DTPM vesicles. While the gross organization of the bilayer is not altered on binding of PLA2 to the outer monolayer of DTPM vesicles, several subtle changes do occur that relate to the nature of the microinterface between the bilayer and the enzyme. For example, unlike the behavior in free PLA2, Trp-3 in PLA2 bound to DTPM vesicles is not quenched by water-soluble quenchers like succinimide or acrylamide, nor is it chemically modified by 2-hydroxy-5-nitrobenzyl bromide. These observations suggest that in the bound enzyme Trp-3 is not accessible to the bulk aqueous phase, and that Trp-3 is located on the face of PLA2 in contact with the bilayer (Fig. 5). Trp-3 is not a conserved residue[36] and PLA2 with chemically modified Trp-3 is catalytically functional on anionic vesicles, which shows that Trp-3 does not take part in the catalytic cycle.

The E to E* step involves ionic interactions as well as the desolvation of the microinterface between the enzyme and the bilayer. A crucial role of ionic interactions in the binding of PLA2 to the bilayer interface is indicated by the fact that the binding affinity of PLA2 to anionic bilayers is about 10 orders of magnitude larger than its binding affinity to zwitterionic bilayers, and that E* bound to anionic vesicles exchanges with excess vesicles only in the presence of salt.[18] Resonance energy transfer between Trp-3 and the dansyl fluorophore in HDNS localized in DTPM bilayer occurs with more than 90% efficiency, which corresponds to a donor to acceptor distance of about 8 Å. On the other hand the efficiency of energy transfer is about 50% from the tryptophan in proPLA2, which has an additional heptapeptide at the N-terminus. This difference is probably responsible for the fact that proPLA2 does not exhibit catalysis in the scooting mode although it is catalytically functional on monomeric substrates and also on anionic vesicles in the hopping mode.[23] A significant difference in the nature of the binding of PLA2 versus proPLA2 is demonstrated by the following experiment. In DTPM + HDNS vesicles coated with PLA2, there is essentially no difference between the fluorescence emission in H_2O and D_2O. However, in the absence of a protein or in the presence of proPLA2 the emission intensity is always higher in D_2O than it is in H_2O. A similar difference is also observed in the fluorescence emission properties of Trp-3.[23] The most likely interpretation of these observations is that in vesicles with PLA2, the microinterface containing the dansyl and Trp-3 fluorophore is desolvated, while this is not the case with proPLA2. The difference in the fluorescence quantum yield in D_2O versus H_2O is due to the collisional energy transfer in the vibrational mode from C–H bonds of the chromophore in the excited state to the O–H bonds of the solvent. Such energy transfer is less probable to O–D bonds, and therefore the quantum yield in D_2O is higher than in H_2O. At the microinterface of E* such energy transfer from the dansyl-chromophore to O–H bonds in water is not possible if the microinterface is desolvated.

The fluorescence quantum yield of Trp-3 as well as that of HDNS at the microinterface between the bound enzyme and the bilayer is higher than that in the aqueous environment. In both cases a blue shift in the emission maximum is observed. Such observations suggest that the polarity and mobility of the fluorophores are substantially altered at the microinterface. For the dansyl fluorophore this conclusion is substantiated by the fact that the life-time of the dansyl fluorophore of HDNS in DTPM bilayer increases from 10.9 to 13.5 nsec, which is consistent with a 70% increase in the emission quantum yield.

Fig. 5. The i-face of PLA2 that is most likely to be in contact with phospholipid bilayer: **(A)** front view seen from the membrane side, **(B)** viewed from one of the sides. This representation is based on the X-ray crystallographic data.[6,7] The residues shown in red are hydrophobic, and those shown in blue are cationic. His-48 and Tyr-69 (shown in yellow) are part of the active site.

The average lifetime obtained from discrete lifetimes of Trp-3 does not change on binding of PLA2;[14,18] however, the steady-state emission quantum yield, the fractional intensities of the lifetime components, and the intensity integral of the decay change by more than 70%. This suggests that on binding to the interface the dynamic quenching by bulk water as well as a significant part of the internal static quenching by neighboring Arg-6 and Lys-10 on the N-terminus helix is released.[18]

A direct structural analysis of the E* form of PLA2 has not been accomplished yet. The fluorescence behavior of PLA2 on the bilayer can be rationalized in terms of X-ray crystallographic features of free PLA2[6,7,28,31] shown in Figure 5. Trp-3 is on the 1,12-α helix on a face of PLA2 (which we call the i-face) from which His-48 is accessible. The amino acid residues located on the i-face form a characteristic pattern of distribution of hydrophobic and polar amino acid residues. In PLA2's studied so far, the cavity leading to the active site is lined with almost invariant hydrophobic residues (Phe-5, Ile-9, Tyr-22, Cys-25, Cys-45, Ala-102, and Ala-103). The top end of this cavity is capped by two helices (residues 39–56 and 92–108), and their opposite sides contain His-48, Tyr-52, Asp-49, and Asp-99, which form an absolutely conserved H-bond network with Tyr-73 and Ala-1, which is involved in the binding of the monomer substrate and catalysis. Many hydrophobic residues make up the collar surrounding the opening (diameter 8 Å) to the cavity on the i-face, and this hydrophobic region (Trp-3; Leu-2, 19, 31, 64; Val-65; Ala-1; Met-20) also contains a patch of cationic residues (Arg-6, Lys-10, His-17). These side chains probably constitute the first line of contact between PLA2 and the substrate interface. The hydrophobic region on the i-face is surrounded by a ring of hydroxylic (Thr-70 and 120, Ser-72), cationic (residues 53, 56, 62, 87, 113, 116, 121, 122) and anionic (residues 59, 114, 119) side chains. This polar ring forms the edge of the i-face and it is somewhat displaced away from the first line of contact (Fig. 5b) along an axis perpendicular to the i-face. We believe that the charge distribution along the polar ring and the topography of the i-face is an important determinant for the binding of PLA2 to the substrate interface. A simplistic interpretation would be that the i-face acts as a "suction-cup" with the charged-ring at the edge and the hydrophobic collar in the middle. The desolvated collar and the cavity help to dislodge the substrate from the interface for the formation of the Michaelis–Menten complex, E*S, at the interface.

BINDING TO THE INTERFACE VS. BINDING TO THE ACTIVE SITE

The mechanistic distinction between interfacial binding and the catalytic steps in Scheme I suggests that a parallel or corresponding functional and topological distinction exists between the interfacial binding region on PLA2 and the catalytic site. For example, the steady-state fluorescence properties of Trp-3 in E* on vesicles of DTPM, *sn*-1-DMPM, and the products of hydrolysis are indistinguishable, although minor differences due to absorbance or fluorescence emission from tyrosine or histidine are noticeable. This suggests that the catalytic susceptibility of an amphiphile at the catalytic site is not a prerequisite for binding of the enzyme to the interface. This is further substantiated by the fact that the presence of a competitive inhibitor of PLA2 at the zwitterionic interface to which PLA2 does not normally bind does not promote the binding of the enzyme. The fact that the binding of the substrate to the catalytic site of PLA2 is not required for its binding to the interface is also demonstrated by the fact that alkylated PLA2, in which His-48 at the catalytic site has been modified and is catalytically inactive, binds to DTPM bilayer with high affinity.

Other possible relationships between binding and catalytic events have been considered in literature. For example, acylation[32] or dimerization[5] of PLA2 has been invoked as a step necessary for the formation of E*. The possibility that acylation of a lysine is required for binding or catalytic action of PLA2 is ruled out by observations such as PLA2 binds to vesicles of DTPM;[17] PLA2 is not acylated in the presence of phospholipid substrates; and AMPA (PLA2 in which all amino groups of lysines are amidinated) has catalytic properties comparable to PLA2.[18] A role for dimerization of PLA2 for catalysis is ruled out by the fact that full catalytic activity is observed when there is at most one enzyme per vesicle, and the turnover number does not change if two or more enzyme molecules are present per vesicle.[17] Self-association of PLA2 during catalysis is further excluded by the fact that there is no intermolecular resonance energy transfer from Trp-3 to an anthranaloxy group on lysine-87 of the modified PLA2, which is catalytically active; however intermolecular resonance energy transfer is observed under conditions where the donor and acceptor molecules are "crowded" at the interface (Jain et al., to be published).

BINDING OF PLA2 TO ZWITTERIONIC VESICLES

The equilibrium dissociation constant for PLA2 from DTPC vesicles is >10 mM, i.e., more than 10^{10}-fold larger than for DTPM vesicles. This difference features prominently in the reaction progress curve for the hydrolysis of DMPC vesicles. As shown in Figure 6, these curves have a latency period which changes with thermotropic and isothermal phase transition characteristics of the substrate bilayer.[1,12,34,35] The latency period is not observed if the products of hydrolysis are present in the vesicles. Such a kinetic behavior is best explained in

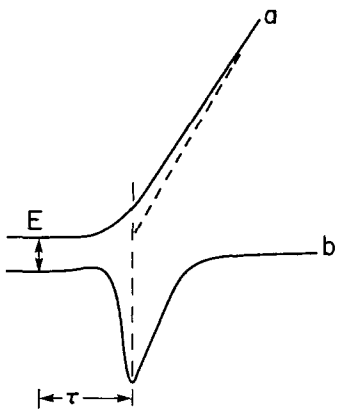


Fig. 6. Reaction progress curve for the hydrolysis of DMPC vesicles by PLA2 as observed by **(a)** pH-stat method or by **(b)** monitoring the fluorescence emission from NK-529 on the surface of these vesicles.[24]

terms of a product-dependent shift in the E to E* equilibrium.[1,12] Binding of PLA2 to DTPC vesicles is not observed at, below, or above the phase transition temperature, nor is it seen in bilayers containing neutral amphiphiles. However, in the range of thermotropic phase transition temperature, the binding affinity increases by several orders of magnitudes in the presence of a few mol% of both the products of hydrolysis or certain anionic amphiphiles.[1,12,24] This explains the shape of the reaction progress curve where the apparent autocatalytic behavior giving rise to the latency period is a consequence of the product dependent shift in the E to E* equilibrium.[25]

The role of the products of hydrolysis and other anionic additives in promoting binding of PLA2 to zwitterionic bilayers is due to complex miscibility and lateral distribution characteristics in mixed lipid bilayers. The lateral distribution of fatty acids in DMPC bilayers has been monitored with the fluorescence emission characteristics of a dicationic dye NK-529. This dye binds to bilayers, and the binding affinity is noticeably higher in the presence of anionic additives.[42] As shown in Figure 6, during the course of hydrolysis of DMPC vesicles the fluorescence emission from this dye undergoes a biphasic change. The sharp decrease, coincident with the latency period, is due to the presence of a small mole fraction of the products of hydrolysis, which are segregated and thus the neighboring dye molecules are self-quenched. As the reaction progresses and the mole fraction of the products increases, the average distance between the dye molecules increases and therefore self-quenching diminishes. Such observations show that above a critical mole fraction (>0.04) the products of hydrolysis of DMPC do not mix ideally in the DMPC bilayer, and clustering of fatty acids could promote binding of PLA2 as is the case for the increased binding of the cationic dye NK-529.[24,42] These results also bear on an interesting question: whether the enzyme is able to recruit anionic amphiphiles in the bilayer. The answer is no because binding and segregation are observed only above a certain mole fraction, and the mole fraction of an anionic amphiphile required for binding depends on the structure of the amphiphile, as well as the phase state of the bilayer.[24,42] This conclusion also accounts for the following related observations on the behavior of PLA2 at zwitterionic interfaces where the E to E* equilibrium is readily perturbed under the conditions commonly used for monitoring interfacial catalysis.

1. The effect of the thermotropic phase transition properties of bilayers on the binding of PLA2 to zwitterionic vesicles containing the products of hydrolysis is most prominent in the complex set of reaction progress curves that are observed in the phase transition range.[35] Since such a behavior is not observed with anionic vesicles, it can be concluded that the binding of PLA2 is not a direct consequence of the organizational and dynamic perturbations induced by temperature in single-component bilayers.

2. An increased rate of hydrolysis by PLA2 in the phase transition region has been reported in single point assays where the product analysis is carried out after a certain time.[29,30] In such studies information about the latency period is lost, and the rate of hydrolysis appears anomalously high when the latency period is short.[1,35] Studies with NK-529 show that segregation of the products of hydrolysis in DMPC vesicles occurs at low mole fractions at the phase transition.[24] Therefore near the phase transition the E to E* equilibrium shifts in favor of E* even in the presence of relatively small amounts of products. Direct binding studies show that a change in the E to E* equilibrium can adequately account for the overall time-course of hydrolysis.[24]

3. Several amphiphiles (such as anesthetics, alkanols, phenothiazines, mepacrine, aristolochic acid) reduce the apparent rate of hydrolysis by PLA2 at zwitterionic[13,16] but not of anionic bilayers.[27] Such results are best interpreted by the observation that lipophilic additives change the thermotropic properties, and therefore the E to E* equilibrium.[13,24]

HOPPING VS. SCOOTING: EPILOG AND PROSPECTS

The Michaelis–Menten formalism adopted within the framework of Scheme I provides a quantitative basis for interfacial catalysis by PLA2.[25] In this minireview we summarized the consequences of the E to E* equilibrium on interfacial catalysis. This is a key step that distinguishes interfacial catalysis from the usual enzymatic catalysis by water-soluble monomeric enzymes acting on water-soluble solitary

substrates. If the equilibrium does not favor E*, as is the case with proPLA2, catalysis occurs exclusively in the hopping mode, where the rate of intervesicle exchange is rapid, and the binding and desorption of the protein from the interface are a part of every catalytic turnover cycle. Thus due to a contribution from the rate constants k_b and k_d, the rate of overall catalytic turnover would be considerably reduced. If the binding equilibrium favors E*, as is the case with virtually all PLA2 molecules on anionic vesicles, catalysis occurs in the scooting mode and the steps involved in the intervesicle exchange do not influence the steady-state catalytic turnover. Under these conditions it is possible to rigorously describe the time course of the reaction, the substrate specificity, competitive inhibition, and related aspects of interfacial catalysis (in preparation).

Specific details of the interactions at the microinterface of E* with bilayers are under investigation. However, available evidence shows that ionic interaction leading to desolvation of the protein–bilayer microinterface is an important step in the overall interfacial catalytic process, i.e., not only for the E to E* step but also for the binding of the substrate monomer to the active site (E* + S to E*S step). The molecular details of the i-face shown in Figure 5 are consistent with the available data. Our future efforts in this direction include dissection of the events leading E to E*, and design of experiments that provide information about the microinterface in E*. Coupled with studies with suitable mutants it should be possible to identify specific functional groups and their interactions required for the stabilization of E* at the interface. To a first approximation such events are reflected in the kinetic and equilibrium constants summarized in the legend to Figure 2. Through a set of such binding and rate parameters we hope to obtain a better understanding of more complex phenomena such as surface dilution, substrate specificity, competitive inhibition, and activation.

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REFERENCES

1. Apitz-Castro, R.J., Jain, M.K., de Haas, G.H. Origin of the latency phase during the action of phospholipase A_2 on unmodified phosphatidylcholine vesicles. Biochim. Biophys. Acta. 688:349–356, 1982.
2. Cevc, G., Marsh, D. "Phospholipid Bilayers." New York: John Wiley, 1987.
3. Dennis, E. A. Kinetic dependence of phospholipase A2 activity on the detergent Triton X-100. J. Lipid. Res. 14: 152–159, 1973.
4. Dennis, E. A. Phospholipase A2 activity towards phosphatidylcholine in mixed micelles: Surface dilution kinetics and the effect of thermotropic phase transition. Arch. Biochem. Biphys. 158:485–493, 1973.
5. Dennis, E. A. Phospholipase A2. The Enzymes 16:307–353, 1983.
6. Dijkstra, B. W., Drenth, J., Kalk, K. H. The active site and the catalytic mechanism of phospholipase A2. Nature 289: 604–606, 1981.
7. Dijkstra, B. W., Kalk, K. H., Hol, W.G.J., Drenth, J. Structure of porcine pancreatic phospholipase A2 at 2.6 A resolution and comparison with bovine phospholipase A2. J. Mol. Biol. 147:97–123, 1983.
8. Jain, M. K., Cordes, E. H. Phospholipase I. Effect of n-alkanols on the rate of hydrolysis of egg phosphatidylcholine. J. Membrane Biol. 14:101, 1973.
9. Jain, M. K., Cordes, E. H. Phospholipases II. Effect of sonication and addition of cholesterol to the rate of hydrolysis of various lecithins. J. Membrane Biol. 14:119, 1973.
10. Jain, M. K., Van Echteld, C.J.A., Ramirez, F., DeGier, J., de Haas, G. H., Van Deenen, L.L.M. Association of lysophosphatidylcholine with fatty acids in aqueous phase to form bilayers. Nature 284:486–487, 1980.
11. Jain, M. K., de Haas, G. H. Structure of 1-acyl-lysophosphatidylcholine and fatty acid complex in bilayers. Biochim. Biophys. Acta 642:203–211, 1981.
12. Jain, M. K., Egmond, M. R., Verheij, H. M., Apitz-Castro, R. J., Dijkman, R., de Haas, G. H. Interaction of phospholipase A_2 and phospholipid bilayers. Biochim. Biophys. Acta 688:341–348, 1982.
13. Jain, M. K., Streb, M., Rogers, J., de Haas, G. H. Action of phospholipase A_2 on bilayers containing lysophosphatidylcholine analogs and the effect of inhibitors. Biochem. Pharmacol. 33:2541–53, 1984.
14. Jain, M. K., Maliwal, B. P. The environment of tryptophan in pig pancreatic phospholipase A_2 bound to bilayers. Biochim. Biophys. Acta 814:134–140, 1985.
15. Jain, M. K., Jahagirdar, D. V. Action of phospholipase A_2 on bilayers: Effect of fatty acid and lysophospholipid additives on the kinetic parameters. Biochem. Biophys. Acta 814:313–318, 1985.
16. Jain, M. K., Jahagirdar, D. V. Action of phospholipase A_2 on bilayers: Effect of inhibitors. Biochim. Biophys. Acta 814:319–326, 1985.
17. Jain, M. K., Rogers, J., Jahagirdar, D. V., Marecek, J. F., Ramirez, F. Kinetics of interfacial catalysis by phospholipase A_2 in intravesicle scooting mode, and heterofusion of anionic and zwitterionic vesicles. Biochim. Biophys. Acta 860:435–447, 1986.
18. Jain, M. K., Maliwal, B. P., de Haas, G. H., Slotboom, A. J. Anchoring of phospholipase A_2: The effect of anions and deuterated water, and the role of N-terminus region. Biochim. Biophys. Acta 860:448–461, 1986.
19. Jain, M. K., Rogers, J., Marecek, J. F., Ramirez, F., Eibl, H. Effect of the structure of phospholipid on the kinetics of intravesicle scooting of phospholipase A_2. Biochim. Biophys. Acta 860:462–474, 1986.
20. Jain, M. K., de Haas, G. H., Marecek, J. F., Ramirez, F. The affinity of phospholipase A_2 for the interface of the substrate and analogs. Biochim. Biophys. Acta 860:475–483, 1986.
21. Jain, M. K., Vaz, V.L.C. Dehydration of the lipid-protein microinterface on binding of phospholipase A_2 to lipid bilayers. Biochim. Biophys. Acta 905:1–8, 1987.
22. Jain, M. K. "Introduction to Biomembranes." New York: John Wiley, 1988:423.
23. Jain, M. K., Rogers, J., de Haas, G. H. Kinetics of binding of phospholipase A_2 to lipid-water interface and its relationship to interfacial activation. Biochim. Biophys. Acta 940:51–62, 1988.
24. Jain, M. K., Yu, B-Z., Kozubek, A. Binding of phospholipase A_2 to zwitterionic bilayers is promoted by lateral segregation of anionic amphiphiles. Biochim. Biophys. Acta 980:23–32, 1989.
25. Jain, M. K., Berg, O. Kinetics of interfacial catalysis by phospholipase A_2 and regulation of interfacial activation and inhibition. Biochim. Biophys. Acta 1002:127–156, 1989.
26. Jain, M. K., Rogers, J. Substrate specificity for interfacial catalysis by phospholipase A_2 in the scooting mode. Biochim. Biophys. Acta 1003:91–97, 1989.

27. Jain, M. K., Yuan, W., Gelb, M. H. Competitive inhibition of phospholipase A_2 in vesicles. Biochemistry 28:4135–4139, 1989.
28. Kuipers, O. P., Thunnissen, T.M.G.M., de Geus, P., Dijkstra, B. W., Drenth, J., Verheij, H. M., de Haas, G. H. Enhanced activity and altered specificity of phospholipase a2 by deletion of a surface loop. Science 244:82–85, 1989.
29. Op den Kamp, J.A.F., de Gier, J., van Deenen, L.L.M. Hydrolysis of phosphatidylcholine liposomes by pancreatic phospholipase a2 at the transition temperature. Biochim. Biophys. Acta 345:253–256, 1974.
30. Op den Kamp, J.A.F., Kaurez, M. T., van Deenen, L.L.M. Action of pancreatic phospholipase A2 on phosphatidylcholine bilayers in different physical states. Biochim. Biophys. Acta 406:169–177, 1975.
31. Renetseder, R., Brunie, S., Dijkstra, B. W., Drenth, J., Sigler, P. B. A comparison of the crystal structures of phospholipase A2 from bovine pancreas and crotalus atrox venom. J. Biol. Chem. 260:11627–11634, 1985.
32. Tomasselli, A. G., Hui, J., Fisher, J., Zurcher-Neely, H., Reardon, I. M., Oriaku, E., Kezdy, F. J., Heinrickson, R. L. Dimerization and activation of porcine pancreatic phospholipase A2 via substrate level acylation of lysine 56. J. Biol. Chem. 264:10041–10047, 1989.
33. Upreti, G. C., Jain, M. K. Effect of the state of phosphatidylcholine on the rate of its hydrolysis by phospholipase A_2 (bee venom). Arch. Biochem. Biophys. 188:364–375, 1978.
34. Upreti, G. C., Rainier, S., Jain, M. K. Intrinsic differences in the perturbing ability of alkanols in bilayer: Action of phospholipase A_2 on the alkanol modified phospholipase bilayers. J. Membrane Biol. 55:97–112, 1980.
35. Upreti, G. C., Jain, M. K. Action of phospholipase A_2 on unmodified bilayers: Organizational defects are preferred sites of action. J. Membrane Biol. 55:113–123, 1980.
36. Van den Bergh, C. J., Slotboom, A. J., Verheij, H. M., de Haas, G. H. The role of Asp-49 and other conserved amino acids in phospholipase A2 and their importance for enzymatic activity. J. Cell. Biochem. 39:379–390, 1989.
37. Verger, R., de Haas, G. H. Enzyme reactions in a membrane model. A new technology to study enzyme reaction in monolayers. Chem. Phys. Lipids 10:127–136, 1973.
38. Verger, R., de Haas, G. H. Interfacial enzyme kinetics of lipolysis. Ann. Rev. Biophys. Bioeng. 5:77–117, 1976.
39. Verheij, H. M., Slotboom, A. J., de Haas, G. H. Structure and function of phospholipase A2. Rev. Physiol. Biochem. Pharmacol. 91:91–203, 1981.
40. Waite, M. "The Phospholipase." New York: Plenum, 1987.
41. Wilshut, J. C., Regts, J., Scherphof, G. Action of phospholipase A2 on phospholipid vesicles. FEBS Lett. 98:181–186, 1979.
42. Yu, B-Z., Jain, M. K. Segregation of anionic lipophiles in bilayers monitored by binding of cationic dye NK-529. Biochim. Biophys. Acta 980:15–22, 1989.