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# The affinity of phospholipase $A_2$ for the interface of the substrate and analogs

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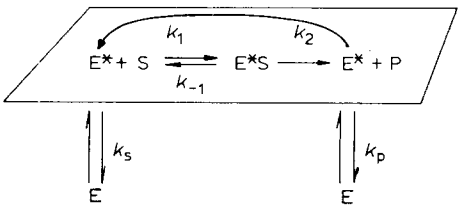
**In the intravesicle scooting mode of interfacial catalysis, the interfacial complex E*S is formed by the interaction of the membrane bound phospholipase $A_2$ (E*) with the substrate monomer (S) in the interface. In the presence of nonhydrolyzable substrate analogs (I) the kinetics of interfacial catalysis is modified. If phospholipase $A_2$ is added to a mixture of the vesicles of L-DMPMe ester and of DTPMe ether or D-DMPMe ester, the extent of hydrolysis, $A$, decreases and the interfacial scooting rate constant, $k_i$, remains unchanged. On the other hand, when the enzyme is added to the vesicles prepared from premixed L-DMPMe ester with D-DMPMe ester or L-DTPMe ether, $k_i$ decreases but $A$ remains constant. Qualitatively, these results are in excellent accord with the Scheme I for interfacial catalysis. However, a quantitative departure has been noted, which suggests that the interfacial dissociation constant for E*S is larger than that for E*I. These results are interpreted to suggest that the catalytic rate constant for decomposition of E*S to E* + P is larger than the rate constant for decomposition of E*S to E* + S. Broader implications of the scooting mode of interfacial catalysis are discussed.**

## Introduction

In the preceding three papers we have examined and elaborated the experimental consequences of interfacial catalysis as related to the kinetics of hydrolysis in the intravesicle scooting mode [1], to effects of anions in the aqueous phase and the bilayer, to the effect of substitution of amino acid residues in the N-terminus region of phospholipase $A_2$ [2], and to the effect of the structure of the head group of the substrate in the bilayer [3]. In this paper we examine yet another assumption implicit in Scheme I. According to




Scheme I. Scheme of interfacial catalysis.

this scheme, a nonhydrolyzable substrate analog (I) in the interface could form a nonproductive

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Abbreviations: DHPMe ether, 1,2-dihexylphosphatidylmethanol; DHPMe ester, 1,2-dihexoylphosphatidylmethanol; DMPMe ester, 1,2-dimyristoylphosphatidylmethanol; DTPMe ether, 1,2-ditetradecylphosphatidylmethanol; D-DMPMe, 2,3-dimyristoylphosphatidylmethanol; cmc, critical micelle concentration. The compound numbers refer to those given in Table I of Ref. 3.

complex, E*I, and therefore act as an inhibitor. We have examined the effect of such analogs on the intravesicle scooting kinetics under two different conditions: (a) When the analog I is present as separate vesicles with the vesicles of the substrate, and the enzyme in the aqueous phase competes for the two populations of the vesicles. (b) When I is codispersed with the substrate before formation of the vesicles, and the enzyme in the aqueous phase competes for the two lipid species in the codispersed vesicles. The results are qualitatively consistent with the predictions of Scheme I. However, quantitative dependence of $k_i$ and $a$ on the mole fraction of I suggests that $k_2$ is relatively rapid compared to $k_{-1}$. The experimental boundary conditions elaborated in this study are also discussed in the general context of interfacial catalysis.

## Materials and Methods

All the experimental protocols and source of reagents and lipids are described in the preceding three papers [1–3]. Appropriate references are also given in the text or in the figure legends.

## Results and Discussion

*Kinetics of hydrolysis of 1,2-dihexanoyl-sn-phosphatidylmethanol (DHPMe ester, No. 5) in the presence of 1,2-dihexyl-sn-phosphatidylmethanol (DHPMe ether, No. 5a)*

Aqueous dispersions of DHPMe ester and DHPMe ether form micelles with critical micelle concentration (cmc) of about 6.5 mM. As shown in Fig. 2 of Ref. 2 DHPMe ester is hydrolyzed by pig phospholipase $A_2$ not only above the cmc, but also below its cmc. This is probably due to formation of microaggregates of phospholipase $A_2$ with anionic amphipaths [2]. Under these conditions the reaction progress curves have a pseudo-zero-order component, and 100% of the substrate in the reaction mixture is ultimately hydrolyzed. The initial rate of hydrolysis depends not only on the substrate and the enzyme concentration, but the initial rate of hydrolysis decreases in the presence of 1,2-dihexylphosphatidylmethanol (No. 5a, DHPMe ether). As shown in Fig. 1, the initial rate of hydrolysis decreases linearly with the increasing

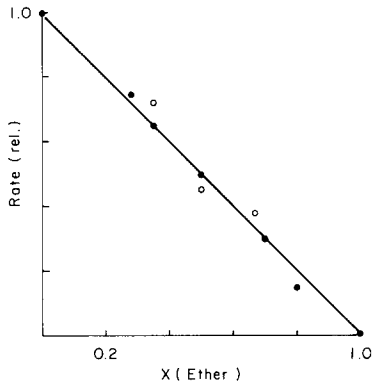


Fig. 1. Effect of varying the mole fraction of dihexylphosphatidylmethanol (DHPMe) ether, No. 5a) on the hydrolysis of 0.15 mM (●) or 1.25 mM (○) dihexanoylphosphatidylmethanol (DHPMe ester, No. 5) by 2 μg phospholipase $A_2$ at pH 8.0 in 0.1 M KCl and 10 mM $CaCl_2$. Specific activity for 0.15 mM substrate alone was 30 I.U., however, the data is normalized. At 1.25 mM DHPMe ester, phospholipase $A_2$ is in a microaggregate with the lipid (see Fig. 2 in Ref. 3).

mole fraction of DHPMe ether in the reaction mixture. These observations show that the relative affinity of the enzyme for the ether and ester analogs are essentially identical, not only in the microaggregates of the enzyme with the substrate, but also when the substrate is in the micellar form, as is the case at high mole fractions of the additive, DHPMe ether.

Effect of the phospholipase $A_2$ concentration on the initial rate of hydrolysis of DHPMe ester dispersions was also examined. As shown in Fig. 2, the initial rate of hydrolysis of DHPMe ester (No. 5) in the presence of equimolar concentration of DHPMe ether (No. 5a) increases linearly with the phospholipase $A_2$ concentration. The slope of this plot is one for the monomer as well as for the microaggregate form of the substrate. The slope of one suggests that the catalytically active form of the enzyme is probably monomeric (see also Ref. 1), or the equilibrium for the aggregated form of the enzyme, if any, is not noticeably perturbed by a 300-fold change in the enzyme concentration.

*Effect of DTPMe ether and D-DMPMe ester on the kinetics of hydrolysis of L-DMPMe ester vesicles in the scooting mode*

According to Scheme I, phospholipase $A_2$ in-

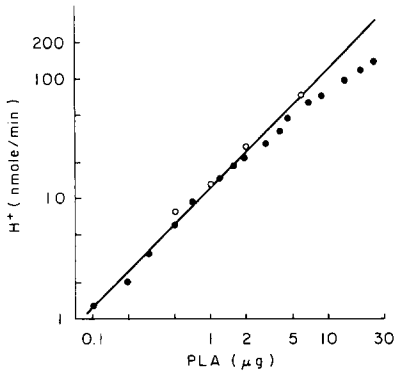


Fig. 2. Effect of phospholipase $A_2$ (PLA) concentration on the hydrolysis of 1.25 mM (●) and 5 mM (○; DHPMe ester, No. 5) codispersed with equimolar dihexylphosphatidylmethanol (DHPMe ether, No. 5a). The line is drawn to slope 1. Note that the total lipid concentration would be double of the substrate concentration plotted in abscissa; the rates of hydrolysis are normalized. Other conditions as in Fig. 1. Thus, the substrate would be in the micellar state above 6.5 mM.

teracts with the substrate interface (E to E*), and with the substrate monomer in the interface (E* to E*S). These two types of affinities of the enzyme can in principle be measured by the protocols outlined in Fig. 4 in Ref. 1 and elaborated below.

(a) When phospholipase $A_2$ is added to a mixture of the vesicles of DTPMe ether and DMPMe ester, the $k_i$ for the first-order reaction progress curve remains constant, whereas the extent of hydrolysis ($A$) decreases with the increasing mole fraction of DTPMe ether. As shown in Fig. 3A, the mole fraction of DTPMe ether vesicles, $X_A$, at which the extent of hydrolysis ($A$) decreases by 50% is about 0.15. It may be recalled that $A$ is related to the number of phospholipid molecules in the outermost monolayer of the vesicle to which E is bound. thus, the kinetics of binding of phospholipase $A_2$ from aqueous phase to the substrate vesicle would determine the value of $A$ in the presence of excess vesicles.

(b) The affinity of the enzyme for the substrate monomer in the interface can be evaluated by following the reaction progress curves for the hydrolysis of DMPMe ester vesicle containing varying mole fractions of DTPMe ether. As epected on the basis of Scheme I and shown in the first paper of this series (Fig. 4 in Ref. 1), under these conditions the extent of hydrolysis, $A$ does not change significantly, but $k_i$ decreases. As shown in Fig. 3B, $k_i$ decreases with the increasing mole fraction of DTPMe ether in DMPMe vesicles, whereas the extent of hydrolysis is not appreciably altered. Of course a correction for the decreasing mole fraction of DMPMe ester in the vesicles has been made in the values of $A$ shown in Fig. 3B. The mole fraction of DTPMe ether, $X_B$, at which $k_i$ decreases by 50% is 0.16.

Although the observations summarized in Figs. 3A and 3B are qualitatively consistent with the

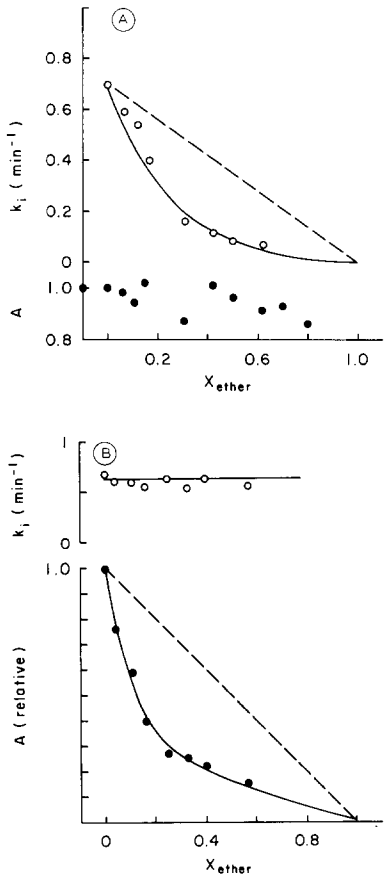


Fig. 3. (A) Effect of varying the mole fraction of DTPMe vesicles mixed with the vesicles of DMPMe (0.3 μmol) on the amplitude ($A$) and the rate constant, $k_i$. (B) Effect of varying the mole fraction of DTPMe ether codispersed with DMPMe ester (0.3 μmol) on the amplitude ($A$) and the rate constant, $k_i$. The reaction mixture contained 4 ml of 0.3 mM $CaCl_2$ and the indicated amount of the lipids. The reaction was initiated by adding 0.4 μg phospholipase $A_2$. Reaction volume 4 ml, 30°C, pH 8.0. Other conditions as described in Ref. 1.

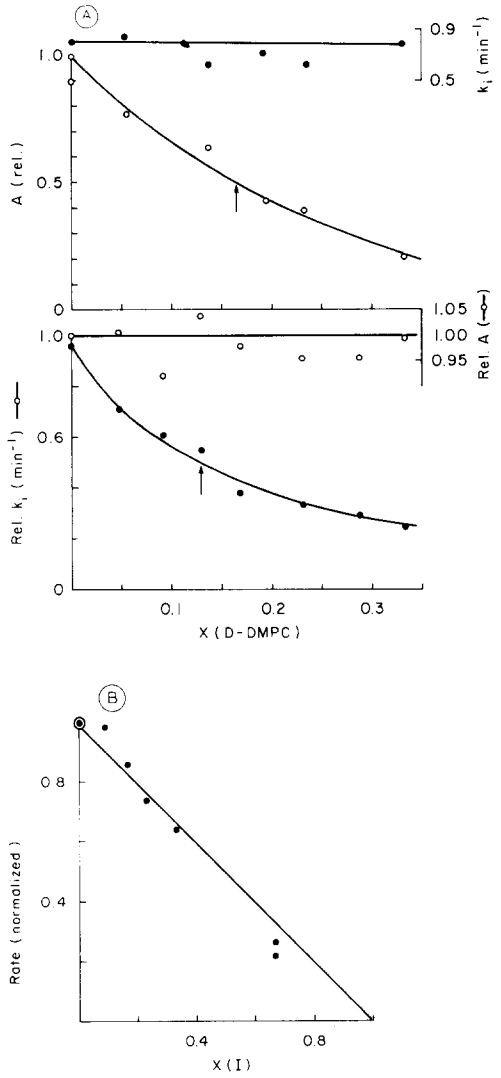


Fig. 4. (A) Effect of the increasing mole fraction of D-DMPMe ester on $k_i$ and $A$ for the hydrolysis of L-DMPMe ester in the scooting mode of interfacial catalysis. Top: for the mixture of vesicle; bottom: for the vesicles prepared from premixed lipids. Conditions as given in Fig. 3. (B) Effect of the varying mole fraction of D-DMPMe ester on the steady-state rate of hydrolysis observed after addition of 0.25 M NaCl at the end of the first-order reaction progress curves from which the data shown in Fig. 3 or Fig. 4A was obtained.

Scheme I, the fact that $X_A = X_B = 0.15$ suggests that the apparent affinity of phospholipase $A_2$ is higher for DTPMe ether than it is for DMPMe ester. The fact that essentially similar results are obtained for $A$ or $k_i$ measured according to the two protocols suggests that a common rate-limiting step must be shared by the two protocols. We suspect it is E* + S to E*S. In order to understand the origin of the bulge in the mole fraction plots of $K_i$ and $A$ (Fig. 3) we studied the effect of D-DMPMe ester on the kinetics of hydrolysis of L-DMPMe. As shown in Fig. 4A, the effect of D-DMPMe on the kinetics of hydrolysis is essentially identical to the effect of DTPMe ether. With both the nonhydrolyzable ether and ester analogs of the substrate (L-DMPMe) the extent of hydrolysis in the mixture of vesicles is inhibited by 50% at the mole fraction of about $X_A = 0.16$; similarly, the $k_i$ for hydrolysis of DMPMe ester in the mixed vesicles is lowered by 50% in the vesicles containing $X_B = 0.13$ mole fraction of D-DMPMe ester in the vesicles prepared from premixed lipids.

These observations show that the effect of D-DMPMe and of DTPMe ether on the kinetics of hydrolysis of L-DMPMe ester in vesicles is essentially identical under two different protocols. Qualitatively the effect of DTPMe ether or that of D-DMPMe ester on the reaction progress curves for the mixture of the vesicles or that for the mixed lipid vesicles is in excellent accord with Scheme I. However, the values of $X_A$ and $X_B$ are not what would be predicted on the basis of Scheme I [3]. The results in Figs. 3 and 4 are also not apparently consistent with the observations summarized in Fig. 1, where it is shown that the inhibitory effect of DHPMe ether is linearly proportional to its mole fraction in the mixed micelles. For example, if the affinity of phospholipase $A_2$ for the DTPMe ether or for D-DMPMe ester is the same as it is for L-DMPMe ester, both $X_A$ and $X_B$ would be 0.5 in each case. As with micellar substrate, under somewhat different conditions one can obtain $X_A = X_B = 0.5$ for D-DMPMe ester vesicles, or L-DTPMe ether vesicles mixed with the L-DMPMe ester vesicles. As shown earlier [1,2], addition of anions at the end of the first-order reaction progress curve leads to a steady-state pseudo-zero-order rate of hydrolysis during which all the excess available substrate is hydrolyzed. The steady-state rate of hydrolysis of L-DMPMe vesicles in the presence of the varying mole fractions of D-DMPMe or DTPMe ether vesicles is shown in Fig. 4b. Under these conditions a linear decrease in the rate of hydrolysis is observed with

the increasing mole fraction of vesicles of the analog. Thus, on the time averaged basis the enzyme encounters the analog vesicles in the same proportion as they are present in the reaction mixture. In conjunction with the data shown in Fig. 1, these observations show that the apparent affinity of the enzyme for the ether or the ester substrate analog is similar to that for the substrate under the conditions where the enzyme is able to exchange readily between the lipid micelles or the vesicles, that is when the rate of interfacial transfer of phospholipase $A_2$ (e.g. during intervesicle exchange) is the rate limiting step.

The bulge in the mole fraction plots for the inhibitory effect of additives (Figs. 3 and 4) is observed only under the conditions of scooting kinetics. A possible explanation for this bulge could be in the design and interpretation of these experiments. For example, for the plots of type shown in Figs. 3 and 4A, the enzyme and the substrate concentration is kept constant, and the analog concentration is varied to change its mole fraction; thus the overall interface concentration also changes. if a step in the overall reaction sequence somehow depends upon the enzyme to interface ratio, a bulge of the type shown in Figs. 3 and 4A could result. As shown in Fig. 5, the extent of hydrolysis, $A$, shows an anomalous dependence on the concentration of D-DMPMe and of L-DMPMe present as separate vesicles in 1 : 1 ratio. As shown earlier [1], with L-DMPMe ester vesicles alone such a decrease in $A$ at higher substrate concentrations is not observed. Also according to Scheme I, it is expected that in the scooting mode the extent of hydrolysis would reach a maximum and remain constant at that level. However, as shown in Fig. 5, the extent of hydrolysis decreases above 80 $\mu$M total lipid concentration, yet the $k_i$ values appear to remain constant above 80 $\mu$M total lipid concentration. Higher $k_i$ at lower lipid concentrations are expected if more than one enzyme molecule is present in each vesicle. These results suggest that a critical ratio of the enzyme to interface is apparently required for a maximum extent of hydrolysis when D-DMPMe ester or DMPMe ether vesicles are also present. This is also demonstrated in the data shown in Fig. 6. At 1 : 1 mole ratios of D- and L-DMPMe vesicles the extent of hydrolysis depends upon the total lipid concentration. At 0.2 mM total lipid concentration, $A$ is smaller than it is for 0.05 mM mixture. However, if additional enzyme is added (cf. curve c, Fig. 6) the extent of hydrolysis increases. Based on the data shown in Fig. 5 or Fig. 6, it appears that the anomalous decrease in $A$ with increasing mole fraction of the additive is observed when the vesicle to the enzyme ratio exceeds one, i.e., when the enzyme molecule

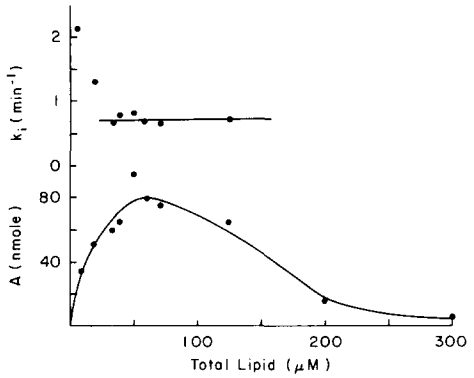


Fig. 5. Effect of 1 : 1 DTPMe ether + DMPMe ester concentration of the extent of hydrolysis ($A$) and $k_i$. Appropriate amounts of vesicles of DTPMe ether and L-DMPMe ester were equilibrated at pH 8.0 in 0.3 mM $CaCl_2$, and the reaction was initiated with 0.4 $\mu$g phospholipase $A_2$.

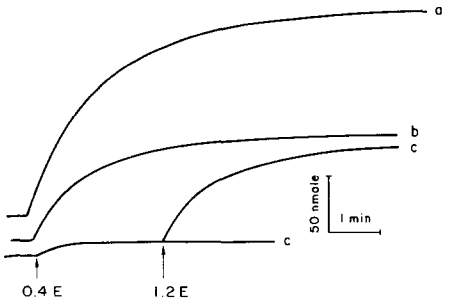


Fig. 6. Reaction progress curves for the hydrolysis of (curve a) 0.1 mM L-DMPMe ester; (curve b) 0.25 mM D-DMPMe with 0.25 mM L-DMPMe; and (curve c) 0.1 mM D-DMPMe vesicles with 0.1 mM L-DMPMe. The reaction in each case was initiated with 0.4 $\mu$g phospholipase $A_2$ (E), and 1.2 $\mu$g phospholipase $A_2$ was also added in curved c as indicated.

competes for the substrate and the ether vesicles. In such a situation the enzyme appears to bind preferentially to vesicles of the analogs rather than the substrate vesicles.

The role of a critical ratio of the enzyme to the vesicles for a maximum extent of hydrolysis is intriguing. One of the simplest interpretations of this data would be that compared to a bound monomer enzyme, an aggregated form of the enzyme can exchange more readily between the vesicles in the absence of the products. In the codispersions of the substrate with the substrate analog the bound aggregated enzyme will be diluted. This situation is however not manifested in the etent of hydrolysis in the presence of the excess substrate; as shown earlier, the extent of hydrolysis does not decrease significantly at high substrate concentration. Similarly, as shown in Fig. 2, in the micellar form of the substrate, the enzyme concentration dependence is linear over a 300-fold enzyme concentration and over at least a 10-fold lipid concentration. Such observations essentially rule out a simple catalytic role for the aggregated enzyme. Similarly, we do not believe that these effects are due to a difference in the nature of the interface of the stereoisomeric lipids, because the phase transition and fusion properties of these lipids are essentially the same (see also Ref. 11).

These results show that the bulge in the mole fraction plots (cf. Figs. 3 and 4A) of the parameters of the scooting kinetics is not an artifact. The extent of bulge depends upon the relative amounts of the enzyme and the substrate in the reaction mixture. The parameters $A$ and $k_i$ do not represent a single kinetic step in the Scheme I, instead these parameters represent a cumulative effect of several thousand turnover cycles. As discussed below, the origin of this apparently higher affinity of phospholipase $A_2$ for the substrate analogs is implicit in the assumptions of Scheme I.

The departure from linearity in the mole fraction plots is observed both in the mixture of the vesicles, as well as with the premixed binary vesicles. Therefore, the origin of the bulge in the mole fraction plots must be related to a step that occurs in the bilayer interface. This step is common in the two protocols and it should occur before the catalytic step. According to Scheme I, the interfacial turnover is characterized by a Michaelis-Menten type of reaction sequence enclosed in the box. As implicit in Scheme I, the interfacial dissociation constant for E*S is given by:

$$K_m = \frac{k_2 + k_{-1}}{k_1}$$

For a nonhydrolyzable substrate analog (I), $k_2 = 0$, therefore, the interfacial dissociation constant, $K_d$, for E*I is $k_{-1}/k_1$. For most enzyme-substrate reactions $k_2$ is assumed to be small and therefore $K_m = K_d$ [10]. If $k_2 \gg 0$, then $K_m > K_d$. Indeed, data shown in Figs. 3 and 4 suggest that for D-DMPMe ester and L-DTPMe ether, $K_d = 0.15\ K_m$. If we assume that $k_1$ and $k_{-1}$ are the same for the two forms of phospholipids, it would imply that $k_2 = 5k_{-1}$. Unfortunately, we do not have any direct method to measure these interfacial rate constants. However, this would not be an unreasonable relationship for an enzyme that remains bound to the bilayer for many catalytic turnover cycles. It may also be noted that the apparent $K_m$ for the ternary codispersions of DMPC is about 3-fold higher than the $K_d$ measured from the binding experiments [4,11]. Similarly, as shown in the preceding paper [3], the apparent $K_d$ is always smaller by a factor of 3 to 7 than the apparent $K_m$ for a variety of lipids.

Detailed consequences of these possibilities are being examined. Meanwhile, certain qualitative inferences can be drawn on the basis of the observations at hand. In the Tabe III of the preceding paper [3] we have shown that the fluorescence intensity of the enzyme bound to micelles and bilayers of the various phospholipids depends upon the structure and conformation of glycerophosphate region of the molecule. These observations imply that the binding detected by fluorescence enhancement is catalytically meaningful, that is, it represents the catalytically active E*S form rather than the E* form of the bound enzyme. The values of $K_d$ in the range of 1 $\mu$M also imply that $k_{-1}$ should be relatively small, which could lead to $k_2 > k_{-1}$. Such intrinsic limitations lead to some very interesting possibilities regarding the nature of interfacial binding equilibria and the interfacial concentrations. Such limitations could be unique to the interaction of proteins in bilayers.

## General discussion

This study provides the first unequivocal experimental demonstration and a phenomenological description of interfacial catalysis in scooting mode. Although our experimental system is a bilayer interface, we believe that these results have a general validity for monolayers, micelles, and biomembranes. Intravesicle scooting and intervesicle exchange of bound phospholipase $A_2$ have profound implications on regulation of phospholipase $A_2$ activity, on the nature of the underlying lipid-protein interactions and the underlying equilibria, and on a detailed understanding of interfacial kinetics in general. In this section we qualitatively eamine the broader implications of the observations reported in this series of papers, as well as those published earlier on the action of phospholipase $A_2$ on the bilayer [4–7] and other forms of the substrate [7–10].

Interfacial catalysis is unusual in the sense that the structure as well as the organization of the substrate influences the catalysis. By controlling a variety of complicating factors we have been able to obtain relatively unambiguous and simple kinetics for hydrolysis in the intravesicle scooting mode. This is possible because unlike any other form of the lipid/water interface, the vesicles of anionic substrate provide a stable interface, i.e., low cmc, very slow rates of intervesicle and transbilayer exchange of the substrate, little or no fusion, no latency phase, and the bilayer matrix is retained even at very high protein-to-lipid ratios. Such conditions reduce the number of possible interpretations for the interfacial rate constant for scooting ($k_i$), for the extent of hydrolysis ($A$), and those for desorption of the bound enzyme ($k_p$ and $k_s$).

Our observations are generally consistent with the Scheme I, but several specific details have emerged. Some of these are articulated in the cartoon shown in Fig. 7 and others are discussed below. Initial binding of the enzyme to the interface (E to E*) is predominantly by site-mediated ionic interactions. While the enzyme is anchored to the bilayer via the anion binding site, the substrate monomer from the bilayer is dislodged by the defects or instabilities. Once in the interface, catalytic turnover by the enzyme is described by a simple first-order process. Based on this qualitative model we begin to understand the comple catalytic behavior of phospholipase $A_2$ on bilayer interface, and thus rationalize a variety of observations: the apparent rate of hydrolysis is stimulated in anionic interface; an apparent activation of hydrolysis at low E/S ratios is observed when the interfacial transfer of the bound enzyme is promoted; anomalous activation of the initial rate of hydrolysis is observed when enzyme to vesicle ratio exceeds one; cationic additives in the interface are inhibitory because they promote desorption of the bound enzyme; hydrophobic solutes promote hydrolysis if the intermolecular interactions of the substrate in the bilayer are weakened, and such solutes could inhibit hydrolysis if they compete with the substrate for E*; the rate of hydrolysis decreases with increasing chain length and increases with increasing unsaturation; effect of anions in the aqueous phase depends upon the position of the various equilibria implicit in Scheme I.

Collectively, these observations and the un-

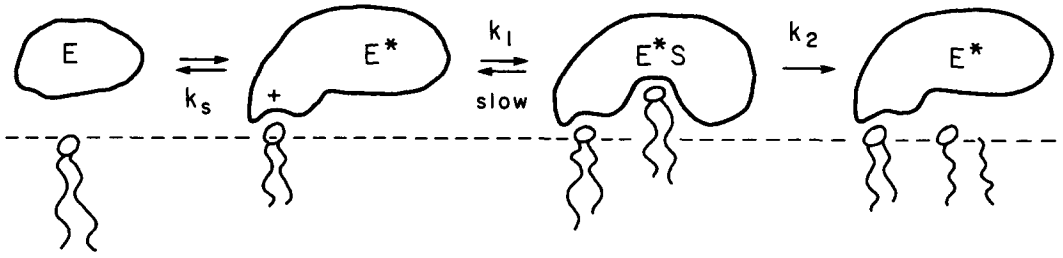


Fig. 7. A cartoon representing some of the major steps involved in the interfacial kinetics in scooting and hopping modes. These steps are implicit in Scheme I.

derlying model shown in Fig. 7 address to the broader questions of specific activity and substrate specificity of phospholipase $A_2$. Obviously, the criteria used for soluble enzyme and soluble monomeric substrate can not be readily adopted to the sequence of events implicit in Scheme I and Fig. 7. Under the conditions where intervesicle exchange of the bound enzyme is significant, the overall rate of hydrolysis is due to a complex interplay of several equilibria. However, it is under these conditions steady-state rates of hydrolysis are obtained. Obviously, the significance of the kinetic parameters like $K_m$ and $V_{max}$ obtained from such data is considerably obscured. The situation becomes far more amenable to a quantitative analysis in terms of $k_i$ and $A$, when the kinetics of hydrolysis are monitored in the intravesicle scooting mode without any intervesicle exchange.

Some of the general experimental boundary conditions for the phenomenon of interfacial catalysis that have evolved from our studies can help in elaborating the implications of Scheme I and the model shown in the cartoon in Fig. 7. These implications are discussed below.

(a) Interfacial activation of phospholipase $A_2$ has not been adequately explained. In the context of Scheme I, interfacial activation has two possible origins. Long residence times in the interface promote hydrolysis in the scooting mode, and therefore lead to first-order kinetics, the extent of which deends upon the number of substrate monomers in the interface. With excess vesicles in the presence of anions, intervesicle transfer of the bound enzyme promotes the extent of hydrolysis by lowering the residence time of the enzyme. Thus, the mode and extent of activation depends upon the protocol and the conditions for the assay.

(b) Perturbations in the bilayer organization that cause a change in any one of the many equilibria implicit in Scheme I would influence the kinetics of hydrolysis. As discussed before, activation or inhibition by lipid-soluble additives could be due to their effect on one of the many steps. The most susceptible of these is the binding equilibrium that depends upon the anion binding site. Thus cation additives [12–16] in the interface and the anions in the aqueous phase act as potential inhibitors by decreasing the residence time of the enzyme in the interface. The effect of chain length, phase properties, and the additives on the kinetics of hydrolysis in intravesicle scooting mode can also be accounted for on the basis of similar considerations.

(c) A catalytic role for the dimeric form of phospholipase $A_2$ in the interface has been suggsted [10]. As discussed earlier [1,2], the experimentally measured values of $A$ and $k_i$ have provided a unique opportunity to answer this question. The data suggests that a single enzyme molecule per vesicle is adequate to hydrolyze all the substrate in the outer monolayer of the vesicle, and with the excess substrate the rate or extent of hydrolysis does not change. If there is a monomer⇌dimer equilibrium for the bound enzyme, presence of excess substrate interface would shift the equilibrium to lower the catalytic activity by the dimeric form of the bound enzyme. This is not the case, which suggests that the formation of dimeric pig phospholipase $A_2$ is not necessary for its catalytic activity in the bilayer inteface.

(d) Anchoring of phospholipase $A_2$ to an anionic interface is of high affinity, yet the enzyme does not penetrate in the hydrophobic region. We suspect that the true dissociation constant for the bound enzyme into a free enzyme and a site of $n$ ($= 40$) lipid molecules is about 0.4 $\mu$M or less. This is in accord with the observation that the rate constant for binding ($E > E^*$) is relatively large ($> 40$ per s), and that the rate constants for desorption ($k_p$ and $k_s$) are small ($< 0.2$ per min). This interpretation may be somewhat simplistic because the bound enzyme can be absorbed and desorbed from a hypothetical binding site without effectively leaving the vesicle. The role of instabilities in the bilayer organization in making the substrate monomer accessible to $E^*$ to form $E^*S$ is a yet unexplored aspect of the same problem. A general discussion of such aspects of lipid-protein interaction is reviewed elsewhere [17].

(e) Studies with zwitterionic phosphatidylcholine vesicles [4–7] are also in accord with the suggestion that the interfacial anionic charge and instabilities in the organization of the bilayer are important for the overall kinetics. The binding affinity of the enzyme for DMPC ester or DTPC ether vesicles are low as indicated by kinetic,

fluorescence, calorimetry, gel filtration, and exchange experiments. When DMPC bilayers contain additives that form defects and impart anionic interfacial charge, the binding of phospholipase $A_2$ to the vesicles increases.

(f) The model underlying Scheme I was originally suggested by Verger and DeHaas [8] to account for their kinetic data for the action of phospholipase $A_2$ on monolayers of medium chain phosphatidylcholines. Agreement of our observations with this model is so striking that we could explain all of our observations in terms of this model. The most significant point of difference arises from the rate-limiting step. The presteady-state rate of incorporation of phospholipase $A_2$ in monolayer was postulated to be slow, with latency period of several minutes, whereas no such latency is observed with monolayers of anionic lipids. In analogy with our results with anionic and zwitterionic lipids we propose that the slow presteady-state phase in monolayers is due to formation of a critical mole fraction of the products that promote binding of the enzyme to the interface, which ultimately leads to interfacial catalysis in the scooting mode. Our data shows that the rate constant $k_2$ for the decomposition of $E^*S$ to $E^* + P$ is large enough (compared to $k_{-1}$ and probably also to $k_1$) to contribute a several-fold increase in the value of $k_m$ compared to $K_d$. In the original Scheme of Verger and DeHaas [8], $k_2$ is considered rate limiting. These considerations are significant for evaluation of the relative affinities of the substrate additives in the interface (cf., Figs. 3 and 4).

To recapitulate, interfacial catalysis in scooting mode has been unambiguously demonstrated for the action of pig pancreatic phospholipase $A_2$ on vesicles of anionic phospholipids. Yet we do not have a fully integrated quantitative interpretation of these observations. Modelling and experimental determination of the various rate constants should ultimately help us in attaining this goal. Essentially irreversible anchoring of phospholipase $A_2$ to bilayer involves ionic as well as hydrophobic interactions. The protocols developed in these papers could provide an opportunity to study the structure of phospholipase $A_2$ in the interface by X-ray crystallographic analysis, the kinetics of inhibition of phospholipase $A_2$, and the nature of the microinterface between the enzyme and the bilayer.

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