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Interfacial Catalysis by Phospholipase A_2: Activation by Substrate Replenishment†

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ABSTRACT: Polymyxin B (Px), a cyclic cationic peptide, was shown to act as a potent activator of interfacial catalysis by phospholipase A_2 (PLA2) acting on dimyristoylphosphatidylmethanol vesicles in the scooting mode. A 7-fold increase in the initial enzymatic velocity was seen with the pig pancreatic PLA2 in the presence of 1 μM Px. Initial experiments including the dependency of the degree of activation by Px on the source of the PLA2 suggested that Px bound to a cationic binding site on the enzyme. However, numerous additional observations led to the conclusion that activation by Px was due to its effects on the substrate interface. For example, the activation by Px was only seen when the PLA2 acted on small vesicles rather than larger ones, and all of the available substrate was eventually hydrolyzed in the presence of a small mole fraction of Px. Px did not promote the intervesicle exchange of PLA2, and it did not alter the binding of inhibitors, calcium, substrate analogues, or the reaction products to the enzyme in the interface. All of the evidence led to the conclusion that Px activated interfacial catalysis by promoting the replenishment of substrate in the enzyme-containing vesicles. When PLA2 was acting on small vesicles in the scooting mode, the observed initial velocity was lower than that measured with large vesicles because the surface concentration of substrate decreased relatively rapidly in the small vesicles. Px promoted the transfer of phospholipids between the vesicles and functioned as an activator by keeping the mole fraction of substrate in the enzyme-containing vesicles close to 1. This effect of Px was consistent with the ability of polycationic peptides to induce the intervesicle mixing of anionic phospholipids in vesicles [Bondeson, J., & Sundler, R. (1990) *Biochim. Biophys. Act 1026*, 186-194]. Activation by substrate replenishment was quantitatively predicted by the theory of interfacial catalysis on vesicles in the scooting mode. The role of substrate replenishment in the kinetics of interfacial catalysis in phospholipid micelles was discussed. Finally, the protocols developed in this paper were outlined in view of their utility in the analysis of activators of interfacial catalysis.

Modulation of interfacial catalysis by PLA2[1] is of interest in understanding the regulation of the eicosanoid pathway. Besides the catalytic manifestations of the direct effect of perturbation of the active site by specific modulators, the apparent catalytic turnover of PLA2 is influenced by up to several orders of magnitude by changes in the organization

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[1] Abbreviations: DMPM, 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol lithium salt; DTPM, 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol lithium salt; PLA2, phospholipase A_2; Px, polymyxin B.

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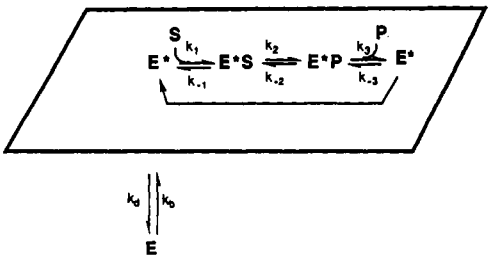
Scheme I



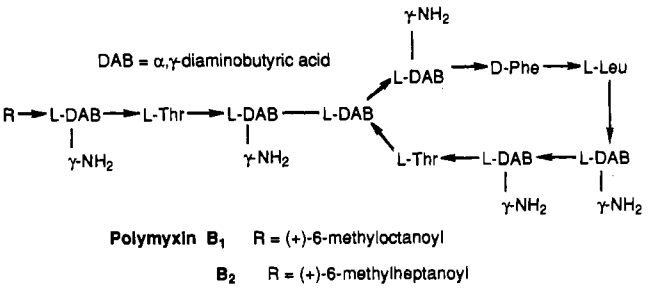
and dynamics of the substrate interface by nonspecific modulators (Jain & Berg, 1989). These effects can be accounted for in terms of the scheme in Figure 1 of Berg et al. (1991) (reproduced in Scheme I), which indicates that the enzyme in the aqueous phase (E) and the enzyme bound to the substrate bilayer (E*) are distinct species (Verger & de Haas, 1976; Verheij et al., 1981; Jain & Berg, 1989).

Changes in the organization and dynamics of the molecules at the interface influence the equilibrium between the enzyme in the aqueous phase and the enzyme in the interface, the E to E* step (Jain et al., 1991a; Jain & Berg, 1989). Since lipolysis occurs within the interface, the overall rate will be proportional to the fraction of the enzyme in the E* form. Moreover, the transfer of the enzyme from and to the interface will influence the overall turnover rate in the case where the enzyme is exchanging between the vesicles. Also, the rate of hydrolysis at a given instance depends on the surface concentration of substrate that the bound enzyme "sees" for the formation of the Michaelis–Menten complex, E*S (Berg et al., 1991).

Interfacial catalysis is an intriguing phenomenon as it is modulated by additives that modify the substrate interface. Within the constraints of Scheme I, it is possible to distinguish several mechanisms that could lead to a change in the observed rate of catalytic turnover. For example, a PLA2-specific activator or inhibitor would bind to the enzyme and modulate its catalytic efficiency for turnover at the interface (Jain et al., 1989). On the other hand, nonspecific modulators of interfacial catalysis could function by changing the E to E* equilibrium during the steady-state turnover (Jain et al., 1991a; Jain & Jahagirdar, 1985). It should also be emphasized that when lipolysis occurs on phospholipid aggregates such as micelles or vesicles, the steady-state concentration of E*S, and hence the reaction velocity, will depend on the rate of replenishment of substrate and the rate of removal of products in the enzyme-containing particles. This latter point will be fully developed in the present paper.

For characterization of inhibitors of interfacial catalysis, we have shown that several possible modes of reduction in catalytic turnover are distinguishable by directly monitoring the E to E* equilibrium (Jain et al., 1984, 1989; Jain & Jahagirdar, 1985; Jain & Berg, 1989), or the E* + I to E*I equilibrium (Jain et al., 1991a), or by monitoring the kinetics of interfacial catalysis in the scooting mode (Jain et al., 1989, 1991a). Thus, under suitably chosen conditions, one can clearly distinguish an active-site-directed specific competitive inhibitor from a nonspecific inhibitor that may influence many other possible steps or parallel processes which are intrinsic in Scheme I. Similar arguments apply to activators of interfacial catalysis and are considered in this paper.

Polymyxin B (Px) is a mixture of two amphiphilic cationic cyclic nonapeptides containing a (+)-6-methyloctyl (Px-B1) or a (+)-6-methylheptyl (Px-B2) chain attached to the N terminus (Storm et al., 1977).



These strongly cationic peptides have been reported to exhibit a wide variety of biological effects (Storm et al., 1977), including activation of PLA2 (Elsbach & Weiss, 1988). Very little is known about the mechanism of activation of PLA2 by compounds such as Px. As already mentioned, modulation of interfacial catalysis can occur by a number of different modes involving either a specific interaction of the additive with the enzyme or an effect of the additive on the dynamics of phospholipids within the substrate aggregate. In this paper we outline a strategy and protocols that were developed to resolve the basis of the effect of activators such as Px on the interfacial catalysis by PLA2 on DMPM vesicles.

Materials and Methods

Unless otherwise indicated, all experiments reported here were carried out with PLA2 from pig pancreas. This and other PLA2s were obtained as in the earlier study (Jain et al., 1991b). The mutant of pig PLA2 in which the tyrosine at position 69 has been changed to phenylalanine, Y69F, was a gift from Prof. H. M. Verheij (University of Utrecht). Px and spermine were from Sigma. The sources of all other reagents and details of protocols used in this study were described in the previous papers (Jain et al., 1986a, 1991a; Berg et al., 1991). The only additional precaution necessary for the kinetic measurements in the presence of Px was that the titration vessel should be washed thoroughly with a detergent such as Micro (Internation Products Inc., Box 118, Trenton, NJ) to prevent carry over. Also, as was the case with other measurements for interfacial catalysis in the scooting mode, the sequence of addition and rapid mixing of reactants and the time delays between additions were critically important for uniform distribution of the components (Berg et al., 1991).

Results

Activation by Px. Elsewhere it was shown that the binding of PLA2 to DMPM vesicles was of such high affinity (K_D less than 0.1 pM) that all of the enzyme present in the reaction mixture was always bound to the bilayer interface (Jain et al., 1986a). Not only was the rate of intervesicle and transbilayer exchange of the phospholipids very small, the rate of intervesicle exchange of the enzyme was also negligible on the time scale of the overall reaction progress curve (Jain et al., 1986a; Jain & Berg, 1989). This accounted for the main characteristics of interfacial catalysis in the scooting mode. For example, as shown in Figure 1 (curve a), the reaction progress curve for the action of PLA2 on these small sonicated vesicles of DMPM had a characteristic first-order appearance (Berg et al., 1991; Jain et al., 1986a). The reaction started immediately after the addition of PLA2 to a suspension of sonicated vesicles of DMPM, and the reaction virtually stopped after a small portion of the total substance in the reaction mixture was hydrolyzed, i.e., about 3% in this case when the vesicle to enzyme ratio was about 20. As shown elsewhere, these effects were not due to inactivation of the enzyme, or due to a shift in the thermodynamic equilibrium for the reaction, or due to a change in the gross organization (polymorphism) of

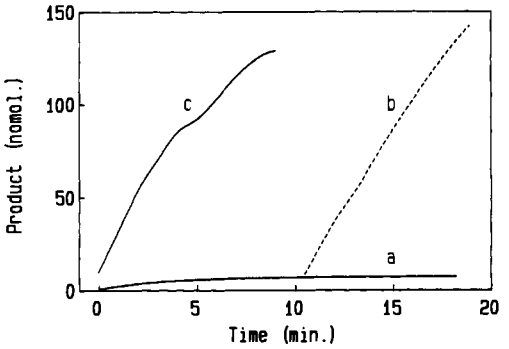


FIGURE 1: Reaction progress curves for the hydrolysis of small sonicated vesicles of DMPM (0.7 mg) by pig pancreatic PLA2 (2.3 pmol) at pH 8.0 and 23 °C in a 4-mL reaction mixture containing 0.6 mM $CaCl_2$ and 1 mM NaCl. (Curve a) The reaction was initiated with PLA2; (curve b) 5 μg of Px was added after the reaction ceased under the conditions for curve a; (curve c) the reaction mixture also contained 5 μg of Px added 30 s before the addition of PLA2. The initial rate of hydrolysis per enzyme molecule, i.e., initial turnover number (s^{-1} E^{-1}) in this paper was measured as the slope near t = 0 [for details see the text and Berg et al., (1991)].

the substrate that made the excess substrate inaccessible to the enzyme (Jain et al., 1986a–d, 1991a,c; Jain & Berg, 1989). In these earlier studies, under the conditions used in Figure 1 (curve a), the reaction ceased because the enzyme remained bound to the vesicle that it just hydrolyzed and was not accessible to additional substrate present in the other vesicles. This processive reaction continued until all of the substrate in the outer monolayer of enzyme-containing vesicles became hydrolyzed. This was further supported by the observation on the activating effects of Px. For example, as shown in Figure 1 (curve b), addition of 1 μM Px caused a dramatic increase in the rate of hydrolysis. Px was a potent activator of PLA2 since as little as 0.001 mole fraction with respect to DMPM substrate caused noticeable activation. Also as shown in this figure (curve c), the reaction initiated in the presence of Px showed a completely different time course. In both cases (curves b and c), in the presence of 1 μM Px all of the available substrate present in the reaction mixture was eventually hydrolyzed. Control experiments (not shown) demonstrated that the Px-mediated release of protons was only due to PLA2 catalyzed hydrolysis. For example, the reaction in the presence of Px was inhibited by specific competitive inhibitors of PLA2 (Jain et al, 1991a, 1989). Furthermore, Px alone did not promote the hydrolysis of DMPM vesicles, nor did it act as a substrate or replace calcium as a cofactor for catalysis. The possibility that Px somehow shifted the position of the thermodynamic equilibrium for the reaction was discounted on the basis of the fact that the PLA2-catalyzed reaction proceeds to completion, as shown by the isotope-exchange method (Ghomaschi et al., 1991a).

The activating behavior of Px was observed with PLA2s from several sources (Table I). In these studies, the activating effect of Px was characterized by measuring the initial rates of hydrolysis of DMPM vesicles, as approximated by drawing a tangent near the beginning of the progress curve. Although this was an approximation, the procedure has been commonly used for expressing specific activities for characterization of activators (Clark et al., 1987; Bomalaski et al., 1989). The initial rates in Table I were expressed as the turnover number, i.e., the velocity per enzyme molecule. This was validated by the fact that under the conditions reported in this paper, essentially all enzymes were bound to the interface. In the absence of Px and at low calcium (0.5 mM), the observed initial turnover numbers (tangent at zero time) were designated

Table I: Initial Rates of Hydrolysis per Enzyme Molecule (Turnover Numbers) with DMPM Vesicles in the Presence of Different Additives and PLA2s from Different Sources[a]

	initial turnover number (s^{-1})		
enzyme source	$N_s k_i$ at 0.5 mM $CaCl_2$	v_0 with 0.5 mM $CaCl_2$ + 5 μg/mL Px	v_0 with 2.5 mM $CaCl_2$
pig pancreas	40	240	280
iso-pig pancreas	28	160	140
Y69F-pig pancreas	17	20	20
pro-pig pancreas		1.1	1.1
bovine pancreas	38	280	300
Crotalus atrox	15	45	55
Agkistrodon halys blom. (basic)	16	30	30
Naja melanoluca (DEI)	12	12	15
Naja melanoluca (DEII)	11	45	50
Naja melanoluca (DEIII)	14	28	30

[a] The sources of these enzymes and the corresponding isozymes are given in Verheij et al. (1981).

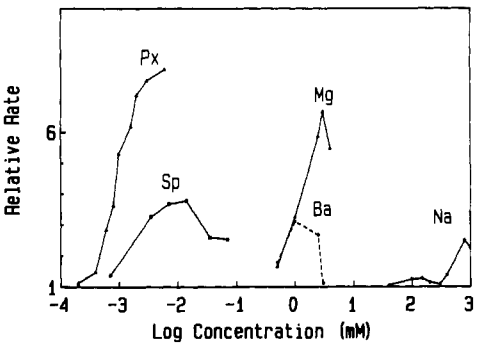


FIGURE 2: Effect of the concentration of activators on the initial rate of hydrolysis of DMPM vesicles relative to that in the absence of activators at pH 8.0 and 22 °C in the presence of 0.5 mM $CaCl_2$. From left the curves are for Px, spermine, barium chloride, magnesium chloride, and sodium chloride.

in Table I as $N_s k_i$, whereas in the presence of Px or at higher calcium (2.5 mM), the initial turnover numbers were expressed as v_0. The meaning of these parameters will be developed under Results. In all studies listed in Table I, virtually all of the substrate was hydrolyzed in the presence of Px under the conditions used for experiments for curves b and c in Figure 1. With the pig PLA2, a 7-fold activation of the initial rate was observed, whereas the activation was less than 1.3-fold with the Y69F mutant (Table I). In addition, as shown in Table I, the degree of activation by Px ranged from 1- to 8-fold depending on the source of the PLA2. Taken together these facts suggested that the activation by Px could be due to a specific interaction of this cyclic peptide with the enzyme.

Activating Effects of Other Cations. Since Px is positively charged, its binding to a putative cation-binding site on PLA2 was considered. The possibility that Px bound to the primary calcium-binding site in PLA2 was discounted on the basis of the observation that activation by Px was not seen in the absence of calcium. This raised the possibility of the presence of a second cation-binding site on the enzyme. In order to understand the specificity for this putative second cation-binding site, the effect of several cations was investigated. For example, the initial rate increased in the presence of varying concentrations of spermine, calcium, magnesium, and barium (Figure 2, Table I). On the basis of the concentrations of activators, the specificity for this site could be in the order Px > spermine > calcium > barium and magnesium > sodium. The apparent decrease in the rates at higher concentrations of magnesium and barium (Figure 2) could be rationalized

on the consideration that these cations competed for the binding of the first calcium to the catalytic site of PLA2. It was also interesting to note that the mutant Y69F was not activated by any of these multivalent ions although the effect of calcium on this mutant was virtually the same as with the wild-type enzyme.

Lack of Evidence for a Direct Px–Enzyme Interaction. Many of these results suggested a specific interaction of multivalent cations with PLA2. Although seductive, this hypothesis was discarded on the basis of numerous observation summarized below. First, Px did not influence the kinetics of alkylation of His-48 on PLA2 in the presence or absence of calcium (Jain et al., 1991a). This indicated that if Px bound to PLA2, such binding did not occur at the catalytic site nor did it modulate the accessibility of the active site histidine toward the alkylating agent. Second, Px did not influence the inhibition constant for several competitive inhibitors of PLA2 (Jain et al, 1991a) obtained by the kinetic method or the protection from alkylation studies (Jain et al., 1991a). Third, Px induced activation was not seen with several structurally different (Jain et al., 1986c; Jain & Rogers, 1989) or organizationally different forms of substrates. For example, the activating effect of Px was not observed with micelles of dioctanoyl-*sn*-glycerol-3-phosphomethanol, or -phosphocholine, or with phospholipids dispersed in detergents like deoxycholate, or with covesicles of dioleoyl-*sn-glycero*-3-phosphate with 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine. Fourth, the activating effect of Px was negligible on the initial rate of hydrolysis of large vesicles, which contrasts the results seen in Figure 1b,c where small sonicated vesicles were used. Fifth, Px did not cause any noticeable difference in the fluorescence and absorption spectral properties of PLA2 in the aqueous phase or when bound to vesicles of the nonhydrolyzable phospholipid DTPM. Sixth, the Px concentration dependence of activation of PLA2 from different sources (analogous to the results shown in Figure 1) was always the same, i.e., activation at 1 μM Px, whereas PLA2s from different sources were activated to different extents (Table I). This suggested either that the affinity of Px for the putative second cation-binding site did not change for PLA2s from different sources or that yet another process related to the effect of Px on the interface was somehow responsible for the activating effect.

Additional observations failed to reveal a specific interaction of Px with PLA2. The elution profile of the pig PLA2 on gel-filtration, reverse-phase, and ion-exchange columns was not altered in the presence of Px. As detailed in a previous paper in this series (Jain et al., 1991a), it was possible to measure the equilibrium dissociation constants for the enzyme in the interface interacting with calcium, substrate, products, or inhibitors, (K_{Ca}, K_S, K_P, and K_I). None of these constants were altered in the presence of Px. In addition, the possibility that Px somehow increased K_P was ruled out on the basis of the observation that the initial rate of hydrolysis of vesicles of DMPM containing 40 mol % products of hydrolysis did not show any increase in the presence of Px. Finally, time-dependent modification of PLA2 by Px was ruled out by the observation that preincubation of the enzyme with concentrations of Px up to 10 μM in the aqueous phase did not result in the activation when the mixture was diluted into an assay mixture with DMPM vesicles so that the final concentration of Px was less than 0.005 μM. Taken together, these results ruled out any direct covalent or noncovalent interaction of Px with E, E*, E*Ca, E*S, and E*P.

Effect of Px on the E to E Equilibrium.* In the presence of Px, all of the DMPM substrate in the reaction became hydrolyzed. This would occur if Px promoted the exchange of enzyme between vesicles. The binding of PLA2 to DMPM vesicles was extremely tight with a dissociation constant of less than 0.1 pM (Jain & Berg, 1989). Thus it was unlikely that Px significantly altered the fraction of enzyme bound to the interface. Indeed, the presence of Px did not produce any detectable change in the binding equilibrium for PLA2 to DMPM or DTPM vesicles as monitored by fluorescence emission from Trp-3 (Jain et al., 1986b) or by the resonance energy transfer method (Jain & Vaz, 1987).

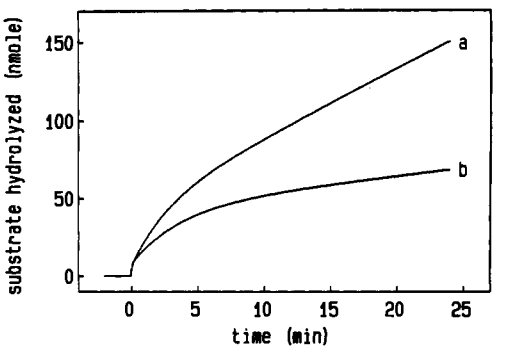


FIGURE 3: Reaction progress curves for the hydrolysis of DMPM vesicles in the presence of 0.3 M NaCl (curve a) and in the absence of salt (curve b). DMPM concentration 0.3 mM; with 10 pmol of PLA2 added at $t = 0$. The reaction mixture (4 mL) contained 0.5 mM $CaCl_2$ at pH 8.0 and 22 °C. These curves were fitted to eq 10 in Jain and Berg (1989) to obtain values of k_d.

It was still possible that Px changed the rate constants for absorption and desorption of the enzyme from the interface by the same factor. If the rate of desorption was increased, this could lead to an increased rate of intervesicle exchange of PLA2 and the resulting hydrolysis of all of the DMPM in the reaction mixture. This was studied further by a comparison of the effects of Px to those induced by high concentrations of NaCl. It has been previously shown that the intervesicle exchange of enzyme is promoted by salts such as NaCl at concentrations greater than about 0.1 M (Jain et al., 1986b). For example, as shown in Figure 3a, in the presence of 0.3 M NaCl, the reaction progress curve for the action of PLA2 on small DMPM vesicles was not of the first-order type seen in the absence of salt (Figure 3b). Rather the product formation continued until all of the substrate in the outer monolayer of the vesicles became hydrolyzed. The curve in Figure 3a was fitted to the equation derived from a kinetic model in which the enzyme underwent intervesicle exchange [eq 10 in Jain and Berg (1989)].

$$\frac{P_t}{P_{max}} = \left[\frac{k_i}{k_i + k_d}\right]\left[k_d t + \frac{k_i}{k_i + k_d}[1 - \exp(-k_i t - k_d t)]\right] \quad (10)$$

$$\text{where } k_i = \frac{k_{cat}}{K_{MS}(1 + 1/K_p)N_s}$$

Here, P_t and P_{max} are the amounts of product formed at time t and infinity, respectively. The rate constant for the dissociation of PLA2 from the vesicle is k_d. The constant k_i is the first-order relaxation rate that describes the exponential reaction progress curve (Figure 3b) and is a function of the maximal velocity per enzyme molecule k_{cat}, the interfacial Michaelis constant K_{MS}, the product dissociation constant K_p, and the number of phospholipids in the outer monolayer of the vesicles N_s. A detailed description of these parameters is given in the Appendix to Berg et al. (1991). In the present study, the values of N_s and k_i were obtained from the first-

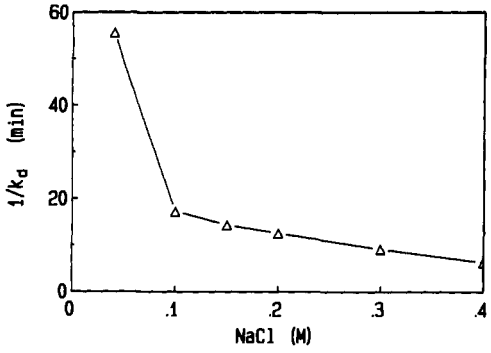


FIGURE 4: Values of dissociation rate constants (k_d) for the dissociation of E* from DMPM vesicles in the presence of varying concentrations of NaCl. The values of k_d were obtained from the reaction progress curves of type shown in Figure 3. See Jain and Berg (1989) for the theoretical details.

order reaction progress curve in the absence of salt (Figure 3b). The whole reaction progress curve in the presence of high salt was fitted to this eq 10 to obtain the value of the dissociation rate constant, k_d, for PLA2 bound to DMPM vesicles. As summarized in figure 4, the value of k_d calculated from such curves increased with increasing concentrations of NaCl.

A comparison of the reaction progress curve in the presence of Px vs NaCl revealed several significant differences. For example, the initial velocity did not change in the presence of NaCl (Figure 3), whereas it increased considerably in the presence of Px (Figure 1b,c). Moreover, the reaction progress curves in the presence of Px even at 0.0005 mol fraction (Figure 1b,c) had a different shape than that seen in the presence of NaCl (Figure 3a). Finally, in the presence of Px, all of the substrate in the reaction mixture was hydrolyzed, whereas with NaCl about 60% was hydrolyzed as the enzymes hopped from vesicle to vesicle. These results provided strong evidence that the effect of Px on interfacial catalysis was not due to a change in the rate of enzyme desorption from the vesicles as predicted by eq 10 above.

Effect of Px on the Substrate Interface. The bulk of the evidence was not in support of a specific interaction of Px with PLA2. In addition, Px did not promote the intervesicle exchange of the enzyme. Several additional experiments to be described next led to the conclusion that the activation by Px was due to its effect on the substrate interface. In particular, the fact that all of the substrate became hydrolyzed in the presence of Px suggested that this peptide somehow increased the amount of substrate that the enzyme "sees" at the vesicle interface. This would be accomplished if Px promoted the intervesicle exchange of lipids either by fusion of vesicles or by other processes.

A key observation that provided the insight into the mode of activation by Px was that the degree of activation was markedly reduced when larger DMPM vesicles were being hydrolyzed. The increase in the initial velocity by 1 μM Px was less than 2-fold for the hydrolysis of large vesicles (N_s = 100000) (not shown) compared to the more than 7-fold effect seen with small vesicles (N_s = 5000) shown in Figure 1. It should be noted that in the absence of Px, the initial velocities observed with large vesicles were always higher than that measured in small vesicles (Berg et al., 1991). This point will be discussed further below. Together these results suggest that the increase in the turnover number with the size of vesicles and the activation by Px seen with small vesicles have a common basis. As elaborated below, such a behavior is a direct consequence of the action of enzymes in heterogeneous systems consisting of an ensemble of particles that are not exchanging their components with each other.

As described in detail in our previous work (Berg et al., 1991), the first-order shape of the reaction progress curve for the hydrolysis of small vesicles (Figures 1a and 3b) was due to the rapid depletion of the amount of substrate that the enzyme "sees" which in turn caused a rapid drop in the steady-state concentration of E*S and the velocity of the reaction. In large vesicles, the decrease in the mole fraction of substrate necessarily occurred relatively slowly, and the first-order nature of the progress curve did not become apparent until much of the substrate had been hydrolyzed. This was quantitatively addressed in the Appendix to Berg et al. (1991) and is elaborated below.

The reaction progress curves for the hydrolysis of vesicles of all sizes containing at most one irreversibly bound enzyme is described by eq A12 [Appendix to Berg et al. (1991)][2]

$$k_i t = -\ln\left(1 - \frac{P_t}{P_{max}}\right) + \left(\frac{k_i N_s}{v_0} - 1\right)\frac{P_t}{P_{max}} \quad \text{(A12)}$$

This equation is the standard integrated Michaelis–Menten equation adopted for interfacial catalysis in the scooting mode. Here, v_0 is the initial turnover number at mole fraction substrate equal to 1. The other terms have been defined above for eq 10. Equation A12 consists of a first-order term (logarithmic in P_t/P_{max}) and a zero-order term (linear in P_t/P_{max}). The shape of the reaction progress curve will depend on the relative contribution of the two terms in eq A12. For vesicles of all sizes, the zero-order term will always dominate at reaction times close to zero. In addition, since the coefficient of the zero-order term is uniquely dependent on N_s, the time over which this term dominates increases with the size of the vesicles. It is important to note that in vesicles of all sizes, the initial velocity per enzyme molecule at mole fraction substrate 1, v_0, at the earliest reaction times was constant; however, the period of time over which the slope of the reaction progress curve, v_t, remained constant depended on N_s. This was predicted by eq A18 of the Appendix to Berg at al. (1991)

$$t_d = \frac{2k_i N_s^2}{v_0^2}\delta \quad \text{(A18)}$$

As the reaction progresses, the slope of the progress curve, v_t, will continuously decrease from its initial value, v_0. The term δ in eq A18 is defined as $\delta = (v_0 - v_t)/v_0$, that is, δ is the relative error that would result if the measured slope at any point along the reaction progress curve, v_t, was approximated as v_0. Using equation A18 and the values of the parameters determined in Berg et al. (1991), it was possible to predict the time over which δ was small. For example, in large vesicles with N_s = 100000, the relative error δ would be less than 50% for reaction times up to about 1 min. In contrast, with small vesicles with N_s = 4000, a 50% error would occur in about 2 s. This was indeed the case for the reaction progress curves shown in Figures 1a and 3b. This analysis underscored a fundamental difference between enzymatic reactions in homogeneous solutions from those that occur on phospholipid interfaces. In the system under present investigation, the enzymatic reaction occurred in a closed system containing a relatively small number of substrates in which the enzyme, substrate, and products did not exchange with excess vesicles. The effects of rapid substrate depletion will be even more severe in smaller aggregates such as micelles, where N_s is typically less than 100. These ideas led to the important conclusion that the observed

[2] Equations numbered with the prefix "A" are numbered identically with those in the Appendix to Berg et al. (1991).

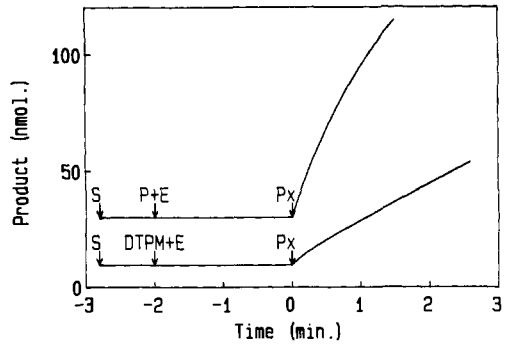


FIGURE 5: Initial portion of the reaction progress curves for the hydrolysis of DMPM vesicles. The sequence of addition was (top) 0.3 mM DMPM vesicles, followed by (after 1 min) vesicles of the products (2 μM) containing 5.3 pmole PLA2 at P + E, and then 1 μM Px after 3 min. (Bottom) Same as above except that PLA was added with DTPM (2 μM final concentration) vesicles. Other conditions are as described in the legend to Figure 2.

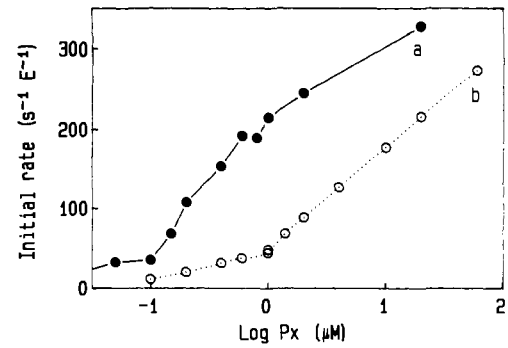


FIGURE 6: Initial rates of hydrolysis per enzyme for the hydrolysis of DMPM vesicles under the exchange conditions of type shown in Figure 5. (Solid line) PLA2 was added to the product vesicles; (dotted line) PLA2 was added to DTPM vesicles.

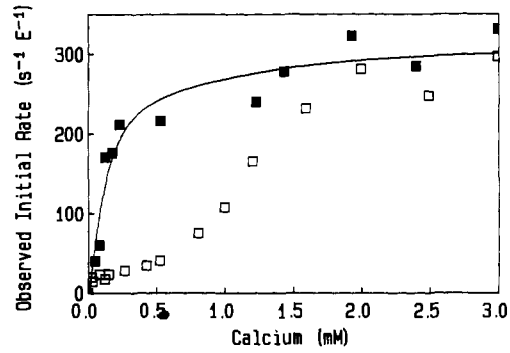


FIGURE 7: Dependence of the observed rate of hydrolysis per enzyme molecule of DMPM vesicles by PLA2 (1–20 pmol depending on the magnitude of the initial rate) at varying concentration of free calcium in the reaction mixture in the absence (open squares) and in the presence (filled squares) of 1 μM Px. The theoretical curve is drawn with the assumptions used to fit the v_0 data in Figure 7 in Berg et al. (1991).

reaction velocities for PLA2-catalyzed lipolysis can be significantly modulated by the size of the vesicles and the rate of substrate replenishment within the aggregates.

On the basis of the above considerations, a likely explanation for the apparent activation in the hydrolysis of small vesicles by Px is that this peptide somehow increased the rate of substrate replenishment so that the initial mole fraction of substrate, equal to 1, was maintained for a longer period of time, thus leading to an apparent activation of PLA2. This idea was consistent with all of the observations described so far which suggested that Px did not bind specifically to PLA2.

An effect of Px on the substrate replenishment at the interface was also suggested from the results shown in Figure 5. In this experiment, the enzyme bound to vesicles composed of reaction products, i.e., a 1:1 mixture of myristate and 1-myristoyl-*sn*-glycero-3-phosphomethanol (top curve, Figure 5) or to vesicles of the nonhydrolyzable substrate analogue DTPM (bottom curve, Figure 5) was able to transfer to DMPM substrate vesicles only in the presence of Px. Under these conditions the reaction started immediately after the addition of Px, and all of the available substrate was hydrolyzed. This simple experiment proved that Px promoted the exchange of substrate from other vesicles to vesicles containing bound enzyme. Px added to one of the two components or Px added to the mixture of the two populations of the components gave essentially identical results, which suggested that Px or the substrate and product molecules were readily exchangeable in the presence of Px. Moreover, on the basis of these experiments, the activating effect of Px could not be ascribed solely to the increased transbilayer movement of the substrate as discussed later.

The initial slope of the reaction progress curve after the addition of Px (Figure 5) was measured as a function of the Px concentration, and the results are presented in Figure 6. In these series of experiments, the enzyme was bound initially to DTPM vesicles or to vesicles composed of the products of the DMPM hydrolysis. However, the same rate was observed with product-containing vesicles at lower Px concentration than with DTPM vesicles. These observations accounted for the difference in the reaction velocities seen in the two curves in Figure 5. These experiments demonstrated that the rate of transfer of phospholipids between vesicles induced by Px was dependent on the concentration of Px as well as on the nature of the lipids undergoing exchange. The results in Figure 5 and 6 were consistent with the conclusion that Px promoted the intervesicle transfer of substrates and products rather than the exchange of enzyme between vesicles.

One of the mechanisms for the increased rate of transfer of lipids between vesicles is fusion of vesicles. Indeed, Px has been reported to induce a variety of changes in the organization of anionic bilayers (Kubesch et al., 1987) including fusion of vesicles (Gad & Eytan, 1983). In previous work, we have shown that concentrations of calcium above 1 mM promoted the rapid fusion of DMPM vesicles (Jain et al., 1986a). It was shown in this previous study that higher concentrations of calcium led to an increase in the observed initial velocity for the hydrolysis of small DMPM vesicles that was remarkably similar to the effect seen with Px (Figure 1). These results warranted a comparison of the effects of Px and high calcium concentrations on the PLA2-catalyzed hydrolysis of small DMPM vesicles. As shown in Figure 7, the measured initial velocity per enzyme molecule with small DMPM vesicles in the absence of Px (open squares) showed a sigmoidal dependence on the concentration of calcium. The initial turnover number increased sharply as the concentration of calcium approached 1 mM (open squares, Figure 7) where rapid fusion of DMPM vesicles was also occurring (Jain et al., 1986a; Berg et al, 1991). In the presence of 1 μM Px (closed squares, Figure 7), the initial rate of hydrolysis increased in a hyperbolic fashion as the calcium concentration was increased. Several features of these results may be noted. First, the calcium dependency of the initial turnover number in the presence of Px was identical with the calcium-dependency measured on large vesicles in the absence of Px (Berg et al., 1991). In both cases, a hyperbolic pattern was seen and was characterized by an apparent dissociation constant for calcium of 0.22 mM and a maximal rate of 320 s^{-1}. As already documented in Berg et al. (1991), the effect of calcium on the hydrolysis of DMPM

vesicles was twofold. It was an essential cofactor for catalytic turnover by PLA2 and at concentrations of above 1 mM it promoted the fusion of small DMPM vesicles. Second, the observed initial velocities in the presence and absence of Px at saturating calcium concentrations were the same, 320 s^{-1} (Figure 7). Third, as seen in Figure 7, the activating effect of Px was seen only in the presence of low concentrations of calcium. For example, at 0.5 mM calcium, before the onset of calcium-promoted fusion, Px induced a 7-fold activation in the hydrolysis of small DMPM vesicles. In contrast, no activation by Px was observed at 2 mM calcium. All of these results can now be quantitatively explained in terms of the formalism developed for the action of PLA2 in the scooting mode [Appendix to Berg et al. (1991)].

On the basis of eq A12, the following conclusions were drawn. It has already been discussed above that, in small vesicles, the reaction progress curve was first-order. This is described by eq A13, which is derived from eq A12 by setting the zero-order term equal to zero [Appendix to Berg et al. (1991a)].

$$P_t = P_{max}[1 - \exp(-k_i t)] \qquad \text{(A13)}$$

Recall that in small vesicles, the true value of the velocity per enzyme at mole fraction of substrate 1, v_0, was only observable for a few seconds (eq A18). Thus, the experimentally observed initial velocity per enzyme in small vesicles will not be v_0 but rather the initial slope of the first-order reaction progress curve divided by the enzyme concentration C_E. This is given by the value of the derivative of eq A13 with respect to time at times near zero divided by the enzyme concentration.

$$\left(\frac{1}{C_E}\right)\left(\frac{dP_t}{dt}\right)_{t=0} = \left[\frac{d(N_s[1 - \exp(-k_i t)])}{dt}\right]_{t=0} = N_s k_i$$

The above equation is valid under the conditions used in the present study, namely with at most one enzyme per vesicle such that P_{max} is given by $C_E N_S$. Thus, the initial velocity per enzyme molecule in small vesicles is $N_s k_i$. This derivation provides the rationale for the use of $N_s k_i$ in Table I for the data in the absence of Px. These results account for the changes in the observed initial velocity induced by calcium in the absence of Px (open squares, Figure 7). As the calcium concentration approaches 1 mM and the vesicles fuse, the initial velocity should change from its value in small vesicles, $N_s k_i$, to its value in large vesicles, v_0. Thus, this analysis predicted that the $v_0/N_s k_i$ ratio should be 7.5 from the data in Figure 7. In the presence of Px, the replenishment of substrate was so rapid that the intitial velocity was close to v_0 for minutes rather than to $N_s k_i$ regardless of whether the reaction was initiated on small or large DMPM vesicles. Now the effect of calcium in the presence of Px (closed squares in Figure 7) was predicted to be the same as the effect of this cofactor on the hydrolysis of large vesicles in the absence of Px, namely, as an essential enzyme cofactor for stabilizing the transition state form of the substrate (Berg et al., 1991; Jain et al., 1991a). Finally, from this analysis, it is clear why the initial turnover numbers in the presence of Px or high calcium were expressed as v_0 in Table I.

Thus, the dual effect of calcium can be discerned and summarized in the following way. In the presence of Px, the effect of calcium was only on the catalytic efficiency of the enzyme as an essential cofactor (closed squares, Figure 7). In the absence of Px, the effect of calcium was twofold. For small vesicles where the initial velocity was $N_s k_i$, it was entirely consistent with its role as an essential cofactor as documented previously [see the plot of $N_s k_i$ vs calcium, Figure 7 in Berg et al. (1991)]. As shown in Figure 7 (open squares), above 1 mM calcium and in the absence of Px, the additional increase in the initial velocity was due to the calcium-dependent increase in the size of the vesicles where the initial rates were given by v_0 rather than $N_s k_i$.

Perhaps, the strongest prediction of the model where Px functions as an activator by promoting substrate replenishment is that the ratio of initial velocities in the presence and absence of Px at low calcium (Figure 7) is postulated to be $v_0/N_s k_i$. Fortunately, this ratio has been independently obtained from eq A15 [Appendix to Berg et al. (1991)]

$$\frac{v_0}{N_s k_i} = \frac{1 + 1/K_P}{1 + 1/K_{MS}} \qquad \text{(A15)}$$

The values of $K_{MS} = 0.3$ and $K_P = 0.025$ have been determined (Jain et al., 1991a), and thus a value of the $v_0/N_s k_i$ ratio of 9.5 can be obtained by this independent method. The correspondence in this ratio obtained from equilibrium data (Jain et al., 1991b) and kinetic data (Figure 7) was gratifying.

With the above analysis, the effect of Px on the Y69F pig PLA2 mutant as well as other PLA2s listed in Table I can now be understood. The overall trend in the data is that the activation by Px or high calcium was much less pronounced for enzymes that have a $v_0/N_s k_i$ close to unity. For example, the $v_0/N_s k_i$ ratio for the Y69F mutant is 1.2, much less than the value of 7 for the wild-type pig PLA2. According to eq A15, this difference is presumably due to the altered values of K_P and K_{MS} for the mutant in comparison to the wild-type enzyme. As discussed above, the ratio of observed initial velocities in the presence and absence of Px is also given by the $v_0/N_s k_i$ ratio. Thus, Px functions as an activator only for enzymes that display a large value of this ratio.

Discussion

The broad significance of PLA2 activators in the regulation of the eicosanoid pathway is the same as that of inhibitors (Bromalaski et al., 1989; Clark et al., 1987; Elsbach & Weiss, 1988; Vold et al., 1988; Conricode & Ochs, 1989). Also the problems encountered in the study of inhibitors of interfacial catalysis (Jain & Jahagirdar, 1985; Jain et al., 1989, 1991a) are inherent in the study of PLA2 activators. In this and previous studies of PLA2 in the scooting mode, we have emphasized two fundamental considerations of importance for the interpretation of the kinetic data for interfacial catalysis. These are the fraction of enzyme at the interface and the mole fraction of the substrate that E* "sees" during the catalytic cycle for the formation of the E*S complex. While this is a restatement of the obvious, in most PLA2 assay systems, it has not been possible to take this into consideration explicitly. Thus the theory developed in the Appendix to Berg et al. (1991) has substantial bearing on virtually all aspects of interfacial catalysis. Many of the problems that are commonly encountered can be readily resolved during catalysis in the scooting mode, and the protocols developed in this paper could be useful for screening specific activators and for establishing the nonspecific activating effects of still others.

In describing our experimental results with Px, we took a rather circuitous route to pose key questions related to the possible modes of activation and to illustrate the protocols that can be used to identify an apparent activator. We believe that such protocols could eventually lead to the discovery of true activators of PLA2, that is, compounds that bind directly to the enzyme and increase its catalytic efficiency. The apparent activation of PLA2-catalyzed hydrolysis by Px is an intriguing phenomenon, and its characteristics are recapitulated here. Many of the observations on the activation by Px such as the

dependency on the source of enzyme could be interpreted by postulating a specific cation-binding site on PLA2 that interacts with Px and other cations. However, the cumulative weight of the evidence led to the conclusion that the activation by Px was due to its effect on the substrate interface, more specifically, Px was found to promote the process of substrate replenishment in enzyme-containing vesicles. This mode of activator function should be a general phenomenon with enzymes that operate on interfaces.

The mechanism by which Px promoted the transfer of lipids between vesicles is not fully understood. It can, however, be ruled out at the outset that Px "solubilizes" or micellizes DMPM vesicles. For example, the 90° light scattering of DMPM vesicles did not change in the presence of up to 0.05 mol fraction of Px. This showed that the vesicles size did not change in the presence of concentrations of Px in excess of that needed to activate the interfacial catalysis (data not shown). This is consistent with the published results on the effect of Px and other cationic peptides on anionic (phosphatidylglycerol and phosphatidic acid) vesicles investigated by a variety of techniques including freeze–fracture electron microscopy and differential scanning calorimetry (Sixl & Galla, 1982; Gad & Eytan, 1983; Kubesch et al., 1987; Boggs et al., 1989; Bondeson & Sundler, 1990). In none of these studies was there any indication of the "solubilization" of phospholipids by Px, even at 0.2 mol fraction. For example, the particle morphology was maintained, and the gel–fluid transition was somewhat altered but retained. These observations would not be possible if mixed micelles of Px were formed with phospholipids.

Px could be functioning as a potent fusogen as reported in previous studies (Gad & Eytan, 1983). Indeed, activation by Px shared a number of features that were also seen with high concentrations of cationic metals such as calcium, barium, and magnesium (Table I, Figure 2). These latter agents are well known to promote the exchange of phospholipids between vesicles by fusion (Jain et al., 1986a). However, the fusion induced by Px displayed some unique features. For example, fusion was observed at extremely low concentrations of Px compared to the metal ions. Second, the Px-induced fusion, unlike that promoted by metal ions, was "leaky" in the sense that the contents of internal aqueous compartment of the vesicles was discharged into the bulk medium. This was observed by examining the release of the trapped fluorescent dye calcein from vesicles following the addition of Px (data not shown). Third, in previous studies on interfacial catalysis by PLA2 on vesicles that have been fused in the presence of calcium, only the phospholipid in the outer monolayer of the vesicles became hydrolyzed, i.e., the enzymatic reaction ceased when roughly 50% of the total substrate became hydrolyzed (Jain et al., 1986a). In contrast, the results of the present study showed that Px induced the transbilayer exchange of phospholipids as well as the intervesicle exchange since essentially all of the substrate in the reaction mixture was hydrolyzed. Although the detailed mechanism for the Px-mediated transfer of substrate and products remains to be established, many interesting hypotheses have been proposed for the effect of this peptide on bilayers (Gad & Eyton, 1983; Sixl & Galla, 1982; Kubesch et al., 1987; Boggs et al., 1989). In the present case, the situation is further complicated by the fact that the dynamics of phospholipids in vesicles are influenced by the presence of calcium, the products of the PLA2 hydrolysis, and Px, and their effects could be synergistic [for example, see Hong and Vacquier (1986) and Pollard et al. (1990)]. The activating effects of Px observed in the present study were consistent with the results recently reported on the intermixing of lipids induced by polycationic peptides (Bondeson & Sundler, 1990). In that study it was shown that oligomers and polymers of basic amino acids such as lysine and arginine induced the intervesicle transfer of phospholipids between vesicles. Peptide-induced transfer was most pronounced in vesicles containing anionic phospholipids and extensive intervesicle mixing with incomplete fusion was seen. In addition, considerable leakage of the vesicle contents was observed in the process. All of these results were seen in the present study with Px and lend further support for the conclusion that Px activated interfacial catalysis by promoting substrate replenishment.

Perhaps the most significant implication of the present study comes from the fact that, in closed systems such as vesicles or micelles, the rate of the enzymatic reaction can be limited by the rate of replenishment of the substrate in the enzyme containing particles. Although this is intuitively obvious, such effects have never been explicitly addressed or theoretically accounted for in previous studies on the kinetics of interfacial catalysis. In the present study, the effects of substrate replenishment have been extensively characterized. The theoretical basis of the effect lies in the basic Michaelis–Menten formalism adopted for the action of an enzyme in closed systems [Appendix to Berg et al. (1991)].

The procedures described here emphasize the role of the rate of substrate replenishment on the rate of hydrolysis by PLA2. In particular, the initial enzymatic velocities per enzyme can be expressed as v_0 or $N_s k_i$. Although both are initial velocities, in the context of interfacial catalysis they have very different significance, and of course the underlying difference would be blurred in single point enzymatic assays. The velocity v_0 is the initial rate per enzyme under the conditions where little or no product is present, i.e., the conditions where the substrate is being rapidly replenished in the interface. Under the conditions where this can be measured directly, Px, higher calcium concentrations, magnesium, or barium had little activating effect. This demonstrated that the activating effects under consideration were not due to the steps intrinsically involved in the catalytic cycle (steps within the box of Scheme I). The effects reported here were predominantly observed under the conditions where the reaction had progressed to some extent in large vesicles or at early times in small vesicles. Under these conditions, the logarithmic term begins to contribute to the initial rate of hydrolysis (eq A12), and the initial rate was approximated by $N_s k_i$. Therefore, depending on the local concentration of the products, the initial rate (tangent at $t = 0$) would change. If the rate of replenishment was rapid, the enzymatic rate would be closer to v_0 and if the replenishment does not occur, the initial rate would be closer to the theoretical $N_s k_i$ value. In order to minimize the effect of the rate of replenishment, we obtained the values of $N_s k_i$ from an analysis of the entire reaction progress curve under conditions where the fusion of vesicles did not occur, that is, in the presence of calcium concentrations up to 2.5 mM (Berg et al., 1991). This analysis was not possible in the presence of Px because all of the substrate in the reaction mixture was hydrolyzed.

In the enzymatic turnover on smaller substrate aggregates such as micelles or detergent–phospholipid mixed micelles, the consequence of substrate replenishment will be even more influential than in larger aggregates such as vesicles. For example, in micelles of size $N_s = 100$, in the absence of any substrate replenishment, the enzymatic velocity will deviate 50% from its initial value, v_0, after only 50 ms (eq A18). *Thus it is clear that unless the rate of substrate replenishment in micelles is fast on the 50-ms time scale, the overall enzymatic*

velocity would be more a reflection of the rate of substrate replenishment than of the intrinsic catalytic efficiency of the PLA2. In all previous studies on the kinetics of action of PLA2 on micelles and mixed micelles, it has been implicitly assumed that the exchange of lipids occurs sufficiently rapidly so as not to influence the steady-state velocities. Recent studies by Nichols and co-workers call this assumption into question (Fullington et al., 1990). In these studies the halftimes for exchange of a variety of different long-chain phospholipids in mixed micelles fell into the range of 0.2–1000 s, which is at least an order of magnitude slower than the rate of substrate replenishment required to avoid significant complications in kinetic studies with mixed micelles.

Yet another mechanism for the replenishment of substrate in micelles is that the enzyme hops rapidly among the ensemble of substrate particles. In this way, the enzyme is not significantly depleting the substrate in a single aggregate. Here again, the exchange of enzyme must be rapid on the same 50-ms time scale. More specifically, for the enzymatic turnover to be free of distortions resulting from substrate depletion, the residency time of the enzyme on the micelle must be less than 50 ms. The residency time is given by the reciprocal of the rate constant for the desorption of E* from the micelle interface, $1/k_d$. Therefore, the value of k_d should be larger than 20 s^{-1}. Although values of k_d have never been directly measured (however, see Figure 4), their values can be estimated from a previous study of the rates of attainment of the E to E* equilibrium (Jain et al., 1988). In that study, the observed rate constant for the approach to equilibrium for the E to E* step, which is the sum of the rate constants for absorption and desorption steps, was found to have a value of $\tau = 4$ s^{-1} (Jain et al., 1988). Since the experimentally determined values of τ were found to be invariant to the nature of the interface, vesicles or micelles composed of a number of different phospholipids yielded similar τ values, the value of k_d cannot exceed 4 s^{-1}. Thus, the assumption of rapid substrate replenishment in micelles, in this case by enzyme exchange, is probably not valid. Although further studies are needed, these ideas strongly suggest the possibility that the rate of substrate replenishment will blur the analysis of the numerous features of interfacial catalysis in small closed systems and that this distortion will be particularly acute in small particles such as micelles. As discussed in this and previous papers, by analyzing PLA2 in vesicles in the scooting mode under the conditions where the enzyme and lipids do not exchange with other vesicles, it has been possible to examine issues such as substrate specificity (Ghomashchi et al., 1991b), inhibition (Jain & Gelb, 1989; Jain et al., 1991a), and activation and to derive information that has the usual mechanistic meaning.

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Registry No. Px, 1404-26-8; PLA2, 9001-84-7; DMPM, 134005-75-7; NaCl, 7647-14-5; Ca, 7440-70-2; dimyristoylphosphatidylmethanol, 60569-01-9; myristic acid, 544-63-8.

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