Interfacial Catalysis by Phospholipase A_2: The Rate-Limiting Step for Enzymatic Turnover†

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ABSTRACT: The kinetics of the phospholipase A_2-catalyzed hydrolysis of bilayer vesicles and mixed micelles of several oxyglycero and thioglycero analogues of phospholipids have been studied. The results with vesicles show that, depending on the source of the enzyme, the rates of hydrolysis of the oxy-containing long-chain phosphatidylmethanols are 2.5- to 28-fold higher compared to the rates of hydrolysis of the analogous thio substrates. The oxygen to sulfur substitution does not significantly alter the affinities of the enzymes for the reaction products or calcium. Since it is unlikely that sulfur substitution changes the rate constants for the formation and dissociation of the enzyme–product complex by the same factor, the element effects seen in the rates of hydrolysis of the oxy- and thioester phospholipids in vesicles are primarily due to a change in the rate constant for the chemical step of the catalytic turnover cycle. For bovine pancreatic phospholipase A_2, various mutants with lower catalytic activity were used to show that the value of the element effect does not increase in the mutants. These results establish that, for the pancreatic phospholipase A_2, the element effect is fully expressed, and the chemical step is fully rate-limiting for both oxyglycero and thioglycero phospholipids in vesicles. It was found that the element effect decreases from 7 to 1 when long-chain phosphatidylmethanols are present in micelles of a neutral diluent. This result suggests that the chemical step is not rate-limiting during the hydrolysis of these mixed micelle substrates.

One of the main problems in the understanding of the mechanism of interfacial catalysis is that the catalytic rates obtained in different assay systems have different meanings since they are composites of different steps. Recently protocols have been developed to determine the values of most of the interfacial equilibrium and rate parameters for the catalytic turnover cycle by phospholipase A_2 (PLA2)[1] on phospholipid vesicles in the scooting mode (Berg et al., 1991; Jain et al., 1991a) according to the scheme shown in Figure 1. During catalysis in the scooting mode, the enzyme does not leave the bilayer interface, and therefore it was possible to study the steps involved in the catalytic cycle at the interface without contributions from the steps in which the enzyme binds to or dissociates from the interface, i.e., the E to E* step.

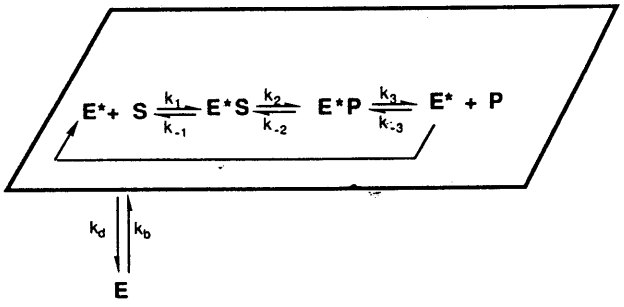

FIGURE 1: Scheme to accommodate key features of interfacial catalysis by PLA2. The species (E*, enzyme; S, substrate; P, products) shown in the box plane are in or bound to the interface, and the enzyme in the aqueous phase is shown as E. During steady-state catalysis in the scooting mode, the E to E* equilibrium is essentially completely in favor of the bound form. For a complete analysis of this formalism see Berg et al. (1991).

A complete deconvolution of the individual rate constants in this scheme requires the knowledge of the relative values of the rate constants k_3 and k_2, i.e., if the release of the reaction products is rate-limiting, or if the chemical step involving the conversion of E*S to E*P is rate-limiting during catalytic turnover at the interface. Such information will also be useful in interpreting the kinetic data of mutants. One way to resolve these two possibilities is to compare the rates of hydrolysis of oxy- and thioester analogues of the substrate. This is based on the idea that the rate constants that do not involve bond formation or cleavage with the oxy- and thio-containing phospholipids are expected to be similar, but the substitution of oxygen by sulfur may cause a significant change in the value of k_2. If the enzymatic turnover number, k_{cat}, is limited by k_2, a large rate effect of thio substitution might be observed. This type of element effect has been used previously to probe the nature of the rate-limiting step in enzymatic processes.

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[1] Abbreviations: DDPM, 1,2-didecanoyl-*sn*-glycero-3-phosphomethanol; DMPM, 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol; monothio-DDPM, 1-thiol-*sn*-glycero analogue of DDPM; dithio-DDPM, 1,2-dithiol-*sn*-glycero analogue of DDPM; monothio-DMPM, 1-thiol-*sn*-glycero analogue of DMPM; dithio-DMPM, 1,2-dithiol-*sn*-glycero analogue of DMPM; deoxy-LPC, 1-*O*-hexadecylpropane-1,3-diol-3-phosphocholine; MG14, 1-octyl-2-phosphonoheptanoyl-*sn*-glycero-3-phosphoethanolamine; MJ33, 1-hexadecyl-3-trifluoroethyl-*sn*-glycero-2-phosphomethanol; PLA2, phospholipase A_2. The individual rate constants are defined in Figure 1. The equilibrium dissociation constants for the enzyme–ligand complexes in the interface are designated by an asterisk.

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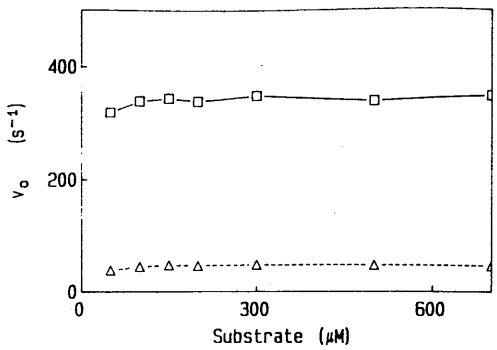

FIGURE 2: Dependence of the initial rate of hydrolysis (v_o) on the concentration of (squares) monothio- and (triangles) dithio-DDPM by porcine pancreatic PLA2. The reaction mixture (4 mL) at 23 °C and pH 8.0 contained 1 mM NaCl, 1 mM $CaCl_2$, 20 μg of polymyxin, and 0.5 or 5 pmol of PLA2. See Materials and Methods for details.

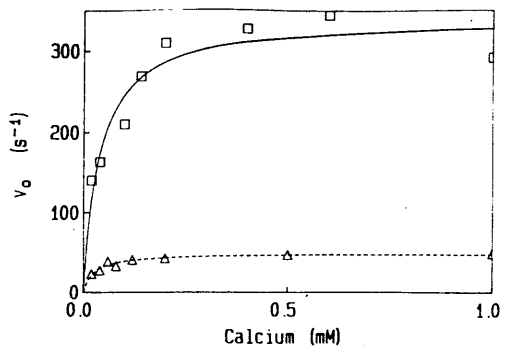

FIGURE 3: Dependence of the initial rate of hydrolysis of (squares) monothio- and (triangles) dithio-DDPM by porcine pancreatic PLA2 as a function of the calcium concentration. Reaction conditions as given in the legend to Figure 2.

For example, the hydrolysis of thioesters by chymotrypsin has been studied extensively (Ingles & Knowles, 1966; Frankfater & Kezdy, 1971; Hirohara et al., 1977). Processive enzymatic reactions such as the utilization of thio-substituted nucleotide analogous by DNA polymerase I have also been studied (Mizrahi et al., 1985). The hydrolysis of thioester-containing phospholipids by PLA2s has been reported previously (Volwerk et al., 1979; Hendrickson & Dennis, 1984; Aarsman et al., 1985), but the effect of the oxygen to sulfur substitution on all of the kinetic and equilibrium parameters has not been previously addressed.

The results reported herein establish that, depending on the source of PLA2, the hydrolysis rates for the oxyester phospholipids in vesicles are 2.5- to 28-fold faster than the rates for the corresponding thioester analogues. In addition, the element effects with the bovine pancreatic PLA2 and some of its active site mutants were all similar in magnitude. Thus, the chemical step is fully rate-limiting for this enzyme in the vesicle assay system. The element effect was then used to probe the rate-limiting step in the hydrolysis of mixed micelles of phospholipid and detergent. The observation that the element effect vanishes in mixed micelles suggests that the chemical step is no longer rate-limiting in this assay system.

MATERIALS AND METHODS

Recombinant bovine pancreatic PLA2 and mutants were obtained as described elsewhere (Dupureur et al., 1990; 1992a,b). Recombinant PLA2 from human synovial fluid (also called human non-pancreatic secreted PLA2) was provided by Dr. Jeffrey L. Browning (Biogen Inc., Cambridge, MA). The protocols for purification of other PLA2s (Jain, 1991b) and the syntheses of DDPM and DMPM (Jain and Gelb, 1991), MG14 (Yuan et al., 1990) and MJ33 (Jain et al., 1991d) have been previously described.

The thiophospholipids used in these studies were synthesized according to the procedures developed elsewhere (Hendrickson & Hendrickson, 1990; Yuan et al., 1990). 1-*S*-Tetradecanoyl-2-*O*-tetradecanoyl-1-thio-*sn*-glycerol and 1,2-bis(*S*-tetradecanoyl)-1,2-dithio-*sn*-glycerol were synthesized as described. These were converted to the *sn*-3-dimethylphosphates by treatment with chlorodimethylphosphite and triethylamine in methylene chloride, followed by oxidation of the phosphite to the phosphate with iodine according to the procedure of Meek et al. (1988). The dimethylphosphates were converted to the monomethylphosphates by refluxing with anhydrous LiBr in acetone (Jain & Gelb, 1991). The 1-*S*-decanoyl-2-*O*-decanoyl-1-thio-*sn*-glycero-3-phosphomethanol (monothio-DDPM) and 1,2-bis(*S*-decanoyl)-1,2-dithio-*sn*-glycero-3-phosphomethanol (dithio-DDPM) were synthesized from the corresponding phosphatidylcholines (Hendrickson & Hendrickson, 1990) by transphosphatidylation with phospholipase D (cabbage) in the presence of methanol using the procedure of Eibl and Kovatchev (1981).

The determination of the equilibrium dissociation constants for the enzyme in the interface bound to Ca^{2+}, reaction products, and competitive inhibitors was carried out using the protection from alkylation method using the neutral diluent deoxy-LPC as described (Jain et al., 1991a,b). The kinetic analysis of PLA2s in the scooting mode and the theoretical rationale for the analytical relationships used in the present study have been reported (Berg et al., 1991). The kinetics of the reaction progress catalyzed by PLA2s were continuously monitored by the pH-stat method at pH 8.0 and 23 °C in the presence of 1 mM NaCl and 0.5 mM $CaCl_2$ unless stated otherwise (Jain et al., 1986a; Jain & Gelb, 1991). Such conditions were well suited for monitoring the entire reaction progress curve. However, with small sonicated vesicles, the true initial velocity lasts for only a few seconds and is therefore difficult to measure (Berg et al., 1991; Jain et al., 1991c). This problem can be overcome if the initial rate of hydrolysis is monitored in the same medium but in the presence of greater than 1.5 mM $CaCl_2$ (Jain et al., 1986a; Berg et al., 1991) or in the presence of 2–5 μg/mL polymyxin B sulfate (Sigma) (Jain et al., 1991c). The estimated relative errors in the kinetic and equliibrium parameters are given in the table legends.

The titration efficiency of the thio substrates was monitored under various conditions. For example, the initial rate of hydrolysis of dithio-DMPM monitored with a pH-stat in the presence of high calcium or in the presence of polymyxin B sulfate was virtually the same as that monitored by a spectroscopic method in which the released thiol is reacted with 5,5′-dithio-bis(2-nitrobenzoic acid) under the same conditions (Volwerk et al., 1979). Similarly, the extent of hydrolysis of small vesicles of DMPM and mono- and dithio-DMPM in the presence of excess enzyme was about 63% of the total substrate present in the reaction mixture. Essentially identical results were also obtained when the reaction progress on codispersions of DMPM together with dithio-DMPM was monitored by the spectroscopic method. Such controls suggest that not only is the integrity of vesicles maintained even after all of the substrate in the outer monolayer of the target vesicles is hydrolyzed but that the thiol product does not contribute protons during the pH-stat titration.

RESULTS

The Element Effect on v_o in Vesicles. The initial velocity (at a mole fraction of substrate equal to 1) per enzyme, v_o,

Table I: Values of v_o for the Action of PLA2s on Vesicles of Oxyester and Thioester Phospholipids

PLA2	monothio-DDPM	dithio-DDPM	element effect[a]
porcine pancreas	350	48	7
bovine pancreas	290	60	5
bee venom	88	29	3
Crotalus atrox	195	7	28
Agkistrodon halys blomhoffi	260	14	18

	DMPM	monothio-DMPM	dithio-DMPM	element effect
human synovial fluid		11.3	4.5	2.5
porcine pancreas	300	280	38	7
bovine pancreas				
wild type	330	240	34	7
D99N	26	19	2.5	7
Y52V	11	7	1.4	5
F106Y	45	30	5	6
F106A	105	70	11	6

[a] Defined as the v_o for the monothio substrate divided by the v_o for the dithio substrate. The relative error in the v_o values is estimated to be 15%, based on conducting each experiment in triplicate. Studies were carried out using the standard conditions with 0.02–0.5 μg of PLA2 but in the presence of 4 μg/mL polymyxin B sulfate. Similar values of v_o were obtained if the polymyxin was replaced by 2.5 mM $CaCl_2$. There relative errors in the ratio of velocities as determined from carrying out each measurement in triplicate are estimated to be about 20%.

for the hydrolysis of bilayer vesicles of DDPM and DMPM as well as their monothio and dithio analogues were obtained from the initial linear region of the reaction progress curves either in the presence of a high calcium concentration or in the presence of polymyxin B. As shown in Figure 2 for the porcine pancreatic PLA2, the initial rates of hydrolysis of the mono and dithio substrates does not change significantly with the bulk concentration of the substrate present as vesicles. This result demonstrates that all of the PLA2 is bound to these vesicles. Also, as shown in Figure 3, the rates of hydrolysis for both substrates changes in a hyperbolic manner with the calcium concentration, and a value of the calcium dissociation constant, K_{Ca}*, of about 0.15 mM was obtained in both cases. This value is identical to the value obtained with DMPM vesicles (Berg et al., 1991).

Values of v_o for the hydrolysis of monothio-DDPM, dithio-DDPM, monthio-DMPM, dithio-DMPM, and DMPM by PLA2s from several sources are summarized in Table I. The results show that, depending on the source of the enzyme, the rates of hydrolysis of the monothio-DMPM are only slightly smaller than those for DMPM. In contrast, the monothio substrates are hydrolyzed 2.5- to 28-fold faster compared to the rate of hydrolysis of the dithio substrates. Since these values were obtained under saturating calcium and vesicle concentrations, it is concluded that the rate of interfacial hydrolysis of the dithio substrates at mole fraction 1 are considerably slower than those for the monothio substrates.

The Equilibrium Dissociation Constants for the Products. The protection from alkylation method (Jain et al., 1991a) was used to measure the dissociation constants for the complex of the enzyme at the interface with the reaction products. As summarized in Table II, values of these dissociation constants, K_P*, for the porcine pancreatic enzyme interacting with the reaction products from the oxyester and thioester substrates are all similar in magnitude.

Values of K_M.* The values of v_o listed in Table I were obtained under the conditions where the mole fraction of the substrate at the interface is 1. Under these conditions, the initial rate is expressed as (Berg et al., 1991)

$$v_o = \frac{k_{cat}}{1 + K_M^*} \quad (1)$$

Here, K_M* is the interfacial Michaelis constant, i.e., when the mole fraction of substrate in the vesicle equals K_M*, 50% of the total enzyme is in the E*S form. A change in the value of v_o could reflect a change in the value of k_{cat} or of K_M*, or both. Elsewhere it has been demonstrated that the chemical step is essentially irreversible, i.e., $k_3 \gg k_{-2}$ (Ghomashchi et al., 1991), and therefore the definition of K_M* is as follows:

$$K_M^* = \frac{k_3(k_2 + k_{-1})}{k_1(k_2 + k_3)} = \frac{(k_2 + k_{-1})}{k_1} \quad \text{if} \quad k_3 \gg k_2 \quad (2)$$

Thus, v_o could depend not only on k_2 but on k_1, k_{-1}, and possibly k_3 as well. The values of K_M* were determined by monitoring the dependence of v_o on the mole fraction of a competitive inhibitor at the interface (Berg et al., 1991):

$$\frac{v_o}{v_I} = 1 + \left(\frac{X_I}{1 - X_I}\right)\left[\frac{1 + (1/K_I^*)}{1 + (1/K_M^*)}\right] \quad (3)$$

Here, v_I is the initial velocity per enzyme in the presence of the inhibitor at mole fraction in the vesicle of X_I and K_I* is the dissociation constant for the enzyme–inhibitor complex in the interface. The parameter $X_I(50)$ is the mole fraction of inhibitor in the interface that reduces v_o by a factor of 2. Values of K_I* for the two different competitive inhibitors of the porcine pancreatic PLA2, MG14 and MJ33, have been determined (Jain et al., 1991a,d). Values of K_M* for the oxyester and thioester substrates were obtained from the values of $X_I(50)$ and K_I* (eq 3) and are summarized in Table III. The K_M* for DDPM and monothio-DDPM are similar, but the

Table II: Values of K_P* (Mole Fraction) for the Dissociation of the Products of Hydrolysis of Oxyester and Thioester Substrates from Porcine Pancreatic PLA2 Bound to Deoxy-LPC Micelles (E*Ca-P) at 23 °C, pH 8.0

products of	K_P*	products of	K_P*
DDPM	0.02	DMPM	0.025[a]
monothio-DDPM	0.026	monothio-DMPM	0.025
dithio-DDPM	0.049	dithio-DMPM	0.044

[a] From Jain et al. (1991a). All studies were carried out using the published conditions and with 0.5 mM $CaCl_2$. The relative errors in the dissociation constants are estimated to be 30%, based on carrying out each measurement in triplicate.

Table III: Values of K_M* for the Action of the Porcine Pancreatic PLA2 on Oxyester and Thioester Substrates[a]

	$X_I(50)$ (K_M*)	
	MG14	rac-MJ33
K_I*	0.0011	0.0016
DDPM	0.05 (0.023)	0.06 (0.027)
monothio-DDPM	0.057 (0.02)	0.065 (0.025)
dithio-DDPM	0.088 (0.013)	0.13 (0.012)
DMPM	0.003 (0.3)	0.006 (0.35)
monothio-DMPM		0.033 (0.06)
dithio-DMPM		0.055 (0.03)

[a] The conditions used were the same as in Table II with polymyxin B sulfate. The relative errors in the K_M* values are estimated to be 30%, based on conducting each measurement in triplicate.

value for dithio-DDPM was about 2-fold smaller. In the case of the DMPM substrates, the K_M* values for both thio substrates were significantly smaller than the value for DMPM.

On the basis of the K_M* values in Table III, it is apparent from eq 1 that the significantly larger values of v_o for the dioxy and monothio substrates compared to that of the dithio substrates (Table I) must arise from a difference in the k_{cat} values for these substrates.

The kinetic constant k_{cat} depends only on the rate constants for the chemical step, k_2, and for the product release step, k_3, according to

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \cong k_2 \quad \text{if} \quad k_3 > k_2 \tag{4}$$

The reverse of the chemical step (k_{-2}) need not be considered (Ghomashchi et al., 1991). Since the values of K_P* for all of the oxy and thio substrates are similar (Table II), it is reasonable to assume that the values of k_3 for the substrates are also similar, i.e., it is unlikely that the element effect on the values of k_3 and k_{-3} is the same. Thus, the differences in the k_{cat} values for the oxy and thio substrate likely arise from the differences in their values of k_2 (eq 4). Therefore, the results suggest that for the dithio-substrates $k_3 > k_2$, i.e., the release of products from E*P is not rate-determining for the maximal turnover number, k_{cat}.

Kinetic Studies with Mutant Forms of Bovine Pancreatic PLA2. Although the above results suggest that the chemical step is rate-limiting for maximal turnover of long-chain phospholipids in vesicles, strictly speaking this applies only to the hydrolysis of the dithio substrates. The data up to this point are insufficient to conclude that the element effect has been fully expressed, i.e., whether the chemical step is rate-limiting for the natural (oxyglycerol) substrate. The latter problem can be evaluated by a complementary approach in which the catalytic properties of the enzyme are altered. When the catalytic rate is lowered due to the mutation of an active site residue, it is more likely that the chemical step instead of a physical step is perturbed. In any case, the element effect of the mutants can be predicted to fall into the following categories (assuming that the intrinsic element effect is not altered by a change in the reaction mechanism): (i) If the chemical step is fully rate-limiting for the wild-type enzyme (with oxyglycerol substrate), then the element effect with the mutant should either remain unchanged (if the chemical step is perturbed in the mutant) or decrease (if a physical step is perturbed in the mutant). (ii) If the chemical step is not rate-limiting or only partially rate-limiting with the wild-type enzyme (with oxyglycerol substrate), the element effect of the mutant should increase (if the chemical step is perturbed and becomes more rate-limiting). As shown by the data in Table I and by the data of other mutants (unpublished results), a mutant has not yet been found that gives an element effect that is greater than 7. It is unlikely that the chemical step is not perturbed in the active site mutants. Therefore, the results suggest that the chemical step is fully rate-limiting for the wild-type PLA2 from bovine pancreas in the hydrolysis of DMPM vesicles.

The Element Effect on Enzymatic Turnover in Vesicles under Substrate-Limiting Conditions. With an excess number of small sonicated DMPM vesicles over the number of enzymes and with low calcium concentrations to avoid vesicle fusion, only a limited amount of substrate is hydrolyzed in the scooting mode since the enzyme does not hop among the vesicles (Jain et al., 1986a). In addition, the reaction progress curve has a first-order appearance (Jain et al., 1986a; Berg et al., 1991) which is typical of enzymatic reactions in which the enzyme is not saturated with substrate during the steady-state turnover. Such progress curves are described by the integrated Michaelis–Menten equation adopted for the hydrolysis of vesicles (Berg et al., 1991):

$$N_s k_i = \frac{k_{cat}}{K_M^*\left(1 + \frac{1}{K_P^*}\right)} = \frac{k_1 k_2}{(k_{-1} + k_2)\left(1 + \frac{1}{K_P^*}\right)} \cong \frac{k_1}{\left(1 + \frac{1}{K_P^*}\right)} \quad \text{if} \quad k_2 > k_{-1} \tag{5}$$

Here, N_s is the total amount of product per enzyme produced at the end of the first-order reaction progress and k_i is the first-order relaxation constant. The parameter $N_s k_i$ gives the apparent second-order rate constant for the enzyme–substrate reaction under substrate-limiting conditions and in the presence of product inhibition. Thus, it is different from v_o, which describes the turnover under conditions in which the enzyme in the interface is nearly saturated with substrate.

First-order reaction progress curves were seen with small vesicles of DMPM and its monothio and dithio analogues. From such curves, the values of $N_s k_i$ for the porcine pancreatic PLA2 acting on DMPM (34 s^{-1}), monothio-DMPM (31 s^{-1}), and dithio-DMPM (25 s^{-1}) were obtained. The similar $N_s k_i$ values for the monothio and dithio substrates is seemingly at odds with the fact that the K_M* and K_P* for these substrates are similar but the k_{cat} for the dithio substrate is 7.4-fold lower than that for the monothio substrate (Table I). In addition, the $N_s k_i$ values of DMPM and dithio-DMPM are also similar despite the fact that the K_M* for the dithio substrate is about 10-fold lower than that for DMPM (Table III). However, with DMPM as substrate, heavy atom isotope effect studies have shown that $k_2 \gg k_{-1}$ (Ghomashchi et al., 1991), and thus k_{cat}/K_M* becomes approximately equal to k_1. If this inequality also holds for monothio-DMPM, which seems reasonable, then the similar values of $N_s k_i$ and K_P* (Table II) for this substrate and DMPM suggest that the k_1 values are similar for these two substrates. In the case of the dithio substrate, using the values of K_M* and K_P* (Tables II and III) and the value k_2 of 38 s^{-1} (Table I), a value of $N_s k_i$ of 53 s^{-1} is obtained. This is within experimental error of the measured number given the uncertainties in the measured values of the dissociation and rate constants. Thus, the element effect on the enzymatic turnover in vesicles under substrate-limiting conditions is much smaller than that measured under substrate-saturating conditions. The significance of these results will be discussed in the next section.

Hydrolysis of Comicellized Substrates. As shown elsewhere (Jain et al., 1991a), the initial rate of hydrolysis of DMPM decreases sharply as the mole fraction of the neutral diluent, deoxy-LPC is increased above 0.3 mole fraction. Under these conditions, the vesicles are comicellized. While the element effect for the hydrolysis of vesicles by the porcine pancreatic PLA2 remains at 7 as the amount neutral diluent is increase from 0 to 0.25 mole fraction, above 0.5 mol fraction the element effect dropped to 0.9 ± 0.1. This suggests that the chemical step is no longer rate-limiting for the hydrolysis of these neutral diluent/phospholipid mixed micelles.

DISCUSSION

The magnitude of the observed element effect on the hydrolysis of phospholipids by PLA2s depends on the effect of oxygen to sulfur substitution on the rate constant for the chemical step, k_2. In addition, the other rate constants shown in Figure 1 may modulate the value of the observed element effect even if they are not effected by the oxygen to sulfur change. Thus, the magnitude of the element effect on a PLA2-catalyzed reaction is difficult to predict. It is fortunate that, in the case of the bovine and porcine pancreatic PLA2s, the observed element effect is large enough (Table I) to be useful as a tool in determining the nature of the rate-limiting steps in the interfacial catalysis. Thus, the discussion that follows is focused on the PLA2s from bovine and porcine pancreas in which the element effect on many of the rate and equilibrium parameters shown in Figure 1 have been determined. In addition, the availability of recombinant bovine pancreatic PLA2 (Dupureur et al., 1990) has permitted the study of the element effect on both the wild-type and mutant forms of the bovine enzyme.

The Nature of the Rate-Limiting Step for Maximal Turnover in Vesicles. The analysis of interfacial catalysis by PLA2 on vesicles in the scooting mode has allowed for most of the rate and equilibrium constants shown in Figure 1 to be determined (Berg et al., 1991). Since the interfacial K_M^* for the action of the pig pancreatic PLA2 on DMPM vesicles is 0.35 mole fraction (Jain et al., 1991a), the enzymatic turnover at the maximal attainable substrate concentration of 1 mole fraction, v_o, is close to the true value of k_{cat} (eq 1). The observed element effects on k_{cat} of 5–7 for the action of the porcine and bovine pancreatic enzymes on vesicles of long-chain monothio and dithio substrates (Table I) together with the fact that the oxygen to sulfur substitution does not change K_{Ca}^* or K_P^* strongly suggest that the chemical step, k_2, and not the product release step, k_3, is rate-limiting for maximal turnover in the scooting mode.

Strictly speaking, the element effect on v_o shows that the chemical step is rate-limiting only for the dithio substrates. Without additional data, the extrapolation of this statement to the turnover of dioxy substrates is not justified. It is possible to imagine the situation where k_2 for the dioxy substrate is slightly larger than k_3, i.e., product release is rate-limiting, and that the reduction in the value of k_2 resulting from the oxygen to sulfur substitution causes a change in the rate-limiting step, i.e., the chemical step is not rate-limiting. This problem has been addressed by investigating the element effect on the scooting mode hydrolysis of vesicles by mutant forms of the bovine pancreatic PLA2. As summarized in Table I, the element effect remains between 5 and 7 for mutants that have a decreased v_o (<10%) compared to wild-type enzyme. In the case of mutants F106A, F106Y, and D99N, previous studies have shown that the reduction in the enzymatic velocity is due primarily to a decrease in k_{cat} (Dupureur et al., 1992b; Dupureur et al., to be published) rather than an increase in K_M^*, whereas, with Y52V, a 10-fold increase in K_M^* and a 5–10-fold decrease in k_{cat} was measured (Dupureur et al., 1992a). Although a decrease in k_{cat} can in principle be caused by a decrease in the chemical step or in the product release step or both, it is much more likely that these active site mutants affect the value of k_2 more than k_3. Thus, in the case of the mutants, it is very likely that the chemical step is rate-limiting for both the dioxy and dithio substrates. The fact that the element effects are the same for the mutants and wild-type enzymes suggest that the chemical step is rate-limiting in the hydrolysis of DMPM by wild-type enzyme as well.

The origin of the lower rate of the chemical step for the dithio substrates may be due to any one or a combination of the following factors in the stabilization of the transition state: (1) geometrical and steric factors such as the bond lengths and bond angles of the R_2CO–S–C structure; (2) altered hydrogen-bonding ability of S versus O; (3) altered polarizability of the C=O due to the less electronegative character of the attached S atom. A significantly lower value of k_{cat} for the dithio substrates compared to that for the oxy analogues is interesting in light of the fact that RS^- is a better leaving group than RO^- as estimated from the rate of breakdown of tetrahedral hemiacetals and thiohemiacetals (Jencks, 1969). The relative rates of hydrolysis of oxyesters versus thioesters by the serine protease chymotrypsin has been studied extensively (Ingles & Knowles, 1966; Frankfater & Kezdy, 1971; Hirohara et al., 1977). Nonspecific ester substrates such as *N*-acetylglycine esters generally show an increase in k_{cat} (typically 10-fold) when oxygen is replaced with sulfur. This suggests that the rate of breakdown of the tetrahedral intermediate is slower than its formation. Alternatively, since nonproductive binding of substrate to the enzyme will lower the observed value of k_{cat}, the element effect with nonspecific substrates may be due to the decrease in the nonspecific binding when O is replaced with S (Hirohara et al., 1977). In contrast, with *p*-nitrophenacyl esters, an effect of O versus S substitution has never been observed with serine proteases. In addition, the specific chymotrypsin substrate *N*-acetyltryptophan ethyl ester shows a 2-fold decrease in k_{cat} accompanying O to S substitution. For these substrates, it has been suggested that the rates of formation and breakdown of the tetrahedral intermediate are similar in magnitude (Hirohara et al., 1977). In the case of PLA2, there is no covalent acyl–enzyme intermediate along the reaction path, and so the magnitude of the O to S element effect on k_{cat} will be sensitive to the relative rates of formation and breakdown of the tetrahedral intermediate. Thus, the relatively large decrease in k_{cat} measured for PLA2-catalyzed hydrolysis of thioester phospholipids in the scooting mode implies that a rate-limiting process in the chemical step exists that does not involve the leaving of the RO^- group.

Given the inability to predict the magnitude of the observed element effect even when the chemical step is fully rate-limiting, the small effects on v_o seen with the bee venom PLA2 and the human synovial fluid PLA2 (Table I) should not be taken as evidence that a step other than the chemical step is rate-limiting. In the case of the human synovial fluid PLA2, recent studies have shown that K_M^* is considerably greater than 1 mole fraction (Gelb et al., to be published), and thus the element effect given in Table I is not on k_{cat} but on the turnover under substrate-limiting conditions, i.e., $N_s k_i$.

The Nature of the Rate-Limiting Step for Turnover under Limiting Substrate Conditions. In small sonicated DMPM vesicles, the reaction velocity is proportional to the mole fraction of substrate in the vesicle (first order), and the $N_s k_i$ parameter that describes the progress curve is a function of k_{cat}/K_M^* and of K_P^* (eq 5). For the wild-type pig pancreatic PLA2 acting on DMPM vesicles, heavy atom isotope studies have shown that the second-order rate constant, k_{cat}/K_M^*, is equal to k_1, i.e., it is limited by the physical step of substrate binding to E* (Ghomashchi et al., 1991). The simplest explanation for the similarity in the observed values of $N_s k_i$ for dioxy-, monothio-, and dithio-DMPM is that all three substrates are limited by the same value of k_1. However, in the absence of direct measurement of the element effect on k_1, other more complicated possibilities cannot be ruled out.

The value of $k_1 = 1350\ s^{-1}$ (Berg et al., 1991) is close to the maximal rate constant for substrate binding, $\approx 10\,000\ s^{-1}$, based on the lateral diffusion of phospholipids (Jain & Berg, 1989). Although it is possible that k_1 is diffusion-limited, this may be discounted on the basis of the observation that both $N_s k_i$ and v_o do not show any anomalous change as the reaction temperature passes through the melting point for the gel to fluid transition of DMPM (Jain et al., 1989), whereas the lateral diffusion coefficients for phospholipids are typically 100-fold larger in the liquid state compared to in the gel state phase (Jain, 1988). The physical processes that cause a decrease in k_1 are not understood. It could be due to a slower diffusion of substrate that is in direct contact with the bound PLA2, or it could be due to a slow intrinsic rate for the dislodgement of the substrate from the plane of the bilayer into the active site slot on the enzyme (Jain et al., 1986; Scott et al., 1990).

The Rate-Limiting Step for Turnover in Neutral Diluent/DMPM Mixed Micelles. Compared to the element effect of 7-fold for the action of the porcine pancreatic PLA2 in vesicles, the effect vanishes when the DMPM substrates are in the form of mixed micelles with the neutral diluent deoxy-LPC. This suggests that the chemical step is no longer rate-limiting in the hydrolysis of these mixed micelles. The binding of PLA2 to either vesicles or micelles can be assessed by monitoring the increase in the fluorescence of tryptophan-3 that accompanies the environment change from aqueous solution to the surface of the interface (Jain et al., 1988). Such spectral studies show that all of the pig pancreatic enzyme is bound to deoxy-LPC/DMPM mixed micelles (data not shown).

The mixed micelles used in the present study contained 0.5 mole fraction of either monothio- or dithio-DMPM, and this is at least 10-fold higher than the K_M^* for these phospholipids (Table III). Although this is true on the global scale where all of the mixed micelles on the average contain saturating amounts of substrate, it is unlikely that this is true on the microscopic scale, i.e., in the enzyme-containing mixed micelles. If the exchange of enzymes or phospholipids among the ensemble of mixed micelles is much faster than the reciprocal of the enzymatic turnover number, then the substrate present in all micelles will be enzymatically degraded in a uniform way. However, it is much more likely that the exchange processes are not fast on the lipolysis time scale (Jain et al., 1991c) and that the observed PLA2 reaction velocities in mixed micelles are limited by the rate of substrate replenishment, i.e., the chemical step, k_2, will not be rate-limiting and the element effect will vanish. Thus, the results of the present study suggest that this is indeed the case, at least for long-chain phospholipids dispersed in micelles of deoxy-LPC. Furthermore, monothio- and dithiophospholipids will be useful tools in examining the nature of the rate-limiting step with other mixed micelle assay systems.

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