Interfacial Catalysis by Phospholipase A_2: Dissociation Constants for Calcium, Substrate, Products, and Competitive Inhibitors†

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ABSTRACT: Interpretation of the kinetics of interfacial catalysis in the scooting mode as developed in the first paper of this series [Berg et al. (1991) *Biochemistry 30* (first paper of six in this issue)], was based on the binding equilibrium for a ligand to the catalytic site of phospholipase A_2. In this paper, we describe direct methods to determine the value of the Michaelis–Menten constant (K_{MS}) for the substrate, as well as the equilibrium dissociation constants for ligands (K_L) such as inhibitors (K_I), products (K_P), calcium (K_{Ca}), and substrate analogues (K_S) bound to the catalytic site of phospholipase A_2 at the interface. The K_L values were obtained by monitoring the susceptibility to alkylation of His-48 at the catalytic site of pig pancreatic PLA2 bound to micellar dispersions of the neutral diluent 2-hexadecyl-*sn*-glycero-3-phosphocholine. The binding of the enzyme to dispersions of this amphiphile alone had little effect on the inactivation rate. The half-time for inactivation of the enzyme bound to micelles of the neutral diluent depended not only on the nature of the alkylating agent but also on the structure and the mole fraction of other ligands at the interface. The K_L values for ligands obtained from the protection studies were in excellent accord with those obtained by monitoring the activation or inhibition of hydrolysis of vesicles of 1,2-dimyristoyl-*sn*-glycerophosphomethanol. Since only calcium, competitive inhibitors, and substrate analogues protected phospholipase A_2 from alkylation, this protocol offered an unequivocal method to discern active-site-directed inhibitors from nonspecific inhibitors of PLA2, such as local anesthetics, phenothiazines, mepacrine, peptides related to lipocortin, 7,7-dimethyleicosadienoic acid, quinacrine, and aristolochic acid, all of which did not have any effect on the kinetics of alkylation nor did they inhibit the catalysis in the scooting mode.

In the first paper in this series (Berg et al., 1991), it was shown that the Michaelis–Menten formalism adopted with interfacial constraints provided an adequate basis for obtaining the rate parameters by the interpretation of the reaction progress curve for the hydrolysis of DMPM vesicles by phospholipase A_2 (PLA2)[1] in the scooting mode. This interpretation was simplified by the demonstration that the reverse reaction in the catalytic step was considerably slower than the rate of dissociation of the products from the enzyme, i.e., $k_{-2} \ll k_3$ (Ghomashchi et al., 1991). The scheme shown in Figure 1 illustrates a further elaboration of the equilibria intrinsic in the interfacial catalytic cycle. In this paper, direct methods are described for obtaining equilibrium dissociation constants for complexes of E* with the substrate (K_S), calcium (K_{Ca}), products (K_P), and inhibitors (K_I).

Binding of a ligand to the catalytic site of PLA2 has been shown to modulate reactivities of the active site residues including His-48 and Asp-49 (Volwerk et al., 1974; Verheij et al., 1980; Fleer et al., 1981). In this paper, we have extended these observations to demonstrate that the alkylation of His-48 was blocked by ligands bound to the active site of PLA2 at the interface. This allowed determination of all the dissociation constants for the equilibria explicitly shown in Figure 1, and it also helped to identify a neutral diluent with a low affinity, i.e., a relatively high K_I value. In a parallel approach, the kinetics of hydrolysis of DMPM vesicles in the scooting mode was monitored in the presence of competitive inhibitors (Jain et al., 1986d, 1989) and interpreted in terms of K_{MS}, K_P, and K_I values.

Materials and Methods

Bromooctanone was prepared as described by Visser et al. (1971). PNBS was purchased from Aldrich and recrystallized from methanol. PNBr was from Sigma. All phospholipids

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[1] Abbreviations: AM-1, peptide MQMKKVLDS; bromooctanone, 1-bromooctan-2-one; DTPC, 1,2-ditetradecyl-*sn*-glycero-3-phosphocholine; DMPM, 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol; DTPM, 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol; H-18, 1-palmitoyl-2-myristoylamino-2-deoxy-*sn*-glycero-3-phosphoglycol; HDNS, dansylated hexadecylphosphoethanolamine; GPC, *sn*-glycero-3-phosphocholine; HPM, hexadecylphosphorylmethanol; 2H-GPC, 2-hexadecyl-*sn*-glycero-3-phosphocholine; GPM, *sn*-glycero-3-phosphomethanol; MG14, 1-octyl-2-phosphonoheptyl-*sn*-glycero-3-phosphoethanolamine; k_b, rate constant for alkylation of PLA2 bound to interface; k_F, rate constant for alkylation of free PLA2; k_L, rate constant for alkylation of PLA2 bound to the interface containing a ligand at the active site; k_O, rate constant for alkylation of PLA2 bound to the interface but no ligand at the active site; K_D, equilibrium dissociation constant for E* to E; K_L, equilibrium dissociation constant for the ligand bound to the active site of PLA2; PC, phosphocholine; PLA2, phospholipase A2 from pig pancreas; PNBr, *p*-nitrophenacyl bromide; PNBS, *p*-nitrobenzene-methylsulfonate; t_b, half-time for inactivation of PLA2 bound to the interface in the absence of a ligand; t_f, half-time for inactivation of PLA2 in the aqueous phase but without a ligand at the active site; t_L, half-time for inactivation of PLA2 at the interface in the presence of a ligand at the catalytic site; t_O, half-time for inactivation of PLA2 at the interface but without a ligand at the active site; the terms K_I, K_{Ca}, K_{MS}, K_P, and K_S have their usual meaning and for their definitions see the Appendix and Table I in Berg et al. (1991).

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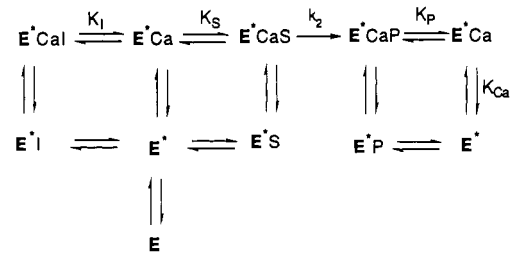

FIGURE 1: Scheme for interfacial catalysis. It emphasizes the various equilibria intrinsic in earlier schemes for interfacial catalysis [e.g., see Jain and Berg (1989) and Berg et al. (1991)]. The species E* (enzyme), I (inhibitor), S (substrate), and P (products) are in the interface, and the enzyme in the aqueous phase is shown as E. The equilibria for the formation of binary and ternary complexes have the usual significance.

used in this study were synthesized by methods described elsewhere (Jain et al., 1986a,c). Aristolochic acid, mepacrine, quinacrine, and dibucaine were from Sigma, and 7,7-dimethyleicosadienoic acid was from Biomol (Philadelphia). AM-1 and related peptides of this series were kindly provided by Dr. Anil Mukherjee (NIH, Bethesda). MG-14 was a gift from Prof. Michael Gelb (University of Washington, Seattle), and H-18 was kindly provided by Professor de Haas (State University, Utrecht). The 2H-GPC used for the initial studies was kindly provided by Dr. Ries, and for subsequent studies it was prepared as described (Ries, 1989).

All studies reported here were carried out with PLA2 from pig pancreas. The kinetics of alkylation of PLA2 was monitored as described elsewhere in detail (Volwerk et al., 1974; Verheij et al., 1980). PNBr, which provided the shortest inactivation time, was used for the alkylation studies; however, many of the studies reported here were also carried out, with essentially identical conclusions, by using bromooctanone or PNBS. Typically, 30–80 μM PLA2, additives (0.02–1 mM, if present), 2H-GPC (16 mM, if present), and the alkylating agent (0.1–4 mM) were incubated at 25 °C and pH 7.3 in 0.05 mL of buffer containing 0.1 M cacodylate, 0.1 M NaCl, and 1 mM $CaCl_2$ or 9 mM EGTA. While some inactivation studies were carried out in the presence of bilayers or micelles of pure amphiphiles, dependences of the rates of inactiation on the mole fraction of inhibitors were determined in comicelles with 2H-GPC (16 mM). Typically, the inactivation reaction was initiated by adding the alkylating agent in acetone or acetonitrile at the final concentrations below their solubility limits in water. At appropriate time intervals, an aliquot of the reaction mixture was diluted 10-fold in 0.2% acetic acid or added directly to the assay mixture. The amount of the unmodified PLA2 remaining in the mixture was assayed by monitoring the hydrolysis of egg yolk suspended in deoxycholate by pH-stat titration (Nieuwenhuizen et al., 1974). The semilog plots for inactivation were always linear, and more than 95% of the enzyme was inactivated at the end of the reaction. The inactivation time (or the pseudo-first-order rate constant) was obtained by a standard curve-fitting program (Biosoft or GraphPad) by a nonlinear regression analysis on 5–10 points spread over more than six half-times or 1500 min (whichever was shorter). The standard deviation in the values of the individual half-times for inactivation was always less than 15%; however, as expected, the uncertainty in values of the ratios of the half-times (t_f/t_b or t_0/t_L) could be considerably larger, depending upon the absolute values of the individual half-times. Thus the estimated uncertainties in the K_L values are about 30%.

As reported for inactivation studies on free PLA2 in the aqueous phase (Pieterson et al., 1974; Volwerk et al., 1974; Verheij et al., 1980), a complete inactivation of PLA2 bound to 2H-GPC also occurred by a single rate constant. Independent control experiments showed that PLA2 incubated for more than 30 h in the presence or absence of amphiphiles (without an alkylating agent) was not inactivated. The second-order inactivation rate constants for the inactivation of PLA2 bound to 2H-GPC were 430 and 70 M^{-1} min^{-1} with PNPBr and bromooctanone, respectively. Control experiments were also carried out to assure that the alkylating agents did not spontaneously decompose in the reaction mixture for well over 18 h. The 2H-GPC concentrations were also high enough to assure that all PLA2 was bound during alkylation studies, and as discussed later the protection from alkylation was from the amphiphilic ligands in the interface.

All fluorescence measurements to ascertain binding of PLA2 to dispersions of amphiphiles were carried out at 23 °C in 100 mM Tris·HCl at pH 8.0 on an SLM 4800S spectrophotometer (Jain et al., 1982, 1986a; Jain & Maliwal, 1985). For these measurements the protein concentration was kept at 3–7 μM. All spectra were uncorrected, and the excitation and emission slit widths were set at 4 nm each. Vesicles of DTPM were used for the binding studies (Jain & Maliwal, 1985; Jain & Vaz, 1987), and the increase in the fluorescence intensity was monitored at 333 nm with excitation at 290 nm. Binding of PLA2 to the interface was also monitored by UV spectroscopy, which exhibits a ground state perturbation of Tyr and Trp near 290 nm (Hille et al., 1983). The results from these three techniques for the binding of PLA2 to an interface were always consistent.

The rate of hydrolysis of DMPM vesicles in the presence of additives at low ($N_S k_i$) and high calcium (v_0) was monitored by pH-stat titration as described by Berg et al. (1991). An inhibitor, if present, was mixed and colyophilized with the substrate before the preparation of the vesicles so as to assure a uniform distribution. Inhibitors that form micellar solutions could also be added to preformed vesicles; however, in such cases for the calculation of the mole fraction, it is necessary to consider that the inhibitor was partitioned only on the outer monolayer of vesicles.

RESULTS

Principles and Experimental Strategy for Determining Equilibria at Interfaces. The kinetics of alkylation of His-48 in PLA2 are influenced by the presence of ligands that bind to the active site (Volwerk et al., 1974), and from such studies it is possible to obtain the equilibrium dissociation constant for the ligand at the active site (Scrutton & Utter, 1965; Baker, 1976). As developed in the Appendix, the overall conceptual strategy for determination of the equilibrium constant for the reaction E* + L to E*L in the interface required establishment of the appropriate equilibrium in the reaction mixture and at the same time elimination of the contribution of other parallel processes. The following considerations facilitated the interpretation of the protection studies in this paper in terms of the formalism developed in the Appendix:

(a) We explicitly distinguish the factors that control binding of PLA2 to the interface, i.e., the E to E* equilibrium, from those that control the binding of a ligand to the enzyme in the interface, i.e., the E* + L to E*L equilibrium. Thus a neutral diluent is defined as an amphiphile that forms aggregates (micelles or bilayers) in aqueous dispersions to which PLA2 binds but does not bind well to the active site of PLA2. As described below, such a neutral diluent was useful in providing an interface to study the binding of ligands to the active site

of E* and for varying the surface concentration of such ligands for the protection and kinetic studies.

(b) For the protection studies on interfaces, it is necessary to assure that the amphiphile used as a neutral diluent is present above its critical micelle concentration so that the enzyme will be present on micelles. Moreover, as discussed in the next section, the lipid to enzyme mole ratio must exceed 50 to assure that there are enough lipid molecules required for the binding of all the enzyme molecules. In addition, the concentration of the neutral diluent should be high enough so that all of the enzyme is in the interface (Jain & Berg, 1989).

(c) The mole ratio of the alkylating agent for our studies is considerably above (10×) the stoichiometric ratio with the enzyme. Also the concentration of the alkylating agents was well below the point at which the pre-equilibrium binding of the agent to E* is saturated, that is, the rate of alkylation was found to increase linearly with increasing concentration of the alkylating agent.

(d) Partitioning of the alkylating agent in the interface does not significantly change the concentration of the alkylating agent in the aqueous phase because the volume of the interface is relatively small.

(e) As described below, the rate of alkylation of PLA2 was similar when the enzyme was in the aqueous phase or fully bound to micelles of the neutral diluent. This has been reported previously with other micelles (Volwerk et al., 1974; de Haas et al., 1990).

(f) The amphiphilic ligands used for the protection studies were virtually completely partitioned in the neutral diluent, and it is assumed that all the components on the interface are uniformly distributed.

Considerations related to the dynamics of equilibration are also important. All the rates of equilibration are assumed to be faster than the rate of alkylation. While the rate of replenishment of the substrate is a key consideration for the interpretation of the rate of interfacial catalysis in micelles (Berg et al., 1991; Jain et al., 1991b), such a concern is not relevant to the presence studies since there is no enzymatic turnover of lipids in the micelles. Also the replenishment of reacted alkylating agent in the enzyme-containing micelles is not a rate-limiting factor since the rate of exchange of the alkylating agent between the bulk and micellar phases is fast on the time scale of the alkylation reaction (several minutes). In addition, the equilibration of free and enzyme-bound forms of the ligand is expected to be much faster than the rate of alkylation so that the protection from alkylation provided by the ligand is a measure of the equilibrium fraction of the bound enzyme that has a ligand at the active site.

Inactivation of PLA2 in the Presence of Amphiphiles. The kinetics of alkylation of His-48 on PLA2 was sensitive to the presence of calcium and other ligands that bind to the active site of PLA2 (Volwerk et al., 1974; Verheij et al., 1980; van Oort et al., 1985; de Haas et al., 1990). For example, as shown in Figure 2, the kinetics of inactivation of PLA2 by bromooctanone were first order, and, as elaborated later in this section, the half-time for inactivation increased not only in the presence of calcium but also in the presence of certain amphiphiles but not others. The dependence of the half-time for inactivation by alkylating agents under a variety of conditions was studied to obtain information about the binding of ligands to the catalytic site of PLA2. As shown in Figure 3, the half-time for inactivation depended on the nature of the amphiphile. For alkyl sulfates, the ratio of inactivation half-times for the free enzyme in the water phase in the absence of a ligand to that for the enzyme bound to the interface, t_f/t_b, decreased with increasing alkyl sulfate to enzyme mole ratio

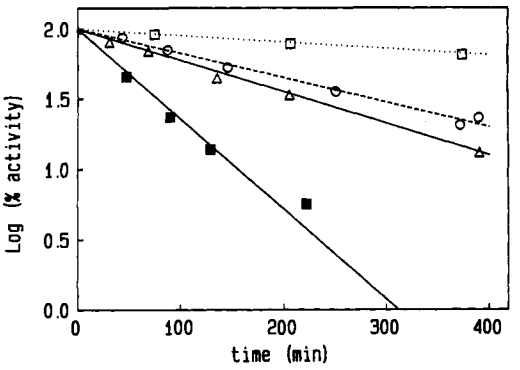

FIGURE 2: Semilogarithmic plots for the kinetics of inactivation of 0.06 mM PLA2 by bromooctanone (4 mM) in the presence of 1 mM (closed squares) and 10 mM $CaCl_2$ (triangle), in the presence of 3.2 mM decyl sulfate (open squares), or in the presence of 3.2 mM (hexadecylphosphoryl)methanol (open circles) with 1 mM calcium chloride. Inactivation studies were carried out at pH 6.8 and 30 °C. See Materials and Methods for other experimental details.

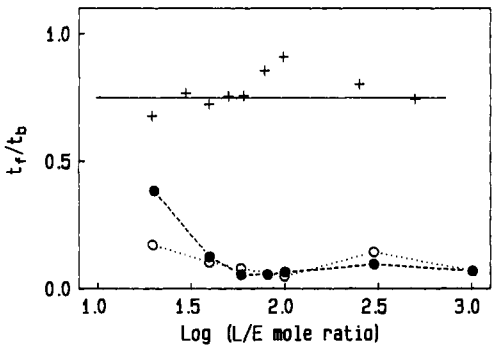

FIGURE 3: Dependence of the relative rate of inactivation (t_f/t_b) of PLA2 (0.06 mM) by bromooctanone (4 mM) at 30 °C in the presence of the varying mole ratio of the amphiphiles with respect to the enzyme (log L/E) of decyl sulfate (open circles), dodecyl sulfate (closed circle), and 2H-GPC (crosses). Other conditions given under Materials and Methods.

(L/E). The t_f/t_b ratio reached a minimum as the L/E ratio approached 50, and beyond that it did not change appreciably. This was due to a confluence of several factors. The two alkyl sulfates have different critical micelle concentrations in the range of 8–15 mM (i.e., $L/E \gg 100$); however, alkyl sulfates form premicellar aggregates with PLA2 (Hille et al., 1983). The results in Figure 3 showed that the maximum protection was observed below 3 mM, i.e., at the L/E ratio of <50 for both alkyl sulfates. This suggested that the mole ratio of the anionic amphiphile to the enzyme, rather than its absolute concentration, was responsible for the protection of PLA2 against alkylation. Furthermore, maximum protection at L/E <50 is consistent with previous studies using a variety of techniques which showed that 30–60 molecules of amphiphiles are required for the binding of PLA2 to the interface (Hille et al., 1983; Jain et al., 1982; Jain & Berg, 1989; Yuan et al., 1990). Thus maximum protection required that the L/E ratio exceed 50 regardless of the critical micelle concentration of the amphiphile.

In contrast to the results with alkyl sulfates, no change in the ratio t_f/t_b was seen with increasing concentrations of 2H-GPC (Figure 3). For dispersions of 2H-GPC, the t_f/t_b ratio remained above 0.7, even though the bulk concentration of the lipid or the lipid to enzyme mole ratio was changed by more than two orders of magnitude or when the concentration of the alkylating agent was varied up to its solubility limit. The degree of protection for free PLA2 by monomeric 2H-GPC (at 5 μM, i.e., below its critical micelle concentration) was

similar to that for PLA2 bound to micelles of 2H-GPC. In addition, the monomeric analogue of 2H-GPC with a hexyl chain offered little or no protection from alkylation. Although 2H-GPC preparations showed no detectable impurities by thin-layer chromatography in several solvent systems, the presence of a potent inhibitor (0.1% impurity with $K_L = 0.001$ mole fraction) could account for a 50% decrease in t_f/t_b. Several other analogues of 2H-GPC, including 1H-GPC, had t_f/t_b ratios of <0.5, suggesting that they have higher affinity for the active site of PLA2 (data not shown). Taken together these studies demonstrate that 2H-GPC, either in aqueous solution or in micellar form, did not bind to the active site of either E or E*. This is a key property of a neutral diluent. The other required feature, the ability of PLA2 to bind to micelles of 2H-GPC, is demonstrated in the next section.

A few additional features of these protection studies may be noted. In these experiments, although the individual values of t_f and t_b are appreciably different for each of the three alkylating agents (bromooctanone, PNBr, PNBS), in all cases the t_f/t_b ratios remained the same. The constant ratio of t_f/t_b with changing bulk concentration of 2H-GPC not only established that this amphiphile had no detectable affinity for the active site of PLA2 but also established that the inactivation rate was independent of whether or not the enzyme was bound to the interface. As developed in the Appendix, this strongly suggested that binding of the enzyme to the micellar interface did not change the affinity of the alkylating agent for the enzyme's active site and vice versa. The implications of this lack of synergism are developed in the Discussion.

Binding of PLA2 to 2H-GPC. For the interpretation of results reported in this paper, it was necessary to show that PLA2 bound to micelles of 2H-GPC. This was ascertained by several techniques. Binding of PLA2 to an interface was most conveniently determined by monitoring the increase in the absorbance at 292 nm (Hille et al., 1983), or the increase in the fluorescence emission of Trp-3 at 333 nm (Jain et al., 1982, 1986b), or by resonance energy transfer to the dansyl group of HDNS present at the interface (Jain & Vaz, 1987). All such changes were observed on binding of PLA2 to micelles of 2H-GPC. For example, in the energy transfer experiments, the fluorescence intensity of HDNS in 2H-GPC micelles in the 450–550-nm region increased appreciably in the presence of PLA2. This increase was accompanied by a corresponding monotonic decrease in the fluorescence emission in the 333-nm region from Trp-3, and the energy transfer efficiency was >50% at >3 mol % HDNS in 2H-GPC micelles. Similarly, the binding of PLA2 to 2H-GPC was accompanied by an 8% increase in the absorbance in the 289-nm region characteristic for Tyr and Trp, and the difference spectrum was similar to that observed for other amphiphiles. Qualitatively, these results were similar to those observed with other zwitterionic amphiphiles such as 1-hexadecyl-*sn*-glycero-3-phosphocholine and hexadecylphosphocholine (Hille et al., 1983; Jain et al., 1986c). Such binding studies showed that the apparent dissociation constant for PLA2 bound in 2H-GPC micelles was about 0.25 mM, i.e., at >3 mM 2H-GPC virtually all PLA2 would be at the interface. Similarly, it was shown that the amount of 2H-GPC up to a mole fraction of 0.8 in DTPM dispersions did not appreciably change the binding characteristics of PLA2, i.e., the apparent dissociation constant for PLA2 on the DTPM interface, K_D, remained below 1 μM.

Binding of Ligands to PLA2 Bound to 2H-GPC. Having established that 2H-GPC functions as a neutral surface diluent, it became feasible to examine the ability of a variety of amphiphiles to protect E* from alkylation. Thus 2H-GPC served as an "inert" matrix in which to incorporate other agents that may interact directly with the active site of PLA2 bound to the same interface. Use of a concentration of 2H-GPC at >3 mM for all protection studies ensured that all PLA2 was bound to micelles, i.e., the interfacial equilibrium was in favor of the E* as shown by the direct binding studies. Under these conditions, even though the enzyme is in a rapid equilibrium between the E and E* forms, it is the protection of E* that is being monitored since the concentration of E is close to zero.

Table I: Compounds That Had No Effect at Mole Fraction 0.1 on Alkylation and on Interfacial Catalysis in the Scooting Mode at 0.3 mM DMPM and 0.6 mM $CaCl_2$, at 22 °C and pH 8.0

alkanols (C_6 to C_{14} including *cis*- and *trans*-tetradec-9-enol)
fatty acids (C_8 to C_{20} including 18:1, 18:2, 18:3, and 20:4), 7,7-dimethyleicosadienoic acid, and long-chain phenols
local anesthetics (dibucaine, tetracaine, procaine, and benzocaine)
antimalarials like mepacrine and quinacrine
ketamine, halothane, chloroform, carbon tetrachloride, and EMD 21657
indomethacin, cortisone and related antiinflammatory agents, alphaxalone, flufenamic acid, and phenothiazines
aristolochic acid and plastatin
butyrophenone (U10029A)
alkylammonium salts (C_{10} to C_{18}), spermine, putrescine, and polymyxin B
antibiotics: gentamycin, tobramycin, and streptomycin
peptide analogues of lipocortin

In principle, deoxycholate could be used instead of 2H-GPC as a matrix for the protection studies because it also offered little protection, i.e., t_f/t_b remained 0.85. However, use of deoxycholate posed other serious problems associated with its anionic character, its ability to bind calcium and thus change the free calcium concentration, its high critical micelle concentration (>2.5 mM), a changing intermicellar concentration in the presence of additives, and a dependence of the dispersity and polymorphism of comicelles on their composition (Nichols, 1988; Fullington, et al., 1990).

As summarized in Table I, many solutes present in the reaction medium did not offer any protection against alkylation of PLA2 in the aqueous phase (E form) or bound to dispersions of 2H-GPC (E* form). These include, mepacrine, aristolochic acid, tetracaine, dibucaine, AM-1, and 7,7-dimethyleicosadienoic acid, all of which have been previously reported to have a PLA2-inhibitory effect under certain assay conditions. Thus the compounds listed in Table I did not bind to the catalytic site of PLA2. These studies also confirmed our earlier results that these nonspecific inhibitors of PLA2 interfere only with the overall kinetics of PLA2 on interfaces where the binding affinity of the enzyme was relatively poor and that such effects are due to the modulation of the organization and dynamics of the interface, which reduces the fraction of the enzyme in the interface (Jain & Jahagirdar, 1985; Jain & Berg, 1989; Jain et al., 1984, 1989a,b,d).

Many solutes offered virtually complete (>95%) protection from alkylation. These include calcium, substrate analogues, products, and competitive inhibitors. The equilibrium dissociation constant for the binding of ligands to the active site of PLA2 were obtained by monitoring the protection from alkylation of the enzyme bound to micelles of 2H-GPC containing varying concentrations or mole fractions of the ligand. The design of the experiments for the determination of the equilibrium dissociation constant at the interface was based on the following relationship developed in the Appendix (compare eq B4):

$$1/(1 - t_0/t_L) = (K_L/X_I + 1)/(1 - k_L/k_0) \quad (1)$$

Here t_0 is the half-time for inactivation of E* by an alkylating agent in the absence of a ligand, and t_L is the half-time in the

Table II: Equilibrium Dissociation Constants[a] for Ligands Bound to the Catalytic Site of PLA2 on 2H-GPC Micelles[a]

complex	K_L	K_{MS} ($X_I(50)$)	K_P ($n_I(50)$)
E*Ca·deoxycholate	>4	0.26	
E*Ca·2H-GPC	>2.3	0.39	
E*Ca·1H-GPC	0.4		
E·Ca	0.28 mM		
E*·Ca		0.16 mM	
E*·DMPM	0.037		
E*DMPM·Ca	0.16 mM (from catalysis data)		
E*·DMPM (*sn*-1)	0.06		
E*Ca·DMPM (*sn*-1)	0.017	0.25 (0.1)	0.11 (0.18)
E*·DTPM	0.024		
E*Ca·DTPM	0.017	0.26 (0.076)	0.08 (0.19)
E*DTPM·Ca	0.11 mM		
E*·prod	0.026		
E*prod·Ca	0.14 mM		
E*Ca·prod	0.025	0.32	
E*Ca·HPOMe	0.04	0.68 (0.087)	0.11 (0.25)
E*Ca·DTPC	0.067		
E*·DTPC	>0.3		
E*Ca·MG-14 (*sn*-1)	>0.06		
E*·MG-14 (*sn*-3)	>0.1		
E*Ca·MG-14 (*sn*-3)	0.0011	0.38 (0.004)	0.036 (0.028)
E*·H-18	>0.06		
E*Ca·H-18	0.0016	0.45 (0.0045)	0.05 (0.03)

[a] The dissociation reaction is for the dissociation of the last species in the complex shown after the dot. The K_b values were determined by from the inactivation studies carried out in 9 mM EGTA or 1 mM $CaCl_2$ at pH 6.8. The $X_I(50)$ or $n_I(50)$ values were determined from experiments designed on the basis of eq 19 or 20, and the K_{MS} or K_P values were also calculated from the slope by using the K_b values. Products refer to both the products of hydrolysis of DMPM in a 1:1 mole ratio. Other abbreviations are given in the first footnote of this paper. The values in parentheses are for the mole fraction of amphiphile required for 50% inhibition of the rate parameter.

presence of a ligand (E*L form) at mole fraction X_I. Dissociation constants ($K_L = K_{Ca}$, K_S, K_P, or K_I) for E*L to E* + L were obtained as the X intercept ($-1/K_L$) in a plot of $1/X_I$ versus $1/(1 - t_0/t_L)$; k_L and k_0 are the intrinsic inactivation rate constants for the reaction of E*L and E* with the alkylating agent, respectively. It may be noted that here the subscripts 0 and L refer to the terms for alkylation of PLA2 at the interface with or without a ligand present. This is to distinguish from the subscripts f and b used earlier, which referred to the enzyme free in the aqueous phase or bound to the interface. However, as shown in the preceding section, PLA2 on 2H-GPC micelles does not have a ligand at the active site. Therefore t_b and t_0 are identical at the interface of such a neutral diluent.

As summarized in Table II, by a suitable combination of experimental conditions, this protocol was used to obtain the dissociation constants shown in Figure 1. For example, the dissociation constant for calcium bound to E* was obtained by comparing the half-time for alkylation of PLA2 on 2H-GPC micelles in the presence (t_L) and in the absence (t_0) of calcium at a given concentration. Similarly, for obtaining the dissociation constant for E*DTPM, the inactivation times in the presence of varying mole fractions of DTPM were compared with the inactivation time in the absence of DTPM. As shown in Figure 4, the plot of $1/(1 - t_0/t_L)$ versus $1/X_I$ for *sn*-3 DTPM dispersed in 2H-GPC (Scrutton and Utter plot) was linear ($r = 0.90$) with a X intercept at $-1/K_L$. The values of K_L for several amphiphiles obtained from such plots are summarized in Table II. In all cases, the values of k_L/k_0 for protection against alkylation in the presence of these amphiphiles were less than 0.03, i.e., the E*L complexes were not susceptible to alkylation as would be expected if the ligand was bound to the active site. These results were obtained with PNBr; however, in some cases protection studies were carried out with other alkylating agents, and the K_L or k_L/k_0 values did not change with the nature of the alkylating agent (data not shown).

For the interpretation of K_L values, it is also necessary to assume that the alkylation of PLA2 occurs on the bound form

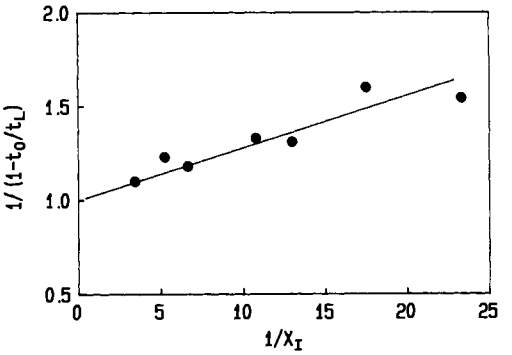

FIGURE 4: Scrutton and Utter plot for the protection of PLA2 on 2H-GPC micelles from alkylation in the presence of DTPM at 1 mM calcium. Other conditions as given in the legend to Figure 2.

of the enzyme, that the ligand binds to the E* form of the enzyme, and that the ligand bound to E* protected PLA2. Support for these assumptions, in addition to that already presented above, came from the observation that inhibitors like MG-14 protected PLA2 bound to 2H-GPC micelles, whereas this compound was considerably less effective on the enzyme in the aqueous phase (Yuan et al., 1990). The fact that the *sn*-1 enantiomer of MG-14 offered little protection also ruled out the possibilities that the effect of these additives is due to a change in the surface pH, charge profile, or the organization of the interface. Probably the strongest evidence in support of these assumptions came from the observation that PLA2 did not bind to vesicles of DTPC (Jain et al., 1982, 1986b) and the kinetics of alkylation were not altered in the presence of DTPC vesicles. This is because free enzyme cannot form the solitary E·DTPC complex in solution as the concentration of monomeric DTPC in the aqueous phase was very low. However in the presence of 2H-GPC micelles, DTPC protected PLA2 from alkylation with a value of K_L comparable to that for DTPM, i.e., 0.06 versus 0.02 (Table II). Under these conditions, the enzyme was bound to the DTPC + 2H-GPC mixed micelles, where the DTPC was able to express its intrinsic affinity by protecting the active site of the bound en-

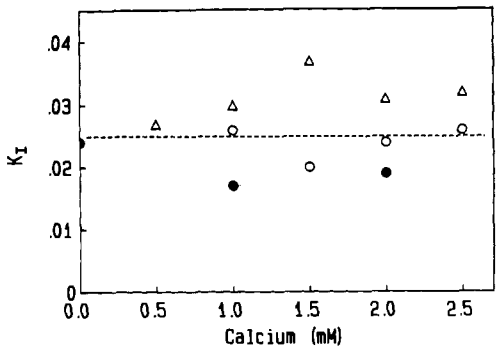

FIGURE 5: Dependence of K_I for DTPM as a function of calcium concentration. The values shown by triangles were obtained from the measurements of $N_S k_i$, and the values shown by open circles were obtained from the measurement of initial rates, v_0; closed circles represent the values obtained from the protection studies (cf. Scrutton–Utter plots as shown in Figure 4).

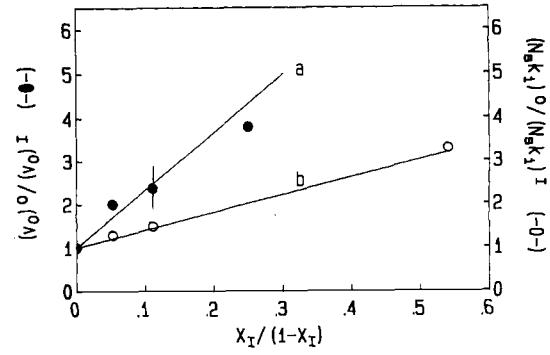

FIGURE 6: Dependence of $(N_S k_i)^0/(N_S k_i)^I$ (open circles) and $(v_0)^0/(v_0)^I$ (closed circles) on the varying mole fraction of DTPM codispersed with DMPM. Such plots are used to obtain the mole fraction of inhibitor required for 50% inhibition, and from these the values of K_{MS} and K_P are computed according to eqs A19 or A20 by using K_I values obtained from the protection experiments.

zyme. This observation also suggests that PLA2 in the aqueous phase does not "solubilize" a substrate molecule from bilayers.

*Dissociation Constant for Calcium Bound to E and to E** (K_{Ca}). Calcium is an obligatory cofactor for interfacial catalysis by PLA2, and this cation is coordinated to Asp-49, which is near His-48 (Verheij et al., 1980; Kuipers et al., 1990). Calcium is not required for the binding of PLA2 to DTPM vesicles (Jain et al., 1986b); however, as shown in Figure 2, calcium protects PLA2 from alkylation in a concentration-dependent manner. Similar results were obtained with PLA2 in the presence of 2H-GPC micelles. As summarized in Table II, the dissociation constant for the E*·Ca complex (0.16 mM, row 5) on 2H-GPC micelles was only slightly smaller than the value of 0.28 mM (row 4) obtained for the dissociation of calcium from the free enzyme in the aqueous phase. In both cases, the k_L/k_0 ratio approached <0.03 at extrapolated infinite concentration of calcium (cf. eq 1). This indicated that the affinity of PLA2 for calcium increased only slightly on binding to 2H-GPC micelles and calcium fully protected His-48 from alkylation.

Dissociation Constants for the Substrate Analogues (K_S) and the Products (K_P). Values of K_L for *sn*-3 DTPM and *sn*-3 and *sn*-1 DMPM (Table II, rows 6–12) appeared relatively insensitive to the presence of the ether versus ester group or the chirality at the *sn*-2 position of the amphiphile, i.e., the respective K_L values were within a factor of two. Protection studies with the other ligands in the presence of calcium were also carried out in order to obtain information about possible synergism in the binding of calcium and the substrate analogues. There is no theoretical difficulty in these experiments as long as the calcium is present below its saturating concentration, and thus there is adequate dynamic range for the inactivation half-times. Such studies showed that the binding of the substrate analogues to the catalytic site did not change noticeably on the binding of calcium. For example, as summarized in Figure 5, K_S (i.e., K_L for DTPM) did not change significantly with changing calcium concentration. Considering all the errors intrinsic in these measurements, our estimate was that the uncertainty in the observed value of K_L was less than 50%. Thus for all practical purposes K_L for analogues of DMPM were in the range 0.02–0.03 in the presence of calcium, and the values were only slightly higher (0.035–0.06) in the absence of calcium. It may be emphasized here that the true K_S value for the substrate, *sn*-3 DMPM, in the presence of calcium cannot be measured because it would be hydrolyzed, and the values given above are approximations with the assumption that the *sn*-1 DMPM or DTPM mimic the substrate at the catalytic site. The K_P value for the mixture of the products of hydrolysis (lysophospholipid + fatty acid) in 1:1 mole ratio was also 0.025, which did not change by varying the concentration of calcium (data not shown).

Dissociation Constants for Inhibitors (K_I). As summarized in Table II, the K_I values for several inhibitors differed significantly. In marked contrast to the K_L values for DTPM and DMPM, the K_I (= K_L) values for the two competitive inhibitors, an *sn*-2 phosphonate MG-14 and an *sn*-2 amide H-18, decreased considerably in the presence of calcium. The significance of these values can be judged from the fact that the K_I values of the various amphiphiles were different by more than 500-fold, depending on their structure and chirality. It is intriguing that the K_I increased appreciably in the presence of calcium and that a large difference in K_I values was observed for the two enantiomeric analogs of MG-14. As discussed next, the values of K_I measured from the protection studies under equilibrium conditions were quantitatively consistent with the kinetic rate and equilibrium parameters obtained from the study of kinetics of hydrolysis in the scooting mode in the presence of these inhibitors.

The Kinetics of Inhibition in the Scooting Mode. The kinetics of hydrolysis of DMPM vesicles in the presence of competitive inhibitors is given by (Berg et al., 1991)

$$(v_0)^0/(v_0)^I = 1 + [(1 + 1/K_I)/(1 + 1/K_{MS})][X_I/(1 - X_I)] \quad \text{(A19)}$$

$$(k_i N_S)^0/(k_i N_S)^I = 1 + [(1 + 1/K_I)/(1 + 1/K_P)][X_I/(1 - X_I)] \quad \text{(A20)}$$

Equation A19 is appropriate with large DMPM vesicles where the initial velocity in the absence $(v_0)^0$ and presence of an inhibitor $(v_0)^I$ can be measured (Berg et al., 1991). In small vesicles, the enzymatic depletion of substrate occurred so rapidly that v_0 can not be easily measured. Rather, the first-order relaxation constant, k_i, is obtained from the analysis of the entire progress curve. In eq A20, N_S is the average number of phospholipids in the outer monolayer of a vesicle, and $(N_S k_i)^0$ and $(N_S k_i)^I$ are the values of these rate parameters in the absence and presence of an inhibitor, respectively. From experiments based on these equations, we obtained values of mole fractions of inhibitors required for a 50% reduction in v_0 [= $X_I(50)$] or in $N_S k_i$ [= $n_I(50)$]. Since these studies could be carried out with several inhibitors with a wide range of K_I values, it was possible to obtain and cross-check the values of K_{MS} and K_P determined by other methods. Also by measuring the v_0 values in the presence of a neutral diluent, it was possible to determine the value of the interfacial K_{MS} for DMPM.

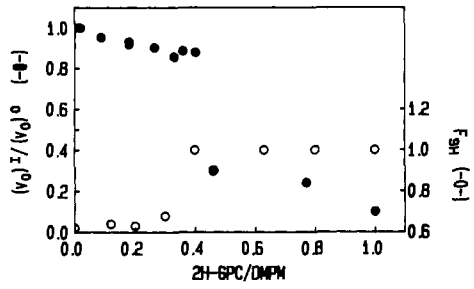

FIGURE 7: Dependence of the initial rate v_0 of hydrolysis at 2.5 mM $CaCl_2$ (closed circles) and the fraction of the substrate hydrolyzed by PLA2 at 0.5 mM $CaCl_2$ as a function of the mole fraction of 2H-GPC in DMPM vesicles (open circles).

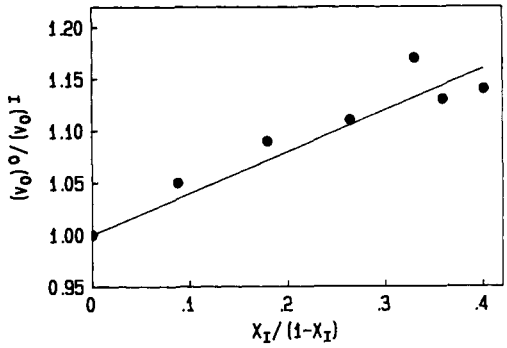

FIGURE 8: Plot of $(v_0)^0/(v_0)^I$ versus $X_I/(1 - X_I)$ for the hydrolysis of DMPM vesicles in the presence of varying mole fraction (X_I) of 2H-GPC. Such plots were used to obtain the value of K_{MS}.

As shown in Figure 6, the ratios of the $N_S k_i$ values in the absence and in the presence of an inhibitor like DTPM were linear (cf. eq A20) with the mole ratio of the inhibitor, $X_I/(1 - X_I)$. From the slope of this line, we obtained the value of $n_I(50)$. Similarly, at higher calcium concentration, which cause the vesicles to fuse into larger ones, the initial rates of hydrolysis, v_0, were measured, and then $X_I(50)$ values were calculated. From the value of $n_I(50)$ for DTPM obtained from the kinetic studies and the value of K_I for DTPM obtained from the protection experiments (Table II), the value of $K_P = 0.08$ was calculated according to eq A20. Similarly, from the vaue of $X_I(50)$ and K_I for DTPM, the value of $K_{MS} = 0.26$ was calculated according to eq A19. As developed in the Discussion, the observation that the mole fraction of an inhibitor required for 50% inhibition (Table II) was different if the inhibition was measured under the initial rate conditions [$X_I(50)$] or from the first-order plots [$n_I(50)$] was a key prediction for competitive inhibition (Berg et al., 1991). As described above, the values of K_{MS} and K_P could be obtained from experiments based on eqs A19 and A20 with several different inhibitors. The results summarized in Table II showed that the average value of K_{MS} was 0.4 and the average value of K_P was 0.07. A larger scatter in the values of K_{MS} and K_P values calculated in Table II was in part due to the data processing strategy. Statistically speaking, all the errors in experimentally measured $X_I(50)$, $n_I(50)$, and K_I values are assigned to K_P or K_{MS}. Since K_P and K_{MS} values could be obtained from the protection and inhibition kinetic data for several inhibitors, their average values would be ultimately more representative. As discussed next, values for K_{MS} and K_P were also obtained by a third independent method.

Yet another cross-check on the consistency of the values of K_{MS} and K_P came from (Berg et al., 1991)

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

The value of the ratio of $k_i N_S/v_0 = 0.13$ was obtained by Berg et al. (1991) from the progress curves for the hydrolysis of DMPM vesicles, which agrees reasonably well with a value of 0.2 calculated from the average values of K_P and K_{MS} given in Table II.

Hydrolysis of DMPM Vesicles in the Presence of 2H-GPC. The usual method for determining K_{MS} is based on the measurement of initial rates at varying substrate concentrations. This could be achieved for interfacial catalysis monitored in the presence of a neutral diluent like 2H-GPC. According to eq A19, the values of v_0 obtained in the presence of a neutral diluent could be used directly to obtain the value of K_{MS}. Since the value of K_L was >2.3, 2H-GPC would have little effect on the calculated values of other more potent ligands, such as the substrate, that bind to the active site. Thus the use of 2H-GPC as a neutral surface diluent enabled a kinetic study of PLA2 on DMPM vesicles in the scooting mode to be carried out in which 2H-GPC was added to vesicles in order to systematically change the surface concentration of the substrate that the bound enzyme "sees".

As shown in Figure 7 (open circles), in codispersions containing >30% 2H-GPC, vesicles were disrupted and all of the substrate present in the reaction mixture was hydrolyzed. However, if the mole ratio of 2H-GPC was kept below 0.3, only 63% of the total substrate was hydrolyzed by an excess of PLA2. This showed that the integrity of the DMPM vesicles was retained in the presence of <30 mol % 2H-GPC. It was intriguing that v_0 for DMPM comicellized with 2H-GPC was considerably smaller than it was for DMPM in bilayers. The broad implications of this observation were pursued elsewhere (Jain et al., 1991b), and the results are consistent with the suggestion that the hydrolysis on these micelles was limited by the rate of replenishment of the substrate rather than by the catalytic step.

As shown in Figure 7, below 30 mol % 2H-GPC, the initial rate of hydrolysis decreased by about 10%. As plotted in Figure 8, below 25 mol % 2H-GPC in DMPM vesicles, eq A19 was obeyed, and the slope of this line is 0.37. On the basis of the protection studies, the estimated K_L was >2.3 for 2H-GPC, which gives $K_{MS} = 0.3$ mol fraction (eq A19). We also carried out similar experiments with deoxycholate as a neutral diluent, where the slope for eq A19 was 0.2, which yields K_{MS} 0.26 if K_d for deoxycholate is >4. Although the scatter in the values of K_{MS} was significant, by at least three different protocols the value of K_{MS} converged in the range of 0.3–0.4 mol fraction. It may also be emphasized here that, in most relations, K_{MS} appeared as the $K_{MS}/(1 + K_{MS})$ term; therefore, any change in K_{MS} reflects a relatively smaller change in this term.

Discussion

The results reported here provide substantial support for the kinetic scheme in Figure 1 for interfacial catalysis by pig pancreatic PLA2. As was the case with earlier versions (Jain et al., 1986a–d), this scheme explicitly distinguishes the binding of the enzyme to the interface (the E to E* step) from the binding of the enzyme in the interface to the ligand in the interface (E* + L to E*L step). However, in this new version, the interfacial equilibria are explicitly elaborated. The dissociation constants K_I, K_P, K_{Ca}, and K_{MS} were obtained from the protection and the kinetic studies, and these values relate to the complexes that are formed during the catalytic cycle. Therefore, along with the rate constant k_2, these equilibria describe the whole Michaelis–Menten space intrinsic in the scheme in Figure 1 of Berg et al. (1991) or in the scheme in

Figure 1 of this paper as elaborated by eqs A12, A15, A19, A20, and A21 from Berg et al. (1991). The general significance of these results is discussed below.

The method for obtaining K_L values should be of general use because all known PLA2s have histidine at the catalytic site (van den Bergh et al., 1989), and the protocol developed in this paper can be applied to impure enzyme preparations. This protocol was adapted from the method developed for determining equilibrium dissociation constants for water-soluble ligands with water-soluble enzymes (Baker, 1976; Scrutton & Utter, 1965; Volwerk et al., 1974). As discussed in the Appendix, there are no theoretical difficulties in such an adaptation. If we assume that the value of $t_f/t_b = 0.7$ for 2H-GPC (Figure 3) is due to its weak affinity for the active site or due to the presence of an impurity, the corrected value of K_L would be obtained by multiplying the observed values by 0.7. Although there was typically less than 30% uncertainty in the value of K_L obtained by the protection studies for each of the inhibitors, the values of K_{MS} and K_P have a somewhat wider scatter because of the data processing strategy. So far there is no other method to determine dissociation constants for the equilibrium reactions in the interface, and therefore no set is available for comparison. However, the results reported here are in complete accord with the expectations based on the kinetic results, and they are internally consistent over the whole Michaelis–Menten space. It is also of general interest to note that the ratio of K_{MS}/K_S is given from the definitions presented in the appendix to Berg et al. (1991):

$$K_{MS}/K_S = (k_2 + k_{-1})/k_{-1} = 12 \quad (2)$$

The experimental value of 12 for this ratio determined in this paper led to the prediction that $k_2 > 10k_{-1}$, which was also in accord with the heavy atom isotope effect reported in the preceding paper in this issue (Ghomashchi et al., 1991).

Broader consequences of the results reported in this paper fall into the following categories. Besides providing a basis for the dissection of the interfacial rate constants (Berg et al., 1991), as developed in sequel the results described here help in (a) evaluation of active-site-directed inhibitors, (b) identification of a surface diluent, and (c) understanding the role of calcium in the binding of ligands to the catalytic site of PLA2.

Evaluation of Active-Site-Directed Inhibitors. Inhibitors of PLA2 have been a topic of some concern since such compounds may be useful for the treatment of inflammatory disorders in man by limiting the availability of arachidonic acid in cells for the biosynthesis of eicosanoids. A shift in the E to E* equilibrium by lipophilic additives has far reaching implications in the evaluation of inhibitors of PLA2 (Jain & Jahagirdar, 1985). Since PLA2 binds tightly to DMPM vesicles, the E to E* equilibrium was not noticeably perturbed by even 50 mol % of most additives, which obviates possible complications arising from the changing quality of the interface. De Haas et al. (1990) have circumvented the problems associated with a shift in the E to E* equilibrium by choosing a saturating bulk lipid concentration of mixed micelles. However, this does not appear to be the case for several other reports in the literature. For example, in their studies with mixed micelles of Triton X-100, Yu et al. (1990) have chosen the bulk lipid concentration considerably below the saturating concentration. As expected, under these conditions an anomalous kinetic behavior would arise if the binding of the enzyme to the Triton X-100 micelles is promoted by changing the concentration of the additive. Similar effects are predicted if the inhibitor modulates the surface charge density of the interface and thus shifts the E to E* equilibrium.

By studying the action of PLA2 in the scooting mode in which all of the enzyme remains tightly associated with the interface, it has been shown that phosphonate-containing phospholipid analogues function as true competitive inhibitors of PLA2 by binding tightly to the catalytic site on the enzyme (Jain et al., 1989). This conclusion is further extended by the protocols based on the ligand-mediated protection of the active site. These protocols have general applicability for the quantitative evaluation of neutral surface diluents, competitive inhibitors, products, substrate analogues, cofactors, and other ligands that bind to the active site of PLA2. The fact that most ligands offer a virtually total protection from inactivation (i.e., $k_L/k_0 < 0.03$) provided a wide dynamic range over which the protective effect could be studied. Moreover, the binding of PLA2 to the interface of a neutral diluent or other nonspecific interactions of solutes with the protein have little effect on the rate of alkylation (Jain & Jahagirdar, 1985; Jain et al., 1984, 1986d, 1989). Indeed, a lack of protection by the putative inhibitors listed in Table I illustrates the difficulties encountered in characterizing inhibitors of PLA2, and such compounds can now be unequivocally categorized as nonspecific modulators of the substrate interface.

Kinetics in the scooting mode (Berg et al., 1991) are capable of distinguishing competitive inhibitors from other types. Equations A19 and A20 distinguish a competitive inhibitor from a noncompetitive inhibitor, because competitive inhibitors change the effective K_{MS} and K_P, but not the k_{cat} value, and thereby give different $n_I(50)$ and $X_I(50)$ values, and their ratios are predicted by eq A21. Other types of inhibitors will change k_{cat} as well and would lead to different results. Thus for all competitive inhibitors codispersed with the substrate, the following ratio should hold [appendix in Berg et al. (1991)]:

$$\frac{[1/X_I(50) - 1]}{[1/n_I(50) - 1]} = \frac{(1 + 1/K_P)}{(1 + 1/K_{MS})} = v_0/N_s k_i \quad (A21)$$

This was indeed the case for the inhibitors listed in Table II, where the ratio $v_0/N_S k_i$ based on the first term of eq A21 for strong inhibitors was about 8, which compares very favorably with the ratio of 7 based on the middle term with the value of $K_P = 0.023$–0.04 and $K_{MS} = 0.3$. Similarly, the $v_0/N_S k_i$ ratio of 7.5 was obtained by fitting the whole progress curve (Berg et al., 1991). This ratio was somewhat lower for weaker inhibitors, probably because they have other effects at the higher mole fractions used, and such effects are probably related to the change in the organization of the interface.

The theoretical basis for characterization of inhibitors as described here also has some advantages over other proposals because the mechanistic basis of Michaelis–Menten parameters is better understood. Recently, de Haas et al. (1990) published a detailed analysis of the kinetics of inhibition of PLA2 on the mixed micelles of substrate with deoxycholate. The inhibitory power (Z) of an inhibitor was expressed with respect to an arbitrarily chosen reference state for an inhibitor that has a K_I value equal to the K_{MS} value for the particular substrate being used. Thus an inhibitor with $K_I > K_{MS}$ would have a negative inhibitory power even though it reduces the enzyme velocity. Although the basic form of their equations (variations on the Dixon plot) was similar to ours within certain limits, it is probably important to recognize the key differences. The design of inhibition experiments as described by de Haas et al. (1990) was based on the fact that in micelles one has to work at high substrate concentration so as to assure that all the enzyme was in the E* form. Furthermore, by varying the mole ratio of inhibitor to substrate, while keeping the sum of the moles of inhibitor and substrate constant, it was assured

that the spurious effects (dispersity, size, surface dilution) of changing interfacial concentrations were minimized. Also, in these experiments one had to match an inhibitor with a structurally similar substrate so that the "quality of the interface", including the number of molecules per unit area, was not altered. With these precautions, one obtained the relative inhibitory power of an inhibitor, which also depended on the nature of the substrate and its mole fraction in the interface. The competitive mechanism for inhibition was postulated only on the basis of the structural similarity with the substrate. In contrast, the present study provides a method for obtaining the value of K_I and for establishing the competitive nature of inhibition by the kinetic and protection protocols. Generally speaking, K_I values for an inhibitor can be compared across the whole spectrum of the experimental conditions. Since K_I values relate to the distribution of the inhibitor between the active site of the enzyme and the bilayer, under certain conditions the interface could influence the value of K_I; however, such nonspecific effects are expected to be minor. Finally, it may also be emphasized that the kinetics in the scooting mode can be monitored on the same interface in the presence of PLA2 from different sources (Jain et al., 1991a). Therefore the kinetic protocols developed above can be adopted without having to match the quality of the interface for each enzyme.

Identification of a Neutral Surface Diluent. In this paper criteria were identified for a neutral surface diluent. Several dozen amphiphiles were examined for their ability to protect His-48 of PLA2 against covalent modification and for their ability to inhibit the hydrolysis of DMPM vesicles. While PLA2 bound to micelles of most of these amphiphiles (Jain et al., 1986c), 2H-GPC was close to an ideal neutral diluent since it offered little protection against alkylation ($K_L > 2.3$ mole fraction). Thus 2H-GPC bound poorly to the active site of PLA2 at the interface, and it did not inhibit catalytic turnover in the scooting mode. Such a functional separation between the binding of the enzyme to the interface and the binding of a ligand to the enzyme at the interface is the hallmark of all the kinetic schemes for interfacial catalysis that we have elaborated (Jain et al., 1986a–d; Jain & Berg, 1989; this series of papers). Thus interfacial binding of PLA2 does not require occupancy of the active site. Similarly, alkylated PLA2 bound to micelles of alkylphosphocholine (Volwerk et al., 1974) and to vesicles of DTPM (Jain et al., 1991a), and high-affinity inhibitors like MG-14 or H-18 did not promote binding of PLA2 to DTPC bilayers to which PLA2 bound with very low affinity ($K_D > 10$ mM). This dissection between the binding to the interface and the binding to the active site was again brought into focus by the observation reported here that PLA2 did not bind to and therefore was not protected by vesicles of DTPC. On the other hand, the K_I for DTPC dispersed in 2H-GPC was almost the same as the K_I for DTPM (Table II). These observations provided virtually absolute evidence for a functional separation of the E to E* step from the E* + L to E*L step, and they called in question any hypothesis that required modification of the active site or the binding of ligands to the active site of PLA2 (E* + S to E*S step) as a prerequisite for the binding of the enzyme to the interface (E to E* step).

A rigorous analysis of interfacial catalysis by PLA2 in terms of the interfacial rate and equilibrium parameters is more difficult than in the case of enzymes that work on water-soluble solitary substrates because the concentration of substrate in the vesicle surface could not be readily manipulated and the values of interfacial rate constants could not be determined by the usual methods. This barrier has been surmounted by developing and characterizing a true neutral surface diluent that conformed to the theory developed in the appendix of Berg et al. (1991). In this context, a neutral surface diluent is defined as an amphiphilic compound that forms dispersions like micelles or bilayers to which PLA2 binds and that is incorporated into substrate vesicles without perturbing the E to E* equilibrium. However, a neutral diluent has little affinity for the catalytic site of the E or the E* forms of the enzyme. In this definition, it is implied that binding of PLA2 to the interface is a distinctly different and independent step from the binding of a ligand to the active site of free or bound PLA2 (Jain et al., 1986d). A neutral diluent is neither a substrate nor an inhibitor of PLA2, but it changes the surface concentration (mole fraction) of other species that participate in the catalytic cycle at the interface. Such a compound will necessarily effect the position of the various interfacial equilibria which are explicitly shown in the scheme in Figure 1. Such a shift in turn would influence the rate parameters for interfacial catalysis by PLA2. Thus *a neutral diluent can alter the mole fraction of the reactants or products in the interface without influencing the E to E* equilibrium; it has no affinity for the catalytic site on the enzyme; and it does not change the gross morphology of the interface so as to minimize the effects associated with the substrate replenishment.*

Phospholipids dispersed in detergent micelles have been extensively used for the study of the kinetics of hydrolysis. In this context, Dennis (1983) has elaborated the concept of surface dilution, which was based on distinctly different criteria from those we have used for a neutral diluent like 2H-GPC. For the use of Triton X-100 as a surface diluent in mixed micelles with phospholipids, they have shown that PLA2 does not bind to Triton X-100 micelles in the absence of phospholipids. Thus, for the interpretation of the kinetics, it was assumed (Hendrickson et al., 1984a,b) that the composition and therefore the binding properties (for the E to E* step) of the mixed micelles changed with increasing mole fraction of the detergent. While this appears reasonable at first glance, for the interpretation of the overall kinetics it was also implicitly necessary to consider additional key factors that control the E to E* step (Jain & Berg, 1989). First, although PLA2 does not bind to Triton X-100 micelles, it is necessary to assume that the composition of the enzyme-containing micelles is the same as the composition of the mixture. Second, it is assumed that the effect of these changes in the composition of the mixed micelles on the E to E* equilibrium can be predicted by a simple hyperbolic relationship. Third, for the interpretation of the kinetic data from mixed micelles, it is also necessary to take into consideration the rate of replenishment of the substrate in a micelle as elaborated in detail elsewhere (Jain et al., 1991b). While the rate of intermicellar exchange of short-chain amphiphiles is rapid because their aqueous concentration is in the millimolar range, replenishment of the long-chain substrate in Triton X-100 mixed micelles probably occurs almost exclusively by fusion–fission of micelles. These rates are reasonably slow (Nichols, 1988), and the rate is expected to depend on the composition as well as the number of micelles. Thus the concentration dependence of the initial rate of hydrolysis would arise from a shift in the E to E* equilibrium with the increasing number of micelles and is due to an increased rate of replenishment of the substrate on the enzyme-containing micelles. This situation was underscored by the results shown in Figure 7, where the rate of hydrolysis decreased sharply as soon as the vesicles are micellized. Even though the total bulk concentration of DMPM remained constant and all the PLA2 was bound to the interface, the rate of hydrolysis on each micelle was effectively reduced because

there were fewer substrate molecules per micelle and the rate of replenishment dropped. This was not the case in vesicles that have considerably more substrate molecules in each aggregate. These arguments follow directly from a key axiom of enzymology that the rate of an enzyme-catalyzed reaction depends on the concentration of the substrates that the enzyme "sees". We are pursuing a rigorous proof for the hypothesis that under certain conditions in micelles the rate of hydrolysis could be limited by the rate of substrate replenishment (Jain & Berg, 1989; Jain et al., 1991b).

Role of Calcium Binding in the Stabilization of the Transition State. The results reported in this paper demonstrate that calcium is not required for the binding of PLA2 to the interface (E to E*), and calcium is probably not required for the binding of the substrate to the enzyme at the interface (E* + S to E*S step). The value of K_{Ca} = 0.16 mM for E* did not change significantly in the presence of the substrate analogues DTPM and *sn*-1 DMPM (Table II), which confirms similar conclusions based on the kinetics of hydrolysis (Volwerk et al., 1979; Teshima et al., 1989). The apparent K_{Ca} for the hydrolysis of DMPM vesicles (Berg et al., 1991; Jain et al., 1991b) is also 0.17 mM. This implies that the binding of the substrate and calcium is not synergistic, which was also supported by the protection experiments. For example, results summarized in Table II showed that the K_S for the substrate analogues and K_P did not change by more than a factor of two in the presence of calcium. Similarly, the value of K_S for the substrate *sn*-3 DMPM in the absence of calcium was identical with the value of K_S for the substrate analogues in the presence or in the absence of calcium. Interestingly, both enantiomers of DTPM and DMPM bound to the enzyme with similar affinities, K_S = 0.03, as also concluded with studies on water-soluble substrate analogues (Volwerk et al., 1979).

The role of calcium in catalysis by PLA2 has been articulated by Verheij et al. (1980, 1981) and Kuipers et al. (1990) as a cofactor that participates in the formation of the transition state. Recent structural data support this notion (Scott et al., 1990; Thunnissen et al., 1990). Thus the *sn*-2 carbonyl group of the substrates with the correct stereochemistry could form the suitable ligand for coordination to calcium in the transition state. Additional support for this conclusion came from the observation that the K_L values for two potent inhibitors of PLA2, with an amide (H-18) and a phosphonate (G-14) at the *sn*-2 position, were appreciably lowered in the presence of calcium, i.e., calcium promoted their binding. Under these conditions the *sn*-1 enantiomer of MG-14 was more than 50-fold weaker than its *sn*-3 enantiomer as a competitive inhibitor for interfacial catalysis (Jain et al., 1989) and also in the protection studies (Table II). Since MG-14 is a transition state analogue, it would appear that as the geometry at *sn*-2 position approaches the transition state, the stabilization by calcium becomes more apparent, and the strength of such interactions depends on the chirality of the glycerol backbone of the inhibitor.

The role of calcium in the stabilization of the transition state as suggested above is in apparent contradiction with the observation that the K_{Ca} for group II PLA2s depended on the presence of substrate (Wells, 1972; Teshima et al., 1989). This can, however, be readily rationalized by the fact that it is not the substrate per se but the conformation of the substrate near the transition state that is liganded to calcium. Thus the transition from E* + S to E* + P in the catalytic cycle can be viewed as a series of discrete steps where the most stable ground state for different ligands may be at different points along the reaction coordinates. X-ray crystallographic data show that calcium in PLA2 is bound by seven ligands: two oxygens from the carboxylate of Asp-49, three carbonyl oxygens from peptides bonds of Tyr-28, Gly-30, and Gly-32, and two water molecules (Dijkstra et al., 1983, Renetseder et al., 1985). Binding of an inhibitor molecule changes only the binding of the two water ligands to the calcium (Scott et al., 1990; Thunnissen et al., 1990). It is likely that the initial binding of the substrate to PLA2 to form the E*S complex occurs through the H-bonding of *sn*-3 phosphate to Tyr-69 [e.g., see Kuipers et al. (1990)]. As a next step in the calcium-containing PLA2, substitution of the two water molecules to form the stable transition state would require two appropriately disposed monodentate ligands. The ligand from the *sn*-2 position of the substrate (polarized carbonyl group) in the transition state or such inhibitors could substitute for one of the water molecules and the *sn*-3 phosphate substitutes for the other. However, substitution of both water molecules may not be obligatory with an inhibitor. On the basis of the data summarized in Table II, it can be calculated that the energy gain in the formation of E*DTPM complex is about 2.1 kcal/mol; and the formation of this complex does not depend on the stereochemistry at *sn*-2 position and does not require calcium. On the other hand the transition state analogue MG-14 in the active site is stabilized further by 1.5 kcal/mol, and the formation of this complex requires appropriate chirality at *sn*-2 position and the presence of an oxygen ligand that can coordinate to calcium. It is interesting to note that MG-14 does not bind to PLA2 in the absence of calcium, presumably because in this case the phosphonate and phosphate groups of MG-14 are liganded to the calcium (Scott et al., 1990). Collectively, these arguments have significant implications on the design of transition state analogues as inhibitors of PLA2 as they imply that under certain conditions the *sn*-3 phosphate and an *sn*-2 oxygen ligand may not be required simultaneously for the binding of an inhibitor to the catalytic site. Indeed, we have designed potent inhibitors of PLA2 without a calcium-binding ligand in the *sn*-3 position (to be published).

To recapitulate, the results summarized in this paper provided a method for resolving the interfacial equilibrium dissociation constants for calcium, substrate, products, and inhibitors bound to PLA2 at the interface (E* form), and they also provided a kinetic basis for unequivocally distinguishing competitive inhibitors from other types. The fact that K_{MS} is close to the maximum possible substrate concentration affirms the possibility that potent inhibitors can be designed. Also, the fact that the rate of alkylation of E and E* is approximately the same suggests that the binding of PLA2 to the interface is most probably not accompanied by a conformational change. These results provided unequivocal evidence for the hypothesis that the catalytic and the interfacial binding steps are kinetically and functionally distinct, which led to determination of the rate and equilibrium parameters (Berg et al., 1991) and to mechanistic insights into substrate specificity (Ghomashchi et al., 1991), catalytic turnover (Berg et al., 1991), interactions leading to stabilization of the transition state, the role of a neutral diluent, and an understanding into the role of interfacial mass transfer and mass balance processes in the kinetics of interfacial catalysis on micelles.

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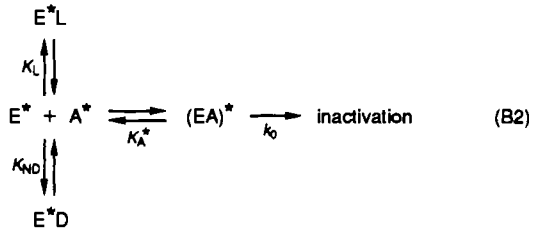
Appendix

Neutral Diluent. The conceptual basis for the protection experiments as described by Scrutton and Utter (1965) will

be extended below for the interfacial reactions. The reaction scheme for the alkylation of enzyme free in solution can be written as

$$E + A \underset{K_A}{\rightleftharpoons} EA \xrightarrow[k_0]{} \text{inactivation} \qquad \text{(B1)}$$

Here EA is a pre-equilibrium complex with alkylating agent bound at the catalytic site before chemical modification takes place. To establish the neutral diluent, this scheme should be compared to the situation where the diluent has been added in sufficient amount to bind all the enzyme at the interface, as in



(B2)

where E* denotes enzyme at the interface and A and A* denote alkylating agent in solution and in the interface, respectively. If the diluent D can bind at the catalytic site, the complex E*D is protected from alkylation. The corresponding dissociation constant K_{ND} refers to the effective concentration (mole fraction) in the interface. The reaction scheme also includes a ligand L present in the interface that can bind to the catalytic site.

The relationship between the concentration of alkylating agent in the interface, c_A^*, and free in solution, c_A, is determined by the partition coefficient P such that $c_A^* = Pc_A$. Furthermore, if there is no synergism between the binding of the alkylating agent at the catalytic site and the surface binding of the enzyme, it must hold that $K_A^* = PK_A$. This is a thermodynamic result based on the fact that the binding free energy is a state function independent of the reaction pathway. In other words, if the surface binds E* and E*A equally well, surface binding will not influence the binding probability for the agent and $K_A/c_A = K_A^*/c_A^*$. This is expected since the catalytic site is well separated from the interfacial binding surface. Thus, although the local concentration of alkylating agent in the interface, c_A^*, is much larger than that in solution (if $P \gg 1$), this is exactly compensated by the difficulty of removing the alkylating agent from the interface for binding to the site on the enzyme.

If the binding steps are equilibrated on the time scale of inactivation and if the pre-equilibrium binding of alkylating agent is not saturated [which is the case in this study since the inactivation rates are proportional to the solution concentration (c_A) of alkylating agent below its solubility limit], one finds for the ratio of the half-times of inactivation for free enzyme (eq B1) and interface-bound enzyme (eq B2 in the absence of a competing ligand L) that

$$t_f/t_b = (K_A c_A^*/K_A^* c_A)/(1 + 1/K_{ND}) = 1/(1 + 1/K_{ND}) \qquad \text{(B3)}$$

As discussed above, the factor $K_A c_A^*/K_A^* c_A$ will be exactly equal to 1 if there is no synergism in the binding of alkylating agent and the enzyme binding to the interface. The finding that t_f/t_b is independent of the type of alkylating agent used brings further support that the factor $K_A c_A^*/K_A^* c_A$ does not contribute to t_f/t_b. Equation B3 requires that t_f and t_b are measured with the same concentration, c_A, of alkylating agent free in solution; this may be an important consideration for very large additions of diluent when the partitioning into the micelles can reduce the free concentration of alkylating agent, but as long as t_f/t_b remains independent of the amount of diluent added (see Figure 3) this is not a problem.

The scheme assumes that the binding of A and D at the catalytic site are mutually exclusive. It can easily be extended to also include the possibility of simultaneous binding; however, as long as the pre-equilibrium binding of alkylating agent does not saturate, this does not influence the resulting t_f/t_b. Thus, when t_f/t_b is close to 1, K_{ND} is large and the ligand binds very weakly to the catalytic site, as expected for a neutral diluent.

Dissociation Constants. In order to determine the dissociation constant of another ligand L in the interface of the neutral diluent, eq B2 will be considered. Thus one finds that the ratio t_0/t_L of the half-times of inactivation for the surface-bound enzyme in the absence and presence of the ligand L can be expressed as

$$\frac{1}{1 - t_0/t_L} = 1 + \frac{K_L}{X_L}\frac{1 + 1/K_{ND}}{1 - K_L/K_{ND}} = 1 + K_L/X_L \qquad \text{(B4)}$$

The last equality in eq B4 holds if D is a true neutral diluent with K_{ND} very large. The expression considers a ligand L that is similar to D and replaces D in the surface structure such that $X_L + X_{ND} = 1$. If L is dispersed without much change in the surface density of the diluent D, it is better to use the concentration c_L rather than the mole fraction X_L as the effective concentration. If the bound complex E*L is not totally protected from alkylation, there will be an extra factor in eq B4 giving eq 1 of the main text. However, since $1/(1 - t_0/t_L)$ plotted as a function of $1/X_L$ extrapolates to 1 at infinite X_L for the compounds described in this study (see Figure 4), they all afford (nearly) complete protection.

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