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Basis for the Anomalous Effect of Competitive Inhibitors on the Kinetics of Hydrolysis of Short-Chain Phosphatidylcholines by Phospholipase A_2[†]

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ABSTRACT: The effect of four specific competitive inhibitors on the kinetics of hydrolysis of short-chain diacyl-*sn*-glycero-3-phosphocholines below their critical micelle concentrations was examined. The kinetics of hydrolysis of short-chain substrates dispersed as solitary monomers were generally consistent with the classical Michaelis–Menten formalism; i.e., hydrolysis began without any latency period, the steady-state rate was observed at higher substrate concentrations, the steady-state initial rate showed a linear dependence on the enzyme concentration, and the hyperbolic dependence of the initial rate on the substrate concentration could be described in terms of K_M and V_{max} parameters. The competitive nature of the inhibitors used in this study has been established by a variety of techniques, and the equilibrium dissociation constants for the inhibitors bound to the enzyme were measured by the protection method [Jain et al. (1991) *Biochemistry 30*, 7306–7317]. The kinetics of hydrolysis in the presence of competitive inhibitors could be described by a single dissociation constant. However, the value of the dissociation constant obtained under the kinetic conditions was comparable to that obtained by the protection method for the inhibitor–enzyme complex bound to a neutral diluent, rather than to the value of the dissociation constant obtained with solitary monomeric inhibitors and the enzyme in the aqueous phase. Spectroscopic methods showed that the effectively lower dissociation constant of an inhibitor bound to PLA2 at the interface is due to the stabilization of the enzyme–inhibitor complex by interaction with other amphiphiles present in the reaction mixture. These results show that the EI complex in the aqueous phase binds other solitary or aggregated amphiphiles to the interfacial recognition region on the enzyme (i-face).

Interfacial catalysis by phospholipase A_2 (PLA2)[1] has been quantitatively described by an adaptation of Michaelis–Menten formalism, where the binding of the enzyme in the aqueous phase to the substrate interface precedes the catalytic turnover by the enzyme in the interface (Berg et al., 1991; Verger & de Haas, 1976). Elsewhere, we have shown that virtually all aspects of the kinetics of action of PLA2 on anionic vesicles are quantitatively described by the catalytic turnover cycle in the interface and the enzyme does not leave the interface between the successive turnover cycles (Jain et al., 1986a; Jain & Berg, 1989). The rate and equilibrium parameters for the catalytic action of PLA2 on DMPM vesicles in this highly processive scooting mode have been determined (Berg et al., 1991; Jain et al., 1991a). The catalysis in the scooting mode has been used to quantitatively characterize the action of PLA2 from different sources (Jain et al., 1991b) as well as the covalently modified enzyme (Ghomashchi et al., 1991), to determine the substrate specificity (Jain & Rogers, 1989; Ghomashchi et al., 1991), and to describe the kinetics in the presence of inhibitors (Jain et al., 1989, 1991a,b) and activators (Jain et al., 1991c). This kinetic model also accounts for interfacial catalysis by PLA2 on micelles and monolayers with the provision that at such interfaces the exchange of the substrate and products must be taken into consideration (Jain & Berg, 1989).

While there appears to be a general consensus about the formation of a specific active-site-directed complex of the enzyme in the interface with the ligands in the interface (Verheij et al., 1981; Jain et al., 1982; Dennis, 1983; Ramirez

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[1] Abbreviations: cmc, critical micelle concentration; deoxy-LPC, 1-hexadecylpropanediol 3-phosphocholine; 2H-GPC, 2-hexadecyl-*sn*-glycero-3-phosphocholine; PC6, PC7, and PC8, diacyl-*sn*-glycero-3-phosphocholines with the indicated acyl chain lengths; MG14, RM2, RM3, MJ33, and MJ72, structures shown in Figure 2; PLA2, phospholipase A_2 from pig pancreas; proPLA, precursor of PLA2.

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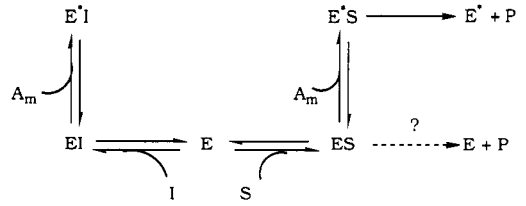

FIGURE 1: Kinetic scheme to account for the hydrolysis of short-chain phosphatidylcholines by PLA2 and the effect of competitive inhibitors. The species marked with an asterisk are formed by interactions of amphiphiles (Am) with PLA2 (E), whereas specific interactions with the substrate or inhibitor involve the active site.

& Jain, 1991), the possibility of formation of a corresponding complex in the aqueous phase has not been adequately addressed. For example, diacylphosphatidylcholines with short acyl chains dispersed as solitary monomerms below their cmc are hydrolyzed by PLA2 (de Haas et al., 1971; Pieterson et al., 1974). In this system, a large change in the rate of hydrolysis at the cmc has been rationalized (Verheij et al., 1981) by the hypothesis that the catalytic turnover at the micellar interface occurs processively whereas below the cmc the hydrolysis of solitary monomeric substrate molecules occurs via a solitary ES complex in the aqueous phase according to the classical Michaelis–Menten scheme: $E + S \rightleftarrows ES \rightarrow EP$; i.e., the binary ES complex is formed and decomposed in the aqueous phase. This possibility is called into question by the observation that the apparent affinity of PLA2 for competitive inhibitors increases appreciably in the presence of other amphiphiles in the reaction medium (Yuan et al., 1990; de Haas et al., 1990).

Due to a broad significance of such observations on the putative "interfacial activation", we have investigated the properties of PLA2–inhibitor complexes in the presence of amphiphiles with weak affinity for the active site. The availability of competitive inhibitors of PLA2 (Jain et al., 1989, 1991d; Scott et al., 1990; Thunnisen et al., 1990) and the ability to independently measure the equilibrium dissociation constant of a ligand bound to the active site of PLA2 (Jain et al., 1991a) have prompted us to investigate the effect of specific competitive inhibitors on the kinetics of hydrolysis of short-chain phosphatidylcholines by pig pancreatic PLA2. In this paper, we examine the effect of four well-established competitive inhibitors on the kinetics of hydrolysis of short-chain phosphatidylcholines below their cmcs. Nevertheless, the results show that even below their cmc the hydrolysis of the short-chain PCs by PLA2 occurs via the formation of an aggregated species in situ according to the kinetic scheme shown in Figure 1; i.e., the enzyme–inhibitor complex binds to other amphiphiles to form a complex that resembles the enzyme–inhibitor complex at the interface of a neutral diluent. Moreover, the rate of hydrolysis of monomeric substrates remains the same whether or not the enzyme is bound to the interface of a neutral diluent. It may be recalled that a neutral diluent molecule does not bind to the catalytic site of the enzyme, although the enzyme binds to the interface of the aqueous dispersions of a neutral diluent (Jain et al., 1991a).

Materials and Methods

All diacylphosphatidylcholines were purchased from Avanti. The inhibitors MG14 and RM3 were kindly provided by Professor Michael Gelb (University of Washington, Seattle, WA) and Dr. Donald Magolda (Du Pont, Wilmington, DE), respectively. Dihexyl- and dioctyl-*sn*-glycero-3-phosphocholines (Jain et al., 1986b), MJ33, MJ72, 2H-GPC, and deoxy-LPC were synthesized (Jain et al., 1991d) as described. All experimental protocols used in the present study are well established in our laboratory. The hydrolysis of diacylphosphatidylcholine substrates by pig pancreatic PLA2 was monitored by pH-stat titration at pH 8.0 and 23 °C in 1 mM NaCl and 0.5 mM $CaCl_2$ (Jain et al., 1986a; Berg et al., 1991). The values of the steady-state initial rate of hydrolysis as well as the V_{max} values are expressed as per second per enzyme molecule, and the values in the more conventional units of micromoles per minute per milligram of enzyme can be obtained by multiplying with 4.1. cmc values summarized in Tables I and III were determined by the fluorometric method using *N*-phenylnaphthylamine (Brito & Vaz, 1986) or adopted from the published literature (Bian & Roberts, 1991; Lin et al., 1987, 1990; Tausk et al., 1974a,b).

As described in detail elsewhere (Jain et al., 1991a), the effective equilibrium dissociation constant for the binding of a ligand (substrate, product, calcium, or inhibitors) to the active site of PLA2 in the interface or in the aqueous phase was obtained by monitoring the half-times for the alkylation of His-48 at the catalytic site in the presence and in the absence of the ligand. The reaction mixture for alkylation contained 2 mM *p*-nitro-phenacyl bromide, 0.1–1 μM PLA2, and 1 mg/mL human γ-globulin in 0.5 mM $CaCl_2$, 50 mM NaCl, and 50 mM cacodylate buffer at pH 7.3 and room temperature. For measurement of K_I^{eff}, deoxy-LPC (1.6 mM) was used as the neutral diluent because it has no affinity for the active site of the bound enzyme, although PLA2 binds to the interface of its dispersions in the aqueous solution (Jain et al., 1991a,d).

Interactions of PLA2 with ligands and amphiphiles were also characterized by spectroscopic method (Hille et al., 1983; Jain et al., 1982, 1986b). The fluorescence measurements on the tryptophan emission were carried out on an SLM 4800S fluorometer with excitation at 285 nm and emission at 333 nm with slit widths set at 4 nm each. The reaction mixture contained 5 μM PLA2 in 20 mM Tris·HCl, 5 mM $CaCl_2$, 10 mM NaCl, and the indicated amounts of amphiphiles and inhibitors. The same reaction conditions were used to monitor the change in the UV absorbance with an HP8452 UV–visible spectrophotometer equipped with a diode array detector and standard data processing software.

Results

According to the classical Michaelis–Menten formalism, the effect of a competitive inhibitor on the kinetics of an enzyme-catalyzed reaction in a homogeneous medium is described by the relationship (Segel, 1976):

$$I(50)/K_I = 1 + [S]/K_M \quad (1)$$

Here $I(50)$ is the concentration of the inhibitor required for 50% inhibition of the steady-state initial rate of hydrolysis at the substrate concentration [S], K_I is the equilibrium dissociation constant of the inhibitor bound to the enzyme, and K_M is the Michaelis–Menten constant for the substrate. In this paper, we examine the validity of eq 1 for the kinetics of hydrolysis of three homologous phosphatidylcholines (PC6, PC7, and PC8) by pig pancreatic PLA2 in the presence of four different specific competitive inhibitors whose structures are shown in Figure 2. These inhibitors were selected for the diversity of their structures and for the range of their cmcs, so that the effects related to their aggregation tendencies and partition coefficients, if any, can be distinguished from their inhibitory effects.

Hydrolysis of Short-Chain Phosphatidylcholines below Their cmc. The progress curves for the hydrolysis of the aqueous solution of the three PCs below their cmcs (Table I) exhibited the kinetic characteristics predicted by the Mi-

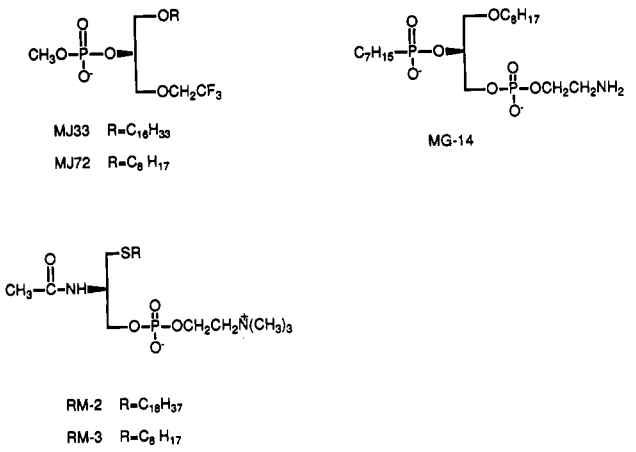

FIGURE 2: Structures of the four specific competitive inhibitors of PLA2 used in the present study.

Table I: Rate and Equilibrium Parameters[a] for the Hydrolysis of Homologous PCs below Their cmcs by PLA2 at pH 8.0 and 23 °C in 1 mM NaCl

parameters	PC6	PC7	PC8
cmc (μM)	15000	1500	240
K_M (μM)	<1000 (35)[b]	290 (26)	80 (11)
V_{max} (s^{-1})	1.3 (18)	7.2 (16)	30 (9)

[a] These values were obtained from the plot of v_0 versus [S] fitted to the Michaelis–Menten hyperbola. [b] Percent standard deviation.

chaelis–Menten formalism for classical homogeneous enzymatic catalysis (Segel, 1976). The reaction starts, without a latency period, immediately after the addition of the enzyme. Once initiated, the reaction progresses at a steady initial rate for a period of time that depends on the concentration of the substrate. All the substrate present in the reaction mixture is hydrolyzed at the end of the reaction, and the whole reaction progress curve can be described by the integrated Michaelis–Menten equation. The initial rate of hydrolysis (v_0) increases linearly with the amount of the enzyme in the reaction mixture. The initial steady-state rate of hydrolysis (v_0) of the short-chain phosphatidylcholines below their cmc (Table I) showed a hyperbolic dependence on the calcium concentration. From such plots, K_{Ca} was calculated as 0.2 mM, which is agreement with the value of 0.28 mM for the hydrolysis of DMPM vesicles and also in agreement with the value of 0.23 mM obtained by direct binding studies (Jain et al., 1991a; Slotboom et al., 1978).

The substrate concentration dependence of the initial rate of hydrolysis at pH 8.0 was virtually similar to that reported at pH 6 (de Haas et al., 1971; Pieterson et al., 1974), i.e., a hyperbolic dependence as predicted by the Michaelis–Menten formalism. As summarized in Tables I and II, we obtained the K_M and V_{max} values under a variety of conditions. The standard deviation in the nonlinear regression of such plots was typically less than 20%; however, the uncertainties in the

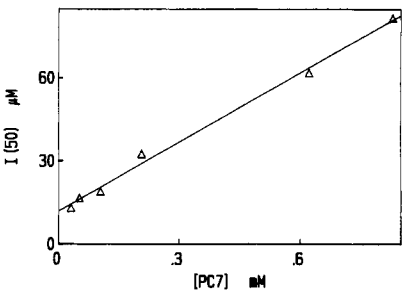

FIGURE 3: Plot of $I(50)$ versus [PC7] for MJ72. The values of K_I and K_M obtained from such plots fitted to eq 1 are summarized in Table III. These measurements were carried out at pH 8.0 and 23 °C in the presence of 1 mM NaCl and 0.5 mM $CaCl_2$.

K_M and V_{max} values were about 30% for the cases where the K_M values are larger. This is because the initial rates tend to be somewhat higher when the substrate concentration approaches the cmc of the substrate. Similarly, the uncertainty in the K_M and V_{max} values for PC6 is large because the rate of hydrolysis is barely above the background rate of spontaneous hydrolysis, and there were indications of systematic deviation from the Michaelis–Menten hyperbola if high enzyme concentrations were used.

Effect of Competitive Inhibitors on Kinetics. In the presence of a competitive inhibitor in the reaction mixture, the initial rate of hydrolysis, v_i, depends on the inhibitor concentration. The Dixon plot, $v_0/v_i - 1$ versus [I], was linear for the monomeric substrate over the whole concentration range of the inhibitor where more than 90% inhibition was achieved. Such an ideal behavior below the cmc of the substrate was observed for the hydrolysis of each of the three PCs with all four inhibitors under a variety of conditions with different pHs and substrate and salt concentrations. The highest inhibitor and substrate concentrations used in these studies were kept below their cmcs, typically in the range of 0.2–2 times K_M or K_I. The $I(50)$ values were obtained from the Dixon plots. Since the $I(50)$ values depend on the substrate concentration, the values of K_I summarized in Table II were calculated with eq 1 by using the K_M values obtained above (Tables I and II) from the substrate concentration dependence of the steady-state initial rate in the absence of an inhibitor.

As shown in Figure 3, the K_M and K_I values were also obtained simultaneously from the dependence of the $I(50)$ values on the substrate concentration. The $I(50)$ values were obtained from the Dixon plots as described above at each of the substrate concentrations. The plots of $I(50)$ versus [S] were linear with all three substrates in the presence of the four inhibitors. According to eq 1, the y intercept of such a plot is K_I, whereas the value of K_M is obtained from the slope. The plot for the inhibition of the hydrolysis of PC7 by MJ72 (Figure 4) gave values of K_I = 13.5 μM and K_M = 163 μM. On the basis of the values of the standard deviation, the uncertainty in each of these values is estimated to be about 30%.

Table II: Effect of Salt and pH on Kinetic Parameters for the Hydrolysis of PC7 below the cmc by PLA2 and ProPLA2

pH	[NaCl] (M)	V_{max} (s^{-1})	K_M (μM)	K_I (μM)			
				MG14	RM3	MJ33	MJ72
			PLA2				
8.0	0.001	7.2	290	3.0	11	0.15	11
8.0	0.1	2.8	90	2.5	5	1.1	13
8.0	1.0	2.1	45	2.8	22	1.9	31
6.0	0.001	6.2	530	0.02	6	0.07	0.9
6.0	0.1	7.8	533	0.02	7	0.05	0.8
			ProPLA2				
8.0	0.001	0.75	340	1.0	11.2	0.12	11
8.0	1.0	0.45	211	3.9	17.4	5.6	64

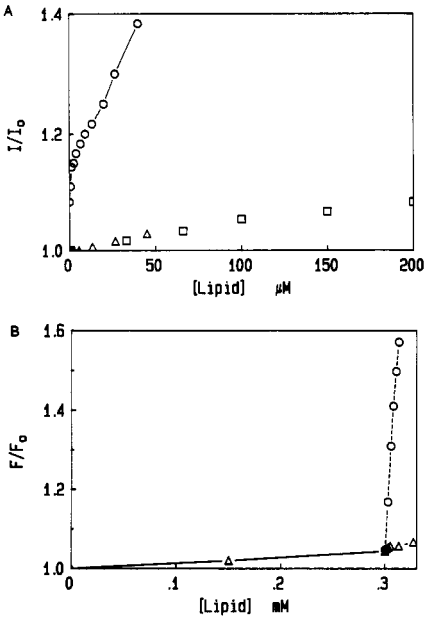

FIGURE 4: (A) Relative fluorescence emission intensity of PLA2 (2.5 μM at pH 8.0 and 23 °C) in the presence of 1 mM calcium as a function of the concentration of (triangles) MG14 alone or (circles) 26 μM MG14 in the presence of 130 μM 1,2-dioctylglycerophosphocholine and (squares) 1,2-dioctylglycerophosphocholine alone at the concentration given on the x axis. (B) Relative fluorescence emission intensity of PLA2 (2.5 μM at pH 8.0 and 23 °C) in the presence of 1 mM calcium as a function of the concentration of (triangles) dihexylglycerophosphocholine and (circles) MJ33 (0.0–0.012 mM) added in the presence of 0.3 mM dihexylglycerophosphocholine. The fluorescence increase in the presence of 0–0.02 mM MJ33 alone was negligibly small.

Table III: K_I (μM) and Calculated K_M (μM) Values[a] (Given in Parentheses) for the Inhibition of Hydrolysis of Homologous PCs (below cmc) in the Presence of Competitive Inhibitors

		K_I (K_M) values with		
inhibitor	cmc	PC6	PC7	PC8
RM3	78	18 (350)	7.3 (167)	7.0 (70)
MG14	58	1.1 (400)	0.43 (138)	1.2 (110)
MJ72	>1000	15 (470)	13.5 (163)	23 (82)
MJ33	8.7	0.43 (400)	0.14 (550)	0.2 (90)

[a] These values were obtained from plots of $I(50)$ versus [S] as shown in Figure 3. All values are given in micromolar. The uncertainty in these values is about 30%.

The K_I values for the four inhibitors and the corresponding K_M values with each of the three PCs are summarized in Table III.

Several interesting conclusions emerge from the values of K_M, K_I, and V_{max} summarized in Tables I–III. First, the K_M values for each of the three substrates obtained by the two different protocols were virtually the same (Table I versus Table III). While the K_M values did not change significantly with pH, they decreased significantly with salt concentration (Table II), and also with the chain length of the substrate (Tables I and III). The effect of the salt concentration on the K_M values followed the same general trend as the effect of the salt concentration on the cmc of PC7 (Tausk et al., 1974a,b). Second, the V_{max} values increased significantly with the chain length of the substrate; however, the effect of the change in the salt concentration or the pH was modest. Third, although the K_I values for the four inhibitors were significantly different, the K_I values for a given inhibitor was approximately the same with all three substrates. Fourth, while the values of K_M and V_{max} changed <3-fold at different pHs and salt concentrations, the values of K_I for MG14, MJ33, and MJ72 changed by more than a factor of 10 between pH 6 and 8, whereas the K_I for RM3 was essentially unchanged. Such a difference between an *sn*-2-amide (RM3) versus *sn*-2-phosphate (MJ33 or MJ72) or phosphonate (MG14) analogs is expected if the functional groups on the *sn*-2-position are hydrogen-bonded with protonated histidine-48 at the catalytic site of the enzyme. Indeed, the X-ray analysis of the cocrystals has shown that the *sn*-2-amide is H-bonded to neutral His-48 (Thunnissen et al., 1990), whereas the *sn*-2-phosphonate is bound to the protonated His-48 (Scott et al., 1990). Fifth, the K_I values for the four inhibitors were similar for PLA2 and for proPLA2 at low salt concentrations (Table II), which suggested that the removal of the N-terminal peptide does not cause any change in the affinity for the inhibitors.

Table IV: Equilibrium Dissociation Constants for the Binding of Inhibitors to the Active Site of Free PLA2 (K_L) or of PLA2 Bound to Micelles of Deoxy-LPC (K_L^{eff})

inhibitor	K_L (μM)	K_L^{eff} (μM)
RM3	83 (14)[a]	6 (18)
MG14	50 (9)	1.7 (40)
MJ72	90 (8)	24 (12)
MJ33	10 (15)	1.5 (35)

[a] Percent standard deviation.

At first glance, it would appear that results summarized above show that the observed kinetic behavior is as predicted by the Michaelis–Menten formalism for a homogeneous system. However, as described below, independent measurements of the equilibrium dissociation constants for the inhibitors show significant departures from ideality and suggest a more complex interplay of the underlying equilibrium and kinetic processes with significant implications on the mechanism of interfacial catalysis.

Dissociation Constants for Inhibitors by the Protection Method. As shown elsewhere, the equilibrium dissociation constant for a ligand bound to the active site of PLA2 can be determined independently by the protection method in the presence or absence of a neutral diluent (Jain et al. 1991a). The K_L^{eff} value for an inhibitor bound to PLA2 is calculated from the half-time for the inactivation of the enzyme by an alkylating agent in the presence (t_L) and in the absence (t_f) of a ligand at concentration [L]:

$$1/(1 - t_f/t_L) = 1 + K_L^{eff}/[L] \quad (2)$$

This relation applies when the enzyme containing the ligand at the active site is virtually completely protected from alkylation. For all cases considered in this paper, this was indeed the case. By definition, the effective dissociation constant of a ligand in the absence of the interface is K_L. The effective dissociation constant obtained in the presence of a neutral diluent at concentration [A_m] is related to the interfacial dissociation constant, K_{L*}, as

$$K_L^{eff} = K_{L*}([A_m] + K') \quad (3)$$

where K' is the concentration of the neutral diluent at which 50% of the total inhibitor present in the reaction mixture is partitioned in the interface.

The protection method was used to determine the equilibrium dissociation constants K_L and K_L^{eff} for the four inhibitors bound to PLA2 as summarized in Table IV. These results show that the K_L values are appreciably higher than the K_L^{eff} values; i.e., the apparent affinity of an inhibitor to PLA2 at the interface is considerably higher than its affinity for free PLA2 in the aqueous phase. It was most surprising and interesting that for all four inhibitors, the K_I values obtained under the kinetic conditions (summarized in Tables II and III)

correlate well with the corresponding K_L^{eff} values, rather than with the K_L values (Table IV) which are severalfold higher. Also, the K_L value was always considerably higher than the $I(50)$ values, which would be inconsistent with eq 1. A possible basis for these observations is developed below.

Effect of Inhibitors on the Spectral Properties of PLA2. Perturbation of the spectral properties of the only tryptophan (Trp-3) residue of PLA2 has been used to characterize interactions of PLA2 with the aqueous dispersions of neutral diluents, substrate analogs, and inhibitors (Jain et al., 1982; Hille et al., 1983). These spectroscopic signatures primarily provide information about the binding of the enzyme to the interface, because Trp-3 is located in the interfacial recognition region (i-face) of PLA2 (Ramirez & Jain, 1991). In order to elaborate processes intrinsic in the E to E* equilibrium in the kinetic scheme shown in Figure 1, we investigated the effect of inhibitors on the fluorescence emission and the UV absorbance properties of PLA2 in the presence and in the absence of amphiphiles whose structure and properties resemble those of the substrates used for kinetic studies.

As shown in Figure 4A, the fluorescence emission of PLA2 at 333 nm increased <10% in the presence of MG14 alone or of 1,2-dioctylglycerophosphocholine alone below their cmcs of 50 and 180 μM, respectively. However, as also shown in this figure, the titration of a mixture of 2.5 μM PLA2 and 130 μM dioctylglycerophosphocholine with MG14 (<50 μM) resulted in a sharp increase in the fluorescence emission. Similar results were obtained with MJ33, MJ72, and RM3 added below their cmcs to dioctylglycerophosphocholine dispersed below its cmc as solitary monomers. A similarly sharp increase in the fluorescence emission in the presence of an inhibitor was also observed with dihexylglycerophosphocholine at concentrations far below its cmc. For example, as shown in Figure 4B, the fluorescence emission of PLA2 increases <5% in the presence of 0.3 mM dihexyl-*sn*-glycero-3-phosphocholine (cmc > 10 mM). Addition of <10 μM MJ33 (cmc = 8.7 μM) caused a >60% increase in the fluorescence emission from Trp-3. Under the same conditions, the fluorescence increase by the addition of 25 μM MJ33 alone was <5%. A large increase in the fluorescence emission was also observed if dihexylglycerophosphocholine was added to this mixture of PLA2 and MJ33; i.e., the order of addition was not important, although quantitative differences were noticeable between different amphiphiles and inhibitors.

Control experiments also showed that such perturbations in the fluorescence emission required calcium and they were not observed in the presence of EGTA or barium, which do not promote binding of a ligand to the active site of PLA2 (Jain et al., 1991a,c). A fluorescence change was also observed on the binding of PLA2 to micelles of a neutral diluent such as deoxy-LPC or 2H-GPC, which suggested that the perturbation of the Trp-3 fluorescence emission does not require the occupancy of the active site; i.e., such measurements do not distinguish between the E* and E*L states of the enzyme. Trp-3 is on the i-face, and the Trp-3 emission was not perturbed by dialkyl-*sn*-glycero-3-phosphocholine or any of the inhibitors dispersed as solitary monomers, which suggests either that the E to EL equilibrium is not favored in the aqueous phase or that the Trp-3 emission is not perturbed in the EL complex. Also, the fluorescence increase in a micellar solution of dioctyl-*sn*-glycero-3-phosphocholine (at 2× cmc) was considerably smaller than that observed with a mixture of an inhibitor and the phosphocholine (both 0.3× their respective cmcs), where there was no indication of comicelle formation. On the other hand, even below the cmc of the amphiphile in the reaction mixture, in the presence of the specific inhibitors the fluorescence emission properties of Trp-3 are virtually identical to those seen on the binding of PLA2 to the interface (Jain et al., 1982; Ramirez & Jain, 1991) or to micelles of a neutral diluent (Jain et al., 1991a). Whether such a perturbation is due to the formation of the E* or the E*L species can not be resolved from the fluorescence measurements; however, the UV absorbance studies described below do distinguish between these species.

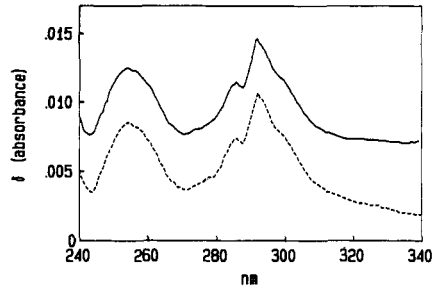

FIGURE 5: Difference absorbance spectrum of 10 μM PLA2 ($E_{1\%}$ = 13.0) obtained with 10 μM MG14 in the presence of (top) 1 mM dihexylglycerophosphocholine and (bottom) 0.6 mM dioctylglycerophosphocholine. Note that the hexyl analog is present below its cmc (cmc >10 mM) while the dioctyl analog is present above its cmc (=0.18 mM). These difference spectra were obtained by subtracting the spectrum of the mixture of PLA2 + dialkylglycerophosphocholine from the spectrum of the mixture containing all three components. This is because no noticeable changes in the absorbance in the 250–300-nm region were observed in the presence of any one of these components alone or in the presence of neutral diluents such as 2H-GPC or deoxy-LPC (data not shown). The spectra are corrected for dilution; however, the base line is shifted for clarity.

The perturbation of Trp-3 and of other aromatic residues in PLA2 was also monitored as a change in the UV absorbance. As shown in Figure 5, the increase in the UV spectrum observed in the presence of MG14 and dihexyl- or dioctyl-GPC below their cmcs showed a characteristic change in the 250–300-nm region. These changes were concentration-dependent, and a small (about 7% at 292 nm) increase in the absorbance of PLA2 was observed in the presence of MG14 and dialkyl-*sn*-glycero-3-phosphocholine. The overall change in the intensities of the UV absorbance spectra was considerably smaller than the changes in the fluorescence emission. Although such changes were not observed with neutral diluents, the characteristic changes were observed if competitive inhibitors were added to a mixture of PLA2 and micelles of the neutral diluents. Also as was the case with the fluorescence change, the changes in the absorbance depended on the concentration of the ligand, and the spectral changes were observed only in the presence of calcium but not barium. Control experiments showed that spectral perturbations were not observed in the presence of any of the inhibitors or dialkylglycerophosphocholines alone below their cmcs. These results suggested that the types of absorbance changes shown in Figure 5 were due to the binding of the inhibitor to the active site of the enzyme at the interface (i.e., E*I form) and that such changes are not observed with the bound enzyme in the absence of a ligand at the active site (E* form) or with the solitary EI complex in the aqueous phase.

The perturbation of the fluorescence emission spectrum from Trp-3 is believed to arise when the i-face of PLA2 interacts with the interface (Ramirez & Jain, 1991), whereas the changes in the UV spectrum in the 280–295-nm region could involve perturbation not only of Trp-3 but also of some tyrosine residues during the formation of the E*I complex at the interface. The results summarized in Figure 4 showed that while dioctylglycerophosphocholine or MG14 alone did not perturb

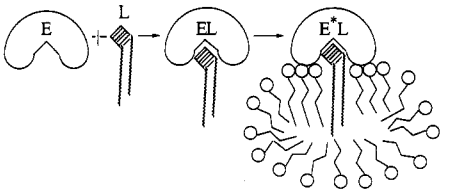

FIGURE 6: Schematical representation of the possible changes that occur during the interaction of PLA2 with a ligand that binds to the active site followed by the formation of an aggregate with amphiphiles.

Trp-3, in the presence of both of these amphiphiles the Trp-3 emission increased significantly. Similar results were obtained with other inhibitors added in the presence of neutral diluents and amphiphiles with weak affinity for the catalytic site of PLA2. Taken together, these observations demonstrate that if the active site of PLA2 contained an inhibitor in the presence of other solitary amphiphiles, in the resulting complex Trp-3 is spectrally perturbed in the same way as if the enzyme is at the interface, i.e., as in the E*L complex.

Discussion

Yuan et al. (1990) have shown by kinetic and direct binding techniques that the apparent affinity of PLA2 for MG14 is considerably higher in the presence of the substrate or a substrate analog than it is for MG14 alone. For example, the $I(50)$ values for MG14 with several substrates were shown to be 10–100 times lower than the values of apparent dissociation constants estimated from the equilibrium dialysis or the protection method. Essentially the same conclusion is reported by de Haas et al. (1990). Studies described in this paper were designed to provide a basis for these observations in terms of the K_I and K_M parameters (eq 1) which primarily relate to the intrinsic affinity of the enzyme in the aqueous phase for the inhibitor or the substrate. The results show that eq 1 is not applicable to this kinetic system; i.e., the observed affinity of the enzyme in the aqueous phase for the solitary inhibitor in the aqueous phase is far too small compared to the value predicted from the kinetic results. In fact, K_I^{eff} values correlate well with the K_I values measured under kinetic conditions. Moreover, spectroscopic results suggest that the complex of PLA2 with an active-site-directed ligand (e.g., an inhibitor) is stabilized by amphiphiles (e.g., substrate analogs or neutral diluent) with weak affinity for the active site of PLA2. Nevertheless, this conclusion is entirely consistent with the hypothesis that the catalytic and interfacial recognition sites of PLA2 are different. Some of the implications of this conclusion are discussed below in the context of the kinetic scheme in Figure 1 and the cartoon shown in Figure 6.

Results summarized in the preceding section show that the effects of specific competitive inhibitors on the kinetics of hydrolysis of three homologous PCs dispersed as solitary monomers exhibit some of the key features of the classical Michaelis–Menten formalism for enzymatic catalysis, i.e., a steady-state initial rate of hydrolysis and the hyperbolic dependence of v_0 on substrate concentration. These results do not necessarily prove that the action of PLA2 occurs on the solitary monomeric substrate or that the solitary ES or EP complex decomposes in the aqueous phase. In fact, the hyperbolic bulk substrate concentration dependence is also predicted if the enzyme was to bind to a preformed interface, or if the occupancy of the active site of the enzyme by a ligand promoted binding to or the formation of an interface with amphiphiles which are otherwise present as solitary monomers. This last possibility is underscored under the present kinetic conditions by the observation that the K_I values obtained from the kinetic measurements correspond to the K_L^{eff} values rather than to the K_L values; i.e., the apparent relationship between K_M and K_I as expected for competitive inhibitors (eq 1) is not obeyed under the kinetic conditions. Evidence for the formation of an aggregated E*L complex involving the i-face of the enzyme in the presence of ether analogs of the substrate was further supported by the observation that even under apparently "monomeric" conditions the fluorescence emission and the UV difference spectra from such complexes resemble the spectra obtained from PLA2 at the interface of neutral diluents in the presence of a ligand that binds to the active site. It may be recalled that such spectral perturbations arise from the Trp-3 residue which is a part of the interfacial recognition site, and it is also structurally and functionally separated from the catalytic active site (Verheij et al., 1981; Ramirez & Jain, 1991).

As summarized in the kinetic scheme in Figure 1, the observations in the preceding section collectively show that somehow the EI or ES complexes of PLA2 in the aqueous solution form a complex in situ with amphiphiles that may or may not have an affinity for the active site. On the basis of the spectral properties and the equilibrium dissociation behavior of such complexes, we postulate the following reaction sequence, which is elaborated in Figure 6 to provide a possible structural and organizational basis:

$$E + L \rightleftharpoons EL + Am \rightleftharpoons E^*L \quad (4a)$$

$$E + Am \rightleftharpoons E^* \rightleftharpoons E^*L \quad (4b)$$

In these sequential equilibria, the binding of a ligand (L) to the active site of PLA2 is stabilized further by the binding of other amphiphiles (Am) to the EL complex. Such amphiphiles may or may not have affinity for the active site, and thus they may be substrates, substrate analogs, inhibitors, or neutral diluents present as solitary monomers or as aggregates. This pair of consecutive equilibria is thermodynamically equivalent, and at this stage, it is not possible to distinguish the sequence in eq 4a from that in eq 4b, although one of these steps can be effectively dissected. The equilibrium dissociation constant for the first step in eq 4a is K_L, as measured by the protection method (Table IV). In the presence of a neutral diluent with a poor affinity for the active site, the overall equilibrium shifts toward E*L, and the effective dissociation constant is given by K_L^{eff}, which as expressed in eq 3 has a contribution not only from K_L^* (for the E* + I* to E*I equilibrium) but also from the partition coefficient of the ligand between water and the interface. Attempts to dissect the relative contribution of these terms are in progress; however, some of the immediate implications of this paradigm are discussed in sequel.

The driving force for the equilibria in eq 4a or eq 4b could come from nucleation, desolvation, or a conformational change. These possibilities are not mutually exclusive; however, certain constraints can be identified. Although E or EL could undergo a conformational change on binding to the interface, the crystal structures of E and EI are virtually superimposable (Scott et al., 1990; Thunnissen et al., 1990), and the rate of alkylation of E is virtually the same as the rate of alkylation of E* at the interface of a neutral diluent (Jain et al., 1991a). For such reasons, at this stage we prefer the possibility that the binding of a ligand to the active site of PLA2 promotes desolvation of the active site as well as the interfacial recognition site (Jain & Vaz, 1987). Thus, the formation of EL acts as a nucleation event for the aggregation of other amphiphiles, if present. On the basis of this paradigm, the formation of the E*L complex could be driven by the relative hydrophil–lipophil balance of the i-face in E and the EI complex.

The distinguishing features of the observations reported in this paper and their broader kinetic consequences arise from

the possibility that the formation of the E*L type of complexes in the absence of a preformed interface may be a net result of nucleation by a ligand at the active site of the enzyme. The gross structural and dynamic character of the E*L complex is expected to depend on the nature and the concentration of the amphiphile. In analogy with the behavior of amphiphiles in aqueous dispersions, the E*L complex can be present in several macroscopic states ranging from a stoichiometric aggregate of the enzyme with amphiphiles (Hille et al., 1983) to a single enzyme scooting on the vesicle surface (Berg et al., 1991; Jain et al., 1991b). In such cases, the spectral characteristics, due to the perturbation of Trp-3, would be indistinguishable, but the gross organization of resulting complexes could be very different. This is probably the case during the formation of premicellar aggregates of snake venom PLA2s with zwitterionic amphiphiles (van Eijk et al., 1983), or those of the pig pancreatic PLA2 with anionic amphiphiles (van Oort et al., 1985; Jain et al., 1986b).

Formation of an E*S type of complex during the hydrolysis of substrates below their cmc eliminates the need for postulating catalysis via an ES intermediate in the aqueous phase (Figure 1). In a reaction mixture containing PLA2 and monomeric substrate molecules, formation of ES would promote the formation of E*S complex by excess substrate in which the substrate is present not only at the active site but also with additional molecules bound to the i-face (Figure 6). In such a complex, catalysis by PLA2 occurs at the interface and processively. Thus, depending on the number of substrate molecules in the E*S complex, the overall steady-state rate of hydrolysis would be determined either by the intrinsic rate of catalytic turnover or by the rate of replenishment of the substrate. Below the cmc, the rate of hydrolysis in the E*S complex is probably limited by the rate of replenishment of the substrate. This is because the intrinsic rate of hydrolysis by the enzyme is rapid, and therefore a small complex containing a few substrate molecules would be rapidly depleted (Jain et al., 1991c). Replenishment of amphiphiles in micelles occurs by exchange with the monomers present in the aqueous phase, or by fusion–fission with other micelles. Below the cmc, the rate of hydrolysis would depend on the fraction of the enzyme in the form of the E*S complex and the rate of replenishment by solitary monomers. Above the cmc, the rate of replenishment would be dominated by the fusion–fission of E*S with the excess substrate micelles by a collisional process. Here the rate of substrate replenishment would be considerably more rapid because scores of substrate molecules are replenished in a single collisional event leading to fusion–fission of micelles. Therefore, the rate of hydrolysis would show a higher order dependence on the concentration of micelles because each fusion–fission event with excess substrate micelle would replenish 10–100 substrate molecules rather than a single molecule replenished during the exchange process. This accounts for the observation that above the cmc the rate increases steeply as a function of the bulk substrate concentration. Such a mechanism also eliminates the need for postulating two catalytic mechanisms for PLA2, i.e., one for the interface via E*S and the other for the aqueous phase via ES. Implications of these suggestions are being investigated.

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