Interfacial Catalysis by Phospholipase A_2: Monomeric Enzyme Is Fully Catalytically Active at the Bilayer Interface†

Mahendra Kumar Jain,*,‡ Girish Ranadive,‡ Bao-Zhu Yu,‡ and H. M. Verheij§

Department of Chemistry, University of Delaware, Newark, Delaware 19716, and Department of Biochemistry, State University, Utrecht, The Netherlands

Received September 17, 1990; Revised Manuscript Received March 26, 1991

ABSTRACT: Interfacial catalysis in the scooting mode by phospholipase A_2 (PLA2) from pancreas and venoms (18 different preparations) was examined on vesicles of 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol under the conditions where the rates of transbilayer and intervesicle exchanges of the enzyme, substrate, and the products of hydrolysis were negligible on the time scale (<30 min) on which all the substrate molecules on the outer monolayer of the target vesicle were hydrolyzed. The reaction progress curves for all PLA2s exhibited no latency period (<3 s). When the vesicle to PLA2 ratio in the reaction mixture was high so that according to the Poissonian distribution model at most one enzyme was bound to a vesicle, the extent of hydrolysis increased linearly with the amount of enzyme in the reaction mixture. However, the extent of hydrolysis per enzyme, N_S, remained the same for all PLA2s, and it corresponded to the size of the target vesicles determined by independent methods. Similarly, the initial rate of hydrolysis increased linearly with the enzyme concentration, and the slope of the log-log plot was one under the conditions of one or more enzyme per vesicle. Such observations showed that monomeric PLA2 is fully catalytically active at the interface. This conclusion was supported by the absence of intermolecular resonance energy transfer from tryptophan-3 donor in the native PLA2 to the anthraniloyl acceptor in An87-PLA2, the catalytically active derivative of PLA2 with an anthraniloyl fluorophore on lysine 87. In this system, intermolecular resonance energy transfer was seen only when the donor-acceptor molecules were "crowded" at a high surface density with a relatively low lipid to protein mole ratio. On the basis of these results, it was concluded that secretory PLA2s from venoms and pancreas are fully catalytically active as monomers. Additional studies reported here showed that acylation of PLA2 was not necessary for catalysis or binding to the interface and that the binding of the substrate to the active site of PLA2 was not necessary for the binding of the enzyme to the interface.

Elsewhere we showed that interfacial catalysis by pig pancreatic phospholipase A_2 (PLA2)[1] was rigorously and explicitly described by a minimal scheme in which the enzyme in the aqueous phase bound to the interface and underwent catalytic turnover with a classical Michaelis-Menten pattern (Jain et al., 1986a-d; 1991; Berg et al., 1991; Ghomashchi et al., 1991a,b). Under the appropriate conditions, the binding of PLA2 to vesicles is sufficiently tight that a processive reaction occurs in which all of the substrate in the outer monolayer of enzyme-containing vesicles becomes hydrolyzed without desorption of the enzyme from the bilayer surface (Jain & Berg, 1989). This is referred to as catalysis in the scooting mode. Furthermore, in the scooting mode, the reaction system was constrained such that the transbilayer movement of lipids and the intervesicle exchange of the substrate, products of hydrolysis, and the enzyme among the ensemble of vesicles did not occur on the time scale of the vesicle hydrolysis (Jain & Berg, 1989). In scooting mode hydrolysis, the observed enzymatic kinetics are not blurred by contributions from the intervesicle movement of enzyme and lipids. Under conditions where the enzyme is bound irreversibly to the vesicles, use of a high vesicle to enzyme ratio insures that there is at most one enzyme bound to a vesicle.

In this paper we show that the extent of hydrolysis per enzyme provided a unique way of addressing a basic question of whether the enzyme was active as a monomer or as a larger aggregate. As shown elsewhere, the amount of product produced per enzyme at the end of the reaction (N_S), was related to the size of target vesicles (Berg et al., 1991), and therefore it provided an unequivocal basis for virtually counting the number of catalytically active PLA2 molecules on vesicles. Interfacial catalysis in the scooting mode on DMPM vesicles was observed with PLA2s from several sources, and in all cases that we have examined the value of N_S was the same and it corresponded to the number of phospholipid molecules in the outer monolayer of the target vesicles. These results showed that the catalytically active enzyme was monomeric and that the aggregation or covalent modification of PLA2 was not required for binding to the interface or for interfacial catalytic activity of PLA2.

Materials and Methods

All reagents and buffers used in this study were analytical grade. *N*-Methylisatoic anhydride was purchased from Mo-

† This work was supported by a grant from the NIH (GM29703).

* Correspondence may be addressed to this author.

‡ University of Delaware.

§ State University, Utrecht.

[1] Abbreviations: AMPA, pig pancreatic PLA2 in which terminal amino groups of all lysines have been amidinated; An87-PLA2, pig pancreatic PLA2 covalently modified at lysine 87 with *N*-methylisatoic anhydride; DMPM, 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DTPM, 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol; DTPC, 1,2-ditetradecyl-*sn*-glycero-3-phosphocholine; HDNS, dansylated hexadecylphosphoethanolamine; Me-PLA2, pig pancreatic PLA2 methylated at His-48 by methyl-4-nitrobenzene sulfonate; N_s, the number of substrate molecules hydrolyzed per enzyme molecule; NOB, 4-nitro-3-octanoyloxy benzoate; NOB-PLA2, pig pancreatic PLA2 to which an octanoyl group has been transferred from NOB; Oct-PLA2, pig pancreatic PLA2 modified at His-48 with 1-bromo-2-octanone; Palm-110 PLA2, pig pancreatic PLA2 with a palmitoyl group at lysine 110; PLA2, phospholipase A_2.

0006-2960/91/0430-7330$02.50/0

lecular Probes. Phospholipids used in this study were synthesized as described (Jain et al., 1986a; Jain & Gelb, 1991). All PLA2s were isolated and identified according to the procedures described elsewhere and reviewed for the purpose of sequence comparison (van den Berg et al., 1989; Verheij et al., 1981). Homogeneity of these preparations was confirmed by HPLC or FPLC on Vydac C4, ODS, Mono Q, or Mono S columns (Pharmacia) in appropriate buffers. PLA2 from the venom of *Naja naja naja* (Pakistan) was kindly provided by Professor Edward Dennis (University of California, La Jolla, CA), and the preparation from venom of *Crotalus atrox* was from Dr. Michael Gelb (University of Washington, Seattle, WA). Peramidinated pig PLA2 (AMPA) and Palm-110 PLA2 (van Scharrenburg et al., 1983; van der Wiele et al., 1988) were kindly provided by Dr. Arend Slotboom (State University, Utrecht). The three isozymes from the venom of *Agkistrodon halays blomhoffii* (Sigma) were isolated by the published procedure (Hanahan et al., 1980). Further purification of the acidic and neutral enzymes was carried out by precipitation with $NdCl_3$ followed by ion-exchange chromatography (Wells, 1975).

Protein concentrations were determined by the method of Bradford (1976). In order to calculate the values of N_S, the absorbance ($E_{1\%}$ for a 1-cm path length) values at 280 nm given in Table II were used for determining the concentration of the enzymes. These values were calculated from the amino acid composition data (Verheij et al., 1981; van den Berg et al., 1989) with standard absorbance values at 280 nm for Trp ($E_{1\%}$ = 4.2), Tyr (= 1.8), and Phe (= 0.4). Most of the PLA2 preparations were lyophilized from 1% acetic acid. Stock solutions of enzymes were prepared at concentrations of 0.05–0.2 mg/mL in water and were kept frozen at −20 °C. For kinetic studies, these solutions were thawed and kept at room temperature for at least 3 h before the kinetic measurements were made. The frozen enzyme solutions kept under these conditions retained their activity for several months even after repeated cycles of freezing and thawing. This protocol eliminated problems with enzymes that aggregate at higher concentrations or in the lyophilized form.

All fluorescence measurements were carried out at 23 °C in 10 mM Tris·HCl and 1 mM $CaCl_2$ at pH 8.0 on an SLM 4800S spectrofluorimeter (Jain et al., 1982, 1986b; Jain & Maliwal, 1985). For these measurements, the protein concentration was kept at 3–7 μM. All spectra are 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. The fluorescence intensities were expressed on an arbitrary scale, and all fluorescence results given in this paper were normalized for the same concentration of protein. Binding of PLA2 to the interface was also monitored by UV spectroscopy, which exhibits a ground state perturbation of Trp and Tyr residues near 292 nm (Hille et al., 1983). The results from these three techniques for the binding of PLA2 to an interface were always consistent.

The rationale, protocols, specific conditions, and controls for the kinetic studies are described elsewhere (Jain & Berg, 1989; Jain et al., 1986a; Jain & Gelb, 1991; Berg et al., 1991). All kinetic studies reported in this paper were carried out on the small sonicated vesicles at 0.5 mM $CaCl_2$ and 1 mM NaCl at pH 8.0 and 23 °C. Under these conditions, the maximum extent of hydrolysis was attained typically in less than 30 min for most enzymes. As shown earlier (Berg et al., 1991), the average size and dispersity of vesicles depended not only on the time of sonication but also on the presence of salt, divalent ions, chain heterogeneity, and the presence of the products of hydrolysis. For the experiments described here, it was necessary was to avoid fusion of vesicles and to minimize the intervesicle exchange of the enzyme. The acyl chain purity of the DMPM preparation was myristoyl (97%), palmitoyl (0.8%), and lauroyl (2.2%). DMPM vesicles with 99.8% chain purity also gave essentially identical results, except for the fact that the size of their sonicated vesicles was somewhat larger (Berg et al., 1991; Jain & Gelb, 1991). Vesicles were prepared by sonication of a suspension of DMPM (sodium or lithium salt) in distilled water in a bath-type sonicator (Sonicor) for 2–4 min. Sonication of the frozen suspension, which thawed during sonication, was found to be considerably more rapid and quite satisfactory to produce an almost water-clear suspension of vesicles. The reaction progress curve for the hydrolysis of anionic phospholipid (typically 1.4 μmol of DMPM) vesicles in the absence of any additive was monitored by the pH-stat (Radiometer) method at pH 8.0 in a 4-mL aqueous phase containing 1 mM NaCl and 0.5 mM $CaCl_2$ (Jain et al., 1986a; Jain & Berg, 1989; Jain & Gelb, 1991). As indicated in the text, under certain conditions the calcium concentration was 1.5 mM for the measurement of initial velocity values per enzyme molecule, v_0 (Berg et al., 1991). The reaction was always initiated by the addition of 10–30 pmol of PLA2 to an equilibrated suspension of the substrate vesicles at pH 8.0 and 23 °C. The titration efficiency for the released fatty acid was routinely monitored by adding a standard solution of myristic acid to DMPM vesicles under appropriate conditions. Such controls showed that the titration efficiency did not change noticeably in the presence of the products of hydrolysis (Jain & Gelb, 1991).

The size of DMPM vesicles was measured and compared to DMPC vesicles by several different procedures described in the literature, including gel-filtration on Agarose or Sepharose 4B columns (Brouillette et al., 1982; Andrews et al., 1975; Barenholz et al., 1977; Huang, 1969, Watts et al., 1978), electron microscopy (Bearer et al., 1982; Miyamoto et al., 1971), dynamic light scattering (Milton et al., 1984), and by comparison with the extruded vesicles as described (Berg et al., 1991). Sonicated vesicles used for these measurements were suspended in 0.5 mM $CaCl_2$, 1 mM NaCl, and 5 mM Tris·HCl at pH 8.0. To the vesicles used for the gel-filtration studies about 0.05 mol % dansyl or fluorescein-labeled DMPE was added before sonication, and the elution profile was monitored by the fluorescence intensity. The freeze–fracture electron microscopy and dynamic-scattering measurements were carried out at Queens University through the courtesy of Dr. Michael Singer (Kingston, Ontario).

Pig pancreatic PLA2 (1 μmol) was modified by *N*-methylisatoic anhydride (10 μmol) in the presence of 60 μmol of hexadecylphosphocholine in 0.5 mL of 0.1 M phosphate buffer at pH 7.0 containing 10 mM $CaCl_2$. After 90 min at room temperature, the reaction mixture was passed through a column of Sephadex G-50 (1.5 × 30 cm) preequilibrated with 1 mM EGTA in phosphate buffer (0.1 M) at pH 7.0. The labeled protein eluted with the void volume, and it was concentrated by ultrafiltration with a 2000 MW cut-off filter (Pharmacia). The concentrated protein solution was purified by FPLC on a Mono Q HR5/5 column (Pharmacia) with a 0–0.8 M NaCl gradient in 10 mM Tris·HCl at pH 8.0. The native PLA2 eluted at 0.29 M salt, whereas An87-PLA2 eluted as a single peak at 0.45 M salt. The molar absorption coefficients of the two peaks in An87-PLA2 were 19 500 (at 280 nm) and 5400 (at 340 nm) compared to 18 200 at 280 nm for

native PLA2 from pig pancreas. The absorption and the fluorescence properties of An87-PLA2 were comparable to those reported for the *N*-methylanthraniloyl derivatives of methylamine and mellitin (Hermetter & Lakowicz, 1986; Kleinfield, 1988), which suggested that 0.95–1.00 mol of *N*-methylanthraniloyl residues were bound to an amino residue of each PLA2 molecule. An87-PLA2 was denatured by incubating 0.4 μmol of protein in 0.2 mL of Tris buffer pH 8.0, containing 8 M urea and 4 μmol of dithiothreitol for 4 h at 37 °C. Then 40 μmol of iodoacetic acid was added and incubated for 30 min, followed by dialysis against 10 mM ammonium bicarbonate at pH 7.5. The mixture was treated with 0.2 μmol of TPCK-trypsin for 1 h and then heated to 100 °C. The lyophilized peptides were separated on a Mono Q column equilibrated with 5 mM Tris, pH 7.5, and developed with 20 mL of salt gradient reaching 0.4 M NaCl. Among the 15 peptide peaks in the eluent from the tryptic digest, only one fragment was found to contain all the fluorescence due to the anthraniloyl label. The position of the label at lysine 87 was identified by sequencing (Applied Biosystems 475A protein sequencer) the fluorescent fragment obtained by trypsin treatment. The first 12 residues of this fragment were FLVDNPYTESYS (positions 63–74 of the primary sequence of pig pancreatic PLA2), which showed that the site of cleavage was K-62 and the site of modification was the next lysine at position 87. The amino acid composition of the completely hydrolyzed peptide was also as expected for the 63–100 peptide, except for the deficiency of one lysine residue. These characteristics of An87-PLA2 showed that it was a homogeneous protein with all the label on K-87. The catalytic characteristics of the labeled protein on DMPM vesicles were virtually indistinguishable from the native pig pancreatic PLA2 (Table II).

For some studies, W-3 in PLA2 and in An87-PLA2 was modified with 2-hydroxy-5-nitrobenzene bromide. This resulted in a complete loss of the fluorescence and the intermolecular resonance energy transfer without any noticeable effect on N_S. For such modifications, the protein (0.014 mM in 2 mL of 0.1 M acetate buffer at pH 5.0) was incubated with a 20-fold excess of the reagent in acetate buffer until the tryptophan fluorescence was completely lost. The reaction was stopped by diluting the reaction mixture with 1 mL of 0.1 M Tris buffer at pH 8.0, and the protein was concentrated by ultrafiltration. It was not necessary to purify this preparation as it showed only one peak on the reverse-phase (ODS) column. The PLA2 modified at Trp-3 showed the same value of N_S, but the catalytic turnover ($N_S k_i$) (Berg et al., 1991) was 50% slower.

Me-PLA2 and Oct-PLA2 were prepared as described by Verheij et al. (1980). PLA2s modified with an alkylating agent at His-48 were purified by FPLC with a Mono Q HR5/5 column (Pharmacia). The elution gradient was 0.0–0.4 M NaCl in 5 mM acetate buffer at pH 5.2. The eluate was concentrated and desalted by ultrafiltration with a 2000 MW cut-off filter (Pharmacia). The protein concentration of the modified enzyme was determined (Bradford, 1976) with the protein assay reagent from Bio-Rad (10-fold dilution, monitored at 596 nm). Native pig pancreatic PLA2 ($E_{1\%}$ = 13.0) was used as a reference standard for calibration. The Me-PLA2 and Oct-PLA2 preparations were also found to be homogeneous by HPLC on ODS or Vydac C4 columns, and both of these preparations showed little residual catalytic activity on DMPM vesicles (Table IV).

The conditions used for acylation of pig pancreatic PLA2 by NOB to prepare NOB-PLA2 and for the kinetic assays with NOB were the same as described by Tomasselli et al. (1989) except that the assays were carried out at 23 °C. The assay mixture (0.75 mL) consisted of 0.088 mM NOB in 10 mM Tris, 5 mM CaCl2, 100 mM NaCl, and 1.33 μM PLA2 at pH 8.0. The progress of the reaction was monitored as an increase in the absorbance at 430 nm. Acylation of the various PLA2 preparations by NOB was carried out at 23 °C by incubating 40 μg of protein with 12 μg of NOB in 0.13 mL of buffer containing 10 mM HEPES and 5 mM $CaCl_2$ at pH 8.0. For analytical purposes, 5–20 μL of the reaction mixture was directly injected for reverse-phase HPLC on a Vydac C4 column (0.46 × 25 cm). The mixture was resolved and eluted in a binary solvent system consisting of solvent A (0.15% aqueous trifluoroacetic acid) and solvent B (0.15% trifluoroacetic acid in 70% aqueous acetonitrile). The gradient program involved an initial 5-min isocratic mode with solvent A, followed by a linear gradient to 100% solvent B over a 15-min period, and then only solvent B for another 20 min. The flow rate was kept at 1 mL/min, and the effluent stream was monitored continuously at 280 nm. Individual peaks were collected and then characterized by assay for catalytic activity on DMPM vesicles (initial rates in 2.5 mM $CaCl_2$), protein content (Bradford, 1976), absorption and fluorescence spectra, and the elution times by comparison with reference standards.

All fluorescence measurements for the binding of PLA2 to DTPM vesicles or related interfaces were done on an SLM 4800S spectrofluorimeter with a 450-W xenon source and interfaced to a microcomputer for data acquisition and manipulation. For these measurements, the excitation was set at 290 nm, slit width for excitation and emission were set at 4 nm each, and the emission spectra were scanned between the 300 to 500 nm range in 1 mM $CaCl_2$ and 10 mM Tris·HCl at pH 8.0 and 22 °C. As described elsewhere (Jain et al., 1982; 1986b; Jain & Vaz, 1987), the binding studies were done with vesicles of DTPM by monitoring Trp-3 fluorescence or, as described here under Results, by monitoring the emission from the anthraniloyl fluorophore of An87-PLA2.

Results

The Size of DMPM Vesicles. There are no known methods to directly obtain the absolute values of the number of phospholipid molecules present in the outer monolayer of a vesicle. Since the fraction of the total substrate in DMPM vesicles accessible for hydrolysis by excess enzyme was typically 63% for the sonicated vesicles used in the present study, it was very likely that these vesicles have a large curvature (Huang & Mason, 1978: Chrzeszczyk et al., 1977). A comparison of the size of DMPM vesicles with those of DMPC vesicles was made by several methods including the calibration with extruded vesicles (Berg et al., 1991). Also as shown in Figure 1, the elution profiles of DMPM and DMPC from the Sepharose 4B column were nearly identical: their elution volumes were the same, while the width of the elution peak for DMPM vesicles was somewhat narrower. These results suggested that the diameter of sonicated DMPM vesicles in 0.6 mM $CaCl_2$ was approximately the same as that of the sonicated DMPC vesicles, i.e., in the range of 200–250 Å (Huang, 1969; Watts et al., 1978). As summarized in Table I, we also obtained an estimate of the diameter of DMPM vesicles by other methods to be 250 Å with 95% of the population dispersed between 210 and 270 Å. This corresponded to the dispersity of about 0.2.

From the diameter of the DMPM vesicles, we calculated the total number of substrate molecules on the outer monolayer. A vesicle of 250-Å diameter has a surface area of 200000 Å^2. The area per phospholipid in small vesicles is not

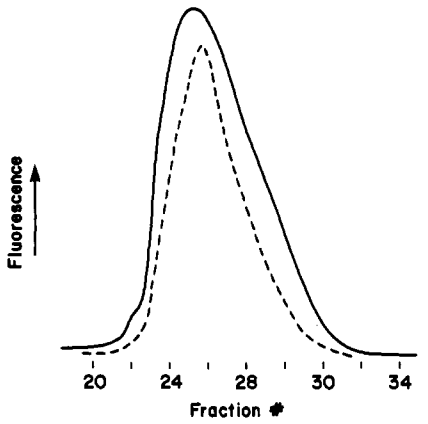

FIGURE 1: Elution profile of DMPC (solid line) and of DMPM (dotted line) vesicles from a 1.2 × 45 cm column of Sepharose 4B. The vesicles were prepared by sonicating the appropriate lipid in 0.5 mM $CaCl_2$, 1 mM NaCl, and 5 mM Tris·HCl, pH 8.0. About 1 μg of dansyl- or fluorescein-labeled dimyristoylglycerophosphoethanolamine was also added before sonication for each milligram of DMPM or DMPC. The elution profile was monitored by measuring the fluorescence intensity of the individual fractions. The flow rate was approximately 7 mL/h, and each fraction was 1.0 mL.

Table I: Diameter of Sonicated Vesicles of DMPM Determined by Different Methods

method	diameter (Å)[a]
freeze–fracture EM	220–350 (n = 580)
light scattering	220–280
gel filtration on Sepharose 4B	200–270
extent of hydrolysis (63%)	230
calibration with extruded vesicles[b]	230 (N_s = 4400)

[a] The values for the diameter of vesicles are for the vesicle population that includes 95% of the total lipid. [b] See Figure 4 in Berg et al. (1991) for details.

known; however, on the basis of the X-ray diffraction and monolayer data, the cross-sectional area per polymethylene chain was expected to be 22–25 Å^2 (Cevc & Marsh, 1987; Jain, 1988). Therefore, the area per phospholipid molecule containing two saturated acyl chains was assumed to be 45–50 Å^2 (Nagle & Wiener, 1988). Thus the average number of phospholipid molecules in the outer monolayer of a DMPM vesicle of 250-Å diameter would be between 4500 and 4000. As shown below, these estimates were in the range of values of N_S obtained from the extent of hydrolysis in the reaction progress curves for PLA2 from several sources.

Reaction Progress Curves for Interfacial Catalysis in the Scooting Mode. The high-affinity binding of PLA2s to DMPM vesicles was best demonstrated by the characteristics of the reaction progress curve (Jain et al., 1986a; Berg et al., 1991). As compared in Figure 2, the extent of hydrolysis for the same preparation of DMPM vesicles by the same amount of PLA2 from *Crotalus atrox* or pig pancreas were virtually identical. In fact, the reaction progress curves of the type shown in Figure 2 were observed with all the PLA2s that we have examined including those from venoms of elapidae and crotalidae. As summarized in Table II, the value of the number of substrates hydrolyzed at the end of the reaction per molecule of enzyme, N_S, was constant within 12% for all the enzymes that we have tested. In these reaction progress curves, the hydrolysis ceased before all of the substrate was hydrolyzed, and the value of N_S was obtained by dividing the moles of product formed at the end of the reaction by the moles of monomeric enzyme in the mixture (Berg et al., 1991). None of the PLA2s or their mutants that we examined (well over 60 including the ones listed in Table II) exhibited any latency period, and the hydrolysis of DMPM vesicles began within 3

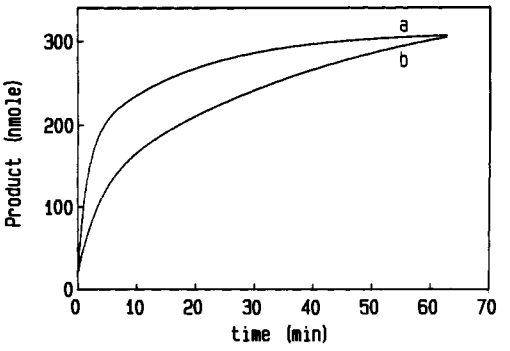

FIGURE 2: Reaction progress curve for the hydrolysis of DMPM vesicles (2 mg or 3 μmol in 4 mL of 0.5 mM $CaCl_2$ and 1 mM NaCl at pH 8.0) by PLA2 from pig pancreas (0.57 μg or 45 pmol, curve a) and *Crotalus atrox* (0.57 μg, curve b). A general quantitative description of the reaction progress curves for catalysis in the scooting mode was given elsewhere (Berg et al., 1991). The values of N_S summarized in Table II were obtained by dividing the extent of hydrolysis in moles by the moles of enzyme in the reaction mixture.

Table II: Catalytic Parameters for the Hydrolysis of Vesicles by Phospholipase A_2 at 21 °C[a]

no.	enzyme	$E_{1\%}$	relative N_s
	pancreas		
1	pig	13	1.0 (= 4300)
1a	pig An87-PLA2	13	0.98
1b	pig Palm-110 PLA2	13	0.97
1c	pig NOB-PLA2	13	0.94
1d	pig Trp-3 modified	7.8	0.93
1e	AMPA	13	1.0
2	iso-pig	13	0.98
3	bovine	13	1.04
4	horse	13	1.13
5	human	13	0.97
6	sheep	13	1.02
	venoms		
7	bee	13	1.02
8	*Naja mosambica* CM2	23	1.02
9	*Naja mosambica* CM3	23	1.09
10	*Naja melanoleuca* DE1	23	1.05
11	*Naja malenoleuca* DE2	23	1.1
12	*Naja melanoleuca* DE3	23	1.04
13	*Naja naja naja*	23	1.1
	Agkistrodon halys blomhoffii		
14	CM1	27	0.96
15	CM2	27	0.97
16	CM3	27	0.91
17	*Crotalus atrox*	23	0.96
18	*Hemachatus hemachatus* DE1	27	1.08

[a] $E_{1\%}$ is the absorbance of the 1% solution of the enzyme in buffer at pH 8.0. N_s is the extent of hydrolysis per enzyme (MW 13900) for the hydrolysis of DMPM vesicles at 21 °C, pH 8.0, with 0.5 mM $CaCl_2$. Its value depends on the size of vesicles (Jain et al., 1990a).

s after the addition of the enzyme. For the objectives set for the present study, we compared only the extent of hydrolysis per enzyme molecule, N_S, for PLA2s from different sources. In Table II we summarize these values relative to those observed with pig PLA2 on the same preparation of DMPM vesicles. The correspondence between the observed value of N_S (4300 for the pig PLA2 when a molecular weight of 14000 is used) and the calculated value of the number of substrate molecules in the outer monolayer of the target vesicles led to the conclusion that the outer monolayer of one vesicle was hydrolyzed by one molecule of PLA2 of molecular weight 14000, irrespective of its source or the state of chemical modification. This conclusion is further reinforced by the following observations and controls.

(a) As was the case with the naturally occurring PLA2s, for the chemically modified enzymes, the value of N_S was

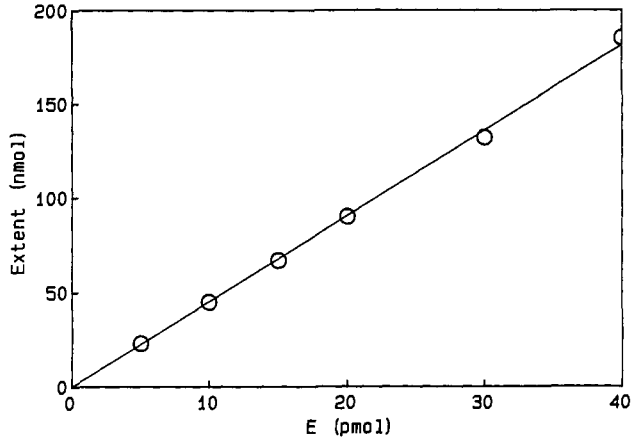

FIGURE 3: Dependence of the extent of hydrolysis of DMPM (1.5 mg) vesicles as a function of the amount of *Naja melanoleuca* DEI PLA2 (E). Other conditions were as given in the legend to Figure 2.

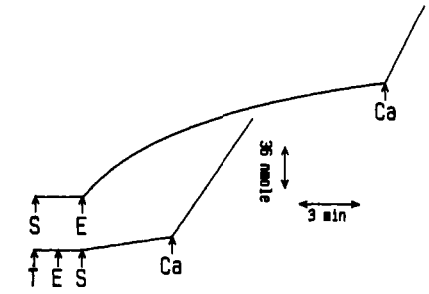

FIGURE 4: Reaction progress curves for the hydrolysis of DMPM (S) vesicles (0.7 mg) by *Naja melanoleuca* DEI PLA2 (20 pmol) in the absence (top) or in the presence (bottom) of DTPM (T) vesicles. The sequence of addition of T, E, and S and calcium is also indicated. Thus, for example, addition of E to DTPM vesicles (bottom curve) bound the enzyme irreversibly, and the addition of DMPM (S) vesicles did not initiate significant proton release. However, on the addition of calcium (2.5 mM final concentration), the lipid vesicles fused and thus the substrate became accessible to the enzyme bound to DTPM vesicles. Other conditions are described in legend to Figure 2.

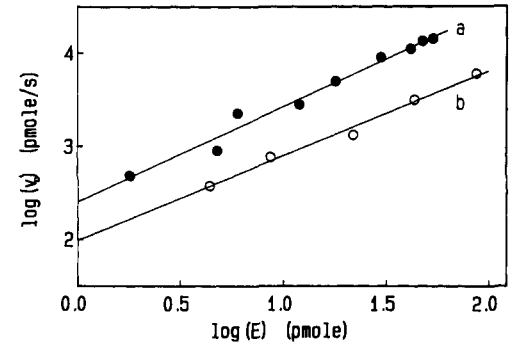

FIGURE 5: Dependence of the initial rate of hydrolysis of DMPM vesicles with varying amounts of PLA2 from pig pancreas (closed circles) and *Crotalus atrox* (open circles). Note that the scales are logarithmic and the slopes are 1.03 and 0.97, respectively.

essentially identical with that for the native PLA2s (Table II, entries 1a–e). The fact that N_S remained unchanged on chemical modification showed that all molecules in the modified enzyme were catalytically active, although their catalytic turnover numbers, $N_S k_i$ values, depended on the nature of the interface. A protocol based on the value of N_S also provided a useful method to ascertain the number of catalytically active PLA2 molecules in a chemically modified preparation or in preparations containing impurities other than PLA2 (Jain & Gelb, 1991).

(b) The value of N_S was obtained under conditions where there is more than a 5-fold excess of vesicles per enzyme. As shown in Figure 3, the extent of hydrolysis by *Naja melanoleuca* DEI PLA2 increased linearly with the amount of enzyme initially added to the reaction mixture, whereas the value of N_S remained constant as long as the total vesicle to enzyme ratio was maintained above 5, which assured with more than 97% Poissonian probability that every enzyme molecule was bound to a different vesicle (Jain & Berg, 1989).

(c) The constraints that defined the experimental boundary conditions for the interpretation of N_S were reaffirmed by several controls for the venom PLA2s as we had shown before for pig pancreatic PLA2 (Jain et al., 1986a–d; Berg et al., 1991). The fact that only a fraction of the total available substrate in the reaction mixture (cf. Figure 2) was hydrolyzed by all PLA2s was consistent with the conclusion that the enzyme, substrate, or the products of hydrolysis did not undergo intervesicle exchange. As shown in Figure 4, like the pancreatic enzymes (Jain et al., 1986a,b), the *Naja melanoleuca* DEI enzyme bound essentially irreversibly to DTPM vesicles and did not exchange significantly with the excess DMPM vesicles added afterward. Thus, addition of venom or pancreatic PLA2 to DTPM vesicles prevented the hydrolysis of DMPM vesicles added later. Hydrolysis would have ensued if PLA2 could hop to DMPM vesicles from DTPM vesicles, or if the phospholipid molecules in these vesicles could exchange, or if the enzyme-containing vesicles could fuse with excess free vesicles. A similar lack of intervesicle exchange of the enzyme was observed in the presence of the products of hydrolysis, i.e., at the end of the reaction progress curve (data not shown). As also shown in Figure 4, addition of higher amounts of calcium [acting as a fusogen as shown in Jain et al. (1986a)] immediately reinitiated hydrolysis of excess vesicles. The fraction of the total substrate accessible for hydrolysis was independent of the source of the enzyme. Such control experiments (Jain et al., 1986a–d; Jain & Gelb, 1991; Berg et al., 1991) served to demonstrate that the characteristic value of N_S for a PLA2 was a function of the number of PLA2 molecules present in the reaction mixture and that a characteristic value of N_S for a vesicle population was not a consequence of the product inhibition, or inactivation of the bound enzyme, or the position of the thermodynamic equilibrium of the underlying esterolytic reaction. Possible artifacts arising from product inhibition and titration efficiency have also been ruled out in these studies as a basis for partial hydrolysis of the total substrate present in the reaction mixture (Jain & Gelb, 1991).

(d) The conclusion that monomeric PLA2 is catalytically active was also supported by the observations summarized in Figure 5. As shown in Berg et al. (1991), the initial rate of hydrolysis of DMPM vesicles can be measured directly or computed from the whole reaction progress curve for the hydrolysis of large vesicles. Thus, by varying the enzyme concentration in the reaction mixture, it was possible to observe the rate of hydrolysis under conditions where there was only one or more than one PLA2 molecule per vesicle. As summarized in Figure 5, the initial rate of hydrolysis of DMPM vesicles at 1.5 mM calcium increased linearly with the amount of enzyme in the reaction mixture. The fact that the log–log plot was linear with a slope of 1 for PLA2 from pig pancreas or *Crotalus atrox* further supported the conclusion that the monomeric PLA2 was fully catalytically active and that there was no anomalous change in the catalytic activity when two or more PLA2 molecules were present on the same surface, i.e., the catalytic activity per subunit would be the same in a dimer if it is formed at all. The y intercepts for the two

enzymes were noticeably different, which underscored the fact that the rates of catalytic turnover by these two enzymes were noticeably different, i.e., about 275 s^{-1} for the pancreatic versus 100 s^{-1} for the *Crotalus atox* PLA2.

(e) As expected, the time required to reach the end of the reaction was different for the different enzymes examined (i.e., as in Figure 2). This reflected the different rate parameters $N_S k_i$ (Berg et al., 1991) and v_0 for PLA2s from different sources which fell into the range of 5–500 s^{-1} (data not show).

(f) Elsewhere (Ghomashchi et al., 1991b) we showed that catalysis in the scooting mode by pancreatic PLA2 was observed with covesicles of zwitterionic and anionic phospholipids. Here again the extent of hydrolysis per enzyme (of MW 14000) measured as N_S was the same for all PLA2s. These results (not shown) demonstrated that the nature of substrate had little effect on the state of aggregation of the bound catalytically active enzyme.

The results summarized in Figures 1–5 showed that the value of N_S provided a useful basis to ascertain the number of catalytically active PLA2 molecules in a preparation, that all the PLA2s that we tested were fully catalytically active as monomers, and that there was no anomalous increase in the rate of catalytic turnover even when it is possible for two PLA2 molecules to be in contact with each other at the substrate interface.

PLA2 Molecules Do Not Aggregate in the Interface. Pig pancreatic PLA2 binds essentially irreversibly ($K_d < 0.1$ pM) to DTPM vesicles (Jain et al., 1986b). Therefore, intermolecular resonance energy transfer between suitably chosen acceptor–donor pairs (Hermetter & Lakowicz, 1986; Kleinfield, 1988) was used to monitor intermolecular interactions between the PLA2 molecules at the interface without any complications from a shift in the E to E* equilibrium. The energy transfer from tryptophan (excitation at 280–290 nm and emission at 320–360 nm) to an anthraniloyl fluorophore on proteins (excitation at 330–360 nm and emission at 420–440 nm) with an energy transfer distance (R_0) of 22 Å was particularly well suited for studying intermolecular interaction in the aqueous phase as well as in the interface (Hermetter & Lakowicz, 1986). This was achieved with An87-PLA2. An examination of the X-ray crystal structure (Protein Data Bank file viewed with the QUANTA display program of Polygen) of pig pancreatic PLA2 (Dijkstra et al., 1983; Kuipers et al., 1989) showed that K-87 is located at the surface of PLA2 that is probably exposed to the aqueous phase and is at the edge of the i face (Jain & Berg, 1989; Ramirez & Jain, 1991), and it is about 17 Å away from Trp-3, which is located in the interfacial binding region of the protein.

As shown in Figure 6A, An87-PLA2 showed an emission maximum at 426 nm when excited at 280 nm (dashed curve), whereas the native enzyme showed only the emission at 345 nm (dotted curve). The emission spectrum of An87-PLA2 obtained on excitation at 345 nm was also compared (solid curve in this figure). In An87-PLA2 the intramolecular energy transfer occurred with about 90% efficiency, i.e., about 90% of the emission from tryptophan was transferred to the anthraniloyl fluorophore present in An87-PLA2. This was consistent with the X-ray crystallographic distance of 16.7 Å between Lys-87 and Trp-3. This conclusion was further supported by the fact that the emission intensity at 426 nm decreased and the intensity at 335 nm increased when An87-PLA2 was denatured and unfolded with urea and mercaptoethanol. The protein was protected from denaturation by lipid vesicles (data not shown). Similarly, as shown in Figure 6B, the intramolecular resonance energy transfer

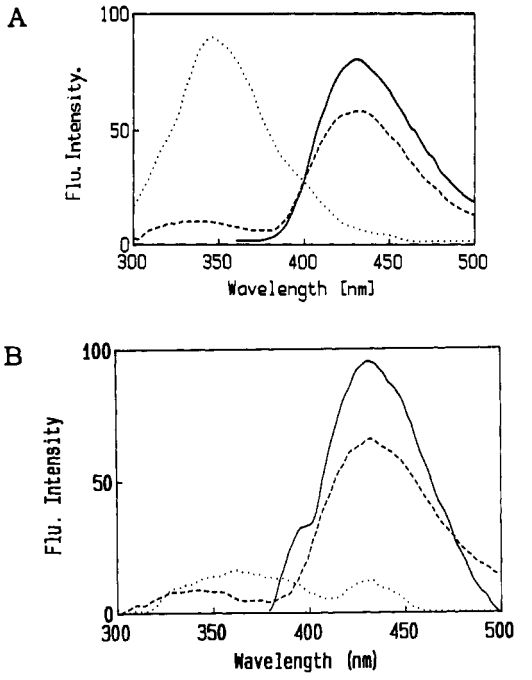

FIGURE 6: (A) Fluorescence emission spectra of pig pancreatic PLA2 (1.73 μM) on excitation at 280 nm (dotted line) and of An87-PLA2 (1.73 μM) on excitation at 280 nm (dashed line) and 345 nm (solid line). All spectra in this and next three figures were recorded in 1 mM $CaCl_2$ and 10 mM Tris·HCl at pH 8.0. The concentration of protein and the ordinate scale were the same for all the measurements in this and the next two figures. (B) Fluorescence emission spectra of An87-PLA2 (dashed line, excitation at 280 nm). After the addition of nitrobenzene bromide for covalent modification of Trp-3, the spectral characteristics are altered (dotted line for excitation at 280 nm and solid line for excitation at 340 nm).

in An87-PLA2 was completely lost on modification of Trp-3 with *N*-bromosuccinimide or 2-hydroxy-5-nitrobenzene bromide. Also Trp-3 in An87-PLA2 bound to the interface was not modified under the same conditions. These results showed that, in An87-PLA2, the resonance energy transfer occurred from the Trp-3 donor to the anthraniloyl acceptor on Lys-87.

As shown under Materials and Methods, An87-PLA2 had only one anthraniloyl fluorophore per PLA2 molecule. The reaction progress curve for An87-PLA2 was essentially identical with that obtained with native pig pancreatic PLA2, which showed that the interfacial binding and the catalytic properties (N_S and k_i) of pig pancreatic PLA2 were not noticeably altered on modification with *N*-methylisatoic anhydride. As shown in Figure 7A, binding of An87-PLA2 to bilayers of DTPM (1:1000 mole ratio) gave an increased emission intensity (compare solid and dashed curves). Such spectral changes were also observed on binding of An87-PLA2 to micelles of 2-hexadecylphosphocholine and decyl sulfate as well as to vesicles of DMPM. However, as expected (Jain et al., 1982, 1986a–d), such binding was not observed with vesicles of DTPC (data not shown). These controls showed that An87-PLA2 was catalytically functional and its binding behavior to the vesicle interface was similar to that of the modified pig pancreatic PLA2.

Besides the intramolecular resonance energy transfer in An87-PLA2 as described above, intermolecular energy transfer between PLA2 and An87-PLA2 in DTPM vesicles was also monitored. As shown in Figure 7A, the fluorescence spectrum of An87-PLA2 on DTPM vesicles showed a major peak at 426 nm and only a relatively small emission in the 330–350-nm region. Under these conditions, each DTPM vesicle contained on the average six An87-PLA2 molecules, which would occupy

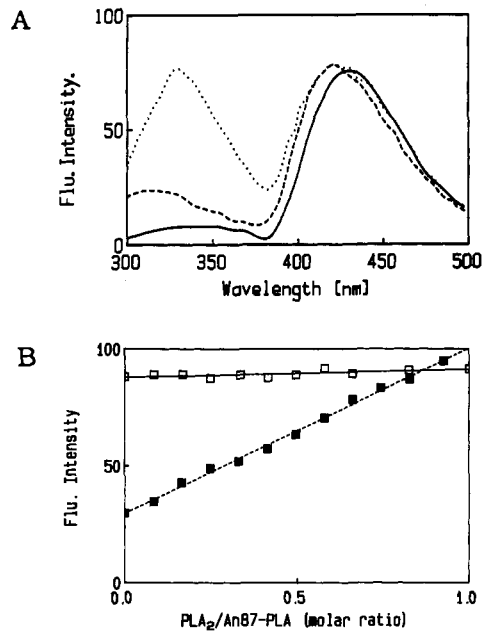

FIGURE 7: (A) Fluorescence emission spectrum of An87-PLA (1.78 μM) in the absence (solid curve) and in the presence (dashed curve) of DTPM (1.73 mM) vesicles. On the addition of 1.73 μM pig pancreatic PLA2 (dotted curve), an additional peak at 333 nm was observed without a change in the 426-nm peak, which showed that there was no intermolecular resonance energy transfer from native PLA to An87-PLA2 even though each vesicle on the average contained six molecules each of labeled and unlabeled PLA2. (B) Change in the fluorescence intensity of An87-PLA2 (open squares) at 426 nm and (closed squares) at 333 nm on the addition of pig pancreatic PLA2 to the mixture of An87-PLA2 (1.73 μM) and 1.7 mM DTPM vesicles. The increase in intensity at 333 nm is due to the emission from pig pancreatic PLA2 bound to DTPM vesicles. Other conditions were as given in the legend to Figure 6.

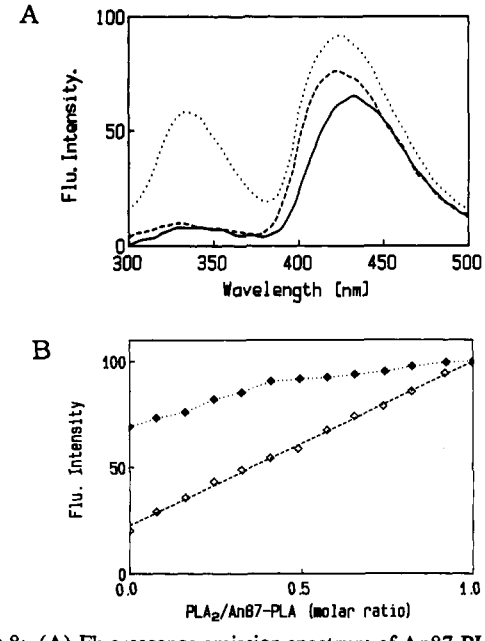

FIGURE 8: (A) Fluorescence emission spectrum of An87-PLA2 (1.73 μM) (solid curve), An87-PLA2 + 170 μM DTPM (1:100 mole ratio or 50:1 enzyme to vesicle ratio) (dashed curve), and An87-PLA2 + PLA2 + DTPM (1:1:100 mole ratios or 50:50:1 enzyme to vesicle ratio (dotted curve). Under these conditions, the density of the enzyme molecules at the vesicle surface was high because each molecule occupies about 0.8% of the surface corresponding to 35 substrate molecules (Jain & Berg, 1989). (B) Increase in the fluorescence intensity at 426 nm (closed diamonds) and at 333 nm (open diamonds) as a function of increasing mole ratio of pig pancreatic PLA2 added to An87-PLA2 and DTPM (1:100 ratio).

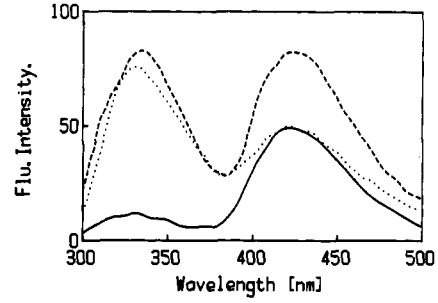

FIGURE 9: (A) Fluorescence emission spectrum of An87-PLA2 (1.73 μM) + decyl sulfate (17.3 mM) (solid curve), An87-PLA2 + PLA2 + decyl sulfate (1:1:100 mole ratio) (dashed curve), and An87-PLA2 + PLA2 + decyl sulfate (1:1:10 000 mole ratio) (dotted curve).

about 4% of the surface area of the vesicles (Jain & Berg, 1989). On the addition of native PLA2 (dotted curve in Figure 7A and also the derived data shown in Figure 7B) to these vesicles the emission at 426 nm did not change, whereas all the increase in the fluorescence intensity at 333 nm was due to the binding of native PLA2 to DTPM vesicles. Similar results were obtained with DMPM vesicles or in the absence of any vesicles (data not shown). A complete lack of intermolecular resonance energy transfer from PLA2 to An87-PLA2 in the aqueous phase or when bound to the same vesicle suggested that pig pancreatic PLA2 molecules in the interface or in the aqueous phase did not have any significant affinity for each other. As described below, this donor–acceptor pair was capable of providing information about close contact of PLA2 and An87-PLA2 as a resonance energy transfer signal if PLA2 and An87-PLA2 approached at the interface under conditions of crowding.

Intermolecular resonance energy transfer from PLA2 to An87-PLA2 in the interface was observed only under conditions where PLA2 and An87-PLA2 were crowded on the interface at a high surface density of the protein. For example, as shown in Figure 8A, incorporation of An87-PLA2 in DTPM vesicles (protein to lipid mole ratio 1:100, corresponding to an enzyme to vesicle ratio of about 50:1) caused an increase in the intensity at 426 nm (dashed curve), which increased further on the addition of PLA2 to these vesicles (dotted curve). The change in the fluorescence intensity at 426 and 333 nm as a function of an increasing mole ratio of PLA2 in An87-PLA2 + DTPM vesicles was shown by results summarized in Figure 8B. Similar results were obtained with DMPM vesicles. Under these conditions of high surface density of donor–acceptor pairs (i.e., "crowding" of An87-PLA2 and native PLA2 on the vesicle interface), the intermolecular resonance energy transfer occurred between Trp-3 and An-87 fluorophores. Such intermolecular energy transfer under crowded conditions was also observed with PLA2 in which His-48 of the catalytic site had been alkylated or with An87-PLA2 in which Trp-3 had been modified with 2-hydroxy-5-nitrobenzene bromide (data not shown). These results showed that functional catalytic site was not required for binding of PLA2 to DTPM or DMPM vesicles or for the intermolecular resonance energy transfer to An87-PLA2.

Intermolecular resonance energy transfer from pig pancreatic PLA2 to An87-PLA2 was also observed in premicellar aggregates with decyl sulfate. Hille et al. (1983) showed that pig PLA2 formed an aggregate with decyl sulfate below the critical micelle concentration of the pure amphiphile. As shown in Figure 9, addition of PLA2 to An87-PLA2 in decyl sulfate (compare the solid curve with the dashed curve) resulted in an increase in the emission intensity at 426 nm. This

Table III: Relative Quantum Yields of PLA2 and of HDNS under a Variety of Conditions in the Presence of PLA2s

conditions[a]	emission (nm)	relative fluorescence intensity		
		PLA2	Oct-PLA2	Me-PLA2
PLA2	342	0.37	0.29	0.43
PLA2	333	0.317	0.24	0.33
P + L (1:100)	333	1.00	0.60	0.72
n		51	40	48
K_d (μM)		<0.1	<0.1	<0.1
L + HDNS + P (40:2:1)	490	1.00	0.62	0.74

[a] DTPM (= L), 670 μM; PLA2 (= P), 3–7 μM; HDNS, 26.8 μM. For all measurements the excitation wavelength was set at 295 nm.

was expected if PLA2 and An87-PLA2 were in the same premicellar aggregate, where emission from PLA2 was accepted by An87-PLA2 in the same aggregate. Also as shown in this figure, such aggregates readily dispersed on the addition of a 1000-fold molar excess of decyl sulfate (dotted curve) where the resonance energy transfer signal at 426 nm disappeared. Similar results were obtained with micelles of 2-hexadecylphosphocholine, which suggested that the PLA2 molecules did not form a specific complex in the micelles. Complementary experiments with An87-PLA2 in which Trp-3 had been inactivated also gave comparable results (data not shown), which showed that intermolecular energy transfer occurred only under the conditions of high surface density or "crowding" of donor and acceptor molecules.

The results on resonance energy transfer between PLA2 and An87-PLA2 (cf. Figures 8 and 9) at high surface density (crowding) showed that pig pancreatic PLA2 and An87-PLA2 in the interface can act as a donor–acceptor pair for resonance energy transfer. However, the energy transfer under such conditions could not be taken as an indication of their propensity to form specific aggregates. In fact, in conjunction with the results in Figures 8 and 9, a lack of intermolecular energy transfer under the conditions of Figure 7 unequivocally ruled out any specific interaction between PLA2 molecules in the micellar or bilayer interfaces of the substrate or a substrate analogue. This was because intermolecular energy transfer between PLA2 and An87-PLA2 was observed only when the surface was virtually completely covered with these enzymes but not under the conditions of lower surface density.

Binding of Alkylated Phospholipase A_2 to Bilayers. We also found that the fluorescence properties of PLA2 alkylated at His-48 in the aqueous phase were similar to those of the native enzyme and the alkylated enzyme bound as readily to the interface as did the native PLA2. Binding of PLA2 to the substrate interface was most conveniently determined by monitoring the fluorescence of W-3. In order to establish whether this fluorescence change was from the bound enzyme containing the substrate at the active site, we studied the binding of the alkylated PLA2 to bilayers by spectroscopic methods (Jain et al., 1982, 1986; Jain & Vaz, 1987). The results summarized in Table III showed that the fluorescence emission spectra of alkylated PLA2 were comparable to that of the native enzyme in the presence and in the absence of DTPM vesicles. In all cases the emission maximum remained at 342 nm for the free protein and at 333 nm for the protein bound to DTPM vesicles. The difference spectra, obtained by subtracting the spectrum in the aqueous phase from that in the presence of DTPM vesicles, were essentially identical for PLA2, Me-PLA2, and Oct-PLA2. From the plot of the change in the emission intensity versus the concentration of DTPM vesicles (binding isotherms), we obtained the values of the relative fluorescence intensity of the bound enzyme (row 3) and the number of phospholipid molecules (= n, row 4) that apparently bind to a PLA2 molecule at the interface. Values of n were obtained as described previously (Jain et al., 1986b,c). Due to the steepness of the binding isotherms, we could not compute the values of apparent dissociation constant of the bound enzyme; however, the upper limit for K_d was estimated to be <100 nM. As was the case with native PLA2, alkylated PLA2 also did not bind to DTPC vesicles. On the basis of the characteristics of these binding isotherms, there was no major change in the binding characteristics of PLA2 modified at the active site residue His-48, although the overall increase in the fluorescence intensity of the alkylated enzymes was somewhat smaller than that of the native enzyme (Table III, row 3).

As summarized in Table III, the binding of PLA2 to an interface was also determined by resonance energy transfer between Trp-3 and a dansyl group present at the bilayer interface. Thus the fluorescence intensity of the fluorescent probe HDNS in DTPM vesicles in the 450–550-nm region increased appreciably in the presence of PLA2. This increase in the fluorescence intensity was accompanied by a corresponding monotonic decrease in the fluorescence emission from Trp-3 in the 333-nm region, and the energy transfer efficiency was >95% at >3 mol % HDNS in DTPM vesicles. The mole percent of HDNS required for a 50% decrease in the fluorescence intensity at 333 nm was approximately the same for the native as well as for the alkylated PLA. These results suggested that the apparent efficiency of energy transfer from Trp-3 of alkylated PLA2 to HDNS at the interface was appreciably lower than it was from Trp-3 of the native enzyme. As elaborated elsewhere (Jain & Vaz, 1987) such differences could be rationalized in terms of the various terms in the Förster energy transfer equation; however, at this stage we were not in a position to dissect their relative contributions.

Acylation of Pig Pancreatic PLA2 by NOB. Acylation of K-56 in PLA2 by 4-nitro-3-octanoyloxy benzoate (NOB) was suggested as a prerequisite for interfacial catalysis (Tomasselli et al., 1989). As summarized in Table II, the value of N_S (as well as the $N_S k_i$ and v_0 values, not shown) for PLA2 palmitoylated at K-110 (Palm-110 PLA2) was essentially identical with the values for other PLA2s. Similarly, an obligatory role of covalent modification of lysine(s) on the catalytic or binding process was unequivocally ruled out by the fact that the binding and the catalytic properties of pig pancreatic PLA2 and AMPA (peramidinated PLA2 in which all lysines were chemically modified) were essentially identical [Table II, see also Jain et al. (1986b)]. The fact that PLA2 from all sources bound to the DTPM interface with a high affinity ruled out the possibility that acylation of K-56 was a prerequisite for the binding of PLA2 to this interface because Lys-56 was not a conserved residue. The latency period for the action of PLA2 on DMPM vesicles was less than 3 s, which ruled out the possibility that a kinetically slow acylation step was required for interfacial activation of the enzyme bound to the interface. Many other possibilities were also ruled out by results summarized in Table IV. The possibility that catalytic activity was necessary for acylation of PLA2 by NOB was ruled out because Me-PLA2 was acylated by NOB, whereas it had very low catalytic activity. The possibility that native PLA2 was acylated by phospholipid was also ruled out by the fact that PLA2 incubated with DMPM or its short-chain homologue was not acylated at all. Finally, the esterolytic activity of PLA2, AMPA, and Me-PLA2 on NOB was comparable to bovine serum albumin (Table IV), which raised the possibility that the hydrophobic domain on these proteins was somehow

Table IV: Rates of Catalytic Turnover by Derivatized PLA2s for the Hydrolysis of NOB or DMPM

enzyme [elution time (min)]	turnover number per min[a] NOB	DMPM
PLA2 (22.73)	2.5	16500
AMPA	1.2	15800
ProPLA2 (19.7)	0.1	110
MePLA2 (22.9)	0.4	150
Oct-PLA2 (24.0)	0.06	<10
acylated (with NOB)		
PLA2 (23.7)	4	14400
MePLA2 (24.5)	0.3	130
Oct-PLA2 (25.6)	0.05	<10
bovine serum albumin	0.8	<0.1

[a] For both substrates the concentration was 88 μM. For DMPM the calcium concentration was 2.5 mM, and the amount of enzyme added was 3 pmol in a 4-mL reaction mixture. For NOB the calcium concentration was 5 mM, and the amount of enzyme added was 0.5–2 nmol in 0.75-mL reaction mixture. The elution times given in parenthese are for chromatography on a reverse-phase column (see Materials and Methods).

responsible for their catalytic activity. The fact that NOB was not hydrolyzed by the proenzyme form of PLA2 and that this enzyme was not acylated by NOB raised the interesting possibility that the hydrophobic region responsible for the action on NOB may be blocked in the precursor PLA2 by the heptapeptide at its N terminus. We also investigated the effect of NOB-PLA2 and of Palm-110 PLA2 on the hydrolysis of DMPC vesicles. In both cases the latency phase and the shape of the reaction progress curve was similar to that observed with native PLA2 (Apitz et al., 1982), which ruled out a role for acylation in the pre-steady-state binding of the enzyme to the interface.

Discussion

In this paper we addressed a key question about the state of catalytically active PLA2 at the interface. The results on interfacial catalysis in the scooting mode on DMPM vesicles with several secretory PLA2s unequivocally demonstrated that, irrespective of their origin, all PLA2s hydrolyze DMPM vesicles without any latency period. Within the constraints of the experimental boundary conditions elaborated earlier for catalysis in the scooting mode (Berg et al., 1991; Jain & Berg, 1989; Jain et al., 1986a), the results reported in this paper lead to the inescapable conclusion that monomeric PLA2 (irrespective of its source) was fully catalytically functional at the interface. Besides ruling out the possibility of dimer formation at the interface, we demonstrated that the binding of an amphiphile to the catalytic site was not required for a high-affinity binding of PLA2 to the interface. Similarly, the results and arguments developed in the preceding section unequivocally demonstrated that acylation of Lys-56 was not required for the high-affinity binding of PLA2 to the interface or for the catalysis at the interface.

Having ruled out the possibilities of specific aggregation, or a covalent modification, or binding of the substrate to the active site of PLA2 as a prelude to the steady-state catalytic turnover at the interface, it is probably worth considering alternative explanations for the observations that gave rise to such possibilities in the first place. The "quality of interface" has been considered a key determinant in the regulation of the catalytic activity by PLA2. Our ability to resolve the E to E* step from the catalytic steps unequivocally demonstrated that virtually all the kinetic manifestations related to critical micelle concentration, pre-steady-state latency period, isothermal and thermotropic gel–fluid transition, surface pressure, and requirements for additives and detergents were due to their effects on the E to E* equilibrium (Jain & Berg, 1989). This was underscored by the fact that PLA2 from all sources and many of their mutants that we have tested (Table II and more than 40 others) hydrolyzed DMPM vesicles or covesicles of zwitterionic phospholipids with anionic phospholipids. Except for the differences associated with substrate specificity and product inhibition, our results leave little doubt that PLA2 from different sources bind with high affinity to anionic interfaces and that such binding was rapid (Jain et al., 1988). As suggested earlier, the electrostatic interactions between the cationic groups around the interfacial binding surface (i-face) of PLA2 and the anionic groups on the substrate interface could be a prelude to the high-affinity binding accompanied by desolvation of the microinterface of E* with the substrate interface (Jain & Vaz, 1987). All processes that interfere with this event would also interfere with the overall catalytic turnover. Thus an apparent activation would be seen whenever the factors responsible for the E to E* equilibrium are modulated in favor of E*. This could be achieved readily if the binding of the enzyme to the interface was relatively poor as elaborated by examples discussed below:

(a) Several reports have considered the possibility that an aggregated form of PLA2 at the interface was more active than monomeric PLA2 (Dennis, 1983; Menashe et al., 1985); however, evidence for the formation of such aggregates was usually derived from dimer formation at higher enzyme concentrations in the aqueous phase or in the premicellar aggregates with certain amphiphiles (Bukowski & Teller, 1986; van Oort et al., 1986, de Haas et al., 1987; Hazlett & Dennis, 1988; van Eijk et al., 1983). Obviously, PLA2s exhibit such a tendency to aggregate because their i-faces are hydrophobic with varying numbers of cationic residues on this face. In these putative dimers or aggregates the contact surface would block the access to the active site, as indeed appears to be the case for dimers observed in the crystallographic unit cells (Dijkstra et al., 1983; Renetseder et al., 1985; Kuipers et al., 1989). It is intriguing to consider the significance of the fact that PLA2 from *Crotalus atrox* is present as a dimer in the aqueous phase with a dissociation constant of about 0.1 nM. Thus in the stock solution (3–5 μM) it would be present as a dimer. If this dimer was a face-to-face dimer, as seen in the crystallographic unit cell (Renetseder et al., 1985), it must dissociate before binding to the interface because, as shown here, during steady-state catalytic turnover there was only one PLA2 per vesicle. This would be expected because the K_D for PLA2s from most sources bound to DMPM vesicles is <1 pM, which is significantly below the equilibrium constant for dimer dissociation. Indeed, a similar situation was encountered in the presence of the specific inhibitor of PLA2 from serum of *Trimeresurus flavoviridis* (Jain et al., to be published). The dissociation constant for the EI complex was 0.1 nM; so when the complex was added to DMPM vesicles, hydrolysis began immediately, showing that the association constant for the binding of the enzyme to this interface was much more favorable. Also, the fact that hydrolysis started immediately after the addition of the complex showed that the dissociation was rapid. On the other hand, this EI complex added to DMPC + product vesicles did not dissociate because the apparent dissociation constant for PLA2 bound to this interface was >10 μM (Jain et al., 1982).

(b) Acylation of PLA2 by NOB does occur. However, it was not necessary for catalysis, and the effect of such acylation was not seen when the binding to the interface was favored. In the "activation" experiments with acylated PLA2, To-

masselli and co-workers (1989) used the monolayer interface where the apparent binding was extremely poor, i.e., less than 3% of the enzyme was in the E* form (Patus et al., 1979). Thus incorporation of the acyl chain at K-56 would alter the equilibrium for the binding of the enzyme between the aqueous and monolayer phases. Thus the overall rate of hydrolysis rather than the catalytic turnover per bound enzyme would be increased. Similarly, the tendency of the acylated enzyme to aggregate in the aqueous phase (Tomasselli et al., 1989) was probably due to an increased hydrophobicity of the i-face (K-56 is at the edge of this face). However, these aggregates dissociated readily before binding to the interface. These conclusions are in accord with the catalytic behavior of K56M and related mutants of PLA2, which are fully catalytically functional although they cannot be acylated (Noel et al., 1990).

(c) We have studied in detail the kinetics of hydrolysis of DMPC vesicles by PLA2 (Jain & Berg, 1989). Here again, all the anomalous kinetic effects in the pre-steady-state are eliminated in the presence of anionic additives such as the products of hydrolysis of anionic phospholipids (Jain et al., 1982, 1989b; Ghomashchi et al., 1991b). Although Biltonen and co-workers have used the dimerization step in the interface to account for the pre-steady-state time course of the reaction (Menasche et al., 1985; Romero et al., 1987), as shown elsewhere, an equally good fit was obtained by invoking only a product-dependent shift in the E to E* equilibrium (Jain & Berg, 1989).

To recapitulate, PLA2s from all sources that we have tested are fully catalytically active as monomers in the interface, and virtually all observations invoked to account for an apparent activation of PLA2 at the interface can be readily accommodated within the constraints of the minimal schemes that we have adapted to account for the kinetic behavior of pig pancreatic PLA2 (Jain & Berg, 1989; Berg et al., 1991). Generally speaking, other interpretations for the apparent interfacial activation have arisen largely due to inadequate appreciation of the E to E* equilibrium at the interface.

ACKNOWLEDGMENTS

We thank Dr. Arend Slotboom for providing AMPA and Palm-110 PLA2, Ms. Y. C. Huang for sequencing the tryptic peptide derived from An87-PLA2, and Drs. Alan Maycock and Dennis Murphy (Sterling Inc.) for providing NOB and for the discussions that ultimately led to the formulation of experiments with the acylated enzyme. We are grateful to Professor Michael Gelb for critically evaluating this manuscript prior to publication.

Registry No. PLA2, 9001-84-7; DMPM, 60569-01-9; DTPM, 105319-70-8; decylsulfate, 142-98-3; hexadecylphosphocholine, 36314-47-3.

REFERENCES

Andrews, S. B., Hoffman, R. M., & Borison, A. (1975) *Biochem. Biophys. Res. Commun 65*, 913–918.

Apitz-Castro, R., Jain, M. K., & de Haas, G. H. (1982) *Biochim. Biophys. Acta 688*, 349–356.

Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., & Carlson, F. D. (1977) *Biochemistry 16*, 2806–2810.

Bearer, E. L., Duzgunes, N., Friend, D. S., & Papahadjopoulos, D. (1982) *Biochim. Biophys. Acta 693*, 93–98.

Berg, O. G., Yu, B., Rogers, J., & Jain, M. K. (1991) *Biochemistry 30* (first paper of six in this issue).

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Brouillette, C. G., Segrest, J. P., Ng, T. C., & Jones, J. L. (1982) *Biochemistry 21*, 4569–4575.

Bukowski, T., & Teller, D. C. (1986) *Biochemistry 25*, 8024–8033.

Cevc, G., & Marsh, D. (1987) *Phospholipid Bilayers: Physical Principles and Models*, John Wiley, Inc., New York.

Chrzeszczyk, A., Wishnia, W., & Springer, C. (1977) *Biochim. Biophys. Acta 470*, 161–170.

de Haas, G. H., van Scharrenburg, G. J. M., & Slotboom, A. J. (1987) *Biochemistry 26*, 3402–3408.

Dennis, E. A. (1983) in *The Enzymes*, Vol. *16*, pp 307–353, Academic Press, New York.

Dijkstra, B. W., Kalk, K. H., Hol, W. G. J., & Drenth, J. (1983) *J. Mol. Biol. 147*, 97–123.

Ghomashchi, F., O'Hare, T., Clary, D., & Gelb, M. H. (1991a) *Biochemistry 30* (second paper of six in this issue).

Ghomashchi, F., Yu, B., Berg, O. G., Jain, M. K., & Gelb, M. H. (1991b) *Biochemistry 30* (fourth paper of six in this issue).

Hanahan, D. J., Joseph, M., & Morales, R. (1980) *Biochim. Biophys. Acta 619*, 640–649.

Hazlett, T. L., & Dennis, E. A. (1988) *Biochim. Biophys. Acta 961*, 22–29.

Hermetter, A., & Lakowicz, J. R. (1986) *J. Biol. Chem. 261*, 8243–8248.

Hille, J. D. R., Egmond, M. R., Dijkman, R., Van Oort, M. G., Jirgensons, B., & de Haas, G. H. (1983) *Biochemistry 22*, 5347–5353.

Huang, C. (1969) *Biochemistry 8*, 344–351.

Huang, C., & Mason, J. T. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 308–310.

Jain, M. K. (1988) *Introduction to Biological Membranes*, 2nd ed., John Wiley, Inc., New York.

Jain, M. K., & Maliwal, B. P. (1985) *Biochim. Biophys. Acta 814*, 134–140.

Jain, M. K., & Vaz, W. L. C. (1987) *Biochim. Biophys. Acta 905*, 1–8.

Jain, M. K., & Berg, O. G. (1989) *Biochim. Biophys. Acta 1002*, 127–156.

Jain, M. K., & Gelb, M. H. (1991) *Methods Enzymol. 197* (in press).

Jain, M. K., Egmond, M. R., Verheij, H. M., Apitz-castro, R. J., Dijkman, R., & de Haas, G. H. (1982) *Biochim. Biophys. Acta 688*, 341–348.

Jain, M. K., Rogers, J., Jahagirdar, D. V., Marecek, J. F., & Ramirez, F. (1986a) *Biochim. Biophys. Acta 860*, 435–447.

Jain, M. K., Maliwal, B. P., de Haas, G. H., & Slotboom, A. J. (1986b) *Biochim. Biophys. Acta 860*, 448–461.

Jain, M. K., Rogers, J., Marecek, J. F., Ramirez, F., & Eibl, H. (1986c) *Biochim. Biophys. Acta 860*, 462–474.

Jain, M. K., de Haas, G. H., Marecek, J. F., & Ramirez, F. (1986d) *Biochim. Biophys. Acta 860*, 475–483.

Jain, M. K., Rogers, J., & de Haas, G. H. (1988) *Biochim. Biophys. Acta 940*, 51–62.

Jain, M. K., Yuan, W., & Gelb, M. H. (1989) *Biochemistry 28*, 4135–4139.

Jain, M. K., Yu., B., Rogers, J., Ranadive, G., & Berg, O. (1991) *Biochemistry 30* (third paper of six in this issue).

Kleinfield, A. (1988) in *Spectroscopic Membrane Probes* (Loew, L. M., Ed.) Vol. 1, pp 63–92, CRC Press, Boca Raton, FL.

Kuipers, O. P., Thunnissen, T. M. G. M., de Geus, P., Dijkstra, B. W., Drenth, J., Verheij, H. M., & de Haas, G. H. (1989) *Science 244*, 82–85.

Menashe, M., Romero, G., Biltonen, R. L., & Lichtenberg, D. (1985) *J. Biol. Chem. 261*, 5328–5333.

Milton, A., Ricka, J., Sun, S. T., Tanaka, T., Nakatani, Y., & Ourisson, G. (1984) *Biochim. Biophys. Acta 777*, 331-333.
Miyamoto, V. K., & Stoecknius, W. (1971) *J. Membr. Biol. 4*, 252-269.
Nagle, J. F., & Wiener, M. C. (1988) *Biochim. Biophys. Acta 942*, 1-10.
Noel, J. P., Deng, T., & Tsai, M.-D. (1990) *J. Am. Chem. Soc.* 112, 3704-3706.
Pattus, F., Slotboom, A. J., & de Haas, G. H. (1979) *Biochemistry 18*, 2691-2697.
Pluckthum, A., & Dennis, E. A. (1985) *J. Biol. Chem. 260*, 11099-11106.
Ramirez, F., & Jain, M. K. (1991) *Proteins 9*, 229-239.
Renetseder, R., Brunie, S., Dijkstra, B. W., Drenth, J., & Sigler, P. B. (1985) *J. Biol. Chem. 260*, 11627-11634.
Romero, G., Thompson, K., & Biltonen, R. L. (1987) *J. Biol. Chem. 262*, 13476-13482.
Tomasselli, A. G., Hui, J., Fisher, J., Zurcher-Neely, H., Reardon, I. M., Oriaku, E., Kezdy, F. J., & Heinrickson, R. L. (1989) *J. Biol. Chem. 264*, 10041-10047.
Van den Bergh, C. J., Slotboom, A. J., Verheij, H. M., & de Haas, G. H. (1989) *J. Cell. Biochem. 39*, 379-390.
Van der Wiele, F. C., Atsma, W., Dijkman, R., Schreurs, A. M. M., Slotboom, A. J., & de Haas, G. H. (1988) *Biochemistry 27*, 1683-1688.
Van Eijk, J. H., Verheij, H. M., Dijkman, R., & de Haas, G. H. (1983) *Eur. J. Biochem. 132*, 183-188.
Van Oort, M. G., Dijkman, R., Hille, J. D. R., & de Haas, G. H. (1985) *Biochemistry 24*, 7993-7999.
Van Scharrenburg, G. J. M., Puijk, W. C., de Haas, G. H., & Slotboom, A. J. (1983) *Eur. J. Biochem. 133*, 83-89.
Verheij, H. M., Volwerk, J. J., Jansen, E. H. J. M., Puijk, W. C., Dijkstra, B. W., Drenth, J., & de Haas, G. H. (1980) *Biochemistry 19*, 743-750.
Verheij, H. M., Slotboom, A. J., & de Haas, G. H. (1981) *Rev. Physiol., Biochem. Pharmacol. 91*, 91-137.
Watts, A., Marsh, D., & Knowles, P. F. (1978) *Biochemistry 17*, 1792-1801.
Wells, M. A. (1975) *Biochim. Biophys. Acta 380*, 357-505.

Interfacial Catalysis by Phospholipase A_2: Activation by Substrate Replenishment†

Mahendra Kumar Jain,*,‡ Joe Rogers,‡ Otto Berg,§ and Michael H. Gelb*,∥

Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716, Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195, and Department of Molecular Biology, Uppsala University Biomedical Center, Uppsala, Sweden

Received September 17, 1990; Revised Manuscript Received March 26, 1991

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

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

† This research was supported by Grants GM-29703 (M.K.J.) and HL-36235 (M.H.G.) from the National Institutes of Health.
* To whom correspondence should be addressed.
‡ University of Delaware.
§ Uppsala University.
∥ University of Washington.

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

0006-2960/91/0430-7340$02.50/0