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### Title

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### Permalink

<https://escholarship.org/uc/item/65z451q9>

### Journal

Biochemistry, 43(13)

### ISSN

0006-2960

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### Publication Date

2004-04-01

### DOI

10.1021/bi035921b

Peer reviewed

Phospholipid Binding and the Activation of Group IA Secreted Phospholipase A<sub>2</sub><sup>†</sup>Scott C. Boegeman,<sup>§</sup> Raymond A. Deems,<sup>‡</sup> and Edward A. Dennis<sup>\*‡</sup>*Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, California 92093-0601, and Department of Chemistry, University of Dallas, Irving, Texas 75062-4736**Received October 28, 2003; Revised Manuscript Received January 14, 2004*

**ABSTRACT:** Equilibrium dialysis was used to study the binding of two nonhydrolyzable, short chain phospholipid analogues to the secreted group IA phospholipase A<sub>2</sub> (PLA<sub>2</sub>), which has been shown to contain several phospholipid binding sites that dramatically affect activity. This study provides new insight into how these activations occur. One analogue contained a phosphorylethanolamine (DiC<sub>6</sub>SNPE) headgroup, while the other contained a phosphorylcholine (DiC<sub>6</sub>SNPC) headgroup. Using phospholipase D, we incorporated tritium into each analogue. No binding of DiC<sub>6</sub>SNPE to PLA<sub>2</sub> was observed under submicellar conditions. Addition of submicellar amounts of Triton X-100 resulted in a linear nonsaturating response to lipid concentration, suggestive of premicellar aggregation of the DiC<sub>6</sub>SNPE with Triton X-100 and PLA<sub>2</sub>. Binding of DiC<sub>6</sub>SNPE when presented as Triton X-100 mixed micelles saturated at 0.93 binding sites per PLA<sub>2</sub> with a K<sub>D</sub> of 38 μM. Addition of sphingomyelin, a potent activator of PLA<sub>2</sub> hydrolysis of phosphorylethanolamine containing compounds, resulted in a 13-fold decrease in the K<sub>D</sub>, to 2.8 μM. This suggests that changes in the catalytic site binding affinity contribute to “phosphatidylcholine activation”. Binding of DiC<sub>6</sub>SNPC with 2.0 mM Triton X-100 showed positive cooperativity (Hill coefficient of 1.7), which saturated at 2.0 binding sites per PLA<sub>2</sub>. No binding of either analogue was observed when the catalytic site was alkylated with *p*-bromophenacyl bromide. Since *p*-bromophenacyl bromide does not physically block the phosphatidylcholine activator site, this indicates that the two phosphatidylcholine binding sites interact. The binding studies show that DiC<sub>6</sub>SNPC binds cooperatively to two sites on group IA PLA<sub>2</sub>, while DiC<sub>6</sub>SNPE binds to only one site.

The superfamily of phospholipase A<sub>2</sub> (PLA<sub>2</sub>)<sup>1</sup> enzymes (1) catalyze the hydrolysis of phospholipids to yield lyso-phospholipids and free fatty acids. Included in this superfamily are several secreted forms of low molecular weight, Ca<sup>2+</sup>-dependent enzymes, including groups I–III, V, and IX (2). These PLA<sub>2</sub>'s typically exhibit the greatest activity on aggregated substrates such as micelles or vesicles (3). This secreted form of PLA<sub>2</sub> is a water-soluble enzyme that has evolved to function in the unique environment present at the lipid–water interface.

For the group IA PLA<sub>2</sub> from cobra (*Naja naja naja*) venom, evidence has accumulated for the existence of multiple distinct phospholipid-binding sites (4). Kinetic studies have shown that the binding of phosphorylcholine (PC) containing compounds substantially increases the activity toward phosphorylethanolamine (PE) containing substrates when the enzyme is sequestered at an interface (5, 6). Roberts et al. (7) have concluded that this activation proceeds through specific interactions of the enzyme with phospholipids and that at least two sites must be present in the catalytically relevant structure, a catalytic site and an activator site. PE containing compounds either do not bind to the PC activator site or do not activate PLA<sub>2</sub> when bound. Use of sphingomyelin (SPM), the most potent PC activator, on PC substrates results in a modest activation, suggesting that PC is capable of self-activation (8). These data formed the basis for the dual phospholipid model (7), that is, two phospholipid molecules are required for the enzyme to achieve full activity and they bind to at least two different binding sites on the enzyme (4). The simplest model that explains this phenomenon is that each monomer of enzyme contains both sites. This is the monomer dual phospholipid model.

However, cobra PLA<sub>2</sub> also exhibits a tendency to aggregate. Numerous studies have confirmed enzyme aggregation in the presence of monomeric lipid even at the low PLA<sub>2</sub> concentrations used in kinetic studies (9–13). This enzyme and lipid induced aggregation suggested that aggregation of the enzyme might be important for catalytic activity, PC

<sup>†</sup> This work was supported by a Grant from the National Institutes of Health, GM 20501. SCB was the recipient of NIH Training Grant 5 T32 CA 09523-06.

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<sup>1</sup> Abbreviations: BSA, bovine serum albumin; BCA, biconchonic acid; CMC, critical micelle concentration; DiC<sub>4</sub>OPC, 1,2-dibutylanoil-*sn*-glycero-3-phosphocholine; DiC<sub>6</sub>OPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; DiC<sub>6</sub>SNPC, 1-(hexylthio)-2-(hexanoylamino)-1,2-dideoxy-*sn*-glycero-3-phosphocholine; DiC<sub>6</sub>SNPE, 1-(hexylthio)-2-(hexanoylamino)-1,2-dideoxy-*sn*-glycero-3-phosphoethanolamine; DiC<sub>6</sub>SPC, 1,2-bis(hexanoylthio)-1,2-dideoxy-*sn*-glycero-3-phosphocholine; DiC<sub>6</sub>SPE, 1,2-bis(hexanoylthio)-1,2-dideoxy-*sn*-glycero-3-phosphoethanolamine; DiC<sub>10</sub>SPC, 1,2-bis(decanylthio)-1,2-dideoxy-*rac*-glycero-3-phosphocholine; DTP, 4,4'-dithiodipyridine or 4-Aldrithiol; IC<sub>50</sub>, inhibitor concentration at 50% activity; *p*-BPB, *p*-bromophenacyl bromide; PC, phosphorylcholine; PE, phosphorylethanolamine; PLA<sub>2</sub>, phospholipase A<sub>2</sub> (EC 3.1.1.4); PLD, phospholipase D (EC 3.1.4.4); SPM, sphingomyelin; TLC, thin-layer chromatography; Tris, tris-(hydroxymethyl)aminomethane.

activation, or interfacial activation. In this scenario, the catalytic and activator sites would reside on different subunits of the aggregate. This is the dimer dual phospholipid model. Despite successfully establishing a causal relationship between lipid, enzyme, and aggregation, we have not established a functional role between enzyme activity and enzyme aggregation.

The complexity of the interaction of cobra PLA<sub>2</sub> with phospholipids has recently increased since site-directed mutagenesis studies have identified three distinct regions on the enzyme that are involved in its activity, that is, the catalytic site, the PC activator site, and an additional interfacial activation site (14).

Previous attempts to distinguish between the monomer and dimer models have centered on linking PC activation and enzyme aggregation. These studies (9–13) have proven inconclusive. In the current study, we employed a different approach. We have explored the number and affinity of the lipid binding sites on the enzyme using the classical method of equilibrium dialysis (15). In many ways, PLA<sub>2</sub> is ideally suited for equilibrium dialysis studies. It is well established that PLA<sub>2</sub> is very stable, alleviating the typical concerns about enzyme denaturation (16). Also the typical conditions used in kinetic assays employ an ionic strength high enough to suppress the Donnan effect (17). To carry out the studies, we employed phospholipase D (PLD) from *Streptomyces* to radiolabel two nonhydrolyzable substrate analogues, which have been shown to be competitive inhibitors of cobra PLA<sub>2</sub> (18). These studies show that each monomer of PLA<sub>2</sub> contains all of the machinery required to produce all of the observed kinetic characteristics of the enzyme and have indicated that the aggregation of the enzyme is superfluous to activity.

## EXPERIMENTAL PROCEDURES

**Materials.** The equilibrium microvolume dialyzer (EMD-101B), the acrylic 0.5 mL by eight chamber dialyzing modules (EMD 1/2), and the dialysis membranes (EMD104, 6–8 kDa cutoff) were purchased from Hoefer Scientific Instruments. Centriprep-3 concentrators were purchased from Amicon. The micro BCA protein assay reagent kit and the BSA protein standard were purchased from Pierce. Cobra venom (*N. naja naja*) was purchased from the Miami Serpentarium. Cobra venom PLA<sub>2</sub> was purified by procedures that yield a homogeneous enzyme free from other PLA<sub>2</sub> isozymes as described elsewhere (16, 19). Purified PLA<sub>2</sub> was stored at 4 °C. The synthesis of the unlabeled substrate analogues, 1-(hexylthio)-2-(hexanoylamino)-1,2-dideoxy-*sn*-glycero-3-phosphocholine (DiC<sub>6</sub>SNPC) and 1-(hexylthio)-2-(hexanoylamino)-1,2-dideoxy-*sn*-glycero-3-phosphoethanolamine (DiC<sub>6</sub>SNPE), has been described elsewhere (18). DiC<sub>6</sub>SNPE was also synthesized from DiC<sub>6</sub>SNPC as described below. Sphingomyelin (SPM) was obtained from Avanti Polar Lipids. The thiol sensitive reagent 4,4'-dithiopyridine (DTP) was purchased from Aldrich under the trade name 4-Aldrithiol. Racemic DiC<sub>10</sub>SPC was a gift from Dr. Neal Roehm of Eli Lilly Research Laboratories. The inhibitor p-BPB (2,4'-dibromoacetophenone) was purchased from Aldrich. Triton X-100 was obtained from Calbiochem. Prosil-28 was purchased from Thomas Scientific. PLD from *Streptomyces* species (type VII) was purchased from Sigma.

Tritiated ethanolamine hydrochloride [HOCH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>·HCl] and choline chloride [HOCH<sub>2</sub>CH<sub>2</sub>N(C<sup>3</sup>H<sub>3</sub>)<sub>3</sub>·Cl] were purchased from American Radiolabeled Chemicals (ARC). The remaining chemicals were at least certified A.C.S. grade and were purchased from Fisher. All deuterated NMR solvents were from Cambridge Isotope Laboratories. Research Products International manufactured Bio-Safe 2 counting cocktail. Analytical thin-layer chromatography (TLC) was carried out on Analtech's Silica Gel GHLF 250 μm glass plates. Preparative TLC was carried out on Analtech's Uniplate tapered silica gel G 250 μm prescored glass plates. Flash chromatography was performed with silica gel 60 (40 μm, Fisher).

**General Methods.** TLC plates were generally visualized by spraying with a molybdate spray that contained 2 M sulfuric acid (20). Phospholipids turned light blue upon heating. Further heating allowed visualization of other compounds by charring. Racemic DiC<sub>10</sub>SPC substrate was purified as described previously (8). The final concentration of all lipids was determined by phosphate assay (21, 22). The substrate and analogue stocks were stored in chloroform at –20 °C. All glass test tubes were silanized prior to use with Prosil-28, according to the manufacturer's instructions. Data were analyzed and fit to the various models with the nonlinear regression package included in SigmaPlot from SPSS Inc.

The buffer employed for both the equilibrium dialysis experiments and the PLA<sub>2</sub> activity assays was 25 mM Tris, pH = 8.0, 10 mM CaCl<sub>2</sub>, and 0.1 M KCl. Lipids were generally resuspended by vortexing, bath sonication (Branson B-12 80 W, Branson Corporation, Danbury, CT) performed for ~1 min, and vortexing again. Enzyme and lipid solutions were generally allowed at least 30 min to equilibrate before use. The water used was always of Milli-Q quality.

<sup>1</sup>H NMR spectra were obtained on an extensively modified Varian 360 MHz NMR spectrometer or a GE QE-300 MHz NMR spectrometer with chemical shifts recorded in parts per million (ppm) from tetramethylsilane. FAB mass spectra were obtained at the University of California Riverside mass spectrometry facility. Tritiated compounds were counted on a 1600 TR Tri-Carb liquid scintillation analyzer from Packard Instruments.

**Preparative TLC.** Preparative TLC plates were developed in both the solvent to be used to run the plate and the solvent used to elute the lipid from the plate before use. Before use the plates were thoroughly dried at 80 °C for at least 1 h in a vacuum oven under reduced pressure. After the plate was developed, the right side of the plate was snapped off along a prescored line and sprayed to visualize the lipids. Ethanolamine compounds were visualized with ninhydrin, and choline compounds were visualized with Dragendorff–Munier reagent. These sprays were made as previously described (23). Using the right side of the plate as a guide, we scraped the silica that contained product on the left side of the TLC plate into a 15 mL sintered glass funnel of medium porosity. The product was then eluted as indicated in the synthesis sections, and the solvent was removed under reduced pressure.

**Synthesis of DiC<sub>6</sub>SNPE.** The DiC<sub>6</sub>SNPE, the nonhydrolyzable substrate analogue, was synthesized from DiC<sub>6</sub>SNPC using PLD from *Streptomyces*, by adapting a published

procedure (24). The PLD was resuspended in buffer (40 mM Ca<sup>2+</sup>, 200 mM sodium acetate, pH = 5.6) to a concentration 0.6 U/ $\mu$ L (as Sigma defines the unit, 1  $\mu$ M h<sup>-1</sup>) and stored at 0 °C. DiC<sub>6</sub>SNPC (2 mL, 57.7 mg, 130  $\mu$ mol) in chloroform was taken to dryness in vacuo. To this was added freshly made buffer (8 mL) containing 1 M ethanolamine hydrochloride (8 mmol). The lipid was resuspended by heating and sonication. The reaction was initiated by adding PLD (32  $\mu$ L, 19.2 U). The reaction was allowed to proceed for 2 h at room temperature with moderate stirring. The reaction was halted by quick freezing in liquid N<sub>2</sub>, and the water was removed by lyophilization. The residue was dissolved in a minimum amount of chloroform/methanol/water (65:25:4) and applied to a preparative TLC plate. The plate was developed in chloroform/methanol/water (65:25:4). The product was eluted off the silica in chloroform/methanol/water (1:1:0.2). The product was further purified on a flash silica gel column equilibrated with chloroform/methanol (65:10). After the sample was loaded, the column was washed with chloroform/methanol/water (65:25:0.25), and the product was eluted with chloroform/methanol/water (65:25:1). The product was dried and resuspended in chloroform and a minimal amount of methanol. The final product was put through a 0.2  $\mu$ m nylon-66 filter to remove particulate matter and stored at -20 °C. The purified yield was 40 mg (75%). The R<sub>f</sub> was 0.57 in chloroform/methanol/water (65:25:4). For NMR, the exchangeable protons were removed using CD<sub>3</sub>-OD. <sup>1</sup>H NMR (CDCl<sub>3</sub>/CD<sub>3</sub>OD 1:1):  $\delta$  0.906 (q, 6H), 1.333 (m, 10H), 1.588 (m, 4H), 2.214 (dt, 2H), 2.566 (t, 2H), 2.704 (dq, 2H), 3.140 (br m, 2H), 3.96–4.13 (m, 5H). HRMS (FAB, MH<sup>+</sup>): *m/z* 413.2236; calculated for C<sub>17</sub>H<sub>38</sub>N<sub>2</sub>O<sub>5</sub>PS 413.2239.

**Tritium Incorporation into DiC<sub>6</sub>SNPE.** <sup>3</sup>H-DiC<sub>6</sub>SNPE was synthesized from unlabeled DiC<sub>6</sub>SNPE by using PLD from *Streptomyces* to exchange unlabeled ethanolamine with radiolabeled ethanolamine. Radioactive ethanolamine hydrochloride (5 mL, 5 mCi, 0.13  $\mu$ mol) in water was concentrated on a SpeedVac to less than 1 mL. DiC<sub>6</sub>SNPE (940  $\mu$ L, 5.3 mg, 13  $\mu$ mol) in chloroform was placed in a 10 mm  $\times$  75 mm test tube, and the chloroform was removed under N<sub>2</sub>. The dried compound was protected from oxygen by storing it under vacuum until used. The labeled ethanolamine was transferred into the tube containing the DiC<sub>6</sub>SNPE, and the mixture was taken to dryness. To the dried residue was added 105  $\mu$ L of buffer (40 mM Ca<sup>2+</sup>, 200 mM sodium acetate, pH = 5.6) containing 119 mM unlabeled ethanolamine hydrochloride. *tert*-Butyl alcohol (25  $\mu$ L, HPLC grade) was added as cosolvent. Control experiments showed that *tert*-butyl alcohol is not a substrate for the exchange reaction catalyzed by PLD. The tube was sealed with a rubber septum. After resuspending the lipid by heating and sonication, the reaction was initiated by addition of 20  $\mu$ L of PLD (6 U) in buffer. The reaction was allowed to proceed tightly sealed at room temperature for 4 h. The final reaction conditions were 104 mM DiC<sub>6</sub>SNPE, 100 mM ethanolamine, and 17% (v/v) *tert*-butyl alcohol (25  $\mu$ L) in 125  $\mu$ L of buffer. The reaction was halted by quick freezing in liquid N<sub>2</sub>, and the solvents were removed on a SpeedVac. The residue was dissolved in a minimum (~100  $\mu$ L) of methanol/water (10:1) and applied to a preparative TLC plate. The plate was developed in chloroform/methanol/water (65:25:4). The product was eluted off the silica in chloroform/

methanol/water (1:1:0.2). The solvent was removed, and the residue was resuspended in chloroform. The chemical recovery was 4.5 mg (1.1  $\mu$ mol, 86%) by phosphate assay, and the radioactive incorporation was 63 mCi/mmol (32%).

**Tritium Incorporation into DiC<sub>6</sub>SNPC:** <sup>3</sup>H-DiC<sub>6</sub>SNPC was synthesized from unlabeled DiC<sub>6</sub>SNPC by using PLD from *Streptomyces* to exchange unlabeled choline with tritiated choline. Radioactive choline chloride (15 mL, 15 mCi, 1.28  $\mu$ mol) in ethanol was concentrated on a SpeedVac to less than 1 mL. DiC<sub>6</sub>SNPC (615  $\mu$ L, 17.73 mg, 39  $\mu$ mol) in chloroform was placed in a 10 mm  $\times$  75 mm test tube, and the chloroform was removed under N<sub>2</sub>. The labeled choline was transferred into the tube containing the DiC<sub>6</sub>SNPC, and the mixture was taken to dryness to yield a light yellow oil. To remove trace amounts of ethanol, the residue was resuspended in 300  $\mu$ L of *tert*-butyl alcohol and dried down again. To the dried residue was added 316  $\mu$ L of buffer (40 mM Ca<sup>2+</sup>, 200 mM sodium acetate, pH = 5.6) containing 119 mM unlabeled choline chloride. *tert*-Butyl alcohol (50  $\mu$ L, HPLC grade) was added as cosolvent. The tube was sealed with a rubber septum. After the lipid was resuspended by heating and sonication, the reaction was initiated by addition of 20  $\mu$ L of PLD (7.2 U) in buffer; additional 20  $\mu$ L aliquots of PLD were added at 1.5 and 3 h. The reaction was allowed to proceed, tightly sealed, at room temperature for 5.5 h. The initial reaction conditions were 104 mM DiC<sub>6</sub>SNPC, 100 mM choline, and 13% (v/v) *tert*-butyl alcohol (50  $\mu$ L) in 335  $\mu$ L buffer. The reaction was halted by quick freezing in liquid N<sub>2</sub>, and the solvents were removed on the SpeedVac. The residue was dissolved in a minimum (~100  $\mu$ L) of methanol/water (10:1) and applied to a preparative TLC plate. The plate was developed in chloroform/methanol/water (65:25:4). The product was eluted off the silica in chloroform/methanol/water/NH<sub>4</sub>OH (15:15:2:4). The solvent was removed, and the residue was resuspended in chloroform. The final product was filtered through a 0.2  $\mu$ m nylon-66 filter to remove colloidal silica and then stored at -20 °C. The chemical recovery was 16.7 mg (3.6  $\mu$ mol, 94%) by phosphate assay, and the radioactive incorporation was 118 mCi/mmol (57%).

**Protein Determination.** The protein concentrations were determined by a micro Lowry procedure or a micro BCA assay. The micro Lowry assay was based on a modification of the original Lowry assay (25, 26). Determined protein values for PLA<sub>2</sub> were adjusted by multiplying by the correction factor (PLA<sub>2</sub> = protein  $\times$  0.66) for PLA<sub>2</sub> with BSA standards (26). The micro BCA assay was performed essentially as described by Pierce. The micro BCA correction factor for PLA<sub>2</sub> using BSA standards was determined to be 0.69  $\pm$  0.03 by using a corrected Lowry assay as reference.

**PLA<sub>2</sub> Activity Assay.** PLA<sub>2</sub> activity was determined by employing an adaptation of the thio assay previously reported (27, 28), except as noted briefly below. DTP (5  $\mu$ L of 69 mM in ethanol) was placed in a quartz cuvette (2 mm  $\times$  10 mm), followed by 400  $\mu$ L of the racemic substrate solution (0.5 mM DiC<sub>10</sub>SPC in 25 mM Tris, pH = 8.0, 0.1 M KCl, 10 mM Ca<sup>2+</sup>, 2.25 mM Triton X-100). The cuvette was placed in the temperature controlled cell holder of the Uvikon 9310 UV-vis spectrophotometer, allowed to equilibrate for 3 min at 30 °C, and then balanced against a blank containing water. The background rate was recorded for 1 min and later subtracted from the observed enzymatic rate. The reaction

was initiated by addition of 5  $\mu\text{L}$  of enzyme (25 mM Tris, pH = 8.0, 0.1 M KCl, 10 mM  $\text{Ca}^{2+}$ , 2.0 mM Triton X-100), and the solution was briefly but vigorously mixed. The enzyme concentration varied. The rate was followed for at least 1 min at 324 nm by monitoring the chromophore 4-thiopyridone (29). Activity was calculated on the basis of the extinction coefficient of 17 100  $\text{M}^{-1} \text{cm}^{-1}$  measured for 4-thiopyridone under similar assay conditions, as determined previously (27).

**Inhibition of PLA<sub>2</sub> with p-Bromophenacyl Bromide.** The inhibition of PLA<sub>2</sub> by reaction with p-BPB was adapted from previous procedures (18, 30). Cobra PLA<sub>2</sub> (7.57 mg) was combined with buffer (final concentration = 25 mM Tris, pH = 8.0, 0.1 mM Triton X-100) in a beaker, and the mixture was stirred gently at room temperature. A stock of p-BPB (16 mM) was made up fresh in absolute EtOH, vortexed, and used immediately. The reaction was started by adding 325  $\mu\text{L}$  of the p-BPB stock to the enzyme solution to yield 0.4 mM p-BPB and 2.5% EtOH, bringing the total volume up to 13 mL. The solution immediately took on a pale yellow hue, which quickly faded. Inhibition of PLA<sub>2</sub> was followed by the thio assay. After 3 h, the PLA<sub>2</sub> had no measurable activity. The buffer and excess p-BPB were removed by repeated concentration in a Centriprep-3 concentrator and subsequent dilution with water, according to the manufacturer's instructions. The concentration of the final PLA<sub>2</sub> stock was determined by the micro BCA assay; PLA<sub>2</sub> recovery was 99%.

**Equilibrium Dialysis.** A typical experiment was run with two dialyzing modules; each module contained eight separate chambers. At least one chamber was reserved for protein determination via the BCA protein assay and contained no substrate analogue. The dialysis membranes were washed thoroughly in water and allowed to equilibrate with buffer (25 mM Tris, pH = 8.0, 10 mM  $\text{CaCl}_2$ , and 0.1 M KCl) for at least 2 h. The dialysis apparatus was assembled essentially according to the manufacturer's instructions, except no grease was used on the O-rings. Each chamber was filled using a gas-tight Hamilton syringe. Experiments were started with equal amounts of ligand plus any additives on both sides of the membrane with enzyme added to only one side. The chambers were sealed with Scotch tape and allowed to equilibrate at room temperature for 48 h. The time needed for equilibration was determined by running a time course and was always less than 48 h. Three aliquots (130  $\mu\text{L}$  each) were removed from each chamber at the end of the experiment, thoroughly mixed with scintillation fluid (Bio-Safe 2, 6 mL) and then counted. Data points are generally the average of the triplicate samplings per chamber with the errors representing the standard error of the mean (sem). Data points near saturation were often run in duplicate. Each complete experiment was repeated at least once. All data shown are from representative experiments. The total ligand concentration in the enzyme chamber ( $L_T$ ) was directly calculated from the cpm in that chamber and the specific activity of the radiolabeled ligand. The free ligand concentration ( $L_F$ ), which is the same in both chambers, was derived from the cpm measured in the chamber that did not contain the enzyme. The ligand bound to the enzyme ( $L_B$ ) was the difference between the total and free ligand, that is,  $L_B = L_T - L_F$ .

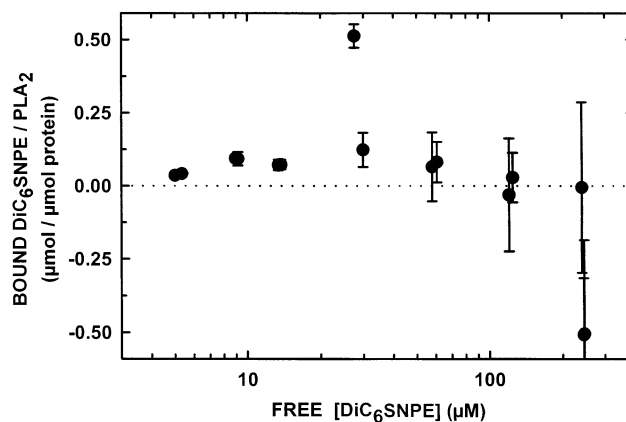


FIGURE 1: Lack of binding of PLA<sub>2</sub> to DiC<sub>6</sub>SNPE up to 240  $\mu\text{M}$ . The standard dialysis buffer was used without additives. The PLA<sub>2</sub> concentration was 3.8  $\mu\text{M}$ . The dotted line represents the 0 binding level. The free DiC<sub>6</sub>SNPE axis is a log scale to better visualize the low concentration range data.

Two binding models were tested. The first model assumes that there are one or more identical independent sites on the enzyme. This is the basic Michealis–Menten binding model and is given by eq 1 (31),

$$\frac{L_B}{E_T} = \frac{nL_F}{K_D + L_F} \quad (1)$$

where  $K_D$  is the dissociation constant,  $n$  is the number of identical independent sites per enzyme molecule, and  $E_T$  is the total enzyme concentration. The second model assumes multiple interacting sites,  $m$ , where the binding of ligand to the first site alters the binding affinity at the other sites. This is basically the Hill formalism and is given by eq 2,

$$\frac{L_B}{E_T} = \frac{mL_F^\alpha}{K_D^\alpha + L_F^\alpha} \quad (2)$$

where  $\alpha$  is the Hill coefficient. This equation assumes that there are no intermediates, that is, there are only two enzyme states, either all binding sites are empty or all are occupied. If this assumption is true,  $\alpha = m$ . The data were fit to these models using the nonlinear regression algorithms supplied with the Sigmaplot graphing software (SPSS Inc.). The coefficient of determination ( $R^2$ ) of the fit and the  $P$  values for the determined parameters were obtained from this software. Only parameters with  $P$  values of less than 0.05 were considered significant.

## RESULTS

**DiC<sub>6</sub>SNPE Binding to PLA<sub>2</sub> under Submicellar Conditions.** Binding was first studied in a submicellar physical state in which only PLA<sub>2</sub> and a short chain substrate analogue (DiC<sub>6</sub>SNPE) were present. The DiC<sub>6</sub>SNPE analogue contains an *sn*-1 thioether, an *sn*-2 amide, and a phosphorylethanolamine headgroup. As shown in Figure 1, essentially no binding was observed up to concentrations of 240  $\mu\text{M}$  DiC<sub>6</sub>SNPE with 3.8  $\mu\text{M}$  PLA<sub>2</sub>. The increasing error as the free ligand concentration increases is typical of equilibrium dialysis experiments. This is because at high  $L_F$  both  $L_T$  and  $L_F$  are very large relative to  $L_B$  and thus there is a larger error due to the subtraction of two large numbers. Controls verified

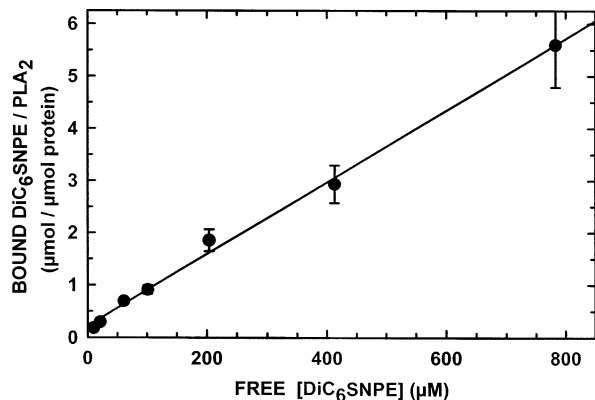


FIGURE 2: Binding of DiC<sub>6</sub>SNPE to PLA<sub>2</sub> in the presence of submicellar Triton X-100. The standard dialysis buffer was used with the addition of 0.2 mM Triton X-100. The PLA<sub>2</sub> concentration was 4.2 μM. Data were fit using linear regression.

that PLA<sub>2</sub> had not crossed the dialysis membrane nor lost any specific activity by the end of the experiment. This indicates that PLA<sub>2</sub> was still fully capable of binding DiC<sub>6</sub>SNPE throughout the experiment, confirming that ligand binding was too weak to measure.

If submicellar amounts (0.2 mM) of Triton X-100 are included with the PLA<sub>2</sub> and DiC<sub>6</sub>SNPE, a dramatic change in the binding occurs as shown in Figure 2. As the concentration of free DiC<sub>6</sub>SNPE increases, a remarkably linear increase in binding is observed. Binding showed no sign of saturating up to 780 μM DiC<sub>6</sub>SNPE and at least 5 mol of DiC<sub>6</sub>SNPE bound per mole of PLA<sub>2</sub>. Control experiments in which Triton X-100 alone was placed on one side of the membrane showed that submicellar amounts of Triton X-100 readily equilibrated across the dialysis membrane. The results shown in Figure 2 are consistent with the formation of premicellar aggregates nucleated by combining an unknown number of PLA<sub>2</sub> molecules with Triton X-100 and DiC<sub>6</sub>SNPE. The apparent continuous increase in DiC<sub>6</sub>SNPE binding is thus a result of partitioning of DiC<sub>6</sub>SNPE into the premicellar aggregate as the concentration of free DiC<sub>6</sub>SNPE increases and does not necessarily indicate a direct binding to PLA<sub>2</sub>.

**Binding of PLA<sub>2</sub> to Micellar Substrate Analogues.** The binding of DiC<sub>6</sub>SNPE and DiC<sub>6</sub>SNPC to PLA<sub>2</sub> was studied when the analogues were present in a mixed micellar physical state. This was accomplished by including 2.0 mM Triton X-100 (CMC = 0.25 mM) in the equilibrium buffer. This not only generated a neutral micellar interface with which the ligands could equilibrate but also established a system free from premicellar aggregates. Previous work has shown that for cobra PLA<sub>2</sub>, Triton X-100 serves as a neutral surface diluent (8, 32) with little or no interaction with the phospholipid binding sites. In addition, PLA<sub>2</sub> does not bind to Triton X-100 micelles in the absence of lipids (7). Controls showed that while Triton X-100 micelles did not equilibrate across the dialysis membrane over the course of the 48-hour experiment, monomer Triton X-100 (~0.25 mM) readily equilibrated. To ensure that equilibrium would be established, these experiments were started with equal amounts of ligand and Triton X-100 on both sides of the membrane. Further controls demonstrated that DiC<sub>6</sub>SNPE and DiC<sub>6</sub>SNPC could readily equilibrate across the membrane even in the presence

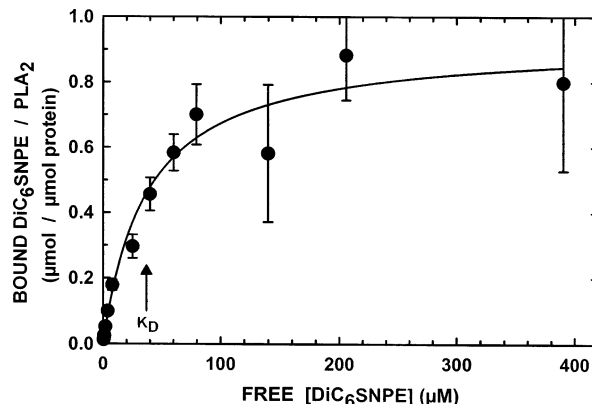


FIGURE 3: Saturation isotherm for the binding of DiC<sub>6</sub>SNPE to PLA<sub>2</sub>. The standard dialysis buffer was used; 2.0 mM Triton X-100 was also included. The data were fit to eq 1, and the line is derived from this fit.  $K_D$  (arrow) was determined to be  $38 \pm 9 \mu\text{M}$ ; saturation was determined to be at  $0.93 \pm 0.07$  mol of DiC<sub>6</sub>SNPE per mole of enzyme. The PLA<sub>2</sub> was at 4.4 μM.

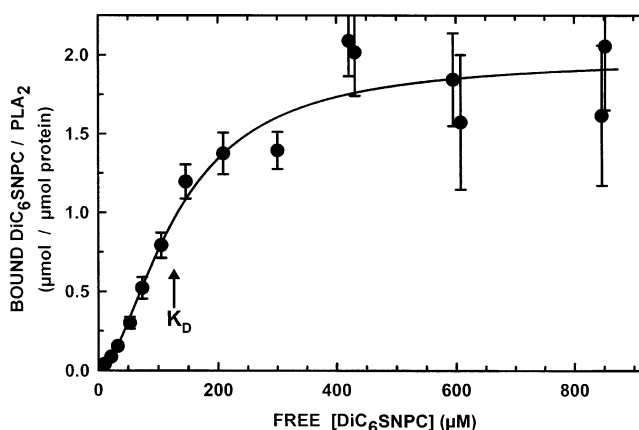


FIGURE 4: Saturation isotherm for the binding of DiC<sub>6</sub>SNPC to PLA<sub>2</sub>. The standard dialysis buffer used included 2.0 mM Triton X-100 as additive. Data were fit to eq 2, and the line is derived from this fit.  $K_D$  (arrow) was  $131 \pm 22 \mu\text{M}$ ; the number of binding sites,  $m$ , was determined to be  $2.0 \pm 0.2$ . The Hill coefficient was  $1.7 \pm 0.4$ . PLA<sub>2</sub> was measured at 10.4 μM.

of Triton X-100 micelles, even though the micelles themselves could not.

A typical saturation isotherm for the binding of micellar DiC<sub>6</sub>SNPE to PLA<sub>2</sub> is shown in Figure 3. The data were fitted to eq 1 using weighted nonlinear regression. The  $K_D$  derived from the curve fit was  $38 \pm 9 \mu\text{M}$ , and the number of binding sites was  $0.93 \pm 0.07$ . The coefficient of determination ( $R^2$ ) for this fit was 0.96, and the  $P$  values for each parameter were less than 0.004. When the data were fitted to eq 2, the  $\alpha$  value derived was close to 1. Thus, eq 2 degenerated to eq 1.

A typical saturation isotherm for the binding of micellar DiC<sub>6</sub>SNPC to PLA<sub>2</sub> is shown in Figure 4. Equation 2 produced the best fit to these data, suggestive of a cooperative interaction. The curve fit to Figure 4 yielded  $2.0 \pm 0.2$  mol of DiC<sub>6</sub>SNPC bound per mole of PLA<sub>2</sub>, for the parameter  $m$ . The apparent  $K_D$  was  $131 \pm 22 \mu\text{M}$ . The Hill coefficient,  $\alpha$ , was  $1.7 \pm 0.4$ . The  $R^2$  for this fit was 0.95, and the  $P$  values for all parameters were less than 0.001. Equation 1 did not fit these data. Equation 2 assumes that no intermediate forms of the enzyme are present, that is, the enzyme has either no ligand bound or two ligands. We also tested the

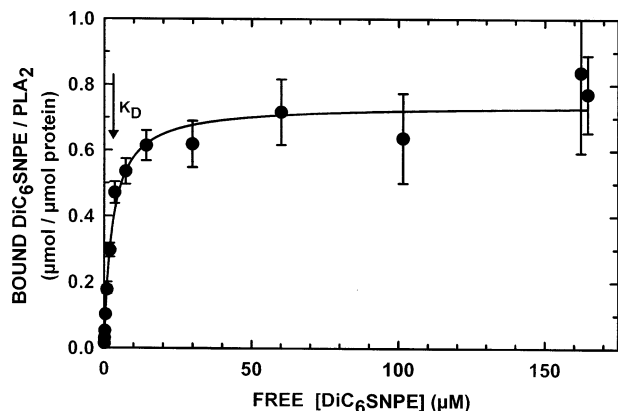


FIGURE 5: Saturation isotherm for the binding of DiC<sub>6</sub>SNPE to activated PLA<sub>2</sub>. The standard dialysis buffer used included 35.0 μM SPM (activator) and 2.0 mM Triton X-100 as additives. PLA<sub>2</sub> was measured at 3.9 μM. Data were fit to eq 1, and the line is derived from this fit.  $K_D$  (arrow) was  $2.8 \pm 0.5 \mu\text{M}$ ; the number of binding sites was  $0.74 \pm 0.02$ .

complete Hill model in which enzyme forms exist where the binding sites are only partially filled. The regression of the data to this equation always reverted to the simple Hill model in which no intermediates are present. The fact that  $\alpha$  and  $m$  are within experimental error of each other also supports this hypothesis (31). These results conclusively demonstrate that there are two PC binding sites per enzyme molecule and that these two sites interact with positive cooperativity.

Addition of SPM, a potent PC activator of cobra PLA<sub>2</sub>, improves PLA<sub>2</sub> activity toward PE substrates by greater than 20-fold (5, 12). To determine whether this activation was due in part to an increase in the binding affinity of the enzyme for PE, we included SPM and repeated the experiment shown in Figure 3. Figure 5 shows the effect that a small amount of SPM (35 μM) has on DiC<sub>6</sub>SNPE in the presence of Triton X-100 micelles. As in Figure 3, the data fit eq 1. In fact, when we attempted to fit the data to eq 2, the value of  $\alpha$  produced was 1.0. In this case, eq 2 becomes eq 1. Clearly the DiC<sub>6</sub>SNPE is still binding to a single site on the enzyme. The  $K_D$  derived from this fit was  $2.8 \pm 0.5 \mu\text{M}$ , and the number of binding sites,  $n$ , was  $0.74 \pm 0.02$  mol of DiC<sub>6</sub>SNPE bound per mole of PLA<sub>2</sub>. The  $R^2$  of this fit was 0.98, and  $P$  values for both parameters were less than 0.0001. Clearly, SPM increases DiC<sub>6</sub>SNPE binding by an order of magnitude. These results also imply that SPM does not compete with DiC<sub>6</sub>SNPE for the catalytic site.

In summary, the  $K_D$  for DiC<sub>6</sub>SNPE binding to PLA<sub>2</sub> decreases 13-fold from 38 to 2.8 μM when the enzyme is activated by SPM. For PC-activated PLA<sub>2</sub>, there are 2.0 binding sites per enzyme molecule where as with the nonactivated DiC<sub>6</sub>SNPE only 0.93 mol of DiC<sub>6</sub>SNPE binds per mole of enzyme.

*Effects on Binding When PLA<sub>2</sub> Is Inactivated with p-Bromophenacyl Bromide.* The treatment of PLA<sub>2</sub> with p-BPB has been shown to alkylate the only histidine of cobra PLA<sub>2</sub>. Modification of the catalytic histidine results in complete and irreversible loss of enzymatic activity, and the histidine is the only residue modified (30). As shown in Figure 6A, inhibition of the catalytic activity of PLA<sub>2</sub> by covalent modification with p-BPB results in no DiC<sub>6</sub>SNPE binding up to concentrations of 300 μM DiC<sub>6</sub>SNPE and 4.5 μM

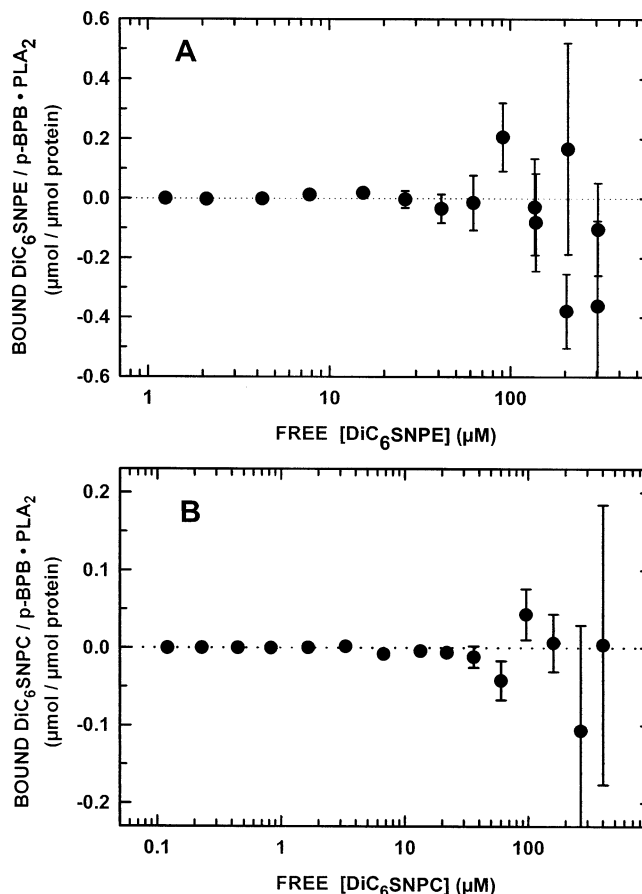


FIGURE 6: Covalent modification of PLA<sub>2</sub> with p-BPB prevents binding to (A) DiC<sub>6</sub>SNPE and (B) DiC<sub>6</sub>SNPC at either the catalytic or activator sites. The standard dialysis buffer used included 2.0 mM Triton X-100. The p-BPB inactivated PLA<sub>2</sub> was (A) 4.5 μM or (B) 13.1 μM, and controls showed no residual enzymatic activity (detection limit < 0.005% residual activity). Dotted line represents the 0 binding point. The free DiC<sub>6</sub>SNPE axis is a log scale to better visualize the low concentration range data.

PLA<sub>2</sub>. This represents a complete inhibition of the binding observed with active enzyme (Figure 3) under the same binding conditions. No binding of p-BPB modified PLA<sub>2</sub> to DiC<sub>6</sub>SNPC was observed up to 400 μM DiC<sub>6</sub>SNPC and 13.1 μM PLA<sub>2</sub> at either of the two binding sites (Figure 6B). These results demonstrate that alkylation of the catalytic histidine disrupts binding at both catalytic and PC activator sites.

## DISCUSSION

*Binding Studies under Submicellar Conditions.* Traditionally, the CMC has been defined as the concentration at which an amphipathic molecule begins to form micelles. At concentrations below the CMC, all of the compound is monomeric, and above this concentration, the monomer concentration remains at the CMC and all additional compound is present in micelles. This model is generally applicable when only amphipathic compounds are present. Several studies have shown, however, that when proteins are present pre-micellar aggregates tend to form (33–37). Pre-micellar aggregates often consist of several enzyme molecules associated with many lipid molecules. These structures appear to form even at lipid concentrations well below the CMC of the lipid alone. Thus, barring experiments

to determine the physical state of all components of an enzyme solution, one should not assume that amphipathic molecules are monomeric when enzyme is present, even when their concentrations are well below their CMCs. Experiments using lipid below the CMC are perhaps best referred to nonspecifically as being submicellar rather than monomeric.

The complexity of the “submicellar systems” is demonstrated in the studies reported here. First, Figure 1 clearly shows that DiC<sub>6</sub>SNPE does not bind to the enzyme even at 240 μM. Yet, DiC<sub>6</sub>SNPE, which is a competitive inhibitor of PLA<sub>2</sub>, was shown to have an IC<sub>50</sub> of less than 20 μM when assayed with submicellar DiC<sub>6</sub>SPC substrate (28). If both experiments contained only monomeric substrate and inhibitor and these compounds were competing for a single site on PLA<sub>2</sub>, the Michaelis–Menten formalism would predict that the IC<sub>50</sub> could not be less than the K<sub>D</sub>, as shown in eq 3.

$$IC_{50} = K_D \left( 1 + \frac{[\text{substrate}]}{K_m} \right) \quad (3)$$

Clearly this is not the case here because the K<sub>D</sub> must be significantly greater than 240 μM since no binding was found below this DiC<sub>6</sub>SNPE concentration. The simplest explanation is that when DiC<sub>6</sub>SNPE is present below 240 μM in the presence of enzyme, it is present as a monomer. However, when 500 μM substrate is included a different environment exists; one in which premicellar aggregates of the enzyme, lipid, and DiC<sub>6</sub>SNPE are formed. Under these conditions, the competition of the DiC<sub>6</sub>SNPE for the enzyme’s catalytic site is significant. This in turn implies that the enzyme was acting on premicellar aggregates even though the substrate was present at submicellar levels. It would appear that PLA<sub>2</sub> catalysis does not occur on truly monomeric DiC<sub>6</sub>SPC or DiC<sub>6</sub>SPE substrates but on premicellar aggregates.

A similar result has been demonstrated for *N. naja naja* and the closely related *N. naja atra* PLA<sub>2</sub>s using a phosphonate-PE as a transition-state analogue (38). In that study, the difference between the binding and kinetic result was even more dramatic with a 400-fold difference (IC<sub>50</sub> = 0.024 μM, K<sub>D</sub> > 10 μM). Taken together the lack of binding to monomeric lipid appears to be generally true for the cobra PLA<sub>2</sub>; neither a ground-state analogue nor a transition-state analogue binds as monomeric lipid.

The same basic conclusion has been reached by Rogers et al. (39) and more recently by Yu et al. (36). Our result adds further weight to the argument (36–39) that PLA<sub>2</sub> is not catalytically competent on truly monomeric substrates but instead functions only on aggregated substrates whether they be premicellar aggregates, bilayers, or micelles.

In fact, Figure 2 appears to show the formation of these premicellar aggregates. When 0.2 mM Triton X-100 (CMC = 250 μM) is added to the system, binding of DiC<sub>6</sub>SNPE is clearly seen from well below 200 up to 800 μM. The facts that the binding is linear, not hyperbolic, and that five molecules of DiC<sub>6</sub>SNPE bind per molecule of enzyme indicate that this binding is not to specific binding sites on the enzyme. Rather binding appears to result from increased nonspecific partitioning of DiC<sub>6</sub>SNPE into premicellar enzyme–lipid aggregates.

*Binding Studies under Micellar Conditions.* Despite ample evidence for both PC activation (5, 6, 40, 41) and PLA<sub>2</sub> aggregation in the presence of phospholipids (9, 11–13, 42), we have not been able to functionally link the two together. This has perpetuated two dual phospholipid models: the dimer model, which links activation and aggregation, and the monomer model, which does not (4). If the dimer model is correct, we would expect to see only one lipid-binding site per enzyme molecule, capable of binding either DiC<sub>6</sub>SNPE or DiC<sub>6</sub>SNPC. If the monomer model is correct, we would expect to see two binding sites for DiC<sub>6</sub>SNPC and either one or two sites for DiC<sub>6</sub>SNPE per enzyme molecule. As these studies have shown, we have determined that cobra PLA<sub>2</sub> has two lipid binding sites; the catalytic site binds to both PE and PC, and the PC activator site binds only PC. The monomer model now clearly accounts for all known characteristics of PC activation. Aggregation appears to simply be coincidental with activity. While dimers or higher order protein aggregates still form, the functional subunit within the protein aggregate is monomeric. Interestingly, the *Crotalus atrox* PLA<sub>2</sub>, which is dimeric under almost all in vitro conditions, has now been found to act at surfaces as a monomer (37). Thus, cobra PLA<sub>2</sub> joins other members of the PLA<sub>2</sub> family in being catalytically competent as a monomer (37, 43).

There has always been the question of whether PE compounds bind to the activator site but are incapable of causing activation or whether they do not bind to this site at all. We have resolved this here. PE compounds bind to only one site (Figure 3), which from NMR results has been proven to be the catalytic site (18). Also, the fact that the enzyme hydrolyzes PE requires that it bind to the catalytic site. A second issue has been does activation affect binding, turnover, or both. SPM is a potent activator of PLA<sub>2</sub>, increasing PLA<sub>2</sub> activity at least 20-fold (5, 12, 44). The 13-fold decrease in the K<sub>D</sub> of DiC<sub>6</sub>SNPE upon SPM activation of PLA<sub>2</sub> demonstrates that PC activation has a large effect on substrate binding and any direct effect on turnover is probably modest. Third, we have assumed that PC compounds are self-activating. Yu and Dennis showed that thio PC compounds were moderately activated by the more potent PC activator, SPM (8). The positive cooperativity observed for DiC<sub>6</sub>SNPC binding confirms that PC is self-activating (Figure 4).

Lefkowitz et al. (14) have used site-directed mutagenesis to identify some of the amino acid residues on the enzyme that are involved in catalytic activity, PC activation, and interfacial activation. They found that in addition to the catalytic site, there were two other regions on the enzyme that were involved in PC and interfacial activation. When mutations were made in one site, the “PC activation site”, only PC activation was affected. The activity of these mutants on PE substrate was the same as the native enzyme. Mutations in the other site, the “interfacial activation site” significantly decrease interfacial activation. These mutants had normal activity on submicellar substrate and still showed PC activation. Interestingly, only one mutation, Tyr63, affected both PC and interfacial activation. The PC activation site is located on one side of the cleft leading to the catalytic site, while the interfacial site is located on the other side of this cleft. While the Tyr63 is located in the interfacial



activation site, it can extend out over the catalytic cleft and interact with Arg30 of the PC site.

Clearly there are three regions of the PLA<sub>2</sub> molecule that are intimately involved in the enzyme's activity, but are all three lipid binding sites? The studies reported here strongly suggest that at least one site is not a binding site. The data reported here are consistent with both PE and PC compounds binding to the catalytic site and with PC alone binding to a second site that is responsible for PC activation. This would presumably correspond to the "PC activation site" identified in the mutation studies. It would appear that the "interfacial activation site" defined in the mutation studies is not a discrete lipid binding site, yet mutations of the amino acids in this site produce dramatic effects on interfacial activation.

The mechanisms of either PC or interfacial activation have yet to be elucidated. We have shown here that PC binding to the PC activator site does dramatically increase the binding of substrate to the catalytic site. Our previous kinetic studies (45) have shown that the catalytic rate constant is also altered when PC binds to the activator site. The most straightforward explanation of these observations would be that binding to the activator site induces a conformational change in the enzyme. This change increases binding at the catalytic site directly and enhances turnover as well. NMR studies of porcine pancreatic PLA<sub>2</sub> provide evidence that PLA<sub>2</sub> does undergo conformational changes upon binding micelles (46–48).

However, enzymes that act at the lipid–water interface, function in a unique environment, one that offers several other means of producing an acceleration of catalysis. Because they act at large interfaces, the orientation of the enzyme relative to the surface can and often does play an additional role in controlling activity. Thus, the binding of PC to the activator site could provide another contact point for the enzyme to the interface. This would anchor the enzyme to the interface, shifting the equilibrium between the free and bound enzyme, and indirectly increase binding at the catalytic site. This anchor could also be holding the enzyme in a specific orientation with respect to the surface that would enhance binding, catalysis, or both. It is also likely that PC binding to the activator site would aid in desolvating the interface; several studies have implicated desolvation as important for catalytic activity (28, 49, 50). Such an "anchoring" mechanism has been demonstrated for phorbol ester binding to protein kinase C in vesicles (51). The conformation change and anchoring mechanisms are not mutually exclusive, and some combination of each is likely.

Similar mechanisms could be responsible for the interfacial activation. However, the studies reported here imply that the interfacial site on the enzyme does not discretely bind lipids. While the key residues in the PC site are aspartic acid and arginine, the key residues in the interfacial site are tryptophan, tyrosine, and phenylalanine. This would imply that while the PC site could interact specifically with the charged moieties on the lipid headgroup, the interfacial site would probably be interacting with the hydrophobic regions of the interface. This could also help desolvation, help orient the enzyme at or in the surface, and help sequester the enzyme to the surface, all without binding to a single lipid moiety but to the "hydrophobic region" of the surface.

The irreversible inhibitor p-BPB has proven to be a useful tool for probing interactions at the catalytic site of PLA<sub>2</sub>s.

It completely blocks catalytic activity in pancreatic and cobra PLA<sub>2</sub> (30, 52). For cobra PLA<sub>2</sub>, p-BPB alkylates only one residue, the catalytic histidine (30). It was anticipated that modification of the catalytic histidine would block substrate binding to the catalytic site as was suggested from NMR work (18). This was confirmed in Figure 6A, when DiC<sub>6</sub>-SNPE no longer bound to the catalytic site in p-BPB modified PLA<sub>2</sub>. We also anticipated that DiC<sub>6</sub>SNPC would not bind to the catalytic site but that it would still bind to the PC site. This was not the case; it did not bind to either site (Figure 6B). Yu and Dennis (41) have demonstrated using protection studies that these two sites are spatially separated and can be characterized by different pH dependencies. Our mutagenesis studies (14) and crystallography studies (53) show that the PC activator-binding site, while adjacent to the catalytic site, is not contiguous with it. These facts would argue that p-BPB modification would not block the PC site.

Comparison of the p-BPB modified and native crystal structures of bovine pancreatic PLA<sub>2</sub> by Renetseder et al. (54) suggests a possible explanation. They observed that for the most part the modified and unmodified structures are the same, including the catalytic site. Since p-BPB is a small molecule that easily fits within the catalytic cavity, this is not surprising. However, the Ca<sup>2+</sup> binding loop showed a substantial change due to loss of the catalytic calcium. As pointed out by Segelke et al. (53), the ordering of the Ca<sup>2+</sup> binding loop through Ca<sup>2+</sup> binding is critical to forming the putative PC activator-binding site. So loss of the catalytic calcium upon p-BPB modification of the enzyme also disrupts the PC activator site, and no DiC<sub>6</sub>SNPC binding is observed at either the catalytic or activator sites.

In the original p-BPB studies, p-BPB modified PLA<sub>2</sub> still bound to mixed micelles of PC and Triton X-100 ( $K_D = 1.8$  mM). These results led to the hypothesis that a second PC binding site existed and that it was not affected by p-BPB modification. It was further assumed that the first binding step, where aqueous PLA<sub>2</sub> is sequestered at the interface, was through this specific interaction with the PC activator site. The present study suggests that the binding to PC mixed micelles was due to nonspecific interactions of the enzyme with the lipid surface, perhaps at the interfacial recognition site proposed by Lefkowitz et al. (14). Whether the initial interaction can be augmented by binding to the PC activator site has yet to be determined. Once PLA<sub>2</sub> is weakly bound to the interface, binding to either the catalytic or PC activator site can proceed as originally proposed.

**Conclusions.** We have used equilibrium dialysis and micellar substrate analogues to show for the first time that PC binds cooperatively to two sites per cobra PLA<sub>2</sub>, while PE binds to only one site. It appears that the aggregation of this enzyme is simply coincidental with activity; the functional subunit within the aggregated protein is a monomer. PC activation of PE hydrolysis now has been shown to be due to both a binding and a catalytic term. In addition, we have shown that the first association with the interface is probably a weak nonspecific enzyme–lipid interaction. The binding studies under submicellar conditions show that cobra PLA<sub>2</sub> is not catalytically competent on truly monomeric substrate but instead catalyzes hydrolysis on premicellar aggregates.

## ACKNOWLEDGMENT

We thank Dr. Laurie Reynolds for her helpful suggestions regarding the equilibrium dialysis work. We also appreciate the fruitful discussions with Dr. Leigh Plesniak and Dr. Brent Segelke. We thank Dr. Michael Gelb for his suggestion to try PLD from *Streptomyces* species for the headgroup exchange. We acknowledge the support of the staff and facilities of the Southern California Regional Mass Spectrometry Facility, a unit of the UC Riverside Analytical Chemistry Instrumentation Facility.

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BI035921B