Kinetic and Inhibition Studies of Phospholipase A_2 with Short-Chain Substrates and Inhibitors†

Wei Yuan,‡ Daniel M. Quinn,§ Paul B. Sigler,∥ and Michael H. Gelb*,‡

Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195, Department of Chemistry, University of Iowa, Iowa City, Iowa 52242, and Department of Molecular Biophysics and Biochemistry and the Howard Hughes Medical Institute, Yale University, New Haven, Connecticut 06511

Received December 4, 1989; Revised Manuscript Received March 7, 1990

ABSTRACT: The action of the phospholipases A_2 (PLA2s) from *Naja naja naja*, *Naja naja atra*, and *Crotalus atrox* venoms as well as the enzyme from porcine pancreas on a number of short-chain, water-soluble substrates was studied. The inhibition of these enzymes by short-chain phosphonate- and thiophosphonate-containing phospholipid analogues was also examined. The kinetic patterns observed for the action of the venom PLA2s on substrates containing phosphocholine head groups all deviated from a classical Michaelis–Menten-type behavior. With a substrate containing an anionic head group, the kinetic pattern observed was more normal. In contrast, Michaelis–Menten-type behavior was observed for the action of the porcine pancreatic PLA2 acting on all of the substrates studied. A short-chain phospholipid analogue in which the enzyme-susceptible ester was replaced with a phosphonate group was found to be a tight-binding inhibitor of the venom PLA2s with IC_{50} values that were some 10^4–10^5-fold lower than the concentration of substrate used in the assay. The degree of inhibition was found to depend dramatically on the stereochemical arrangement of substituents in the inhibitor which strongly suggests that the inhibitors are binding directly to the active site of the PLA2s. By comparison, the phosphonate analogue functioned as a poor inhibitor of the porcine pancreatic PLA2. Direct inhibitor binding studies indicated that the short-chain phosphonate inhibitor bound weakly to the venom enzymes in the absence of the short-chain substrates. Several other unusual features of the inhibition were also observed. The data are interpreted in terms of a model in which the enzyme and substrate form a lipid–protein aggregate at substrate concentrations below the critical micelle concentration (cmc). Possible reasons for the selective binding of the inhibitor to the enzyme–substrate microaggregate are discussed.

PLA2 is probably one of the best studied enzymes that catalyzes a reaction at the lipid/water interface (Jain & Berg, 1989; Dennis, 1983; Volwerk & de Haas, 1981). For most lipolytic enzymes, including PLA2, the hydrolytic activity on aggregated forms of the substrate (micelles, mixed micelles, monolayers, and bilayers) is much higher than the activity on soluble substrates that are monomolecularly dispersed in solution. It has been established by numerous approaches that the PLA2s bind to the substrate interface via an interfacial binding surface on the enzyme that is topologically and functionally distinct from the site on the enzyme where the esterolysis occurs (Jain et al., 1986a; Dennis, 1983; Verger & de Haas, 1976).

The inhibition of PLA2 continues to be of medicinal interest since it is currently believed that the liberation of free arachidonic acid for the biosynthesis of the eicosanoids is the result of the action of this enzyme on the pool of phospholipids in the cell membrane (Dennis, 1987; Irvine, 1982). In the case of PLA2, the medicinal chemist is faced not only with the task of designing small molecular weight inhibitors that bind tightly to the enzyme but also with the challenge of evaluating the inhibitors in a meaningful way. With an enzyme that operates on a lipid/water interface, there is always concern that the inhibition by a class of putative inhibitors is the result of one of a number of possible nonspecific effects. For example, we and others have recently shown that many of the previously reported inhibitors of PLA2 function not by binding directly to the catalytic site on the enzyme but rather by partitioning into the substrate bilayer and promoting the desorption of the enzyme from the interface (Jain et al., 1989; Jain & Jahagirdar, 1985). This problem can be overcome by examining the action of PLA2 on vesicles in the "scooting mode" in which the enzyme remains irreversibly bound to the bilayer surface and hydrolyzes all of the substrate in the outer layer of vesicles (Jain et al., 1986a, 1989). The action of inhibitors of PLA2 has also been examined in mixed micelles in which the substrate and inhibitor are present in the surface of a detergent micelle such as Triton X-100 or deoxycholate (de Haas et al., 1989; Yuan et al., 1987, 1989b; Yuan & Gelb, 1988; Davidson et al., 1986); however, because of the dynamic nature of micelles, these studies are much more difficult to interpret than studies with vesicles in the scooting mode (Jain & Berg, 1989; Jain et al., 1989).

For this paper, kinetic and inhibition studies were carried out with substrates and inhibitors that contain short alkyl chains and are therefore soluble in water. In this way, an attempt was made to study the inhibition of PLA2 in the absence of a substrate interface.

Materials and Methods

Materials. *p*-Bromophenacyl bromide and 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) were obtained from Aldrich. 1-*O*-Hexadecyl-2-deoxy-2-*S*-thiohexadecanoyl-*sn*-glycero-3-phosphocholine was prepared by a modification of the previously reported procedure for similar compounds (Aarsman et al., 1985) and will be described elsewhere (Yuan,

† Supported by Grants HL-36235 to M.H.G. and GM-22324 to P.B.S. from the National Institutes of Health.

* To whom correspondence should be addressed.
‡ University of Washington.
§ University of Iowa.
∥ Yale University.

0006-2960/90/0429-6082$02.50/0

1990). 1,2-Dihexyl-*sn*-glycero-3-phosphocholine was a generous gift from Professor Mahendra Jain (University of Delaware). 1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine (diC_6-PC) was purchased from Avanti Polar Lipids. PLA2 from *Naja naja naja* venom was obtained as a generous gift from Professor Edward Dennis (University of California, San Diego) or purified as described (Hazlette & Dennis, 1985). PLA2 from *Naja naja atra* venom was purified essentially as described (Yang et al., 1981) except that the last chromatograpic step with SP-Sephadex was omitted. The first, second, and third columns were run with SP-Sephadex C-50, DEAE-Sephadex, and CM-Sephadex C-25, respectively (all from Pharmacia). In our hands, this PLA2 came off the first column in the flow-through fraction. PLA2 from *Crotalus atrox* venom was purified as described previously (Keith et al., 1981). The enzyme from porcine pancreas was purchased from Boehringer. Concentrations of enzymes were determined by measuring the absorbance at 280 nm by using the published extinction coefficients (Darke et al., 1980; de Haas et al., 1968) for the *N. naja naja* and porcine pancreatic enzymes. For the PLA2s from *N. naja atra* and *C. atrox*, the extinction coefficients, $A^{1\%}_{280}$, were calculated from the amino acid sequences to be 25 and 17.2, respectively, by using the published formula (Perkins, 1986).

General Synthetic Methods. Chromatographic techniques, drying of the solvents, and spectroscopic methods have been described previously (Yuan et al., 1987, 1989a). All inhibitors were characterized by a combination of ^1H NMR, ^{31}P NMR, and liquid secondary ion mass spectrometry (LSIMS). All final compounds gave single spots on silica gel thin-layer chromatography (TLC) plates. The following TLC solvents were used: C, chloroform; EA, ethyl acetate; M, methanol; and PE, low-boiling petroleum ether. The TLC staining procedures have been previously described: $KMnO_4$ (Yuan et al., 1987); AMA; ninhydrin; and PAA (Yuan et al., 1989a). The syntheses of inhibitors **8**–**11** were carried out in a manner similar to that of the related long-chain phospholipid analogues (Yuan et al., 1989a). The synthesis of compound **8** is described in detail below. The syntheses of compounds **9**–**11** will be described in detail elsewhere (Yuan, 1990).

n-Heptylphosphonic Acid Dimethyl Ester (**1**). *n*-Heptyl bromide (17.9 g) and trimethyl phosphite (12.4 g) were heated together under reflux in an oil bath at 150–160 °C for 14 h. The reaction mixture was distilled in vacuo. The product (8.4 g, 40%) was collected as a colorless liquid: bp 132 °C/13.5 mmHg; TLC (R_f = 0.2, 50% EA in PE, $KMnO_4$).

n-Heptylphosphonic Acid Monomethyl Ester (**2**). A solution of dimethyl ester **1** (8.2 g, 39 mmol) and sodium hydroxide (2.0 g) in methanol (30 mL) was refluxed in an oil bath at 100 °C for 20 h. The methanol was removed in vacuo, and the residue was dissolved in water (100 mL). The solution was extracted with ether until no organic material remained in the ether extract (detected by TLC, 50% EA in PE). The water layer was neutralized with concentrated HCl to pH 1 and extracted with ether (2 × 100 mL). The extracts were combined and dried over $MgSO_4$, and the ether was removed in vacuo to give the product (6.2 g, 81%) as a colorless oil: TLC (R_f = 0.2, 25% M in C, $KMnO_4$); NMR ($CDCl_3$, 300 MHz) δ 0.90 (t, 3 H), 1.24–1.44 (m, 8 H), 1.58–1.82 (m, 4 H), 3.74 (d, 3 H), 10.4 (br s, 1 H).

1-Octyl-sn-glycerol (**3**). The preparation of compound **3** was carried out essentially as described previously for 1-hexadecyl-3-*sn*-glycerol (Yuan et al., 1989a). The 1-octylglycerol acetonide was purified on silica (first with PE followed by 10% EA in PE). The 3-octyl-*sn*-glycerol was purified on silica (25% EA in PE followed by 50% EA in PE). The inversion of configuration at carbon 2 was carried out exactly as described (Yuan et al., 1989a) except that the ditosylate was not purified on silica. The replacement of tosylate with acetate was monitored by TLC and required 36 h. The saponification step was also monitored by TLC and required 2 h. Product **3** was purified as described above for its enantiomer. The enantiomeric purity of **3** was determined as described (Yuan et al., 1989a; Yuan, 1990).

1-Octyl-3-trityl-sn-glycerol (**4**). This compound was prepared essentially as described (Yuan et al., 1989a), but the tritylation required a longer reaction time. Compound **3** (5.2 g, 25.1 mmol) in pyridine (75 mL) was treated with trityl chloride (10.4 g, 37.5 mmol) at room temperature for 36 h. The reaction was followed by TLC. Most of the pyridine was removed in vacuo, and the residue was dissolved in ether (200 mL) and washed with water (3 × 100 mL). The ether was removed in vacuo, and the residue was dissolved in petroleum ether (100 mL) and filtered to remove the white solid. The filtrate was concentrated in vacuo, and the pale yellow oil was purified on silica (first with PE, followed by 5% EA in PE, then 10% EA in PE) to give the product (6.8 g, 60%): TLC (R_f = 0.31, 10% EA in PE, ultraviolet lamp).

Phosphonylation of **4** (**5**). Compound **2** (1.2 g, 6 mmol) was added to a flask equipped with a condenser protected with a $CaSO_4$-filled dry tube. Freshly distilled thionyl chloride (4 mL) was added, and the solution was refluxed for 5 h. The thionyl chloride was removed in vacuo. A solution of **4** (1.35 g, 3 mmol), triethylamine (1.5 g, 15 mmol), and 4-dimethylaminopyridine (20 mg, Aldrich) in dry chloroform (12 mL) was added to the phosphonyl chloride. The mixture was stirred at room temperature overnight in a tightly stoppered flask. Petroleum ether was added, and the salt was removed by filtration. The filtrate was concentrated in vacuo, and the residue was purified on silica (first with PE, followed by 10% EA in PE) to afford the product (1.1 g, 59%) as a pale yellow oil: TLC (R_f = 0.5, 25% EA in PE, ultraviolet lamp); NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, 6 H), 1.15–1.35 (m, 18 H), 1.45–1.65 (m, 4 H), 1.65–1.85 (m, 2 H), 3.15–3.45 (m, 4 H), 3.55–3.72 (m and dd, 5 H), 4.72 (br s, 1 H), 7.18–7.50 (m, 15 H).

Detritylation of Compound **5** (**6**). Compound **5** (1.1 g) was detritylated essentially as described previously (Yuan et al., 1989a) with BF_3–methanol (1.0 mL, 14%, Sigma) in dichloromethane (15 mL) to give compound **6** (0.50 g, 75%) as a pale yellow oil after purification on silica (25% EA in PE, followed by 50% EA in PE): TLC (R_f = 0.14 and 0.25, mixture of diastereomers, 50% EA in PE, PAA); NMR ($CDCl_3$, 500 MHz) δ 0.90 (t, 6 H), 1.20–1.45 (m, 16 H), 1.50–1.75 (m, 6 H), 1.75–1.90 (m, 2 H), 3.40–3.65 (m, 6 H), 3.78 (dd, 3 H), 4.55 (br s, 1 H).

Phosphatidylethanolamine Analogue (**7**). The preparation of **7** was carried out essentially as described previously (Yuan et al., 1989a) starting with **6** (290 mg). Compound **7** (170 mg, 42%) was obtained as a white powder after purification on silica (10% M in C, followed by 20% M in C) and lyophilization from benzene: TLC (R_f = 0.24 and 0.38, mixture of diastereomers, 25% M in C, ninhydrin); NMR (10% CD_3OD in $CDCl_3$, 500 MHz) δ 0.89 (two t, 6 H), 1.20–1.45 (m, 18 H), 1.55–1.70 (m, 4 H), 1.80–1.95 (m, 2 H), 3.3 (br s, 2 H), 3.40–3.55 (m, 2 H), 3.60 (br s, 2 H), 3.82 (d, 3 H), 4.0 (br s, 1 H), 4.15 (br s, 1 H), 4.26 (br s, 2 H), 4.87 (br s, 1 H).

Phosphatidylethanolamine Analogue (**8**). Compound **7** (110 mg, 0.22 mmol), thiophenol (240 mg, 2.2 mmol), tri-

ethylamine (0.49 mL), and dioxane (1.3 mL) were mixed together in a test tube which was tightly stoppered with a screw cap and stirred in an oil bath at 60 °C. The reaction was followed by TLC (50% M in C, ninhydrin) and was finished in about 5 h. The reaction mixture was transferred to a flask with the aid of a few milliliters of methanol/chloroform (1/1), and the solvent was removed in vacuo. The residue was purified on a silica column (1 × 5 cm, first with 10% M in C, followed by 25% M in C and then 50% M in C) to give the product as a white solid (59 mg, 55%) after lyophilization from benzene containing a few drops of methanol: TLC (R_f = 0.2, 50% M in C, ninhydrin); NMR (10% CD_3OD in $CDCl_3$, 500 MHz) δ 0.89 (t, 6 H), 1.25–1.42 (m, 18 H), 1.52–1.65 (br s, 6 H), 3.05–3.15 (br s, 2 H), 3.46 (br s, 2 H), 3.51 (br s, 2 H), 3.98 (br s, 1 H), 4.12 (br s, 3 H), 4.48 (br s, 1 H); LSIMS (negative ion mode, 488, M − 1); ^{31}P NMR (10% CD_3OD in $CDCl_3$, 121 MHz) δ −0.90 (br s), 25.8 (br s).

^3H-Labeled Inhibitor (**8**). Compound **8** was prepared in radiolabeled form by phospholipase D catalyzed exchange of the ethanolamine in the head group with tritiated ethanolamine. The phospholipase D was partially purified from Savoy cabbage as described (Davidson & Long, 1958) except that the step with the calcium phosphate gel was omitted. After the acetone precipitation step, the precipitate was dissolved in water as described and centrifuged. The supernatant was lyophilized to give the enzyme powder. [1-^{3}H]Ethan-1-ol-2-amine hydrochloride (1 mCi, 19 Ci/mmol, Amersham) was supplied by the manufacturer in water. The solution was placed in a small glass tube, and most of the water was removed with a stream of N_2. Compound **8** (2.5 mg) was added followed by 200 μL of ether and 50 μL of a solution containing 0.2 M unlabeled ethanolamine in 100 mM sodium acetate and 100 mM $CaCl_2$, pH 5.6. A stock solution of 7.6 mg of enzyme powder in 200 μL of buffer was prepared, and 50 μL was added to the reaction mixture. The tube was stoppered, and the solution was stirred vigorously with a small magnetic stir bar at room temperature. Additional ether was added if necessary due to losses by evaporation, and three additional 50-μL aliquots of the same enzyme stock solution were added every 24 h. Finally, the reaction was kept an additional 40 h at room temperature. The water and ether were removed with a stream of air, and the residue was dissolved in 0.6 mL of $CHCl_3$/MeOH (1/1). The solution was centrifuged to remove solid, and the supernatant was purified by preparative TLC (silica gel, 20 × 20 cm plate, 0.25 mm). The plate was developed with methanol/$CHCl_3$/29% aqueous ammonia (9/1/1). A thin strip was cut from the plate and sprayed with ninhydrin to visualize the inhibitor **8** (R_f = 0.76) and ethanolamine **8** (R_f = 0.36). The silica containing **8** was scrapped from the plate. The product was eluted by washing the silica with the same solvent (6 × 2 mL) in a sintered glass funnel. The solvent was removed with a stream of air, and the residue was taken up in a small amount of benzene containing a few drops of $CHCl_3$/MeOH (1/1) and lyophilized. A stock solution of radiolabeled **8** was made in $CHCl_3$/MeOH (1/1), and the concentration of **8** was obtained by comparison of the PLA2 inhibition potency of a known volume of this solution against a known amount of unlabeled **8**. The final specific activity of the labeled **8** was approximately 54 mCi/mmol.

(n-Octanoylthio)glycollecithin (*C_8-thioPC*). This compound was prepared by a modification of the previously reported procedure (van Oort et al., 1985a). 2-(Octanoylthio)ethanol was prepared from octanoyl chloride and 2-mercaptoethanol as described (Hille et al., 1983). The material was purified on silica (PE followed by 5% EA in PE and then 20% EA in PE). The phosphorylcholine group was introduced by using ethylene chlorophosphate followed by treatment with trimethylamine (Thuong & Chabrier et al., 1974). The final product was purified on silica (10% M in C, followed by 25% M in C and then 50% M in C).

Racemic 2,3-Bis(hexanoylthio)propylphosphocholine (*diC_6-thioPC*). This material was prepared from 2,3-dimercapto-1-propanol (Aldrich) according to a modification of the previously described procedure (Volwerk et al., 1979; Yuan, 1990). The final product was purified on silica (25% M in C, followed by 50% M in C).

Racemic 2,3-Bis(hexanoylthio)propylphosphomethanol Lithium Salt (*diC_6-thioPMe*). 2,3-Bis(hexanoylthio)-1-propanol was converted to the phosphatidylmethanol analogue as described (Wooley & Eibl, 1988). The final product was purified on silica (50% EA in PE, followed by EA and then 5% M in C) and lyophilized from benzene: TLC (R_f = 0.35, 25% M in C, AMA); NMR ($CDCl_3$, 300 MHz) δ 0.89 (t, 6 H), 1.30 (m, 8 H), 1.65 (m, 4 H), 2.55 (t, 4 H), 3.18 (m, 1 H), 3.42 (m, 1 H), 3.62 (d, 3 H), 3.89 (m, 2 H), 4.06 (m, 1 H).

Enzyme Assays. The components of all buffers are given in the figure legends. Assays with thiolester substrates were carried out as follows. The substrate was dissolved in the buffer at the highest concentration used and filtered through a 0.22-μm disposable filter unit (Millipore) to remove dust. Solutions containing the desired lower substrate concentrations were prepared by dilution with filtered buffer. Ellman's reagent (50 mM in ethanol) was added to all buffer solutions to give a final concentration of 0.8 mM. The solutions of substrate were kept on ice until use. The substrate cocktail (0.3 mL) was added to a microcuvette and placed in a Perkin-Elmer Lambda-3 spectrophotometer with a thermostated cuvette holder maintained at 25 °C. The absorbance at 412 nm was monitored. The reactions were initiated by the addition of enzyme. For those samples that contained inhibitor, a stock solution of inhibitor in water was added to give the desired final concentration of inhibitor.

A coupled colorimetric assay of the PLA2-catalyzed hydrolysis of diC_6-PC was also developed (Allgyer & Wells, 1979). The reaction mixture contained 0.9 mM sodium *p*-nitrophenoxide, 0.09 M NaCl, 9 mM $CaCl_2$, pH 7.9, and various concentrations of diC_6-PC substrate **8**. PLA2 reactions were initiated by adding enzyme to assay cocktail (0.5 mL) in a cuvette. The reactions were monitored at 25 °C by following the absorbance at 456 nm. All enzyme assays were conducted in triplicate.

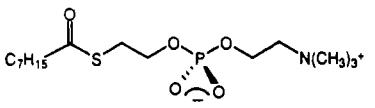
Enzyme Alkylation Studies. PLA2 from *N. naja naja* venom was diluted to a concentration of 0.7 μM in buffer (25 mM Tris-HCl, 0.1 M KCl, pH 8.5). Ninety-five microliters of this solution was mixed with 5 μL of *p*-bromophenacyl bromide in acetone. The concentrations of other additives were as follows: no calcium and no **8** (1 mM EDTA, 50 μM inactivator); no calcium with **8** (1 mM EDTA, 50 μM inactivator, 9.5 μM **8**); calcium alone (10 mM $CaCl_2$, 300 μM inactivator); calcium plus **8** (10 mM $CaCl_2$, 300 μM inactivator, 9.5 μM **8**). The mixture was incubated at 21 °C, and 4-μL aliquots were periodically removed and diluted into 0.5 mL of a mixed-micelle assay solution present in a microcuvette [4 mM Triton X-100, 0.5 mM 1-*O*-hexadecyl-2-deoxy-2-*S*-thiohexadecanoyl-*sn*-glycero-3-phosphocholine, 25 mM Tris-HCl, 0.1 M KCl, 10 mM $CaCl_2$, 0.8 mM Ellman's reagent, pH 8.5, prepared as described by Hendrickson and Dennis (1984)]. The increase in absorbance at 412 nm was monitored in a spectrophotometer with temperature control at 30 °C.

Equilibrium Dialysis Binding Studies. Equilibrium dialysis was performed with a multichamber device with 1-mL wells (Hoeffer Scientific) and fitted with 6000–8000-Da cutoff membranes. A stock solution of PLA2 was made to the desired final concentration (typically 2–3 μM) in buffer (25 mM Tris-HCl, 0.1 M KCl, 10 mM $CaCl_2$, pH 8.0), and 1-mL portions were placed on one side of the dialysis membrane. The wells on the other side of the membrane were loaded with 1-mL portions of buffer containing variable concentrations of radiolabeled inhibitor **8** (typically 2.5–10 μM). In some experiments, 1,2-dihexyl-*sn*-glycero-3-phosphorylcholine was added at a concentration of 1 mM. The cells was agitated for 48 h at room temperature. The liquid on both sides of the membrane was withdrawn and mixed with 20 mL of scintillation fluid. Each well was washed with additional portions of buffer (4 × 0.1 mL), and the washings were transferred to the counting vials. The amount of radioactivity was determined in a Beckman LS 1801 scintillation counter. Control experiments in which buffer without enzyme was added to one side of the membrane and buffer plus inhibitor to the other side showed that the inhibitor distributed uniformly on both sides of the membrane. In addition, PLA2 in buffer was added to one side of the membrane and buffer without inhibitor was added to the other side. Assay of the enzyme at the beginning and at the end of this experiment showed that less than 20% of the activity was lost and that the enzyme did not cross the membrane.

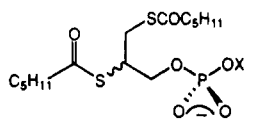
cmc Measurements. cmc values for C_8-thioPC, diC_6-thioPC, diC_6-thioPMe, and **8** were determined by measuring the fluorescence of the dye 8-anilino-1-naphthalenesulfonic acid (Aldrich) as a function of the phospholipid concentration (Wells, 1974). For diC_6-thioPC and diC_6-thioPMe, experiments were performed in 25 mM Tris-HCl, 0.1 M KCl, and 10 mM $CaCl_2$, pH 8.0. For inhibitor **8**, the measurement was made in 0.1 mM Tris-HCl, pH 8.0, and either 0 or 10 mM $CaCl_2$. All experiments used a dye concentration of 0.1 mM and a fluorescence excitation at 375 nm and detection at 495 nm.

Results

Kinetic and Inhibition Studies of PLA2 Acting on Single-Chain Phosphorylcholine-Containing Substrate, C_8-thioPC. Thioester-containing substrates C_8-thioPC, diC_6-thioPC, and diC_6-thioPMe were prepared as described under Materials and



C_8-thioPC



diC_6-thioPC, X = $CH_2CH_2N(CH_3)_3^+$
diC_6-thioPMe, X = CH_3

Methods. The cmc values for the thioester substrates were measured by the fluorescent dye partitioning method (Wells, 1974). All of the measurements were made in the same buffer that was used in the kinetic studies (25 mM Tris-HCl, 0.1 M KCl, 10 mM $CaCl_2$, pH 8.0). The cmc value for diC_6-thioPC was found to be 2.0 mM, and the solution remained clear both below and above the cmc. In the case of diC_6-thioPMe, which contains an anionic head group, the solution became cloudy

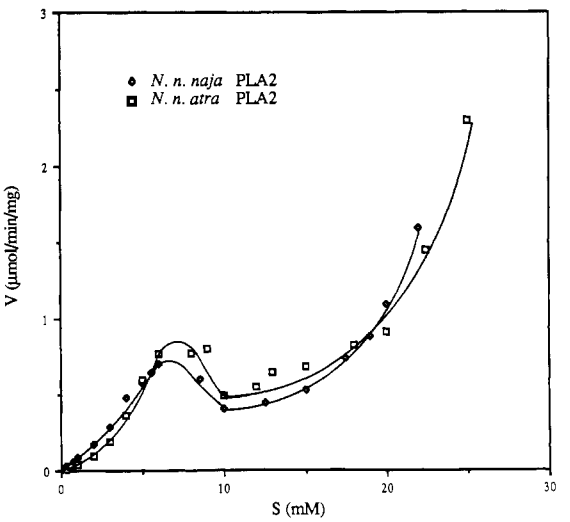


FIGURE 1: Velocity versus substrate C_8-thioPC concentration. Enzyme concentrations were 0.3 μM for the *N. naja naja* PLA2 and 0.2 μM for the *N. naja atra* enzyme. The buffer was 50 mM HEPES and 10 mM $CaCl_2$, pH 7.0.

and the dye fluorescence increased when the concentration reached 2.5 mM. Below this cloud point, the dye fluorescence remained constant, indicating that the compound was not aggregating at concentrations below 2.5 mM. The cmc for C_8-thioPC has been previously reported to be 19.5 mM (Hille et al., 1983), and this value was confirmed in the present study.

The hydrolysis of the single-chain substrate C_8-thioPC by the PLA2s from *N. naja naja* and *N. naja atra* venoms was studied. The initial reaction velocity as a function of substrate concentration is shown in Figure 1. With concentrations of C_8-thioPC below the cmc, a hyperbolic, Michaelis–Menten-type curve was not observed. Rather, there was a sharp increase in the velocity at substrate concentrations below the cmc. With higher substrate concentrations, the velocity first decreased and then increased steadily as the concentration approached the cmc. A similar kinetic pattern was seen with both enzymes. The peak of activity at low substrate concentration occurred at about 0.3 times the cmc. The anomalous kinetic behavior seen in Figure 1 was reproducible in several series of experiments. It was also seen with batches of substrate that were purified by chromatography on DEAE-cellulose rather than on silica gel, and it is therefore not due to some type of surface-active phenomenon that could have resulted from the contamination of the substrate with trace amounts of silica.

Kinetic patterns similar to those shown in Figure 1 have been reported for the action of the porcine pancreatic PLA2 on anionic, sulfate-containing phospholipid substrates (van Oort et al., 1985a,b). In this case, large lipid–protein aggregates, consisting of about 6 enzyme molecules and about 40 lipid molecules, form below the cmc of the pure substrate and the activity of the enzyme is higher in these pre-cmc aggregates. Apparently these lipid–protein aggregates exist in a very narrow range of substrate concentration, and they tend to fall apart at slightly higher and lower substrate concentrations. The formation of pre-cmc enzyme–lipid aggregates has also been reported for the porcine pancreatic PLA2 operating on the anionic substrate dihexanoylphosphatidylmethanol (Jain et al., 1986b).

Formation of PLA2–lipid aggregates at concentrations of substrate below the cmc is the most likely explanation for the unusual kinetic patterns seen in Figure 1. Gel filtration studies with the PLA2s from *Naja melanoleuca* and *N. naja naja* venoms have shown that the large lipid–protein aggregates

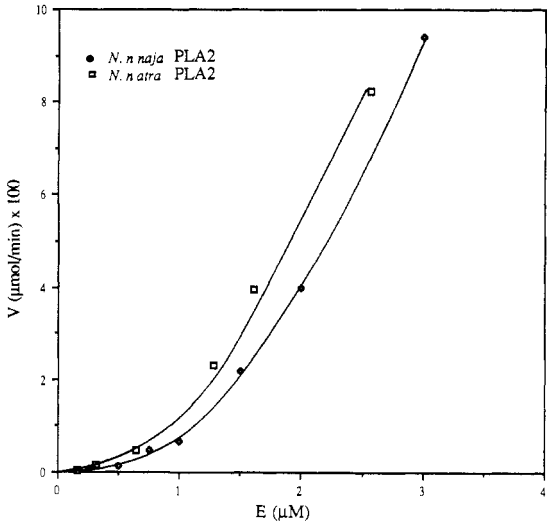


FIGURE 2: Velocity versus enzyme concentration in the presence of substrate C_8-thioPC (13.5 mM). The buffer was 25 mM Tris-HCl, 0.1 M KCl, and 10 mM $CaCl_2$, pH 8.0.

form in the presence of phosphorylcholine-containing detergents such as dodecylphosphorylcholine below the cmc (Van Eijk et al., 1983; Plückthun & Dennis, 1985). Sedimentation studies with the cobra venom enzyme in the presence of concentrations of dihexanoylphosphatidylcholine substrate below the cmc have detected the presence of large lipid–protein complexes that contain about 68 lipid molecules per enzyme (Bukowski & Teller, 1986). Although it has been previously proposed that the lipid induces the formation of enzyme–enzyme aggregates (Plückthun & Dennis, 1985), the phenomenon is probably better described as an enzyme-induced aggregation of the substrate (Bukowski & Teller, 1986; van Oort et al., 1985a,b). In a sense, the enzyme is designed to bind to a phospholipid interface, and it therefore may tend to collect a number of phospholipid molecules from the solution onto its interfacial binding surface which then causes the lipid to form an aggregate below the cmc. Apparently a major difference between the PLA2s from porcine pancreas and the cobra venoms is that with the former the aggregation occurs with sulfate-containing substrates, whereas with the latter the aggregation is seen with zwitterionic substrates.

As the substrate concentration was increased above 0.3 times the cmc, the velocity dropped (Figure 1). The reason for this is not clear. Since the activity of the enzyme is enhanced in the lipid–protein aggregate, the falloff in activity may be due to a decrease in the extent of aggregation as the lipid-to-protein ratio increases. A second possibility is that there is a change in the rate-limiting step with higher lipid concentrations. It is possible that the overall rate of lipolysis in these enzyme–lipid aggregates is limited by the rate of exchange of substrate and products in and out of the aggregate. At higher lipid concentrations, the extent of aggregation may actually be higher and the rate of substrate replenishment might now be limited by collisions between larger aggregates. Further experiments will be necessary to shed light on this difficult aspect of the kinetics. As the cmc of the pure lipid was approached, the velocity increased dramatically (Figure 1). This increase near the cmc is well-known in the field of lipolysis (Volwerk & de Haas, 1981; Verger & de Haas, 1976) and is due to the much higher activity of these enzymes on substrate micelles compared to substrate monomers. The molecular basis for this interfacial activation is not understood.

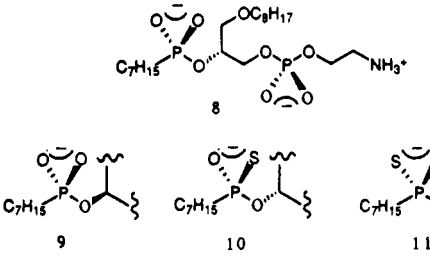
Plots of the initial velocity as a function of the enzyme concentration for the action of the *N. naja naja* and *N. naja atra* enzymes on C_8-thioPC are given in Figure 2. The experiments were conducted with a constant concentration of substrate, 13.5 mM, which is below the cmc. Nonclassical behavior is seen in that the plots were nonlinear. The highly curved plots are consistent with the idea that a lipid–protein aggregate is forming and that the enzyme is more active in this aggregate. Since the degree of aggregation depends critically on the substrate/enzyme ratio (Figure 1), a nonlinear plot for the velocity versus enzyme concentration was expected.

Table I: Inhibition of PLA2 by Phosphonate Inhibitor **8**

	IC_{50}[a] (μM)		
enzyme source	C_8-thioPC	diC_6-thioPC	diC_6-thioPMe
N. naja naja	0.5 (12) (0.2)	0.025 (1.0) (0.02)	30 (0.5) (0.12) 18 (1.9) (0.12)
N. naja atra	0.7 (12) (0.2)	0.1 (1.0) (0.03)	20 (0.5) (0.16) 12 (1.9) (0.16)
porcine pancreas	50 (12) (1.2)	40 (1.0) (0.45)	20 (1.0) (0.09)

[a] The concentrations of substrate used are given in millimolar in parentheses following the IC_{50} values. Enzyme concentrations are given in micromolar in parentheses following the substrate concentrations. All inhibition studies were performed in buffer (25 mM Tris-HCl, 0.1 M KCl, 10 mM $CaCl_2$), pH 8.5 for C_8-thioPC and pH 8.0 for diC_6-thioPC and diC_6-thioPMe.

The inhibition of PLA2 by short-chain phosphonate-containing phospholipid analogue **8** was studied. The structures of this and related analogues (**9**–**11**) are shown below.



8

9 **10** **11**

Compound **8** contains a tetrahedral phosphonate group that replaces the enzyme-susceptible ester. Since the phosphonate is a structural mimic of the tetrahedral intermediate that forms during the enzyme-catalyzed hydrolysis of substrates, compound **8** was expected to bind tightly to the active site of PLA2. The cmc of this analogue was measured to be 0.23 mM in 0.1 mM Tris-HCl, pH 8, and in the presence of 10 mM $CaCl_2$. In the absence of $CaCl_2$, no aggregation was seen with concentrations of **8** up to 1 mM. Thus, calcium promotes the aggregation of the inhibitor as expected for a compound with an anionic phosphonate group. The inhibition of the *N. naja naja* PLA2 by **8** was tested in the presence of a fixed concentration of C_8-thioPC substrate, 12 mM, which is below the cmc. The reaction velocity was reduced to zero with increasing concentrations of **8** in the range of 0–10 μM (data not shown). These inhibitor concentrations used are all well below the cmc for the inhibitor. The concentration of **8** that produced 50% inhibition (IC_{50}) was 0.5 μM (Table I). Thus, compound **8** is a very potent inhibitor of this enzyme, since the inhibition occurs with concentrations of **8** that are some (2×10^4)-fold below the concentration of substrate.

The stereoconfiguration of inhibitor **8** at carbon 2 of the glycerol backbone is the same as in the naturally occurring phospholipids (2-*R* configuration). Since PLA2 stereospecifically hydrolyzes phospholipids with the 2-*R* configuration, it was expected that the enantiomer of **8**, compound **9**, would be a much poorer inhibitor. The IC_{50} value for **9** was measured to be 25 μM, which is 50-fold higher than the value for **8**. Analysis of the enantiomeric purity of **9** was carried

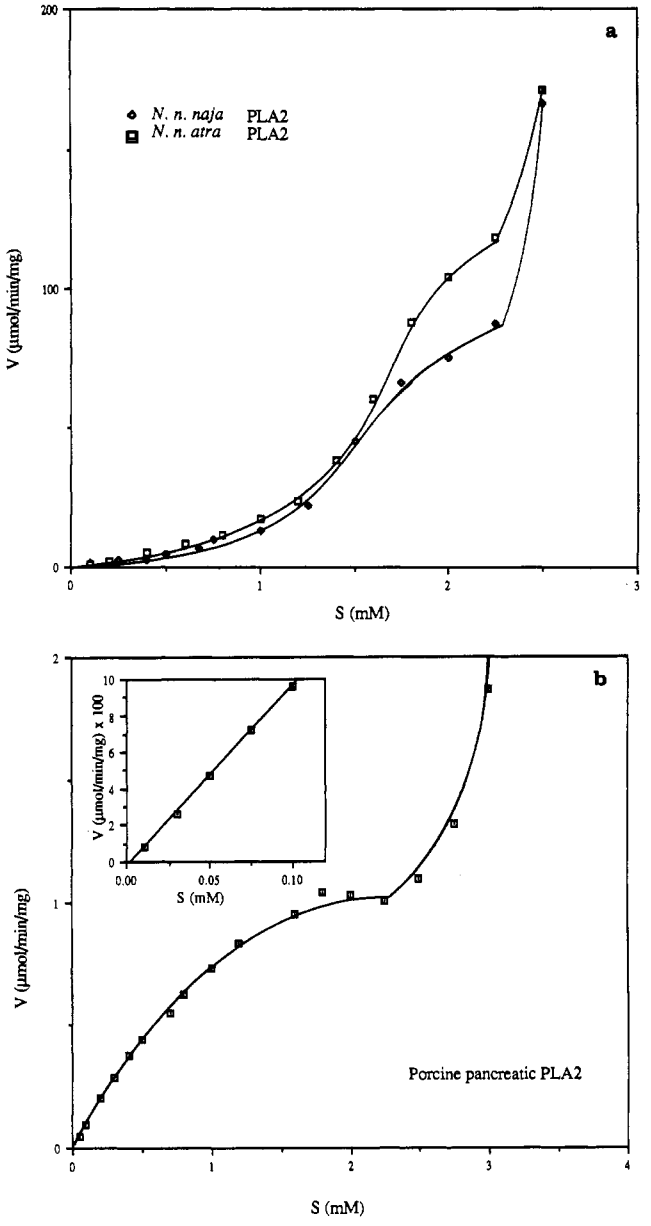


FIGURE 3: (a) Velocity versus substrate diC_6-thioPC concentration. Enzyme concentrations were 0.09 μM for the *N. naja naja* PLA2 and 0.06 μM for the *N. naja atra* enzyme. (b) Same as (a) except with the porcine pancreatic enzyme (1.2 μM). The inset shows the velocity at very low substrate concentrations. The buffers were the same as in Figure 2.

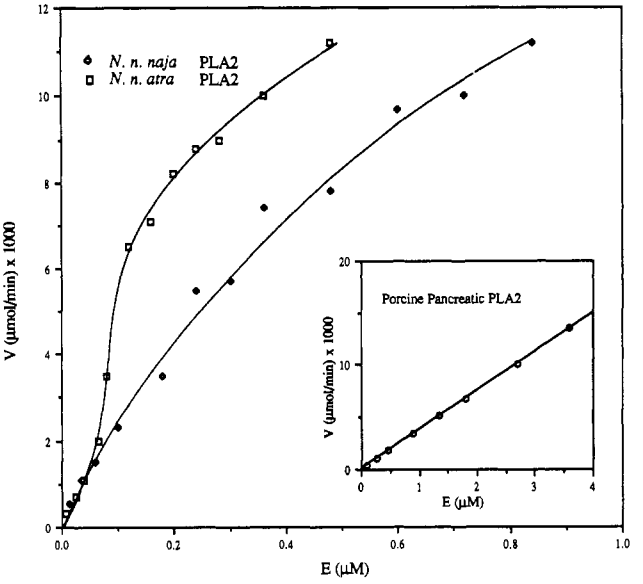


FIGURE 4: Velocity versus enzyme concentration with diC_6-thioPC (0.75 mM) as substrate. The buffer was the same as in Figure 2.

out as described under Materials and Methods, and it was found to contain approximately 0.5% of compound **8**. With such a high degree of enantiomeric purity, it is extremely difficult to get a more accurate determination of the amount of **8** present in **9**. For this reason, the enantioselectivity in the inhibition of PLA2 by **8** vs **9** may actually be greater than 50-fold. Analogues of **8** were prepared that contain sulfur in place of one of the nonbridging phosphonate oxygens (compounds **10** and **11**). Both compounds have the 2-*R* configuration at the glycerol backbone but differ in the stereochemistry of atoms about the chiral thiophosphonate group. The absolute stereochemistry at phosphorus for these compounds has not yet been determined. Compounds **10** and **11** inhibited the *N. naja naja* PLA2 acting on the C_8-thioPC substrate. The IC_{50} values were 0.5 and 8.0 μM, respectively. Thus, there is a 16-fold difference in the inhibition potencies, depending on the stereoconfiguration of the thiophosphonates. The results of these structure/activity studies are similar to the trends seen with the phosphonate and thiophosphonate inhibitors tested in vesicles (Jain et al., 1989) and in mixed micelles (Yuan et al., 1989b). The dramatic dependency of the inhibition on the stereochemical arrangement of substituents in the inhibitors strongly suggests that these compounds are binding tightly to the active site of the enzyme (Jain et al., 1989; Yuan et al., 1989b).

Kinetic and Inhibition Studies of PLA2 Acting on Two-Chain Phosphorylcholine-Containing Substrate diC_6-thioPC and diC_6-PC. The action of the PLA2s from *N. naja naja* and *N. naja atra*, as well as the enzyme from porcine pancreas, was studied on the two-chain, phosphorylcholine-containing substrate diC_6-thioPC. Figure 3 shows the initial reaction velocities as a function of substrate concentration both below and above the cmc of the substrate. Again, the kinetic patterns with the cobra venom enzymes were similar to each other and were nonhyperbolic (Figure 3a). Below the cmc, the velocity increased in an accelerating manner. At the cmc of the substrate, the velocity increased abruptly. In marked contrast, the kinetic pattern observed with the enzyme from porcine pancreas (Figure 3b) was of the hyperbolic, Michaelis–Menten type at substrate concentrations below the cmc. Again, the velocity increased abruptly at the cmc. Below the cmc, the data could be fit to the Michaelis–Menten equation with a K_m of 1.5 mM and a V_{max} of 1.8 μmol min^{-1} mg^{-1}. Note also that the specific activity of the porcine pancreatic enzyme is some 100-fold lower than the specific activity of the cobra venom enzymes acting on the same substrate. The kinetic pattern seen in Figure 3b is very similar to that reported previously (Volwerk et al., 1979). The inset to Figures 3b shows that the velocity versus substrate concentration is linear at very low substrate concentrations (0–0.04 times the cmc) for the porcine pancreatic enzyme. Such a linear behavior is consistent with normal, Michaelis–Menten-type behavior. With the cobra PLA2s, the velocity falls rapidly to zero as the substrate concentration goes far below the cmc.

Figure 4 shows the velocity for the hydrolysis of diC_6-thioPC by the cobra venom PLA2s as a function of enzyme concentration. Again, a nonlinear, non-Michaelis–Menten behavior is observed. In contrast, the velocity versus enzyme concentration plots for the porcine pancreatic enzyme (inset to Figure 4) shows a normal linear response. The non-Michaelis–Menten nature of the action of the cobra venom enzymes on

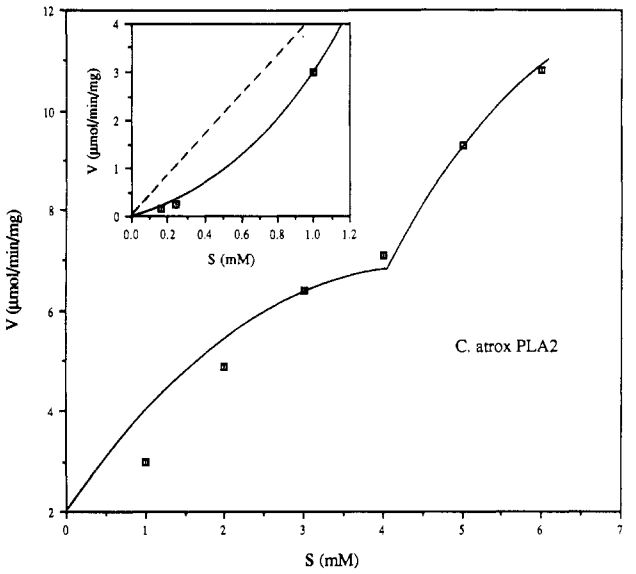


FIGURE 5: Velocity versus substrate concentration for the hydrolysis of diC_6-PC by the PLA2 from *C. atrox* venom. The cmc of this substrate is about 10 mM. The inset shows the velocity at very low substrate concentrations. The conditions used are described under Materials and Methods.

diC_6-thioPC substrate is again likely due to enzyme-induced formation of premicellar lipid–protein aggregates since aggregation is seen with the *N. naja naja* and *N. melanoleuca* enzymes acting on short-chain phosphatidylcholines (Bukowski & Teller, 1986; van Eijk et al., 1983).

The action of the PLA2 from *C. atrox* venom on the two-chain oxyester containing substrate diC_6-PC was also studied. Figure 5 shows the initial velocities as a function of the substrate concentration both below and above the cmc of the substrate. Again, a large increase in the reaction velocity is seen at the cmc of the substrate. With concentrations of diC_6-PC in the range 1–4 mM, the data deviated somewhat from a hyperbolic pattern. The best fit to the Michaelis–Menten equation gave a K_m value of 4 mM and a V_{max} value of 14 μmol min^{-1} mg^{-1}. However, as shown in the inset to Figure 5, the initial velocities measured at low substrate concentrations were much lower (3–4-fold) than the values calculated from the Michaelis–Menten equation (dotted line in Figure 5) using the kinetic constants determined at the higher substrate concentrations. The concentrations of diC_6-PC in the assay solution were verified by phosphate analysis, which indicated that the substrate was not absorbed onto the glass walls of the cuvette. In this case, the deviation of the data from the normal Michaelis–Menten-type behavior is less obvious than in the examples shown in Figures 1 and 3a.

The inhibition of the cobra venom and porcine pancreatic enzymes acting on diC_6-thioPC by inhibitor **8** was studied, and the results are summarized in Table I. The substrate concentrations were well below the cmc and kept at a constant value of 1.0 mM. Compound **8** was again a potent inhibitor of the cobra venom enzymes. Note, however, that the IC_{50} values are significantly smaller than those measured in the presence of the single-chain C_8-thioPC substrate. This result would not be expected for a soluble inhibitor and a soluble substrate competing with each other for the binding to the enzyme. A possible explanation for this nonclassical behavior is given below. In the case of the *N. naja naja* enzyme, the IC_{50} value of 25 nM is (2×10^4)-fold below the substrate concentration of 0.5 mM (1 mM total racemic diC_6-thioPC). Compound **8** was, by comparison, a rather poor inhibitor of the porcine pancreatic enzyme (Table I). The discussion of this point is also given below.

The Presence of Substrate Is Required for the Inhibition by **8**. From the above results, it is clear that, in many cases, the action of PLA2 on short-chain substrates below the cmc is not a simple reaction between a monomeric enzyme and a monomeric substrate. Rather, the enzyme and substrate form a lipid–protein aggregate; however, the precise structure of this aggregate remains to be characterized. Even though, in kinetic studies, the inhibition by **8** is seen with concentrations of inhibitor that are orders of magnitude below the cmc of the inhibitor, the inhibitor will be bound to the enzyme within this lipid–protein aggregate. It was therefore of interest to determine whether the inhibitor binds tightly to the enzyme in the absence of substrate, that is, under conditions in which this lipid–protein aggregate does not form. Two approaches were taken to assess the direct binding of **8** to PLA2. In the first approach, the inactivation of PLA2 by *p*-bromophenacyl bromide was studied in the presence and absence of **8**. This reagent is known to alkylate the catalytically important histidine residue in the active site of PLA2 (Roberts et al., 1977; Volwerk et al., 1974). Binding of the inhibitor to the active site of the enzyme should protect the enzyme from inactivation by the bromomethyl ketone. The *N. naja naja* enzyme was incubated with *p*-bromophenacyl bromide, and aliquots of the reaction mixture were periodically withdrawn and diluted into an assay cocktail containing substrate present in a detergent micelle (see Materials and Methods). This assay is very sensitive, and the reaction velocity was shown to depend in a linear fashion on the concentration of enzyme (in contrast to the results with the short-chain substrates), and thus the initial velocities accurately measure the amount of remaining active enzyme. Under all conditions, the loss of enzyme activity with time displayed pseudo-first-order kinetics (data not shown). In the absence of inhibitor **8**, the rate of inactivation in the absence of calcium, k_{inact} = 610 M^{-1} s^{-1}, was much larger than in the presence of calcium, k_{inact} = 7.8 M^{-1} s^{-1}. This protection by calcium has been previously documented (Roberts et al., 1977; Volwerk et al., 1974). The recent structure elucidation of the porcine pancreatic PLA2 inactivated by *p*-bromophenacyl bromide suggests that the bromine atom attached to the aromatic ring of the inactivator would sterically clash with the enzyme-bound hydrated calcium ion (Renetseder et al., 1988). In the absence of calcium, the rate of inactivation was unchanged by the addition of 9.5 μM **8**, k_{inact} = 580 M^{-1} s^{-1}. In the presence of calcium, addition of **8** (9.5 μM) caused the rate of inactivation to decrease below that measured with calcium alone, k_{inact} = 5.4 M^{-1} s^{-1}. However, the protection afforded by the inhibitor was not complete, even when the inhibitor concentration was 9.5 μM, which is more than a sufficient amount to completely inhibit the action of this enzyme on the C_8-thioPC and diC_6-thioPC substrates. This result suggested that, although the inhibitor was binding to the active site of the enzyme, the binding was much weaker than that seen in the kinetic experiments described above. This was the first clue that the tight binding of **8** to the enzyme occurred only in the presence of substrate.

In a second approach, use was made of radiolabeled **8** so that the interaction of the enzyme with the inhibitor in the absence of substrate could be studied by equilibrium dialysis. Studies with the *N. naja naja* PLA2 were conducted with enzyme concentrations of 0.5, 1.0, and 5.0 μM and concentrations of **8** up to 10 μM. No measurable accumulation of the inhibitor on the enzyme side of the dialysis chamber was seen. This suggests that the dissociation constant for the

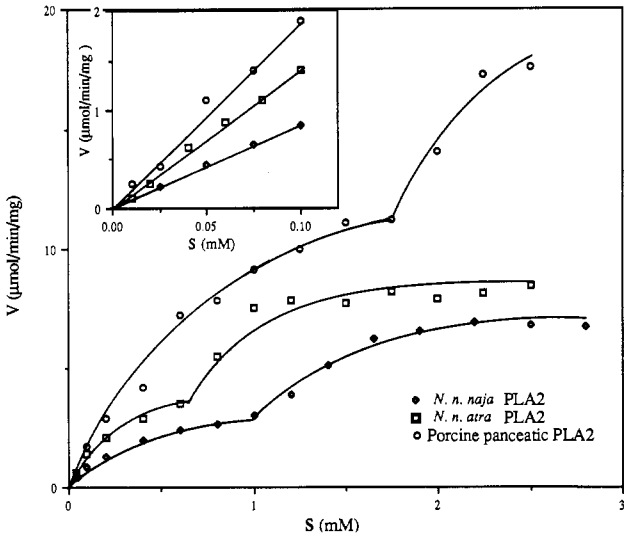


FIGURE 6: Velocity versus substrate diC_6-thioPMe concentration. Enzyme concentrations were 0.1 μM for the *N. naja naja* PLA2 and 0.07 μM for both the *N. naja atra* and porcine pancreatic enzymes. The buffer was the same as in Figure 2. The inset shows the results obtained with very low substrate concentrations.

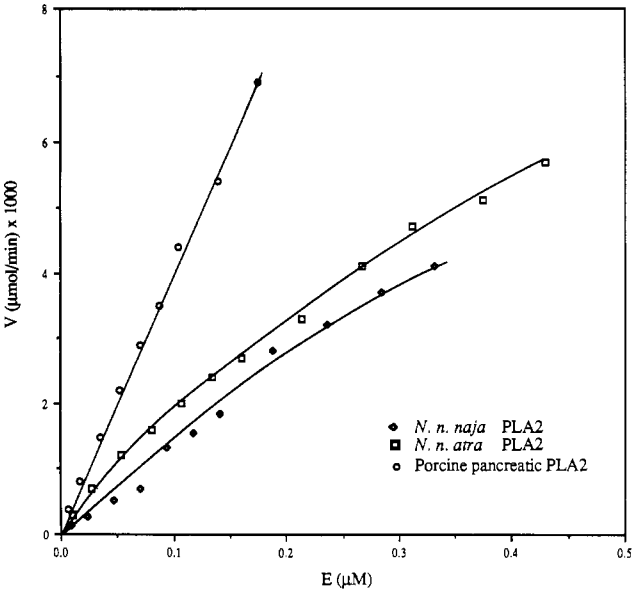


FIGURE 7: Velocity versus enzyme concentration with substrate diC_6-thioPMe. The substrate concentrations were 0.75 mM for both the *N. naja naja* and the porcine pancreatic PLA2s and 0.53 mM for the *N. naja atra* enzyme. The buffer was the same as in Figure 2.

interaction of **8** with PLA2 was greater than approximately 50 μM (for a dissociation constant of 50 μM, an 8% difference in radioactivity on both sides of the membrane would have been seen with the highest concentrations of enzyme and inhibitor used). Similar results were obtained with the PLA2 from *N. naja atra* venom. These results demonstrate that the affinity of PLA2 for inhibitor **8** is weak in the absence of substrate. Equilibrium dialysis experiments were also carried out as described above but also in the presence of 1,2-dihexyl-*sn*-glycero-3-phosphorylcholine. This diether phospholipid was used as a substrate analogue since the thioester substrates would suffer enzymatic hydrolysis during the dialysis experiment. Since dodecylphosphorylcholine has been shown to form a microaggregate with the *N. naja naja* PLA2 (Plückthun & Dennis, 1985), the same type of aggregation was expected in the presence of the dihexyl phospholipid analogue. The concentration of the substrate analogue was 1–2 mM, which is well below the cmc of 6 mM for this compound. In the presence of the analogue, a dramatic improvement in the binding of **8** to PLA2 was observed. The data fit nicely to the Scatchard equation (Segel, 1975) (data not shown). For the *N. naja naja* enzyme, a dissociation constant of 0.8 μM and the number of bound inhibitors per enzyme of 1.2 were obtained. This suggests that a single inhibitor molecule is binding to the enzyme. A similar Scatchard plot was seen with the enzyme from *N. naja atra* venom (not shown). In this case, a dissociation constant of 3.9 μM and a binding stoichiometry of 1.2 were obtained from the plot.

Kinetic and Inhibition Studies with diC_6-thioPMe Substrate. DiC_6-thioPMe contains an anionic head group and was tested as a substrate for PLA2. Figure 6 shows the velocity versus substrate concentration curves for the hydrolysis of diC_6-thioPMe by the *N. naja naja*, *N. naja atra*, and porcine pancreatic enzymes. With the cobra venom PLA2s, a normal hyperbolic pattern is seen with substrate concentrations up to approximately 0.7–1.0 mM. Above 0.7–1.0 mM, the velocity climbs rapidly with increasing substrate concentration. This break in the curve occurs significantly below the cloud point of 2.5 mM for this substrate. The inset to Figure 6 shows that the velocity increases in a linear behavior with increasing concentrations of diC_6-thioPMe far below the cmc of the substrate. This normal behavior is in marked contrast to the unusual kinetic patterns seen with these two enzymes and substrates C_8-thioPC and diC_6-thioPC. The kinetic pattern for the porcine pancreatic PLA2 acting on this anionic substrate is also shown in Figure 6. Again, a normal hyperbolic pattern is seen with substrate concentrations below the cmc. In this case, the jump in the velocity is seen at a substrate concentration of 1.8 mM, which is very close to the cloud point of the substrate. This kinetic pattern for the porcine pancreatic PLA2 is significantly different from the nonhyperbolic behavior of this enzyme acting on sulfate-containing substrates and on dihexanoylphosphatidylmethanol (van Oort et al., 1985a,b; Jain et al., 1986b). Apparently, with the cobra venom enzymes, an enzyme-induced aggregation of the substrate occurs below the cloud point, but this aggregation is much less pronounced when the substrate is diluted well below its cloud point.

Figure 7 shows the velocity versus enzyme concentration curves given by a constant concentration of diC_6-thioPMe, 0.75 mM, which is below the cloud point. For the porcine pancreatic enzyme, a linear response is seen. For the cobra venom enzymes, the response deviates slightly from linearity; however, the curvature in the pattern is much less dramatic than the velocity versus enzyme concentration patterns seen with C_8-thioPC and diC_6-thioPC substrates (Figures 2 and 4). With diC_6-thioPMe, the specific activities for all three enzymes are similar. This is in marked contrast to the results seen with diC_6-thioPC, where the cobra venom enzymes were 100-fold more active than the porcine pancreatic PLA2.

The inhibition of all three enzymes by phosphonate **8** was studied in the presence of concentrations of diC_6-thioPMe below the cloud point. The results are given in Table I. Interestingly, **8** was a much poorer inhibitor of the cobra venom enzymes in the presence of diC_6-thioPMe than in the presence of either C_8-thioPC or diC_6-thioPC. For the *N. naja naja* enzyme, the IC_{50} was 30 μM when the concentration of diC_6-thioPMe, 0.5 mM, was below the break point in the velocity seen in Figure 6. With a substrate concentration of 1.9 mM, which is above the break point, the IC_{50} dropped to 18 μM. Again, these results are incompatible with simple

competitive inhibition, where the IC_{50} value would be expected to increase as the concentration of substrate is increased. Similar results were seen with the enzyme from *N. naja atra* venom (Table I). Compound **8** was also a weak inhibitor of the porcine pancreatic PLA2 acting on diC_6-thioPMe (Table I).

Discussion

Kinetics of Hydrolysis of Short-Chain Substrates. The hydrolysis of phosphatidylcholine substrates C_8-thioPC (Figure 1) and diC_6-thioPC (Figure 3a) by the cobra venom enzymes at concentrations of substrate below the cmc displayed complicated kinetic patterns that deviated significantly from normal hyperbolic, Michaelis–Menten-type behavior. The dependencies of the reaction velocities on the enzyme concentration also displayed a nonclassical trend (Figures 2 and 4). Nonclassical kinetic behavior has been previously reported for the action of PLA2 on short-chain substrates (Bukowski & Teller, 1986; Jain et al., 1986b; van Oort et al., 1985a,b; van Eijk et al., 1983). In all of these cases, it is clear that the enzyme causes the lipid to aggregate at concentrations below the cmc of the pure lipid. It is possible that the enzyme is simply nucleating the formation of substrate micelles at substrate concentrations below the cmc. However, from the kinetic results seen in Figures 1 and 3, it is clear that a large increase in the reaction velocity at the cmc of the pure substrates still occurs. Thus, there must be at least two types of substrate aggregation; microaggregation below the cmc and normal micelle formation at the cmc. Although very little is known about the structure of the microaggregates that form below the cmc, they probably contain a higher protein/lipid ratio than the aggregates that form at the cmc. In the region where microaggregates are forming, the degree of aggregation is critically dependent on the protein/lipid ratio. In the case of the substrate C_8-thioPC (Figure 1), microaggregation seems to reach a maximum below the cmc and then to decrease before the cmc of the pure substrate is reached. This is similar to the previously reported behavior of the porcine pancreatic PLA2 acting on sulfate-containing substrates (van Oort et al., 1985a,b). With the substrate diC_6-thioPC, the region of maximal microaggregation is apparently much closer to the cmc so that no pre-cmc velocity maximum is observed (Figure 3a). A similar pattern is seen for the action of the PLA2 from *C. atrox* acting on diC_6-PC (Figure 5), except that the anomalous kinetic behavior is much more subtle. For this enzyme, a previous study suggested that the behavior on diC_6-PC monomers below the cmc obeyed the Michaelis–Menten equation (Achari et al., 1987); however, the velocity pattern at very low substrate concentrations was not studied. The kinetic patterns seen in Figures 3a and 5 are similar to those reported for the porcine pancreatic PLA2 acting on the sulfate-containing substrates at high pH [Figure 4 of van Oort et al. (1985a)] and to the pattern seen with the PLA2 from *N. melanoleuca* acting on diheptanoylphosphatidylcholine (van Eijk et al., 1983).

If the enzyme and lipid form a microaggregate below the cmc, why does the velocity still increase significantly at the cmc of the substrate (Figures 1 and 3a)? One possible explanation relies on the fact that the lipid molecules within the aggregate are in a state of dynamical exchange with other lipid molecules present in other aggregates. Consider a microaggregate containing two or three enzymes and perhaps 50 lipid molecules. After a short period of time, most of the substrates in the microaggregate will become hydrolyzed. More substrate can be hydrolyzed only after the lipid products have left the microaggregate and new lipid substrates have been taken up. This can occur by exchange of lipids between microaggregates. If the rate of the lipid exchange is comparable to the rate of the enzymatic lipolysis, the overall enzymatic reaction velocity may be limited by the dynamics of lipid exchange. The rates of intermicellar exchange are of the order of 1000 s^{-1}, which is close to the catalytic rate constant for PLA2, and thus it cannot be safely assumed that the rate of lipid exchange within the microaggregates is very fast compared to the catalytic turnover (Jain & Berg, 1989). At the cmc of the substrate, much larger micelle aggregates form which contain, on the average, only a single enzyme. Now, many more catalytic cycles can occur without the need for exchange of substrates and products in and out of micelles. This may be the reason that the enzymatic velocity continues to increase at the cmc of the substrate. With the *N. melanoleuca* PLA2 acting on diheptanoylphosphatidylcholine, no additional increase in reaction velocity was seen at the cmc of the substrate (van Eijk et al., 1983). In this case, the microaggregates that form below the cmc may have a much larger lipid/protein ratio so that the exchange of lipids is never rate limiting. Alternatively, the dynamics of lipid exchange in the microaggregate in this case may be intrinsically higher than for the microaggregates formed in the present study. Even if larger substrate micelles form at the cmc, this would not result in any further rate acceleration. This proposal is consistent with the 10-fold higher specific activities observed with the *N. melanoleuca* PLA2 (van Eijk et al., 1983) compared to the results in the present study (Figure 3a).

In marked contrast to the kinetic patterns seen with the cobra venom PLA2s acting on the zwitterionic substrates, the action of the porcine pancreatic PLA2 on diC_6-thioPC displayed normal, hyperbolic kinetics (Figure 3b; Volwerk et al., 1979). In addition, the velocity versus enzyme plot was linear (Figure 4). Finally, the specific activity of the porcine pancreatic PLA2 was about 100-fold lower than the specific activities of the cobra venom enzymes acting on the same substrate. All of these results suggest that the porcine pancreatic PLA2 hydrolyzes diC_6-thioPC in a classical Michaelis–Menten fashion in which monomeric substrate binds to the enzyme to form a enzyme–substrate complex which goes on to products. However, it should be pointed out that the hyperbolic kinetic pattern shown in Figure 3b, although consistent with a simple Michaelis–Menten kinetic model, does not constitute proof for such a model. Hyperbolic kinetics of the type shown in Figure 3b will be observed for any kinetic model that includes a change in the rate-limiting step for the enzymatic reaction with increasing substrate concentration. As explained below, it is possible that the porcine pancreatic PLA2 is forming a microaggregate with the diC_6-thioPC substrate below the cmc. Above the cmc, the velocity increases abrubtly and eventually reaches a maximal value with increasing substrate concentrations (de Haas et al., 1971).

For the ideas that follow, it is useful to consider the process of micelle formation in more detail since it may lead to a clue about the nature of the interaction of PLA2 with the substrate below the cmc. As the concentration of lipid is increased above the cmc, the concentration of micelles increases but the concentration of monomers in equilibrium with the micelles remains roughly constant. Thus, micelle formation is better viewed as a form of phase separation and is best described by a collection of equilibria between particles of different aggregation number, eqs 1, rather than as a single-step aggregation involving only monomers and micelles, eq 2 (Jain, 1988). When the number of equilibria of the type given in

$$L + L \rightleftharpoons L_2 \rightleftharpoons L_3 \rightleftharpoons L_4 \ldots \quad (1)$$

$$nL \rightleftharpoons L_n \quad (2)$$

eqs 1 becomes large, as is the case for micelles with aggregation numbers greater than 50, a process of cooperative phase separation occurs as stated by the Gibbs phase rule. It is important to point out that, at lipid concentrations between zero and just greater than the cmc, numerous aggregates containing different numbers of lipid molecules will be present in the solution. In the presence of PLA2, the formation of microaggregates may proceed along a similar path as described by eqs 1. At low substrate concentrations below the cmc, much of the enzyme will be present either uncomplexed to lipid or present in a protein–lipid microaggregate, $E(L_n)$, where n is a small number. The enzymatic velocity will be small since much of the enzyme will not be bound to lipid, and the activity of the enzyme within the small microaggregates may be low because of slow exchange of lipids in and out of the particle. As the concentration of substrate is increased, the amount of free enzyme decreases with a concomitant increase in the reaction velocity. The reaction begins to saturate below the cmc of the substrate as the concentration of free enzyme falls to zero. At this point, a further increase in the concentration of substrate below the cmc does not produce a further increase in the reaction velocity. This picture is very different from the classical Michaelis–Menten formalism where saturation results when all of the enzyme contains substrate monomer bound to the catalytic site during the steady-state turnover. In this microaggregation model, saturation kinetics will be observed when all of the enzyme is bound in a protein–lipid microaggregate, regardless of what fraction of enzyme catalytic sites is occupied by substrate. It is still possible that the enzymatic reaction is still limited by exchange of substrates and products in and out of the microaggregate. Thus, the enzymatic velocity may still not be at the maximal value. Near the substrate cmc, much larger micelles start to form and the bound enzyme can now undergo many more catalytic cycles without the need for substrate replenishment by exchange processes. This mode of hydrolysis is much faster than that which occurs in small enzyme–lipid microaggregates. As the substrate concentration is increased above the cmc, the number of micelles continues to increase. The rate of lipid exchange between micelles will continue to increase since, at high micelle concentrations, the exchange is promoted by collision and fusion of micelles with other micelles. Eventually, the rate-limiting step will change from a lipid-exchange process to a step that is part of the enzymatic catalytic cycle. Once this occurs, the reaction velocity will again stop increasing with increasing substrate concentration. However, even at this point, it is still possible that the catalytic site of the enzyme is not saturated with monomeric substrate in the steady-state turnover. This final V_{max} value represents the point at which all of the enzyme is bound to the lipid interface and the enzymatic velocity is no longer limited by the replenishment of substrate.

Additional studies will be required to determine whether the velocity versus substrate concentration curves shown in Figure 3b and in a previous study (Volwerk et al., 1979) are the result of a truly monomeric interaction between the porcine pancreatic PLA2 and solitary monomeric substrate or whether the interaction always occurs in an aggregate. In this context, some previously reported results are worth noting. The enzymatic velocities measured on micelles of a series of short-chain phosphatidylcholines were found to increase with increasing fatty acid chain length (de Haas et al., 1971). It is known that the size of these micelles increases with increasing fatty acid chain length (Tausk et al., 1974a,b). For any given concentration of micelles, the enzymatic velocity will be larger with the larger micelles where exchange of lipids is less rate limiting. In a second example, the ratio of the apparent K_m value for the hyperbolic kinetics below the cmc to the cmc remains fairly constant for a series of phospholipid substrates (Volwerk et al., 1979; Verheij et al., 1981). If the enzymatic reaction below the cmc of the substrate is truly monomeric in nature, one would not expect such a strong correlation between the cmc and the apparent K_m. For example, it should be possible to find a substrate that displays hyperbolic kinetics with a K_m value well below the cmc of the substrate, and yet such a substrate has not yet been reported. On the other hand, if the enzymatic reaction is necessarily linked to microaggregation of the substrate, one would always expect the velocity of the enzymatic reaction below the cmc to increase as the substrate concentration is increased near its cmc, since only in this region of substrate concentrations will the population of enzyme–lipid aggregates start to form. Evidence against the microaggregation model for the action of the porcine pancreatic PLA2 comes from binding studies in which it was shown by equilibrium dialysis that a single molecule of decylphosphocholine binds to the enzyme (Volwerk et al., 1979). However, in the same study, a nonclassical kinetic pattern was reported for the action of the porcine pancreatic PLA2 on (*n*-decanoylthio)glycollecithin at substrate concentrations below the cmc. This suggests that microaggregates are capable of forming with the porcine pancreatic PLA2 acting on phosphocholine-containing substrates. Whatever the nature of the reaction of PLA2s with short-chain substrates below the cmc, it is clear that the degree of microaggregation that occurs in the case of the porcine pancreatic enzyme operating on phosphatidylcholine substrates (Figure 3a) is much less pronounced than for the action of the venom enzymes on the same substrates (Figure 1 and 3a). In addition, the degree of microaggregation of the cobra venom enzymes is much less pronounced in the presence of the anionic substrate, diC_6-thioPMe (Figure 6). For the moment, it should be stated that the nature of the kinetics for the action of PLA2s on short-chain substrates at concentrations below the cmc is highly variable and will depend on the source of the enzyme, the chemical structure of the substrate, and the details of the assay condition employed. *On the basis of the available evidence, in no case should it be assumed that PLA2 hydrolyzes short-chain phospholipids via the formation of a classical Michaelis complex involving a single enzyme and a single substrate molecule.*

Inhibition Studies with Short-Chain Phospholipid Analogues. Several points must be addressed in a discussion of the inhibition of PLA2 by the short-chain phospholipid analogues prepared in this study. Phosphonate **8**, in the presence of certain substrates, is an extremely potent inhibitor of the enzymes from cobra venom; however, the compound is a weak inhibitor of the porcine pancreatic PLA2. With the cobra venom enzymes, the IC_{50} values for the inhibitor were found to be critically dependent on the presence of substrate and on the structure of the substrate. For example, in the case of the *N. naja naja* PLA2, the IC_{50} value for **8**, when tested in the presence of the two-chain substrate diC_6-thioPC, is small (20 nM) but increases to 0.5 μM in the presence of single-chain substrate C_8-thioPC and increases even further to 20 μM in the presence of anionic substrate diC_6-thioPMe. In the presence of the substrate analogue 1,2-dihexyl-*sn*-glycero-3-phosphorylcholine, the dissociation constant for **8** was measured to be 0.8 μM by equilibrium dialysis. The observation

that the binding of the inhibitor can be strongly promoted by a diether phospholipid analogue rules out the possibility that acylation of the enzyme by the substrate (Cho et al., 1988) is responsible for the enhanced inhibitor binding. Similar trends were seen with the PLA2 from *N. naja atra* venom. In the absence of substrate, the dissociation constant for the interaction of **8** with the cobra venom enzymes was at least 50 μM. Such a large variation in the potency of inhibition with the nature of the substrate is inconsistent with simple competitive inhibition in which a water-soluble inhibitor and a water-soluble substrate compete for the binding to the active site of PLA2.

There is a striking correlation between the potency of inhibition by **8** and the nature of the pre-cmc substrate aggregates. In the case of the cobra venom enzymes, where large lipid–protein, pre–cmc aggregation occurs, potent inhibition is seen. In marked contrast, relatively poor inhibition by **8** is seen with low concentrations of the anionic substrate diC_6-thioPMe. With this substrate, massive microaggregation does not occur. The variation in the IC_{50} value of inhibitor **8** by 3 orders of magnitude for the same enzyme but with two different substrates is dramatic. It is also interesting that the inhibition of the porcine pancreatic enzyme by **8** is always weak and that the kinetic pattern with this enzyme shows no evidence for the formation of large lipid–protein microaggregates. There is nothing unusual above the active site of the porcine pancreatic enzyme which precludes the binding of **8** since this compound was found to exhibit similar inhibition potencies against both the cobra venom and porcine pancreatic enzymes when tested in vesicles (Jain et al., 1989). *Thus, one is forced to conclude that the potent inhibition of PLA2 by phosphonate* **8** *is somehow dependent on the enzyme being bound to a large phospholipid aggregate, either to a vesicle (Jain et al., 1989) or in a lipid–protein microaggregate of the type seen in the present study.* This proposal is also substantiated by the studies in which the direct binding of **8** to the enzyme was measured by equilibrium dialysis. In these experiments, a tight, stoichiometric enzyme–inhibitor interaction was seen only in the presence of a short-chain phospholipid analogue, which presumably formed a lipid–protein aggregate. In the absence of the phospholipid analogue, little binding could be detected by equilibrium dialysis or by protection studies using *p*-bromophenacyl bromide.

Several lines of evidence suggest that inhibitor **8** is binding in a specific manner to the active site of the cobra venom enzymes. It has already been mentioned that the Scatchard plots indicated that the inhibitor forms a 1:1 complex with the enzyme. In addition, the degree of inhibition was found to depend dramatically on the stereochemical arrangement of substituents on the glycerol backbone. In the case of the thiophosphonates, the IC_{50} values of the inhibitors varied with the chirality of the thiophosphonate group. The fact the some protection from alkylation of the active-site histidine was seen in the presence of **8** also suggested that the inhibitor is binding to the active site of the enzyme and that this binding required calcium ion.

To fully understand the inhibition phenomenon reported in the present study, further work will be required. A working hypothesis is schematically presented in Figure 8. Apparently, the direct binding of inhibitor **8** to the enzyme in the absence of substrate is not nearly as favorable as the binding of the inhibitor to the enzyme within the enzyme–substrate aggregate. It might be imagined that, in both cases, portions of the inhibitor will form favorable interactions with groups in the active site of the enzyme. Possible contacts would include the

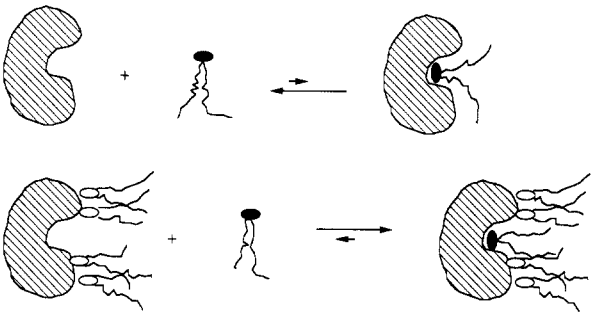


FIGURE 8: Schematic depiction of the binding of inhibitor **8** to PLA2 in the absence and in the presence of a cluster of substrate phospholipids bound to the interfacial binding surface on the enzyme.

binding of one of the phosphonate oxygens to the calcium ion and the other phosphonate oxygen to the histidine residue (Volwerk & de Haas, 1981). In addition, the phosphate group may be also bound to the calcium (Tsai et al., 1985). However, as shown in Figure 8, the presence of a cluster of substrate molecules bound to the interfacial binding surface of the enzyme might allow for substantial hydrophobic interactions between the hydrocarbon chains of the inhibitor and the hydrocarbon chains of the blanketing substrate molecules. Such interactions cannot occur in the presence of enzyme and inhibitor alone (Figure 8). According to this model, the large fluctuations seen in the IC_{50} values for **8** as a function of the structure of the substrate used in the assay are due to the differences in the energetics of the interactions between the substrate and the inhibitor within the aggregate (Figure 8). Although it is possible that, in the absence of substrate, the inhibitor can provide its own lipid cluster on the surface of the enzyme, such a nucleation phenomenon would not be expected to occur at the low inhibitor concentrations used in the present study.

A second possibility for the selective binding of the inhibitor in the presence of the substrate interface is worth noting. Jain and co-workers have provided evidence indicating that the microinterface region of the substrate aggregate that is in direct contact with the interfacial binding surface of the enzyme is not accessible to bulk solvent (Jain & Vaz, 1987). This desolvation of the phospholipid head groups in the microinterface may facilitate the dislodging and binding of the substrate to the catalytic site on the enzyme (Jain & Berg, 1989). The proenzyme form of the porcine pancreatic PLA2 is able to bind to anionic phospholipid vesicles; however, this binding is not accompanied by the desolvation of the interface (Jain & Vaz, 1981). It is likely that a phospholipid molecule must first be desolvated before it can bind to the catalytic site on the proenzyme. If desolvation is slow relative to other steps in the catalytic cycle, this step could limit the rate of lipolysis and may be the reason that the proenzyme exhibits small turnover numbers. In this context, it is possible that the solvated form of the phosphonate inhibitor must become desolvated before binding to the catalytic site on the enzyme. By analogy with the observations with vesicles (Jain & Vaz, 1987), it is likely that the microaggregates described in this and previous studies are also desolvated at the site of contact between enzyme and lipid. If this desolvation occurs, it would be expected to promote the desolvation of the inhibitor that is present in the aggregate. This would facilitate the binding of the phosphonate to the catalytic site on the enzyme and account for the requirement of an interface for tight inhibitor binding. In this model, the high turnover numbers seen in the enzyme–lipid aggregates (interfacial activation) described in this and other studies and the tight binding of the phosphonate

inhibitor to the catalytic site on the enzyme are facilitated by the same process, the desolvation of the interface in the vicinity of the enzyme. The validity of this interpretation depends critically on the nature of the interaction of the enzyme with the substrate below the cmc. If the hyperbolic kinetics seen in Figure 3a represents the formation of a classical Michaelis complex between a single enzyme and a single substrate, then the low turnover number for this soluble enzyme–substrate complex as the saturation point (V_{max}) below the cmc must be an intrinsic property of this complex and the large increase in velocity at the cmc (interfacial activation) must involve the transformation of the enzyme into a more efficient catalysis, for example, by a conformational change as the protein binds to the micelle interface. On the other hand, in the microaggregation model detailed above, the catalytic site on the enzyme does not become saturated with monomeric substrate below the cmc and the interfacial activation seen at the cmc is the result of a change in the rate-limiting step of the enzymatic turnover. In this case, the intrinsic catalytic efficiency of the enzyme has not changed. For example, in the desolvation model of Jain, the aggregation of the lipid substrate on the surface of the enzyme increases the rate of the disloding of the substrate from the interface into the catalytic site on the enzyme. The same process may also promote the binding of the inhibitor **8** to the enzyme.

Although an attempt was made in the present study to examine the inhibition of PLA2 by using water-soluble inhibitors in the presence of water-soluble substrates, it is clear that a simple picture does not emerge. It is not yet known whether the unusual aspects of the inhibition seen in the present study will be generally observed with all lipolytic enzymes. The present results do establish the need to carry out a complete analysis of the inhibition of PLA2 by soluble compounds, using both kinetic and direct binding methods, to fully appreciate the nature of the inhibition in the context of the subtleties of interfacial catalysis.

Acknowledgments

We are grateful to Professor Mahendra Jain for his insightful discussions and to Lurilla Lee for technical assistance. Mass spectra were obtained at the UCSF resource (A. L. Burlingame, Director, NIH RR01614).

References

Aarsman, A. J., Roosenboom, C. F. P., van der Marel, G. A., Shadid, B., van Boom, J. H., & van den Bosch, H. (1985) *Chem. Phys. Lipids 36*, 229.

Achari, A., Scott, D., Barlow, P., Vidal, J. C., Otwinowski, Z., Brunie, S., & Sigler, P. B. (1987) *Cold Spring Harbor Symp. Quant. Biol. 52*, 441.

Allgyer, T. T., & Wells, M. A. (1979) *Biochemistry 18*, 5348.

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

Cho, W., Tomasselli, A. G., Heinrikson, R. L., & Kezdy, F. J. (1988) *J. Biol. Chem. 263*, 11237.

Darke, P. L., Jarvis, A. A., Deems, R. A., & Dennis, E. A. (1980) *Biochim. Biophys. Acta 626*, 154.

Davidson, F. F., Hajdu, J., & Dennis, E. A. (1986) *Biochem. Biophys. Res. Commun. 137*, 587.

Davidson, F. M., & Long, C. (1958) *Biochem. J. 69*, 458.

de Haas, G. H., Postema, N. M., Nieuwenhuizen, W., & van Deenen, L. L. M. (1968) *Biochim. Biophys. Acta 159*, 118.

de Haas, G. H., Bonsen, P. P. M., Pieterson, W. A., & van Deenen, L. L. M. (1971) *Biochim. Biophys. Acta 239*, 252.

de Haas, G. H., van Oort, M. G., Dijkman, R., & Verger, R. (1989) *Biochem. Soc. Trans. 17*, 274.

Dennis, E. A. (1983) *Enzymes (3rd Ed.) 16*, Chapter 9.

Dennis, E. A. (1987) *Drug Dev. Res. 10*, 205.

Hazlett, T. L., & Dennis, E. A. (1985) *Toxicon 23*, 457.

Hendrickson, H. S., & Dennis, E. A. (1984) *J. Biol. Chem. 259*, 5734.

Hille, J. D. R., Egmond, M. R., Dijkman, R., van Oort, M. G., Sauve, P., & de Haas, G. H. (1983) *Biochemistry 22*, 5353.

Irvine, R. F. (1982) *Biochem. J. 204*, 3.

Jain, M. K. (1988) *Introduction to Biological Membranes*, Wiley, New York.

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

Jain, M. K., & Jahagirdar, D. V. (1985) *Biochim. Biophys. Acta 814*, 319.

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

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

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

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

Keith, C., Feldman, D. S., Deganello, S., Glick, J., Ward, K. B., Jones, E. O., & Sigler, P. B. (1981) *J. Biol. Chem. 256*, 8602.

Perkins, S. J. (1986) *Eur. J. Biochem.*, 170.

Plükthun, A., & Dennis, E. A. (1985) *J. Biol. Chem. 260*, 11099.

Renetseder, R., Dijkstra, B. W., Huizinga, K., Kalk, K. H., & Drenth, J. (1988) *J. Mol. Biol. 200*, 181.

Roberts, M. F., Deems, R. A., Mincey, T. C., & Dennis, E. A. (1977) *J. Biol. Chem. 252*, 2405.

Segel, I. H. (1975) *Enzyme Kinetics*, Wiley, New York.

Tausk, R. J. M., Karmiggelt, J., Voordouw, G., & Overbeek, J. Th. G. (1974a) *Biophys. Chem. 1*, 175.

Tausk, R. J. M., Van Esch, J., Karmiggelt, J., Voordouw, G., & Overbeek, J. Th. G. (1974b) *Biophys. Chem. 2*, 53.

Thuong, N. T., & Chabrier, P. (1974) *Bull. Soc. Chim. Fr.*, 667.

Tsai, T.-C., Hart, J., Jiang, R.-T., Bruzik, K., & Tsai, M.-D. (1985) *Biochemistry 24*, 3180.

van Eijk, J. H., Verheij, H. M., Dijkman, R., & de Haas, G. H. (1983) *Eur. J. Biochem. 132*, 183.

van Oort, M. G., Dijkman, R., Hille, J. D. R., & de Haas, G. H. (1985a) *Biochemistry 24*, 7987.

van Oort, M. G., Dijkman, R., Hille, J. D. R., & de Haas, G. H. (1985b) *Biochemistry 24*, 7993.

Verger, R., & de Haas, G. H. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 77.

Verheij, H. M., Slotboom, A. J., & de Haas, G. H. (1981) *Rev. Physiol. Biochem. Pharm. 91*, 91.

Volwerk, J. J., & de Haas, G. H. (1981) in *Lipid-Protein Interactions*, Vol. 1, p 69, Wiley, New York.

Volwerk, J. J., Pieterson, W. A., & de Haas, G. H. (1974) *Biochemistry 13*, 1446.

Volwerk, J. J., Dedieu, A. G. R., Verheij, H. M., Dijkman, R., & de Haas G. H. (1979) *Recl. Trav. Chim. Pays-Bas 98*, 214.

Wells, M. A. (1974) *Biochemistry 13*, 2248.

Wooley, P., & Eibl, H. (1988) *Chem. Phys. Lipids 47*, 55.

Yang, C. C., King, K., & Sun, T. P. (1981) *Toxicon 19*, 645.

Yuan, W. (1990) Ph.D. Thesis, University of Washington.

Yuan, W., & Gelb, M. H. (1988) J. Am. Chem. Soc. 110, 2665.
Yuan, W., Berman, R. J., & Gelb, M. H. (1987) J. Am. Chem. Soc. 109, 8071.
Yuan, W., Fearon, K., & Gelb, M. H. (1989a) J. Org. Chem. 54, 906.
Yuan, W., Fearon, K., & Gelb, M. H. (1989b) UCLA Symp. Mol. Cell. Biol., New Ser. 106, 351.

Activities of Native and Tyrosine-69 Mutant Phospholipases A_2 on Phospholipid Analogues. A Reevaluation of the Minimal Substrate Requirements†

Oscar P. Kuipers, Nicolaas Dekker, Hubertus M. Verheij,* and Gerard H. de Haas
Department of Biochemistry, University of Utrecht, CBLE, P.O. Box 80.054, NL-3508 TB Utrecht, The Netherlands
Received November 27, 1989; Revised Manuscript Received February 26, 1990

ABSTRACT: The role of Tyr-69 of porcine pancreatic phospholipase A_2 in substrate binding was studied with the help of proteins modified by site-directed mutagenesis and phospholipid analogues with a changed head-group geometry. Two mutants were used containing Phe and Lys, respectively, at position 69. Modifications in the phospholipids included introduction of a sulfur at the phosphorus (thionophospholipids), removal of the negative charge at phosphorus (phosphatidic acid dimethyl ester), and reduction (phosphonolipids) or extension (diacylbutanetriol choline phosphate) of the distance between the phosphorus and the acyl ester bond. Replacement of Tyr-69 by Lys reduces enzymatic activity, but the mutant enzyme retains both the stereospecificity and positional specificity of native phospholipase A_2. The Phe-69 mutant not only hydrolyzes the R_p isomer of thionophospholipids more efficiently than the wild-type enzyme, but the S_p thiono isomer is hydrolyzed too, although at a low (~4%) rate. Phosphonolipids are hydrolyzed by native phospholipase A_2 about 7 times more slowly than natural phospholipids, with retention of positional specificity and a (partial) loss of stereospecificity. The dimethyl ester of phosphatidic acid is degraded efficiently in a calcium-dependent and positional-specific way by native phospholipase A_2 and by the mutants, indicating that a negative charge at phosphorus is not an absolute substrate requirement. The activities on the phosphatidic acid dimethyl ester of native enzyme and the Lys-69 mutant are lower than those on the corresponding lecithin, in contrast to the Phe-69 mutant, which has equal activities on both substrates. Our data suggest that in porcine pancreatic phospholipase A_2 fixation of the phosphate group is achieved both by an interaction with the phenolic OH of Tyr-69 and by an interaction with the calcium ion. In the mutant Y69K the ϵ-NH_2 group can play a role similar to that of the Tyr OH group in native PLA_2. The smaller side chain of the Y69F mutant can interact with more bulky head groups, allowing for relatively high enzymatic activities on modified phospholipids. On the basis of these results, a reevaluation of the minimal substrate requirements of phospholipase A_2 is presented.

The lipolytic enzyme phospholipase A_2 (PLA_2) specifically cleaves the 2-acyl ester bond of phosphoglycerides in a calcium-dependent reaction. The mechanism by which the calcium ion activates this enzyme is in debate. For some phospholipases from snake venoms it has been suggested that the enzyme binds calcium ions and substrate in an ordered way [for a review, see Verheij et al. (1981a)]. For PLA_2s isolated from mammalian pancreatic tissue the situation is more complex. At pH values of 6 or lower, all pancreatic phospholipases readily bind to micelles of substrates or substate analogues in the absence of calcium ions, although no hydrolysis occurs as long as no calcium ion is bound in the active site. Above pH 6 the binding of some PLA_2s to lipid aggregates becomes calcium dependent (van Dam-Mieras et al., 1975). Comparison of naturally occurring isozymes as well as site-specific mutagenesis studies revealed that two aspartate residues, i.e., Asp-66 and Asp-71, are involved in the binding of a second calcium ion (Donné-Op den Kelder et al., 1983; van den Bergh et al., 1989a). Hence, it was assumed that the role of this second calcium ion is to improve the binding of PLA_2 to micelles and thereby to increase the turnover number of the enzyme (van den Bergh et al., 1989a).

Chemical modification studies as well as X-ray analyses have shown that the "catalytic" calcium ion is bound to Asp-49 in the vicinity of the active site histidine-48 (Fleer el al., 1981; Dijkstra et al., 1983). Although several di- and trivalent cations bind stoichiometrically to PLA_2s from pancreatic tissue or snake venom, the enzymatic activity in these cases is only a few percent at the most. These observations suggested (Verheij et al., 1980) that the calcium ion in the active site serves two functions: the fixation of the phosphate of the polar head group and the polarization of the carbonyl of the susceptible ester bond. An X-ray structure of the ternary complex between phospholipase A_2, the Ca^{2+} ion, and a substrate analogue molecule could elucidate the relative position of these molecules. Unfortunately, the crystallization of such a complex has not yet been achieved. There is, however, experimental evidence accumulating that sheds more light on these interactions. The fixation of the phosphate by the Ca^{2+} ion was merely suggested by intuition, until Tsai et al. (1985) showed that the Ca^{2+} ion is probably in contact with the *pro-R* oxygen

†This work was supported by the Biotechnology Action Program of the EEC (Grant BAP-0071-NL).

0006-2960/90/0429-6094$02.50/0