

- Rooney, E. K., East, J. M., Jones, O. T., McWhirter, J., Simmonds, A. C., & Lee, A. G. (1983) *Biochim. Biophys. Acta* 728, 159-170.
- Stoeckenius, W. H., & Kunau, W. H. (1968) *J. Cell Biol.* 38, 337-357.
- Stoeckenius, W. H., & Bogomolni, R. A. (1982) *Annu. Rev. Biochem.* 51, 587-616.

- Tornabene, T. G., Kates, M., Gelpi, E., & Oro, J. (1969) *J. Lipid Res.* 10, 294-303.
- Trewhella, J., Anderson, S., Fox, R., Gogol, E., Khan, S., Engelmann, D., & Zaccai, G. (1983) *Biophys. J.* 42, 233-241.
- Tsuda, M., Govindjee, R., & Ebrey, T. G. (1984) *Biophys. J.* 44, 249-254.

## Synthesis of Prodan-Phosphatidylcholine, a New Fluorescent Probe, and Its Interactions with Pancreatic and Snake Venom Phospholipases A<sub>2</sub><sup>†</sup>

H. S. Hendrickson,\* E. J. Dumdei, A. G. Batchelder, and G. L. Carlson

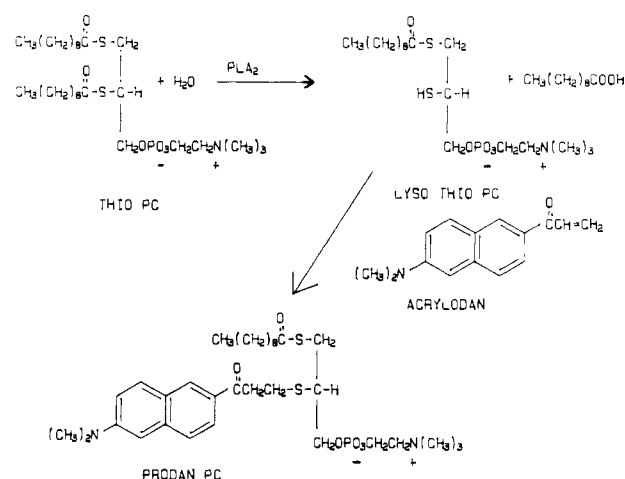
Department of Chemistry, St. Olaf College, Northfield, Minnesota 55057

Received December 16, 1986; Revised Manuscript Received February 24, 1987

**ABSTRACT:** A new fluorescent probe, prodan-PC, was synthesized by incubating thio-PC, a thiol ester analogue of phosphatidylcholine [1,2-bis(decanythio)-1,2-dideoxy-*sn*-glycero-3-phosphocholine], with acrylodan, a fluorescent thiol-reactive reagent [6-acryloyl-2-(dimethylamino)naphthalene], in the presence of phospholipase A<sub>2</sub>, which served to generate lysothio-PC in situ. Prodan-PC (PPC) showed maximum absorption in ethanol at 370 nm. The fluorescence emission spectrum showed maximum emission at 530 nm in water and at 498 nm in ethanol. In the presence of a saturating amount of phospholipase A<sub>2</sub>, the emission maximum shifted to about 470 nm. PPC showed a critical micellar concentration around 5 μM, with evidence of premicellar aggregation above 1 μM. Binding of PPC to *Crotalus adamanteus* phospholipase A<sub>2</sub> was evidenced by an increase in emission at 480 nm and an increase in fluorescence anisotropy. An apparent dissociation constant of 0.323 μM was calculated for this enzyme complex. Binding was dependent on the presence of calcium ion and was abolished by blocking the active site with *p*-bromophenacyl bromide. Binding was also followed by energy transfer from tryptophan in the enzyme to PPC. Apparent dissociation constants for PPC complexes with phospholipases A<sub>2</sub> from *Naja naja naja* and porcine pancreas and the pro-phospholipase A<sub>2</sub> from porcine pancreas were 0.509, 0.107, and 0.114 μM, respectively. PPC was shown to inhibit the activity of pancreatic phospholipase A<sub>2</sub> in thio-PC-sodium cholate mixed micelles. Inhibition studies were complicated because PPC can also serve as an activator of the snake venom enzymes. The unusually high affinity of PPC for phospholipase A<sub>2</sub> is discussed in terms of its use as a fluorescent probe and in the design of high-affinity inhibitors of the enzyme.

Several years ago we synthesized a chiral thiol ester analogue of phosphatidylcholine (thio-PC)<sup>1</sup> as a substrate for a continuous spectrophotometric assay of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) (Hendrickson et al., 1983). The availability of a very polarity-sensitive thiol-reactive fluorescent reagent, acrylodan (Prendergast et al., 1983), suggested the synthesis of a new fluorescent phosphatidylcholine analogue by treatment of thio-PC with PLA<sub>2</sub> in the presence of acrylodan (Scheme I). The new fluorescent probe, prodan-PC (PPC), seemed attractive for the study of phosphatidylcholine interaction with PLA<sub>2</sub>. This probe has good absorbance, has high quantum yield, and is very sensitive to the polarity of its environment. Emission of the prodan moiety shows a dramatic shift to shorter wavelength in nonpolar environments due to charge separation in the excited state (Weber & Farris, 1979). Its absorption maximum at 370 nm is sufficiently close to the emission maximum of tryptophan (about 344 nm) so that it might be useful in energy-transfer studies with PLA<sub>2</sub>. PLA<sub>2</sub> from porcine pancreas has a single tryptophan in or near the active site. PLA<sub>2</sub> from *Crotalus adamanteus* and *Naja naja naja* have more than two tryptophan residues, some of which may be in a hydrophobic surface area of the enzyme sur-

Scheme I



rounding the active site (Verheij et al., 1981). Here we report the synthesis of prodan-PC, some of its properties, and some

<sup>†</sup> These studies were supported by Grant GM-33606 from the National Institutes of Health.

\* Author to whom correspondence should be addressed.

<sup>1</sup> Abbreviations: PC, phosphatidylcholine; thio-PC, 1,2-bis(decanythio)-1,2-dideoxy-*sn*-glycero-3-phosphocholine; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; acrylodan, 6-acryloyl-2-(dimethylamino)naphthalene; prodan, 6-propionyl-2-(dimethylamino)naphthalene; PPC, prodan-PC, a thioether adduct of acrylodan and lysothio-PC; HPLC, high-performance liquid chromatography; cmc, critical micellar concentration; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

interactions with PLA<sub>2</sub> from porcine pancreas and the venoms of *C. adamanteus* and *N. naja naja*.

## EXPERIMENTAL PROCEDURES

**Materials.** Acrylodan was obtained as a generous gift from Dr. Franklyn G. Prendergast, Mayo Foundation, Rochester, MN. Acrylodan and prodan were also purchased from Molecular Probes, Eugene, OR. 1,2-Bis(decanylthio)-1,2-dideoxy-*sn*-glycero-3-phosphorylcholine (thio-PC) was synthesized as described for the bisheptanoyl homologue by Hendrickson et al. (1983). PLA<sub>2</sub> was purified from *Crotalus adamanteus* venom according to the method of Wells (1975). PLA<sub>2</sub> and pro PLA<sub>2</sub> were purified from porcine pancreas by the method of Nieuwenhuizen et al. (1974). PLA<sub>2</sub> from cobra (*Naja naja naja*) venom was obtained in homogeneous form as a generous gift from Dr. Edward A. Dennis, University of California at San Diego. Reduced Triton X-100 (X-100R) was purchased from Sigma Chemical Co., St. Louis, MO.

**Protein Determination.** Protein concentrations were determined by UV absorption with extinction coefficients of  $\epsilon_{280} = 1.23 \text{ mL mg}^{-1} \text{ cm}^{-1}$  and  $\epsilon_{280} = 1.30 \text{ mL mg}^{-1} \text{ cm}^{-1}$  for the pancreatic PLA<sub>2</sub> and pro-PLA<sub>2</sub>, respectively (Nieuwenhuizen et al., 1974),  $\epsilon_{278} = 2.20 \text{ mL mg}^{-1} \text{ cm}^{-1}$  for the cobra venom PLA<sub>2</sub> (Darke et al., 1980), and  $\epsilon_{280} = 2.27 \text{ mL mg}^{-1} \text{ cm}^{-1}$  for the *C. adamanteus* PLA<sub>2</sub> (Wells & Hanahan, 1969). Protein concentrations are expressed in terms of monomer concentration.

**Analytical Methods.** Phosphorus was determined by the method of Eaton and Dennis (1976). UV absorption spectroscopy was performed on a Perkin-Elmer Lambda 3B spectrophotometer. NMR spectra were obtained on an IBM 200-AF Fourier-transfer spectrometer at 200 MHz.

**Reaction of *p*-Bromophenacyl Bromide with PLA<sub>2</sub>.** To a solution of 3.1 mg of *C. adamanteus* PLA<sub>2</sub> in 3 mL of buffer (0.1 M NaCl, 20 mM Tris-HCl, pH 7.5) was added 6 mg of *p*-bromophenacyl bromide in 100  $\mu\text{L}$  of acetone. At various times, aliquots were removed for assay of enzyme activity (Hendrickson et al., 1983). After 3 h the activity decreased to less than 1% of the original. The blocked enzyme was separated from excess reagent by passage through a small column of Bio-Gel P-6-DG (Bio-Rad, Richmond, CA).

**Fluorescence Studies.** Corrected fluorescence spectra and steady-state anisotropies were obtained on a Perkin-Elmer MPF-66 fluorescence spectrophotometer thermostated at 25 °C. Fluorescence binding studies were carried out in 50 mM Tris-HCl, 100 mM NaCl, and 10 mM CaCl<sub>2</sub>, pH 7.5, except where otherwise noted. Corrected fluorescence emission spectra were used to obtain fluorescence quantum yields relative to that of quinine sulfate dissolved in 1.0 N sulfuric acid (quantum yield = 0.55; Hercules, 1966).

**Computer Analysis of Binding Data.** Fluorescence binding data were fit to a hyperbolic binding equation ( $h = 1$ ) or Hill equation ( $h > 1$ ) with a nonlinear regression analysis program adapted from Kinfitt (Knack & Röhm, 1981):

$$\Delta F = \Delta F_{\max} K_d^h / (K_d^h + [P]^h)$$

where  $\Delta F$  and  $\Delta F_{\max}$  are the experimental and maximal changes in fluorescence emission,  $K_d$  is the protein concentration giving half-maximal fluorescence change,  $[P]$  is the concentration of free protein, and  $h$  is the Hill coefficient. The data set,  $\Delta F$  and  $[P]$ , is entered into the computer along with an estimate of  $h$ . The program then calculates an estimate of  $\Delta F_{\max}$  and  $K_d$  by a linear regression and then refines those values and  $h$  by successive iterations until a minimal sum of the squares of the deviations is achieved with the Gauss-

Newton algorithm. Corrections for free unbound protein (or ligand) concentrations were obtained from total concentrations by a series of iterative approximations. After each calculation, a better approximation of the free concentration was obtained from the calculated  $K_d$  value until successive  $K_d$  values were the same (converged).  $K_d$  values are not interpreted as actual dissociation constants but rather are apparent dissociation constants or, more simply, the concentrations of protein (or ligand) giving half-maximal fluorescence change. Hill coefficients have no intrinsic significance but are used to fit curves that appear to be sigmoidal and do not fit a simple hyperbolic function.

From a titration of PLA<sub>2</sub> into PPC, the maximum moles of PPC bound per mole of protein  $N$  was calculated by a nonlinear regression analysis of  $n$  as a function of  $[\text{PPC}]$ :

$$n = N[\text{PPC}] / (K_d + [\text{PPC}])$$

where  $n$  is the average moles of PPC bound per mole protein and  $[\text{PPC}]$  is the concentration of free PPC. The value of  $n$  was calculated from  $n = (\Delta F / \Delta F_{\max}) [\text{PPC}]_t / [P]_t$ , where  $[\text{PPC}]_t$  and  $[P]_t$  are the total concentrations of PPC and protein, respectively. The value of  $[\text{PPC}]$  was calculated from  $[\text{PPC}] = (1 - \Delta F / \Delta F_{\max}) [\text{PPC}]_t$ .

**Synthesis of Prodan-Phosphatidylcholine (PPC).** Acrylodan (20 mg, 89  $\mu\text{mol}$ ) and 80  $\mu\text{mol}$  of thio-PC in CHCl<sub>3</sub> were placed in a tapered reaction vessel. The solvent was evaporated under nitrogen and the residue dried under vacuum. The residue was dissolved in 10 mL of 2% methanol in diethyl ether, and 0.8 mL of 0.1 M, pH 7.4, borate buffer (containing 4 mg/mL calcium acetate) was added. PLA<sub>2</sub> (200  $\mu\text{L}$  containing 117 units, 1 mg/mL, No. P-6139, Sigma Chemical Co., St. Louis, MO) was added, and the vessel sealed and stirred vigorously at room temperature. After being stirred overnight, the thio-PC and lysothio-PC had disappeared as monitored by HPLC. The reaction mixture was separated into aqueous and ether layers. PPC was found entirely in the aqueous layer by HPLC. The aqueous layer was extracted with a mixture of 8 mL of methanol, 5 mL of chloroform, and 4 mL of water. The chloroform layer was separated and the aqueous layer extracted with another 5 mL of chloroform. The chloroform extracts were combined, concentrated, and purified by preparative HPLC on a 10 mm  $\times$  50 cm silica gel (Analtch 10050, 10  $\mu\text{m}$ ) column with hexane-2-propanol-water (6:8:1.6) at a flow rate of 10 mL/min. Detection was accomplished by both fluorescence ( $\text{ex} = 370 \text{ nm}$ ,  $\text{em} > 430 \text{ nm}$ ) and UV absorption at 235 nm.

**Assay of PLA<sub>2</sub> Activity.** A spectrophotometric assay of porcine pancreatic PLA<sub>2</sub> activity in the presence and absence of PPC was performed according to the procedure described by Hendrickson and Dennis (1984a) with thio-PC as the substrate. The assay solution contained 0.1 M KCl, 10 mM CaCl<sub>2</sub>, 25 mM Tris-HCl, pH 8.0, 0.5 mM thio-PC, 2.5 mM sodium cholate, 0.8 mM dithiopyridine, and varying amounts of PPC.

## RESULTS

**Synthesis and Characterization of PPC.** PPC was readily synthesized by incubating thio-PC with PLA<sub>2</sub> to generate lysothio-PC in situ, which then reacts with acrylodan by a Michael addition. It was purified by preparative HPLC. The absorption spectrum of PPC in ethanol showed a maximum at 370 nm with an extinction coefficient of  $29\,500 \text{ M}^{-1} \text{ cm}^{-1}$ , calculated on the basis of phosphorus analysis. The NMR spectrum was consistent with the structure given for PPC as compared to the spectra for lecithin (Birdsall et al., 1972) and prodan [6-propionyl-2-(dimethylamino)naphthalene]. It

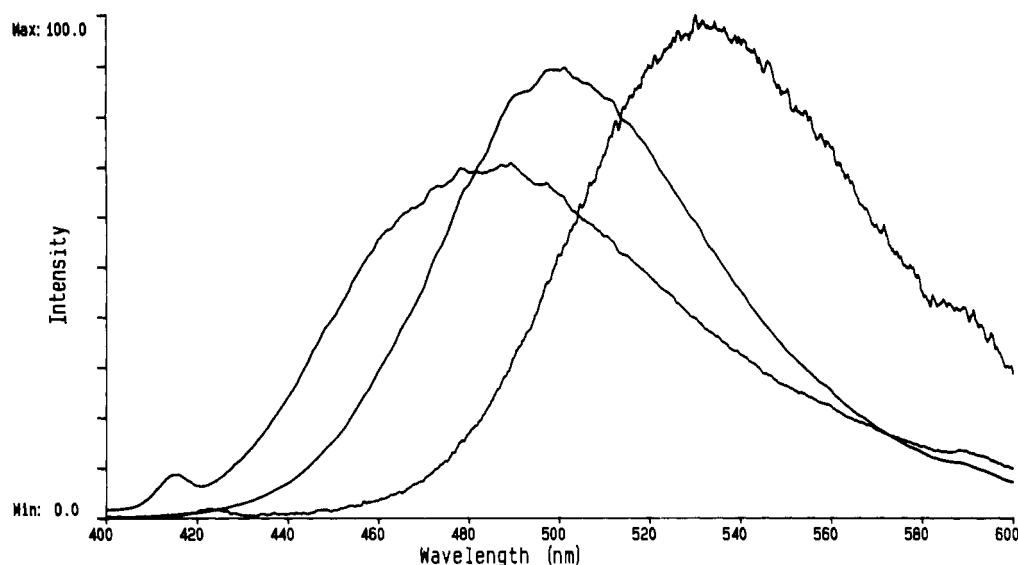


FIGURE 1: Corrected fluorescence emission spectra of PPC. Peak at 530 nm: 0.928  $\mu$ M PPC in water; slit widths, 2 nm/2 nm; relative intensity scale, 0–1. Peak at 498 nm: 1.1  $\mu$ M PPC in ethanol; slit widths, 5 nm/5 nm; relative intensity scale, 0–100. Peak at 484 nm: 1.1  $\mu$ M PPC in hexane; slit widths, 5 nm/5 nm; relative intensity scale, 0–2. Excitation at 370 nm.

showed a triplet (6.4 Hz) at 0.78 ppm for the acyl  $-\text{CH}_3$ , a multiplet at 1.15–1.17 ppm for the acyl C-4 to C-9  $-\text{CH}_2-$ , a multiplet at 1.46–1.56 ppm for the acyl C-3  $-\text{CH}_2-$ , a triplet (7.6 Hz) at 2.43 ppm for the acyl C-2  $-\text{CH}_2\text{CO}-$ , a triplet (5.9 Hz) at 2.96 ppm for the Ar  $\text{COCH}_2-$  [quartet (7.3 Hz) at 3.06 ppm for prodan], a singlet at 3.02 ppm for the  $\text{N}(\text{CH}_3)_3$ , a singlet at 3.10 ppm for the Ar  $\text{N}(\text{CH}_3)_2$  (singlet at 3.08 ppm for the prodan), a multiplet at 3.3–3.4 ppm for the choline  $-\text{CH}_2\text{N}$ , a multiplet at 3.4–3.5 ppm for the glycerol *sn*-3  $-\text{CH}_2-$ , doublets (10.6 Hz) of doublets (5.5 Hz) at 3.88 and 4.04 ppm for the nonequivalent glycerol *sn*-1  $-\text{CH}_2-$ , and multiple peaks from 6.7 to 8.3 ppm for aromatic protons (same pattern from 6.8 to 8.3 ppm for prodan). The peaks for  $-\text{CH}_2\text{SCH}$  are not identified but may be in the region of 3.1–3.2 ppm. Solvent peaks include OH at 3.22 ppm,  $\text{HCD}_2\text{OD}$  [quintet (1.6 Hz)] at 3.29 ppm, and  $\text{CHCl}_3$  at 7.24 ppm.

The fluorescence emission spectrum of PPC (Figure 1) showed maximum emission at 530 nm in water, at 498 nm in ethanol, and at 484 nm (with a small peak at about 416 nm) in hexane. A quantum yield of 0.34 was calculated for PPC in ethanol relative to quinine.

The cmc of PPC was determined by observing the shift in emission wavelength as a function of concentration. At high concentrations, micellar PPC showed maximum emission at 480 nm as compared to 530 nm at low concentrations. A plot of the ratio of emission intensities at 480 nm/530 nm (Figure 2) showed a large break around 5  $\mu$ M and a smaller break around 1  $\mu$ M.

**Interactions of PPC with *C. adamanteus* PLA<sub>2</sub>.** PPC in the presence of a saturating amount of *C. adamanteus* PLA<sub>2</sub> showed a shift in maximum emission from 530 to 472 nm and an increase in intensity by a factor of 3.6. Titration of PPC into this PLA<sub>2</sub> showed a sigmoidal increase in emission intensity at 480 nm, indicative of binding (Figure 3A). This binding was abolished in the absence of calcium ion and also when the PLA<sub>2</sub> active site was blocked by reaction with *p*-bromophenacyl bromide. Titration of PLA<sub>2</sub> into 2.78  $\mu$ M PPC (Figure 3B) showed a biphasic increase in emission intensity (>450 nm): a plateau at about 1  $\mu$ M followed by an increase up to 5  $\mu$ M PLA<sub>2</sub>. Steady-state anisotropy measurements in the same experiment showed a single hyperbolic increase that followed the initial increase in fluorescence intensity.

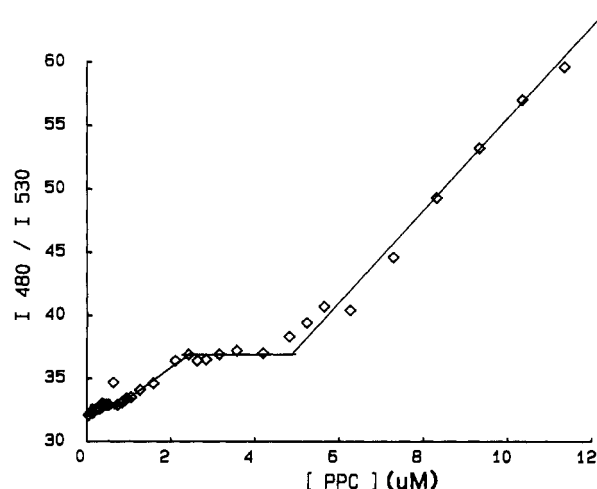


FIGURE 2: Determination of cmc for PPC. Ratio of emission intensities at 480 nm/530 nm vs. concentration of PPC ( $\mu$ M) in standard buffer. Excitation at 370 nm; slit widths, 5 nm/5 nm.

Table I: Apparent Dissociation Constants and Stoichiometries of Complexes of PPC with Phospholipases A<sub>2</sub> from Various Sources

enzyme source	$K_d$ ( $\mu$ M)	$N$ (mol of PPC/mol of PLA <sub>2</sub> )
<i>C. adamanteus</i>	$0.323 \pm 0.042$	nd <sup>a</sup>
<i>C. adamanteus</i> <sup>b</sup>	$0.361 \pm 0.096$	nd <sup>a</sup>
<i>N. naja naja</i>	$0.509 \pm 0.006$	nd <sup>a</sup>
porcine pancreas	$0.107 \pm 0.007$	$0.64 \pm 0.08$
porcine pancreas (pro) <sup>c</sup>	$0.114 \pm 0.009$	$0.87 \pm 0.20$

<sup>a</sup> Not determined. Data were not sufficient to calculate  $N$ . <sup>b</sup> With Triton X-100R micelles (PPC/Triton, 1:100 mol ratio). <sup>c</sup> Pro-PLA<sub>2</sub>.

Binding of PPC with *C. adamanteus* PLA<sub>2</sub> could also be followed by energy transfer as indicated by quenching of the tryptophan fluorescence of PLA<sub>2</sub> by PPC. Excitation of PLA<sub>2</sub> tryptophan at 290 nm in the presence of PPC resulted in quenching of tryptophan emission at 344 nm and the appearance of PPC emission at 472 nm. PPC excited alone at 290 nm gave negligible emission. Titration of PPC into PLA<sub>2</sub> (Figure 3C) showed a hyperbolic increase in the quenching of tryptophan emission at 344 nm with excitation at 290 nm. The  $K_d$  value calculated from this titration was 0.323  $\mu$ M.

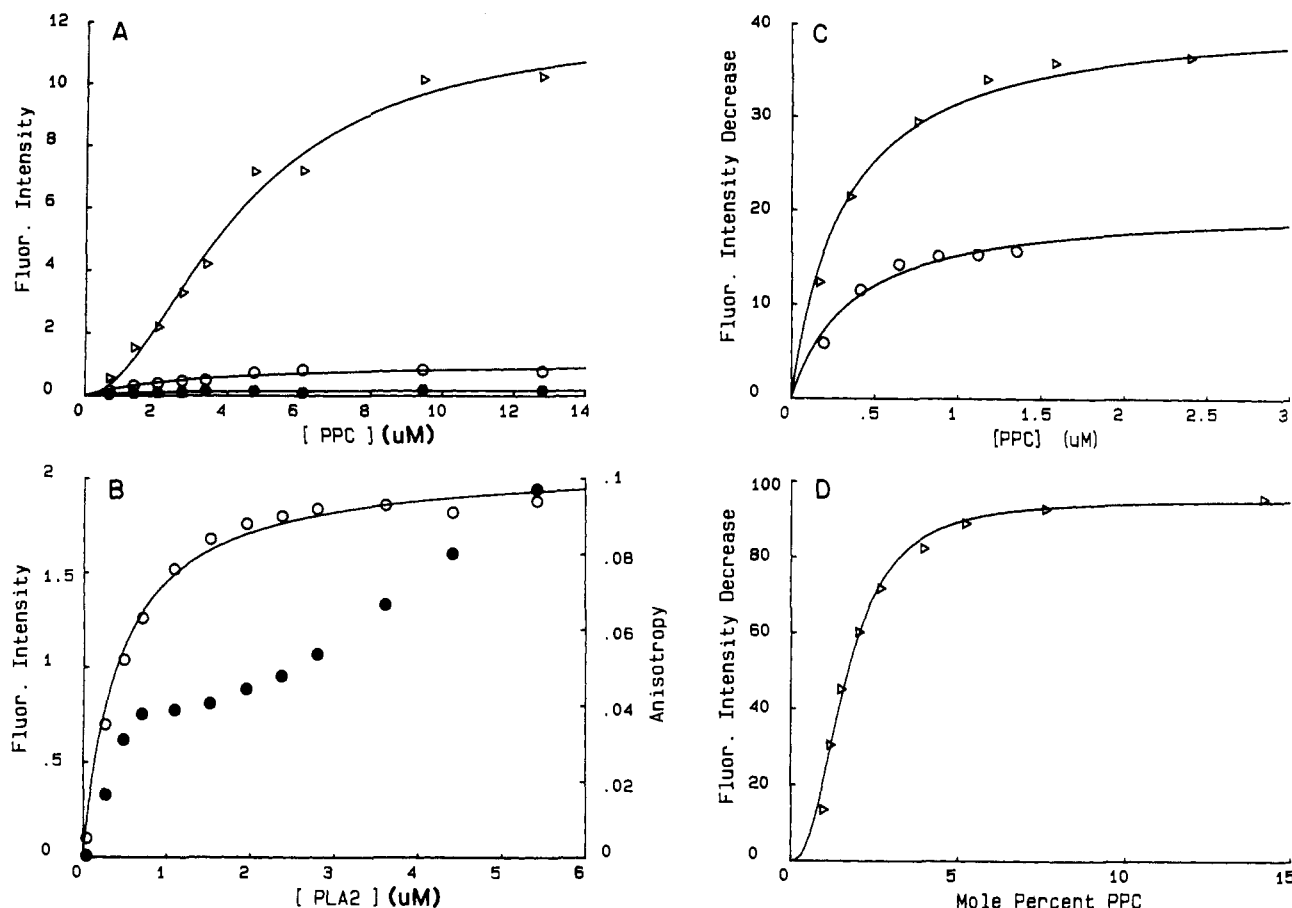


FIGURE 3: Interactions of PPC with *C. adamanteus* PLA<sub>2</sub>. (A) Titration of PPC into *C. adamanteus* PLA<sub>2</sub>. Increase in fluorescence emission intensity at 480 nm vs. total concentration of PPC with 3.06  $\mu$ M PLA<sub>2</sub> present. (Open triangles) In standard buffer with 10 mM CaCl<sub>2</sub>; (open circles) in standard buffer with 1 mM EDTA and no CaCl<sub>2</sub>; (closed hexagons) PLA<sub>2</sub> blocked with *p*-bromophenylacetyl bromide, in standard buffer with 10 mM CaCl<sub>2</sub>. Excitation at 370 nm. (B) Titration of *C. adamanteus* PLA<sub>2</sub> into 2.78  $\mu$ M PPC. Total concentration of PLA<sub>2</sub>. (Closed circles) Increase in fluorescence emission intensity (>450 nm); (open circles) increase in PPC steady-state fluorescence anisotropy (>450 nm). Anisotropy in absence of PLA<sub>2</sub> was 0.058. Excitation at 370 nm. (C) Titration of PPC into *C. adamanteus* PLA<sub>2</sub> in the presence and absence of Triton X-100R (reduced). Decrease (quenching) in fluorescence emission intensity at 344 nm; excitation at 290 nm (slit widths: 2-nm excitation, 4-nm emission). Concentration of free PPC. (Triangles) Titration of PPC without Triton into 0.161  $\mu$ M PLA<sub>2</sub>; (circles) titration of PPC-Triton at a constant mole ratio of 1:100 (PPC/Triton) into 0.161  $\mu$ M PLA<sub>2</sub> containing the cmc (0.25 mM) of Triton. (D) Interaction of *C. adamanteus* PLA<sub>2</sub> with PPC in Triton X-100R (reduced) micelles at constant bulk concentration of PPC as a function of surface concentration. Decrease (quenching) in fluorescence intensity at 344 nm; excitation at 290 nm (slit widths: 2-nm excitation, 4-nm emission). Titration of Triton into 0.160  $\mu$ M PLA<sub>2</sub>, 5.22  $\mu$ M PPC, and 0.25 mM Triton. Surface concentration of PPC expressed as mole percent in Triton micelles. Data fit to Hill equation with  $h = 2.44$ .

(Table I). Binding of PLA<sub>2</sub> to PPC in reduced Triton X-100 micelles was determined by titration of PPC-Triton (1:100 mol ratio) into 0.161  $\mu$ M PLA<sub>2</sub> containing the cmc (0.25 mM) of Triton (Figure 3C). The  $K_d$  value calculated from this titration [ $K_d = 0.361 \mu$ M (Table I)] was similar to that calculated in the absence of Triton micelles. Binding of PPC to PLA<sub>2</sub> within the micelle interface was determined by measuring the tryptophan quenching as a function of the mole percent of PPC in Triton micelles at constant bulk concentration of PPC and PLA<sub>2</sub>. This was accomplished by titrating Triton into a solution of 5.22  $\mu$ M PPC, 0.160  $\mu$ M PLA<sub>2</sub>, and the cmc (0.25 mM) of Triton. It is assumed that PPC is completely incorporated into the Triton micelles. The micellar concentration of Triton is equal to the total Triton concentration minus the concentration of monomolecular Triton. The mole percent of PPC in the micelles is thus equal to

$$100\{[\text{PPC}]/([\text{Triton}]_{\text{total}} - [\text{Triton}]_{\text{mono}} + [\text{PPC}])\}$$

where  $[\text{Triton}]_{\text{mono}} = 0.25 \text{ mM}$  (cmc). The data fit a sigmoidal binding curve (Figure 3D) with the PPC concentration at half-quenching equal to 1.67 mol % ( $\pm 0.06$ ).

**Interactions of PPC with Cobra Venom PLA<sub>2</sub>.** Titration of cobra venom PLA<sub>2</sub> into PPC resulted in a blue shift in the

PPC emission maximum from 530 to 471 nm with a decrease in intensity. The emission spectrum in the absence of added enzyme, multiplied by an appropriate dilution factor, was subtracted from each emission spectrum in the presence of enzyme. In this way, a series of difference emission spectra were constructed. An overlay of these difference spectra showed a single isoemissive point at about 505 nm and positive peaks at 471 nm. A hyperbolic increase in emission intensity at 471 nm was observed as a function of PLA<sub>2</sub> concentration (Figure 4A). A  $K_d$  value equal to 0.509  $\mu$ M (Table I) was calculated from this titration. Steady-state anisotropy measurements of PPC carried out during this titration showed a similar hyperbolic increase as a function of concentration.

**Interactions of PPC with Porcine Pancreatic PLA<sub>2</sub> and Pro-PLA<sub>2</sub>.** Titration of porcine pancreatic PLA<sub>2</sub> into PPC resulted in a shift in the PPC emission maximum from 530 to 472 nm and a slight increase in intensity. Difference emission spectra were constructed as explained in the previous paragraph. Overlays of these difference spectra showed one defined isoemissive point around 515 nm for concentrations of PLA<sub>2</sub> up to 0.45  $\mu$ M. At higher concentrations the isoemissive point was not well-defined but decreased steadily to about 510 nm. A hyperbolic increase in emission intensity

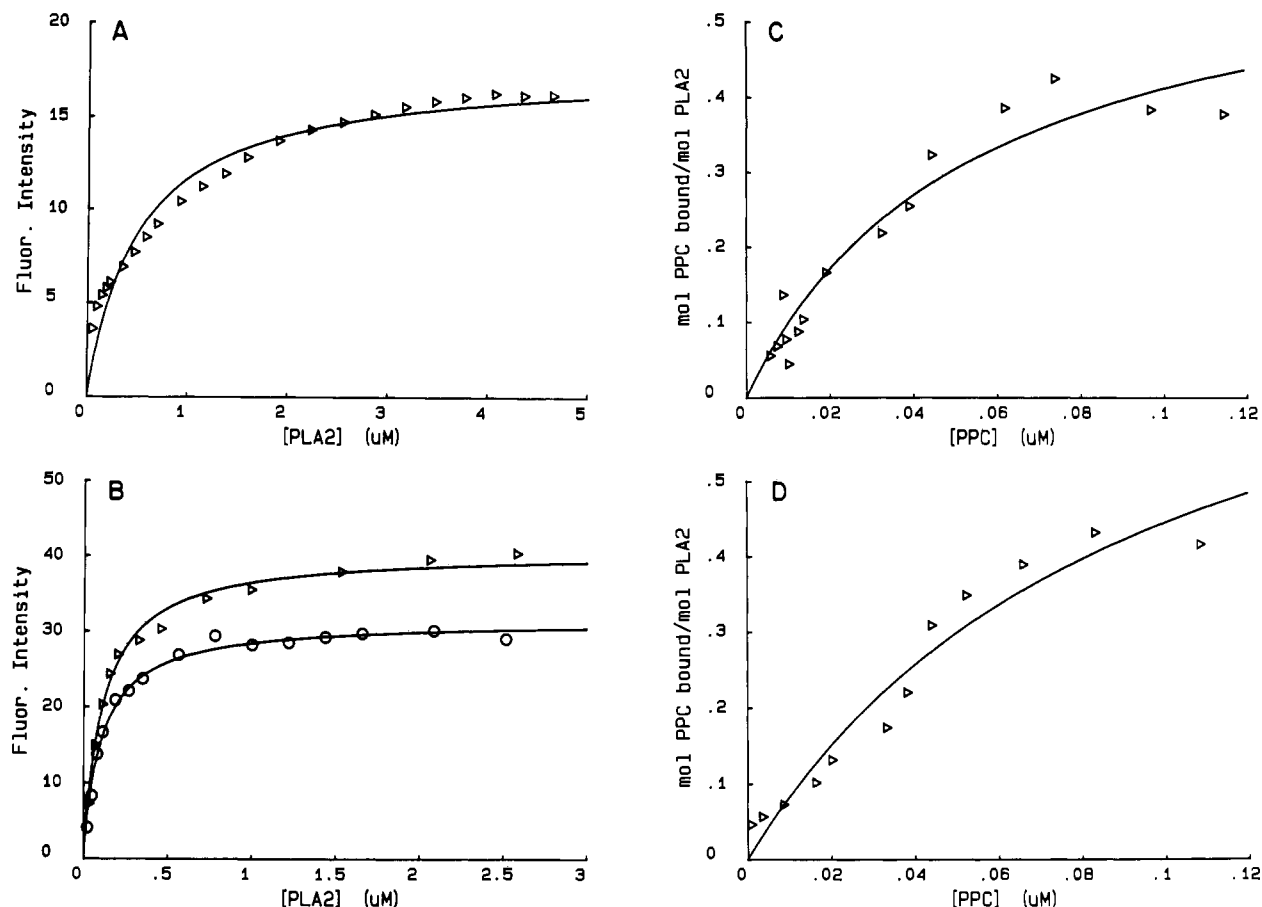


FIGURE 4: Interactions of PPC with cobra venom and porcine pancreatic  $\text{PLA}_2$ . (A) Titration of cobra venom  $\text{PLA}_2$  into  $0.441 \mu\text{M}$  PPC. Concentration of free  $\text{PLA}_2$ . Increase in fluorescence emission intensity at  $471 \text{ nm}$  taken from corrected difference spectra. Excitation at  $370 \text{ nm}$  (slit widths:  $5 \text{ nm}/5 \text{ nm}$ ). (B) Binding of porcine pancreatic  $\text{PLA}_2$  and pro- $\text{PLA}_2$  to PPC. Titration of  $\text{PLA}_2$  into  $0.132 \mu\text{M}$  PPC. Concentration of free  $\text{PLA}_2$ . Increase in fluorescence emission intensities at  $474 \text{ nm}$  taken from corrected difference spectra. (Triangles) Pro- $\text{PLA}_2$ ; (circles)  $\text{PLA}_2$ . Excitation at  $370 \text{ nm}$  (slit widths:  $6 \text{ nm}/6 \text{ nm}$ ). (C) Binding of PPC to porcine pancreatic  $\text{PLA}_2$ . Recalculation from (B). Nonlinear regression computer fit. (D) Binding of PPC to porcine pancreatic pro- $\text{PLA}_2$ . Recalculation from (B). Nonlinear regression computer fit.

at  $480 \text{ nm}$  was observed as a function of  $\text{PLA}_2$  concentration (Figure 4B). A  $K_d$  value equal to  $0.107 \mu\text{M}$  (Table I) was calculated. The maximum moles of PPC bound per mole of PLA was calculated to be  $0.64$  (Figure 4C, Table I). Steady-state anisotropy measurements showed a similar hyperbolic increase as a function of  $\text{PLA}_2$  concentration.

Titration of the zymogen, pro- $\text{PLA}_2$ , into PPC resulted in a hyperbolic increase in emission intensity at  $474 \text{ nm}$  (Figure 4B). Overlays of difference emission spectra showed a single isoemissive point at about  $510 \text{ nm}$ . The  $K_d$  value calculated from this titration was equal to  $0.114 \mu\text{M}$  (Table I). The maximum moles of PPC bound per mole of pro- $\text{PLA}_2$  was calculated to be  $0.87$  (Figure 4D, Table I).

**Inhibition of  $\text{PLA}_2$  Activity by PPC.** The inhibition of porcine pancreatic  $\text{PLA}_2$  by PPC is shown in Figure 5. The fraction of inhibition was a hyperbolic function of PPC concentration with maximal inhibition of  $0.69 (\pm 0.02)$  and half-maximal inhibition at  $6.38 \mu\text{M} (\pm 0.76)$  PPC. With the cobra venom enzyme, PPC was observed to give little or no inhibition of  $\text{PLA}_2$  activity. For example, in thio-PC-Triton mixed micelles ( $1:25 \text{ mol ratio}$ ,  $0.3 \text{ mM thio-PC}$ ), addition of PPC up to  $80 \mu\text{M}$  resulted in no significant inhibition (data not shown).

## DISCUSSION

PPC is conveniently synthesized from acrylodan and lyso-thio-PC, the latter generated in situ by the action of  $\text{PLA}_2$  on thio-PC. Like prodan, its emission maximum is quite sensitive to the polarity of the environment. It ranges from  $530 \text{ nm}$  in

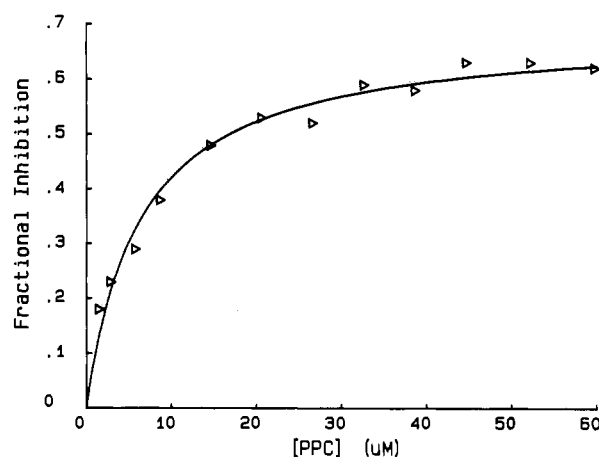


FIGURE 5: Inhibition of activity of porcine pancreatic  $\text{PLA}_2$  by PPC. Thio-PC ( $0.5 \text{ mM}$ ) with  $2.5 \text{ mM}$  sodium cholate and varying amounts of PPC. The activity of the uninhibited reaction was  $171 \mu\text{mol min}^{-1} \text{ mg}^{-1}$ . Assay conditions described in text.

water to  $498 \text{ nm}$  in ethanol. In hexane there is a broad peak at  $484 \text{ nm}$  with a small peak at  $416 \text{ nm}$ . Emission at  $484 \text{ nm}$  may be due to reverse micelles since there is some residual water in the sample. The small-peak at  $416 \text{ nm}$  may be due to monomolecular PPC at the cmc since Weber and Farris (1979) reported an emission maximum of  $401 \text{ nm}$  for prodan in cyclohexane. PPC appears to have a definite cmc at about  $5 \mu\text{M}$  and shows evidence of pre-micellar aggregation at about  $1 \mu\text{M}$  (Figure 2). In most of the titrations of  $\text{PLA}_2$  into PPC,

the PPC concentration was kept well below 1  $\mu\text{M}$  (in the monomolecular region) to avoid complications due to interfacial effects.

Binding of PPC to  $\text{PLA}_2$  is accompanied by a shift in the PPC emission maximum from 530 to about 480 nm. This is about the same shift as seen in the binding of prodan to bovine serum albumin (Weber & Farris, 1979). This binding appears to be at the active site since it is abolished in the absence of calcium ions and calcium is known to bind at the active site. Binding is also abolished by blocking the active site with *p*-bromophenacyl bromide, which reacts with a histidine imidazole group at the active site (Volwerk et al., 1974).

Titration of *C. adamanteus*  $\text{PLA}_2$  into PPC showed a biphasic increase in emission intensity (Figure 3B). This enzyme is known to be dimeric (Smith & Wells, 1981). At low concentrations of  $\text{PLA}_2$ , where there is a high ratio of PPC to  $\text{PLA}_2$ , more than one PPC molecule bound to an enzyme dimer may result in self-quenching. At higher concentrations of  $\text{PLA}_2$ , the PPC redistributes to only one bound PPC molecule per enzyme dimer. The reduction in self-quenching may then explain the increase in emission seen above 1  $\mu\text{M}$   $\text{PLA}_2$ . Fluorescence anisotropy, which is also an indication of binding of PPC to protein, coincides with the initial increase in fluorescence emission.

Binding of PPC to *C. adamanteus*  $\text{PLA}_2$  can also be followed by quenching of the tryptophan fluorescence of the protein. In this way, binding of PPC to  $\text{PLA}_2$  in the presence and absence of Triton X-100R micelles was determined. Similar  $K_d$  values were calculated for the PPC- $\text{PLA}_2$  complex in the presence and absence of micelles (Table I). The initial binding of  $\text{PLA}_2$  to PPC thus appears to be independent of the presence of interface. Once the *C. adamanteus*  $\text{PLA}_2$  is bound to the interface, it appears to interact with additional PPC molecules, since tryptophan quenching was also a function of the interfacial concentration of PPC (Figure 3D). At the high bulk concentration of PPC it would appear that the active site is already saturated; however, additional binding of PPC to  $\text{PLA}_2$  in the micelle surface may occur in a region surrounding the active site of  $\text{PLA}_2$  at the higher local concentration of PPC experienced in the interface. This may result in the quenching of additional tryptophan residues around the active site or enhance the original quenching of tryptophan at the active site by a protein conformational change. This effect is similar to the kinetics of cobra venom  $\text{PLA}_2$  observed as a function of interfacial substrate concentration (Hendrickson & Dennis, 1984a). In the latter case, the enzymatic activity of  $\text{PLA}_2$  was dependent both on the bulk substrate concentration at constant surface concentration and on the surface concentration of substrate at constant bulk concentration. These results are consistent with the dual phospholipid model for  $\text{PLA}_2$  action (Dennis, 1983), where  $\text{PLA}_2$  binds first to a substrate molecule at the interface and then binds a second substrate molecule within the interface before catalysis takes place. One phospholipid acts as an activator, enhancing the catalytic activity toward the other phospholipid at the active site.

Interactions of PPC with cobra venom and pancreatic  $\text{PLA}_2$  were followed by recording emission spectra during the titration of enzyme into PPC. Computer subtraction of the spectrum of PPC in the absence of enzyme from the spectra in the presence of enzyme yielded a series of difference spectra. For the cobra venom  $\text{PLA}_2$  and the pancreatic pro- $\text{PLA}_2$ , these difference spectra showed a single isoemissive point, indicating the presence of only two species of PPC: free PPC and a single PPC- $\text{PLA}_2$  complex. In the case of the

active pancreatic  $\text{PLA}_2$ , a single isoemissive point was seen only up to 0.45  $\mu\text{M}$   $\text{PLA}_2$ . Beyond this concentration the isoemissive point drifted to lower wavelengths. This may indicate more than one PPC- $\text{PLA}_2$  complex above that concentration. Titration of *C. adamanteus*  $\text{PLA}_2$  into PPC showed no defined isoemissive point. In the case of this dimeric enzyme, there may be at least two complexes: one and two bound PPC molecules per enzyme dimer.

The stoichiometry of the PPC- $\text{PLA}_2$  complex was calculated from fluorescence data of the titration of  $\text{PLA}_2$  into PPC. Values of  $N$ , maximum moles of PPC bound per mole of  $\text{PLA}_2$ , were 0.64 and 0.87 for complexes with pancreatic  $\text{PLA}_2$  and pancreatic pro- $\text{PLA}_2$ , respectively. Data from the titration of cobra venom  $\text{PLA}_2$  did not cover high enough values for  $n$ , average moles of PPC bound, to calculate  $N$ . These values approach a 1:1 stoichiometry for the complexes. This stoichiometry should, however, be confirmed by an independent method.

If PPC binds to the active site, binding of PPC with similar affinity to pancreatic  $\text{PLA}_2$  and pro- $\text{PLA}_2$  is consistent with the fact that pro- $\text{PLA}_2$  has a functional site but a reduced affinity for lipid interfaces. The change in isoemissive point seen with pancreatic  $\text{PLA}_2$  above 0.45  $\mu\text{M}$  but not seen with pro- $\text{PLA}_2$  may indicate some additional interaction with another part of the enzyme in the former case.

Since PPC binds to  $\text{PLA}_2$  with a much greater affinity than does the substrate, one would expect to observe inhibition of  $\text{PLA}_2$  activity with PPC present. This is a complex situation, however, since PPC may also function as an activator of  $\text{PLA}_2$  from snake venoms. Davidson et al. (1986) observed activation of cobra venom  $\text{PLA}_2$  activity toward phosphatidylethanolamine-Triton mixed micelles by an *sn*-2 amide analogue of phosphatidylcholine. The same analogue inhibited  $\text{PLA}_2$  activity toward phosphatidylcholine-Triton mixed micelles. With interfacial substrate and an enzyme that is specific for zwitterionic lipids or is activated by zwitterionic lipids as in the case of cobra venom  $\text{PLA}_2$  (Hendrickson & Dennis, 1984b), PPC in the interface will enhance enzyme binding to the interface where it then experiences a much higher local concentration of substrate. This will result in activation. To the extent that PPC competes with substrate in the interface for binding to the enzyme active site, then inhibition will occur. This competition is dependent on the relative two-dimensional dissociation constants for enzyme-inhibitor or enzyme-substrate complexes within the interface. In the case of the venom  $\text{PLA}_2$ , they only differ by about 1 order of magnitude [for cobra venom  $\text{PLA}_2$ ,  $K_d$  for thio-PC- $\text{PLA}_2$  = 15 mol% (Hendrickson & Dennis, 1984a); a similar value was obtained for *C. adamanteus*  $\text{PLA}_2$  (Hendrickson and Hampton, unpublished results)]. In order to observe inhibition with PPC, we chose pancreatic  $\text{PLA}_2$ , an enzyme that prefers anionic substrates and appears to be activated by anionic lipids. In this case we observed half-inhibition at about 6  $\mu\text{M}$  PPC with cholate-thio-PC mixed micelles. Maximal inhibition, however, was only about 69%. These results illustrate the complexity of the system and the fact that an inhibitor that interacts reversibly with a phospholipase can act both as an inhibitor and as an activator depending on its presence in the interface.

A remarkable aspect of the interactions of PPC with  $\text{PLA}_2$  is the high affinity. Apparent dissociation constants ( $K_d$  values) for PPC- $\text{PLA}_2$  complexes calculated from fluorescence experiments ranged from 0.1 to 0.5  $\mu\text{M}$ . This is in contrast to dissociation constants for the thio-PC-cobra venom  $\text{PLA}_2$  complex of 0.1–0.2 mM calculated from kinetic data (Hendrickson & Dennis, 1984a) and dissociation constants for

phosphatidylcholine-PLA<sub>2</sub> complexes on the order of a few millimolar. Substitution of the more hydrophobic sulfur for oxygen at the *sn*-1 and *sn*-2 positions of glycerol accounts for the increased affinity for PLA<sub>2</sub> of thio-PC over phosphatidylcholine. In addition, the tetrahedral geometry of the carbon attached to the sulfur at the *sn*-2 position of PPC may resemble more the transition-state intermediate and result in some increased affinity. The most significant factor, however, may be an effect of the aromatic prodan ring. A rigid hydrophobic moiety, as compared to a flexible hydrocarbon chain, may be much more effective in close interaction with a hydrophobic portion of the protein. This would not be reflected in the free energy change for transfer from aqueous to nonpolar solvent since solvent molecules do not provide a rigid restricted binding site. Thus, a nonrigid molecule and a rigid molecule might have similar free energies of transfer from aqueous to nonpolar solvents [and similar hydrophobicities as defined by Tanford (1980)] but very different free energies for transfer to a hydrophobic site on a protein. Enhanced binding of a rigid hydrophobic moiety to a hydrophobic site on a protein could be a function of both entropy, in terms of restricted motion, and enthalpy, in terms of van der Waals interactions. This is a theoretical consideration that needs further development. In any case, the high-affinity binding of PPC at the active site of PLA<sub>2</sub> makes this a potentially useful probe for studies with this enzyme and also suggests some strategy in the design of high-affinity enzyme inhibitors. In addition, this probe may also be useful in studies with other phospholipid-binding proteins such as phospholipid transfer proteins.

#### ACKNOWLEDGMENTS

We thank Dr. Lynn Buffington, Carleton College, Northfield, MN, for running the NMR spectra on the Carleton College IBM 200-AF NMR spectrometer. We thank Dr. Franklyn G. Prendergast, The Mayo Foundation, Rochester, MN, for the gift of acrylodan and very helpful discussions during the course of this work. We thank Peter Callahan, The Mayo Foundation, for help in preparing the figures.

#### REFERENCES

- Birdsall, N. J. M., Feeney, J., Lee, A. G., Levine, Y. K., & Metcalf, J. C. (1971) *J. Chem. Soc.*, 1441-1445.
- Darke, P. L., Jarvis, A. A., Deems, R. A., & Dennis, E. A. (1980) *Biochim. Biophys. Acta* 626, 154-161.
- Davidson, F. F., Hajdu, J., & Dennis, E. A. (1986) *Biochem. Biophys. Res. Commun.* 137, 587-592.
- Dennis, E. A. (1983) *Enzymes (3rd Ed.)* 16, 307-353.
- Eaton, B. R., & Dennis, E. A. (1976) *Arch. Biochem. Biophys.* 176, 604-609.
- Hendrickson, H. S., & Dennis, E. A. (1984a) *J. Biol. Chem.* 259, 5734-5739.
- Hendrickson, H. S., & Dennis, E. A. (1984b) *J. Biol. Chem.* 259, 5740-5744.
- Hendrickson, H. S., Hendrickson, E. K., & Dybvig, R. H. (1983) *J. Lipid Res.* 24, 1532-1537.
- Hercules, D. M. (1966) *Fluorescence and Phosphorescence Analysis: Principles and Applications*, pp 60-61, Wiley-Interscience, New York.
- Knack, I., & Röhm, K.-H. (1981) *Hoppe-Seyler's Z. Physiol. Chem.* 362, 1119-1130.
- Nieuwenhuizen, W., Kunze, H., & de Haas, G. H. (1974) *Methods Enzymol.* 32, 147-154.
- Prendergast, F. G., Meyer, M., Carlson, G. L., Iida, S., & Potter, J. D. (1983) *J. Biol. Chem.* 258, 7541-7544.
- Smith, C. M., & Wells, M. A. (1971) *Biochim. Biophys. Acta* 663, 687-694.
- Tanford, C. (1980) *The Hydrophobic Effect*, 2nd ed., Chapter 2, Wiley-Interscience, New York.
- Verheij, H. M., Slotboom, A. J., & de Haas, G. H. (1981) *Rev. Physiol. Biochem. Pharmacol.* 91, 91-203.
- Volwerk, J. J., Pieterse, W. A., & de Haas, G. H. (1974) *Biochemistry* 13, 1446-1454.
- Weber, G., & Farris, F. J. (1979) *Biochemistry* 18, 3075-3078.
- Wells, M. A. (1975) *Biochim. Biophys. Acta* 380, 501-505.
- Wells, M. A., & Hanahan, D. J. (1969) *Biochemistry* 8, 414-424.