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Synthesis of Prodan–Phosphatidylcholine, a New Fluorescent Probe, and Its Interactions with Pancreatic and Snake Venom Phospholipases A_2†

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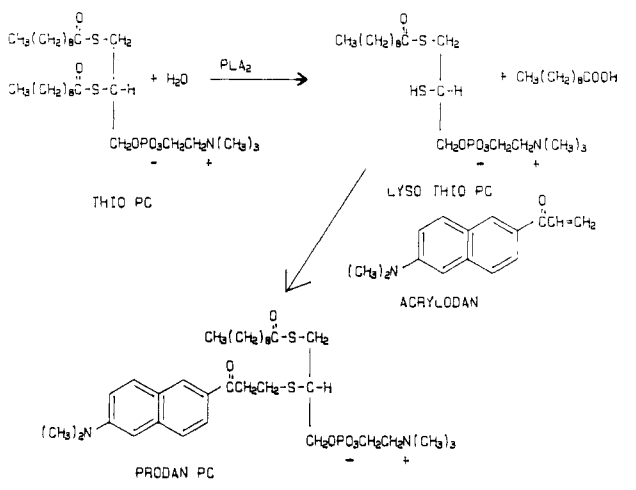
ABSTRACT: A new fluorescent probe, prodan–PC, was synthesized by incubating thio-PC, a thiol ester analogue of phosphatidylcholine [1,2-bis(decanoylthio)-1,2-dideoxy-*sn*-glycero-3-phosphocholine], with acrylodan, a fluorescent thiol-reactive reagent [6-acryloyl-2-(dimethylamino)naphthalene], in the presence of phospholipase A_2, 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_2, 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_2 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_2 from *Naja naja naja* and porcine pancreas and the prophospholipase A_2 from porcine pancreas were 0.509, 0.107, and 0.114 μM, respectively. PPC was shown to inhibit the activity of pancreatic phospholipase A_2 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_2 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)[1] as a substrate for a continuous spectrophotometric assay of phospholipase A_2 (PLA_2) (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_2 in the presence of acrylodan (Scheme I). The new fluorescent probe, prodan–PC (PPC), seemed attractive for the study of phosphatidylcholine interaction with PLA_2. 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_2. PLA_2 from porcine pancreas has a single tryptophan in or near the active site. PLA_2 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 surrounding the active site (Verheij et al., 1981). Here we report the synthesis of prodan–PC, some of its properties, and some

Scheme I



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[1] Abbreviations: PC, phosphatidylcholine; thio-PC, 1,2-bis(decanoylthio)-1,2-dideoxy-*sn*-glycero-3-phosphocholine; PLA_2, phospholipase A_2; 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.

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interactions with PLA_2 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(decanoylthio)-1,2-dideoxy-*sn*-glycero-3-phosphorylcholine (thio-PC) was synthesized as described for the bisheptanoyl homologue by Hendrickson et al. (1983). PLA_2 was purified from *Crotalus adamanteus* venom according to the method of Wells (1975). PLA_2 and pro PLA_2 were purified from porcine pancreas by the method of Nieuwenhuizen et al. (1974). PLA_2 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 ϵ_{280} = 1.23 mL mg^{-1} cm^{-1} and ϵ_{280} = 1.30 mL mg^{-1} cm^{-1} for the pancreatic PLA_2 and pro-PLA_2, respectively (Nieuwenhuizen et al., 1974), ϵ_{278} = 2.20 mL mg^{-1} cm^{-1} for the cobra venom PLA_2 (Darke et al., 1980), and ϵ_{280} = 2.27 mL mg^{-1} cm^{-1} for the *C. adamanteus* PLA_2 (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_2. To a solution of 3.1 mg of *C. adamanteus* PLA_2 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 μL 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 $CaCl_2$, 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 Kinfit (Knack & Röhm, 1981):

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

where ΔF and ΔF_{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, ΔF and [P], is entered into the computer along with an estimate of h. The program then calculates an estimate of ΔF_{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 algorithum. 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_2 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 [PPC]:

$$n = N[PPC]/(K_d + [PPC])$$

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

Synthesis of Prodan–Phosphatidylcholine (PPC). Acrylodan (20 mg, 89 μmol) and 80 μmol of thio-PC in $CHCl_3$ 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_2 (200 μL 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 × 50 cm silica gel (Analtech 10050, 10 μm) column with hexane–2-propanol–water (6:8:1.6) at a flow rate of 10 mL/min. Detection was accomplished by both fluorescence (ex = 370 nm, em > 430 nm) and UV absorption at 235 nm.

Assay of PLA_2 Activity. A spectrophotometric assay of porcine pancreatic PLA_2 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 $CaCl_2$, 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_2 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 M^{-1} 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

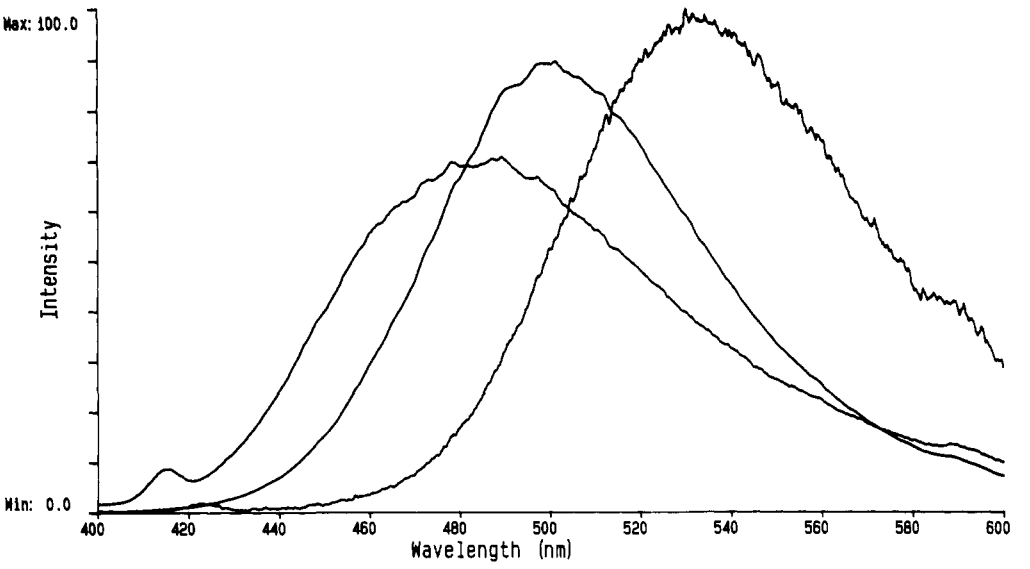

FIGURE 1: Corrected fluorescence emission spectra of PPC. Peak at 530 nm: 0.928 μM PPC in water; slit widths, 2 nm/2 nm; relative intensity scale, 0–1. Peak at 498 nm: 1.1 μM PPC in ethanol; slit widths, 5 nm/5 nm; relative intensity scale, 0–100. Peak at 484 nm: 1.1 μM PPC in hexane; slit widths, 5 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 $-CH_3$, a multiplet at 1.15–1.17 ppm for the acyl C-4 to C-9 $-CH_2-$, a multiplet at 1.46–1.56 ppm for the acyl C-3 $-CH_2-$, a triplet (7.6 Hz) at 2.43 ppm for the acyl C-2 $-CH_2CO-$, a triplet (5.9 Hz) at 2.96 ppm for the Ar $COCH_2-$ [quartet (7.3 Hz) at 3.06 ppm for prodan], a singlet at 3.02 ppm for the $N(CH_3)_3$, a singlet at 3.10 ppm for the Ar $N(CH_3)_2$ (singlet at 3.08 ppm for the prodan), a multiplet at 3.3–3.4 ppm for the choline $-CH_2N$, a multiplet at 3.4–3.5 ppm for the glycerol *sn*-3 $-CH_2-$, doublets (10.6 Hz) of doublets (5.5 Hz) at 3.88 and 4.04 ppm for the nonequivalent glycerol *sn*-1 $-CH_2-$, 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 $-CH_2SCH$ are not identified but may be in the region of 3.1–3.2 ppm. Solvent peaks include OH at 3.22 ppm, HC-D_2OD [quintet (1.6 Hz)] at 3.29 ppm, and $CHCl_3$ 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 μM and a smaller break around 1 μM.

Interactions of PPC with C. adamanteus PLA$_2$. PPC in the presence of a saturating amount of *C. adamanteus* PLA_2 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_2 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_2 active site was blocked by reaction with *p*-bromophenacyl bromide. Titration of PLA_2 into 2.78 μM PPC (Figure 3B) showed a biphasic increase in emission intensity (>450 nm): a plateau at about 1 μM followed by an increase up to 5 μM PLA_2. Steady-state anisotropy measurements in the same experiment showed a single hyperbolic increase that followed the initial increase in fluorescence intensity.

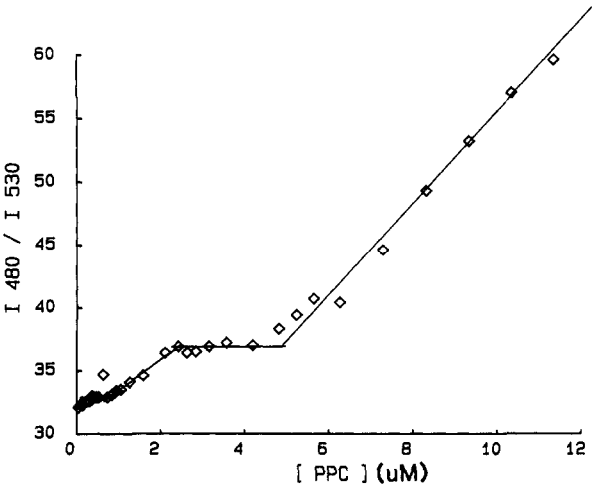

FIGURE 2: Determination of cmc for PPC. Ratio of emission intensities at 480 nm/530 nm vs. concentration of PPC (μM) in standard buffer. Excitation at 370 nm; slit widths, 5 nm/5 nm.

Table I: Apparent Dissociation Constants and Stoichiometries of Complexes of PPC with Phospholipases A_2 from Various Sources

enzyme source	K_d (μM)	N (mol of PPC/mol of PLA_2)
C. adamanteus	0.323 ± 0.042	nd[a]
C. adamanteus[b]	0.361 ± 0.096	nd[a]
N. naja naja	0.509 ± 0.006	nd[a]
porcine pancreas	0.107 ± 0.007	0.64 ± 0.08
porcine pancreas (pro)[c]	0.114 ± 0.009	0.87 ± 0.20

[a] Not determined. Data were not sufficient to calculate N. [b] With Triton X-100R micelles (PPC/Triton, 1:100 mol ratio). [c] Pro-PLA_2.

Binding of PPC with *C. adamanteus* PLA_2 could also be followed by energy transfer as indicated by quenching of the tryptophan fluorescence of PLA_2 by PPC. Excitation of PLA_2 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_2 (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 μM

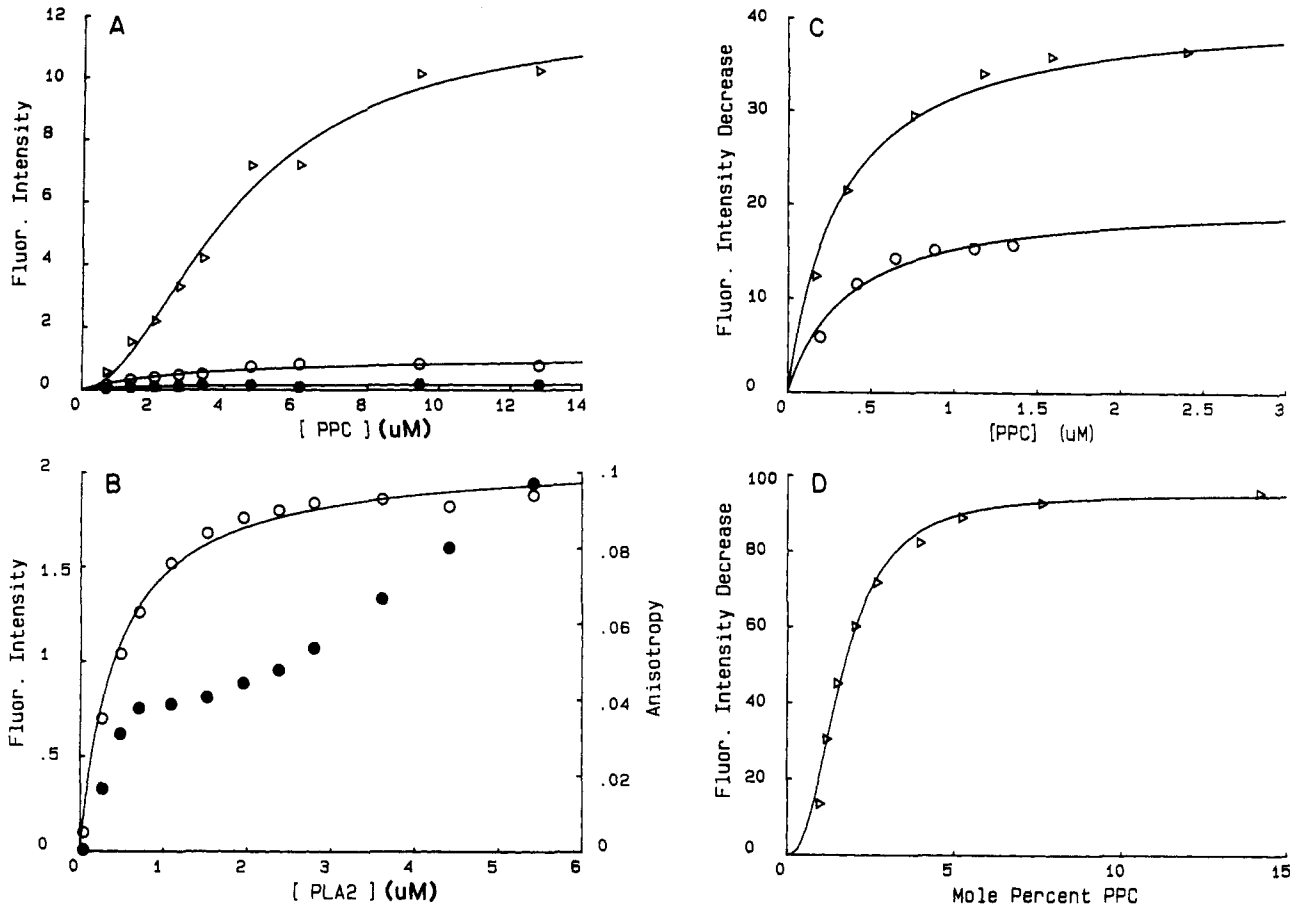

FIGURE 3: Interactions of PPC with *C. adamanteus* PLA_2. (A) Titration of PPC into *C. adamanteus* PLA_2. Increase in fluorescence emission intensity at 480 nm vs. total concentration of PPC with 3.06 μM PLA_2 present. (Open triangles) In standard buffer with 10 mM $CaCl_2$; (open circles) in standard buffer with 1 mM EDTA and no $CaCl_2$; (closed hexagons) PLA_2 blocked with *p*-bromophenylacyl bromide, in standard buffer with 10 mM $CaCl_2$. Excitation at 370 nm. (B) Titration of *C. adamanteus* PLA_2 into 2.78 μM PPC. Total concentration of PLA_2. (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_2 was 0.058. Excitation at 370 nm. (C) Titration of PPC into *C. adamanteus* PLA_2 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 μM PLA_2; (circles) titration of PPC–Triton at a constant mole ratio of 1:100 (PPC/Triton) into 0.161 μM PLA_2 containing the cmc (0.25 mM) of Triton. (D) Interaction of *C. adamanteus* PLA_2 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 μM PLA_2, 5.22 μM 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_2 to PPC in reduced Triton X-100 micelles was determined by titration of PPC–Triton (1:100 mol ratio) into 0.161 μM PLA_2 containing the cmc (0.25 mM) of Triton (Figure 3C). The K_d value calculated from this titration [K_d = 0.361 μM (Table I)] was similar to that calculated in the absence of Triton micelles. Binding of PPC to PLA_2 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_2. This was accomplished by titrating Triton into a solution of 5.22 μM PPC, 0.160 μM PLA_2, 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

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

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

Interactions of PPC with Cobra Venom PLA_2. Titration of cobra venom PLA_2 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_2 concentration (Figure 4A). A K_d value equal to 0.509 μM (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_2 and Pro-PLA_2. Titration of porcine pancreatic PLA_2 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_2 up to 0.45 μM. At higher concentrations the isoemissive point was not well-defined but decreased steadily to about 510 nm. A hyperbolic increase in emission intensity

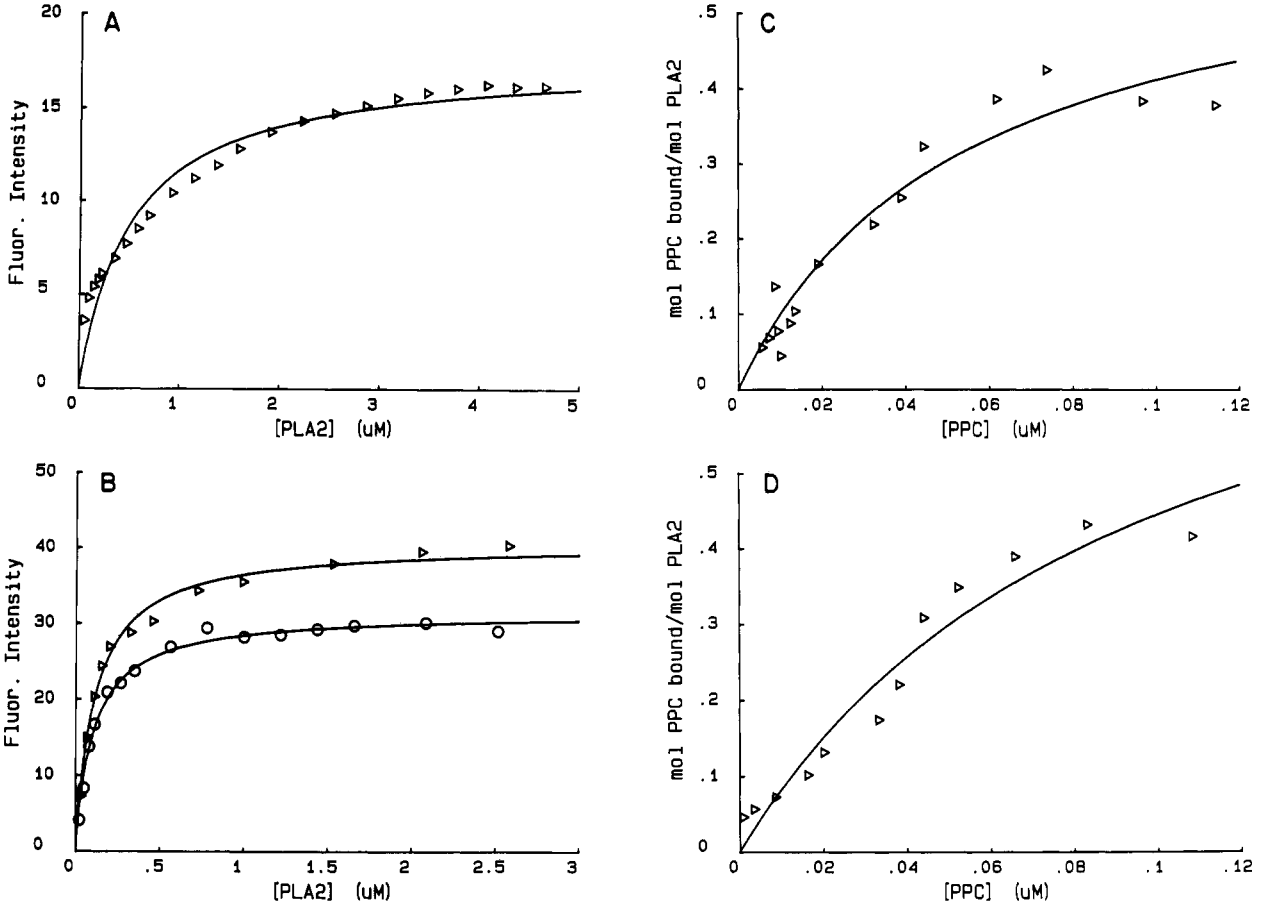

FIGURE 4: Interactions of PPC with cobra venom and pancreatic PLA_2. (A) Titration of cobra venom PLA_2 into 0.441 μM PPC. Concentration of free PLA_2. Increase in fluorescence emission intensity at 471 nm taken from corrected difference spectra. Excitation at 370 nm (slit widths: 5 nm/5 nm). (B) Binding of porcine pancreatic PLA_2 and pro-PLA_2 to PPC. Titration of PLA_2 into 0.132 μM PPC. Concentration of free PLA_2. Increase in fluorescence emission intensities at 474 nm taken from corrected difference spectra. (Triangles) Pro-PLA_2; (circles) PLA_2. Excitation at 370 nm (slit widths: 6 nm/6 nm). (C) Binding of PPC to porcine pancreatic PLA_2. Recalculation from (B). Nonlinear regression computer fit. (D) Binding of PPC to porcine pancreatic pro-PLA_2. Recalculation from (B). Nonlinear regression computer fit.

at 480 nm was observed as a function of PLA_2 concentration (Figure 4B). A K_d value equal to 0.107 μM (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 PLA_2 concentration.

Titration of the zymogen, pro-PLA_2, into PPC resulted in a hyperbolic increase in emission intensity at 474 nm (Figure 4B). Overlays of difference emission spectra showed a single isoemissive point at about 510 nm. The K_d value calculated from this titration was equal to 0.114 μM (Table I). The maximum moles of PPC bound per mole of pro-PLA_2 was calculated to be 0.87 (Figure 4D, Table I).

Inhibition of PLA_2 Activity by PPC. The inhibition of porcine pancreatic 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 (±0.02) and half-maximal inhibition at 6.38 μM (±0.76) PPC. With the cobra venom enzyme, PPC was observed to give little or no inhibition of PLA_2 activity. For example, in thio-PC–Triton mixed micelles (1:25 mol ratio, 0.3 mM thio-PC), addition of PPC up to 80 μM resulted in no significant inhibition (data not shown).

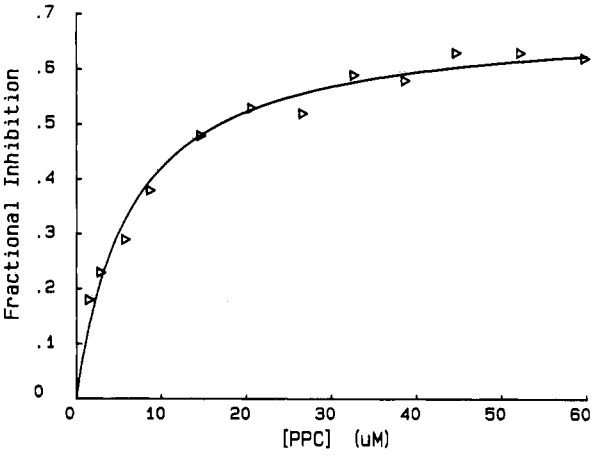

FIGURE 5: Inhibition of activity of porcine pancreatic PLA_2 by PPC. Thio-PC (0.5 mM) with 2.5 mM sodium cholate and varying amounts of PPC. The activity of the uninhibited reaction was 171 μmol min^{-1} mg^{-1}. Assay conditions described in text.

Discussion

PPC is conveniently synthesized from acrylodan and lyso-thio-PC, the latter generated in situ by the action of PLA_2 on thio-PC. Like prodan, its emission maximum is quite sensitive to the polarity of the environment. It ranges from 530 nm in water to 498 nm in ethanol. In hexane there is a broad peak at 484 nm with a small peak at 416 nm. Emission at 484 nm may be due to reverse micelles since there is some residual water in the sample. The small-peak at 416 nm may be due to monomolecular PPC at the cmc since Weber and Farris (1979) reported an emission maximum of 401 nm for prodan in cyclohexane. PPC appears to have a definite cmc at about 5 μM and shows evidence of premicellar aggregation at about 1 μM (Figure 2). In most of the titrations of PLA_2 into PPC,

the PPC concentration was kept well below 1 μM (in the monomolecular region) to avoid complications due to interfacial effects.

Binding of PPC to 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* 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 PLA_2, where there is a high ratio of PPC to PLA_2, more than one PPC molecule bound to an enzyme dimer may result in self-quenching. At higher concentrations of 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 μM PLA_2. Fluorescence anisotropy, which is also an indication of bindig of PPC to protein, coincides with the initial increase in fluorescence emission.

Binding of PPC to *C. adamanteus* PLA_2 can also be followed by quenching of the tryptophan fluorescence of the protein. In this way, binding of PPC to PLA_2 in the presence and absence of Triton X-100R micelles was determined. Similar K_d values were calculated for the PPC–PLA_2 complex in the presence and absence of micelles (Table I). The initial binding of PLA_2 to PPC thus appears to be independent of the presence of interace. Once the *C. adamanteus* PLA_2 is bound to the interace, 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 PLA_2 in the micelle surface may occur in a region surrounding the active site of PLA_2 at the higher local concentration of PPC experienced in the interace. 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 PLA_2 observed as a function of interfacial substrate concentration (Hendrickson & Dennis, 1984a). In the latter case, the enzymatic activity of 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 PLA_2 action (Dennis, 1983), where 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 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 prescence of enzyme yielded a series of difference spectra. For the cobra venom PLA_2 and the pancreatic pro-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–PLA_2 complex., In the case of the active pancreatic PLA_2, a single isoemissive point was seen only up to 0.45 μM PLA_2. Beyond this concentration the isoemissive point drifted to lower wavelengths. This may indicate more than one PPC–PLA_2 complex above that concentration. Titration of *C. adamanteus* 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–PLA_2 complex was calculated from fluorescence data of the titration of PLA_2 into PPC. Values of N, maximum moles of PPC bound per mole of PLA_2, were 0.64 and 0.87 for complexes with pancreatic PLA_2 and pancreatic pro-PLA_2, respectively. Data from the titration of cobra venom 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 PLA_2 and pro-PLA_2 is consistent with the fact that pro-PLA_2 has an functional site but a reduced affinity for lipid interfaces. The change in isoemissive point seen with pancreatic PLA_2 above 0.45 μM but not seen with pro-PLA_2 may indicate some additional interaction with another part of the enzyme in the former case.

Since PPC binds to PLA_2 with a much greater affinity than does the substrate, one would expect to observe inhibition of PLA_2 activity with PPC present. This is a complex situation, however, since PPC may also function as an activator of PLA_2 from snake venoms. Davidson et al. (1986) observed activation of cobra venom PLA_2 activity toward phosphatidylethanolamine–Triton mixed micelles by an *sn*-2 amide analogue of phosphatidylcholine. The same analogue inhibited 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 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 PLA_2, they only differ by about 1 order of magnitude [for corbra venom PLA_2, K_d for thio-PC–PLA_2 = 15 mol% (Hendrickson & Dennis, 1984a); a similar value was obtained for *C. adamanteus* PLA_2 (Hendrickson and Hampton, unpublished results)]. In order to observe inhibition with PPC, we chose pancreatic 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 μM PPC with cholate–thio-PC mixec 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 PLA_2 is the high affinity. Apparent dissociation constants (K_d values) for PPC–PLA_2 complexes calculated from fluorescence experiments ranged from 0.1 to 0.5 μM. This is in contrast to dissociation constants for the thio-PC–cobra venom PLA_2 complex of 0.1–0.2 mM calculated from kinetic data (Hendrickson & Dennis, 1984a) and dissociation constants for

phosphatidylcholine–PLA_2 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_2 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_2 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.

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