Polymerizable Phosphatidylcholines: Importance of Phospholipid Motions for Optimum Phospholipase A_2 and C Activity†

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Received March 12, 1993; Revised Manuscript Received June 7, 1993®

ABSTRACT: Cross-linkable short-chain phosphatidylcholines with thiols at the chain terminus have been synthesized and characterized. These micelle-forming species were used to investigate two water-soluble phospholipases. When reduced, the thiol lipids were excellent substrates for phospholipase A_2. Once cross-linked, they became extremely poor substrates. This is consistent with a mechanism in which a key step is the partial extraction of the substrate phosphatidylcholine from an aggregate. In contrast, phospholipase C activity was slightly enhanced if the product diglyceride was tethered to the aggregate through disulfide formation. For this enzyme such a kinetic effect is consistent with the hydrophobic diglyceride biasing the enzyme to the interface.

Water-soluble phospholipases must bind to substrate at an interface before the appropriate ester bond can be hydrolyzed. As recent phospholipase A_2 crystal structures show, there is a hydrophobic pocket near the active center where the monomeric substrate is anchored (White et al., 1990; Scott et al., 1990; Thunnissen et al., 1990). It has been proposed that when phospholipase A_2 binds to an aggregate surface, the substrate must leave the aggregate matrix to some degree and enter the pocket (White et al., 1990). This could then be a key component of the observed kinetic "interfacial activation" of this enzyme toward aggregated substrate (Scott et al., 1990). An alternative explanation of the rate increases observed toward micellar substrate is that the enzyme acts in a processive or scooting mode without desorbing from the surface (Berg et al., 1991; Jain et al., 1991a,b). Even with this interpretation, the enzyme must extract the lipid from the aggregate to some degree to position it in the active site. The kinetic relevance of such lipid vertical motions to phospholipase A_2 action is unknown. For phospholipase C from *Bacillus cereus* even less is known about what is critical in interfacial behavior. Although a crystal structure exists for phospholipase C (Hough et al., 1989), there is considerable ambiguity about how phospholipid is bound to the enzyme. Perhaps for this interfacially active enzyme as well as phospholipase A_2 a large part of the substrate must be removed from the aggregate prior to hydrolysis by the enzyme.

To address the importance of phospholipid extraction or isolation from an interface, we have synthesized a PC[1] analogue, 1,2-bis(8-mercaptooctanoyl)-*sn*-glycero-3-phosphocholine (diC_8SH-PC), which can be polymerized at the terminus of the acyl chains in the micellar state through the formation of –S–S– cross-links. For further investigation of the importance of access of the PC for phospholipase activity, two asymmetric PC species which can form *sn*-2 linked PC dimers, 1-hexanoyl-2-(8-mercaptooctanoyl)PC (1-C_6-2-C_8SH-PC) and 1-hexanoyl-2-(10-mercaptodecanoyl)PC (1-C_6-2-C_{10}SH-PC), and 1-(8-mercaptooctanyl)-2-hexanoyl-PC (1-C_8SH-2-C_6PC), which upon cross-linking forms an *sn*-1 linked dimer, were also synthesized. These molecules and their cross-linked products are schematically shown in Figure 1. In the cross-linked dimers the molecules are linked via a disulfide at the terminus of the fatty acyl chains. The cross-link is significantly removed from the region of the phospholipid interacting with the enzymes. These substrates should show different enzyme kinetics if the substrate must be extracted (either partially or fully) from the micelle and bound to the enzyme prior to the catalytic step.

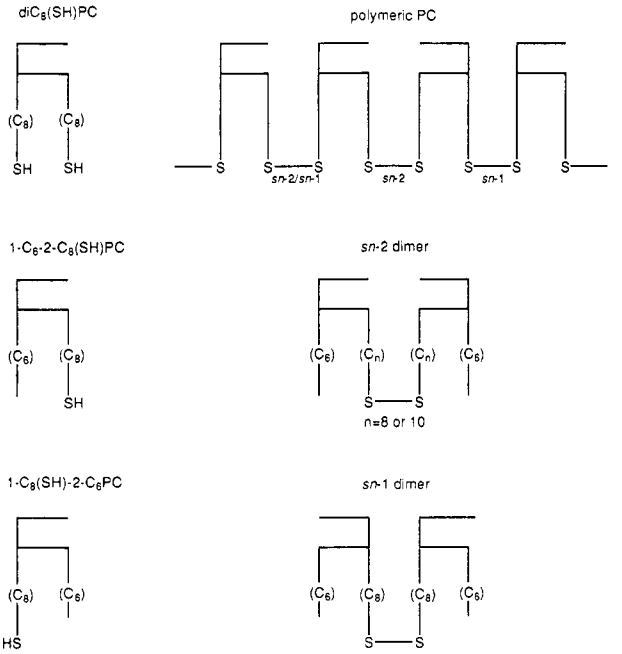

FIGURE 1: Schematic structures of thiol PCs and their cross-linked species.

MATERIALS AND METHODS

Chemicals. All chemicals and reagents were obtained commercially and used without further purification unless

† This work was supported by NIH GM 26762.

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® Abstract published in *Advance ACS Abstracts,* September 1, 1993.

[1] Abbreviations: PC, phosphatidylcholine; diC_nPC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; diC_8SH-PC, 1,2-bis(8-mercaptooctanoyl)-*sn*-glycero-3-phosphocholine; 1-C_6-2-C_8SH-PC, 1-hexanoyl-2-(8-mercaptooctanoyl)-*sn*-glycero-3-phosphocholine; 1-C_6-2-C_{10}SH-PC, 1-hexanoyl-2-(10-mercaptodecanoyl)-*sn*-glycero-3-phosphocholine; 1-C_8SH-2-C_6PC, 1-(8-mercaptooctanoyl)-2-hexanoyl-PC; cmc, critical micelle concentration; T_1, spin–lattice relaxation time; QLS, quasi-elastic light scattering; DTT, dithiothreitol.

0006-2960/93/0432-9545$04.00/0

stated otherwise. Diheptanoyl-PC and 1-hexanoyl-PC were obtained from Avanti Polar Lipids either in chloroform or as a solid powder. Dithiothreitol (99%), hydrogen peroxide (30%), catalase (bovine liver), 8-bromooctanoic acid, 10-bromodecanoic acid, thiourea, and 4-(dimethylamino)pyridine were obtained from Aldrich. *N,N'*-Dicyclohexylcarbodiimide and *sn*-glycero-3-phosphorylcholine ($CdCl_2$ complex) were obtained from Sigma. Phospholipase A_2 (*Naja naja naja*) and C (*Bacillus cereus*) were obtained as powders from Sigma. Chloroform and methanol were of HPLC grade from Aldrich and Fluka. Deionized water (10 megohms·cm) doubly distilled from potassium permanganate was used in surface tension measurements and in making certain lipid solutions.

Synthesis of 1,2-Bis[8-(ethyldithio)octanoyl]-sn-glycero-3-phosphocholine (diC8SSE-PC). The synthesis of this compound followed the procedure of Samuel et al. (1985). All of the reagents were dried over P_2O_5 in vacuum for a week, and chloroform was refluxed over P_2O_5 for more than 3 h and then distilled. Commercially available 8-bromooctanoic acid upon treatment with thiourea and absolute ethanol was converted to 8-mercaptooctanoic acid. The protecting agent ethyl ethanethiosulfinate (initially obtained from Dr. Tracey M. Handel, California Institute of Technology, and then from Dr. Anthony Curtis, Boston College) and triethylamine were then added to the acid to yield the protected thiol acid [8-(ethyldithio)octanoic acid]. Glycero-3-phosphorylcholine was added to a chloroform solution of 8-(ethyldithio)octanoic acid activated with a mixture of *N,N'*-dicyclohexylcarbodiimide and 4-(dimethylamino)pyridine. The mixture was stirred in the dark under argon at room temperature for a week. The solution was subsequently mixed with Dowex 50-X8–100 resin. This slurry was then filtered within 30 min. After evaporation of the solvent at reduced pressure, the material was loaded onto a silicic acid column with a chloroform/methanol solvent system. TLC showed only one spot for the collected material. A ^1H NMR spectrum of the species of $CDCl_3$ confirmed its identity with the published data (Samuel et al., 1985).

Synthesis of 1,2-Bis(8-mercaptooctanoyl)-sn-glycero-3-phosphocholine (diC8SH-PC). DiC_8SSE-PC was deprotected by thiol exchange between dithiothreitol (DTT) and the protected lipid. Generally, 50 mg of the short-chain lipid was dissolved in 2 mL of ethanol with about 1000 mg of DTT. The solution was degassed before and after the solutes were added by blowing Ar gas through it for several minutes. The mixture then was sealed immediately and kept in the dark at around 40–45 °C for several days. The incubation time for the deprotection reaction was critical. If this incubation was too long, e.g., 6 days, a small amount of lipid was produced with both sulfhydryls cleaved (resulting in a terminal $-CH_3$ resonance detected at 0.8 ppm in the ^1H NMR spectrum of the mixture in $CDCl_3$). If the time for the reaction was too short, e.g., 2 days, the exchange of HS– group was not complete as detected by ^1H NMR. The optimized time for the deprotection reaction of this compound was between 3 and 4 days. At the end of this incubation time, the solvent was evaporated by a stream of Ar or by rotary evaporation. The residue was then chromatographed under an Ar atmosphere in a glovebox on a silicic acid column with a chloroform/methanol linear gradient. The deprotected short-chain PC (diC_8SH-PC) eluted near pure methanol.

Synthesis of 1-Hexanoyl-2-(8-mercaptooctanoyl)PC, 1-Hexanoyl-2-(10-mercaptodecanoyl)PC, and 1-(Mercaptooctanoyl)-2-hexanoyl-PC. The syntheses of the first two asymmetric compounds followed the procedure of Samuel et al. (1985) using 1-hexanoyl-PC and the appropriate protected thiol fatty acid [10-(ethyldithio)decanoic acid was synthesized from 10-bromodecanoic acid as described above for the 8-carbon compound] activated with *N,N'*-dicyclohexylcarbodiimide and 4-(dimethylamino)pyridine. Reaction mixtures were stirred for 48 h at 37 °C in the dark. After removal of the solvent by rotary evaporation, the residue was applied to an AGMP-50 resin (50–100 mesh, hydrogen form) to remove the catalyst. Resulting fractions were combined and passed over a silicic acid column. The identity of the protected thiol phospholipids was confirmed by ^1H and ^{13}C NMR spectroscopy (Burns & Roberts, 1980). Both $1\text{-}C_6\text{-}2\text{-}C_8$SSE-PC and $1\text{-}C_6\text{-}2\text{-}C_{10}$SSE-PC were deprotected by incubation with excess DTT for 3–4 days. Excess DTT was removed by passage of the incubation mixture over a silicic acid column kept in an anaerobic environment. Fractions containing the deprotected PC were combined and stored anaerobically. The other isomer, 1-(8-mercaptooctanoyl)-2-hexanoyl-PC ($1\text{-}C_8\text{SH-}2\text{-}C_6$PC), was prepared from 1,2-bis[8-(ethyldithio)octanoyl]PC which was treated with cobra venom phospholipase A_2 to produce the lyso species. The $1\text{-}C_8$SH-PC was reacylated with hexanoic acid. After deprotection with DTT and removal of excess reducing agent, the thiol phospholipid solution was stored anaerobically at 4 °C.

Cross-Linking of Thiol Phospholipids. For polymerization, 0.064 mL of H_2O_2 (30%) was added to 1 mL of an aqueous solution of diC_8SH-PC (25 mM). The reaction mixture was stirred at 45 °C for 6 h. With this polymerized material, two methods were used to remove unreacted H_2O_2: (i) repeated lyophilization of the cross-linked material and (ii) incubation with catalase. For the first, the solvent was blown off, 2 mL of water added, and the sample lyophilized. The hydration/lyophilization procedure was repeated three times. H_2O_2 and H_2O have an azeotropic point at about 30%. Hence, three hydration/lyophilizations should remove all of the unreacted H_2O_2. An alternate method for removing the H_2O_2 was to add excess catalase (Sigma) to the aqueous solution. This was done directly to pH-stat samples and had no effect on the phospholipase activity.

Synthesis of 1-Hexanoyl-2-(1-pyrenebutyroyl)-PC. 1-Hexanoyl-2-(1-pyrenebutyroyl)-PC ($1\text{-}C_6\text{-}2\text{-}C_4$Pyr-PC) was synthesized from 1-hexanoyl-PC and the corresponding pyrene fatty acid using 1,1'-carbonyldiimidazole (Aldrich) as described previously (Burns & Roberts, 1980). ^{13}C chemical shifts of the pure short-chain species are diagnostic as to the identity of the fatty acids on the *sn*-1 and *sn*-2 positions and were used to confirm the identity of the asymmetric pyrene-labeled PC. The ^{13}C chemical shifts (in $CDCl_3$, referenced to the central peak of ^{13}C in $CDCl_3$ as 77.00 ppm) for *sn*-1 chain carbons of this compound include 33.94, C(2); 26.80, C(3); 32.56, C(4); 22.14, C(5); and 13.76, C(6). Shifts (parts per million) for the *sn*-2 chain carbons include 33.99, C(2); 24.44, C(3); 31.12, C(4); and a complex aromatic pattern from 135.64 to 124.83 ppm. (Thirteen resonances belonging to the pyrene ring include the following: 135.64, 131.34, 130.81, 129.92, 128.63, 126.69, 125.87, and 123.22, all tertiary carbons, and 127.42, 127.33, 124.93, 124.83, and 124.79, representing CH species. Carbonyl resonances were observed at 173.50 and 172.78 ppm. Shifts for backbone and headgroup carbons were the same as in other short-chain PC species (Burns & Roberts, 1980).

Surface Tension Measurement. Both the drop volume method, as described previously (Bian & Roberts, 1991), and the ring method using a DeNuoy tensiometer were used to measure surface tensions of reduced and cross-linked PC

species. In the determination of the surface tension measurements for the thiol species, 2 mM DTT was added to the samples to keep the substrates in the reduced form.

pH-Stat Assays. Activities of phospholipase A_2 (*N. naja naja*) and phospholipase C (*B. cereus*) toward these short-chain lecithins were measured by the pH-stat technique (Dennis, 1973) with NaOH as the titrant and an endpoint of pH 8. The instrument was calibrated daily with 2.45 mM potassium acid phthalate to determine exact base concentration. Base concentration was kept at 3 or 0.5 mM NaOH, depending on the substrate concentration, and the base chamber was protected from CO_2 absorption with ascarite (Fluka) to prevent bicarbonate formation. Protein concentrations of stock phospholipase solutions were determined by Bradford (1976) assay. Lecithin sample concentrations were determined by phosphorus assay (Chen et al., 1956; Turner & Rouser, 1970). Most of the experiments were run in duplicate or triplicate and kept under a N_2 atmosphere. $CaCl_2$ was added to a concentration of 1 mM for all runs with phospholipase A_2. The enzyme specific activity toward 5 mM diC_7PC at pH 8 was 2250 μmol min^{-1} mg^{-1} for phospholipase A_2 and 1500 μmol min^{-1} mg^{-1} for phospholipase C (El-Sayed & Roberts, 1985; Gabriel et al., 1987). For the kinetic analyses of the reduced thiols, a stream of argon was passed into the titration vessel to keep the samples in the reduced form.

Quasi-Elastic Light Scattering. The average hydrodynamic radii of diC_8SH-PC micelles were measured in a QLS system with a Brookhaven Instruments digital correlator and a 50 mW He–Ne laser as the light source (632.8 nm) in the Nuclear Engineering Department, Massachusetts Institute of Technology. Samples were filtered through a Millex-GV 0.22-mm filter (Millipore Corp.) into small (1-mL capacity) test tubes. The sample was incubated for ~5 min prior to data acquisition. The diffusion coefficient was extracted as the time constant in an exponential fit of the decay curve. An average hydrodynamic radius, R_H, was calculated from the measured diffusion coefficient, D, using the Stokes–Einstein relation [$D = kT/6\eta R_H$, where T is the absolute temperature and η is the solvent viscosity (Eum et al., 1989)].

NMR Spectroscopy. Natural abundance ^1H WALTZ decoupled ^{13}C natural abundance NMR spectra (125.7 MHz) were obtained for samples dissolved in 0.5 mL of D_2O. T_1 relaxation times were measured with an inversion–recovery sequence on a Varian Unity 500-MHz spectrometer using a 5-mm broadband probe with a 25-kHz sweep width, 32 768 data points, 12-μs (90°) pulse width, 1.0-s recycle delay, 8 τ values (time between the 180 and 90 °C pulses), and 200 transients per τ value. Free induction decays were processed with a 4-Hz line broadening.

Fluorescence. Fluorescence measurements were performed with a Shimadzu RF5000V spectrofluorometer. To excite the short-chain pyrene-PC probe, the excitation wavelength was set at 352 nm, and the emission was monitored from 360 to 550 nm. Almost all of the fluorescence information for both excimer and monomer bands was included in this range. The excitation bandwidth was 1.5–3 nm, and the emission bandwidth was 3 nm or larger.

RESULTS

Physical Properties of Thiol PCs. The terminal thiol PCs behaved much like the comparable saturated short-chain PCs with respect to cmc. DiC_8SH-PC had a cmc of 0.8 mM, slightly higher than that of diC_8PC. DiC_8PC phase-separates above the cmc; diC_8SH-PC does not phase-separate but formed very large micelles which grew with increasing PC concentrations (Figure 2). There was no detectable change in the average hydrodynamic radius, R_H, upon cross-linking, indicating no large change in aggregate morphology. The asymmetric thiol PCs had cmc values which were also close to the values for the symmetric saturated PCs. 1-C_6-2-C_8-SH-PC has a total of 14 carbons and might be considered comparable to diC_7PC in hydrophobicity. The cmc of the reduced thiol PC (2.0 mM) was comparable to that of diheptanoyl-PC (1.5 mM) (Bian & Roberts, 1992). In a similar fashion, the 16-carbon asymmetric thiol PC had a cmc similar to that of diC_8PC (Table I). In contrast to these reduced lipids, the disulfide-linked dimers had cmc values much lower than expected. For symmetric PCs, the observed cmc is higher than would be expected for a lyso-PC with the same total number of acyl chain carbons (Bian & Roberts, 1992), indicating that in the diacyl species the chains pack together to exclude water. While the reduced thiol species exhibited this behavior, the considerably lower cmc of the cross-linked dimers suggested that with the two headgroups effectively linked via a dicarboxylic acid moiety, the four fatty acyl chains cannot pack well together. More of the hydrocarbon chain area must be in contact with water, thus lowering the cmc. Minimum areas at the air–water interface per phosphocholine moiety (Table I) were also larger for both reduced and dimeric PC (85 ± 6 Å^2) phosphocholine moieties compared to those of diacyl-PCs (50–60 Å^2) (Bian & Roberts, 1992). This indicates that the sulfur atoms at the end of the acyl chains have some influence on molecular packing at the air–water interface, but the surface area is only dependent on

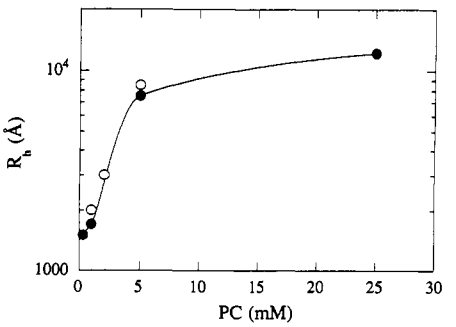

FIGURE 2: R_H (determined by QLS) as a function of diC_8SH-PC concentration. The open circles are for cross-linked PC.

Table I: Surface Properties of Reduced and Polymerized Phosphatidylcholines

PC	cmc (mM)	phosphocholine area[a] (Å^2)
diC_7PC[b]	1.5	55.6
1-C_6-2-C_8SH-PC	2.0	85.0
1-C_6-2-C_8S-PC | 1-C_6-2-C_8S-PC	0.025	96.0
1-C_8S-2-C_6PC | 1-C_8S-2-C_6PC	0.011	–[c]
diC_8PC[b]	0.27	51.4
1-C_6-2-C_{10}SH-PC	0.15	78.4
1-C_6-2-C_{10}S-PC | 1-C_6-2-C_{10}S-PC	0.025	80.2
diC_8SH-PC	0.8	–[c]

[a] Minimum area of each phosphocholine headgroup at the air–water interface at the cmc extrapolated from the slope of the surface tension vs ln(PC) curve (Bian & Roberts, 1992). Values for the area of headgroups in a dimeric molecule would be twice as large. [b] Values taken from Bian and Roberts (1992). [c] Not enough data points to extrapolate the area at the interface.

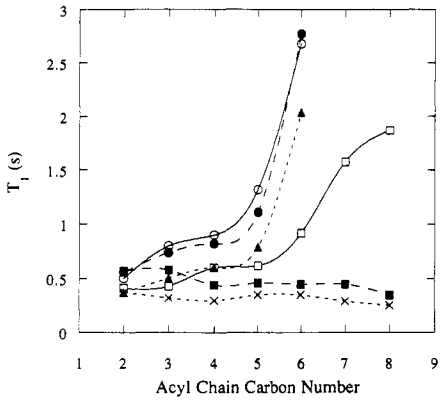

FIGURE 3: ^{13}C T_1 values for acyl chain carbons (numbered from the carboxyl carbon as C-1): (○) diC_6PC; (□) diC_8PC; (●) *sn*-1 chain and (■) *sn*-2 chain of 1-C_6-2-C_8SH-PC; (▲) *sn*-1 chain and (×) *sn*-2 chain of cross-linked 1-C_6-2-C_8SH-PC. Values for the symmetric diacyl PC carbons are taken from Burns and Roberts (1980).

having a terminal sulfur group, not on whether it is reduced or oxidized.

The effect of the sulfur substitution on acyl chain segmental motion could be examined by looking at ^{13}C T_1 values for reduced and cross-linked 1-C_6-2-C_8SH-PC. Values for headgroup and glycerol backbone T_1 were comparable to those reported previously (Burns & Roberts, 1980). Values for acyl chain carbons in reduced (1-C_6-2-C_8SH-PC) and cross-linked compounds showed some striking differences from the normal diacyl analogues (Figure 3). The *sn*-1 chain carbons had T_1 values comparable to those of diC_6PC, but the T_1 values of the *sn*-2 reduced 8-mercaptooctanoyl chain actually decreased toward the terminal thiol. This indicates dramatically damped segmental motion at the chain terminus. Forming the cross-linked dimer reduced these values for the *sn*-2 carbons further (although the change was small compared to the decrease observed between saturated and thiol-containing chains) and had a small but detectable effect on the *sn*-1 chain carbons.

One explanation for the damped motion could be that the thiol group in this lipid is oriented at the micelle surface. This was explored by examining the effect of Pr^{3+}, a lanthanide shift reagent, on proton shifts of 1-C_6-2-C_8SH-PC (Figure 4). Choline and glycerol CH_2 groups were significantly shifted downfield. In contrast, the α-CH_2 and CH_2SH groups were not shifted downfield, indicating they were not accessible to the Pr^{3+} ions. The same relative shifts were observed with the cross-linked *sn*-2 dimeric species (data not shown). This, coupled with the altered segmental motion characteristics of the *sn*-2 chain in 1-C_6-2-C_8SH-PC, suggests that the sulfhydryl acts as an anchor at the end of the chain and damps segmental motion.

Reduced and Polymerized PC as Substrates for Phospholipase A_2. Micellar diC_8SH-PC was a good substrate for phospholipase A_2 (almost as good as diC_7PC or diC_8PC) when it was in a nonpolymerized state (Figure 5). It had an apparent K_M around 2 mM, comparable to observed apparent K_M values for phospholipase A_2 acting on other phosphatidylcholines (Dupureur et al., 1992). After polymerization of the species at 25 or 10 mM (where almost all of the molecules are in very large micelles) and subsequent dilution to assay concentrations, this PC became a very poor substrate for phospholipase A_2 (Figure 5, inset). The apparent K_M toward the polymerized material was 3–4 mM in phosphocholine units.

Molecules such as $lysoC_{12}PC$ and the ether-linked species diheptyl-PC which have been shown to enhance observed activity toward PE and other phospholipid substrates in

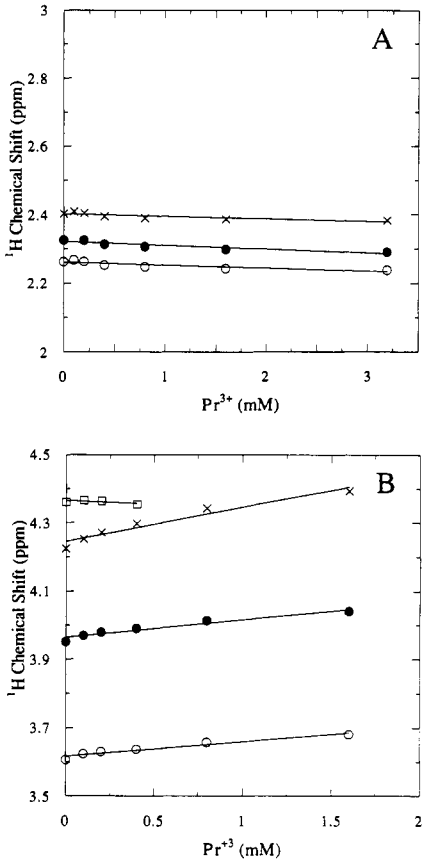

FIGURE 4: ^1H chemical shifts (referenced with respect to HDO at 4.75 ppm from external TMS) of 10 mM 1-C_6-2-C_8(SH)-PC as a function of added Pr^{3+} ions: (A) acyl chain protons including (○) *sn*-1 and (●) *sn*-2 α-CH_2 groups and (×) the *sn*-2 CH_2SH; (B) glycerol backbone and headgroup protons including (□) *sn*-1 CH_2O, (×) glycerol CH_2OP, (●) choline CH_2OP, and (○) CH_2N.

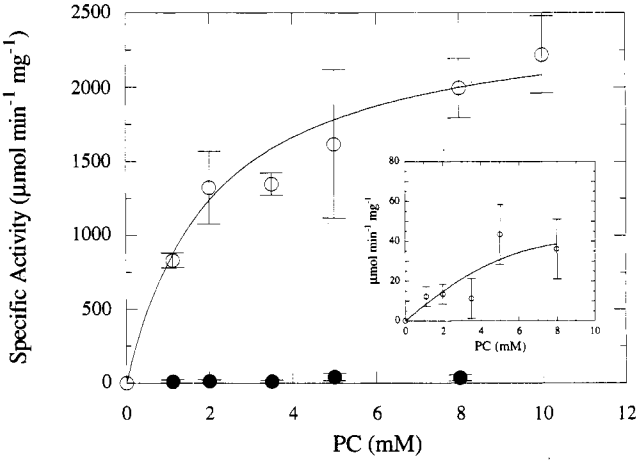

FIGURE 5: Phospholipase A_2 activity toward diC_8SH-PC as a function of substrate concentration: (○) nonpolymerized substrate; (●) polymerized substrate. Assays include 5 mM $CaCl_2$. The inset shows a blowup of the activity of the enzyme toward polymerized PC as a function of phosphocholine unit concentration. Error bars (determined from measurements in duplicate) are indicated on the experimental points.

detergent mixed micelles (Roberts et al., 1989) were added to the polymerized substrate. These activator molecules could function either by increasing the partitioning of the enzyme to the micelle surface (Ramirez & Jain, 1991) or by occupying a specific regulatory site on the enzyme (Dennis & Pluckthun, 1986). Up to 3 mM diheptyl-PC or 2 mM 1-C_{12}PC added to a system containing 2 mM polymerized substrate had no effect on the enzyme activity (either further inhibition or

Table II: Phospholipase A_2 (*N. naja naja*) Activity toward Reduced and Polymerized (for Example, Dimeric) Asymmetric PC Species[a]

substrate	specific activity (μmol min^{-1} mg^{-1})	
	reduced PC	cross-linked PC
diC_7PC	2460 ± 300	
1-C_6-2-C_8SH-PC	1685 ± 226	6.5 ± 1.5
1-C_6-2-C_{10}SH-PC	2500 ± 157	49 ± 19[b]
1-C_8SH-2-C_6PC	1044 ± 7[c]	73 ± 16[d]

[a] Assay conditions include 1 mM Ca^{2+}, 5 mM phospholipid (unless otherwise indicated). [b] 2 mM *sn*-2 dimer. [c] 3 mM PC. [d] 1 mM *sn*-1 dimer.

activation) toward the polymerized PC.

The asymmetric thiol lipid 1-C_6-2-C_8SH-PC was also a good substrate for phospholipase A_2 (Table II). Once cross-linked to form the dimer (the smallest cross-linked unit), phospholipase activity decreased dramatically. Varying the Ca^{2+} concentration from 1 to 10 mM had no effect on the low activity of the cross-linked substrate. One possible explanation for these results is that the cross-linked lipid no longer fits into the active site of the enzyme. Phospholipase A_2 crystal structures suggest that the maximum number of carbons that can fit into the active site is eight (Scott et al., 1990). Therefore, terminal cross-linking of an eight-carbon PC may prevent the substrate from adopting the correct conformation in the active site. To address this question, a cross-linkable substrate with a longer fatty acyl chain, 1-C_6-2-C_{10}SH-PC, was synthesized. As shown in Table II, the incorporation of a longer *sn*-2 chain with a terminal thiol produced a substrate comparable to diC_7PC. Upon cross-linking to form *sn*-2 disulfide-linked dimers, the phospholipase A_2 activity still dropped dramatically.

The presence of a cross-link between the *sn*-2 chains could prevent substrate binding to the enzyme because of steric reasons (e.g., the linked *sn*-2 chains might misalign the *sn*-2 carbonyl ester group and thus prevent efficient hydrolysis). As a probe of any specific effects due to the cross-linking between the *sn*-2 chains, an *sn*-1 dimer from 1-C_8SH-2-C_6-PC was also synthesized. In this species, the disulfide cross-link is between two noncatalytic chains; the *sn*-2 chains are untethered and should be conformationally accessible to the enzyme. The reduced lipid was a slightly poorer substrate for phospholipase A_2, presumably because the *sn*-2 chain is only six carbons in length. Previous work (Lewis et al., 1990) showed that when comparing phospholipase A_2 activity toward substrates with the same total number of acyl carbons, a hexanoyl *sn*-2 chain was cleaved at around 70%, the rate of a seven- or eight-carbon chain. Once polymerized, the activity of the substrate was significantly suppressed but not to quite as great an extent for the *sn*-2 dimers. The significant reduction in phospholipase A_2 activity toward the *sn*-1 as well as the *sn*-2 dimers strongly suggests that molecule vertical motions play a role in catalysis.

The question remains as to whether the cross-linked molecules can still bind (albeit not catalytically effectively) to the enzyme (in which case they would be inhibitory toward better substrates) or whether they can no longer be extracted from the aggregate matrix (in which case they would not be inhibitory by virtue of not partitioning the enzyme to the surface). In Figure 6 is shown the pH-stat profile of phospholipase A_2 hydrolysis of polymerized diC_8SH-PC (5 mM) in the presence of 1 mM Ca^{2+}. After 25 min and no measurable hydrolysis by the enzyme, an equal volume of 6 mM nonpolymerized diC_8SH-PC was added to the reaction mixture, making the final lipid concentrations 2.5 mM polymerized and 3 mM reduced diC_8SH-PC. The added lipid

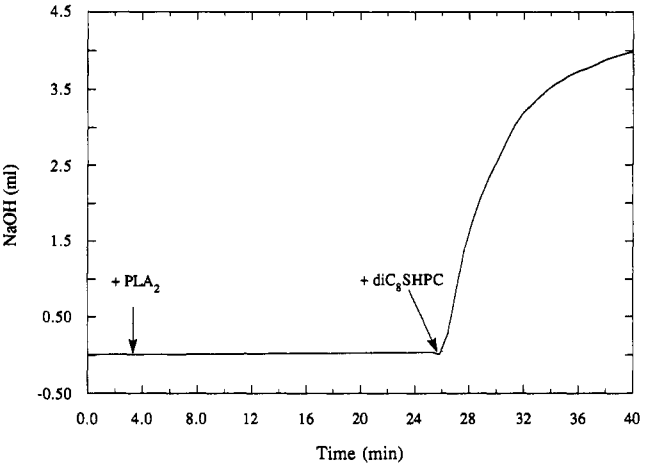

FIGURE 6: pH-stat profile for phospholipase A_2 hydrolysis of polymerized diC_8SH-PC. The first arrow indicates the addition of 1.5 μg of phospholipase A_2 to 5 mM polymerized PC (1 mM Ca^{2+}). The second arrow indicates the dilution of the polymerized PC sample with an equal volume of nonpolymerized diC_8SH-PC to a final concentration of 3 mM.

was an excellent substrate for the enzyme already present in the assay mixture. The specific activity calculated was 1271 ± 118 μmol min^{-1} mg^{-1} in this mixed system. For comparison, phospholipase A_2 activity toward 3 mM reduced diC_8SH-PC alone was 1740 ± 110 μmol min^{-1} mg^{-1}. There is some inhibition of phospholipase A_2 activity since the specific activity has dropped to 73% of the value for pure reduced PC at that concentration. If one assumes for the moment that the polymerized lipid is a competitive inhibitor and uses an apparent K_M of 2 mM for the reduced compound, the K_I would be estimated as 2.7 mM, i.e., slightly higher than the K_M of the reduced lipid. Interestingly, this value is consistent with the apparent K_M one estimates from Figure 5 (inset) for the polymerized diC_8SH-PC. Under these conditions the polymerized micelle is not a good substrate, yet it does exhibit some inhibitory ability with a bulk K_I comparable to its apparent K_M. The added diC_8SH-PC molecules cannot mix randomly with the extensively cross-linked micelle—they could even form a separate micelle phase. Hence, it is difficult to separate whether they are poor substrates and mediocre inhibitors because the interface adsorption step is altered by cross-linking or the catalytic site binding step.

The cross-linked *sn*-1 and *sn*-2 dimeric PCs, which were poor substrates for phospholipase A_2, are expected to form mixed micelles with monomeric lipids (e.g., diC_7PC or diC_8-PC). As such, they can then be examined as inhibitors in the mixed short-chain PC micelle system. DiC_8PC (5 mM) was used as the substrate since its cmc [0.2 mM (Bian & Roberts, 1992)] was closer to that of the cross-linked dimeric lipids (~0.05 mM) than to that of diC_7PC. While the polymeric cross-linked PC showed only moderate inhibition of phospholipase A_2, both *sn*-1 and *sn*-2 dimers were very effective inhibitors of the hydrolysis of diC_8PC (Figure 7). Further analysis of this observed inhibition requires information on lipid mixing of the dimer PC with the substrate molecule.

The extent to which the dimer PC lipids mix with other PC species can be judged using steady-state fluorescence measurements of a micellar short-chain pyrene-labeled PC (1-C_6-2-C_4Pyr-PC) mixed with the dimeric PC and comparing this to the spectrum generated from mixing 1-C_6-2-C_4Pyr-PC with diC_8PC. 1-C_6-2-C_4Pyr-PC has a cmc of ~0.01–0.02 mM as judged by monitoring the excimer and monomer fluorescence bands. Micelles of this lipid at 0.03 mM exhibited

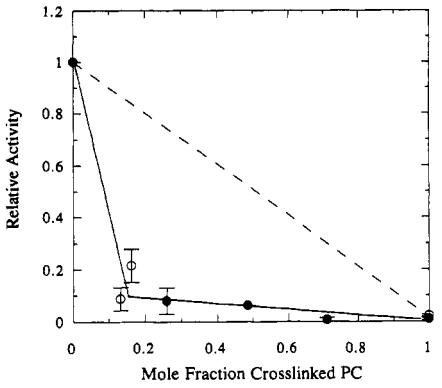

FIGURE 7: Inhibition of phospholipase A_2 activity toward diC_8PC by cross-linked (●) 1-C_6-2-C_8SH-PC and (○) 1-C_8SH-2-C_6PC. The total PC concentration was maintained at 5 mM.

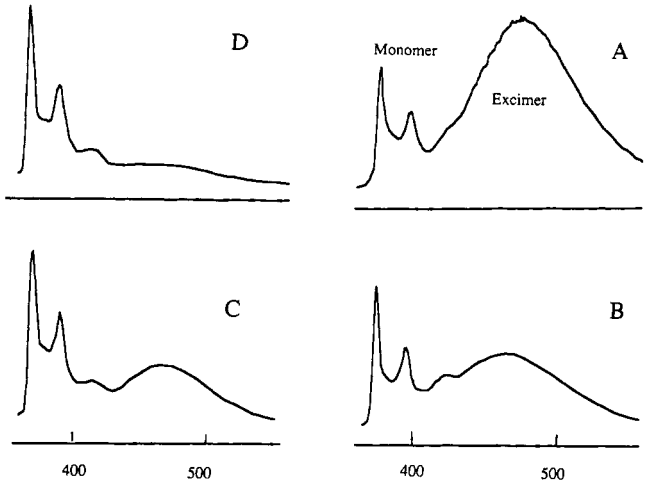

FIGURE 8: Fluorescence spectra of 0.03 mM 1-C_6-2-C_4Pyr-PC (A) alone, (B) mixed with 1.1 mM disulfide-linked dimer of 1-C_6-2-C_8-SH-PC, (C) mixed with 1.25 mM total diC_8PC (1.05 mM micellar diC_8PC), and (D) mixed with 2.2 mM total diC_8PC. Monomer and excimer bands are labeled.

a fluorescence spectrum dominated by the excimer band ~450 nm (Figure 8A). The total pyrene-PC concentration was kept low because this lipid tends to form extremely large micelles (Bian & Roberts, 1991). The spectrum of the same concentration of 1-C_6-2-C_4Pyr-PC mixed with 1.1 mM *sn*-2 dimer PC (e.g., 2.2 mM tethered phosphocholine units) is shown in Figure 8B. There was an immediate decrease in the excimer band and an increase in monomer bands. No changes in the spectrum occurred as a function of incubation time, indicating the mixing occurred rapidly. This spectrum was comparable to what was observed for 0.03 mM 1-C_6-2-C_4Pyr-PC mixed with 1.25 mM diC_8PC (Figure 8C). In the diC_8PC sample the micelle concentration available for dilution of the pyrene-PC is 1.05 mM since the cmc is 0.2 mM. In both mixtures the ratio of unlabeled PC (either diC_8PC or polymerized 1-C_6-2-C_8SH-PC) to pyrene-PC was kept high to have mixed micelles with at most one to three fluorophores to minimize the excimer band in the mixed micelle. The total unlabeled PC concentration was kept ~1 mM to sense any differences in mixing efficiencies of a dimeric PC molecule compared to diC_8PC. Higher PC concentrations (e.g., a factor of 2) will readily dilute the pyrene-labeled probe and lead to little detectable excimer (Figure 8D). Examining the changes in the fluorescence spectrum caused by mixing the lower PC concentrations would be most sensitive to any differences in micelle partitioning of the *sn*-2 dimer vs monomer PC. While these experiments do not address any differences in the time scale of micelle mixing, they indicate that both the dimeric 1-C_6-2-C_8SH-PC unit and diC_8PC form mixed micelles with 1-C_6-2-C_4Pyr-PC with presumably the same degree of randomness.

Table III: Phospholipase C (*B. cereus*) Activity toward Reduced and Polymerized (for Example, Dimeric) Asymmetric PC Species[a]

substrate	specific activity (μmol min^{-1} mg^{-1}) reduced PC	polymerized PC
diC_7PC	1329 ± 64	
1-C_6-2-C_8SH-PC	937 ± 69	1099 ± 3
1-C_6-2-C_{10}SH-PC	1232 ± 52	1934 ± 77[b]
1-C_8SH-2-C_6PC	577 ± 19[c]	1520 ± 70[d]
diC_8SH-PC		
4 mM		994 ± 61
2 mM	660 ± 7	810 ± 19
0.7 mM		669 ± 39

[a] Assay conditions include 5 mM phospholipid (unless otherwise indicated). [b] 2 mM *sn*-2 dimer. [c] 3 mM PC. [d] 1 mM *sn*-1 dimer.

Assuming these dimers mix as randomly with the diC_8PC micelles as they do with the 1-C_6-2-C_4Pyr-PC micelles, one can get a sense of the inhibition potency (Figure 7) by examining the relative activity (at fixed total PC) as a function of the mole fraction of the poor substrate (Ransac et al., 1990). An inhibitor with an interfacial dissociation constant (K_I*) comparable to the substrate interfacial Michaelis–Menten constant (K_M*) would yield a linear decrease in activity as indicated by the dotted line. An inhibitor with $K_I^* < K_M^*$ would produce a rapid drop-off in activity with increasing inhibitor content. This latter is the trend that is observed for both *sn*-1 and *sn*-2 dimeric PCs. The molar fraction of *sn*-1 or *sn*-2 dimer which reduces the enzyme activity toward micellar diC_8PC to 50% of its value is <0.1 phosphocholine unit. This value is a measure of the inhibitory capacity of these dimeric PCs, and its low value suggests that the dimeric PC can bind tightly to phospholipase A_2 but not in a catalytically productive fashion.

Reduced and Polymerized PC as Substrates for Phospholipase C. Quite different kinetic results were obtained from a comparison of phospholipase C activity toward reduced and cross-linked thiol PCs. The addition of the sulfhydryl groups at both chain termini made diC_8SH-PC micelles poorer substrates than either diC_7PC or diC_8PC. The initial specific activity was ~660 μmol min^{-1} mg^{-1}, about 50% that towards 5 mM diC_7PC. Upon cross-linking, the micelles became slightly better substrates for phospholipase C (~1.2–1.5-fold increase). If diglyceride removal from the enzyme is rate-limiting, as has been suggested previously (El-Sayed & Roberts, 1985), anchoring that product to other micellar molecules (which occurs in the cross-linked diC_8SH-PC system) may aid in release of enzyme for another round of catalysis and hence increase the observed initial specific activity. In that case, one might expect a smaller enhancement for the enzyme acting on the phospholipid species with a single terminal thiol. Initial activities of phospholipase C toward 1-C_6-2-C_8SH-PC, 1-C_6-2-C_{10}SH-PC, and 1-C_8SH-2-C_6PC were 50–90% of the value toward diC_7PC (Table III). Upon cross-linking to form the dimeric PC, observed initial phospholipase C activities increased 1.20–2.6-fold compared to the parent reduced compound. There are comparable enhancements to that observed with the diC_8SH-PC material. Nonetheless, in all cases, formation of the chain-linked substrate enhanced the observed phospholipase C activity compared to that of the parent reduced compound.

DISCUSSION

Short-chain ω-thiol phospholipids that can be polymerized are useful species for investigating water-soluble phospholipase activity. When thiol functions are located at the terminus of long-chain PCs, cross-linking of small vesicles formed from these structures causes dramatic changes in aggregate morphology. Vesicles are converted into nonentrapping tubule structures (Handel, 1989). This makes a comparison of enzyme activity toward reduced and cross-linked material difficult to interpret since both aggregate morphology and chain motions have been altered. With the shorter chain analogues, such phase changes are avoided. Furthermore, since the polymerization is at the terminus of the acyl chains, the headgroup of these polymerized species should still have a relatively large range of motions. Chain segmental motions are affected, but the change is most dramatic in comparing the thiol PCs with normal diacyl PCs. Thus, introduction of a terminal thiol has a much more potent effect on lipid chain motions than cross-linking the thiol lipids. Such segmental chain motions are not critical to phospholipase activity since the lipid with the free thiols is a good substrate. The slight further changes in chain segmental mobility upon disulfide formation are small by comparison. Cross-linking lipid molecules has a more pronounced damping effect on two other types of lipid motions: (i) lateral diffusion and (ii) the motion of the molecules along the normal of the micelle surface (vertical displacements of the molecule from the micelle surface). Thus, a comparison of phospholipase activity toward reduced and oxidized thiol PCs probes the importance of vertical and lateral motions in enzyme activity. If it is necessary for the actual substrate to leave the matrix surface (at least partially) for optimal hydrolysis by enzyme, damping vertical motions should have a profound effect on enzyme activity. Hampering lateral diffusion can alter how an enzyme processes substrates once it is anchored to the interface. Lateral diffusion is proportional to molecular weight; therefore, the larger the cross-linked unit, the lower the diffusion, and there should be a significant difference in dimer vs polymer PCs.

Cross-linking terminal thiol phospholipids suppresses cobra venom phospholipase A_2 activity. While this is consistent with lateral diffusion or vertical PC displacements as critical to enzyme activity, other explanations should also be examined. Suppose substrate rotation about its molecular axis is important for enzyme activity. This means the substrate orientation relative to enzyme in the two-dimensional matrix is important for hydrolysis. If this is the case, phospholipase A_2 activity toward polymerized substrate should be lower since the motion of substrate has been limited (Thuren et al., 1984). However, this cannot explain the fact that activity toward polymerized substrate decreases to about 1–5% of that of the unpolymerized monomer molecule because the headgroup and initial part of the *sn*-2 chain in the polymerized substrate molecules should still be able to adopt the required conformation for enzymatic hydrolysis. Another possibility is that the hydrolysis products cannot be released either from the enzyme or from the matrix. Buildup of inhibitory products in the matrix can only be important for later hydrolysis times. Since the activities in Figure 3 and Table II are initial rates, there should be little product in the two-dimensional matrix. Furthermore, for the *sn*-1 dimer, the product hexanoic acid will partition to a large extent in the aqueous phase and should not build up in the interface. A third explanation for the low activity is that the conformation of the substrate must be changed after it is bound to the enzyme for catalysis to occur. Modification at the substrate at C-8 or C-10 should have a minimal effect on the initial segment of the *sn*-2 chain. Furthermore, modification of one chain should have no effect on conformations and motions of the other. This is in fact what is observed in the ^{13}C T_1 values for 1-C_6-2-C_8SH-PC: the chain without the thiol group has segmental motions comparable to those of diC_6PC. Therefore, for the *sn*-1 dimer, the *sn*-2 chain motions and conformations should be unaltered. The most reasonable explanation for the low activity of phospholipase A_2 toward polymerized substrate (either multimeric cross-linked diC_8-SH-PC or the cross-linked dimers) is that substrate lateral or vertical motions are critical for enzyme binding to the lipid.

A distinction between vertical and lateral motions can be made by examining the cross-linked vs polymer activities. Lateral diffusion in cross-linked diC_8SH-PC has clearly been dramatically reduced. However, the diffusion constant for the *sn*-1 and *sn*-2 dimeric molecules will be only slightly smaller than that for the un-cross-linked PC in the micelle. If lateral diffusion were the critical parameter, then one would expect the dimers to be significantly more active than the polymeric PC. Since this is not the case, it is more likely that lipid vertical motions are the important parameter for phospholipase A_2 activity. This enzyme must be able to partially extract and isolate a substrate molecule from its micelle milieu.

There has been considerable effort to explain phospholipase A_2 kinetics in terms of a two-step binding model of interfacial catalysis. The present data, interpreted in view of that model, place constraints on which steps are most affected by damping vertical substrate motions. Since the polymeric micelles are very poor substrates and mediocre inhibitors, one could postulate that the initial step in the reaction, enzyme adsorption to the polymeric micelles (which cannot mix randomly with other monomeric substrate molecules), is impaired compared to adsorption on the unpolymerized molecules. It is the vertical or lateral motions of the PCs that have been altered by cross-linking. This implies that the initial adsorption to a PC surface is contingent on these motions. In contrast to the polymeric micelle, the *sn*-1 and *sn*-2 dimers are poor substrates and very good inhibitors. Fluorescence evidence indicated that dimeric PC molecules should mix well with other micellar PC molecules. Therefore, adsorption to a mixed micelle with only a small percentage of dimer should not reflect a large change in enzyme partitioning between soluble and adsorbed states. The strong inhibition caused by these dimers must then reflect an effect on the second step of the reaction—active site binding and catalysis. The potent inhibition by the dimers, both *sn*-1 and *sn*-2 molecules, suggests they interact with the enzyme in a nonproductive but very specific fashion. Perhaps attempts at docking such dimers to known structures of the enzyme can shed light on what specific interactions can occur that would prevent hydrolysis.

Previous work has shown that for optimum activity phospholipase C requires an *sn*-2 carbonyl and some degree of hydrophobicity in the chains, although there is no specificity for either *sn*-1 or *sn*-2 chain length (El-Sayed et al., 1985; Lewis et al., 1990). For catalysis to occur the enzyme must bind to the interface, bind a substrate molecule in the correct orientation, cleave the phosphodiester bond, and release products. Since the cross-link occurs at the chain terminus, far from the region of the molecule undergoing transformation, and since the "dimeric" PCs form micelles which behave similarly to the reduced species, it is unlikely that interface has been significantly altered. By constraining the chains through the formation of disulfide cross-links, both the vertical and lateral mobilities of the substrate and diglyceride product at the interface have been impaired. Substrate headgroup

areas and hence accessibility of the phosphodiester bond to the enzyme are not affected. Since all the substrate is accessible in micelles and >15% diglyceride generated causes micelle aggregation and fusion (K. Lewis and M. F. Roberts, unpublished results), the distinction of the enzyme dissociating from one micelle and adsorbing to another between catalytic events and the enzyme processing many substrate molecules while anchored to a given micelle is moot except at the earliest stages of hydrolysis with normal short-chain PC micelles. A key result of cross-linking the substrate via the acyl chains is to keep the hydrophobic diacylglycerol product in the interface. If the enzyme has a reasonable affinity for the diacylglyceride [as has been suggested by other studies of this enzyme (El-Sayed & Roberts, 1985)], then activity should increase when that product is constrained to the micelle and cannot phase-separate and remove the enzyme from the PC micelle surface.

In summary, these experiments demonstrate that phospholipid vertical motions are important for optimal phospholipase A_2 (*N. naja naja*) activity in terms of both adsorption and catalytic binding steps, while they are less critical for phospholipase C (*B. cereus*) activity.

ACKNOWLEDGMENT

We thank Ms. Karen Lewis for critical comments and useful discussions and Dr. Pierro Lo Nostro (Department of Nuclear Engineering, MIT) for help with the QLS measurements.

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