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Asymmetric Short-Chain Phosphatidylcholines: Defining Chain Binding Constraints in Phospholipases†

Karen A. Lewis, Jinru Bian, Ann Sweeney, and Mary F. Roberts*
Department of Chemistry, Boston College, Chestnut Hill, Massachusetts 02167
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ABSTRACT: Several short-chain asymmetric lecithins with a total of 14 carbons in the acyl chains (ranging from 1-lauroyl-2-acetylphosphatidylcholine to 1-hexanoyl-2-octanoylphosphatidylcholine) have been synthesized and characterized. The specific activities of phospholipase A_2 from cobra venom, phospholipase A_2 from porcine pancreas, and phospholipase C from *Bacillus cereus* toward these lecithins as micelles have been determined. The results of these kinetic studies allow the definition of hydrophobic binding requirements in the active sites of these water-soluble phospholipases. For phospholipase C, with the exception of monomyristoylphosphatidylcholine, each of the asymmetric short-chain lecithins exhibits high activity, comparable to the 14-carbon symmetric short-chain species, diheptanoylphosphatidylcholine. Therefore, for phospholipase C, in addition to the acyl linkages, only a certain degree of hydrophobicity in the fatty acyl chains is requisite for substrate binding and appreciable hydrolysis; there is no chain specificity. The activity of phospholipase A_2 from cobra venom toward the same asymmetric lecithins is quite different. As the *sn*-2 chain lengthens, activity is increased to a maximum for diheptanoyl-PC. Further increase in the number of carbons in the *sn*-2 chain has no effect on hydrolysis rates. For this enzyme, seven carbons in the *sn*-2 chain are necessary for optimal activity. In contrast, porcine pancreatic phospholipase A_2 activity shows very little dependence on *sn*-2 chain length.

Symmetric short-chain lecithins have been used extensively as substrates for water-soluble phospholipases (DeHaas et al., 1971; Bonsen et al., 1972; Wells, 1974; Verger & De Haas, 1976; Little, 1977). These molecules can be monomeric in aqueous solution and hence are soluble substrates for lipolytic enzymes. As their concentration is increased, they form micelles rather than bilayer structures (Tausk et al., 1974a,b). Short-chain phospholipids are a good choice for an assay system because (i) both monomeric and aggregated substrates can be formed depending on the fatty acyl chain lengths, (ii) micelles have no sidedness as do vesicles, and (iii) the individual lecithin molecules have many of the same conformational, dynamic, and packing features of naturally occurring lecithins in bilayers (Burns & Roberts, 1980; Burns et al., 1983; Lin et al., 1986, 1987a). The detailed structures of several short-chain lecithins have been elucidated with small-angle neutron scattering (SANS)[1] (Lin et al., 1986, 1987a,b). Dihexanoylphosphatidylcholine (diC_6PC)[1] forms nearly spherical micelles that do not grow in length with increasing concentration. The fatty acyl chains in these small micelles form a highly disordered core structure (Lin et al., 1986). For a species with more than 12 carbons in the fatty acyl chains, rod-shaped micelles (spherocylinders) are formed that grow in the longitudinal direction with increasing lipid concentration. A series of asymmetric PC's could be fit to spherocylinder models with the same radial structure but different lengths; their growth characteristics (i.e., the length of the rod) can be predicted by a simple thermodynamic model of micelle growth.

Short-chain lecithin substrate analogues have played an important role in determining the structural features of the substrate critical to the activity of phospholipases (Roberts, 1991; Bonsen et al., 1972). The linear symmetric species were used to show that phospholipases preferentially hydrolyze substrate in an aggregated form, i.e., micellar PC is a better substrate than monomeric PC (Roholt & Schlamowitz, 1961; Wells, 1972). In addition to the aggregation state of the lipid (monomer, micelle, or vesicle), chain packing and substrate geometry are factors that also can affect phospholipase activity to some degree. This can often be probed by modifications of the substrate molecule as emphasized by Bonsen et al. (1972) where phospholipids with different backbone configurations, headgroups, and some chain modifications were examined as substrates for phospholipase A_2. For phospholipase C, short-chain ether-linked lecithins were shown to be poor substrates for the enzyme, even though the substrate

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* To whom correspondence should be addressed.

[1] Abbreviations: diC_nPC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; 1-C_n-2-C_m-PC, 1-(*n*)acyl-2-(*m*)acyl-PC; CMC, critical micelle concentration; SANS, small-angle neutron scattering.

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modification occurred far from the phosphodiester bond to be hydrolyzed (Burns et al., 1981; El-Sayed et al., 1985). These results suggested a carbonyl is necessary in binding substrate to the enzyme active site. Substrate lecithins with methyl substituents on both acyl chains as close as one carbon and as far as five carbons away from the carbonyl moiety are poor substrates and good inhibitors of cobra venom phospholipase A_2 activity (DeBose et al., 1985). When either the *sn*-1 or *sn*-2 chain is modified with one methyl substituent in any position along the chain, phospholipase A_2 activity is increased to that for the linear lecithin, indicating that substrate steric factors are important for phospholipase A_2. Lecithins that are too bulky may either block access to the active site or bind in the wrong orientation for catalysis to occur.

In light of these prior observations with short-chain lecithins, we have synthesized and characterized a series of asymmetric short-chain lecithins with a total number of 14 carbons in the acyl chains. The *sn*-1 and *sn*-2 chain lengths have been varied to observe the effects of perturbing the number of carbons in a given chain while holding the number of carbons in the whole phospholipid constant. Because all species have a total of 14 carbons in the fatty acyl chains, the CMC's and overall packing of these lipids into aggregates should be similar to those of diheptanoyl-PC, the symmetric short-chain homologue (Tausk et al., 1974b), which can be well modeled as a spherocylinder (Lin et al., 1987a). The asymmetric lecithins synthesized include 1-lauroyl-2-acetyl-PC (1-C_{12}-2-C_2-PC), 1-decanoyl-2-butyroyl-PC (1-C_{10}-2-C_4-PC), 1-nonanoyl-2-valeroyl-PC (1-C_9-2-C_5-PC), 1-octanoyl-2-hexanoyl-PC (1-C_8-2-C_6-PC), and 1-hexanoyl-2-octanoyl–PC (1-C_6-2-C_8-PC). Investigation of the physical properties and kinetic behavior of these lipids then allows a refined definition of chain binding requirements for phospholipase A_2 (cobra venom and pancreatic sources) and C (*Bacillus cereus*).

Experimental Procedures

Materials. Diheptanoyllecithin, monomyristoyllecithin, and bovine brain sphingomyelin were obtained from Avanti Polar Lipids, Inc. The nonionic detergent Triton X-100 was purchased from Sigma Chemical Co. All other reagents mentioned in the text were of high purity and used without further purification unless otherwise indicated. Egg phosphatidylethanolamine (PE) (Avanti) was purified by silicic acid column chromatography to remove residual phosphatidylcholine contaminants before use. A linear gradient of $CHCl_3/CH_3OH$ (Aldrich) was used as the eluent. Organic solvents were removed from the purified PE with a stream of N_2, and the dried lipid film was lyophilized to remove traces of solvent. The dried PE was resuspended in $CHCl_3$ and stored at −20 °C until use. Phospholipase A_2 from *Naja naja naja* and porcine pancreas and phospholipase C from *Bacillus cereus* were the highest purity available from Sigma.

Synthesis of Asymmetric Short-Chain Lecithins. The asymmetric short-chain lecithins 1-octanoyl-2-hexanoyl-PC (1-C_8-2-C_6-PC), 1-hexanoyl-2-octanoyl-PC (1-C_6-2-C_8-PC), 1-hexanoyl-2-phenylbutyroyl-PC, and 1-hexanoyl-2-phenylvaleroyl-PC were synthesized by the fatty acid imidazole method using the appropriate lysolecithin (obtained from Avanti) and fatty acid (obtained from Sigma or Aldrich) in a ratio of 1:5:6 lysolecithin/fatty acid/1,1′-carbonyldiimidazole (Aldrich) (Burns & Roberts, 1980; Burns et al., 1983). Since the nine-carbon lysolecithin was not commercially available, 1-nonanoyl-2-valeroyl-PC (1-C_9-2-C_5-PC) was synthesized by initially cleaving the *sn*-2 chain of dinonanoyl-PC (Avanti) with phospholipase A_2 (using an ether/aqueous borate buffer with Ca^{2+} emulsion system), and reacylating with valeric acid (Aldrich). 1-Lauroyl-2-acetyl-PC (1-C_{12}-2-C_2-PC) and 1-decanoyl-2-butyroyl-PC (1-C_{10}-2-C_4-PC) were synthesized from the appropriate lysolecithin and fatty acid anhydride in a ratio of 1:5:1 lysolecithin/fatty acid anhydride/4-pyrrolidinopyridine (Mason et al., 1981). Lipid purity and reaction progress for all syntheses were monitored by thin-layer chromatography (TLC) in a solvent system of $CHCl_3/CH_3OH$/10.5 M NH_4OH (60:35:8) and detected by iodine vapor staining as described previously (Burns & Roberts, 1980). Each of the asymmetric short-chain lecithins was purified by silicic acid (Bio-Rad) column chromatography. Lecithin solutions were quantified by a colorimetric phosphate assay (Chen et al., 1956; Turner & Rouser, 1970). The ^{13}C NMR chemical shifts of methylene and methyl carbons are diagnostic for the fatty acid identities on the synthetic lecithin and for any contaminating lipid isomers at the *sn*-1/*sn*-2 positions (Burns & Roberts, 1980). For 1-C_6-2-C_8-PC, the ^{13}C chemical shifts (ppm) (in CD_3OD and referenced to internal tetramethylsilane) for *sn*-1 chain carbons were the following: 34.93, C(2); 26.01, C(3); 32.96, C(4); 23.70, C(5); 14.44, C(6). Shifts (ppm) for the *sn*-2 octanoyl chain were as follows: 35.13, C(2); 25.68, C(3); 32.47, C(4); 30.22, C(5) and C(6); 23.41, C(7); 14.30, C(8). For 1-C_8-2-C_6-PC, the chemical shifts (ppm) for *sn*-1 chain carbons were the following: 34.25, C(2); 25.07, C(3); 29.52, C(4); 31.76, C(5) and C(6); 23.07, C(7); 13.81, C(8). Shifts (ppm) for the *sn*-2 hexanoyl chain were as follows: 34.41, C(2); 25.38 C(3); 29.52, C(4); 22.80, C(5); 13.67, C(6). For 1-C_{12}-2-C_2-PC, the chemical shifts (ppm) for carbons in the *sn*-1 chain were the following: 34.20, C(2); 25.36, C(3); 29.56, C(4); 29.97, C(5); 29.81, C(6), C(7), C(8), and C(9); 32.4, C(10); 23.12, C(11); 13.84, C(12). The chemical shift (ppm) for the *sn*-2 methyl carbon was 43.43, C(2). 1-C_{10}-2-C_4-PC had the following chemical shifts (ppm) for the carbons in the *sn*-1 chain: 34.24, C(2); 25.37, C(3); 29.57, C(4); 29.86, C(5); 29.86, 29.89, and 30.14, C(6) and C(7); 32.47, C(8); 23.13, C(9); 13.85, C(10). Chemical shifts (ppm) for the *sn*-2 chain methylene and methyl carbons were the following: 36.33, C(2); 18.80, C(3); 13.35, C(4).

Gas Chromatography. Fatty acid content at the *sn*-2 position was analyzed by gas chromatography by preparing methyl esters [using BF_3/methanol catalysis (Metcalfe & Schmitz, 1961)] from the fatty acids generated by phospholipase A_2 cleavage. Gas chromatography of the methyl esters was performed on a Hewlett-Packard 5890A gas chromatograph with a Hewlett-Packard 3393A integrator used to determine the peak areas of the representative fatty acids in the mixture. Experimental conditions for gas chromatographic analysis of methyl esters were used as described previously with the exception of a 12 mL/min flow rate of helium (Paulson et al., 1974).

Kinetic Analyses. Activities of phospholipase A_2 and phospholipase C were measured toward the asymmetric lecithins by the pH-stat technique (Dennis, 1973) with NaOH as the titrant and an end point of pH 8. The instrument was calibrated daily with 5 mM potassium acid phthalate to determine exact base concentration. The base concentration was kept between 3 and 5 mM NaOH, 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 assay (Bradford, 1976). Lecithin samples ranged in concentration from 2 to 25 mM as determined by phosphorus assay. All experiments were run in duplicate or triplicate and kept under a N_2 atmosphere; 5 mM $CaCl_2$ was added to the sets of

Table I: Characterization of Short-Chain Lecithins: CMC, R_H, and ΔCH_3 (Chain Methyl Chemical Shift Nonequivalence)

lecithin	CMC[a] (mM)	R_H (Å)[b]	ΔCH_3 (Hz)[c]
1-C_{14}-PC	0.05 ± 0.01[d]	34	
1-C_{12}-2-C_2-PC	0.41 ± 0.06	24	376.9 ± 0.5
1-C_{10}-2-C_4-PC	0.8 ± 0.3	24	29.8 ± 0.3
1-C_9-2-C_5-PC	1.3 ± 0.2		17.4 ± 0.3
1-C_8-2-C_6-PC	1.5 ± 0.2[e]	65	8.8 ± 1.2
diC_7PC	1.5 ± 0.1	75	2.4 ± 0.6
1-C_6-2-C_8-PC	1.9 ± 0.5[e]	70	5.5 ± 1.1

[a] Determined in D_2O by ^1H NMR spectroscopy, unless otherwise indicated. [b] All samples 50 mM. [c] Chemical shift difference at 300 MHz between *sn*-1 and *sn*-2 terminal methyl groups. [d] Kramp et al. (1984); Nakagaki et al. (1986). [e] Lin et al. (1987b).

samples that were hydrolyzed by phospholipase A_2 enzymes, as this divalent cation was necessary for activity. This is sufficient Ca^{2+} to optimize activity of the pancreatic enzyme at pH 8.0 (De Haas et al., 1971). While pH 8 is not the optimum pH for pancreatic phospholipase A_2 and the specific activities are lower than at pH 6, the more basic pH is better for pH-stat assays since all the fatty acid will be ionized. The values we have observed for diC_7PC at pH 8 are comparable to what others have reported under similar conditions at pH 8.0 (DeHaas et al., 1971; Bonsen et al., 1972).

CMC Determination. The critical micellar concentration (CMC) of each synthetic lecithin in D_2O was measured by ^1H NMR (at 300 MHz) as described previously (Lin et al., 1987b). Accumulation times ranged from 4 to 800 transients depending on lipid concentration.

Quasi-Elastic Light Scattering. R_H, the average hydrodynamic radius, of micelles was obtained from the autocorrelation functions of the intensity of scattered light measured in a QLS spectrometer of standard design. The methods for extraction of D, the diffusion constant, and use of this in the Stokes–Einstein equation to estimate R_H have been described in detail previously (Eum et al., 1989). R_H values of the short-chain lecithin micelles were compared at 50 mM, a concentration where the symmetric homologue diheptanoyl-PC forms moderately polydisperse rod-shaped micelles.

Results

Physical Characterization of Short-Chain Lecithin Micelles. Table I shows critical micellar concentrations (CMC's) determined by ^1H NMR spectroscopy. If the *sn*-2 chains in the 14-carbon lecithins were completely independent of each other in the monomer state, the CMC should be the same for all of them. All the species with 14 carbons total in the fatty acyl chains have CMC values in the range of 0.4–1.9 mM. The longer the *sn*-1 chain, the lower the CMC for these molecules. A smaller CMC indicates either that PC's with longer *sn*-1 chains are more hydrophobic than species with more equivalent length chains or that they pack better into a micelle. The values are also consistent with the initial 2 carbons of the *sn*-2 chain oriented at the interface in contact with water, hence not contributing much to the hydrophobicity of the molecule, since the CMC for 1-C_{12}-2-C_2-PC (0.41 ± 0.06 mM) is essentially the same as that of the 12-carbon lyso compound (for 1-lauroyl-PC, the CMC is 0.66 ± 0.02 mM; J. Bian and M. F. Roberts, unpublished results) and an order of magnitude higher than the 14-carbon lyso-PC. A plot of log CMC vs carbon number in the *sn*-1 chain is shown in Figure 1A. The dependence is nonlinear, indicating that the two fatty acyl chains are not completely independent of each other in the monomeric state and their hydrocarbon surfaces must contact each other to some extent and prevent contact with water. The effectiveness of that interaction will of course depend on the

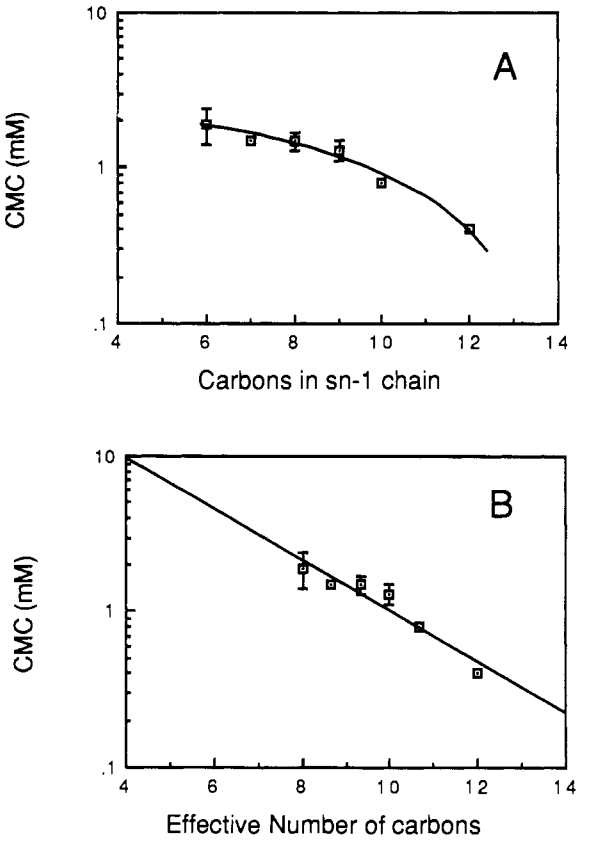

FIGURE 1: Plot of log CMC vs (A) number of carbons in the *sn*-1 chain and (B) N, the effective number of CH_2/CH_3 groups estimated from $N = 12 - (n - 2)(2/3)$, for a series a short-chain lecithins with 14 carbons total in the fatty acyl chains. The CMC values were determined by ^1H NMR spectroscopy; error bars for each CMC are also indicated.

relative chain lengths of the two fatty acids. If one assumes that when two chains are in contact about one-third of each contacting CH_2 group is excluded from water, then an effective number of carbons (N) can be computed, $N = 12 - (n - 2)(2/3)$, where n is the number of carbons in the *sn*-2 chain and it is assumed that the first two carbons in the *sn*-2 chain are at the interface and cannot interact with the *sn*-1 chain. A plot of N versus the log of the CMC should be linear (Figure 1B) and the slope used to estimate the increment in free energy for the CH_2 groups interacting with one another. From the slope, that free energy increment is 222 cal/mol. If the CH_2 groups were always interacting with one another, one would expect a value of 740 cal/mol; since only a third of that is detected, it implies that the chains interact with each other in the monomeric state only about one-third of the time.

Average sizes for these asymmetric short-chain lecithins at 50 mM lecithin have been determined by quasi-elastic light scattering (Table I). At this concentration, the symmetric PC compound, diC_7PC, is moderately polydisperse with an average hydrodynamic radius, R_H, of 75 Å; this decreases to 35 Å for 5 mM PC (Burns et al., 1983). Micelles of the most asymmetric short-chain lecithins are smaller, even at the higher PC concentration. R_H is 24 Å for 50 mM 1-C_{12}-2-C_2-PC and 1-C_{10}-2-C_4-PC. For the first of these, an extended lauroyl chain length is about 13 Å; adding the 10 Å for the backbone/headgroup region (Lin et al., 1986, 1987a,b) and ~2 Å for hydration predicts an R_H ~25 Å for spherical micelles and suggests that both of these micelles do not grow appreciably with increasing lipid concentration. Thus, at assay concentrations (~5 mM), all the short-chain PC micelles will have 25–35-Å R_H.

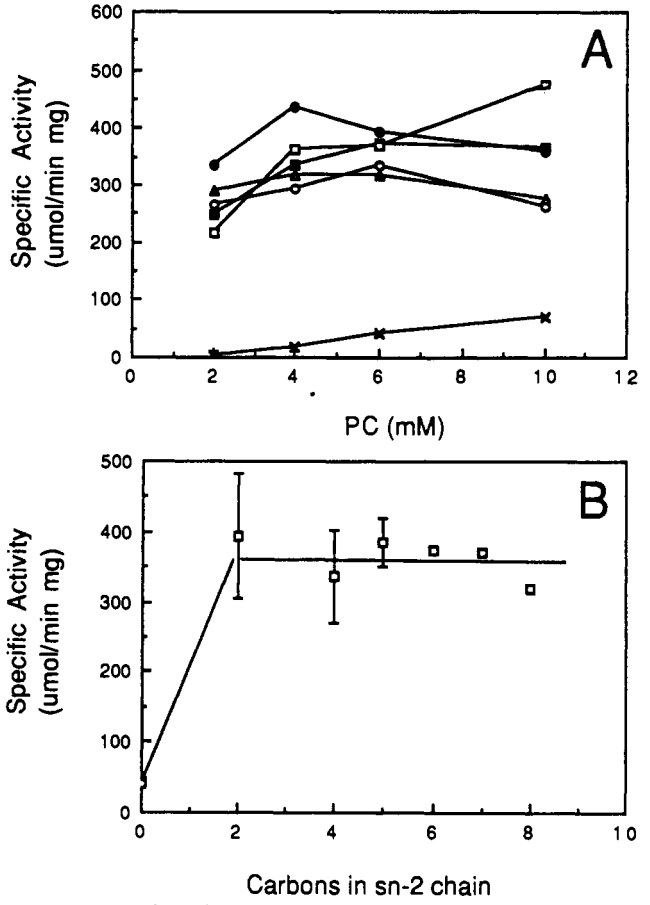

FIGURE 2: (A) Specific activity of *Bacillus cereus* phospholipase C toward short-chain lecithins containing 14 carbons total in both acyl chains as a function of lecithin concentration: 1-C_{14}-PC (×); 1-C_{12}-2-C_2-PC (●); 1-C_{10}-2-C_4-PC (○); 1-C_8-2-C_6-PC (■); diC_7PC (□); 1-C_6-2-C_8-PC (▲). Assay conditions were pH 8.0, 25 °C; all activities shown are the mean of triplicate samples. (B) Dependence of the specific activity of *Bacillus cereus* phospholipase C on the number of carbons in the *sn*-2 fatty acyl chain for 6 mM PC. The error bars show the standard deviation from the mean of assays done in triplicate.

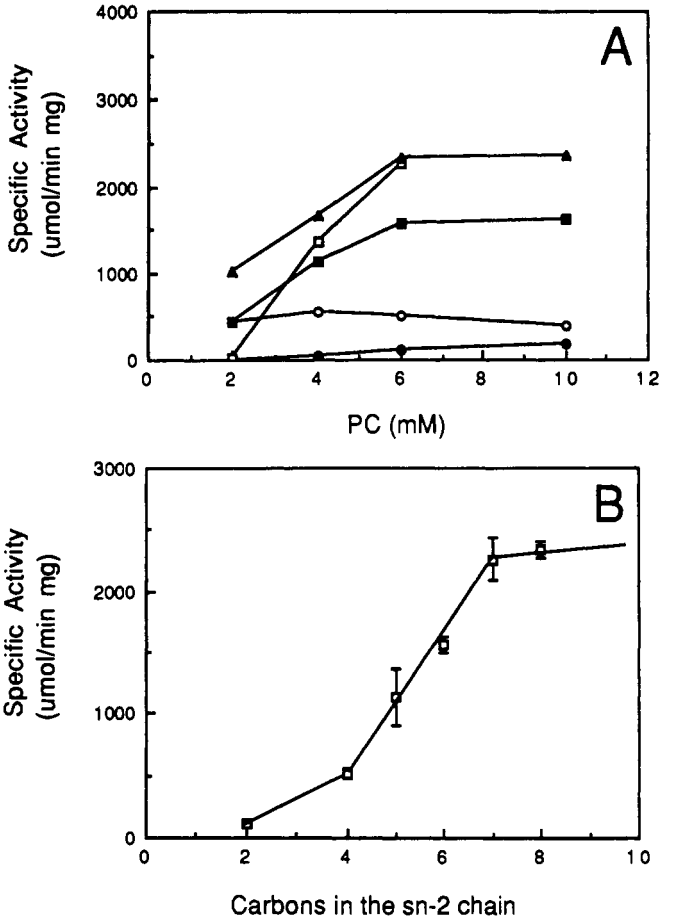

FIGURE 3: (A) Specific activity of phospholipase A_2 (*Naja naja naja*) toward short-chain lecithins containing 14 carbons total in both acyl chains as a function of lecithin concentration. The identification of symbols and assay conditions are given in the legend of Figure 2, $CaCl_2$ (5 mM) was added to all samples. (B) Dependence of the specific activity of cobra venom phospholipase A_2 on the number of carbons in the *sn*-2 fatty acyl chain for 6 mM PC.

Averaged conformational properties of these short-chain PC micelles can be compared by examining glycerol $J_{H(1)-H(2)}$ coupling constants (Hauser et al., 1980). In diheptanoyl-PC micelles, $^2J_{AB}$ = 12.8 Hz, $^3J_{AC}$ = 8.1 Hz, and $^3J_{BC}$ = 3.4 Hz (Debose et al., 1985). All the asymmetric species synthesized have essentially identical backbone coupling patterns (±0.1 Hz), indicating that the conformational disposition of the fatty acyl chains on the glycerol backbone is unaffected by different lengths at the *sn*-1 and *sn*-2 positions. Differences in magnetic environment can be detected for the terminal methyl groups of both chains. The chemical shift difference between terminal methyl groups (Lin et al., 1987b) is smallest for the symmetric compound (diC_7PC), and increases as the *sn*-2 chain becomes shorter relative to the *sn*-1 (Table I).

The net result from these physical studies is that at the concentrations used in enzyme assays, all the 14-carbon short-chain PC micelles will either be small spherocylinders or be close to spheres with similar backbone and headgroup conformations.

Susceptibility to Phospholipases. All the novel asymmetric lecithins examined have CMC's in the range of 1 mM; therefore, for phospholipase assays with 2–20 mM PC, almost all of the activity will reflect hydrolysis of micellar substrate. Figure 2A illustrates typical activities for phospholipase C (*B. cereus*) toward different asymmetric PC's as a function of substrate concentration. Errors in specific activities are typically 10–15% of the averaged value. For all the double-chain amphiphiles, enzyme activity is more or less constant from 2 to 10 mM, consistent with the low K_m for this enzyme acting on micellar diC_7PC (El-Sayed et al., 1985). The only 14-carbon PC species demonstrating poor activity with phospholipase C is monomyristoyl-PC, which has a free hydroxyl group at the *sn*-2 glycerol carbon. This is consistent with previous studies of ether-linked lipids that showed that a carbonyl is required for enzyme activity (Burns et al., 1981; El-Sayed et al., 1985). If the phospholipase C specific activity is compared for all the different species at 6 mM PC, any dependence on the number of carbons in the *sn*-2 chain can be detected. As shown in Figure 2B, there is no dependence on the number of carbons in the *sn*-2 chain other than the need for a carbonyl moiety which was indicated earlier by examining ether-linked substrates. Phospholipids with as few as two carbons and as many as eight carbons in the *sn*-2 chain are comparable substrates for this enzyme.

Phospholipase A_2 (*N. naja naja*) shows a strikingly different dependence on the number of carbons in the *sn*-2 chain. Enzyme activities observed toward 1-C_{12}-2-C_2-PC and 1-C_{10}-2-C_4-PC are considerably lower than for diC_7PC or PC's with longer *sn*-2 chains (Figure 3A). Apparent K_m values for these PC's are in the range of 2–3 mM total PC (1–2 mM micellar PC). The same relative activities of the asymmetric PC's to diheptanoyl-PC are seen in Triton X-100 mixed micelles: 5 mM 1-C_{10}-2-C_4-PC/20 mM Triton X-100 micelles display 25.8% of the activity of mixed micelles with 5 mM diC_7PC; 5 mM 1-C_{12}-2-C_2-PC/20 mM Triton X-100 is 13.2%

as good a substrate for phospholipase A_2 as the symmetric homologue. If specific activities of phospholipase A_2 toward the pure short-chain PC micelles are compared for 6 mM substrate, a pronounced dependence on *sn*-2 chain length is observed (Figure 3B). Once the number of carbons in the *sn*-2 chain reaches seven or eight, activity levels off and remains high. The same relative activity trends with the asymmetric lecithins (5 mM) are seen in Triton X-100 (20 mM) mixed micelles. This confirms that the lower activity for shorter *sn*-2 chains is a property of the PC's and not the type of micelles. This indicates that seven carbons in the *sn*-2 chain are necessary for optimal phospholipase A_2 (*N. naja naja*) activity. The data presented here also suggest that part of the poor substrate characteristics originally seen with symmetric short-chain diC_4PC and diC_6PC (Roholt & Schlamowitz, 1961; Wells, 1972) must be attributed to lack of a long enough stretch of CH_2 groups in the *sn*-2 chain as well as the monomeric state of the substrate.

^{13}C NMR analysis of the preparation of 1-C_9-2-C_5-PC in CD_3OD showed that more than 1 PC species was present. The ^{13}C NMR shifts (ppm) for the carbons in the *sn*-1 chain were as follows: 26.01, C(3); 30.42, C(4); 30.32, C(5); 30.21 C(6) and C(7); 23.74, C(8); 14.13, C(9); shifts (ppm) for the *sn*-2 valeroyl chain carbons were 28.13, C(3); 23.25, C(4); and 14.13, C(5). The C(2) position is extremely sensitive to chain length and *sn*-1/*sn*-2 orientations (Burns & Roberts, 1980). For the 1-C_9-2-C_5-PC sample, that region exhibited four distinct resonances (35.07, 34.88, 34.81, and 34.62 ppm with the middle two ~3 times as intense as the outer resonances) and suggested the presence of a mixture of chains at both positions; i.e., some amount of 1-C_5-2-C_9-PC had been synthesized. None of the other PC's showed any heterogeneity or more than two C(2) carbons, indicating purities based on the ^{13}C NMR spectra of >98% of the appropriate species. The presence of both 1-C_9-2-C_5-PC and 1-C_5-2-C_9-PC was quantified by GC analysis of the fatty acids liberated by exhaustive phospholipase A_2 treatment of the asymmetric compound. The free fatty acids, isolated by column chromatography from the lyso-PC/phospholipase A_2 sample, run on GC and quantified, were 78% valeric acid and 22% nonanoic acid. Thus, the 1-nonanoyl-PC originally generated by phospholipase A_2 hydrolysis of diC_9PC had apparently equilibrated to 78% 1-lyso-PC and 22% 2-lyso-PC under the reaction conditions used for synthesis of the asymmetric species. The presence of both PC isomers needs to be taken into account when determining the specific activity of phospholipase A_2 toward 1-C_9-2-C_5-PC. If one assumes that (i) 1-C_5-2-C_9-PC is a substrate comparable in activity to diheptanoyl-PC [for comparison, in Triton X-100 mixed micelles, activities toward symmetric PC's with ≥7 carbons have been shown to be identical (Roberts et al., 1977b)], (ii) both isomers have comparable K_m values, and (iii) both species form ideal mixed micelles, then the observed specific activity (assuming saturation of the enzyme) is the appropriately weight-averaged value of the activities for each of the isomers: $v_{obs} = 0.78(v, \text{1-}C_9\text{-2-}C_5\text{-PC}) + 0.22(v, \text{1-}C_5\text{-2-}C_9\text{-PC})$ where v for the latter is equal to that for diC_7PC. With this treatment, an upper limit for the specific activity toward 1-C_9-2-C_5-PC is 1141 μmol min^{-1} mg^{-1}, as shown in Figure 3B.

The strong dependence of phospholipase A_2 activity on *sn*-2 chain length does not appear to be universal for all such enzymes. Enzyme activities of porcine pancreatic phospholipase A_2 toward 1-C_{12}-2-C_2-PC, 1-C_{10}-2-C_4-PC, and 1-C_8-2-C_6-PC are shown in Figure 4A. The activity toward the most asymmetric of these is ~50% of that for diheptanoyl-PC. The

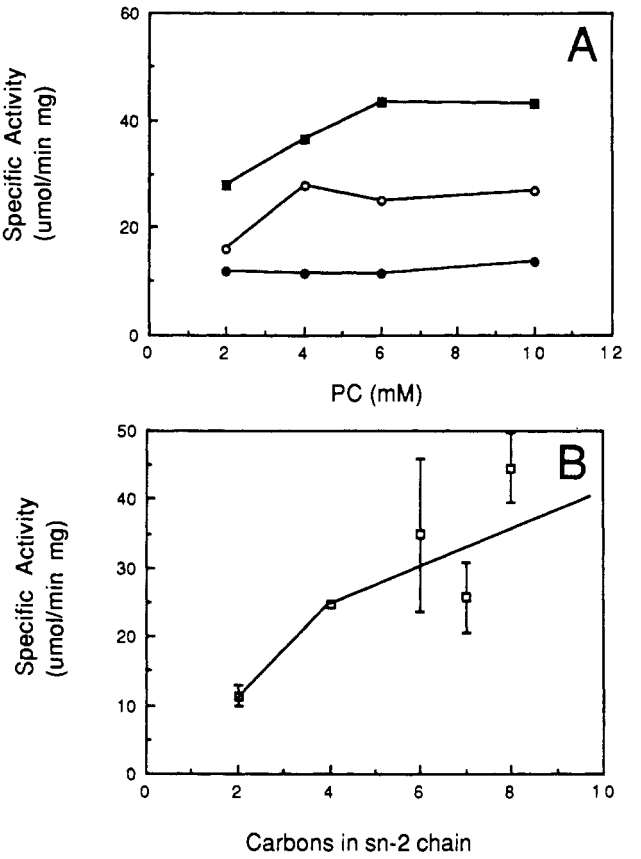

FIGURE 4: (A) Specific activity of porcine pancreatic phospholipase A_2 toward 1-C_{12}-2-C_2-PC (●), 1-C_{10}-2-C_4-PC (○), and 1-C_8-2-C_6-PC (■) as a function of PC concentration. Assay conditions were pH 8.0, 5 mM $CaCl_2$, 25 °C. (B) Dependence of the specific activity of pancreatic phospholipase A_2 on the number of carbons in the *sn*-2 fatty acyl chain for 6 mM PC.

dependence of activity on *sn*-2 chain length for 6 mM PC is shown in Figure 4B. There is a trend for increased specific activity with increasing chain length, but for this phospholipase A_2, the requirement for a linear hydrophobic stretch appears two to three carbons shorter. It is interesting to compare enzyme activity toward 6 mM 1-C_8-2-C_6-PC (43.4 μmol min^{-1} mg^{-1}) to that observed with 20 mM diC_6PC (14.6 μmol min^{-1} mg^{-1}). While both species have six carbons in the *sn*-2 chain, the symmetric PC has a considerably higher CMC (~14 mM). Even at a sample concentration of 20 mM diC_6PC, monomers predominate in solution, and the effective micellar PC concentration is ~6 mM. The monomers bind and are hydrolyzed by the enzyme at a lower V_{max}. In essence, they act as competitive inhibitors of the micellar PC, thus lowering the observed rate.

Activity of Phospholipase A_2 toward Short-Chain PC Mixed Micelles. The most asymmetric PC's were the poorest substrates for cobra venom phospholipase A_2. To determine whether or not these species were binding to the active site with the same affinity as 14-carbon lecithins with 7 or more carbons in the *sn*-2 chain, the activity of the enzyme was measured toward mixed micelles of diheptanoyl-PC and the asymmetric PC's. Figure 5A illustrates phospholipase A_2 activity toward 5 mM diC_7PC with varying concentrations of 1-C_{12}-2-C_2-PC. Inhibition of enzymatic activity toward diC_7PC is proportional to the mole fraction of 1-C_{12}-2-C_2-PC in the mixed micelles, suggesting that 1-C_{12}-2-C_2-PC binds to the enzyme active site with the same affinity as the longer *sn*-2 compounds. Once bound, it must not be in the proper orientation for efficient hydrolysis of the *sn*-2 ester bond. In mixed micelles of diC_7PC with 1-C_{10}-2-C_4-PC (Figure 5B),

Table II: Effect of Asymmetric Lecithins on Phospholipase A_2 (*Naja naja naja*) Catalyzed Hydrolysis of Egg Phosphatidylethanolamine in Triton X-100 Mixed Micelles[a]

	PE hydrolysis (μmol min^{-1} mg^{-1})[b]					
	+0.02 mM PC			+0.2 mM PC		
added lecithin[c]	−PC	+PC	% activation[d]	−PC	+PC	% activation
diC$_7$PC	42 (10)	55 (12)	32	40 (17)	116 (7)	193
1-C$_{10}$-2-C$_4$-PC	50 (6)	64 (7)	29	104 (30)	302 (139)	191
1-C$_{12}$-2-C$_2$-PC	50 (8)	55 (4)	10	115 (43)	280 (97)	143

[a] Assay conditions are 5 mM egg phosphatidylethanolamine, 20 mM Triton X-100, and 5 mM Ca^{2+}, pH 8.0. [b] 0.65 μg of phospholipase A_2 (*Naja naja naja*) used per assay; standard deviations are given in parentheses. [c] Abbreviations: diC$_7$PC, diheptanoyl-PC; 1-C$_{10}$-2-C$_4$-PC, 1-decanoyl-2-butyroyl-PC; 1-C$_{12}$-2-C$_2$-PC, 1-lauroyl-2-acetyl-PC. [d] Activation (percent of PE control) is determined by subtracting the value −PC from the value +PC for a given sample and then dividing that number by the −PC value.

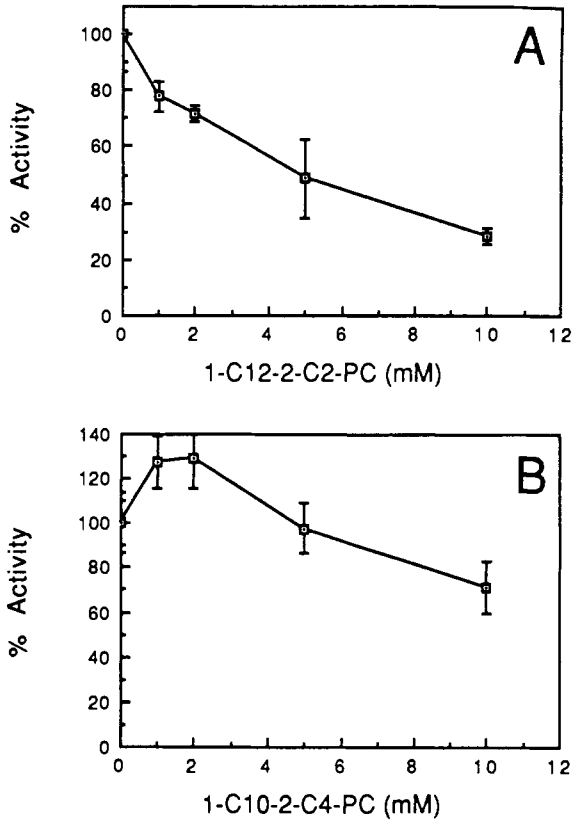

FIGURE 5: Inhibition of phospholipase A_2 (*Naja naja naja*) catalyzed hydrolysis of diheptanoyl-PC (5 mM) by (A) 1-C$_{12}$-2-C$_2$-PC and (B) 1-C$_{10}$-2-C$_4$-PC. Enzyme specific activities are expressed as the percentage of a control sample of 5 mM diheptanoyl-PC alone. $CaCl_2$ (5 mM) is present; the pH of the assay was maintained at 8.

phospholipase A_2 activity is only noticeably inhibited at higher asymmetric PC concentrations (when the mole fraction of 1-C$_{10}$-2-C$_4$-PC is >0.5). 1-C$_{10}$-2-C$_4$-PC has ~20% of the activity of diC$_7$PC, and since it is a better substrate than 1-C$_{12}$-2-C$_2$-PC, at low concentrations its effect on observed enzymatic activity will be less. The reduced inhibition may also suggest that the binding of this asymmetric PC is weaker than that for the symmetric homologue or 1-C$_{12}$-2-C$_2$-PC.

Activation of Egg Phosphatidylethanolamine Hydrolysis. A wide variety of phosphocholine-containing species (including short-chain PC's) have been shown at low concentrations to activate phospholipase A_2 (*N. naja naja*) toward PE in Triton X-100 mixed micelles (Roberts et al., 1979; Adamich et al., 1979; Pluckthun & Dennis, 1982; DeBose et al., 1985). It has been suggested that these lipids activate the enzyme by binding to a functionally distinct site on the enzyme. Both 1-C$_{12}$-2-C$_2$-PC and 1-C$_{10}$-2-C$_4$-PC were investigated for their ability to activate phospholipase A_2 (*N. naja naja*) toward 5 mM phosphatidylethanolamine/20 mM Triton X-100 mixed micelles. At a concentration of 0.02 mM, 1-C$_{10}$-2-C$_4$-PC activates PE hydrolysis 29% and is as effective as the comparable amount of diC$_7$PC (32% activation) (Table II). At this same concentration, 1-C$_{12}$-2-C$_2$-PC activates PE hydrolysis by only 10%, about one-third less than diheptanoyl-PC. At a higher lecithin concentration (0.2 mM), diC$_7$PC and 1-C$_{10}$-2-C$_4$-PC activate phospholipase A_2 hydrolysis of PE 193% and 191%, respectively, while 1-C$_{12}$-2-C$_2$-PC increases the PE hydrolysis rate slightly less, 143%. A concentration of 0.2 mM diC$_7$PC in 20 mM Triton X-100 micelles (without PE) shows essentially no hydrolytic activity by phospholipase A_2. Thus, both asymmetric species can effectively bind to the activator site.

Table III: Phospholipase-Catalyzed Hydrolysis of Short-Chain Lecithins (5 mM) as Micelles and in Gel-State Long-Chain Phospholipid (20 mM) Bilayer Aggregates[a]

	specific activity (μmol min^{-1} mg^{-1})	
phospholipid system	PLA$_2$[b]	PLC[c]
diC$_7$PC	2119 (123)[d]	2236 (76)
diC$_7$PC/SPM[e]	1129 (146)	1369 (99)
1-C$_{12}$-2-C$_2$-PC	16 (12)	2066 (143)
1-C$_{12}$-2-C$_2$-PC/SPM	67 (65)	1911 (351)
1-C$_{10}$-2-C$_4$-PC	412 (128)	2096 (160)
1-C$_{10}$-2-C$_4$-PC/SPM	1064 (127)	1616 (62)

[a] Bilayer aggregates were formed by cosolubilizing both lecithins in organic solvent, removing the solvent, lyophilizing, rehydrating, warming samples to 40 °C, and bath-sonicating samples in an E/MC Model 250 bath sonicator for 5 min. [b] 0.65 μg of phospholipase A_2, PLA$_2$ (*Naja naja naja*), was used per assay. [c] 1.2 μg of phospholipase C, PLC (*Bacillus cereus*), was used per assay. [d] Values in parentheses represent standard deviations. [e] Abbreviations: SPM, sphingomyelin (bovine brain); otherwise as in Table I.

Phospholipase-Catalyzed Hydrolysis of Short-Chain Lecithins in Bilayer Aggregates. Very asymmetric PC's as micelles are poor substrates for phospholipase A_2. The same short-chain species can be incorporated into small bilayer aggregates with gel-state long-chain phospholipids such as dipalmitoyl-PC or bovine brain sphingomyelin (Gabriel & Roberts, 1986; Eum et al., 1989). In essence, this provides a bilayer lipid assay system with a nonsubstrate long-chain lipid as the major species. Bovine brain sphingomyelin (20 mM) forms bilayer aggregates with 5 mM diC$_7$PC which are small (R_H ~90 Å, J. Bian, unpublished results) and nonlytic to erythrocytes [hence, classical micelles do not coexist and all the short-chain PC is partitioned in the bilayer particle (Bian & Roberts, 1990)]. In these particles, the long-chain component is a nonsubstrate for the phospholipases A_2 and C (Gabriel et al., 1987). Activity of phospholipases toward the short-chain PC in these particles can be compared to that toward the pure lecithin micelles at the same concentration (Table III). Whether in a micelle or a bilayer, 1-C$_{12}$-2-C$_2$-PC is a poor substrate for phospholipase A_2 from cobra venom, although the activity increases ~4-fold compared to pure 1-C$_{12}$-2-C$_2$-PC micelles. In contrast 1-C$_{10}$-2-C$_4$-PC becomes comparable to diC$_7$PC in these binary lipid bilayer particles. For phospholipase C (*B. cereus*), activity toward the asym-

Table IV: Activity of Phospholipase A_2 (*Naja naja naja*) toward Phenylalkanoyl-PC Micelles[a]

lecithin	specific activity (μmol min^{-1} mg^{-1})	CMC[b] (mM)
diC_7PC	1684 (82)[c]	1.5
1-C_6-2-C_4(phenyl)-PC[d]	935 (112)	3.9 ± 0.4
diC_4(phenyl)-PC	308 (154)	1.6[e]
1-C_6-2-C_5(phenyl)-PC	2410 (122)	1.47 ± 0.04
diC_5(phenyl)-PC	492 (400)	0.5[e]

[a] Lecithin concentration in assays was 5 mM, with 5 mM Ca^{2+}, pH 8.0, and 0.65 μg of phospholipase A_2 (*Naja naja naja*) added. [b] CMC values determined from ^1H NMR spectroscopy. [c] Values in parentheses are standard deviations from assays performed in triplicate. [d] Abbreviations: 1-C_6-2-C_4(phenyl)-PC, 1-hexanoyl-2-phenylbutyroyl-PC; 1-C_6-2-C_5(phenyl)-PC, 1-hexanoyl-2-phenylvaleroyl-PC. [e] El-Sayed et al. (1985).

metric short-chain PC's in the bilayer aggregates is comparable to the micellar control sample and diC_7PC.

Activity of Phospholipase A_2 toward Phenylalkanoyl Substrates. Phospholipase A_2 has previously been shown to be sensitive to steric bulk in both acyl chains (DeBose et al., 1985). Because of this and the well-developed requirement for ≥6 carbons in the *sn*-2 chains for optimal activity, the enzyme was examined for its activity toward lecithins with phenylalkanoyl chains. While these species have >14 carbons in the fatty acyl chains, they possess unsaturated carbons which are considerably less hydrophobic than saturated species; the asymmetric species synthesized were designed to have similar CMC values to diC_7PC. Four substrates were synthesized with a phenyl group introduced after four or five carbons in the fatty acyl chain. The specific activities of phospholipase A_2 (*N. naja naja*) toward these mono- and diphenylalkanoyl substrates are shown in Table IV. The diphenylalkanoyl-PC's were hydrolyzed at ~20% of the rate observed for diC_7PC. Monophenylalkanoyls, with hexanoic acid esterified at the *sn*-1 carbon, were better substrates for phospholipase A_2. The short-chain PC with five carbons (four CH_2 groups) before the phenyl ring (1-C_6-2-C_5-phenyl-PC) was equivalent as a substrate to diC_7PC. The specific activity of the enzyme toward a 5 mM sample of the asymmetric PC with three CH_2 groups before the phenyl ring (1-C_6-2-C_4-phenyl-PC) was half that toward diheptanoyl-PC. The 1-C_6-2-C_4-phenyl-PC has a higher CMC than the other phenylalkanoyl-PC's, and hence the lower specific activity at 5 mM substrate could reflect the lower micelle concentration of the sample. When phospholipase A_2 activity was examined with 10 mM substrate, the ratio of enzyme activity toward 1-C_6-2-C_4-phenyl-PC to diC_7PC was maintained at ~0.5. These observations are consistent with the proposed requirement of phospholipase A_2 for a reasonable number of unhindered CH_2's in the *sn*-2 chain for optimal activity.

Discussion

Asymmetric Short-Chain PC's: Well-Defined Micelles. The asymmetric lecithins with 14 carbons in the acyl chains form a useful set of substrates to probe chain specificity of phospholipases. Because of the same total number of carbons, they have similar CMC's. The differences in the CMC values that are detected reflect the fact that both chains are not independent in interacting with water in a monomeric state, but rather interact with each other to some extent. Since this is more probable for two chains with nearly equivalent length fatty acids, those species will be slightly less likely to form micelles. Micelle size also decreases as the lecithin become more asymmetric in structure (i.e., 1-C_{12}-2-C_2-PC and 1-C_{10}-2-C_4-PC). This follows from packing considerations of headgroup area and hydrocarbon chain volume. While there are no SANS data for 1-C_{12}-2-C_2-PC, the series 1-C_8-2-C_6-PC, diC_7PC, and 1-C_6-2-C_8-PC have been examined (Lin et al., 1987b). At high PC concentrations, all three form rod-shaped micelles with 1-C_8-2-C_6-PC forming the smallest structures. As the lipid concentration is decreased to 5 mM, the particles approach minimum size micelles, estimated to be ~30 monomers for all 3 species. Thus, all structures will be similar for this series of micelles at assay concentrations. The averaged conformations of these PC's around the glycerol backbone are also similar. This ensures that they will be excellent probes for specific chain binding requirements of the phospholipases.

Interactions of Phospholipase C with Fatty Acyl Chains. Phospholipase C from *Bacillus cereus* is a monomeric, 245-residue protein that catalyzes the hydrolysis of the glycerophosphate bond in phospholipids to liberate diacylglycerol and a phosphate ester. The high-resolution crystal structure of phospholipase C from *Bacillus cereus* has been determined at 1.5 Å using molecular replacement methods (Hough et al., 1989). Phospholipase C is quite unique as it appears to be the only member in a novel class of enzymes reported to be entirely α helical in its conformation. Two groups of residues are present in the active site of phospholipase C: an acidic pocket formed by Glu-4, Asp-55, Tyr-56, and Glu-146, and a second group containing Ser-64, Thr-65, Phe-66, Phe-70, Ile-80, Thr-133, Asn-134, Leu-135, and Ser-143 (Hough et al., 1989) which appears to be moderately hydrophobic. Previous work had shown that diC_4PC is a poor substrate (with a high K_m and a low V_{max}) for phospholipase C (*B. cereus*) (El-Sayed et al., 1985). The low specific activity could not be attributed to the predominance of monomers in solution since when diC_6PC and diC_7PC were assayed as monomers they were found to be good substrates for this enzyme (El-Sayed et al., 1985). The dramatic improvement in phospholipase C activity observed for diC_6PC compared to diC_4PC indicated that more than four carbons in each chain or more than eight total in both chains are required to anchor the enzyme to the PC (presumably by hydrophobic interactions). Whether this was a general hydrophobic effect or specific to the *sn*-2 chain was unclear. The present results allow unambiguous interpretation of those data: the low activity observed for diC_4PC is due to an insufficient total number of carbons and hence a general lack of hydrophobicity of the chains rather than to a lack of carbons in a specific chain. This conclusion is refined by the observation that all the asymmetric short-chain lecithins with a total of 14 carbons in the acyl chains display high activity, comparable to the symmetric homologue diC_7PC, with phospholipase C. This indicates that other than a carbonyl group, the *sn*-2 chain (which ranges from two to eight carbons) is not necessary for high phospholipase C activity. To be a good substrate for phospholipase C, a lipid requires a phosphoester bond, an ester linkage (with the *sn*-2 carbonyl better than the *sn*-1 carbonyl) for binding interactions with the enzyme (Burns et al., 1981; El-Sayed et al., 1985), and a general hydrophobicity of the acyl chains (apparently satisfied by ≥12 carbons total in both chains).

Interactions of Phospholipase A_2 with Fatty Acyl Chains. Phospholipase A_2 is a ubiquitous lipolytic enzyme responsible for the cleavage of the *sn*-2 ester bond in phospholipids. There exists a high degree of homology in the sequences between different sources of the enzyme with the proposed catalytic residues His-48, Tyr-52, Tyr-73, and Asp-99 conserved in all phospholipase A_2 proteins (Renetseder et al., 1985). The *Naja naja naja* enzyme appears to be active as a dimer, while the pancreatic enzyme may be monomeric [for a discussion, see Roberts and Dennis (1989)]. Kinetically, phospholipase A_2

from cobra venom displays high V_{max} values, consistent with the observation that it is the most highly "penetrating" snake venom currently known (Van der Wiele et al., 1988). Porcine pancreatic phospholipase A_2 is not a very effective "penetrating" enzyme; this kinetic effect has been attributed (among other reasons) to the absence of a hydrophobic region that serves as a lipid binding domain and which is present in phospholipase A_2 from *N. naja naja* (Van der Wiele et al., 1988). In the present work, we have kinetically shown specific differences in hydrophobic binding of the cobra venom and pancreatic enzymes. The former specifically binds a larger segment of the *sn*-2 chain.

Information on steric bulkiness and its effect on substrate processing can also be gained by examining phospholipase A_2 activity toward modified short-chain lecithin molecules. For diphenylalkanoyl substrates, there is essentially no difference in activity whether three or four carbons separate the carbonyl and phenyl groups. The two phenyl groups render the lipids too bulky to fit optimally into the proposed "dual phospholipid" active site (Dennis, 1987). Activity improves on eliminating one of the phenyl groups. Bulkiness of the substrate is less of a factor with the monophenylalkanoyl-PC's. Activity is lower for the monophenylalkanoyl-PC with only three CH_2's separating the phenyl and carbonyl groups because it does not fit as well into an active site designed to hold six CH_2 groups. When four CH_2's separate the carbonyl and phenyl groups, activity is high, comparable to diC_7PC. The presence of the extra carbon in the latter species seems to be enough to make it fit properly into the active site (if one considers the first carbon in the phenyl ring).

In sum, these experiments indicate both the steric limits of phospholipase A_2 (*Naja naja naja*) and *sn*-2 chain requirements. While seven carbons in the *sn*-2 chain of the substrate are necessary for optimal hydrolysis by phospholipase A_2, anything after the fourth carbon in this chain can be part of an aromatic ring, or possess a branch methyl (DeBose et. al., 1985) as long as the *sn*-1 chain is linear.

Short-Chain PC's in Bilayers. Previous studies (Gabriel et al., 1986) have shown that when 20 mol % short-chain lecithin (diheptanoyl-PC) is mixed with a long-chain non-substrate phospholipid (sphingomyelin) below its gel-to-liquid-crystalline phase transition temperature, bilayer aggregates are the major species in solution. Furthermore, the short-chain components are preferentially hydrolyzed in this assay system (Gabriel et al., 1987). These bilayer particles contain <0.1 mM monomeric or micellar short chain; hence, the substrate is in an aggregated form. In general, specific activities of both phospholipases A_2 and C decrease 2-fold in going from pure micelle to bilayer. Some of this drop may be the result of competitive inhibition by the long-chain lipid (Gabriel et al., 1987). That drop in specific activity does not occur with the two most asymmetric PC's. In fact, with phospholipase A_2, there is a distinct increase in specific activity when the short-chain PC is organized in the bilayer particles (although the values for 1-C_{12}-2-C_2-PC are still much lower than for diC_7PC). While the short-chain PC's (e.g., diC_7PC) in these binary PC aggregates are partially phase-separated from the gel-state long-chain phospholipid, there must be some lipid–lipid interactions (probably involving the fatty acyl chains) that affect enzymatic processing of the short-chain PC. Because of the shortened *sn*-2 chains, such interactions may be missing for 1-C_{12}-2-C_2-PC and 1-C_{10}-2-C_4-PC; hence, an apparent increase in activity is observed. An alternative explanation could involve the long-chain PC as well as the short-chain PC binding to the enzyme, perhaps with the long-chain molecule in the activator site. In this case, the *sn*-2 chain of 1-C_{12}-2-C_2-PC would still be too short for productive binding at the catalytic site, but the 1-C_{10}-2-C_4-PC may now fit in with better binding properties.

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Inhibitors Directed to Binding Domains in Neutrophil Elastase†

Suresh C. Tyagi‡ and Sanford R. Simon*,§
Department of Biochemistry and Cell Biology and Department of Pathology, State University of New York at Stony Brook, Stony Brook, New York 11794

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ABSTRACT: Human neutrophil elastase (HNE) can be inhibited by unsaturated fatty acids, including oleic acid [Ashe, B. M., & Zimmerman, M. (1977) *Biochem. Biophys. Res. Commun. 75*, 194-199; Cook, L., & Ternai, B. (1988) *Biol. Chem. Hoppe-Seyler 369*, 627-631], but is not affected by saturated fatty acids. We have shown that the interaction of oleic acid with HNE can be characterized by two apparent inhibitory modes: a high-affinity mode (K_i = 48 ± 3 nM), resulting in partial noncompetitive inhibition (87% residual activity), and a competitive inhibitory mode of lower affinity (K_i = 16 ± 1 μM). Binding of oleate in the high-affinity mode induces a blue shift in the endogenous fluorescence arising from the tryptophan residues in HNE. This shift is maximal in the presence of 1 μM oleate; higher concentrations of fatty acid have no further effect on the fluorescence spectrum. The negatively charged fluorescent ester of oleic acid and hydroxypyrenetrisulfonate (HPTSoleate) interacts with HNE at an apparent single site (K_i = 44 ± 3 nM), resulting in competitive inhibition. A blue shift in the emission maximum of the pyrene fluorescence at 410 nm and a decrease in the ratio of the intensities of the maximum at 388 and 410 nm indicate that upon binding to HNE the environment of the pyrene ring in HPTSoleate becomes more hydrophobic. In order to probe further the roles of nonpolar and electrostatic interactions in binding of negatively charged hydrophobic inhibitors to HNE, the enzyme was modified with the arginine-specific reagent pyreneglyoxal (PYG). Under conditions in which only two arginines are modified by PYG, the catalytic activity of HNE is eliminated. Upon reaction with HNE, the two maxima in the emission spectrum of PYG are both blue-shifted, and the ratio of the intensities around 378 and 395 nm is decreased, indicating increased hydrophobicity of the environment surrounding the pyrene ring. An additional blue shift of both maxima and a further change in intensity ratio are seen in the presence of oleic acid, but only at high concentrations (200 μM), suggesting that the apparent high-affinity mode of binding for oleate may no longer be accessible after reaction of HNE with PYG. These results suggest a role for at least one arginine residue in a hydrophobic environment in regulating substrate binding and catalysis by HNE. Inhibitors which interact with both this positively charged center and the neighboring hydrophobic environment should be especially potent and selective for HNE.

Human neutrophil elastase (HNE)[1] is believed to be responsible for much of the damage to connective tissues associated with inflammatory processes (Travis & Salvesen, 1983; Janoff, 1985; Bieth, 1986). Interactions between this protease and the proteins of the extracellular matrix are dominated by hydrophobic interactions (Lonky & Wohl, 1983; Lestienne & Bieth, 1980). The extended substrate binding pocket of HNE contains an abundance of hydrophobic amino acid side chains, including a number of phenylalanine rings (Bode et al., 1986).

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* Address correspondence to this author at the Department of Pathology, State University of New York at Stony Brook.
‡ Department of Biochemistry and Cell Biology.
§ Department of Biochemistry and Cell Biology and Department of Pathology.

[1] Abbreviations: HNE, human neutrophil elastase; DMF, dimethylformamide; PYG, pyreneglyoxal; HPTSoleate, ester of oleic acid and 8-hydroxypyrene-1,3,6-trisulfonate; MeOSucAAPVpNa, methoxysuccinylalanylalanylprolylvalyl-*p*-nitroanilide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; PPE, porcine pancreatic elastase; IEF, isoelectric focusing; PBS, phosphate-buffered saline.

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