Fluorescence Studies of Phosphatidylcholine Micelle Mixing: Relevance to Phospholipase Kinetics†

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ABSTRACT: Two fluorescent micellar phospholipid probes (1-hexanoyl-2-(1-pyrenebutyroyl)phosphatidylcholine and 1-octanoyl-2-(1-pyrenebutyroyl)phosphatidylcholine) have been synthesized, characterized, and used to monitor the dynamics of lipid/amphiphile exchange in a variety of detergents and phospholipid micelles using both steady-state and stopped-flow fluorescence techniques. The ratio of the pyrene monomer to excimer band is a good indicator of the extent of lipid mixing at equilibrium. Following the time dependence of increase in the monomer band with stopped-flow methodology provides a rate constant for this exchange process (most systems were well fit with a single exponential). Short-chain pyrene-labeled phosphatidylcholine mixing with Triton X-100 micelles is extremely fast and follows a concentration dependence indicative of the importance of micelle collisions for the exchange process. Submicellar amounts of Triton have no effect on the fluorescent dynamics of the probe molecule. Other detergents such as β-octyl glucoside and deoxycholate are also effective at higher concentrations, although significant differences exist in the extent of probe mixing. Short-chain diacylphosphatidylcholine and lysophosphatidylcholine mixing rates are moderately fast with mixing times that decrease as the hydrophobicity/chain length of the diluent matrix increases. The rate constants for lipid exchange can be compared to turnover rates of several phospholipases in these assay systems. Anomalous mixing behavior of unusual micelle forming lipids [bolaforms and ω-carboxylate phosphatidylcholines [Lewis, K. A., Bian, J., Sweeny, A., & Roberts, M. F. (1994) *Biochemistry 29*, 9962–9970] and polymerizable phosphatidylcholines [Soltys, C. E., Bian, J., & Roberts, M. F. (1993) *Biochemistry 32*, 9545–9551] is particularly helpful in understanding kinetics of water-soluble phospholipases on these systems.

Water-soluble lipases (e.g., phospholipases A_2 and C) must bind to substrate at an interface before the appropriate ester bond can be hydrolyzed. These enzymes which act on the outer surface of a bilayer have key roles in signal transduction and membrane remodeling. While bilayer phospholipids are the natural substrates for these enzymes, they are often poorly hydrolyzed. For diacyl-PC[1] substrates, packing the phospholipid into a micelle rather than a bilayer array leads to a higher rate of hydrolysis (El-Sayed et al., 1985; Kensil & Dennis, 1979; Op den Kamp et al., 1975; Wells, 1972; Roholt & Schlamowitz, 1961). Both detergent/phosphatidylcholine mixed micelles (Lichtenberg et al., 1983; Verger, 1980; Dennis, 1973a,b) and pure short-chain lecithins (Lewis et al., 1990; DeBose et al., 1985; Little, 1977; Verger & De Haas, 1976; Wells, 1974; Bonsen et al., 1972b; De Haas et al., 1971) have, therefore, been used extensively in the investigation of water-soluble phospholipases. In the latter systems, the individual lecithin molecules have many of the same dynamic features of naturally occurring lecithins in bilayers with the exception that the area per headgroup is considerably larger and intermolecular interactions with the aggregate are looser (Bian & Roberts, 1992; Lin et al., 1986, 1987a; Burns et al., 1983; Burns & Roberts, 1980). An implicit assumption in using these micellar phospholipid systems for phospholipase kinetics is that there is a rapid reorganization of lipid components when the micelle concentration is decreased or when other amphiphilic molecules are added. This has been questioned in recent kinetic studies of phospholipase A_2 (Jain et al., 1991; Rogers et al., 1992). Clearly, a direct measurement of phospholipid exchange is necessary to evaluate whether or not substrate replenishment and/or product exchange has a pronounced effect on the enzyme kinetics.

Two fluorescent micellar phospholipid probes, 1-hexanoyl-2-(1-pyrenebutyroyl)-PC (1-C_6-2-C_4pyr-PC) and the more hydrophobic 1-octanoyl-2-(1-pyrenebutyroyl)-PC (1-C_8-2-C_4-pyr-PC), have been synthesized and characterized in order to monitor the rate of phospholipid exchange in a variety of detergents and phospholipid micelles using both steady-state and stopped-flow fluorescence techniques. In the steady-state fluorescence experiment, the pyrene emission spectrum is characterized by several discrete bands: monomer emissions at 380 and 395 nm and an emission at longer wavelength (~470 nm) from an excited state dimer, or excimer, produced by the collisional interaction of an excited state pyrene molecule and a ground state pyrene molecule. The photophysics of pyrene fluorescence has been described in detail previously and is applicable to the present phospholipid pyrene probes (Forster, 1969; Chen & Chen, 1988). At low 1-C_6-2-C_4pyr-PC concentrations (below the CMC) all of the fluorescence emission appears between 365 and 425 nm originating from the lowest excited singlet state. At higher concentrations the fluorescent band associated with excimer formation (maximum intensity at 470 nm) appears. Thus, the ratio of excimer intensity to monomer intensity is high for aggregated phosphatidylcholine and shows a sharp increase at the CMC. If nonfluorescent micellar molecules are added to the pyrene-labeled phosphatidylcholine micelles, the excimer band will decrease as the collisional cross-sectional interaction of one pyrene–phosphatidylcholine with another pyrene

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[1] Abbreviations: diacyl-PC or diC_nPC, 1,2-diacyl-3-phosphocholine; 1-C_x-2-C_y-PC, 1-acyl-2-acyl-3-phosphocholine; 1-acyl-PC or 1-C_n-PC, 1-acyl-3-phosphocholine; CMC, critical micelle concentration.

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molecule decreases. Following the time dependence of the increase in intensity of the monomer band with stopped-flow fluorescence methodology provides a rate constant for this mixing process (Charlton et al., 1976; Sengupta et al., 1976; Galla et al., 1979; Homan, & Pownall, 1988). The excimer/monomer ratio is proportional to the pyrene concentration only in systems with homogeneously distributed probes; otherwise that ratio reflects a complicated function characterizing the intensity ratio as a function of probe concentration (Roseman et al., 1980). However, if an excess of acceptor particles is used, the time dependence of excimer decrease or monomer increase can be fit to a single or multiexponential function to obtain transfer rates (Homan & Pownall, 1988; Sengupta et al., 1976; Galla et al., 1979). One of the key assumptions is that excimer formation of pyrene-labeled lipids is predominantly diffusion controlled. This has been verified in liquid crystalline membranes and, therefore, should also be applicable to mixed micelles (Lemmetyinen et al., 1989). Therefore, under equilibrium conditions, relative changes in these emission bands are good indicators of the extent of lipid mixing.

The results in the present work show that the mixing of short-chain pyrene-labeled phosphatidylcholine micelles with Triton X-100 micelles is extremely fast compared to the other detergents examined. Furthermore, the concentration dependence of the rate constant is indicative of the importance of micelle collisions for the exchange process. Mixing rates with diacyl-PCs as well as lysophosphatidylcholines are moderately fast and decrease as the hydrophobicity of the probe increases. For a good micellar substrate such as dihexanoyl-PC or diheptanoyl-PC, micelle reorganization should occur rapidly as product is produced, although rates depend on the hydrophobicity of the micelle molecules. A comparison of micelle mixing rate constants with the phospholipase turnover numbers strongly argues that substrate depletion and replenishment is not, in general, rate limiting with these systems. However, in some systems containing modified phosphatidylcholine species, relatively slow mixing rates were observed which correlate well with a dramatically reduced susceptibility to the phospholipases.

MATERIALS AND METHODS

Chemicals. All chemicals and reagents were obtained commercially and used without further purification unless stated otherwise. Dihexanoyl-PC (diC_6PC), diheptanoyl-PC (diC_7PC), dioctanoyl-PC (diC_8PC), 1-hexanoyl-PC, 1-octanoyl-PC, 1-myristoyl-PC, and 1-lauroyl-PC in chloroform were obtained from Avanti Polar Lipids. 1-Palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine was obtained from Molecular Probes, Inc. 1-Pyrenebutyric acid and 4-dimethylaminopyridine were obtained from Aldrich; *N,N'*-dicyclohexylcarbodiimide was obtained from Sigma. Deionized water (10 MΩ·cm) doubly distilled from potassium permanganate was used in surface tension measurements and in making certain lipid solutions.

Synthesis of Pyrenebutyroyl-PC Probes. 1-Hexanoyl-PC was added to a chloroform solution of 1-pyrenebutyric acid activated with a mixture of *N,N'*-dicyclohexylcarbodiimide and 4-dimethylaminopyridine. 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 chromatographed on a silicic acid column using a chloroform/methanol gradient. Fractions were checked for purity by thin layer chromatography and purified a second time over silicic acid. The identity and purity of 1-C_6-2-C_4pyr-PC was confirmed by ^1H and ^{13}C spectroscopy (Soltys et al., 1993; Bian, 1991). The same synthetic scheme was followed in the preparation of 1-C_8-2-C_4pyr-PC.

Synthesis of Other Phosphatidylcholines. 1-Decanoyl-2-butyroyl-PC (1-C_{10}-2-C_4-PC) and 1-octanoyl-2-heptanoyl-PC (1-C_8-2-C_7-PC) were synthesized and purified as described earlier (Lin et al., 1987b; Lewis et al., 1990). Likewise, the synthesis of 1,2-bis(6-mercaptohexanoyl)-PC (diC_6SH-PC), 1,2-bis(8-mercaptooctanoyl)-PC (diC_8SH-PC), and 1-hexanoyl-2-(8-mercaptooctanoyl)-PC (1-C_6-2-C_8SH-PC) has been described in detail previously (Soltys et al., 1993). Micellar concentrations of the sulfhydryl-containing phospholipids were cross-linked with H_2O_2 to form polymers (dimers in the case of the asymmetric thiol phosphatidylcholine); excess H_2O_2 was removed by the addition of catalase (Soltys et al., 1993).

Surface Tension Measurements. A Fisher Surface Tensiomat 21 utilizing the du Nouy ring method was used in measuring the surface tension of phosphatidylcholine and detergent species to determine CMC values when needed (Findlay, 1972). The break in the plot of surface tension versus ln(amphiphile) was used to estimate the CMC.

Fluorescence Measurements. Steady-state fluorescence measurements were made with a Shimadzu RF5000V spectrofluorimeter. The short-chain pyrene-labeled phosphatidylcholine was excited at 350 nm with a 1.5–3-nm bandwidth; probe emission was monitored from 360 to 550 nm using a 3-nm bandwidth. Almost all of the fluorescence information for both excimer and monomer bands was included in this emission range.

Stopped-flow fluorescence measurements were performed with a KinTeK Inc. spectrophotometer operating at a constant temperature of 25 °C. The dead time of this instrument was approximately 1 ms. One syringe contained the pyrene-labeled molecule, while the other syringe contained the diluent of interest. A colorimetric phosphate assay was used to determine exact phosphatidylcholine concentrations (Chen et al., 1956; Turner & Rouser, 1970). The probe was excited at 350 nm, and fluorescence intensity was monitored at 380 nm using a 1 in. filter band-pass via a National Instruments analog/digital interface. For each sample, at least five successive runs were made in order to ensure reproducibility. The mixing rate constant reported represents an average of these values.

RESULTS

Monomer–Micelle Reorganization of Pyrene-PC Probe. The 1-C_6-2-C_4pyr-PC probe has a CMC of 0.01–0.025 mM (Bian, 1991) and when present at 0.12 or 0.06 mM (Figure 1A,B) will exhibit a large excimer band at 470 nm. The increase in the monomer band and decrease in the excimer band as the phosphatidylcholine micelles are diluted from 0.12 to 0.06 mM reflects the larger proportion of probe present as monomers. The increase in the ratio of the monomer to excimer band is from 0.184 to 0.519. When the 0.06 mM solution is diluted further to 0.03 mM (as would be done in a stopped-flow experiment by mixing the phosphatidylcholine micelles 1:1 with a syringe containing buffer), there will be another redistribution of molecules increasing the ratio of monomeric to micellar phosphatidylcholine (Figure 1C). The change for this dilution is larger because of the closeness to the CMC of the 1-C_6-2-C_4pyr-PC. Thus, the fluorescence of the probe micelles is sensitive to changes in micelle distribution. The time course of this change can be followed in a stopped-

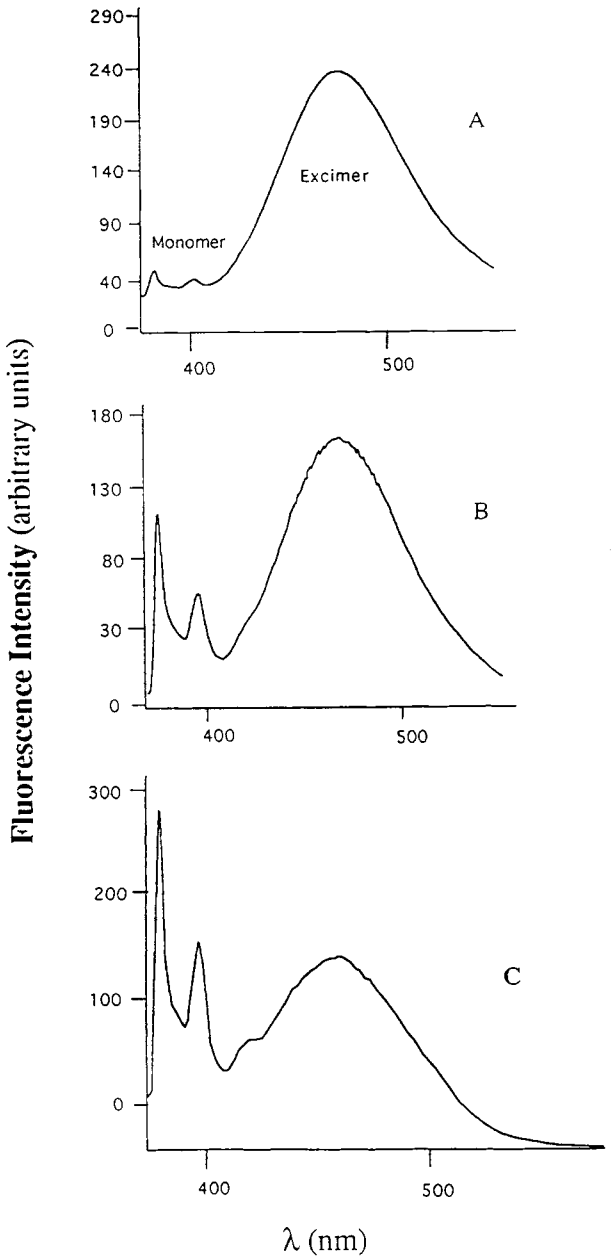

FIGURE 1: Fluorescence spectra of (A) 0.12 mM, (B) 0.06 mM, and (C) 0.03 mM 1-C_6-2-C_4pyr-PC. The excitation wavelength was 350 nm.

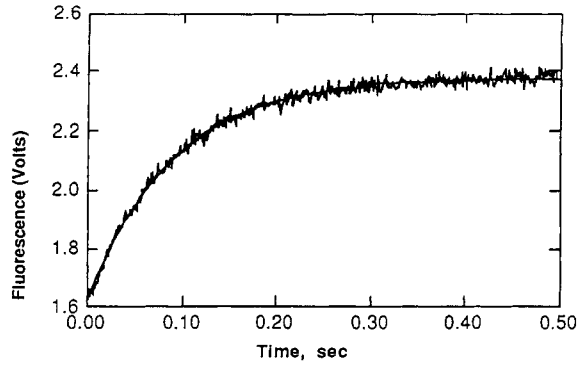

FIGURE 2: Fluorescence intensity of 1-C_6-2-C_4pyr-PC at 380 nm as a function of time after dilution of a 0.06 mM solution with water to 0.03 mM. The line through the data is a fit to $A_0e^{-k/t} + B$ (k = 11.5 ± 0.1 s^{-1}).

flow experiment (Figure 2). The data are well fit by a pseudo-first-order rate constant, 11.5 ± 0.1 s^{-1}, which reflects redistribution mixing of this particular pyrene-containing short-chain lecithin species. If we introduce a monomeric diluent and it does not insert into the 1-C_6-2-C_4pyr-PC micelle, then the micelle dilution reorganization rate should be the same as for 1-C_6-2-C_4pyr-PC micelles alone. If a micellar diluent is added, mixing of the fluorescent micelles with the nonfluorescent micelles can be accurately followed with this probe if the rate constant $k > 11$ s^{-1}.

The more hydrophobic short-chain pyrene-PC probe, 1-C_8-2-C_4pyr-PC, was also examined in a 1:1 dilution experiment. The CMC for this lipid is an order of magnitude lower, and hence a 2-fold dilution from 0.06 to 0.03 mM had no effect on the monomer or excimer intensities. Therefore, mixing processes slower than the micelle/monomer dilution ($k < 11$ s^{-1}) can be followed accurately with this probe.

Detergent Mixing with 1-C_6-2-C_4pyr-PC. Long-chain phospholipids dispersed in detergent mixed micelles are a popular assay system for water-soluble enzymes (Dennis, 1973a,b; Lichtenberg et al., 1983). These kinetic studies involving detergent mixed micelles have thus far assumed ideal, rapid mixing of the detergent with phospholipid molecules. Three detergents, Triton X-100, β-D-octyl glucoside, and sodium deoxycholate, at both monomer and micellar concentrations were mixed with 1-C_6-2-C_4pyr-PC micelles. There was a negligible effect on 1-C_6-2-C_4pyr-PC spectral properties when the detergent was mixed below its CMC with pyrene-labeled micelles. Slightly below the CMC, some mixing was detected by a slightly increased pyrene monomer band, although the extent of mixing was small as measured by the ratio of monomer to excimer band intensity (I_{380}/I_{470}). Above the CMC, micellar detergent interacted with probe micelles to reduce the excimer intensity (compare Figure 3 panels A and B showing the effect of mixing 1-C_6-2-C_4pyr-PC micelles with monomeric and micellar β-octyl glucoside). The I_{380}/I_{470} ratios, which monitor the efficiency of micelle mixing, are summarized in Table 1. Triton X-100 micelles were most effective in the extent to which they reduced the 1-C_6-2-C_4-pyr-PC excimer band. The other nonionic detergent, β-octyl glucoside, was distinctly less effective even when correcting for its high CMC. Not until a micelle concentration of 11.8 mM β-octyl glucoside was the extent of micelle mixing (I_{380}/I_{470} = 40.3) close to that of Triton X-100 at 2.20 mM micelles (I_{380}/I_{470} = 43.5). This difference could reflect differences in average micelle sizes or nonrandom mixing in the case of the β-octyl glucoside. Alternatively, the larger headgroup of β-octyl glucoside may affect the way that molecule packs with the 1-C_6-2-C_4pyr-PC molecules. Triton X-100 micelles were also more effective in mixing with pyrene-labeled micelles than deoxycholate (for example, see the steady-state spectra in Figure 3C,D).

The time scale for reaching this equilibrium, measured by fitting the stopped-flow data with a first-order rate constant (Figure 4), was very short for all micellar detergents. Furthermore, the mixing rate increased as the diluent micelle concentration increased. A model to account for the transfer of phospholipids into bile salt micelles (Nichols, 1988; Fullington et al., 1990) proposed that, at low micelle concentrations, transfer occurred predominantly by aqueous diffusion of individual monomeric molecules and, at high micelle concentrations, transfer occurred via a collision-dependent process. This is supported by the stopped-flow analyses of 1-C_6-2-C_4pyr-PC mixing with the three detergents. The dependence of k on both total detergent concentration and micellar detergent concentration is shown in Figure 5. When comparable concentrations of micellar detergent are

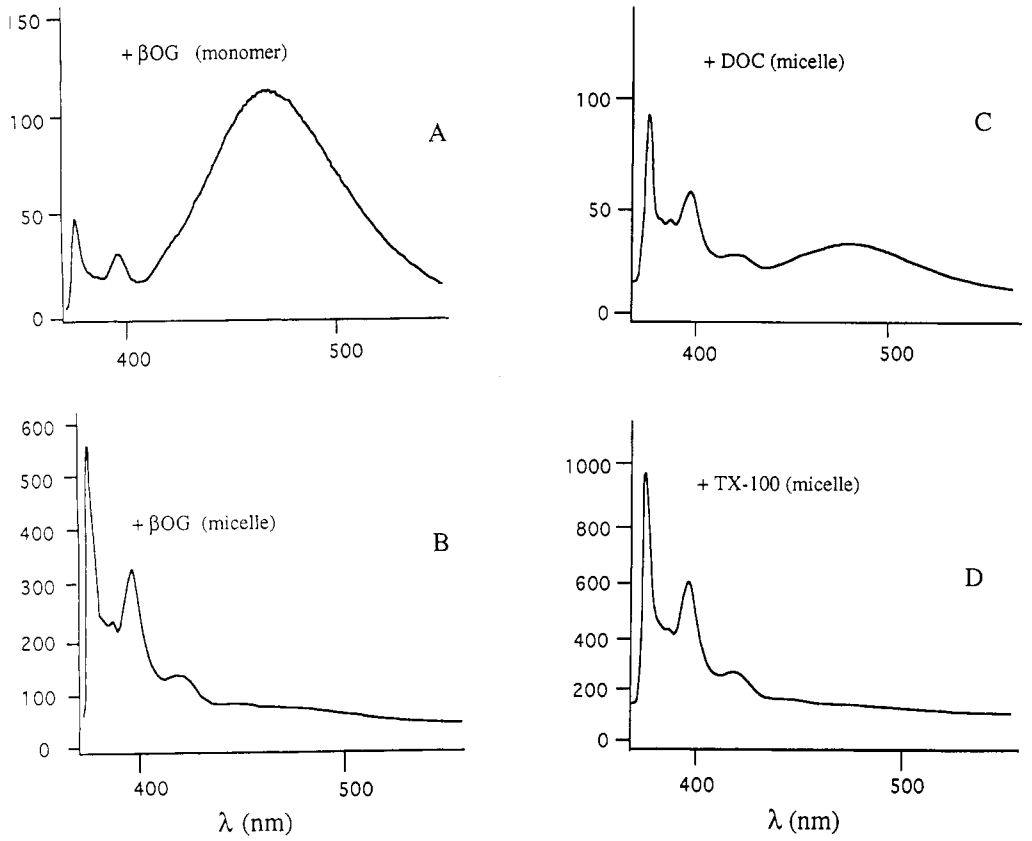

FIGURE 3: Fluorescence spectra of 0.06 mM 1-C_6-2-C_4pyr-PC mixed with (A) monomeric β-octyl glucoside (15.0 mM), (B) 11.8 mM micellar β-octyl glucoside (70.0 mM original detergent concentration), (C) 2 mM micellar DOC (10.0 mM original DOC concentration), and (D) 2.2 mM micellar Triton X-100 (5 mM original TX-100 concentration).

Table 1: Mixing of Detergents with 1-C_6-2-C_4pyr-PC[a] and 1-C_8-2-C_4pyr-PC[a] As Monitored by Fluorescence

[detergent]$_{final}$[b] (mM)	CMC (mM)	[micelle][c] (mM)	k (1-C_6-2-C_4pyr-PC) (s^{-1})	I_{380}/I_{470}	k (1-C_8-2-C_4pyr-PC) (s^{-1})	I_{380}/I_{470}
TX-100	0.3					
0.5		0.2	544 ± 53	2.16	46.4 ● 1.3	14.7
2.5		2.2	456 ± 33	43.5	114 ± 3	
10		9.7	735 ± 152	83.0		
βOG	23.2					
15			7.4 ± 0.1	0.41		
20			13.8 ± 1.5	1.18	5.5 ± 0.1	17.5
25		1.8	49.8 ± 5.2	17.5		
30		6.8	242 ± 34	23.6		
35		11.8	372 ± 25	40.3	38.4 ± 0.9	
DOC	3.0					
0.5			17.3 ± 0.4	0.67		
2.5			38.8 ± 1.2	0.59		
5.0		2.0	105.6 ± 5.6	4.43	9.3 ● 0.1	
10		7.0	284 ± 53	48.3	112.2 ● 1.2	

[a] Initial pyrene-labeled phosphatidylcholine concentration is 0.06 mM. [b] Abbreviations: TX-100, Triton X-100; βOG, β-octyl glucoside; DOC, deoxycholate. [c] The micelle concentration is the bulk detergent concentration minus the CMC.

compared, both β-octyl glucoside and deoxycholate behave similarly. However, Triton X-100 micelles mixing with 1-C_6-2-C_4pyr-PC are initially more effective in the speed with which they reduce the monomer band. Interestingly, the slope of k versus micellar Triton concentration is the same as that for the other two detergents. Why Triton is initially more effective in promoting micelle mixing is unclear at this time.

As the hydrophobicity of the pyrene probe increased (i.e., 1-C_8-2-C_4pyr-PC versus 1-C_6-2-C_4pyr-PC), the rate of mixing with detergent micelles decreased markedly, although the decrease in mixing rates was most pronounced for β-octyl glucoside. Furthermore, the concentration dependence of the mixing rate with detergent concentration was more pronounced with the more hydrophobic probe (compare the ratio of k for 0.2 and 2.2 mM micellar Triton X-100 and 2.0 and 7 mM micellar deoxycholate mixing with both pyrene-labeled probes). This is consistent with micelle collisions rather than monomer exchange dominating the exchange process. Because the detergent has not changed, it also suggests that monomer diffusion of the pyrene probe contributes significantly to the exchange rates observed with 1-C_6-2-C_4pyr-PC.

Thus, while the mixing rates clearly depend on micelle characteristics of the diluent, the hydrophobicity of the pyrene-labeled phosphatidylcholine also affects the mixing. In order to examine mixing behavior of a much more hydrophobic pyrene-labeled lecithin with Triton X-100 micelles, a mixed micelle sample of 0.06 mM 1-C_6-2-C_4pyr-PC or 1-C_{16}-2-C_{10}-pyr-PC and 1.45 mM Triton X-100 was placed in one syringe with 5 mM Triton X-100 placed in the second syringe. The rate for the 1-C_6-2-C_4pyr-PC/Triton X-100 mixed micelles

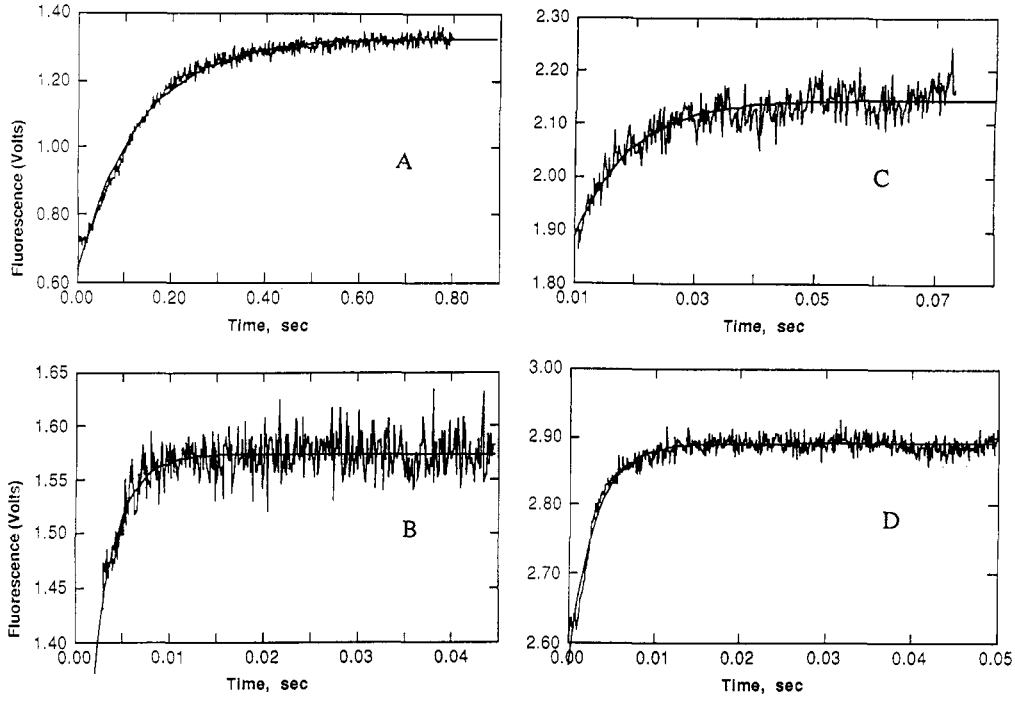

FIGURE 4: Fluorescence intensity of 1-C_6-2-C_4pyr-PC at 380 nm as a function of time after dilution of a 0.06 mM solution to 0.03 mM with (A) monomeric or (B) micellar β-octyl glucoside, (C) micellar DOC, and (D) micellar Triton X-100. The final concentrations of the detergents are the same as in Figure 3. The lines through the data are best fits to $A_0e^{-k/t} + B$. Note the differences in time scale for monomeric versus micellar detergents.

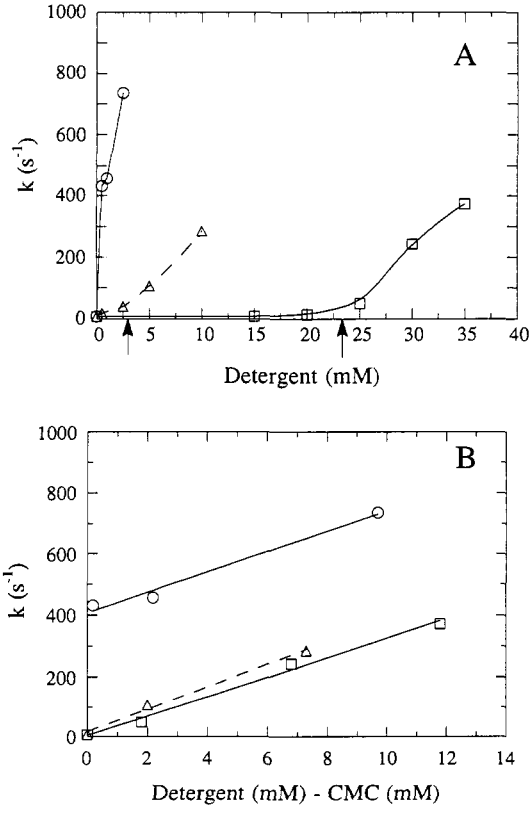

FIGURE 5: Dependence of k, the first-order rate constant for monomer formation of 1-C_6-2-C_4pyr-PC, on (A) total detergent concentration or (B) micellar detergent concentration (total detergent minus the CMC): (○) Triton X-100; (△) sodium deoxycholate; (□) β-octyl glucoside. The arrows indicate the CMC values for deoxycholate and β-octyl glucoside.

reorganization with pure Triton X-100 micelles was 395 ± 120 s^{-1}. Errors are larger since the monomer band is initially higher in the Triton mixed micelle than in experiments with pure 1-C_6-2-C_4pyr-PC micelles. The rate constant for the process is similar to the mixing rate for pure 1-C_6-2-C_4pyr-PC with Triton X-100 (456 ± 33, the entry in Table 1 for a final Triton concentration of 2.5 mM). For the same experiment with the more hydrophobic 1-C_{16}-2-C_{10}pyr-PC mixed micelles, the rate constant was 69 ± 0.9 s^{-1}. The lower rate observed for the 1-C_{16}-2-C_{10}pyr-PC/Triton X-100 mixed micelles further mixing with Triton X-100 is the result of the increase in hydrophobicity of the probe.

Short-Chain PC Mixing with 1-C_6-2-C_4pyr-PC. It has been suggested that when short-chain lecithin micelles have been used as substrates for phospholipases, substrate replenishment in the micelles may limit the observed activity (Jain et al., 1991). If the short-chain lecithin micelles do not rapidly reorganize when individual molecules are hydrolyzed to form water-soluble fatty acids and lysophosphatidylcholine, then lower enzymatic rates could be observed. With this in mind we have looked at mixing efficiencies (via steady-state measurements) and rates (via stopped-flow measurements) for a series of short-chain symmetric and asymmetric lecithin molecules differing in CMC, chain length, and micelle size, with 1-C_6-2-C_4pyr-PC and 1-C_8-2-C_4pyr-PC micelles. The results are summarized in Table 2. diC_6PC has the highest CMC of the lipids studied (14 mM) and forms nearly spherical micelles that do not grow in length with increasing concentration (Lin et al., 1986). Below the CMC, there was virtually no effect of this lipid on the micelle reorganization of 1-C_6-2-C_4pyr-PC (Figure 6A), either on the I_{380}/I_{470} or the mixing rate constant. However, right around the CMC (final diluted concentration of 13 mM), the rate was too fast (>1000 s^{-1}) to measure accurately. The increase in I_{380}/I_{470} was large and indicated that excellent mixing of probe and diC_6PC micelles had occurred (Figure 6B). The effect of a more hydrophobic micellar fluorescent probe, 1-C_8-2-C_4pyr-PC, on mixing rates with diC_6PC micelles was also examined (Table 2). With the more hydrophobic pyrene probe, the diC_6PC still mixed very rapidly, although the mixing slowed down enough to be measurable ($k \approx 389 \pm 47$ s^{-1}).

Table 2: Mixing of Short-Chain PCs with 1-C_6-2-C_4pyr-PC[a] and 1-C_8-2-C_4pyr-PC[a] As Monitored by Fluorescence

$[PC]_{final}$ (mM)	CMC (mM)	[micelle][b] (mM)	k (1-C_6-2-C_4pyr-PC) (s^{-1})	I_{380}/I_{470}	k (1-C_8-2-C_4pyr-PC) (s^{-1})	I_{380}/I_{470}
DiC_6PC	14.0					
3.3			9.5 ± 0.2	0.49		
8.3			2.14 ± 0.05	0.50		
13.0			>1000	1.3		
17.6		3.6	>1000	21.2		
30.5		16.5	>1000	42.9		
40.6		26.6	>1000		389 ± 47	22.0
DiC_7PC	1.5					
3.5		2.0	56.3 ± 4.1	10.7	23.2 ± 0.4	3.86
4.9		3.4	98.6 ± 10.3	15.5		
5.0		3.5			27.5 ± 0.4	
6.4		4.9	144.4 ± 8.8			
DiC_8PC	0.2					
1.5		1.3	23.1 ± 0.4	7.8		
2.5		2.3	0.74 ± 0.01	18.6		
1-C_{10}-2-C_4-PC	0.8					
0.04			38.4 ± 0.6	0.30		
2.2		1.8	78.9 ± 1.6	10.2		
3.5		3.1	82.2 ± 1.4	23.7		
1-C_8-2-C_7-PC	0.2					
2.5		2.3	33.0 ± 0.5	5.3		
5.0		4.8	35.4 ± 0.5			

[a] Initial pyrene-labeled phosphatidylcholine concentration is 0.06 mM. [b] The micelle concentration is the bulk detergent concentration minus the CMC.

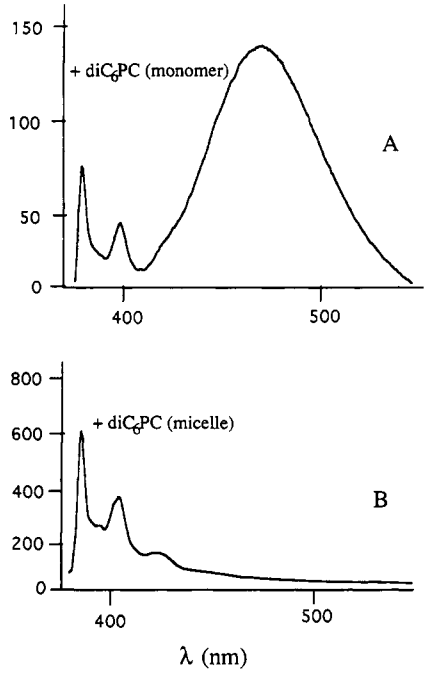

FIGURE 6: Fluorescence spectra of 1-C_6-2-C_4pyr-PC after dilution of a 0.06 mM solution to 0.03 mM with (A) monomeric (final concentration 8.33 mM) or (B) micellar diC_6PC (final concentration 30.0 mM total PC, 16.5 mM micellar PC).

DiC_7PC aggregates above its CMC of 1.5 mM (Bian & Roberts, 1991, 1992; Tausk et al., 1974) to form rod-shaped micelles (or spherocylinders) that increase in size with increasing concentration of PC (Lin et al., 1987a). If this short-chain phosphatidylcholine was mixed at monomer concentrations with the probe micelles, there was no effect on the observed rate constant for 1-C_6-2-C_4pyr-PC reorganization upon dilution. However, at final diC_7PC concentrations immediately below and immediately above the CMC, interesting mixing behavior with 1-C_6-2-C_4pyr-PC micelles was observed. For a final concentration after the 2-fold dilution just below the CMC, steady-state fluorescence showed a small amount of mixing with the pyrene-PC probe ($I_{380}/I_{470} = 0.69$). Complementary stopped-flow experiments showed an immediate small drop in monomer fluorescence intensity followed by a relatively slow exponential increase (Figure 7, panels A and C, respectively). This phenomenon may reflect an initial decrease in the CMC of the probe lipid leading to a drop in the monomer intensity followed by a redistribution and increase in monomer as some diC_7PC monomers solubilize in the probe micelles and inhibit excimer formation to a small degree. For final concentrations right above the CMC of the diluent phosphatidylcholine (Figure 7B,D), an immediate burst in monomer fluorescence was observed followed by a rapid decrease. Upon initial mixing of the two lipids, the diC_7PC is micellar but will redistribute to mostly monomer as a function of time. Therefore, the probe micelles are initially diluted with micellar diC_7PC and then reorganize to micelles with less diC_7PC in them in accordance with more of those diC_7PC molecules existing as monomers. At concentrations well above its CMC, diC_7PC and 1-C_6-2-C_4pyr-PC micelles mixed well and exhibited moderate mixing rates in comparison to the detergents. The inclusion of 1–5 mM Ca^{2+} in the diluent solution had no effect on the mixing rates with the 1-C_6-2-C_4pyr-PC micelles. When the final diC_7PC micellar concentration was ~5 mM, the mixing rate with 1-C_6-2-C_4pyr-PC micelles (final concentration 0.03 mM) was 144 s^{-1}.

The most hydrophobic of the symmetric PCs which still forms micelles is diC_8PC which has a CMC of ~0.2 mM (Bian & Roberts, 1992). Solutions of this short-chain PC phase separate at concentrations greater than a few millimolar. For a final diC_8PC concentration of 1.5 mM mixing with 0.03 mM 1-C_6-2-C_4pyr-PC, the mixing rate was 23 s^{-1}; mixing efficiency as measured by I_{380}/I_{470} was quite good. Increasing the final diC_8PC concentration to 2.25 mM led to further dilution of the probe in mixed micelles as evident by a high I_{380}/I_{470} (Table 2). However, the rate of this mixing was extremely slow, $k = 0.74 \pm 0.01$ s^{-1}. Therefore, for diC_8PC, the change in aggregate morphology from simple rod-shaped micelles in solution to a second discrete phase has a dramatic effect on the kinetics of micelle mixing. This suggests that removal of products and diC_8PC micelle reorganization upon enzyme action may be limited by the micelle dynamics, particularly if one is at concentrations where the diC_8PC tends to phase separate. A comparison of mixing rate constants as

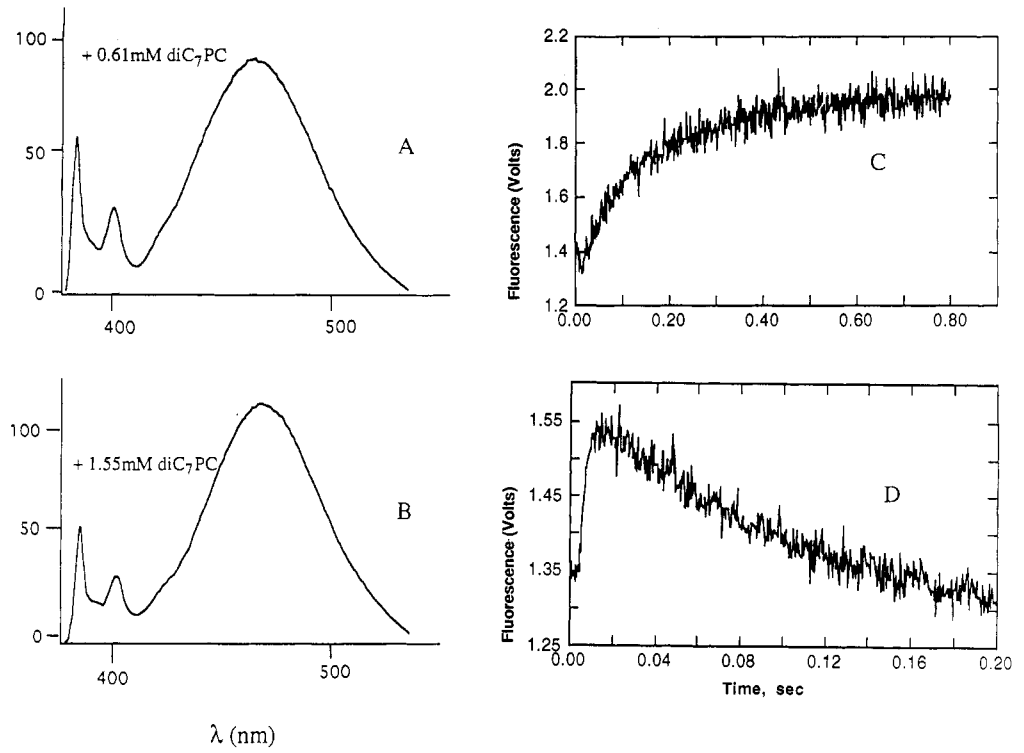

FIGURE 7: Fluorescence spectra of 0.06 mM 1-C_6-2-C_4pyr-PC with (A) monomeric (0.61 mM) or (B) micellar diC_7PC (1.55 mM total PC, 0.05 mM micellar PC). The time dependences of the fluorescence changes shown in panels A and B are shown in panels C and D.

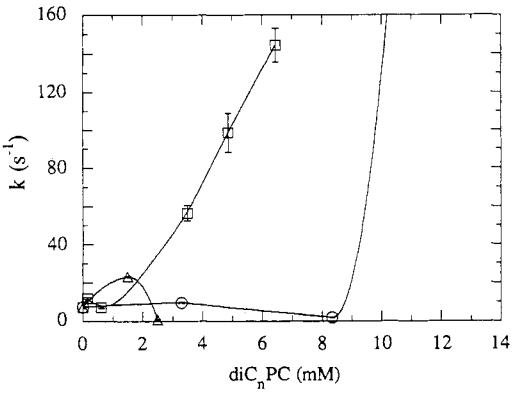

FIGURE 8: Dependence of k, the first-order rate constant for monomer formation of 1-C_6-2-C_4pyr-PC, on total short-chain PC concentration: (○) diC_6PC; (□) diC_7PC; (△) diC_8PC. Error bars are shown for the diC_7PC mixing data.

a function of total concentration of diluent phosphatidylcholine is shown in Figure 8. Both the increase in mixing when the diluent is micellar and the differences between the micellar species are obvious in this plot.

Two asymmetric lecithins were examined to see if micelle size had an effect on short-chain phosphatidylcholine mixing with 1-C_6-2-C_4pyrPC. 1-C_{10}-2-C_4PC has a CMC of 0.4 mM and forms smaller micelles than diC_7PC (Bian & Roberts, 1991); it is more akin to diC_6PC in its aggregation behavior (its micelles are nearly spherical) but has the hydrophobicity of diC_7PC. Steady-state fluorescence analyses showed that the extent of mixing for this molecule (I_{380}/I_{470} = 23.7 at 3.1 mM micelle) is similar to that of diC_7PC (I_{380}/I_{470} = 15.5 at 3.0 mM micelle) and diC_6PC (I_{380}/I_{470} = 21.2 at 3.6 mM micelle). Mixing rates from the stopped-flow analysis were comparable to diC_7PC at comparable micelle concentrations (Table 2). 1-C_8-2-C_7PC has a CMC of 0.2 mM and forms very large rod-shaped micelles (Lin et al., 1987b). It is closer to diC_8PC in hydrophobicity, and its mixing rates were closer to that of symmetric short-chain lecithin. Thus, differences

Table 3: Mixing of Lysophosphatidylcholine with 1-C_6-2-C_4pyr-PC[a] As Monitored by Fluorescence

[lyso-PC]$_{final}$ (mM)	CMC (mM)	[micelle][b] (mM)	k_1 (1-C_6-2-C_4pyr-PC) (s^{-1})	I_{380}/I_{470}
1-C_{12}PC	0.7			
0.96		0.26	385 ± 52	1.85
4.6		3.9	210 ± 4	3.69
15.3		14.6	236 ± 5	
1-C_{14}PC	0.07			
0.28		0.21	287 ± 16	1.57
0.56		2.9	68.7 ± 0.9	14.8

[a] Initial 1-C_6-2-C_4pyr-PC concentration is 0.06 mM. [b] The micelle concentration is the bulk detergent concentration minus the CMC.

in micelle size appear to be secondary effects in micelle mixing; molecule hydrophobicity appears to be the major force.

The effect of chain number on mixing parameters was also examined with two lysophosphatidylcholine species. At low micelle concentrations, the lysolecithin solutions mixed rapidly with 1-C_6-2-C_4pyr-PC (Table 3) with rates comparable to those of the detergents examined (Triton X-100, β-octyl glucoside, and deoxycholate). For 1-C_{12}PC, the rate showed little change as the micelle concentration was increased from 3.9 to 14.6 mM. For 1-C_{14}-PC, there appeared to be a dramatic drop in the mixing rate at 2.8 mM. On the basis of how the phase change of diC_8PC affected the micelle mixing rate, we can suggest that 1-C_{14}-PC must also undergo a change in aggregate morphology as the concentration of the molecule increases. This results in an aggregate that does not mix as rapidly with other micelles.

A summary of mixing rates and efficiencies (as measured by I_{380}/I_{470}) for all lecithin and lysolecithin species is presented in Figure 9. Most of the phosphatidylcholines examined had 14 total carbons in the acyl chains. These showed a remarkable similarity in the dependence of k values on micelle concentration. Less (12 acyl chain carbons) or more (15 or 16 acyl chain carbons) hydrophobic diluent species did not fall within the range for the 14 carbon lipids. This indicates that it is

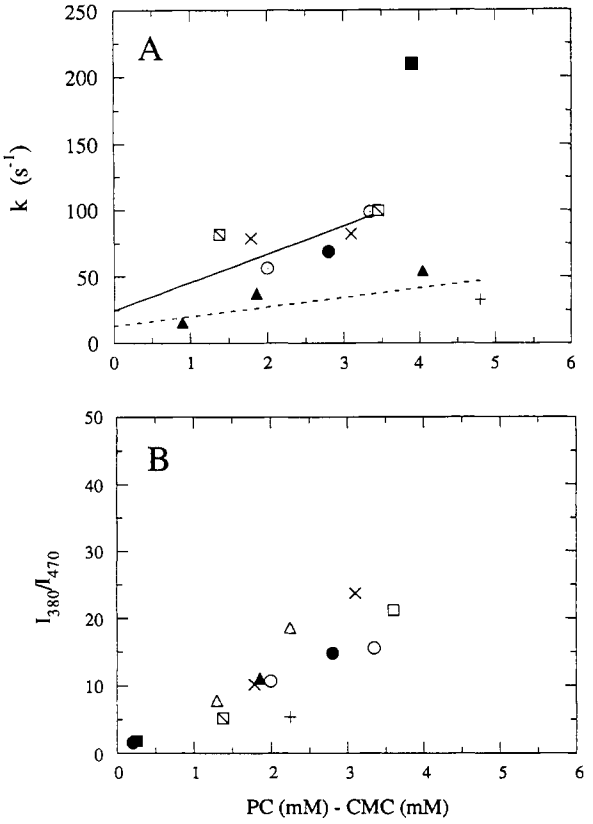

FIGURE 9: Effect of micellar short-chain PC and lyso-PC concentration (total PC concentration minus CMC) on (A) k and (B) I_{380}/I_{470}: (□) diC_6PC; (○) diC_7PC; (△) diC_8PC; (×) 1-C_{10}-2-C_4-PC; (+) 1-C_8-2-C_7-PC; (⊡) 1-C_6-2-C_8(SH)-PC; (▲) diC_8(SH)PC; (■) 1-C_{12}-PC; (●) 1-C_{14}-PC. In panel A the solid line is the least-squares fit through k values for all PC species with 14 total acyl chain carbons; the dotted line is through k values for PCs with 15 or 16 carbons. Errors in k values in panel A are typically <15%; these are given in Tables 2, 3, and 4.

the hydrophobicity of the diluent phosphatidylcholine which controls the mixing rates and mixing efficiencies.

Mixing of Polymerizable Phosphatidylcholine Micelles with 1-C_6-2-C_4pyr-PC. We have synthesized a series of short-chain thiol substituted lecithin molecules which can be polymerized at the terminus of the fatty acyl chain in the micellar state through the formation of disulfide cross-links (Soltys et al., 1993). These molecules have shown interesting kinetic behavior with both phospholipase A_2 and C. Both steady-state and stopped-flow fluorescence measurements were performed with these micelles to see how well they were able to mix with the 1-C_6-2-C_4pyr-PC molecules. The asymmetric thiol 1-C_6-2-C_8(SH)-PC showed a mixing rate comparable to that of diC_7PC at similar micelle concentrations (Table 4). With the polymerized *sn*-2 dimer micelles, there was a slight decrease in the observed mixing rate—presumably caused by the doubled size of the monomeric phosphatidylcholine unit and its more hydrophobic nature [the CMC has dropped dramatically for this dimer (Soltys et al., 1993)]. Mixing efficiency, as monitored by I_{380}/I_{470}, has been reduced upon cross-linking to form the dimers, while the mixing rate is only modestly affected. For the diC_8(SH)PC (4.0 mM micelle concentration) the mixing rate with 1-C_6-2-C_4pyr-PC micelles was 54 s^{-1}; for diC_6(SH)PC (25 mM micelles) the mixing rate was 693 s^{-1}. Each of these mixing rates is similar to that of the short-chain lecithin without the -SH group. However, once either system is cross-linked to form a polymeric micelle, the mixing rate was dramatically reduced to 4–6 s^{-1}. The time dependence of the pyrene monomer band is shown for

Table 4: Mixing of Polymerizable Phosphatidylcholine Micelles with 1-C_6-2-C_4pyr-PC[a] As Monitored by Fluorescence

$[PC]_{final}$ (mM)	CMC (mM)	[micelle][b] (mM)	k (1-C_6-2-C_4pyr-PC) (s^{-1})	I_{380}/I_{470}
1-C_6-2-C_8(SH)-PC	2.0			
1.5		–	26.1 ± 0.4	
3.4		1.4	82.0 ± 3.0	5.22
5.5		3.5	100 ± 12	
sn-2 dimer	0.06			
0.62		0.56		1.09
1.13		1.07		1.85
1.40		1.34	66.5 ± 5.2	
5.47		5.41	98 ± 19	4.3
diC_8(SH)PC	0.1			
1.0		0.9	15.5 ± 0.3	
2.0		1.9	37.0 ± 1.1	11.1
4.1		4.0	54.4 ± 4.0	
diC_8(-S-S-)PC	–[c]			
1.5		1.5	4.19 ± 0.15	
4.2		4.2	4.05 ± 0.10	11.0
diC_6(SH)PC	10.0[d]			
35		25.0	693 ± 88	7.90
diC_6(-S-S-)PC	5.0[e]			
0.6		–[f]	5.4 ± 0.2	1.28
4.0		–[f]	13.1 ± 0.7	
7.7		2.8	6.1 ± 0.5	6.44

[a] Initial 1-C_6-2-C_4pyr-PC concentration is 0.06 mM. [b] The micelle concentration is the bulk diluent concentration minus the CMC. [c] Upon polymerization, basically all of the phosphatidylcholine is aggregated, i.e., micellar. [d] Determined from the surface tension versus ln(phospholipid) curve in the presence of 20 mM dithiothreitol. [e] The surface tension versus ln(phospholipid) curve shows a break at 5 mM; above this concentration it is assumed that cross-linked units aggregate to form micelles and below this concentration the individual cross-linked units are soluble.

1-C_6-2-C_4pyr-PC micelles mixing with the cross-linked *sn*-2 dimer and the polymerized micelle (Figure 10). This indicates that the presence of a large cross-linked unit impedes the collisional interactions (as well as amphiphile "monomer" exchange) between the pyrene probe micelles and the polymerized phosphatidylcholine micelles.

DISCUSSION

Enzymes acting on surfaces often display complex kinetic behavior which is difficult to interpret. Previous work has alluded to this fact, particularly in regard to the action of phospholipase A_2 acting toward micellar substrates. As the enzyme converts substrate into product, the composition of the micelle will change rapidly. It has been implicitly assumed that the exchange of lipids occurs rapidly between micelles and does not influence steady-state velocities by either substrate depletion or product inhibition. If on the other hand most of the substrate molecules in an individual micelle are hydrolyzed before significant reorganization of the micelle mixture can occur and the enzyme stays associated with a given micelle on this time scale, the observed rate of hydrolysis will be decreased. Recent studies have shown that the rate of exchange of long chain phospholipids in bile salt mixed micelles are often fast but depending on conditions can be considerably slower (Fullington et al., 1990; Nichols, 1988). Therefore, in order for an accurate interpretation of the phospholipase kinetic data, exchange of lipids must be fast on the time scale of the catalytic reaction, i.e., lipid exchange rate should be comparable or greater than the enzyme turnover number. Otherwise a steady state will develop where the apparent activity is primarily determined by the exchange rate of the lipid between the micelles (Berg et al., 1991; Jain et al., 1991).

Phospholipase A_2 has an apparent turnover number (derived from the apparent V_{max}) of 500–600 s^{-1} toward micellar diC_7-

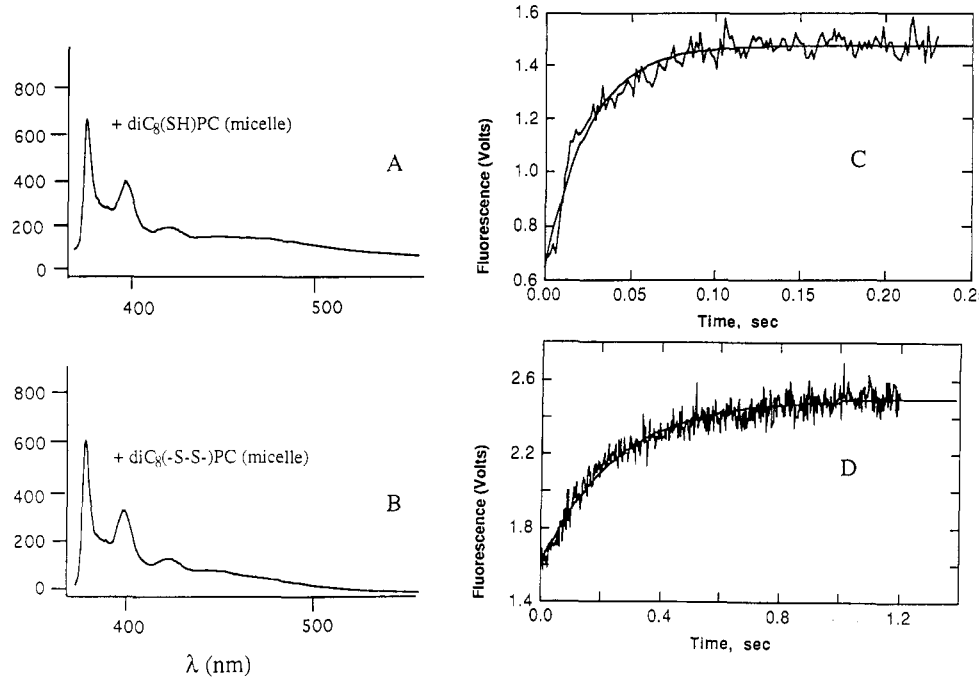

FIGURE 10: Fluorescence spectra of 1-C_6-2-C_4pyr-PC after dilution of a 0.06 mM solution to 0.03 mM with (A) 3.9 mM diC_8(SH)PC (final diluant concentration of 1.95 mM) or (B) 3.0 mM diC_7PC (1.5 mM final nonfluorescent PC). The corresponding time dependences of the fluorescence intensity at 380 nm as a function of time after mixing for panels A and B are shown in panels C and D, respectively.

PC (Lewis et al., 1990); phospholipase C has an apparent turnover number of ~1200 s^{-1} toward the same lipid micelles (El-Sayed & Roberts, 1985). DiC_7PC micelles, while initially small (27 ± 1 molecules per minimum size micelle), grow rapidly above the CMC. At 5 mM (a typical assay concentration) and room temperature, there are 100–120 molecules per micelle (Lin et al., 1987a, 1990). The phosphatidylcholine mixing rate constant of 144 s^{-1} for 1-C_6-2-C_4pyr-PC with this concentration of diC_7PC suggests that substrate replenishment could pose problems for both phospholipase A_2 and C. The considerably higher rate constant for the probe mixing with diC_6PC (>1000 s^{-1} for micellar substrate) indicates that there should be no problem with substrate replenishment with these micelles. Therefore, if substrate replenishment is rate-limiting for either enzyme, there should be a dramatic decrease in enzymatic activity comparing the increasingly hydrophobic series diC_6PC, diC_7-PC, and diC_8PC. For phospholipase A_2, all three substrate micelles give rise to similar observed specific activities with the highest activity reported for diC_8PC (Lewis et al., 1990; Roberts et al., 1978). Jain and co-workers have suggested that for phospholipase A_2, unless substrate replenishment is fast on a 10–50-ms time scale (which translates to k of 100 to 20 s^{-1}), overall enzyme activity will reflect the rate of substrate replenishment and not the intrinsic catalytic efficiency (Berg et al., 1991; Jain et al., 1991). The mixing rates determined with the pyrene lecithin probes in the present work, therefore, rule out substrate replenishment as a major kinetic factor for phospholipase A_2 acting on micellar diC_6PC and diC_7PC substrates. Previous evidence to support substrate depletion as a kinetic problem included initial velocities toward diC_8PC and diC_8-phosphatidylmethanol (Berg et al., 1991) with specific activities about 4-fold higher for the anionic lipid. DiC_8PC phase separates at mM concentrations, and, as shown in the present fluorescent study, this adversely affects micelle exchange rates (to <1 s^{-1} for concentrations above 2 mM); there is no information on whether or not a similar phase separation is observed with the phosphatidylmethanol system. If dioctanoylphosphatidylmethanol does not phase separate, the difference in observed phospholipase A_2 activities could then be related to slow exchange of lipids in the diC_8PC mixture. Perhaps a better short-chain micellar system to quantify the importance of substrate depletion in phospholipase kinetics without the complication of phase separation would be diC_7PC versus diC_7-phosphatidylmethanol.

Phospholipase C apparent V_{max} values decrease slightly for diC_6PC and diC_7PC [2900 and 2650 μmol min^{-1} mg^{-1}, respectively (El-Sayed et al., 1985)]; the apparent V_{max} decreases further for diC_8PC, (~2200 μmol min^{-1} mg^{-1}). For the same series of short-chain PC micelles, lipid mixing rates decrease from >1000 s^{-1}, to 100 s^{-1}, to 20 s^{-1}, while the micelle sizes increase dramatically [for 5 mM micellar PC from 16 to 100 to >4000 molecules per micelle (Lin et al., 1987)]. The two effects of micelle exchange and growth oppose one another. The role of micelle size in enzyme kinetics can be assessed by comparing enzyme rates toward diC_7PC and 1-C_{10}-2-C_4-PC (Lewis et al., 1990). The asymmetric phosphatidylcholine forms spherical micelles with 27 ± 5 molecules, while the rod-shaped diC_7PC micelles have ~100 molecules per micelle. The micelle mixing rate is comparable for both of these micelles. Phospholipase C activity is also the same toward both these micelles, even though there is a large difference in size (Bian & Roberts, 1991). This suggests that micelle growth is less relevant in determining observed specific activities than lipid exchange rates. The slightly lowered specific activity of phospholipase C toward diC_7PC and diC_8PC could reflect an influence of the slower rate of micelle mixing; however, the changes in activity are relatively small compared to changes in the mixing rate.

Cross-linked PC micelles have dramatically reduced mixing rates with 1-C_6-2-C_4pyr-PC micelles. When initial rates are examined, phospholipase C shows an enhanced activity toward cross-linked diC_8(SH)PC compared to the reduced diC_8(SH)-PC substrate micelles (Soltys et al., 1993). This is exactly opposite of what would be predicted if lipid exchange is impaired and substrate depletion is kinetically limiting. Again, it is consistent with other factors controlling the observed rate of phospholipase C. In contrast, the cross-linked micelles are

poor substrates for phospholipase A_2, but the *sn*-2 dimer is also a poor substrate (Soltys et al., 1993) and lipid mixing rates for it are comparable for reduced and cross-linked species. Therefore, the lowered activity of phospholipase A_2 toward the cross-linked lipids cannot be ascribed to substrate depletion.

The Triton X-100 detergent system displays rapid mixing with both pyrene-labeled phosphatidylcholine micelles. The observed rate constants for 0.5–10 mM Triton X-100 mixing with 1-C_6-2-C_4pyr-PC are comparable to the phospholipase A_2 turnover number. This suggests that substrate replenishment cannot be a severe problem in this assay system as long as short-chain lecithin substrates are used. However, as the hydrophobicity of the pyrene-labeled phosphatidylcholine increases, the lipid mixing rates decrease. The 1-C_{16}-2-C_{10}-pyr-PC/Triton X-100 system is a good analog for a long-chain lecithin in a Triton X-100 micelle. The lipid reorganization rate was $\sim 70\,s^{-1}$. Under these conditions, lipid mixing is slower than enzyme turnover indicating that substrate depletion could possibly occur in a Triton X-100/long-chain phosphatidylcholine mixed micelle assay system. The prediction based on mixing studies is that the observed enzyme activity should increase dramatically in a Triton X-100 mixed micelle system as the chain length of the phosphatidylcholine substrate is shortened. Values in the literature suggest that this is not the case (Roberts et al., 1978). Therefore, lipid reorganization in a Triton X-100/phosphatidylcholine mixed micelle system does not appear to be kinetically limiting. The same conclusion was reached previously with kinetic studies of Triton X-100/long-chain phosphatidylcholine mixed micelles as substrates for phospholipase A_1 (Kucera et al., 1988). That work also used 12-doxylphosphatidylcholine broadening of phosphatidylcholine resonances in a Triton X-100 mixed micelle system to estimate a 0.2-ms lifetime for exchange of the lecithins. There was ambiguity about whether this represented lecithin exchange within or between micelles. The disparity between the mixing time of the spin label study (which leads to a k of $5000\ s^{-1}$) and that observed in the present work (typically $k \sim 500\ s^{-1}$) suggests that the earlier work was primarily monitoring lecithin exchange within micelles. In any event, both studies discount substrate depletion as a rate-limiting factor for phospholipase activities in a Triton/phosphatidylcholine mixed micelle system.

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