Kinetics of *Bacillus cereus* Phosphatidylinositol-Specific Phospholipase C with Thiophosphate and Fluorescent Analogs of Phosphatidylinositol†

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ABSTRACT: Thiophosphate analogs (C–S–P bond) of phosphatidylinositol (C_n-thio-PI: racemic hexadecyl-, dodecyl-, and octylthiophosphoryl-1-*myo*-inositol) and a fluorescent analog (pyrene-PI: *rac*-4-(1-pyreno)-butylphosphoryl-1-*myo*-inositol) were all substrates for phosphatidylinositol-specific phospholipase C from *Bacillus cereus*. Hydrolysis of thio-PI was followed by coupling the production of alkylthiol to a disulfide interchange reaction with dithiobispyridine. Hydrolysis of pyrene-PI was followed using a HPLC-based assay with fluorescence detection. The activity of PI-PLC with thio-PI analogs showed an interfacial effect. C_{16}-Thio-PI, which had a critical micelle concentration (CMC) of 7 μM, gave a hyperbolic activity versus concentration curve between 0 and 2 mM, while C_8-thio-PI, which had a CMC above 10 mM, showed very low activity which increased greatly upon introduction of an interface in mixed micelles with hexadecylphosphocholine (HDPC). Pyrene-PI, which aggregates above 0.3 mM, gave a sigmoidal activity curve with much higher activity above the CMC. All three thio-PI homologs as mixed micelles with HDPC gave hyperbolic activity curves with PI–PLC that were a function of bulk concentration of substrate at constant surface concentration and surface concentration of substrate at constant bulk concentration. The maximal activity of PI-PLC with pure C_{16}-thio-PI micelles was 6.25 μmol min^{-1} mg^{-1}, while that with pyrene-PI was estimated to be 68 μmol min^{-1} mg^{-1}. With pure C_{16}-thio-PI micelles, 0.022 mM substrate gave half V_{max}, similar to that in mixed micelles with HDPC.

Phosphatidylinositol-specific phospholipase C (PI-PLC;[1] EC 3.1.4.10) from *Bacillus cereus* catalyzes the cleavage of phosphatidylinositol to a diglyceride and D-*myo*-inositol 1,2-(cyclic)phosphate. The latter is subsequently hydrolyzed slowly by the same enzyme to D-*myo*-inositol 1-phosphate (Griffith et al., 1991; Volwerk et al., 1990). This enzyme also catalyzes the release of a number of enzymes linked to glycosylphosphatidylinositol (GPI) membrane anchors (Low & Saltiel, 1988). The mammalian PI-PLC plays a key role in the phosphoinositide cascade system of signal transduction (Dennis et al., 1991). The smaller, less complex bacterial PI-PLC may serve as a paradigm for the mammalian enzyme.

Kinetic studies of PI-PLC have, until recently, been hampered by the lack of a continuous assay. An early kinetic study of *B. cereus* PI-PLC using the discontinuous radioisotope assay was reported by Sundler et al. (1978). Shashidhar et al. (1991a) recently developed a continuous fluorometric assay using synthetic 2-naphthyl-*myo*-inositol 1-phosphate. However, the specific activity of PI-PLC for this substrate was only about 0.003% that for the natural substrate. They also developed an assay using a chromogenic substrate, 4-nitrophenyl-*myo*-inositol 1-phosphate (Shashidhar et al., 1991b). The specific activity with this substrate was higher (77 μmol min^{-1} mg^{-1} at 1 mM) but it had a rather low affinity for the enzyme active site (K_M > 15 mM). Since it is water soluble and cannot be incorporated into interfaces, it is limited to kinetic studies in monomolecular systems. Recently we reported the synthesis of a thiophosphate analog (C–S–P bond) of phosphatidylinositol, C_{16}-thio-PI (*rac*-hexadecylthiophosphoryl-1-*myo*-inositol; see Chart I), and its use in a continuous spectrophotometric assay of PI-PLC (Hendrickson et al., 1991a). We also reported the synthesis of a fluorescent analog, pyrene-PI [*rac*-4-(1-pyreno)butylphosphoryl-1-*myo*-inositol; see Chart I), and its use in a discontinuous, HPLC-based assay of PI-PLC (Hendrickson et al., 1991b). Here we report kinetic studies of *B. cereus* PI-PLC with these two substrates.

MATERIALS AND METHODS

Recombinant *B. cereus* PI-PLC was expressed in *Escherichia coli* and purified according to the procedure of Koke et al. (1991) using the pIC expression plasmid (a generous gift from Dr. J. J. Volwerk, Institute of Molecular Biology, University of Oregon, Eugene, OR). The protein concentration was determined using an extinction coefficient of 1.83 mL mg^{-1} cm^{-1} at 280 nm (personal communication, J. J. Volwerk). HDPC was synthesized as described by van Dam-Mieras et al. (1975). DTP (Aldrithiol-4), DTNB, and tetrabutylammonium dihydrogen phosphate (1 M in water) were purchased from Aldrich Chemical Co., Milwaukee, WI. Racemic C_{16}-thio-PI, C_{12}-thio-PI, and C_8-thio-PI were synthesized according the procedure of Hendrickson et al. (1991a) for C_{16}-thio-PI. Racemic pyrene-PI was synthesized according to the procedure of Hendrickson et al. (1991b). Pyrene-PI is now available from Molecular Probes, Inc., Eugene, OR.

PI-PLC was assayed with thio-PI using the spectrophotometric method described by Hendrickson and Dennis (1984). A measured amount of thio-PI (in chloroform/methanol solution) was dried under a stream of argon and then in vacuo. Buffer (50 mM MES, pH 7) and detergent (if used) were added and the solution was vortexed and sonicated (bath-type sonicator) to completely disperse the lipid. Substrate solution (225 μL) was placed in a microcuvette (2 × 10 mm) and 5

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTP, 4,4′-dithiobispyridine; GPI, glycosylphosphatidylinositol; HDPC, hexadecylphosphocholine; MES, 2-(*N*-morpholino)ethanesulfonic acid; PI-PLC, phosphatidylinositol-specific phospholipase C; pyrene-PI, *rac*-4-(1-pyreno)butylphosphoryl-1-*myo*-inositol; C_{16}-thio-PI, *rac*-hexadecylthiophosphoryl-1-*myo*-inositol; C_{12}-thio-PI, *rac*-dodecylthiophosphoryl-1-*myo*-inositol; C_8-thio-PI, *rac*-octylthiophosphoryl-1-*myo*-inositol.

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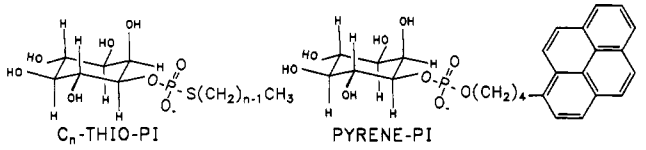
Chart I: Structures of Phosphoinositide Analogs



μL of 50 mM DTP (in ethanol) was added. The cuvette was placed in the cell compartment of a Perkin-Elmer Lambda-3b spectrophotometer at 25 °C and the absorbance at 324 nm was recorded to obtain a stable base line. PI-PLC (5 μL containing 10–50 ng of protein) was added and the absorbance was recorded for several minutes. Enzyme activity was linear with respect to the amount of enzyme used in these assays and the time courses were initially linear with no lag. Activity was calculated using an extinction coefficient for thiopyridine of 19 800 M^{-1} cm^{-1} (Yu & Dennis, 1991). Dilute solutions of enzyme (about 0.05 mg/mL) were prepared from a stock solution (about 1 mg/mL) and used immediately or stabilized in the presence 5 mM HDPC.

PI-PLC was assayed with pyrene-PI by HPLC separation (Abidi et al., 1991) of pyrene-PI and 4-(1-pyreno)butanol with fluorescence detection using the general method described by Hendrickson (1991) for phospholipase A_2 assays. The reaction mixture consisted of lipid dispersed in 50 mM MES buffer, pH 7. It was incubated at 25 °C, and at various time intervals 5-μL aliquots were removed and diluted to 100 μL with acetonitrile/methanol/water (70:15:15). Twenty microliters of diluted sample was chromatographed on a reverse-phase silica column (5-μm Spherisorb ODS, 25 cm × 4.6 mm) with 5 mM tetrabutylammonium dihydrogen phosphate in acetonitrile/methanol/water (70:15:15) at 1 mL/min, and the eluate was analyzed by fluorescence detection (excitation, 343 nm; emission > 370 nm). Retention times for pyrene-PI and pyrenebutanol were 2.4 and 6.2 min, respectively. Dilute enzyme solutions were prepared in the presence of 0.1% bovine serum albumin.

Enzyme activity as a function of substrate concentration was analyzed by nonlinear regression fitting of data to the Michaelis–Menten equation using the program ENZFITTER (Elsevier-BIOSOFT, Cambridge, England).

The critical micelle concentrations (CMC) of thio-PI homologs were determined in 50 mM MES, pH 7, by measuring the surface tension (Wilhelmy plate method, using a rough-surfaced platinum plate attached to a Cahn RTL electrobalance; Adamson, 1982) as a function of concentration. A break in the curve occurs at the CMC. The CMC of pyrene-PI was determined by fluorescence spectroscopy. The ratio of excimer/monomer emission (I_{485}/I_{377}; excitation, 343 nm) was measured as a function of lipid concentration in 50 mM MES, pH 7. An increase in the ratio above 0 indicates aggregation.

RESULTS

PI-PLC was assayed spectrophotometrically with 0.2 mM C_{16}-thio-PI, 0.4 mM HDPC, 50 mM MES, pH 7, and 1 mM DTP. Activity was linear with respect to the amount of enzyme up to 70 ng (0.3 μg/mL). The reaction rate was independent of DTP concentration above 0.1 mM and no lag time was seen above 0.4 mM. With DTNB in place of DTP, the rate was considerably less with a long lag time. With 1 mM substrate and 0.16% (w/v) sodium deoxycholate, the activity was considerably less than that observed with HDPC. Activity as a function of pH showed an optimum from pH 6.5 to 7.5 and was somewhat higher in MES than in Tris buffer.

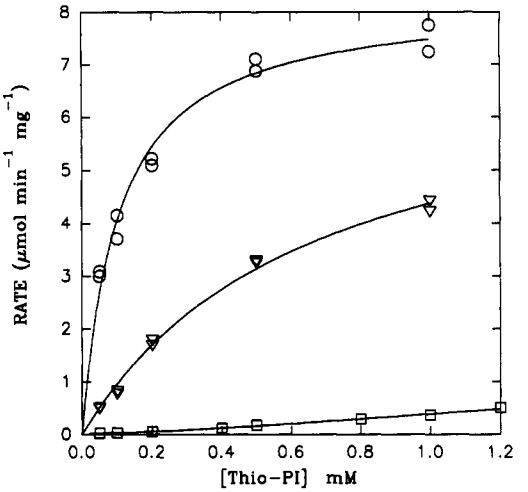


FIGURE 1: Activity of PI-PLC as a function of bulk concentration of thio-PI. Circles, C_{16}-thio-PI; triangles, C_{12}-thio-PI; squares, C_8-thio-PI. No detergent; 50 mM MES, pH 7.

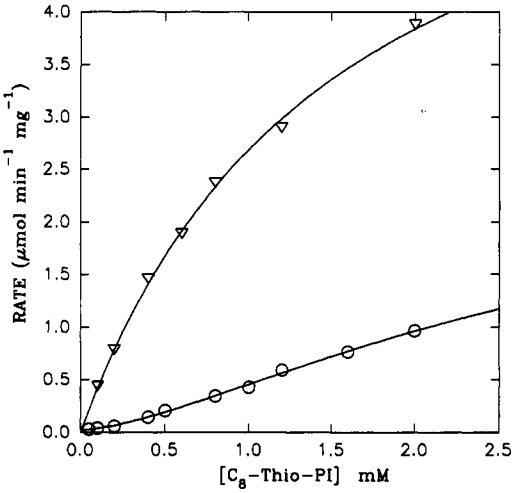


FIGURE 2: Activity of PI-PLC as a function of bulk concentration of C_8-thio-PI. Circles, C_8-thio-PI in the absence of detergent; triangles, C_8-thio-PI/HDPC (1:4) mixed micelles; 50 mM MES, pH 7.

PI-PLC gave hyperbolic activity versus concentration curves with pure C_{16}- and C_{12}-thio-PI (Figure 1). With C_8-thio-PI the activity was much lower and the curve seemed to be sigmoidal with no indication of saturation even at 2 mM substrate. With C_8-thio-PI/HDPC mixed micelles (1:4 ratio),[2] the activity increased considerably and was a hyperbolic function of substrate concentration (Figure 2). With C_{12}-thio-PI/HDPC mixed micelles (1:4 ratio), the concentration of substrate giving half-maximal velocity (K_s) decreased considerably (Figure 3). The kinetic parameters are shown in Table I. For C_{16}-thio-PI, K_s values were similar in pure and mixed micelles. In mixed micelles with 1:2, 1:6, and 1:8 ratios of thio-PI to HDPC the values of K_s were 0.018, 0.022, and 0.018 mM respectively.

The activity of PI-PLC was a hyperbolic function of surface concentration of thio-PI at constant bulk concentration of substrate (Figure 4). Both the maximal rate and apparent surface K_M increased with increasing chain length (Table II).

The CMC values for C_{16}- and C_{12}-thio-PI were determined from surface tension measurements. Surface tension as a

[2] All concentrations of HDPC used were above the CMC. The stated ratios of HDPC to substrate include the amount of monomeric species in solution, but this has only a negligible effect on the ratios of micellar species.

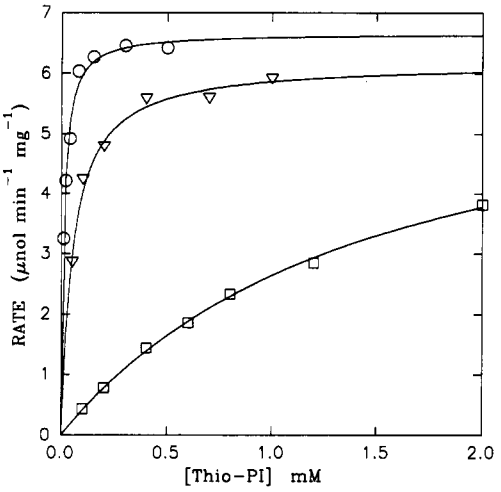


FIGURE 3: Activity of PI-PLC as a function of bulk concentration of thio-PI/HDPC (1:4) mixed micelles. Circles, C_{16}-thio-PI; triangles, C_{12}-thio-PI; squares, C_8-thio-PI; 50 mM MES, pH 7.

Table I: PI-PLC Activity with Pure C_n-Thio-PI and C_n-Thio-PI/HDPC Mixed Micelles

	pure C_n-thio-PI		C_n-thio-PI/HDPC (1:4) mixed micelles	
n	V_{max} (μmol min^{-1} mg^{-1})	K_S[a] (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_S[a] (mM)
16	6.25	0.022	6.56	0.011
12	6.17	0.25	6.09	0.053
8	*b*		6.67[c]	1.51[c]

[a] Data were fit to the Michaelis–Menten equation where K_S is simply the bulk concentration of thio-PI which gives half V_{max}. [b] Nonhyperbolic; 0.8 μmol min^{-1} mg^{-1} at 2 mM thio-PI. [c] Estimate.

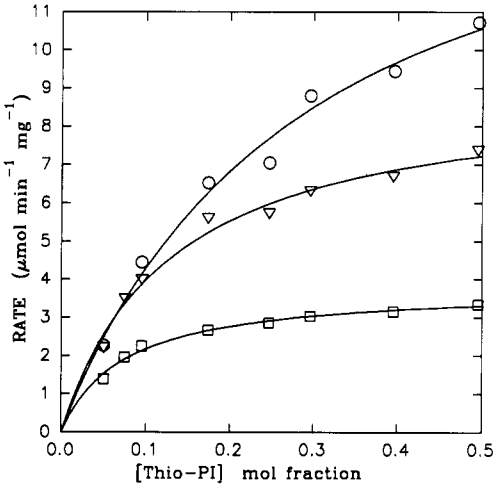


FIGURE 4: Activity of PI-PLC as a function of surface concentration of thio-PI in mixed micelles with HDPC. Constant bulk concentration of thio-PI, 1 mM; 50 mM MES, pH 7. These data were obtained with a different batch of enzyme having a lower specific activity. These specific activities were normalized with those in the other figures by multiplying by a factor of 1.45.

function of C_{16}-thio-PI concentration showed breaks at 7 and 20 μM. Similarly, C_{12}-thio-PI showed breaks at 0.4 and 1.6 mM.

PI-PLC was assayed with pyrene-PI in a HPLC-based assay with pure lipid in 50 mM MES buffer, pH 7. The rate as a function of substrate concentration was sigmoidal and fit the Hill equation $\{v = V_{max}[S]^h/(K^h + [S]^h)\}$ with V_{max} = 68 μmol min^{-1} mg^{-1}, K = 0.87 mM, and h = 1.9 (Figure 5). Substrate aggregation was followed by fluorescence spectroscopy. The excimer/monomer ratio (I_{485}/I_{377}) as a function

Table II: PI-PLC Activity with C_n-Thio-PI/HDPC Mixed Micelles[a]

n	V_{max} (μmol min^{-1} mg^{-1})	K_M[b] (mole fraction)
16	16.7	0.29
12	9.06	0.13
8	3.78	0.074

[a] Constant bulk concentration of thio-PI, 1 mM; 50 mM MES, pH 7. Data from Figure 4. [b] Data were fit to the Michaelis–Menten equation where K_M is simply the mole fraction of substrate which gives half V_{max}.

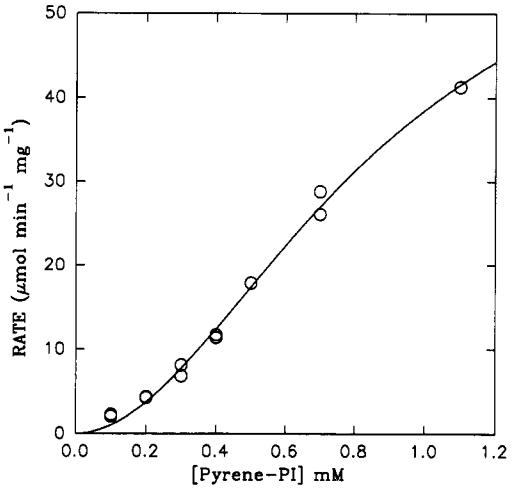


FIGURE 5: Activity of PI-PLC as a function of bulk concentration of pyrene-PI. No detergent; 50 mM MES, pH 7.

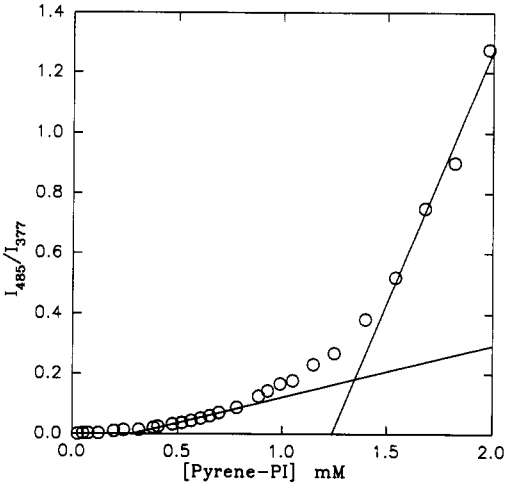


FIGURE 6: Aggregation of pyrene-PI as determined by fluorescence spectroscopy. Excitation, 343 nm; emission, 377 and 485 nm; 50 mM MES, pH 7.

of pyrene-PI concentration showed a gradual increase above 0 at about 0.3 mM and a much steeper increase at about 1.2 mM (Figure 6).

DISCUSSION

Thio-PI is a substrate for PI-PLC and its hydrolysis can be followed spectrophotometrically by coupling the production of alkylthiol to a disulfide interchange reaction with DTP. When DTNB was used in place of DTP, the reaction was very slow with a long lag time. This is probably due to electrostatic repulsion of negatively charged DTNB from the negatively charged thio-PI interface. The maximal activity of PI-PLC with C_{16}-thio-PI was about 16 μmol min^{-1} mg^{-1} in mixed micelles. This is about 1% of the activity with the natural substrate (Griffith et al., 1991). This difference could be due to the replacement of oxygen with sulfur and/or differences

in hydrophobicity and interfacial packing of substrate in the interface. Thiolester analogs of phosphatidylmethanol are hydrolyzed by phospholipase A_2 at about a 7-fold lower rate as compared to the oxy analogs (Jain et al., 1992). The lower activity may also be due to surface depletion in micelles if substrate or enzyme does not exchange rapidly between micelles. PI-PLC, however, gave a much lower apparent K_s value (bulk concentration) with thio-PI; thus, kinetic studies can be done with micromolar concentrations of substrate rather than the low millimolar concentrations required with natural phosphatidylinositol.

The activity of PI-PLC with PI analogs showed an interfacial effect. C_{16}-Thio-PI, which aggregates above 7 μM, gave a hyperbolic activity versus concentration curve, while C_8-thio-PI, which must be monomolecular below 2 mM,[3] gave very low activity which best fits a sigmoidal curve (Figure 1). Introduction of an interface with mixed micelles of C_8-thio-PI and HDPC (CMC = 10 μM; van Dam-Mieras et al., 1975) resulted in a large increase in activity and gave a hyperbolic curve (Figure 2). Pure C_{12}-thio-PI gave a hyperbolic activity curve and intermediate rates between 0 and 1 mM, even though its CMC is about 0.4 mM (Figure 1). Its higher activity below the CMC (hyperbolic curve) may be due to premicellar aggregation of lipid (below the CMC), possibly induced by the presence of enzyme. It gave a V_{max} similar to that for C_{16}-thio-PI but a much larger K_s value (Table I). Pyrene-PI, which aggregated above 0.3 mM (Figure 6), gave a sigmoidal activity curve in the range of 0–1.2 mM, with much higher activity above the CMC (Figure 5). At this point one can only speculate on the source(s) for the effect of interface on enzyme activity. One factor may be a rate-limiting release of product in the absence of an interface. The very hydrophobic alcohol product may be more readily released into a hydrophobic interface than into water.

In the presence of mixed micelles with HDPC, all three thio-PI homologs gave similar values for V_{max} with PI-PLC. The values of K_s (bulk substrate concentration giving half V_{max}) for C_{16}-thio-PI were similar with mixed and pure micelles (Table I) and did not change significantly between substrate/detergent ratios of 1:2 and 1:8. This indicates that HDPC is an inert dilutor which does not bind PI-PLC. This is consistent with Sundler et al. (1978), who showed that PI-PLC does not bind to phosphatidylcholine vesicles. The K_s values markedly increased with decreasing alkyl chain length. This may reflect a hydrophobic effect in substrate binding to the enzyme or differences in interfacial packing which may affect enzyme activity.

PI-PLC activity was not only a function of bulk substrate concentration in micelles but also a function of surface concentration in mixed micelles with HDPC (Figure 4). This is similar to the effect seen with phospholipase A_2 in mixed micelles (Hendrickson & Dennis, 1984). Contrary to these results, Sundler et al. (1978) reported no effect of surface dilution of substrate in mixed micelles of phosphatidylinositol and Triton X-100 with phosphatidylcholine. Their data covered a limited range of surface concentrations and they utilized a more complex mixed-micelle system. HDPC may thus prove to be a good inert dilutor for the study of enzyme activity in mixed micelles. Both the V_{max} and K_M values decrease with decreasing alkyl chain length (Table II). The reasons for this are not clear.

PI-PLC was also active with pyrene-PI, an analog in which the diacylglycerol moiety of the natural substrate is replaced with a pyrenebutanol group. Bruzik et al. (1992) demonstrated a complete lack of stereospecificity of PI-PLC with regard to the configuration of the diacylglycerol moiety. They suggest that this moiety "is probably not involved in recognition or binding by the enzyme". The maximal activity (estimated to be 68 μmol min^{-1} mg^{-1}) was higher than that observed with thio-PI; this difference may reflect more the element effect (sulfur versus oxygen). This activity, however, was still about 20 times less than that observed with the natural substrate. This may be due to differences in interfacial packing and hydrophobicity.

This is the first reported continuous assay of PI-PLC with a substrate which can readily form an interface. Preliminary studies with a mammalian PI-PLC (a generous gift from Dr. Sue Goo Rhee, NIH) showed low activity (about 0.1 μmol min^{-1} mg^{-1}) with both C_{16}-thio-PI and pyrene-PI (H. S. Hendrickson, unpublished results).

The studies reported here were all done with racemic substrates. The enzyme is presumed to be specific for the naturally occurring D isomer of inositol; Volwerk et al. (1990) showed that the cyclic phosphodiesterase activity of PI-PLC is specific for the D isomer of inositol 1,2-(cyclic)phosphate. We are in the process of synthesizing chiral analogs and plan to extend these studies to pure chiral substrates. We are also in the process of synthesizing the more natural thiophosphate analog with a diacylglycerol moiety and plan to compare its activity with that of the natural (oxyphosphate) phosphatidylinositol. The diglyceride phospholipid will also be useful in studies of enzyme activity in bilayer vesicle systems.

[3] The CMC for C_8-thio-PI was not measured since excessive amounts of lipid would be required for the minimum 2.5 mL of solution required. However, since C_{12}-thio-PI has a CMC of 0.4 mM, about 2 orders of magnitude greater than that for C_{16}-thio-PI, we estimate that C_8-thio-PI has a CMC greater than 10 mM.

REFERENCES

Abidi, S. L., Mounts, T. L., & Rennick, K. A. (1991) *J. Liq. Chromatog. 14*, 573–588.

Adamson, A. W. (1982) *Physical Chemistry of Surfaces*, 4th ed., pp 24–27, John Wiley & Sons, New York.

Bruzik, K. S., Morocho, A. M., Jhon, D.-Y., Rhee, S. G., & Tsai, M.-D. (1992) *Biochemistry 31*, 5183–5193.

Dennis, E. A., Rhee, S. G., Billah, M. M., & Hannun, Y. A. (1991) *FASEB J. 57*, 2068–2077.

Griffith, O. H., Volwerk, J. J., & Kuppe, A. (1991) *Methods Enzymol. 197*, 493–502.

Hendrickson, H. S. (1991) *Methods Enzymol. 197*, 90–94.

Hendrickson, H. S., & Dennis, E. A. (1984) *J. Biol. Chem. 259*, 5734–5739.

Hendrickson, E. K., Johnson, J. L., & Hendrickson, H. S. (1991a) *Bio/Med. Chem. Lett. 1*, 615–618.

Hendrickson, E. K., Johnson, J. L., & Hendrickson, H. S. (1991b) *Bio/Med. Chem. Lett. 1*, 619–622.

Jain, M. K., Yu, B.-Z., Rogers, J., Gelb, M. H., Tsai, M.-D., Hendrickson, E. K., & Hendrickson, H. S. (1992) *Biochemistry 31*, 7841–7847.

Koke, J. A., Young, M., Henner, D. J., Volwerk, J. J., & Griffith, O. H. (1991) *Protein Expression Purif. 2*, 51–58.

Low, M. G., & Saltiel, A. R. (1988) *Science 239*, 268–275.

Shashidhar, M. S., Volwerk, J. J., Keana, J. F. W. & Griffith, O. H. (1991a) *Anal. Biochem. 198*, 10–14.

Shashidhar, M. S., Volwerk, J. J., Griffith, O. H., & Keana, J. F. W. (1991b) *Chem. Phys. Lipids 60*, 101–110.

Sundler, R., Alberts, A. W., & Vagelos, P. R. (1978) *J. Biol. Chem. 253*, 4175–4179.

van Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., & de Haas, G. H. (1975) *Biochemistry 14*, 5387–5394.

Volwerk, J. J., Shashidhar, M. S., Kuppe, A., & Griffith, O. H. (1990) *Biochemistry 29*, 8056–8062.

Yu, L., & Dennis, E. A. (1991) *Methods Enzymol. 197*, 65–75.