Substrate Requirements of Bacterial Phosphatidylinositol-Specific Phospholipase C†

Karen A. Lewis, Venkata R. Garigapati, Chun Zhou, and Mary F. Roberts*

Department of Chemistry, Boston College, Chestnut Hill, Massachusetts 02167

Received March 25, 1993; Revised Manuscript Received June 10, 1993

ABSTRACT: A series of symmetric short-chain phosphatidylinositols (PI), including dihexanoyl-PI, diheptanoyl-PI (racemic as well as D and L forms), and 2-methoxy inositol-substituted diheptanoyl-PI, have been synthesized, characterized, and used to investigate key mechanistic questions about phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis*. Key results include the following: (i) bacterial PI-PLC exhibits a 5–6-fold "interfacial activation" when its substrate is present in an interface as opposed to existing as a monomer in solution (in fact, the similarity to the activation observed with nonspecific PLC enzymes suggests a similarity in activation mechanisms); (ii) the 2-OH must be free since the enzyme cannot hydrolyze diheptanoyl-2-*O*-methyl-PI (this is most consistent with the formation of inositol cyclic 1,2-phosphate as a necessary step in catalysis); (iii) the inositol ring must have the D stereochemistry (the L-inositol attached to the lipid moiety is neither a substrate nor an inhibitor); and (iv) the presence of noninhibitory L-PI with the D-PI substrate relieves the diacylglycerol product inhibition detected at ~30% hydrolysis.

Phosphatidylinositol-specific phospholipase C (PI-PLC)[1] is responsible for the cleavage of membrane phosphatidylinositols into diacylglycerol and inositol phosphate(s). PI-PLC enzymes exist both intracellularly and extracellularly in a wide variety of tissues and organisms. Extracellular PI-PLCs have been isolated from the culture media of several microorganisms (Ikezawa & Taguchi, 1981; Low, 1981), while intracellular PI-PLC isozymes are prevalent in mammalian cells (Rhee et al., 1989; Takenawa & Nagai, 1981; Homma et al., 1988; Ryu et al., 1986, 1987; Bennett & Crooke, 1987; Banno et al., 1988) and are involved in second messenger metabolism (Berridge, 1984, 1986). The extracellular PI-PLC enzymes are water-soluble and relatively specific for nonphosphorylated PI. They are involved in the release of proteins tethered to the plasma membrane by cleavage of their glucosylphosphatidylinositol (GPI) anchors (Griffith et al., 1991).

The phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis* catalyzes the cleavage of the glycerophosphate linkage of phosphatidylinositol to yield diacylglycerol and D-*myo*-inositol cyclic 1,2-monophosphate (Figure 1). This same enzyme can also slowly catalyze the hydrolysis of the latter product to D-*myo*-inositol 1-monophosphate (Volwerk et al., 1990; Campbell & Thatcher, 1991). Several key questions must be addressed to understand this enzyme. (i) Is PI-PLC activated when the substrate is present in an interface? (ii) Is formation of the inositol cyclic 1,2-phosphate a necessary step in catalysis? (iii) What are the stereochemical requirements for the inositol ring?

^{31}P NMR spectroscopy has been previously used to detect and quantify long-chain substrate PI (–0.4 ppm) and the products *myo*-inositol cyclic 1,2-monophosphate (16.3 ppm) and *myo*-inositol 1-monophosphate (3.6 ppm) as well-resolved resonances (Volwerk et al., 1990). Unlike other methods which can only quantify the amount of inositol phosphate produced, this method can also identify the exact inositol phosphate species produced from the chemical shift. ^{31}P NMR studies have shown that the bacterial PI-PLC enzyme exhibits both intrinsic phosphotransferase and cyclic phosphodiesterase activities (Volwerk et al., 1990; Griffith et al., 1991). The second reaction has been reported to require 1000-fold higher concentrations of PI-PLC to be observable by this method. The ^{31}P NMR method has also been previously used to demonstrate that the bacterial PI-PLC phosphodiesterase reaction is regiospecific in that only *myo*-inositol 1-monophosphate is produced (no *myo*-inositol 2-monophosphate was detected), and it is stereospecific in that only D-*myo*-inositol cyclic 1,2-monophosphate was hydrolyzed (Volwerk et al., 1990; Griffith et al., 1991).

Short-chain PIs form micellar aggregates which can be well characterized in terms of physical properties such as critical micelle concentration (CMC) and micelle size. These properties are requisite to address the question of whether or not PI-PLC is an "interfacially active" enzyme like other water-soluble phospholipases. Studies were also performed with diC_7PI micelles in the presence and absence of Triton X-100 (2:1 ratio of detergent to PI) to address whether detergent inhibits the enzyme [as has been previously reported with the nonspecific PLC (Eaton & Dennis, 1975)] or merely acts as an inert spacer (surface dilution). An interesting possibility to address with this system is whether the conversion of *myo*-inositol cyclic 1,2-monophosphate to *myo*-inositol 1-monophosphate via the cyclic phosphodiesterase activity of PI-PLC (Volwerk et al., 1990) is low due to detergent inhibition or to sequestering of the enzyme to the interface. If the enzyme is inhibited or trapped by the detergent at the interface, it may not be efficient in catalyzing the conversion of the two water-soluble species.

† This work has been supported by NIH Grant GM26762.

* Author to whom correspondence should be addressed.

[1] Abbreviations: PI, phosphatidylinositol; PI-PLC, PI-specific phospholipase C; diC_7PI, diheptanoyl-PI; diacyl-PC, 1,2-diacyl-*sn*-3-glycerophosphocholine; CMC, critical micelle concentration; diC_7(2-*O*-methyl)PI, diheptanoylphosphatidyl-2-*O*-methylinositol; NPCl, 5,5-dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphorinan; GPI, glucosylphosphatidylinositol; diC_6PI, dihexanoyl-PI; FID, free induction decay; diC_8PI, dioctanoyl-PI; lyso-PI, 1-acyl-*sn*-3-glycerophosphoinositol; Ins(1:2cyc)P, inositol cyclic 1,2-phosphate.

0006-2960/93/0432-8836$04.00/0

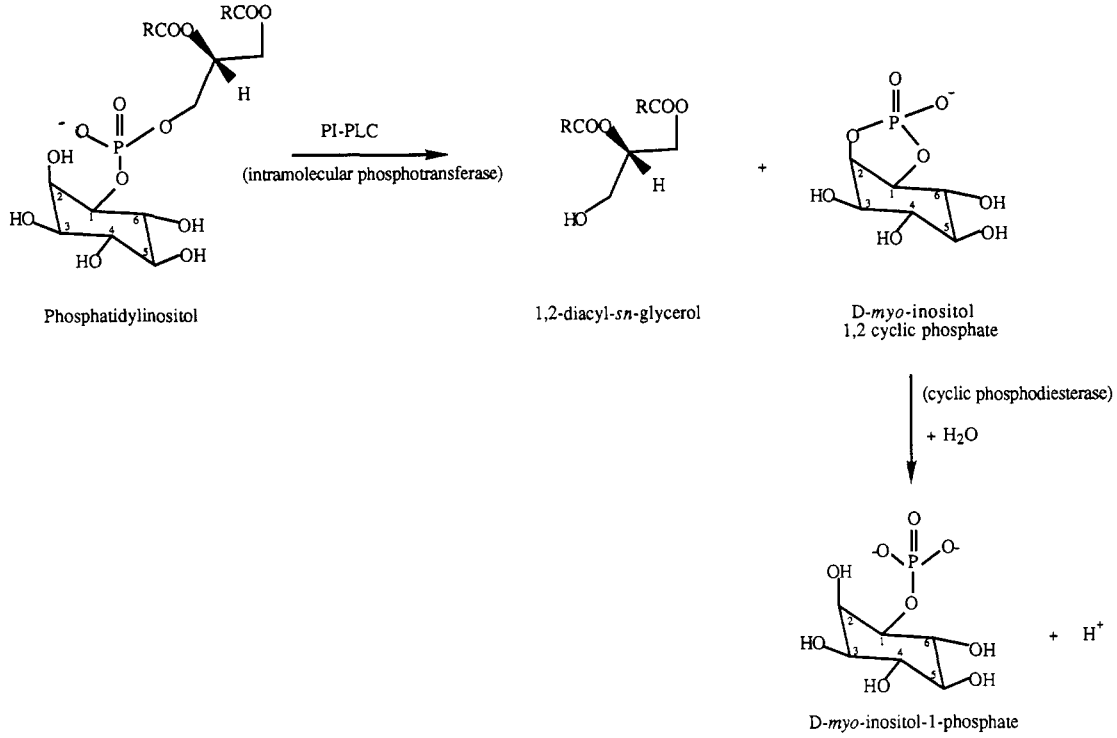

FIGURE 1: Degradation scheme of short-chain phosphatidylinositol by phosphatidylinositol-specific phospholipase C.

MATERIALS AND METHODS

Synthesis of Short-Chain Phosphatidylinositols. Racemic and L and D forms of diC_7PI were synthesized by condensing the appropriate benzyl-protected precursor, 2,3,4,5,6-pentabenzylinositol, with the H-phosphonate of diheptanoylglycerol (Garigapati & Roberts, 1993a). The diacylglycerol was prepared enzymatically by treating diheptanoyl-PC at concentrations above its CMC with nonspecific PLC from *Bacillus cereus* in Tris buffer at pH 8.0. For the synthesis of diC_6PI, a comparable scheme was used. The corresponding H-phosphonates were readily prepared from the diacylglycerols using PCl_3 and imidazole as discussed previously (Garigapati & Roberts, 1993a). ^1H NMR spectral characteristics are diagnostic for the structure and identification of intact diacyl phospholipids. In particular, the acyl chain resonances differ in integrated intensity rather than chemical shift between samples with different chain lengths (Burns & Roberts, 1980). The acyl chain and backbone protons (in ppm from internal TMS) were as follows: 0.79 (ω-CH_3), 1.21 ($(CH_2)_n$), 1.53 (β-CH_2), multiplet centered at 2.35 (α-CH_2), 4.01 (CH_2OP), and 4.36 (CHO). The degradation of the diacyl-PIs into lyso-PI and fatty acid was not observed, but would be noted by the presence of an additional α-CH_2 resonance representing ionized fatty acid $\sim$0.25 ppm upfield from the lipid α-CH_2 resonance (K. Lewis and M. F. Roberts, unpublished results). The assignments for the inositol head group have been previously reported (Lin et al., 1990) and were confirmed with COSY experiments. ^{31}P NMR spectroscopy was also used to check PI purity. Final assignment of the D- and L-inositol forms of diC_7PI relied on a comparison of the specific rotation [measured in $CHCl_3$–CH_3OH (1:1) on a Perkin-Elmer 241 Polarimeter] of the synthetic short-chain materials with that of authentic PI from bovine liver [$[\alpha]_D = +18.3°$ in $CHCl_3$–CH_3OH (1:1)] obtained from Avanti: $[\alpha]_D = +17.7°$ for the D-inositol diC_7PI isomer and $[\alpha]_D = -14.3°$ for the L-inositol diC_7PI in $CHCl_3$–CH_3OH (1:1).

Diheptanoylphosphatidyl-2-*O*-methylinositol [diC_7(2-*O*-methyl)PI] was synthesized using the H-phosphonate approach with 5,5-dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphorinan (NPCl) as the condensing agent. 2-*O*-Methyl-3,4,5,6-tetrabenzylinositol was obtained from *myo*-inositol by a series of reactions described in detail elsewhere (Garigapati & Roberts, 1993b). The suitably protected inositol was condensed with the H-phosphonate of diheptanoylglycerol using NPCl in pyridine in the ratio of 1.2:1.5 equiv and subsequently oxidized in I_2–pyridine–water to give the diheptanoylphosphatidyl-2-(*O*-methyl-3,4,5,6-tetrabenzyl)inositol. Benzyl groups were removed by hydrogenolysis in ethanol with 10% Pd–C to yield diheptanoyl-2-*O*-methylinositol. This phospholipid was purified on silica gel using 20–25% CH_3OH in $CHCl_3$. Appropriate fractions were pooled and lyophilized to give a white powder.

Critical Micellar Concentration Determination. CMC values were determined by the measurement of apparent surface tension in dynes/centimeter (mN/m) with a Fisher Scientific Surface Tensiomat 21. The initial sample prepared for each PI in the series was sufficiently concentrated to be above the CMC and was serially diluted so that at least five points were used to fit the lines in the both monomer and micellar regions of the plot. CMC determinations were generally carried out in H_2O and also in Tris–acetate buffer (pH 7.5) for diC_6PI to ascertain the CMC value under the conditions of the ^{31}P NMR kinetic assay. A value for the surface area per head group at the CMC was extracted from the monomer region of the curve using methods which have been previously described (Bian & Roberts, 1992).

Quasi-Elastic Light Scattering (QLS). QLS measurements were performed on a spectrometer of standard design in the Massachusetts Institute of Technology Department of Nuclear Engineering. The QLS system used consisted of a Brookhaven Instruments digital correlator BI-2030 AT with 72 channels and a 50-mW He–Ne laser as the light source (632.8 nm). The methods for extraction of D, the diffusion constant, and the use of this in the Stokes–Einstein equation ($D = kT/6\eta R_h$), where T is the absolute temperature and η is the solvent viscosity, to estimate R_h have been described in detail previously

(Eum et al., 1989). R_h values of synthetic short-chain PI micelles were compared at concentrations well above their respective CMCs where the reference compound, diheptanoyl-PC, forms moderately polydisperse rod-shaped micelles. Samples of 1–2 mL were filtered through a Millex-GV 0.22-μm filter (Millipore Corp.) into glass scintillation vials and incubated for ~5 min prior to data acquisition. Samples were also diluted with water or the appropriate buffer to check for concentration-dependent growth of particles.

Phosphatidylinositol-Specific Phospholipase C Purification. PI-PLC, isolated from culture supernatants of *Bacillus subtilis* (BG2320) transfected with the PI-PLC gene from *Bacillus thuringiensis* (Henner et al., 1988) and purified as previously described (Low et al., 1988), was provided by Dr. Martin Low of Columbia University (New York). For storage purposes, the purified enzyme was dialyzed against 50 mM Tris–acetate, pH 7.4 (4 °C), and 0.02% sodium azide and then mixed with an equal portion of glycerol. The activity of this preparation was measured to be 1700 μmol/min·mL using [^{3}H]phosphatidylinositol as the substrate. The enzyme is stable in 50% glycerol (v/v) when stored at –20 °C. This enzyme has no divalent cation requirements and is relatively insensitive to inhibition by isotonic salt solutions (Low et al., 1988).

^{31}P NMR Assays of PI-PLC. For PI-PLC kinetic studies, the ^{31}P NMR parameters were based on those previously used by Griffith and co-workers (Volwerk et al., 1990). ^{31}P NMR experiments were run at 202.3 MHz on a Unity 500 spectrometer; 5-mm sample tubes were used. For all kinetic determinations, a control spectrum (t = 0 min) was performed prior to the addition of enzyme. PI-PLC (*B. thuringiensis*) was added to initiate hydrolysis. The amount of enzyme added varied between 45 ng and 7.5 μg as determined by the Bradford assay (Bradford, 1976), depending on whether the phosphatidylhydrolase or the cyclic phosphodiesterase activity was being monitored. After the addition of the appropriate amount of PI-PLC, an arrayed experiment which contained at least 8 × 300 transient collections (each FID was ~15 min) was conducted. ^{31}P NMR T_1 measurements (Vold et al., 1968) for diC$_7$PI micelles in 50 mM Tris–acetate buffer (pH 7.5), *myo*-inositol cyclic 1,2-monophosphate, and *myo*-inositol 1-monophosphate (which are the water-soluble products of PI-PLC cleavage of diC$_7$PI) were performed prior to conducting the kinetic assays to determine the optimal pulsing parameters. T_1 values for the short-chain PI starting material and the water-soluble hydrolysis products *myo*-inositol cyclic 1,2-monophosphate and *myo*-inositol 1-phosphate were 0.72, 2.09, and 2.04 s, respectively. The hydrophilic products have longer T_1's than the amphipathic starting material because they are water-soluble and do not form aggregates. Optimal pulsing conditions included a 5.5-μs pulse width (70°), a recycle time of 2.7 s, a 5339-Hz sweep width, 10 688 points, and an ambient probe temperature (20 °C). ^{1}H–^{31}P decoupling was not used in these experiments to avoid complications from differences in substrate and product NOEs. Free induction decays (FIDs) were weighted with 20-Hz line broadening unless otherwise stated. Reported chemical shifts (ppm) were referenced to an external capillary of phosphoric acid at 0 ppm.

The specific activity for PI-PLC toward short-chain PI substrates was calculated from the integrated intensity of water-soluble inositol cyclic 1,2-phosphate (Ins(1:2cyc)P) relative to the initial integrated intensity of substrate PI (micromoles). This value is then divided by the milligrams of protein added and the incubation time to generate a value for specific activity which is expressed in μmol min^{-1} mg^{-1}. Rates for the conversion of *myo*-inositol cyclic 1,2-monophosphate to *myo*-inositol 1-phosphate were calculated in an analogous fashion. The decrease in substrate PI intensity could also be used to calculate rates, as long as <20% of the substrate was utilized.

Table I: Surface Properties of Short-Chain Phosphatidylinositols Relative to Comparable Length Phosphatidylcholines

lipid	buffer	CMC	A_{CMC} (Å^2)
diC$_6$PI	H_2O	12.3[a]	46
diC$_6$PI	Tris–acetate, pH 7.5	9.0[a]	58
diC$_7$PI	H_2O	1.5[a]	61
diC$_6$PC	H_2O	14[b]	59[d]
diC$_7$PC	H_2O	1.5[c]	56[d]

[a] CMC values were determined by the du Nouy ring detachment method. [b] Lin, T.-L., Chen, S.-H., Gabriel, N. E., & Roberts, M. F. *J. Am. Chem. Soc.* (1986) *108*, 3499. [c] Lin, T.-L., Chen, S.-H., Gabriel, N. E., & Roberts, M. F. *J. Phys. Chem.* (1987) *91*, 406. [d] Bian, J., & Roberts, M. F. (1992) *J. Colloid Interface Sci. 153*, 420.

RESULTS AND DISCUSSION

Surface and Physical Properties of Short-Chain Phosphatidylinositols. Short-chain PIs aggregate spontaneously to form micelles which can be characterized by critical micellar concentration, the surface area per head group, and micelle size. With such detailed physical characterization of the substrate, the enzyme kinetics is often easier to interpret. Table I lists the critical micellar concentrations (CMC) and the surface areas per head group at the CMC (A_{CMC}) determined for diC$_6$PI and diC$_7$PI as compared to PCs of the same chain length. The CMCs were determined in H_2O, and also in Tris–acetate buffer at pH 7.5 for diC$_6$PI, to determine the CMC under the conditions of the ^{31}P NMR PI-PLC kinetic assay. The CMC values obtained for the chemically synthesized short-chain PIs in water are very similar to what has been previously reported for the short-chain PCs (Lin et al., 1986, 1987; Bian & Roberts, 1992). For diC$_7$PI, the CMC was measured to be 1.5 mM in H_2O, identical to the value for diC$_7$PC. This suggests that the chain length of both phospholipids is what dominates the CMC values. diC$_6$PI displayed a CMC of 12.3 mM in pure water, a value that is very close to the value reported for diC$_6$PC in H_2O (14 mM). The CMC value for diC$_6$PI is suppressed to 9.0 mM under the buffering conditions (50 mM Tris–acetate) of the NMR assay. Both of these values were significantly higher than the value reported for D-diC$_6$PI prepared enzymatically (Rebecchi et al., 1993). In that study the CMC was determined as 2.6 mM in a higher ionic strength medium (100 mM KCl and 25 mM HEPES); such a drop in CMC would be expected since the PI compound is negatively charged. The molecular surface areas at the CMC (A_{CMC}) of the PI species are comparable to those of the PC series (Bian & Roberts, 1992). This suggests that the acyl chain packing is the major contributor to micellization.

diC$_7$PI micelles were examined by QLS to determine their average size. This experiment showed that even at low concentrations PI micelles were large ($R_h \approx 600$ Å ~ 1.5 mM) and grew with increasing PI concentration (up to ~1200 Å at 2.5 mM). The micelles were also relatively uniform in size at the low concentration, but became more polydisperse (0.08–0.27) as the PI concentration increased to 2.5 mM. While these values are much higher than what is generally observed for PC micelles, the sizes are quite similar to what has previously been observed for comparable concentrations of phosphatidic acid micelles ($R_h \approx 400$ Å), which may reflect the influence of repulsive interactions of the negatively charged

Table II: Rates of PI-Specific Phospholipase C Hydrolysis of Synthetic Short-Chain Diheptanoyl-PI (5 mM) Species

substrate	phosphotransferase activity (μmol min^{-1} mg^{-1})	relative activity[a]			
		phosphotransferase		c-phosphodiesterase	
			+Triton X-100		+Triton X-100
D,L-diC$_7$PI	405 ± 95	1.00	0.92	0.0024	0.0019
D-diC$_7$PI	454 ± 70	1.00		0.003	
L-diC$_7$PI	0				
D,L-2-(OCH$_3$)diC$_7$PI	0				

[a] The activity is compared to that of pure diC$_7$PI at the same concentration.

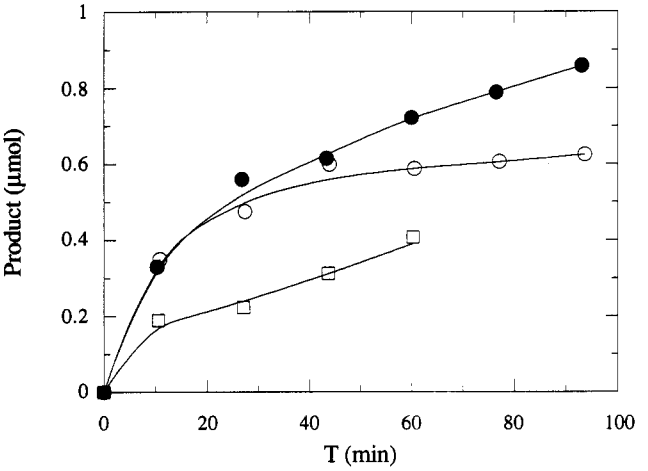

FIGURE 2: Reaction time course for PI-PLC (0.072 μg) production of inositol cyclic 1,2-phosphate (μmol) from 0.4 mL of 4.5 mM D-diC$_7$-PI alone (○), mixed with 3 mM L-diC$_7$PI (●), or 0.75 mM diC$_7$-(2-*O*-methyl)PI (□) in 50 mM Tris–acetate, pH 7.5. Amounts of product were estimated by measuring the ^{31}P peak intensity of the water-soluble product (as described in the text) compared to total PI intensity before the addition of enzyme.

head groups for the latter cases (V. Garigapati, K. Lewis, J. Bian, and M. F. Roberts, unpublished results).

Substrate Specificity of PI-PLC. Short-chain PIs provide an advantageous approach to the study of the substrate requirements and mechanism of action of water-soluble PI-specific phospholipase C, since they can be presented to the enzyme as monomers, micelles, and detergent-mixed micelles. Like other short-chain phospholipid species which have been previously used to study phospholipases (Bonsen et al., 1972), short-chain PIs can be synthesized with a variety of chain substitutions (such as altering chain length or unsaturation) or backbone modifications. Head-group modifications (e.g., D- or L-inositol, 2-methoxy-PI) can also be used to probe substrate requirements. Rates were measured from ^{31}P NMR spectra of the PI solutions as a function of PI concentration, as described previously (Volwerk et al., 1990). Each species of interest (short-chain PI substrate at –0.6 ppm, water-soluble hydrolysis products D-*myo*-inositol cyclic 1,2-phosphate at 16.0 ppm and D-*myo*-inositol 1-phosphate at 3.9 ppm) was observable with the cyclic product detected when there was ≥2–3% hydrolysis of a millimolar sample (Roberts, 1991a).

The activity of PI-PLC was examined toward 5 mM racemic, D-, and L-diC$_7$PI. The time course for PI-PLC is initially linear, but levels off after 20–30% product generation [suggestive of product inhibition which has been postulated to be rate-limiting for the nonspecific PI-PLC enzyme (El-Sayed & Roberts, 1985)]. Only the linear region was used to generate a specific activity for the enzyme. As shown in Table II, the phosphotransferase rates observed for the D form and the racemic mixture were essentially the same. The L-diC$_7$-PI was neither a substrate nor an inhibitor. Previous studies (Volwerk et al., 1990) suggested that the L-diacyl-PI isomer is neither a substrate nor an inhibitor of PI-PLC, although this was based on the cyclic phosphodiesterase activity and on kinetics with a water-soluble inositol nitrophenyl phosphate (Leigh et al., 1992), not with a phospholipid substrate. The present results confirm that the phosphotransferase activity also requires the D-phosphoinositol isomer. Also of note is that the presence of the nonsubstrate L-PI relieves the product inhibition observed at later time periods with the pure D-inositol PI (Figure 2). Presumably, the higher concentration of lipid matrix solubilizes more diacylglycerol.

The most reasonable mechanism proposed for the action of PI-PLC toward its substrate involves an obligate transfer of the phosphate group from the glycerol moiety to the 2-OH of the inositol ring (Lin et al., 1990; Volwerk et al., 1990). If this is a required part of the enzyme-catalyzed hydrolysis, then a PI derivative which no longer has a free hydroxyl group at that position should be inert to PI-PLC. Racemic diC$_7$-(2-*O*-methyl)PI was examined as a potential substrate for PI-PLC (Table II). It was clearly not a substrate for the enzyme, remaining unhydrolyzed after 2 weeks in the presence of 0.5 μg of enzyme. This modified PI has a ^{31}P chemical shift slightly downfield from that of the substrate (Figure 3); the action of PI-PLC toward a 1:1 mixture of D-diC$_7$PI and diC$_7$(2-*O*-methyl)PI can be followed by NMR spectroscopy. Racemic diC$_7$(2-*O*-methyl)PI was an inhibitor of D-diC$_7$PI hydrolysis by the enzyme. The rate toward 5 mM pure D-diC$_7$-PI was 454 ± 70 μmol min^{-1} mg^{-1}; this value decreased by 72% to 126 ± 3 μmol min^{-1} mg^{-1} in a 5 mM D-diC$_7$PI–5 mM diC$_7$(2-*O*-methyl)PI mixture, suggesting that the nonsubstrate methoxy compound bound more tightly to the enzyme than the substrate. Around 50% inhibition was observed in a mixed micelle sample of 4.5 mM D-diC$_7$PI and 0.75 mM diC$_7$(2-*O*-methyl)PI (Figure 2). Under these assay conditions, both PI species have similarly low CMCs and the bulk of the lipids are micellar, with the inhibitor mole fraction approximating the surface mole fraction of the inhibitor. This is the first phospholipid inhibitor based on the mechanism of action of the bacterial PI-PLC and potentially can be used to screen other PI-PLC activities to determine whether the Ins(1:2cyc)P is an obligatory intermediate in PI hydrolysis.

Interfacial Activation of PI-PLC toward Dihexanoyl-PI. A common feature in lipolytic enzymes such as phospholipases is the preference they display toward substrates in aggregated as opposed to monomeric forms, a phenomenon referred to as "interfacial activation". This activation was first noted by Roholt and Schlamowitz (1961) and later was investigated in detail by de Haas and co-workers with pancreatic phospholipase A$_2$. In the latter case, hydrolysis was optimal in micellar systems and very poor toward monomeric systems (de Haas et al., 1971; Pieterson et al., 1974). Interfacial activation may be the end result of several different factors. Furthermore, the mechanism responsible for observed rate increases upon the aggregation of substrate may not be unique to all phospholipases, but may be quite different depending on the

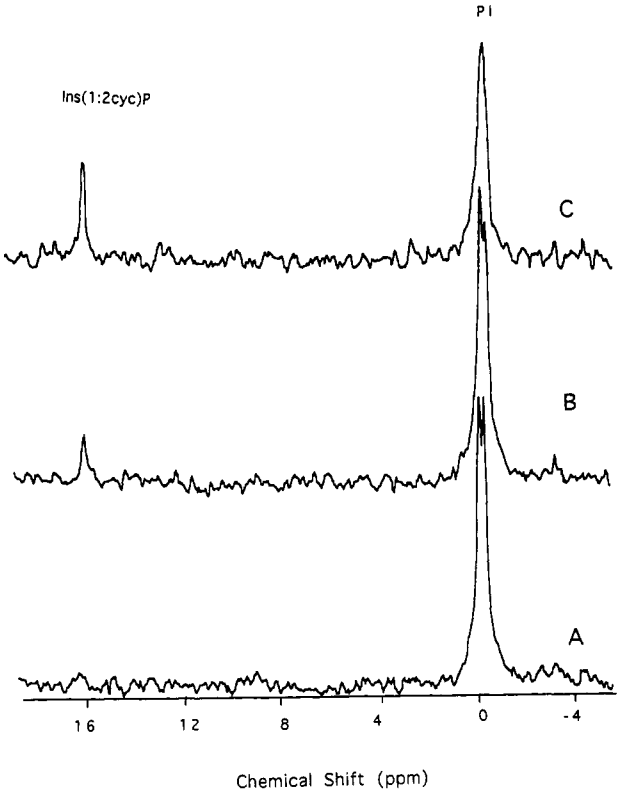

FIGURE 3: ^{31}P NMR spectra (202.3 MHz) of 5 mM diC$_7$(2-*O*-methyl)PI–5 mM D-diC$_7$PI incubated with 67.5 ng of PI-PLC for (A) 15 min, (B) 30 min, and (C) 75 min. Both substrate and inhibitor PI resonances can be distinguished in initial time points: D-diC$_7$PI occurs at –0.5 ppm while diC$_7$(2-*O*-methyl)PI occurs at –0.3 ppm. The product Ins(1:2cyc)P resonance occurs at ~16 ppm.

individual phospholipase and its specificity. The degree of activation observed toward phospholipid aggregates varies between sources of the enzyme (i.e., pancreatic versus cobra venom PLA$_2$), specificity of the phospholipase (phospholipase A$_2$ exhibits a 20–100-fold activation while nonspecific bacterial phospholipase C exhibits only a 2–3-fold activation upon micellization), and type of aggregate employed (micelle versus bilayer) (Roberts, 1991b; Roberts & Dennis, 1989; El-Sayed et al., 1985).

diC$_6$PC was chosen for those pH–stat studies because it has a fairly high CMC in Tris–acetate, allowing good measurements in both the monomer region (0–9 mM) and the micellar region (>9 mM). The interfacial activation curve for PI-PLC toward diC$_6$PI is shown in Figure 3, with the CMC (obtained under buffered conditions) denoted by the arrow. The monomer V_{max} can be extrapolated as ~100 μmol min^{-1} mg^{-1}, while the micellar V_{max} can be extrapolated to be ~560 μmol min^{-1} mg^{-1}. This represents a 5–6-fold activation of PI-PLC toward aggregated substrates compared to monomeric substrates. This activation is similar in magnitude to that observed for phospholipase C-δ1 acting on diC$_8$PI (although, because of the suppressed CMC in that case, the activation rests on a single data point), as well as similar to the ~3-fold activation seen with the nonspecific bacterial PLC acting toward diC$_6$PC (El-Sayed et al., 1985), which may suggest that the underlying determinant for enhancement might result from a common mechanism. Figure 4 shows that PI-PLC activation occurs slightly prior to the CMC, which may reflect a premicellar aggregation phenomenon generated by the presence of the phospholipase, an observation which has been previously reported for phospholipase D hydrolysis of short-chain PC (Allgyer & Wells, 1974). Single-chain thiophosphate and fluorescent analogs of PI which form micelles have also been examined as substrates for PI-PLC (Hendrickson et al., 1992). These are poor substrates compared to the symmetric short-chain PI species, but they exhibited some extent of interfacial activity.

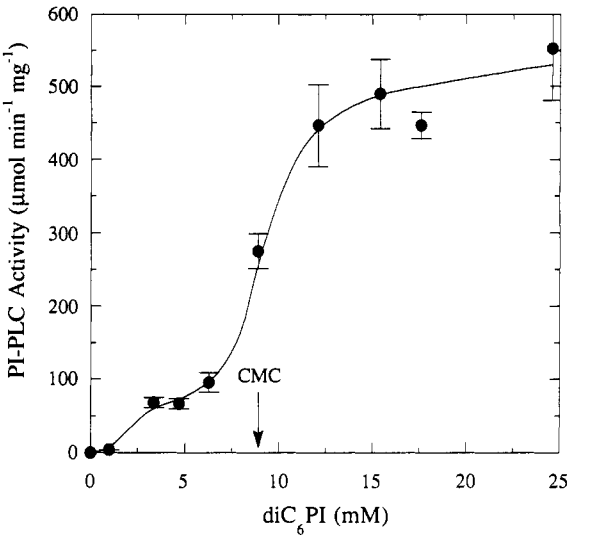

FIGURE 4: Specific activity (μmol min^{-1} mg^{-1}) of phosphatidylinositol-specific PLC (*Bacillus thuringiensis*) toward racemic diC$_6$PI as a function of substrate concentration. The CMC of this lipid in 50 mM Tris buffer (determined by surface tension) is indicated by an arrow.

Effect of Detergent on the Activity of PI-PLC toward Diheptanoyl-PI. Assay systems that have been employed prior to the short-chain PI system described here generally require the use of detergents, such as Triton X-100 or deoxycholate, to solubilize long-chain PI substrates (Sundler et al., 1978; Volwerk et al., 1990). It is not known whether or not the presence of detergents resulted in a decrease in either the phosphotransferase or cyclic phosphodiesterase activity of PI-PLC compared to pure lipid substrates, as the only reported experiments with pure substrate PI utilized unilamellar vesicles which were minimally hydrolyzed (<5% in 24 h) (Volwerk et al., 1990). It seems especially suspect that the cyclic phosphodiesterase activity, which is more likely to occur in the water phase rather than at the interface, might be hampered by the presence of detergents, which could potentially explain the 1000-fold lower activity observed relative to the phosphotransferase activity (Volwerk et al., 1990). To address this concern, we performed experiments with racemic diC$_7$PI, which forms micelles, with and without a 2-fold excess of Triton X-100 present. The relative specific activity for the phosphotransferase activity toward 10 mM diC$_7$PI decreased only slightly under these conditions (Table II) to 92% of the original activity. This suggests that Triton X-100 does not alter PI-PLC activity in this system, and the detergent acts merely as an inert spacer. The second, cyclic phosphodiesterase activity, which converts D-*myo*-inositol cyclic 1,2-phosphate to D-*myo*-inositol 1-phosphate was measured as 450-fold lower than the phosphotransferase activity (Table II). This is similar to the drop in activity observed with the PI-specific phospholipase C from *Bacillus cereus*. The presence of the detergent decreased this to ~80% of the activity without Triton X-100. While the presence of a 2-fold excess of detergent does not dramatically inhibit the cyclic phosphodiesterase activity, it does have a small effect on it. This could indicate that under these conditions the presence of substrate micelles in the assay sequesters the enzyme to the interface in the same manner that detergent would, thereby

generating similar rate values.

In summary, the short-chain PI systems indicate (i) an absolute stereochemical requirement for the D-inositol PI isomer, (ii) a 5–6-fold increase in activity as the substrate is aggregated, and (iii) perhaps most importantly, the 2-hydroxy group is necessary for enzyme activity, but is not required for lipid binding to the enzyme. Modification of the inositol ring at this position can be used to develop specific lipophilic enzyme inhibitors.

ACKNOWLEDGMENT

We thank Dr. Martin Low of Columbia University for his generous gift of PI-specific phospholipase C and Professor Sow-Hsin Chen and Mr. Bruce Carvalho of the Massachusetts Institute of Technology for access to the QLS system and help with the QLS measurements of micelle sizes.

REFERENCES

Allgyer, T. T., & Wells, M. A. (1979) *Biochemistry 18*, 5348–5353.

Banno, Y., Yada, Y., & Nozawa, Y. (1988) *J. Biol. Chem. 263*, 11459–11465.

Bennett, C. F., & Crooke, S. T. (1987) *J. Biol. Chem. 262*, 13789–13797.

Berridge, M. J. (1984) *Biochem. J. 220*, 345–360.

Berridge, M. J. (1986) Phosphoinositides and Receptor Mechanisms (Putney, J. W., Ed.) in *Receptor Biochemistry and Methodology* (Venter, J. C., & Harrison, L. C., Eds.) Vol. 7, pp 25–45, Alan Liss, New York.

Bian, J., & Roberts, M. F. (1992) *J. Colloid Interface Sci. 153*, 420–428.

Bonsen, P. P., Burbach-Westerhuis, G. J., de Haas, G. H., & van Deenen, L. L. M. (1972) *Chem. Phys. Lipids 8*, 199–208.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Burns, R. A., Jr., & Roberts, M. F. (1980) *Biochemistry 19*, 3100–3106.

Campbell, A. S., & Thatcher, G. R. J. (1991) *Tetrahedron Lett. 32*, 2207–2210.

De Haas, G. H., Bonsen, P. P. M., Pieterson, W. A., & Van Deenen, L. L. M. (1971) *Biochim. Biophys. Acta 239*, 252–266.

Eaton, B. R., & Dennis, E. A. (1976) *Arch. Biochem. Biophys. 176*, 604–609.

El-Sayed, M. Y., DeBose, C. D., Coury, L. A., & Roberts, M. F. (1985) *Biochim. Biophys. Acta 837*, 325–335.

Eum, K.-M., Riedy, G., Langley, K. H., & Roberts, M. F. (1989) *Biochemistry 28*, 8206–8213.

Gabriel, N. E. (1986) Ph.D. Dissertation, Massachusetts Institute of Technology, Cambridge, MA.

Garigapati, V. R., & Roberts, M. F. (1993a) *Tetrahedron Lett. 34*, 769–772.

Garigapati, V. R., & Roberts, M. F. (1993b) *Tetrahedron Lett.* (in press).

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

Hendrickson, H. S., Hendrickson, E. K., Johnson, J. L., Khan, T. H., & Chial, H. J. (1992) *Biochemistry 31*, 12169–12172.

Henner, D. J., Yang, M., Chen, E., Hellmiss, R., Rodriguez, H., & Low, M. G. (1988) *Nucleic Acids Res. 16*, 10383–10383.

Homma, Y., Imaki, J., Nakanishi, O., & Takenawa, T. (1988) *J. Biol. Chem. 263*, 6592–6598.

Ikezawa, H., & Taguchi, T. (1981) *Methods Enzymol. 71*, 731–741.

Leigh, A. J., Volwerk, J. J., Griffith, O. H., & Keana, J. F. W. (1992) *Biochemistry 31*, 8978–8983.

Lin, T.-L., Chen, S.-H., Gabriel, N. E., & Roberts, M. F. (1986) *J. Am. Chem. Soc. 108*, 3499–3507.

Lin, T.-L., Chen, S.-H., Gabriel, N. E., & Roberts, M. F. (1987) *J. Phys. Chem. 91*, 406–413.

Lin, G., Bennett, C. F., & Tsai, M.-D. (1990) *Biochemistry 29*, 2747–2757.

Low, M. G. (1981) *Methods Enzymol. 71*, 741–746.

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

Low, M. G., Stiernberg, J., Waneck, G. L., Flavell, R. A., & Kincade, P. W. (1988) *J. Immunol. Methods 113*, 101–111.

Pieterson, J. C., Vidal, J. C., Volwerk, J. J., & de Haas, G. H. (1974) *Biochemistry 13*, 1455–1460.

Rebecchi, M., Eberhardt, R., Delaney, T., Ali, S., & Bittman, R. (1993) *J. Biol. Chem. 268*, 1735–1741.

Rhee, S. G., Suh, P.-G., Ryu, S.-H., & Lee, S. Y. (1989) *Science 244*, 546–550.

Roberts, M. F. (1991a) *Methods Enzymol. 197*, 31–48.

Roberts, M. F. (1991b) *Methods Enzymol. 197*, 95–112.

Roberts, M. F., & Dennis, E. A. (1989) in *Phosphatidylcholine Metabolism* (Vance, D. E., Ed.) pp 121–142, CRC Press, Boca Raton, FL.

Roholt, O. A., & Schlamowitz, M. (1961) *Arch. Biochim. Biophys. 94*, 364–379.

Ryu, S. H., Cho, K. S., Lee, K. Y., Suh, P. G., & Rhee, S. G. (1986) *Biochem. Biophys. Res. Commun. 141*, 137–144.

Ryu, S. H., Cho, K. S., Lee, K. Y., Suh, P. G., & Rhee, S. G. (1987) *J. Biol. Chem. 262*, 12511–12518.

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

Takenawa, T., & Nagai, Y. (1981) *J. Biol. Chem. 256*, 6769–6775.

Vold, R. L., Waugh, J. S., Klein, M. P., & Phelps, D. E. (1968) *J. Chem. Phys. 48*, 3831–3839.

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