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Kinetics of phosphatidylinositol-specific phospholipase C with vesicles of a thiophosphate analogue of phosphatidylinositol

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Abstract

1,2-Dimyristoyloxypropane-3-thiophospho(1D-1-*myo*-inositol) (D-thio-DMPI) was synthesized as a substrate for the continuous spectrophotometric assay of phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus*. Release of thio-diglyceride is followed by a coupled reaction with 4,4′-dithiopyridine to produce a chromophore, 4-thiopyridine, measured by its absorption at 324 nm. Sonicated vesicles of D-thio-DMPI gave sigmoidal Michaelis-Menten kinetics with PI-PLC as a function of bulk concentration of substrate (Hill plot: $V_{max} = 132$ μmol min^{-1} mg^{-1}, apparent $K_m = 0.115$ mM, $h = 1.8$). Addition of dimyristoyl phosphatidylcholine (DMPC) or dimyristoyl phosphatidylmethanol to vesicles of D-thio-DMPI resulted in an initial increase in rate followed by a decrease at higher concentrations of non-substrate lipid. Binding of PI-PLC to vesicles of DMPC with 10 mol% of *N*-dansyl phosphatidylethanolamine was demonstrated by fluorescence resonance energy transfer from tryptophan in the enzyme to the dansyl lipid probe. Copyright © 1996 Elsevier Science Ireland Ltd

Keywords: *Bacillus cereus*; Phosphatidylinositol; Phospholipase C; Thiophosphate; Vesicle; 1,2-Dimyristoyloxypropane-3-thiophospho(1D-1-*myo*-inositol)

Abbreviations: D-thio-DMPI, 1,2-dimyristoyloxypropane-3-thiophospho(1D-1-*myo*-inositol); *rac*-thio-DMPI, 1,2-dimyristoyloxypropane-3-thiophospho(*rac*-1-*myo*-inositol); DMPC, dimyristoyl phosphatidylcholine; DMPM, dimyristoyl phosphatidylmethanol; DTP, 4,4′-dithiopyridine; MES, 2-[*N*-morpholino]ethanesulfonic acid; *N*-dansyl-PE, *N*-dansyl phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PM, phosphatidylmethanol.

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1. Introduction

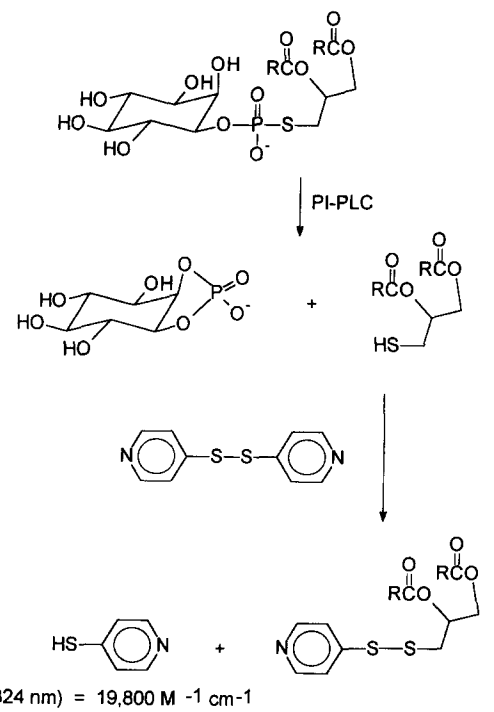
Phosphatidylinositol-specific phospholipase C (PI-PLC; EC 3.1.4.10) from *Bacillus cereus* catalyzes the cleavage of phosphatidylinositol to a diglyceride and 1D-*myo*-inositol 1,2-(cyclic)phosphate. The latter is subsequently hydrolyzed, at a slower rate by the same enzyme, to 1D-*myo*-inositol-1-phosphate (for a recent review see Bruzik and Tsai [1]). The traditional assay of PI-PLC involves the determination of water-soluble inositol phosphate from radiolabeled phosphatidylinositol [2]. Kinetic studies of the enzyme have been hampered by the lack of a simple continuous assay with phospholipids closely resembling the natural substrate in bilayer membrane systems. Earlier, we developed a continuous spectrophotometric assay using a thiophosphate substrate analogue, hexadecylthiophospho-1-*myo*-inositol, in which the release of a thiol (hexadecylmercaptan) was coupled to the formation of a chromophore with 4,4'-dithiopyridine [3]. This substrate, however, was hydrolyzed at a rate (16.9 μmol min^{-1} mg^{-1}) only about 10% that with the natural substrate, and the enzyme reaction could only be studied in micelles, but not bilayers [4]. Here we report the synthesis of an optically-active diglyceride-containing thiophosphate analogue of phosphatidylinositol in which the oxygen between the phosphorus and glycerol carbon is substituted by a sulfur 1,2-dimyristoyloxypropane-3-thiophospho(1D-1-*myo*-inositol) (D-thio-DMPI). The synthesis of a dipalmitoyl homologue of this lipid, was first reported by Alisi et al. [5]; however, it was racemic and they made no report of its hydrolysis by PI-PLC. D-thio-DMPI facilitates a continuous spectrophotometric assay of PI-PLC (Scheme 1). This substrate is hydrolyzed at a much higher rate than that observed with hexadecylthiophospho-1-*myo*-inositol.

Like many phospholipases, PI-PLC acts preferentially at lipid-water interfaces. This 'interfacial activation' was shown for *B. cereus* PI-PLC with alkylthiophospho-1-*myo*-inositol and pyrenobutylphosphoryl-*myo*-inositol substrate analogues above and below their critical micellar concentrations by Hendrickson et al. [4], and with dihexanoyl phosphatidylinositol by Lewis et al. [6]. The minimal mechanism for the enzyme reaction involves classical Michaelis-Menten enzyme-substrate binding and catalysis within the two-dimensional interface, preceded by an equilibrium between free soluble enzyme in the bulk phase and enzyme bound to the interface [7–9]. This mechanism was recently studied by Volwerk et al. [10] using water-soluble analogues of the substrate and vesicles containing phosphatidylinositol (PI), phosphatidylcholine (PC), and phosphatidylmethanol (PM). Here we report on the PI-PLC-catalyzed hydrolysis of a membrane phospholipid substrate analogue in a bilayer vesicle system.

2. Experimental

2.1. Materials

Recombinant *Bacillus cereus* PI-PLC was expressed in *Escherichia coli* and purified according to the procedure of Koke et al. [11] using the pIC



Scheme 1. Spectrophotometric assay of PI-PLC with D-thio-DMPI.

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 1.84 ml mg^{-1} cm^{-1} at 280 nm [10]. Dilute solutions of enzyme were stabilized in 0.1% *S*-carboxymethyl bovine serum albumin (Sigma Chemical Co., St. Louis, MO). DTP (Aldrithiol-4) was purchased from Aldrich Chemical Co. (Milwaukee, WI). DMPC, DMPM, and N-dansyl-PE were purchased from Sigma. Racemic 1,2-dimyristoyloxypropane-3-phthalimide was synthesized according to the procedure of Müller and Roth [12].

2.2. Synthesis of 1,2-dimyristoyloxypropane-3-thiophospho(1D-1-myo-inositol) (D-thio-DMPI)

Optically-active 1,2-dimyristoyloxypropane-3-thiophospho(1D-1-*myo*-inositol) (D-thio-DMPI) was synthesized by a procedure similar to that used to synthesize hexadecylthiophospho(1D-1-*myo*-inositol) (see scheme I in Bushnev et al. [13]) with slight modifications. 2,3-*O*-(D-1′,7′,7′-Trimethyl[2,2,1]bicyclohept-2′-ylidene)-4,5,6-*O*-tris(methoxymethyl)-1D-1-*myo*-inositol dimethyl phosphite was synthesized as previously reported [13], using the procedure of Bruzik and Tsai [14] starting from *myo*-inositol and D-camphor. The addition of dimethyl chlorophosphite was done at −75°C rather than −15°C, as the reaction was much cleaner at the lower temperature. The product was then coupled to racemic 1,2-dimyristoyloxypropane-3-thio-phthalimide under conditions similar to those published for the hexadecyl analogue. The final product was isolated as the triethylammonium salt by treatment with triethylamine prior to the final chromatography on silica gel. It gave one spot by TLC on silica gel (R_f = 0.40; $CHCl_3$–CH_3OH–H_2O, 65:35:3) and had a ^{1}H-NMR spectrum consistent with its structure.

1,2-Dimyristoyloxypropane-3-thiophospho(1-*rac*-*myo*-inositol) (*rac*-thio-DMPI) was prepared in a similar procedure using racemic 2,3,5,6-bis-cyclohexylidino-4-methoxymethyl-1-*rac*-*myo*-inositol dimethyl phosphite (prepared according to Hendrickson et al. [3]).

2.3. Enzyme assays

A measured amount of phospholipid (in chloroform/methanol solution) in a small glass culture tube was dried under a stream of nitrogen and then under high vacuum. MES buffer (50 mM, pH 7) was added and the suspension was vortexed and briefly sonicated in a bath sonicator (Model G112SPIT, Lab Supplies, Hicksville, NY). The cloudy solution was frozen and then sonicated for 15-s intervals until an almost water-clear dispersion was obtained. This solution was annealed at room temperature for at least 20 min before use.

Substrate vesicle solution (225 μl) and 5 μl of 50 mM DTP (in ethanol) were added to a semimicro (2 × 10 mm) cuvette. 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 (no background). PI-PLC (10 μl containing up to 100 ng of enzyme) was added and the absorbance recorded for several min. The time courses were initially linear with no lag, and activity was linear with respect to the amount of enzyme added. Activity was calculated using an extinction coefficient for thiopyridine of 19 800 M^{-1} cm^{-1} [15]. Enzyme activity as a function of substrate concentration was analyzed by nonlinear regression fitting of data to the Hill equation using the program SigmaPlot (Jandel Scientific, San Rafael, CA).

2.4. Fluorescence spectroscopy

Fluorescence resonance energy transfer from tryptophan of PI-PLC to *N*-dansyl-PE (10 mol%) in DMPC vesicles was measured with a Perkin-Elmer LS50B spectrofluorometer. PI-PLC (72 mg/ml) was titrated into 1 mM DMPC, 0.1 mM *N*-dansyl-PE in 50 mM MES (pH 7) buffer at 21°C. Excitation was at 290 nm and emission was measured at 520 nm.

3. Results and discussion

3.1. Synthesis of thio-DMPI

Optically-active D-thio-DMPI and *rac*-thio DMPI were synthesized using a procedure similar

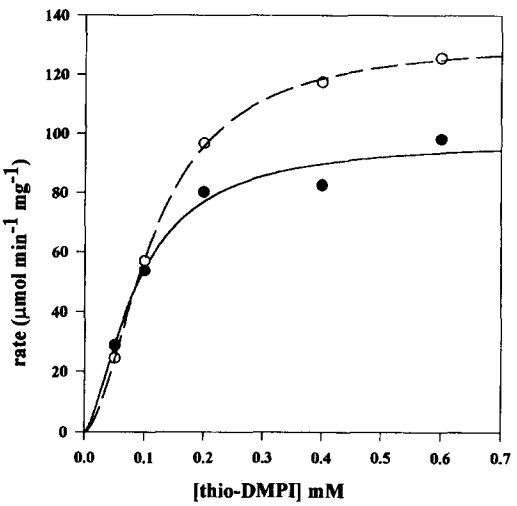


Fig. 1. Activity of PI-PLC as a function of bulk concentration of thio-DMPI. Closed circles, *rac*-thio-DMPI; open circles, D-thio-DMPI. Data fitted to the Hill equation by nonlinear regression analysis.

to that reported for hexadecylthiophospho-1-*myo*-inositol [13], with minor modifications. The thiophosphate was made by reacting 1,2-dimyristoyloxypropane-3-thio-phthalimide in an Arbuzov-type reaction with the protected inositol dimethyl phosphite. The final product was isolated as a triethylammonium salt, which was considerably more soluble in chloroform/methanol than the lithium salt previously obtained.

rac-Thio-DMPI was synthesized by a similar procedure, reacting racemic 1,2-dimyristoyloxypropane-3-thio-phthalimide with 2,3,5,6-bis-cyclohexylidino-4-methoxymethyl-1-*rac*-*myo*-inositol dimethyl phosphite.

3.2. Kinetics of PI-PLC with pure thio-DMPI vesicles

Small sonicated vesicles of thio-DMPI were prepared and used as substrate for PI-PLC. Michaelis-Menten kinetics were observed with sigmoidal plots of rate versus bulk concentration of thio-DMPI (Fig. 1). With racemic substrate (*rac*-thio-DMPI), V_{max} was 98.1 ($\pm$9.7) μmol min^{-1} mg^{-1}, the apparent K_m was 0.087 ($\pm$0.017) mM, and the Hill coefficient was 1.5 ($\pm$0.5). With optically-active substrate (D-thio-DMPI), V_{max} and the apparent K_m were 132 ($\pm$2) μmol min^{-1} mg^{-1} and 0.115 ($\pm$0.003) mM, respectively, and the Hill coefficient was 1.78 ($\pm$0.07). From the latter value for V_{max}, the calculated value for k_{cat} is 76 s^{-1}; this is about one-third that with natural substrate vesicles (220 s^{-1} [10]). The value for the apparent K_m is about an order of magnitude smaller than that with the natural substrate.

The ratio of k_{cat} values for the oxy-phosphate and thio-phosphate substrates is similar to that seen for the natural and thiolester (fatty acid) analogues of phospholipid substrates with phospholipase A_2 [16]. Like phospholipase A_2, this may reflect a difference in the catalytic step; however, more detailed studies are needed in order to substantiate that hypothesis. Further studies are planned using substrates that differ only by an oxygen to sulfur substitution in the phosphodiester group. The smaller value for the apparent K_m is also seen with thiolester substrate analogues and phospholipase A_2, and may be due to enhanced enzyme binding with the more hydrophobic sulfur atom.

The fact that V_{max} for the optically-active substrate is less than twice that with the racemic substrate may indicate that the interfacial K_m is less than 0.5 mol fraction. The interfacial K_m for PI-PLC in mixed vesicles of PI and PC was determined by Volwerk et al. [10] to be 0.26 mol fraction of PI. We have recently synthesized optically-active L-thio-DMPI (with 1L-*myo*-inositol) and plan to study the interfacial kinetics as a function of the ratio of D- to L-isomers. Substrates containing 1L-*myo*-inositol have been shown to be neither substrates nor inhibitors of PI-PLC [2,6,17].

3.3. Kinetics of PI-PLC with mixed-lipid vesicles of thio-DMPI and either DMPC or DMPM

The kinetics of PI-PLC with vesicles of D-thio-DMPI in the presence of DMPC or DMPM, at a constant bulk concentration of 0.5 mM substrate, are shown in Fig. 2. Addition of either DMPC or DMPM resulted in an initial increase in rate, followed by a decrease at higher mol fractions. The effect was greatest with DMPC, which increased the rate by about 50% at 0.6 mol fraction; DMPM resulted in an increase of only about 20% at 0.1 mol

fraction before the rate began to decrease. This apparent activation may be a result of more lipid in the interface and increased interfacial binding of enzyme, and/or intrinsic activation by the non-substrate lipid. The concentration of substrate may be in the region of half-maximal interfacial binding of enzyme, in which case the increase in activity may just be due to increased phospholipid interface. Volwerk et al. [10] observed no increase in the activity of PI-PLC upon the addition of PC or PM to mixed micelles with PI, but only a decrease. Their experiments were done at a much higher bulk concentration of PI (2 mM) where the interface is already saturated with enzyme.

The decrease in rate at higher mol fraction of DMPC is probably due to surface dilution of substrate. The greater decrease in rate with DMPM may be due to competitive inhibition by binding of this anionic lipid at the active site [10]. Further studies are needed to understand these effects. The changes in rate shown in Fig. 2 could also be due to changes in the size or nature of the vesicles. We plan to further study the kinetics with well-characterized vesicles of homogeneous size.

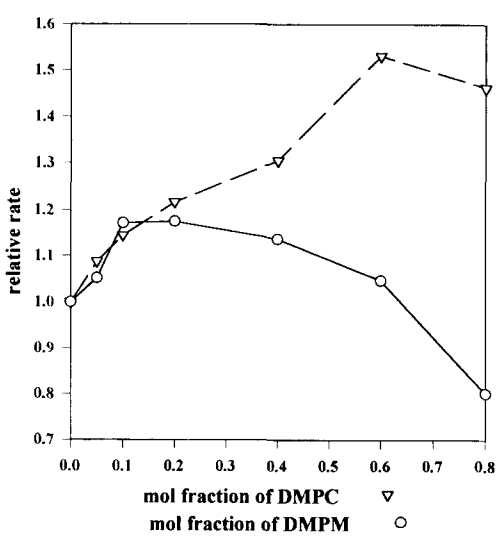


Fig. 2. Activity of PI-PLC with D-thio-DMPI in mixed-lipid vesicles as a function of added DMPC or DMPM. Constant bulk concentration of D-thio-DMPI, 0.5 mM.

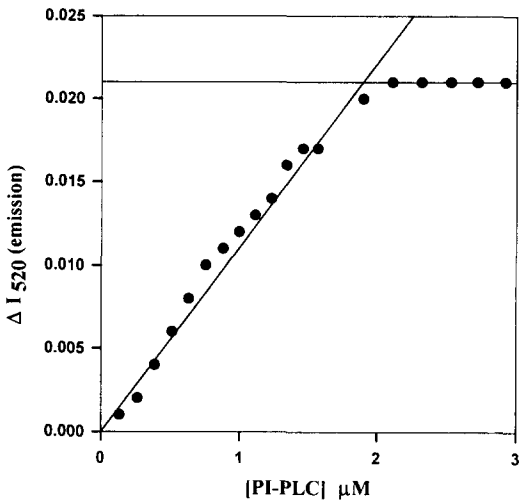


Fig. 3. Fluorescence resonance energy transfer from PI-PLC to *N*-dansyl-PE in DMPC vesicles as a function of PI-PLC concentration. [DMPC] = 1 mM, [*N*-dansyl-PE] = 0.1 mM. Excitation = 290 nm, emission = 520 nm.

3.4. Fluorescence resonance energy transfer studies of PI-PLC binding to vesicles

Binding of PI-PLC to DMPC vesicles was confirmed by fluorescence spectroscopy. Fluorescence resonance energy transfer from tryptophan in PI-PLC to a *N*-dansyl-PE probe (0.1 mol fraction) in sonicated DMPC vesicles is shown in Fig. 3. Titration of PI-PLC into the vesicles (1 mM DMPC) showed a linear increase in dansyl emission at 520 nm (excitation of tryptophan at 290 nm) up to 2 μM PI-PLC, at which point no further increase was observed. This seems to indicate stoichiometric binding, so it was not possible to calculate a dissociation constant. PI-PLC contains seven tryptophans, two of which (Trp47 and Trp242) are exposed at the surface [18]. Volwerk et al. [10] showed that these two tryptophans were sensitive to binding to vesicles and micelles by UV- and fluorescence-difference spectroscopy. They calculated dissociation constants between 0.04 and 0.2 mM for PC vesicles with different fatty acid composition. Our results confirm that there is indeed tight binding of PI-PLC to phospholipid vesicles. In addition, gel filtration studies (H.S. Hendrickson and T.P. Weiss, unpublished data) give direct evidence for PI-PLC binding to pure DMPC vesicles.

Acknowledgements

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