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# Continuous spectrophotometric assay of mammalian phosphoinositide-specific phospholipase C $\delta_1$ with a thiophosphate substrate analog

H. Stewart Hendrickson *

*Department of Chemistry, University of Washington, P.O. Box 351700, Seattle, WA 98195-1700, USA*

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**Abstract**

1,2-Dimyristoyloxypropane-3-thiophospho(1D-1-*myo*-inositol) (D-thio-DMPI) was used as a substrate for the continuous assay of phosphoinositide-specific phospholipase C (PI-PLC). Its activity with a $\Delta(1-132)$ deletion mutant of mammalian PI-PLC $\delta_1$ is about one-fourth that with PI under similar conditions. Optimal conditions for the assay include 0.2 mM substrate, 0.2 mM $Ca^{2+}$, and a mole ratio of hexadecylphosphocholine detergent to substrate of 2.0. A minimum of about 60 ng of pure enzyme can be detected. The apparent bulk $K_m$ for PI-PLC with D-thio-DMPI under these conditions is about 6 $\mu$M. Enzyme activity as a function of surface concentration of substrate shows no sign of saturation up to the maximum mole fraction. © 1998 Elsevier Science B.V.

*Keywords:* 1,2-Dimyristoyloxypropane-3-thiophospho(1D-1-*myo*-inositol); Phosphoinositide-specific phospholipase C; Assay; Micelle; Thiophosphate

## 1. Introduction

Phosphoinositide-specific phospholipases C (PI-PLC; EC 3.1.4.11) from mammalian cells represent a large family of closely related enzymes that play a key role in signal transduction through the hydrolysis of phosphatidylinositol bisphosphate to two second messengers, inositol trisphosphate and diacylglycerol [1–4]. They can be grouped into three major classes, $\beta$, $\gamma$ and $\delta$, based on their size and primary structure. The 2.4-Å crystal structure of the isozyme PI-PLC $\delta$1 was recently reported by Essen et al. [5]. It is a multi-domain protein containing a catalytic domain in addition to other domains, including an N-terminal pleckstrin homology (PH) domain, shared by many signaling proteins [6]. The structure suggests a catalytic mechanism similar to that of ribonuclease, involving a 1,2-cyclic-inositol phosphate intermediate.

PI-PLC, like other water-soluble phospholipases, is subject to 'interfacial activation', the preference for

Abbreviations: D-thio-DMPI, 1,2-dimyristoyloxypropane-3-thiophospho(1D-1-*myo*-inositol); L-thio-DMPI, 1,2-dimyristoyloxypropane-3-thiophospho(1L-1-*myo*-inositol; D-thio-DPPI, 1,2-dipalmitoyloxypropane-3-thiophospho(1D-1-*myo*-inositol); DTP, 4,4′-dithiobispyridine; HDPC, hexadecylphosphocholine; MES, 2-(*N*-morpholino)ethanesulfonic acid; PC, phosphatidylcholine; PH, pleckstrin homology; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; $PIP_2$, phosphatidylinositol-4,5-bisphosphate; PI-PLC, phosphoinositide-specific phospholipase C

* Corresponding author. Fax: +1-206-685-8665; E-mail: hend@u.washington.edu


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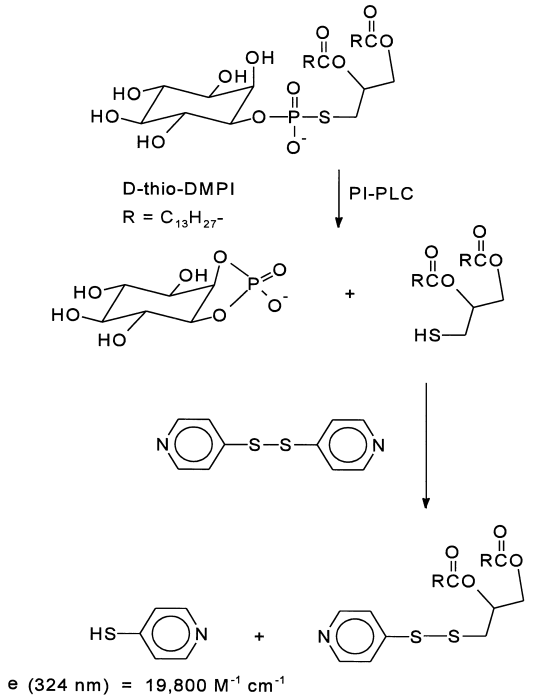
aggregated over monomeric substrate. This was shown for bacterial PI-PLC by Hendrickson et al. [7] with various PI analogs, and by Lewis et al. [8] with short-chain PI, and also with short-chain PI for mammalian PI-PLC$\delta$1 by Rebecchi et al. [9]. This activation, in which the activity increases several fold, may involve a change in enzyme conformation [10–12]. The simplest kinetic mechanism involves reversible binding of the soluble enzyme to the lipid interface, followed by an interfacial Michaelis–Menten mechanism [13].

Kinetic studies of PI-PLC have been hampered by the lack of a continuous simple assay with phospholipids closely resembling the natural substrate in bilayer membranes. Traditional assays involving the use of radioisotopes [14] are tedious and time consuming. Continuous assays have been developed for the bacterial PI-PLC. A water-soluble substrate analog, *myo*-inositol-1-(4-nitrophenylphosphate) [15] was used in a kinetic analysis of PI-PLC from *Bacillus cereus* [10]. Since this substrate does not form micelles nor readily incorporate into micelles or bilayers, its use is mostly limited to a study of the enzyme in the absence of an interface. We have developed thiophosphate analogs, with sulfur in the bridging position between the glycerol carbon and phosphorus. This facilitates a continuous assay when coupled to the colorimetric detection of free thiol. The first of these analogs, a simple *n*-alkyl inositol thiophosphate, was used in monomolecular and micellar systems [7,16]. However, the activity with PI-PLC from *B. cereus* was only about 10% that with the natural substrate, and bilayer structures could not be formed. We then reported the synthesis of an optically active thiophosphate analog of dimyristoyl phosphatidylinositol, D-thio-DMPI, that may form bilayer structures [17]. It has an activity with PI-PLC about one-third that with the natural substrate. Recently, Hondal et al. [18] reported the use of a dipalmitoyl thiophosphate analog of PI, D-thio-DPPI, for the assay of bacterial PI-PLC. It did not readily disperse in water and required detergents for its use. In mixed micelles with diheptanoyl PC, it gave only about half of the activity of sonicated dispersions of D-thio-DMPI with PI-PLC. The activity of *B. cereus* PI-PLC with D-thio-DMPI, the non-substrate stereoisomer L-thio-DMPI, and dimyristoyl phosphatidylmethanol was recently reported by Hendrickson et al. [13].




Scheme 1. Reaction scheme for the assay of PI-PLC using D-thio-DMPI as a substrate.

Mammalian PI-PLCs preferentially catalyze the hydrolysis of $PIP_2$, although many also catalyze the hydrolysis of PIP and PI at much slower rates. They also require $Ca^{2+}$ for activity, unlike the bacterial enzymes which require no $Ca^{2+}$. The first chromogenic substrate for the continuous assay of mammalian PI-PLC was a $PIP_2$ analog, D,L-myo-inositol-4,5-bisphosphate-1-(4-nitrophenyl phosphate) [19]. This is a water-soluble substrate, which does not incorporate into lipid interfaces. Wu et al. [20] recently reported the activity of PI-PLC$\delta$1 with micellar substrates, determined by $^{31}$P NMR analysis. Here we report the use of our thiophosphate analog D-thio-DMPI to assay (Scheme 1) a deletion variant of PI-PLC$\delta$1, $\Delta$(1–132), in which the N-terminal PH domain is absent.

## 2. Materials and methods

### 2.1. Chemicals

HDPC was synthesized as described by van Dam-Mieras et al. [21] (it is also available from Sigma, St.

Louis, MO). D- and L-Thio-DMPI were synthesized as described by Hendrickson et al. [17]. MES, *S*-carboxymethyl bovine serum albumin, and Triton X-100 were obtained from Sigma, and DTP (Aldrithiol-4) was obtained from Aldrich (Milwaukee, WI). The recombinant $\Delta(1-132)$ deletion variant of rat PI-PLC$\delta$1 was a generous gift from Professor Roger Williams, MRC, Cambridge, UK. Diluted solutions of the enzyme ($< 0.2$ mg ml$^{-1}$) were stabilized in 0.1% *S*-carboxymethyl bovine serum albumin.

### 2.2. Lipid dispersions

Stock solutions of phospholipids (about 10 mM) in chloroform–methanol were stored at $-20$°C. D-Thio-DMPI is quite stable for over a year under these conditions. The concentration of phospholipid was determined by phosphorus analysis [22]. Aliquots of lipid were dried under a stream of nitrogen and then under high vacuum. Buffer (50 mM MES, pH 7, 0.1 M NaCl) and $CaCl_2$ were added, and the solution was vortexed and sonicated to give a clear dispersion. In the case of lipid in the absence of detergent, buffer was added first, the lipid was dispersed by vortexing and sonication, then $Ca^{2+}$ was added and the dispersion was again sonicated.

### 2.3. Enzyme assay

The standard assay solution contained 0.2 mM D-thio-DMPI, 0.4 mM HDPC, 0.2 mM $CaCl_2$, 0.1 M NaCl, and 50 mM MES, pH 7. An aliquot (225 $\mu$l) of this solution and 5 $\mu$l of 50 mM (in ethanol) DTP were placed in a 2 mm $\times$ 10 mm microcuvette. The cuvette was placed in a Perkin-Elmer Lambda 3b spectrometer, thermostated at 30°C. The wavelength was set to 324 nm, absorbance to zero, and the temperature allowed to equilibrate for 4 min. The background absorbance was recorded for 30–40 s, after which 5 $\mu$l of enzyme (0.5–1 $\mu$g) was added and quickly mixed with a Drummond Model 210 positive displacement pipette. The absorbance was then recorded for several minutes. Data were collected through a computer interface and analyzed by Perkin-Elmer's PECSS software. The maximum initial rate ($\Delta A$ per second, corrected for background) was multiplied by the factor 712 (60 s/min $\times$ the total volume, 0.235 ml, divided by the extinction coefficient for thiopyridine, 0.0198 ml nmol$^{-1}$ [23]) to give the activity in units of nanomoles per minute.

### 2.4. Curve fitting

Plots of enzyme activity vs. lipid concentration were fitted to the Michaelis–Menten equation (or Hill modification, $v = V_{max}[S]^h/(K_m^h + [S]^h)$) by nonlinear least-squares regression using the computer program Sigma Plot (Jandel, San Rafael, CA).

## 3. Results

### 3.1. Optimization of assay conditions

Clear sonicated dispersions of D-thio-DMPI became cloudy upon the addition of 2 mM $Ca^{2+}$, which rendered the dispersions unsuitable for spectroscopic measurements. Addition of HDPC to form mixed-lipid micelles completely removed any cloudiness. Opti-

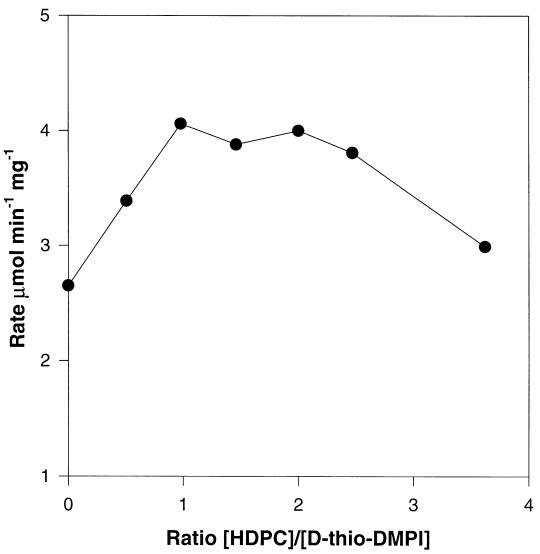



Fig. 1. Activity of $\Delta(1-132)$-PI-PLC as a function of the ratio of HDPC to D-thio-DMPI. Constant total lipid concentration, 0.2 mM; 0.2 mM $CaCl_2$; 0.1 M NaCl; 50 mM MES, pH 7.0; $T = 30$°C; 0.8 $\mu$g of enzyme added.

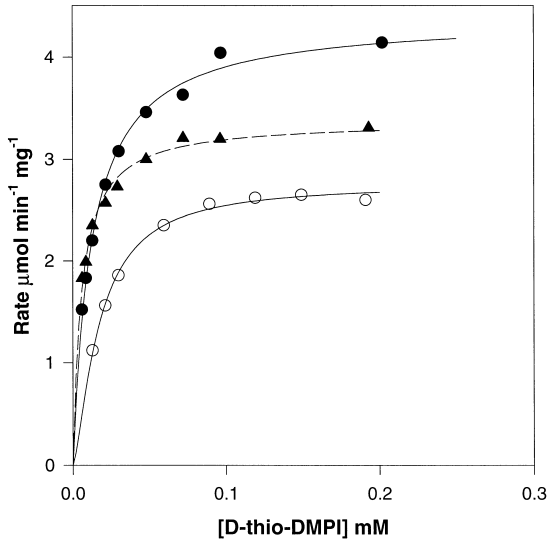



Fig. 2. Activity of $\Delta(1-132)$-PI-PLC as a function of substrate concentration. Closed circles: at a constant ratio of [HDPC]/[D-thio-DMPI] = 2.0; 2 mM $CaCl_2$; 0.1 M NaCl; 50 mM MES, pH 7.0; $T = 30°C$; 0.8 $\mu$g of enzyme added. Closed triangles: at a constant ratio of [HDPC]/[D-thio-DMPI] = 2.0; 0.2 mM $CaCl_2$; 0.1 M NaCl; 50 mM MES, pH 7.0; $T = 30°C$; 0.8 $\mu$g of enzyme added. Open circles: in the absence of HDPC; 0.2 mM $CaCl_2$; 0.1 M NaCl; 50 mM MES, pH 7.0; $T = 30°C$; 0.8 $\mu$g of enzyme added.

mal enzyme activity was seen at ratios of HDPC/D-thio-DMPI between 1 and 2 (Fig. 1). Enzyme activity as a function of lipid concentration in mixed-lipid micelles (Fig. 2) gave $V_{max} = 4.39 \pm 0.07$ $\mu$mol $min^{-1}$ $mg^{-1}$ and an apparent $K_m = 12.4 \pm 0.7$ $\mu$M D-thio-DMPI. Decreasing the $Ca^{2+}$ concentration from 2 to 0.2 mM resulted in only a small reduction in activity (Table 1). With Triton X-100, at a mole ratio of detergent to D-thio-DMPI of 2, rates were very similar to those with HDPC (Table 1). At 0.2 mM $Ca^{2+}$, 0.2 mM thio-DMPI dispersions usually remained clear without added detergent; however, results with these dispersions were not as consistent as those with detergent present. Enzyme activity as a function of substrate concentration in the absence of HDPC also gave a saturation curve (Fig. 2) (best fit with slight cooperativity and a Hill coefficient $h = 1.4$) with $V_{max} = 2.76 \pm 0.05$ $\mu$mol $min^{-1}$ $mg^{-1}$ and an apparent $K_m = 17.3 \pm 0.7$ $\mu$M D-thio-DMPI (Table 1). DTP did not appear to affect the enzyme since addition of DPI about 50 s after the enzyme resulted in a similar rate.

### 3.2. Dependence of activity on surface concentration of substrate

In order to assess the dependence of activity on surface concentration of substrate, the mole fraction of D-thio-DMPI was varied by changing the ratio of D-thio-DMPI/L-thio-DMPI at a constant ratio of HDPC to total (D- and L-) thio-DMPI and concentration of HDPC. Analogs containing L-*myo*-inositol are neither substrates nor competitive inhibitors of PI-PLC [8,24,25]. Activity increased exponentially with increasing mole fraction of D-thio-DMPI in the presence of HDPC (Fig. 3), and showed no evidence of saturation up to the maximum 0.35 mole fraction. Racemic thio-DMPI (at 0.175 mole fraction D-thio-DMPI in the presence of HDPC) would give an activity 42% (from Fig. 3) that with optically active substrate. In the absence of HDPC, an exponential increase in activity was also seen (Fig. 3), again with no evidence of saturation up to 1.0 mole fraction.

Table 1
Activities of $\Delta(1-132)$-PI-PLC$\delta_1$ with D-thio-DMPI

| Detergent mole ratio | $[Ca^{2+}]$ (mM) | $V_{max}$ ($\mu$mol $min^{-1}$ $mg^{-1}$) | $K_m$ ($\mu$M) |
|---|---|---|---|
| 2 HDPC | 2.0 | $4.39 \pm 0.07$ | $12.4 \pm 0.7$ |
| 2 HDPC | 0.2 | $3.38 \pm 0.05$ | $5.8 \pm 0.4$ |
| 2 Triton X-100 | 0.2 | $3.75 \pm 0.12$ | $7.5 \pm 1.1$ |
| 0 | 0.2 | $2.76 \pm 0.05$[a] | $17.3 \pm 0.7$[a] |

[a] Hill coefficient equals 1.4.
The errors are shown as S.D., defined as the square root of the mean of the square of the differences from their mean of the data samples for each kinetic curve.

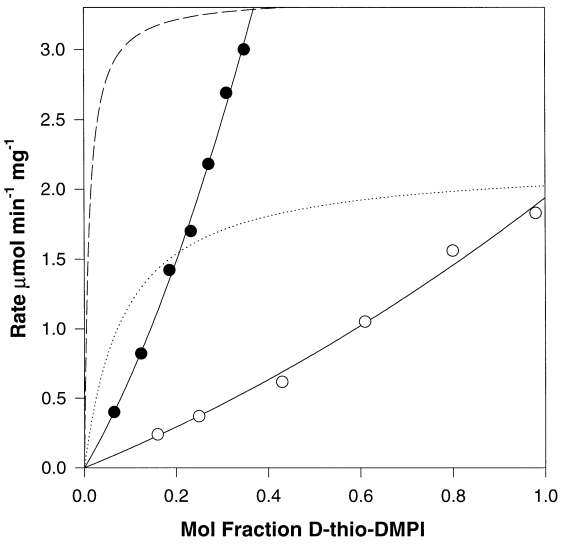



Fig. 3. Activity of $\Delta$(1–132)-PI-PLC as a function of mole fraction of D-thio-DMPI. Closed circles: at a constant [HDPC] = 0.35 mM and total [D- and L-thio-DMPI] = 0.2 mM; 0.2 mM $CaCl_2$; 0.1 M NaCl; 50 mM MES, pH 7.0; $T = 30$°C; 0.8 $\mu$g of enzyme added. Open circles: in the absence of HDPC; constant total [D- and L-thio-DMPI] = 0.2 mM; 0.2 mM $CaCl_2$; 0.1 M NaCl; 50 mM MES, pH 7.0; $T = 30$°C; 0.8 $\mu$g of enzyme added. Dashed line: theoretical curve for no surface dilution effect and enzyme binding only to D-thio-DMPI in the presence of HDPC. Dotted line: theoretical curve for no surface dilution effect and enzyme binding only to D-thio-DMPI in the absence of HDPC.

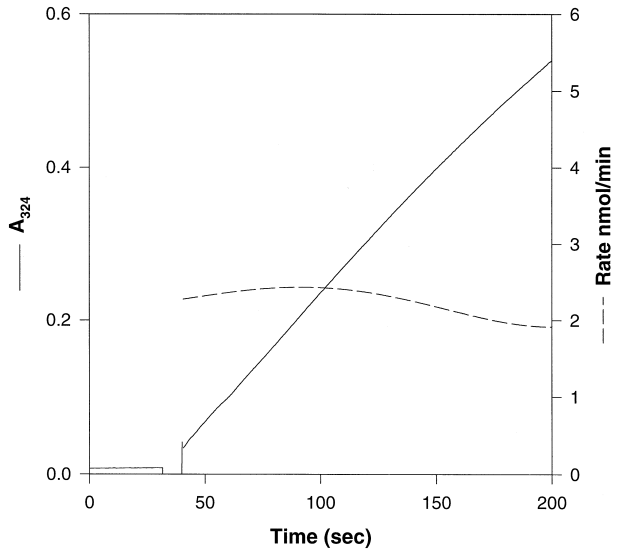



Fig. 4. Time course for standard assay of $\Delta$(1–132)-PI-PLC. Standard conditions: 0.2 mM D-thio-DMPI; 0.4 mM HDPC; 0.2 mM $CaCl_2$; 0.1 M NaCl; 50 mM MES, pH 7.0; $T = 30$°C; 0.8 $\mu$g of enzyme was added and mixed between 32 and 40 s. Data ($A_{324}$ vs. time) were collected at a rate of 0.5 s and fitted to a fifth-order polynomial; the rate (dashed line) was then calculated from the first derivative and plotted using Sigma Plot.

### 3.3. Standard assay

In view of these results, the standard assay conditions were selected as 0.2 mM D-thio-DMPI, 0.4 mM HDPC, and 0.2 mM $Ca^{2+}$ in order to minimize the amount of lipid and $Ca^{2+}$ used. Under these conditions, the time course for the assay showed an initial linear rate with no lag (Fig. 4); also, no lag was observed in the absence of detergent. There was a linear relation between activity and amount of enzyme (Fig. 5), and the specific activity of the enzyme was 3.3 $\mu$mol $min^{-1}$ $mg^{-1}$. The minimum detectable rate (about ten times the background noise level) was 0.2 nmol $min^{-1}$, which corresponds to about 60 ng of pure enzyme. To assure that replenishment of substrate by micelle–micelle exchange of lipid was not rate limiting, the standard assay was repeated in the presence of 17 $\mu$g $ml^{-1}$ of polymyxin B. Polymyxin B has been shown to mediate the exchange of anionic phospholipids between lipid interfaces [26]. There

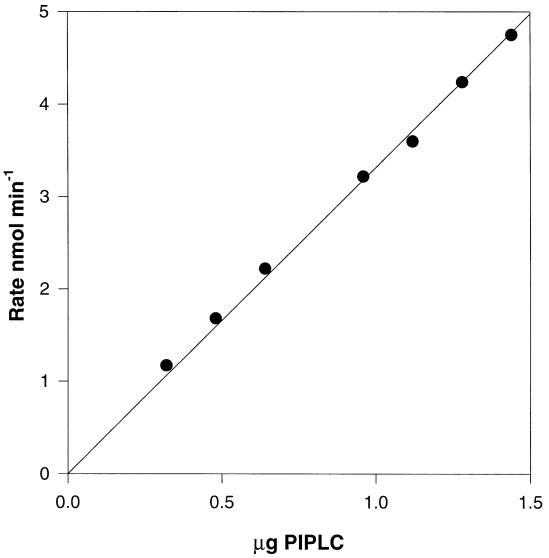



Fig. 5. Activity of $\Delta$(1–132)-PI-PLC as a function of amount of enzyme added. Standard assay conditions (see Fig. 4). Specific activity (slope) equals 3.32 $\mu$mol $min^{-1}$ $mg^{-1}$.

was no significant difference in the kinetics of the standard assay with and without polymyxin B.

## 4. Discussion

D-Thio-DMPI is a suitable substrate for the continuous assay of mammalian PI-PLC. Its activity with $\Delta(1-132)$-PI-PLC$\delta_1$ is about one-fourth that with PI under similar conditions. Wu et al. [20] measured a specific activity of 16 $\mu$mol min$^{-1}$ mg$^{-1}$ for $\Delta(1-132)$-PI-PLC$\delta_1$ with PI/Triton X-100 mixed micelles; the full-length enzyme gave an activity of 36 $\mu$mol min$^{-1}$ mg$^{-1}$. The PH domain, absent in the $\Delta(1-132)$ variant, has a high affinity for $PIP_2$ and appears to be involved in membrane tethering in addition to possible enzyme regulation [6].

D-Thio-DMPI will tolerate at least 0.2 mM $Ca^{2+}$ without becoming cloudy; above that concentration a detergent (HDPC) is necessary to prevent precipitation of substrate. Optimal conditions for the assay include 0.2 mM substrate, 0.2 mM $Ca^{2+}$, and a mole ratio of HDPC to substrate of 2.0. Similar rates were seen using Triton X-100 as a detergent. While the catalytic subunit contains 11 cysteine residues, these appear to be mostly inaccessible to the surface; the thiol reagent DTP, necessary for this coupled assay, does not appear to affect catalytic activity.

The apparent bulk $K_m$ for PI-PLC with D-thio-DMPI indicates fairly strong binding of enzyme to the lipid interface. The fact that it is not significantly affected by the presence of zwitterionic detergent, suggests that this binding is influenced mainly by the negatively charged inositol phosphate head groups. Enzyme activity also shows a significant surface dilution effect. In the absence of any surface dilution effect, assuming the enzyme only binds to D-thio-DMPI, the activity would follow hyperbolic saturation curves (dashed and dotted lines) as shown in Fig. 3. Enzyme activity as a function of surface concentration of substrate was considerably less than that expected for no surface dilution effects, and showed no sign of saturation up to the maximum mole fraction. This seems to indicate that D-thio-DMPI has a much lower affinity for interfacial substrate binding at the active site of the mammalian enzyme. This is not surprising since the more-phosphorylated $PIP_2$ is the preferred substrate for the mammalian enzyme. With *B. cereus* PI-PLC, where PI rather than $PIP_2$ is the preferred substrate, an apparent interfacial Michaelis constant of 0.22 mole fraction for D-thio-DMPI was observed [13].

The use of D-thio-DMPI in the assay of mammalian PI-PLC has the advantage providing a lipid interface, in contrast to the water-soluble substrate used in a continuous chromogenic assay by Rukavishikov et al. [19]. It should provide useful kinetic data on the activity of these enzymes at lipid interfaces. Since the catalytic domains of all mammalian PI-PLCs are quite similar, as is also that of the bacterial PI-PLC, this substrate should also provide a useful assay for the other isozymes.

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