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Binding of phosphatidylinositol-specific phospholipase C to phospholipid interfaces, determined by fluorescence resonance energy transfer

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Abstract

Dissociation constants for binding of phosphatidylinositol-specific phospholipase C from *Bacillus cereus* (*bc*PI-PLC) and the mammalian phosphatidylinositol-specific phospholipase C-δ_1 to lipid interfaces containing phosphoinositol, phosphocholine, and phosphomethanol head groups were determined by fluorescence resonance energy transfer. Dansyl-labeled lipid probes were used as acceptors, with intrinsic tryptophan of the enzyme as the donor. Titration of protein into lipid provided data from which K_d and N, the limiting number of lipid molecules per protein bound, were calculated by non-linear regression analysis of exact binding equations. These results were compared with apparent K_m values from kinetic data. K_d values in the low μM range in terms of lipid monomers or low nM range in terms of binding sites were calculated with good fits of experimental data to theoretical binding curves. *bc*PI-PLC binds with high affinity to PI interfaces, slightly lower affinity to PC interfaces, and much lower affinity to PM interfaces. The mammalian enzyme also binds with high affinity to PI interfaces, but shows little or no binding with PC interfaces under similar concentration conditions. These K_d values correlate reasonably with apparent K_m values from kinetic experiments. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Fluorescence resonance energy transfer; Phosphoinositide-specific phospholipase C; Dissociation constant; *Bacillus cereus*; Mammalian; Kinetics; Lipid interface

1. Introduction

Phosphatidylinositol-specific phospholipase C from *Bacillus cereus* (*bc*PI-PLC) and the mammalian PI-PLC-δ_1 catalyze the hydrolysis of phosphatidylinositol to diacylglycerol and *myo*-inositol 1,2-(cyclic)phosphate, which is subsequently hydrolyzed to *myo*-inositol 1-phosphate [1]. *bc*PI-PLC, an extracel-

Abbreviations: CMC, critical micelle concentration; DMPC, 1,2-dimyristoyl phosphatidylcholine; DMPM, 1,2-dimyristoyl phosphatidylmethanol; DnDPC, 1-(11-dansylundecyl)-2-decanoyl-*sn*-glycero-3-phosphocholine; L-DnDPI, 1-(11-dansylundecyl)-2-decanoyl-*sn*-glycero-3-phospho(1L-1-*myo*-inositol); FRET, fluorescence resonance energy transfer; HDPC, hexadecylphosphocholine; L-HDPI, hexadecylphospho(1L-1-*myo*-inositol); HDPM, hexadecylphosphomethanol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; *bc*PI-PLC, phosphatidylinositol-specific phospholipase C from *Bacillus cereus*; PI-PLC-δ_1, full-length recombinant mammalian phosphatidylinositol-specific phospholipase C-δ_1; Δ-(1–132)PI-PLC-δ_1, deletion mutant of phosphatidylinositol-specific phospholipase C-δ_1; D-thio-DMPI, 1,2-dimyristoyloxypropane-3-thiophospho(1D-1-*myo*-inositol); L-thio-DMPI, 1,2-dimyristoyloxypropane-3-thiophospho(1L-1-*myo*-inositol)

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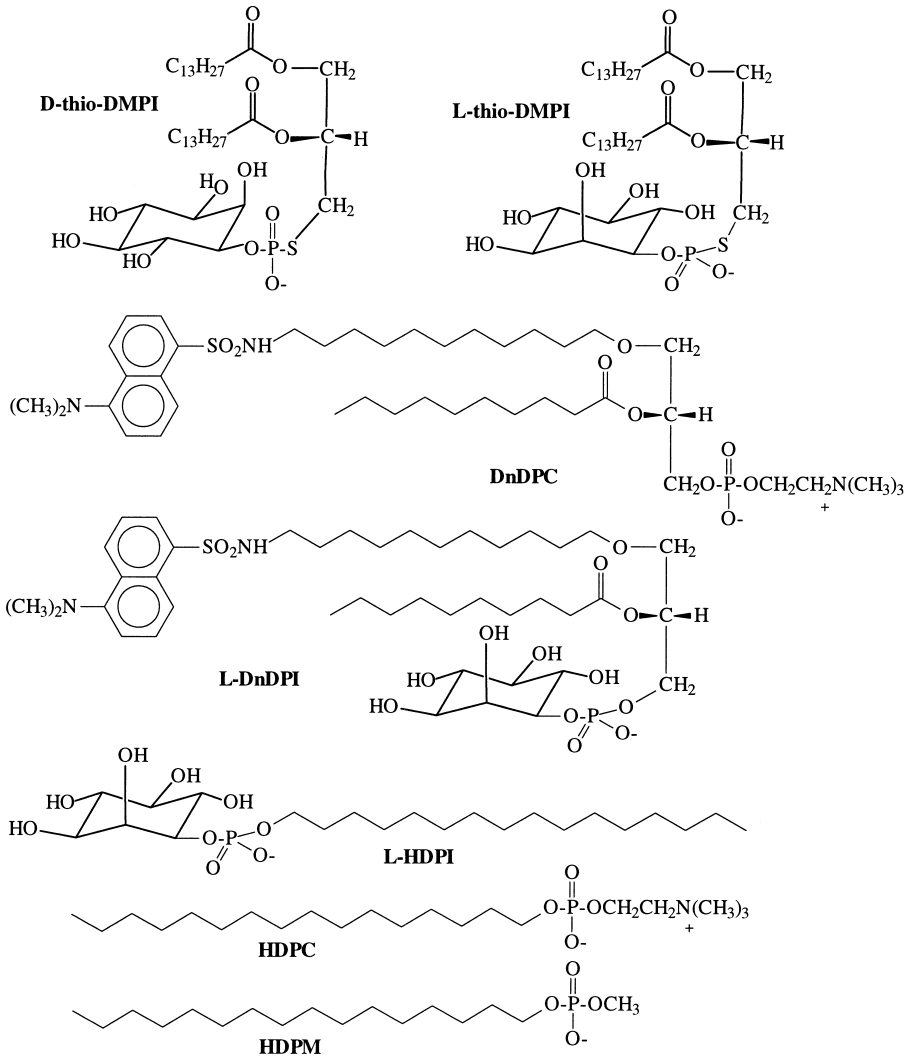

Scheme 1. Structures of phospholipid analogs.

lular enzyme, is specific for non-phosphorylated PI and also catalyzes the release of a number of enzymes and other proteins linked to glycosylphosphatidylinositol membrane anchors [2,3]. PI-PLC-δ_1, a cytosolic enzyme, shows preference for the hydrolysis of PIP and PIP_2, and plays a key role in signal transduction through the hydrolysis of PIP_2 to two second messengers, diacylglycerol and inositol 4,5-bisphosphate [4,5]. The X-ray crystal structures of *bc*PI-PLC [6] and PI-PLC-δ_1 [7] have been reported. PI-PLC-δ_1 contains a N-terminal pleckstrin homology domain connected to the catalytic domain by an EF-hand domain, and a C-terminal calcium-binding C2 domain [8]. Both bacterial and mammalian enzymes share a structurally similar catalytic domain in the form of a distorted $(\beta\alpha)_8$ barrel [9]. The enzymes specifically bind substrates that contain 1D-*myo*-inositol; isomers with 1L-*myo*-inositol are neither substrates nor inhibitors [10–12].

PI-PLCs, like other water-soluble phospholipases, are subject to 'interfacial activation', the preference for aggregated over monomeric substrates. This was shown for bacterial enzymes by Hendrickson et al. [13] and Lewis et al. [11], and for PI-PLC-δ_1 by Rebecchi et al. [14]. The simplest mechanism involves reversible enzyme binding to the interface followed by an interfacial Michaelis-Menten mechanism [15]. The kinetics of bacterial PI-PLC appear sigmoidal with respect to bulk lipid concentration. Allosteric activation of bacterial PI-PLC, both towards PI

cleavage and hydrolysis of *myo*-inositol 1,2-(cyclic)-phosphate, was proposed to be due to specific binding of a single PC molecule to the enzyme, which then anchors the protein to a lipid interface [16,17].

Quantitative data on the interactions of soluble PI-PLC with lipid interfaces are necessary to understand the first step in the mechanism of these enzymes and the process of interfacial activation. Some data are available on binding of *bc*PI-PLC to lipid interfaces from changes in intrinsic tryptophan fluorescence [16,17,20]. These experiments involve adding lipid to enzyme and are difficult to analyze since the curves are often biphasic and may involve multiple changes in tryptophan environments during and after the process of binding. Other techniques involve enzyme binding to lipid-coated beads and gel filtration [17]. These experiments are difficult and often limited to particular lipids and the strength of binding. Binding of PI-PLC-δ_1 to phospholipid interfaces was studied by sedimentation of sucrose-loaded vesicles [18]. Those results were not quantitative, and were difficult to interpret since in many cases only a small fraction of enzyme bound to the vesicles even at high lipid concentrations.

Here we present a method based on fluorescence resonance energy transfer (FRET) to determine both dissociation constants and the binding stoichiometry. The phospholipid analogs used in these studies are shown in Scheme 1. The dansyl lipids, DnDPC and L-DnDPI, are used as acceptors; intrinsic tryptophan in the protein is the donor. We use phosphoinositide analogs with 1L-*myo*-inositol (L-thio-DMPI and L-HDPI) to avoid lipid binding at the substrate active site and hydrolysis of lipid. Good fits are obtained between experimental data and theoretical binding curves, and dissociation constants are determined in the nM range in terms of lipid interface binding sites and low μM range in terms of lipid monomers. These results are reasonable when compared to apparent Michaelis constants obtained from kinetic studies.

2. Materials and methods

2.1. Enzymes

Recombinant *bc*PI-PLC was expressed in *Escherichia coli* and purified by the procedure of Koke et al. [19]. Recombinant mammalian PI-PLC-δ_1 and Δ-(1–132)PLC-δ_1 (a mutant with the N-terminal pleckstrin homology domain deleted) were generous gifts from Dr. Roger Williams (MRC, Cambridge, UK). The mammalian enzymes were dialyzed into 50 mM MES, pH 7, 100 mM NaCl to remove added dithiothreitol which interfered with fluorescence measurements. Enzyme concentrations were determined by absorption at 280 nm using extinction coefficients of 1.84 mg ml^{-1} cm^{-1} [20], 1.02 mg ml^{-1} cm^{-1} [21], and 1.14 mg ml^{-1} cm^{-1} [22] for *bc*PI-PLC, Δ-(1–132)PI-PLC-δ_1, and PI-PLC-δ_1 respectively.

2.2. Lipids

HDPC was synthesized as described by van Dam-Mieras et al. [23] (also available from Sigma, St. Louis, MO). D- and L-thio-DMPI were synthesized as described by Hendrickson et al. [24]. DnDPC was synthesized as described by Schindler et al. [25] (also available from Molecular Probes, Eugene, OR). L-DnDPI was synthesized from 1-(11-dansyl-undecyl)-2-decanoyl-*sn*-glycerol [25] and 2,3:5,6-*O*-diisopropylidene-4-*O*-(*tert*-butyldimethylsilyl)(1L-1-*myo*-inositol) [26] by the phosphoramidite coupling method described by Hendrickson et al. [27]. L-HDPI was synthesized in a similar manner from hexadecanol. All L-*myo*-inositol-containing phospholipids were treated with *bc*PI-PLC to remove any residual D-*myo*-inositol-containing stereoisomers and then repurified. HDPM was synthesized from hexadecanol and dimethyl chlorophosphite followed by iodine oxidation and mono-demethylation with LiBr in acetone.

2.3. Preparation of lipid dispersions

Aliquots of lipids (in chloroform-methanol solution) in a small glass tube were evaporated to dryness under a stream of nitrogen and then under high vacuum. Buffer (50 mM MES, pH 7 for experiments with *bc*PI-PLC; 50 mM MES, pH 7, 100 mM NaCl for experiments with PI-PLC-δ_1) was added and the lipid was dispersed by vortexing and sonication in a bath-type sonicator; optically clear dispersions were easily obtained.

2.4. Fluorescence spectroscopy

FRET was measured in a Perkin-Elmer 650-10 or Perkin-Elmer MPF-44B spectrofluorometer at 23°C with stirring. Emission was measured at 520 nm with excitation at 285 or 290 nm. Excitation and emission bandwidths were set at 3 or 5 nm and a 310 nm cutoff filter was placed in the emission beam to reduce effects of light scattering. Enzyme was titrated into lipid dispersions containing 3 mol% dansyl-labeled lipid probe.

2.5. Critical micelle concentration (CMC) determination

The CMCs of lipid dispersions containing 3 mol% dansyl-labeled lipid probe were determined by fluorescence spectroscopy. Lipid was titrated into buffer and emission was measured at 520 nm (excitation at 345 nm). The emission increased sharply and became linear with respect to lipid concentration above the CMC.

2.6. Enzyme kinetics

Activity of *bc*PI-PLC was determined by a spectrophotometric method using D-thio-DMPI as substrate [15]. Data were fitted to the Michaelis-Menten or Hill equation using the non-linear regression program SigmaPlot (SPSS, Chicago, IL).

3. Results

3.1. Calculations

Binding of PI-PLC to the lipid interface can be represented by the following equilibrium and dissociation constant: E+L⇋EL, where E represents free enzyme and L represents the lipid interface binding site (containing N lipid molecules);

$$K_d = \frac{[E][L]}{[EL]} \quad (1)$$

and

$$[L] = [TL]/N \quad (2)$$

where TL is total lipid and N is the limiting number of lipid molecules per enzyme molecule bound. The experimental data, change in emission intensity and volume of enzyme solution added (ΔI and V_c), were analyzed according to the method of Wang et al. [28] as follows:

$$E_t = [E] + [EL] \text{ and } L_t = [L] + [EL] \quad (3)$$

where E_t and L_t represent the total concentrations of enzyme and lipid interface binding sites respectively. The dissociation constant

$$K_d = \frac{(E_t - [EL])(L_t - [EL])}{[EL]} \quad (4)$$

Eq. 4 can be rearranged using the mathematical solution to a quadratic equation to give

$$[EL] = \frac{E_t + L_t + K_d - \sqrt{(E_t + L_t + K_d)^2 - 4E_tL_t}}{2} \quad (5)$$

Using the maximum molar change in emission intensity, $\Delta I_{max} = \Delta I/[EL]$, Eq. 5 can be rearranged to

$$\Delta I = \Delta I_{max} \frac{(E_t + L_t + K_d - \sqrt{(E_t + L_t + K_d)^2 - 4E_tL_t})}{2} \quad (6)$$

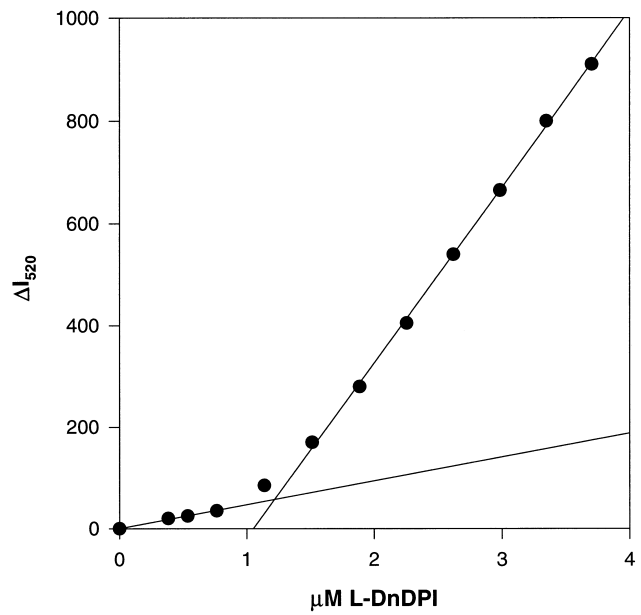

Fig. 1. Determination of CMC for L-DnDPI. Titration of L-DnDPI into 20 mM MES, pH 7. Excitation at 345 nm, emission at 520 nm.

If the initial volume and concentration of lipid interface binding sites are V_0 and $[L]_0 = [TL]_0/N$ respectively, and the concentration of stock enzyme solution is $[E]_0$, then the total concentration of interface binding sites and enzyme can be obtained by accounting for the total volume of enzyme, V_c, added during the titration:

$$E_t = \frac{[E]_0\ V_c}{V_0 + V_c} \tag{7}$$

and

$$L_t = \frac{([TL]_0/N)\ V_0}{V_0 + V_c} \tag{8}$$

The experimental data, ΔI and V_c, along with values for $[E]_0$, $[TL]_0$, and V_0, can be fitted to Eqs. 6–8 by non-linear regression analysis (SigmaPlot) using estimates of ΔI_{max}, K_d, and N. Best results are obtained when $[TL]_0$ is several times the value of $N \times K_d$. In the initial phase of the titration of enzyme into lipid, where $[TL] \gg [E]$, all or most of the enzyme is bound to the interface. N can be estimated from extrapolation of the initial linear slope of the plot of ΔI vs. V_c to the estimated maximum value of ΔI. The total lipid concentration at this intersect divided by the concentration of enzyme equals N.

3.2. CMC determinations

The CMCs for fluorescent lipids and lipid mixtures containing 3 mol% fluorescent lipid were determined by fluorescence spectroscopy as a break in the titration curve of lipid into buffer. L-DnDPI and DnDPC had CMCs of 1.2 μM and <0.5 μM respectively. The titration curve for L-DnDPI is shown in Fig. 1. The CMCs for the lipid mixtures are shown in Table 1.

3.3. FRET titration of bcPI-PLC into lipid dispersions

Titrations of *bc*PI-PLC into HDPC, HDPM, and L-HDPI micelles, and sonicated L-thio-DMPI dispersions are shown in Figs. 2 and 3. Enzyme tryptophan was excited at 290 nm and emission from the dansyl probe was measured at 520 nm. The calculated values of K_d and N are shown in Table 1. Titration of enzyme into L-HDPI or L-thio-DMPI interfaces

Table 1
Dissociation constants for PI-PLC-lipid complexes from FRET experiments[a]

Lipid[b] enzyme	CMC (μM)[c]	K_d (μM) (binding sites)	$K_d \times N$ (μM) (lipid monomers)	N lipid molecules per protein	R^2_{adj} [f]
41.1 μM L-HDPI/L-DnDPI *bc*PI-PLC	<0.5	0.018 ± 0.003	3 ± 1	173 ± 3	0.9995
41.6 μM L-thio-DMPI/L-DnDPI *bc*PI-PLC	<0.5	0.013 ± 0.006	4 ± 2	270 ± 20	0.9967
40.3 μM HDPC/DnDPC *bc*PI-PLC	6.3	0.21 ± 0.04[d]	7 ± 2[d]	36 ± 1[d]	0.9991
42.1 μM HDPM/L-DnDPI *bc*PI-PLC	8.4	0.7 ± 0.3[e]	50 ± 30[e]	80 ± 40[e]	0.9976
21.1 μM L-thio-DMPI/L-DnDPI Δ-(1–132)PI-PLC-δ_1	<0.5	0.022 ± 0.006	11 ± 3	500 ± 100	0.9944
21.1 μM L-thio-DMPI/L-DnDPI Δ-(1–132)PI-PLC-δ_1, 0.2 mM Ca^{2+}	<0.5	0.0074 ± 0.0003	8.9 ± 0.4	1200 ± 50	0.9998
21.1 μM L-thio-DMPI/L-DnDPI PI-PLC-δ_1	<0.5	0.037 ± 0.005	18 ± 3	500 (fixed)	0.994

[a]Enzyme titrated into lipid in buffer at 23°C. Data were fitted to Eqs. 6–8 by non-linear regression analysis (SigmaPlot); 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 curve.
[b]Lipid contains 3 mol% dansyl probe.
[c]Estimated errors for CMC values, ±0.3 μM.
[d]Uncorrected for CMC = 6.3 μM monomeric lipid. With 34 μM interfacial lipid: K_d (binding sites) was unchanged, $N = 30$, $K_d \times N = 6.3$ μM.
[e]Uncorrected for CMC = 8.4 μM monomeric lipid. With 33.7 μM interfacial lipid: K_d (binding sites) was unchanged, $N = 64$, $K_d \times N = 41.6$ μM.
[f]The adjusted coefficient of determination is a measure of how well the regression model describes the data (1.0 indicates a perfect fit), which also takes into account the number of independent variables (calculated in SigmaPlot regression report).

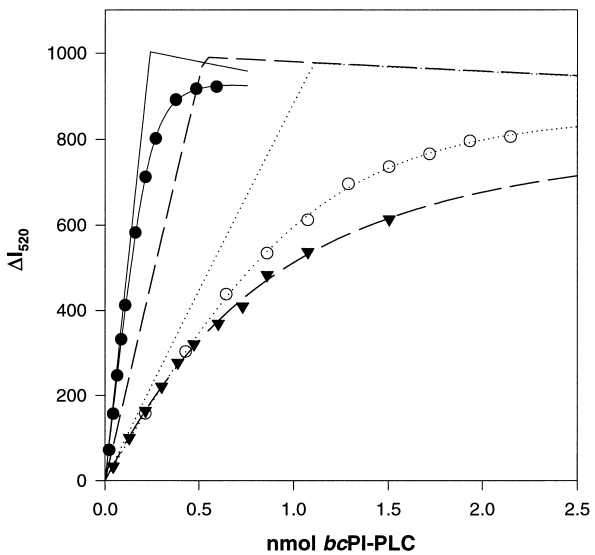

Fig. 2. Titration of *bc*PI-PLC into L-HDPI/L-DnDPI, HDPC/DnDPC, or HDPM/L-DnDPI. Enzyme (10.8 or 43.2 μM) titrated into 1 ml of lipid (about 40 μM with 3 mol% dansyl probe, see Table 1); excitation at 290 nm, emission at 520 nm; data fitted to Eqs. 6–8 (curved lines) with parameters shown in Table 1; straight lines are theoretical curves for stoichiometric binding ($K_d = 0$). ●, L-HDPI/L-DnDPI; ○, HDPC/DnDPC; ▼, HDPM/L-DnDPI. Ordinate, arbitrary units.

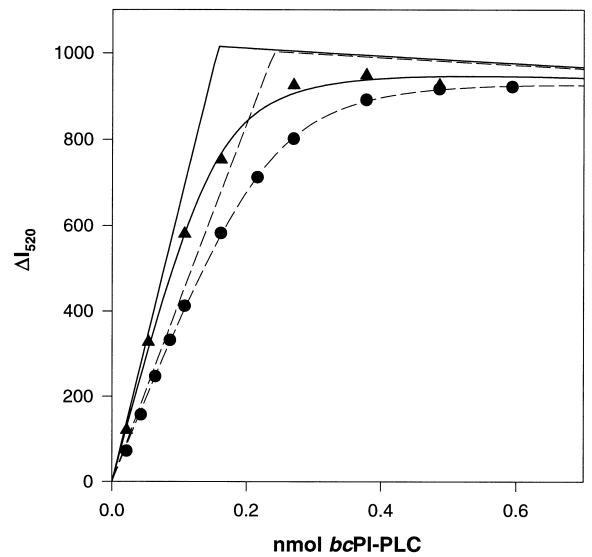

Fig. 3. Titration of *bc*PI-PLC into L-HDPI/L-DnDPI, or L-thio-DMPI/L-DnDPI. Enzyme (10.8 μM) titrated into 1 ml of lipid (about 40 μM with 3 mol% dansyl probe, see Table 1); excitation at 290 nm, emission at 520 nm; data fitted to Eqs. 6–8 (curved lines) with parameters shown in Table 1; straight lines are theoretical curves for stoichiometric binding ($K_d = 0$). ●, L-HDPI/L-DnDPI; ▲, L-thio-DMPI/L-DnDPI. Ordinate, arbitrary units.

showed the highest affinity in terms of binding sites (K_d values equal 0.018 μM and 0.013 μM respectively), followed by HDPC ($K_d = 0.21$ μM) and HDPM ($K_d = 0.7$ μM). There was a range of N values (lipid molecules/protein) from 270 for L-thio-DMPI to 36 for HDPC. Titration of enzyme into a low concentration of L-thio-DMPI (Fig. 4) gave a biphasic binding curve. Up to about 0.2 nmol of added enzyme, the data fitted a binding curve with a single K_d value (0.013 μM binding sites) similar to that seen in Fig. 3, with a high value of N. Above 0.2 nmol of enzyme, the emission increased indicating additional binding of enzyme to the interface at a much lower affinity, which showed no indication of saturation.

3.4. FRET titration of PI-PLC-δ_1 and Δ-(1–132)PI-PLC-δ_1 into lipid dispersions

Titrations of PI-PLC-δ_1 and Δ-(1–132)PI-PLC-δ_1 into sonicated L-thio-DMPI dispersions are shown in Fig. 5. The calculated values of K_d and N are shown in Table 1. Binding was similar for both PI-

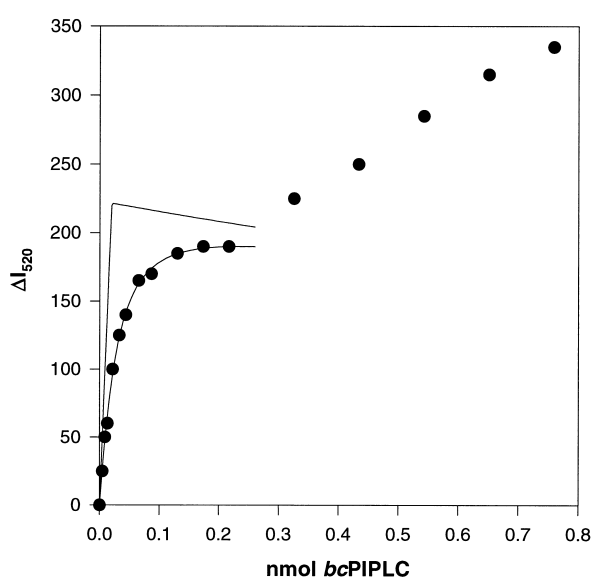

Fig. 4. Titration of *bc*PI-PLC into L-thio-DMPI/L-DnDPI. Enzyme (2.17 μM) titrated into 1.3 ml of lipid (10.4 μM with 3 mol% dansyl probe); excitation at 285 nm, emission at 520 nm; data (up to 0.22 nmol enzyme) fitted (curved line) to Eqs. 6–8 with $K_d = 0.013 \pm 0.002$ μM, $N = 700 \pm 100$; straight line is the theoretical curve for stoichiometric binding ($K_d = 0$). Ordinate, arbitrary units.

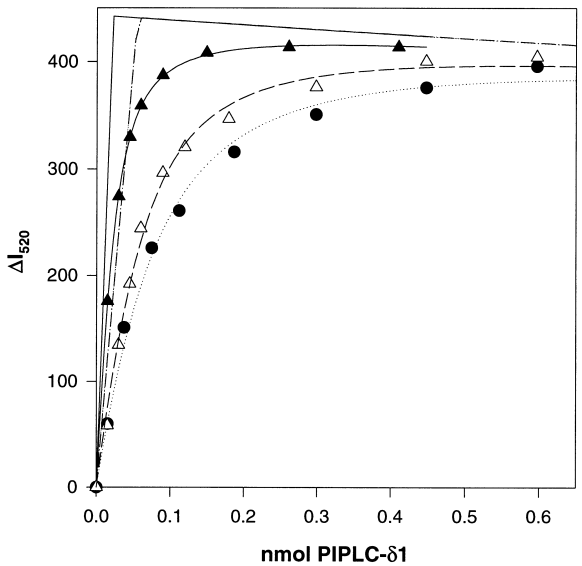

Fig. 5. Titration of PI-PLC-δ_1 and Δ-(1–132)PI-PLC-δ_1 into L-thio-DMPI/L-DnDPI. Enzyme titrated into 1.3 ml of lipid (21.1 μM with 3 mol% dansyl probe); excitation at 285 nm, emission at 520 nm; data fitted to Eqs. 6–8 (curved lines) with parameters shown in Table 1; straight lines are theoretical curves for stoichiometric binding ($K_d = 0$). ●, PI-PLC-δ_1 (7.46 μM); △, Δ-(1–132)PI-PLC-δ_1 (7.6 μM); ▲, Δ-(1–132)PI-PLC-δ_1 (7.6 μM) with Ca^{2+}. Ordinate, arbitrary units.

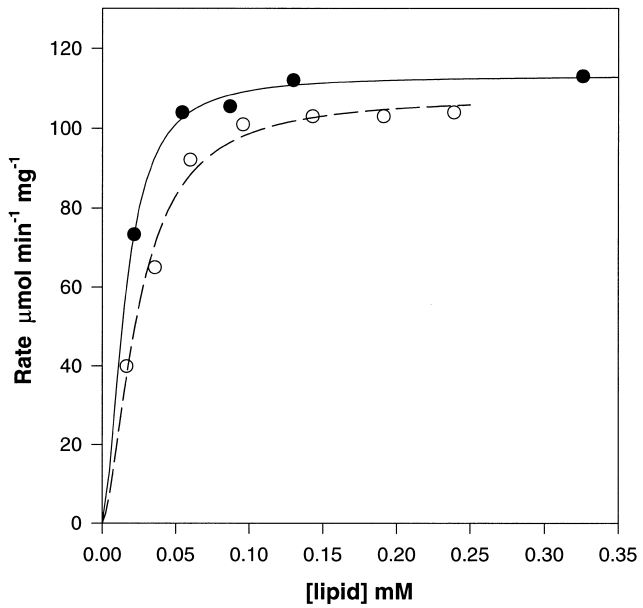

Fig. 6. Activity of *bc*PI-PLC as a function of total lipid concentration. ●, D-thio-DMPI/HDPC (1:4); fit to Hill equation $\{v = V_{max}[TL]^h/(K_m^h + [TL]^h)\}$: $K_m = 15$ μM, $V_{max} = 113$ μmol min^{-1} mg^{-1}, $h = 1.8$; ○, D-thio-DMPI/L-HDPI (1:1); fit to Hill equation: $K_m = 24$ μM, $V_{max} = 108$ μmol min^{-1} mg^{-1}, $h = 1.7$. Enzyme, 36 ng; $T = 30°C$; 50 mM MES, pH 7.

PLC-δ_1 and Δ-(1–132)PI-PLC-δ_1 in the absence of added Ca^{2+}; however, in the presence of 0.2 mM Ca^{2+}, Δ-(1–132)PI-PLC-δ_1 bound with higher affinity. Values for N were high, although there was greater uncertainty, and in the case of PI-PLC-δ_1, N could not be fitted as a parameter, but was arbitrarily fixed at a constant of 500. Varying values of N had little effect on K_d in terms of binding sites, but had a definite effect on K_d in terms of lipid monomers. Binding of Δ-(1–132)PI-PLC-δ_1 to HDPC/DnDPC micelles showed no FRET under similar conditions (concentrations of lipid and enzyme) indicating little or no binding, with a K_d value probably in excess of 1 mM in terms of lipid binding sites.

3.5. Kinetics of bcPI-PLC with D-thio-DMPI

The Michaelis-Menten curves for *bc*PI-PLC with D-thio-DMPI in mixed micelles with HDPC and L-HDPI are shown in Fig. 6. In the presence of HDPC and L-HDPI, apparent K_m values of 12.5 μM and 24 μM respectively were determined (Table 2). With D-thio-DMPI in the presence of both HDPC

Table 2
Kinetic parameters[a] for *bc*PI-PLC and Δ-(1–132)PI-PLC-δ_1

Substrate/detergent (mol ratio)	Enzyme	Apparent K_m (μM)	Apparent V_{max} (μmol min^{-1} mg^{-1})	Hill coefficient	Reference
D-thio-DMPI/HDPC (1:4)	*bc*PI-PLC	15 ± 1	113 ± 2	1.8 ± 0.4	This paper
D-thio-DMPI/L-HDPI (1:1)	*bc*PI-PLC	24 ± 3	108 ± 4	1.7 ± 0.3	This paper
D-thio-DMPI/DMPM (1:1)	*bc*PI-PLC	210 ± 10	91 ± 5		[15]
D-thio-DMPI [Ca^{2+}] = 0.2 mM	Δ-(1–132)PI-PLC-δ_1	17.3 ± 0.7	2.76 ± 0.05		[35]

[a]Data were fitted to the Michaelis-Menten or Hill equation by non-linear regression analysis (SigmaPlot); 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 curve.

and L-HDPI, slight apparent cooperativity was observed and the data best fitted a Hill equation with $h = 1.8$ and 1.7 respectively.

4. Discussion

4.1. Use of FRET in binding studies

*bc*PI-PLC has seven tryptophan residues that contribute to its absorption at about 285 nm and fluorescence emission at 337 nm [6]; the catalytic domain of PI-PLC-δ_1 also contains seven tryptophan residues [7]. FRET involves transfer of excited state energy from a donor such as tryptophan to a suitable acceptor molecule without the appearance of a photon [29]. The rate of energy transfer depends on the extent of overlap between the donor emission and acceptor absorption spectra, the relative orientation of the donor and acceptor transition dipoles, and the distance between the two molecules. The latter dependence, which is proportional to the inverse sixth power of the characteristic Förster distance, has been used to measure distance in biochemical systems in a critical range of 20–50 Å.

The dansyl group of lipid probes provides a useful acceptor since its absorption occurs at about 335 nm with good overlap with the emission of donor tryptophan, and it exhibits fluorescence emission at 520 nm. Not all of the seven tryptophans of *bc*PI-PLC will be equal in FRET depending on their relative orientation and distance to the probe when enzyme is bound to the interface; however, the observed FRET will be an average of all possible orientations and distances in the interface-bound enzyme molecules, and should thus provide a useful measure of the amount of bound enzyme. Xu and Nelsesteun used FRET between intrinsic enzyme tryptophan and a dansyl-lipid probe to study the association of α-PI-PLC [30] and protein kinase C [31] with phospholipid vesicles. Earlier we showed FRET between *bc*PI-PLC and *N*-dansyl PE in DMPC vesicles as a function of added enzyme [24]; this was also reported by Volwerk et al. [20].

4.2. CMCs of lipid dispersions

The CMCs for L-DnDPI and L-thio-DMPI/L-DnDPI were negligible (< 0.5 μM) when compared to the concentrations used in FRET-binding or kinetic studies. The CMC for HDPC is 10 μM [23]; in the presence of 3 mol% DnDPC a CMC of about 6 μM was determined. HDPM in the presence of 3 mol% L-DnDPI showed a CMC of about 8 μM. In the kinetic studies the presence of D-thio-DMPI probably lowers the CMC of the lipid mixture to a level that is insignificant. The K_d values for *bc*PI-PLC binding to HDPC/DnDPC and HDPM/L-DnDPI in terms of binding sites were unchanged when recalculated using the corrected concentrations of interfacial lipid; values for N and $N \times K_d$ were only slightly lower (see footnotes to Table 1).

4.3. Binding of bcPI-PLC to lipid interfaces

Volwerk et al. [20] used changes in tryptophan fluorescence emission to estimate K_d for *bc*PI-PLC binding to various phospholipid interfaces by titration of lipid into protein. This was complicated by the fact that fluorescence emission appeared to be biphasic, particularly with anionic lipids where binding to the interface resulted in an increase while binding at the active site resulted in a decrease in fluorescence emission. Estimates of K_d were only possible with zwitterionic PC lipids and varied from 10 to 200 μM in terms of lipid monomers.

Zhou et al. [16] also observed a biphasic increase in tryptophan fluorescence for bacterial PI-PLC binding to short-chain PC molecules: a small increase attributed to monomer binding and a large increase above the CMC due to interfacial binding. The latter change increased at lipid concentrations above that necessary to activate the enzyme towards hydrolysis of *myo*-inositol 1,2-(cyclic)phosphate, which indicates further changes in tryptophan environments. Dissociation constants for lipid monomer binding were calculated from ^{32}P-NMR line widths to be between 0.3 mM and 0.5 mM for short-chain PCs below their CMCs. Binding of bacterial PI-PLC to phospholipid vesicles was also studied by different techniques [17]. Dissociation constants of 80 μM for binding to DMPC vesicles from intrinsic fluorescence emission, and 22 μM for binding to PC-coated beads were determined. Binding to anionic phosphatidic acid vesicles was weaker and could only be estimated

from gel filtration studies to have a dissociation constant of about 2 mM.

Our FRET experiments, which involve titration of protein into lipid, give good fits to a single binding equation, indicative of a single process. These experiments allow the determination of both K_d in terms of lipid binding sites and N, the limiting number of lipid molecules per bound protein. The K_d values obtained are in the low μM range and not unreasonable when compared with the estimates of Volwerk et al. [20] and the apparent K_m values from kinetic studies (see discussion below). Dissociation constants for enzyme binding to PI interfaces are even lower than those with PC interfaces, particularly in terms of lipid binding sites. With HDPM, however, binding was much weaker, as was also evident from gel filtration studies with phosphatidic acid vesicles [17].

Values of N, the limiting number of lipid molecules per enzyme bound, seem reasonable for interfaces of HDPC and HDPM, but rather high for PI-containing interfaces (Table 1). Volwerk et al. [20] estimated about 60 ditetradecyl phoshatidylmethanol molecules per bound *bc*PI-PLC at the interface by FRET experiments compared to the value of 80 that we calculate for HDPM. A geometrically determined value of N can be calculated as follows. *bc*PI-PLC has a molecular mass of 34.5 kDa; as a globular protein with a partial specific volume of 0.73 cm^3/g it has a cross-section area of 1.46×10^{-13} cm^2/molecule. If the cross-section area of a lipid molecule in the interface is 7.5×10^{-15} cm^2/molecule (75 $Å^2$/molecule), then the limiting number of lipid molecules per protein binding site is about 20. Our calculated value of 36 for PI-PLC binding to HDPC micelles seems reasonable by comparison. The calculated value of 270 for enzyme binding to L-thio-DMPI dispersions seems high. However, titration of excess *bc*PI-PLC into L-thio-DMPI dispersions at low lipid concentrations (Fig. 4) showed additional binding of enzyme molecules at high protein/lipid ratios, above the initial saturation plateau used to calculate binding parameters. This indicates that binding of enzyme to N lipid molecules occurs preferentially (with higher affinity), but at higher protein concentration more enzyme molecules may bind to the interface (with lower affinity). This may have something to do with the ability of enzyme molecules to pack closely on a negatively charged interface.

In these experiments (addition of enzyme to lipid) binding is observed in the opposite direction from usual experiments in which lipid is added to enzyme. In the latter case, titrations begin with a high ratio of enzyme to lipid where initially the maximum amount of enzyme is bound to the lipid, and as the titration proceeds the amount of bound enzyme per lipid decreases. In our experiments, we begin with a high ratio of lipid to enzyme, and the maximum amount of bound enzyme per lipid is only seen towards the end of the titration. Thus, if some enzyme molecules bind more easily (at a high N value or low density, and low K_d) this binding is apparent in the initial portion of the titration curve. Fig. 4 shows additional enzyme molecules binding at higher enzyme concentrations. If this titration was done in the opposite direction (lipid added to enzyme) we would probably observe a lower value of N and a greater value of K_d overall, and would probably not observe any preference for enzyme binding at a lower density (high N value).

4.4. Binding of PI-PLC-δ_1 and Δ-(1–132)PI-PLC-δ_1 to lipid interfaces

Pawelczyk and Lowenstein [18] studied binding of PI-PLC-δ_1 to phospholipid interfaces by sedimentation of sucrose-loaded vesicles. They added phospholipid vesicles to protein and measured the percentage of enzyme activity bound. Saturation occurred at different percentages of enzyme activity bound, which varied from about 5% with PI vesicles to 10% with PC vesicles and 22% with PE/PC (8:2) vesicles. They concluded that the strength of binding was of the order PE/PC > PC > PI, even though PC and PI vesicles showed half-saturation at much lower concentrations of lipid. It is unclear why 100% saturation could not be achieved, even at high lipid concentrations, and why strength of binding should be a function of the saturation value rather than the concentration of lipid giving half-saturation.

Our studies using FRET show that PI-PLC-δ_1 and Δ-(1–132)PI-PLC-δ_1 bind to L-thio-DMPI interfaces with high affinities comparable to that of *bc*PI-PLC (Table 1). There is little difference between PI-PLC-δ_1 and Δ-(1–132)PI-PLC-δ_1, although the addition of 0.2 mM Ca^{2+} to Δ-(1–132)PI-PLC-δ_1 seems to slightly increase its binding affinity, at least in terms

of binding sites. The latter effect could be due to the presence of a C2 domain with two distinct Ca^{2+}-binding sites [32]. The C2 domain in PI-PLC-δ_1, however, appears to play only a minor role while the pleckstrin homology domain plays a major role in membrane binding [33]. The K_d value for Δ-(1–132)PI-PLC-δ_1 with L-thio-DMPI compares reasonably with the apparent K_m value from kinetic studies (Table 2). The values of N are high and less reliable than those calculated for the bacterial enzyme; this is probably a result of the lower total lipid concentration used. However, the value of K_d in terms of binding sites was not significantly affected by differences in N. No binding of Δ-(1–132)PI-PLC-δ_1 to HDPC micelles could be seen by FRET under similar concentration conditions. It is interesting to note that PC activates the full-length PI-PLC-δ_1 towards hydrolysis of D-*myo*-inositol 1,2-(cyclic)phosphate, but has no effect on Δ-(1–132)PI-PLC-δ_1 [34]. This suggests that the pleckstrin homology domain has the ability to bind PC in the interface.

4.5. Kinetic studies

The apparent K_m from kinetic data of PI-PLC as a function of bulk lipid substrate concentration may be a rough measure of the K_d for initial binding of enzyme to lipid interface. It is, however, complicated by coupling of the equilibrium between free and interface-bound enzyme to subsequent equilibria between interfacial enzyme and substrate as represented by the interfacial K_m*, and possible allosteric or apparent cooperative effects within the interface. Thus, apparent K_m values should be compared with K_d values obtained in other types of experiments.

Kinetic experiments gave apparent K_m values for *bc*PI-PLC in the low μM range for D-thio-DMPI in the presence of HDPI or HDPC (24 and 15 μM respectively, Table 2), somewhat higher but not unreasonable as compared to the K_d values (3 and 7 μM respectively, Table 1) obtained in terms of lipid monomers from FRET experiments. With DMPM the apparent K_m was much higher (210 μM, Table 2), consistent with weaker binding of enzyme to HDPM micelles calculated by FRET (50 μM, Table 1). The kinetic experiments in the presence of HDPC and L-HDPI are pushing the lower limits for determination of K_m since at these low substrate concentrations initial rates do not stay linear very long, but rapidly decrease due to substrate depletion; for this reason they may result in somewhat higher values of K_m. The apparent K_m value for Δ-(1–132)PI-PLC-δ_1 with D-thio-DMPI dispersions (17.3 μM) was also in reasonable agreement with the K_d value (8.9 μM).

4.6. Conclusions

We have shown that binding of phospholipases to lipid interfaces, with K_d values in the low μM range in terms of lipid monomers or low nM range in terms of binding sites, can be calculated from FRET experiments with good fits of experimental data to theoretical binding curves. Under suitable conditions values of N, the limiting molecules of lipid per enzyme bound, can also be calculated from the same experiment. *bc*PI-PLC binds with high affinity to PI interfaces, slightly lower affinity to PC interfaces, and much lower affinity to PM interfaces. These K_d values correlate reasonably with apparent K_m values from kinetic experiments. Dissociation constants obtained by titration of protein into lipid, rather than the reverse, should provide a better comparison with kinetic parameters since both are determined under a high ratio of lipid to protein. Δ-(1–132)PI-PLC-δ_1 and PI-PLC-δ_1 also bind with high affinity to PI interfaces, but Δ-(1–132)PI-PLC-δ_1 shows little or no binding by FRET with PC interfaces under similar concentration conditions.

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