The Role of Interfacial Binding in the Activation of *Streptomyces chromofuscus* Phospholipase D by Phosphatidic Acid*

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The *Streptomyces chromofuscus* phospholipase D (PLD) cleavage of phosphatidylcholine in bilayers can be enhanced by the addition of the product phosphatidic acid (PA). Other anionic lipids such as phosphatidylglycerol, phosphatidylglycerol, or phosphatidyl ethanolamine do not activate this PLD. This allosteric activation by PA could involve a conformational change in the enzyme that alters PLD binding to phospholipid surfaces. To test this, the binding of intact PLD and proteolytically cleaved isoforms to styrene divinylbenzene beads coated with a phospholipid monolayer and to unilamellar vesicles was examined. The results indicate that intact PLD has a very high affinity for PA bilayers at pH ≥ 7 in the presence of EGTA that is weakened as Ca$^{2+}$ or Ba$^{2+}$ are added to the system. Proteolytically clipped PLD also binds tightly to PA in the absence of metal ions. However, the isolated catalytic fragment has a considerably weaker affinity for PA surfaces. In contrast to PA surfaces, all PLD forms exhibited very low affinity for PC interfaces with an increased binding when Ba$^{2+}$ was added. All PLD forms also bound tightly to other anionic phospholipid surfaces (e.g. phosphatidylserine, phosphatidylethanolamine, phosphatidyl ethanolamine). However, this binding was not modulated in the same way by divalent cations. Chemical cross-linking studies suggest that a major effect of PLD binding to PA-Ca$^{2+}$ surfaces is aggregation of the enzyme. These results indicate that PLD partitioning to phospholipid surfaces and kinetic activation are two separate events and suggest that the Ca$^{2+}$ modulation of PAPL binding involves protein aggregation that may be the critical interaction for activation.

Mammalian phospholipase D enzymes have a complex intermediary role in many well characterized signal transduction pathways involving membrane-linked and cytosolic soluble signaling pathways (1). Most PLD enzymes have a high affinity for anionic phospholipids, and all appear to require Ca$^{2+}$ for catalytic activity. Regulation of this class of enzymes is complex: protein kinase C (2), ARF (3), and Rho (2) proteins are activators of PLD. The lipophilic product of PLD cleavage, phosphatidic acid (PA), acts as a second messenger in cells and has been shown to activate phosphatidylglycerol-specific phospholipase C-γ1 (4), inhibit adenylate cyclase (5), and mobilize intracellular Ca$^{2+}$ (6, 7).

The PLD secreted by *Streptomyces chromofuscus* is considerably smaller than the eukaryotic enzymes, although like those enzymes it requires Ca$^{2+}$ for activity (8). The bacterial PLD is not involved in signal transduction but has a role in phosphate retrieval; it may also play a role in promoting infections of the organism. The *S. chromofuscus* PLD is an unusual phospholipase in that it does not exhibit "interfacial activation" (preference for micellar rather than monomeric short chain phospholipid substrate (9)) or "surface dilution" (dependence of enzyme specific activity on the mole fraction of substrate in a micelle or bilayer surface (10)) kinetics (11). A ping-pong-like ordered binding mechanism has been proposed for the enzyme in which the substrate (e.g. phosphatidylcholine) binds and is converted to a covalent phosphatidyl-enzyme with release of the free base (e.g. choline), followed by water attacking the PLD-PA covalent adduct to release phosphatidic acid (12). The nucleophilic attack of water on the distal phosphate ester bond results in cleavage of the P-O bond. The phosphatidyl-enzyme intermediate can be decomposed to a different phospholipid in the presence of a high concentration of a primary alcohol such as ethanolamine, serine, methanol, butanol, etc., a reaction used to generate different head group phospholipids (13, 14).

*S. chromofuscus* PLD is dramatically activated for hydrolysis of PC packed in vesicles by the incorporation of PA (lyso-PA or phosphatidylglycerol 4-phosphate) in the vesicle. The presence of PA increases the apparent $V_{max}$ and has little effect on the apparent $K_m$ (11). Other nonsubstrate anionic phospholipids such as PI or fatty acids do not activate the enzyme. This activation is allosteric because PA bilayers can activate the PLD toward diC$_4$PC, a water-soluble substrate with no tendency to partition into bilayers (11). Two forms of *S. chromofuscus* PLD have been isolated from culture supernatants (15): intact PLD that is a monomer with a molecular mass of 57 kDa (PLD$_{57}$) and a tight complex of PLD that has been proteolytically cleaved near the C-terminal portion of the protein (PLD$_{42}$20, named for the apparent size of the two subunits on SDS-PAGE). Interestingly, PLD$_{42}$20 is more active than intact enzyme toward PC vesicles and toward monomeric substrates (dihexanoyl-phospholipids). Only the intact PLD$_{57}$ can be activated by PA. One possible explanation for PA activation is that the negatively charged product anchors the enzyme to the zwitterionic PC surface for processive catalysis, although this cannot be the only explanation because PA bilayers enhance PLD$_{57}$ cleavage of diC$_4$PC (11).

The present work is aimed at examining the binding affinity of *S. chromofuscus* PLD for PA, PC, and other phospholipid...
surfaces. Two different types of surfaces were used to measure protein binding to phospholipids: (i) hydrophobic beads (composed of styrene divinylbenzene (SDVB)) coated with a monolayer of phospholipid and (ii) unilamellar vesicles. The results show that PLD$_{57}$ has a pH-dependent high affinity for POPA bilayers in the presence of EGTA that is weakened (at pH ≥ 7) as Ca$^{2+}$ or other divalent cations are added to the system. The proteolytically clipped PLD$_{42/20}$ also binds tightly to POPA in the absence of metal ions at pH 8; isolated PLD$_{57}$ has a considerably weaker affinity for POPA surfaces.

In contrast to PA surfaces, all PLD forms exhibited very low affinity for PC interfaces with an increased binding when Ba$^{2+}$ was added. The binding of PLD$_{57}$ to PC was enhanced by preincubation of the enzyme with diC$_4$PA, consistent with PA binding to an allosteric site and causing a conformational change of the protein to a form with a higher affinity for bilayer surfaces. All PLD forms also bound tightly to other anionic phospholipid surfaces (e.g., PS, PMe, and PI); however, this binding was not modulated by the addition of divalent cations. These results indicate that PLD partitioning to phospholipid surfaces and kinetic activation are two separate events.

**Materials and Methods**

**Chemicals**—Crude *S. chromofuscus* PLD (lyophilized powder) was purchased from Sigma. DiC$_4$PC, POPC, POPA, POPS, PI, and dioleoylphosphatidylmethanol were purchased from Avanti and used without further purification. Hitrap HIC and Hitrap Q columns and phenyl-Sepharose were purchased from Amersham Pharmacia Biotech. DiC$_4$PA and diC$_4$PMe were synthesized from diC$_4$PC as described previously (11, 16). The reaction progress was monitored by $^{31}$P NMR spectroscopy (17), and the final product was characterized by $^1$H NMR spectroscopy. SDVB beads were purchased from Seradyn and prepared for coating with phospholipids as described by Cho and co-workers (18).

**Purification of PLD Isoforms**—Crude PLD from *S. chromofuscus* was fractionated to yield PLD$_{57}$, PLD$_{42/20}$, and PLD$_{20}$ using Hitrap HIC and Hitrap Q columns as described previously (15). In addition, chromatography on palmitoyl cellulose equilibrated with 10 mM Tris, pH 8.0, was used to isolate PLD$_{57}$ from the other species (20). Protein was eluted with a Triton X-100 gradient from 0 to 0.5 mM; PLD activity of fractions was assayed toward diC$_4$PC using the pH-stat technique. The PLD species responsible for the activity were identified by SDS-PAGE. Fractions containing PLD were pooled and dialyzed against 10 mM Tris, pH 8.0, to remove the Triton X-100. PLD$_{57}$ and PLD$_{42}$ were cleanly isolated from this column; PLD$_{20}$ was not detected in the eluants from the palmitoyl cellulose column. An alternate purification scheme to isolate PLD$_{57}$ and PLD$_{42}$ used chromatography of *S. chromofuscus* supernatant on a phenyl-Sepharose column. PLD fractions were eluted with a gradient of 1.0 to 0 mM ammonium sulfate.

**Preparation of Vesicles**—An appropriate aliquot of phospholipid dissolved in chloroform was evaporated under argon for 10–20 min. The resulting film was lyophilized and then resuspended in 1 mM EGTA (or other divalent cations) and then weighed out in tared Eppendorf tubes so that when resuspended in 250 μl of buffer the final concentration of phospholipid would range between 60 and 600 μM.

For binding experiments where the ligand was varied, a fixed amount of enzyme was added to each tube (e.g. for PLD$_{57}$, 2.5 μg/500 μl). PMe–, PI–, and POPA-coated beads were incubated with PLD with gentle rocking for 10 min at room temperature. PLD binding to POPC and POPS vesicles could not be examined in the presence of Ca$^{2+}$, because the enzyme would be active and generate PA under those conditions. Instead, Ba$^{2+}$ was used and compared with vesicle binding of the enzymes in the presence of EGTA. Free PLD (E$_f$) was separated from bound PLD (E$_b$) by centrifugation; the supernatant was analyzed for free PLD activity in the supernatant after centrifugation (E$_f$). When the enzyme concentration was fixed and phospholipid concentration was varied, K$_d$ was derived as indicated above. In experiments with fixed phospholipid but varied enzyme, K$_d$ was derived from E$_f$ = L/E + K$_d$. Cooperative binding was not considered. All datum points were done in at least duplicate (and often in triplicate).

For PLD$_{57}$ beads were not saturated with phospholipid at 60 μM but were saturated when prepared in 600 μM bulk phospholipid (bead surface saturation by phospholipids occurred between 120–300 μM bulk phospholipid). Therefore, a qualitative comparison of PLD binding to SDVB beads at comparable total phospholipid concentration but different surface saturation was used to check for large differences in PLD affinity that could indicate PLD binding directly to uncoated beads. For these experiments the phospholipid concentration of 420 μM was set in order to achieve a slight decrease in PLD$_{57}$ specific activity in the supernatant (indicating slightly more PLD$_{57}$ binding to the beads) when beads were coated with 600 versus 60 μM phospholipid. Although PLD$_{57}$ binds slightly more tightly to saturated beads, the difference is small and consistent with PLD$_{57}$ interacting with the phospholipid monolayer rather than the bead surface. The proteolytically clipped PLD$_{42/20}$ bound to beads with a much stronger dependence on the bead coverage. To avoid complications of the PLD$_{42/20}$ interacting differentially with the subsaturated beads, binding experiments were run at fixed ligand (420 μM) and varying PLD$_{42/20}$.

**Cross-linking of PLD**—Both PLD$_{57}$, and proteolytically cleaved fragments of PLD were examined for aggregate formation in the absence and presence of phospholipids and metal ions using the chemical cross-linking agent EDC, a heterobifunctional imide, obtained from Pierce. The cross-linking reaction was carried out in 100 mM MES, 100 mM NaCl, with the pH between 6 and 7 (average 6.5) in the absence and presence of cations. PLD, ranging from 25 to 75 μg, and 10 mM EDC were incubated for various times at 30 °C or room temperature. Cross-linking studies with PLD and POPA or PI vesicles used incubation of the same PLD concentration and varied [Ca$^{2+}$ or Ba$^{2+}$] to minimize production of PA by the high concentration of PLD, which is inhibited by Ba$^{2+}$ but not totally). Excess reagent was quenched by increasing the pH to 9. Samples were analyzed using either a 6.5% denaturing gel or a 7.5–15% gradient denaturing gel.

**Protein Concentration**—The amount of protein free and bound as well as protein lost (trapped in the centricron filters) was measured with texted, centrifuged, and filtered through an Amicon centrifron-100 concentrator. PLD bound to vesicles stayed on the filter; free PLD passed through the filter and into the supernatant. There was always some protein “lost” during filtration (not accounted for on the filter or in the filtrate and presumably adhered to the filter and not easily eluted). This amount of free PLD was measured using a calibration curve of the total protein concentration of the sample before and after filtration was analyzed by SDS-PAGE and by pH-stat for activity toward 5 mM diC$_4$PC (the pH-stat of short chain PA is 6.8 so that most of the product PA is titratable at pH 8.0) to measure the amount of PLD bound to the vesicles. Bound PLD (E$_b$) was calculated by measuring total activity of PLD toward diC$_4$PC (5 mM) in the absence of free PLD (E$_f$) and compared with free PLD activity in the supernatant after filtration (E$_f$); E$_b$ = E$_f$ – E$_f$. Alternatively, intensities of PLD bands on SDS-PAGE before (E$_f$) and after filtration (E$_b$) were compared to estimate E$_b$. The K$_d$ was derived from fitting the data to the equation E$_f$/E$_b$ = L/(L + K$_d$), where L is the total phospholipid concentration, and E$_b$ is bound enzyme.
PLD Binding to Phospholipid Surfaces

TABLE I

| Enzyme | Ca^{2+} mM | Ba^{2+} mM | K_D μM |
|--------|------------|------------|--------|
| PLD_{57} | 0 | 0 | <2 |
| | 2 | 0 | 12 | 1.3 |
| | 5 | 0 | 215 | 43 | 294 | 20 |
| | 0 | 5 | 462 | 21 | 235 | 21 | 1220 | 180 |
| PLD_{42} | 0 | 0 | <10 |
| | 5 | 0 | 536 | 53 |
| | 0 | 5 | 459 | 31 |
| PLD_{42} | 0 | 0 | >757 |
| | 2.0 | ± | 0.2 |

* EGTA (1 mM) was present in the binding buffer along with the indicated concentration of metal ion.

NanoOrange Protein Quantitation Kit (Molecular Probes N-6666). The NanoOrange reagent (bound to protein) was excited at 480 nm, and the emission scanned from 570–590 nm. A known amount of PLD_{57} or some proteolytic fragment was used to construct a standard curve.

**PLD Assays**—Two methods were used to measure PLD specific activity. To quantify free PLD in the binding studies, a pH-stat assay was used. Hydrolysis of 5 mM diC_{4}PC to diC_{4}PA was monitored with a Radiometer pH-stat model Vit90 as described previously (15) using 5 mM NaOH as the titrant. For each phospholipid concentration, assays were run in duplicate or triplicate. For assays with unilamellar vesicles as the substrate, either ^31P or ^1H spectra were acquired to monitor PLD activity as described previously (11, 15). The assay buffer used was 50 mM imidazole in D_2O buffer, pH 7.2 (meter reading).

**RESULTS**

**PLD_{57} Binding to PA Interfaces**—In the presence of EGTA and at pH 8.0, PLD_{57} bound quite tightly to the PA-coated SDVB beads; no activity for free PLD_{57} was detected in the supernatant under these conditions. Given the error in determining PLD specific activity (15%) and the smallest concentration of PA examined (10 μM), K_D < 2 μM (Table I). Inclusion of Ca^{2+} (or Ba^{2+}) in the binding buffer dramatically weakened the interaction of PLD with the PA-coated beads, and more free enzyme could be detected in the supernatant (Fig. 1). At a fixed metal ion concentration but with varying PA, the amount of enzyme bound to the beads was quantified and used to calculate a K_D (Fig. 2). Increasing the Ca^{2+} concentration from 2 to 5 mM (in the presence of 1 mM EGTA) increased the PLD_{57} K_D for the PA from 12 to 215 μM (Fig. 2). In the presence of 5 mM Ba^{2+} instead of Ca^{2+}, the PLD_{57} K_D for PA-coated beads was 462 ± 21 μM (Table I). However, not all divalent ions were effective in releasing PLD_{57} from PA surfaces. In the presence of Mg^{2+}, PLD remained tightly bound to the PA-coated beads. The apparent affinity of Ca^{2+} for PA has been described in the literature as ranging from 0.5 to 5 mM. A large number of factors affect this interaction including pH, solution ionic strength, and the presence of divalent cations or cationic peptides. The change in PLD binding to PA in the presence of Ca^{2+} suggested that Ca^{2+} may compete very effectively with PLD for binding to PA surfaces.

The tight interaction of PLD_{57} with PA surfaces in the presence of EGTA at pH 8.0 was also examined with POPA vesicles and a filtration assay. In Fig. 3, lane 1 shows total enzyme (PLD_{57}) prior to filtration, and lane 5 shows free enzyme after incubation with 0.5 mM POPA vesicles. Clearly, most of the PLD_{57} was bound to the vesicle surface in the absence of divalent metal ions.

The effects of pH and ionic strength on PLD partitioning to the bilayer surface were also monitored. The amount of free PLD was estimated from residual PLD activity in the filtrate toward diC_{4}PC or by SDS-PAGE of the filtrate compared with sample prior to filtration. As the solution pH was decreased from 8 to 6, there was a significant decrease in the amount of PLD bound to the PA surface (Table II). Because pK_a for PA is ~8.5 in pure PA bilayers (21), the majority of the PA is monoanionic over this pH range and not changing significantly. Between pH 5 (16% bound) and 7 (78% bound), the PA is nearly all monoanionic: hence the large change in binding is not likely to result from changes in PA ionization. A more likely explanation is that a group on the enzyme (possibly a histidine) must be deprotonated for efficient binding of PLD to PA in the absence of Ca^{2+}. Adding Ca^{2+} to the solution of PA vesicles enhanced PLD binding at acidic pH values (Table II). This suggests that a group(s) on the enzyme interacts with the metal...
ion to lower the $pK_a$ of the group that must be deprotonated for optimal binding of the protein to PA.

PLD$_{57}$ binding to POPA in the absence of divalent cations was also examined as a function of added NaCl. Increasing the NaCl from 0.1 to 1 mM caused a large decrease in the amount of enzyme bound to the PA vesicles (Table III). The ability of high NaCl to inhibit PLD binding to PA indicates that the interaction has a large electrostatic component.

**PLD$_{57}$ Binding to Other Anionic Phospholipid Interfaces**—Intact PLD also bind tightly to other anionic phospholipid surfaces, both phospholipid-coated SDVB beads and unilamellar vesicles, in the presence of 1 mM EGTA (Table I). PLD$_{57}$ bound to PI, PMe, and PS surfaces with $K_D < 2 \mu M$. Again, as Ca$^{2+}$ (or Ba$^{2+}$ in the case of substrates PS and PMe) was added, the PLD binding affinity decreased for the phospholipid. With the nonsubstrate and noninhibitor PI, PLD$_{57}$ binding at pH 8.0 was the same whether Ca$^{2+}$ or Ba$^{2+}$ was added (e.g., $K_D$ values of 294$^\pm$20 and 235$^\pm$21 $\mu M$ were obtained for PI at pH 4 M (total cation minus EGTA concentration) Ba$^{2+}$ and Ca$^{2+}$, respectively). As with PA, the interaction of the enzyme with PI was weaker at acidic pH and metal ions enhanced binding at low pH (Table II). Interestingly, PI, PMe, and PS do not activate PLD$_{57}$ toward PC vesicles (11). Thus, binding to phospholipid surfaces alone is not sufficient for kinetic activation.

**Proteolytically Clipped PLD Binding to PA Interfaces**—The proteolytically clipped PLD where the two fragments are still associated was not activated by PA for hydrolysis of PC vesicles (15). How it interacts with PA surfaces could shed light on the mechanism for the kinetic activation. When incubated with 0.5 mM PA vesicles in the absence of divalent metal ions, PLD$_{42/20}$ bound well to the PA (Fig. 3, compare lane 4 with lane 8). Using PA-coated SDVB beads (Table I), the $K_D$ of PLD$_{42/20}$ for PA in the presence of EGTA was considerably higher ($K_D = 130$ $\mu M$) than that of intact PLD$_{57}$ ($K_D < 2 \mu M$). However, the $K_D$ of the clipped enzyme for PMe and PS, both substrates of PLD$_{42/20}$ was still low (<50 $\mu M$) in the presence of EGTA. Addition of Ba$^{2+}$ reduced the affinity of the PLD fragments for these anionic phospholipid surfaces as it did for the intact PLD.

The 42-kDa fragment alone exhibited much weaker binding to anionic lipid surfaces irrespective of phospholipid identity and surface (Fig. 3, for vesicles compare lane 2 (total PLD$_{42}$) with lane 6 (PLD$_{42}$ in the filtrate); for SDVB beads see Table I). For example, the PLD$_{42}$ $K_D$ for a PS monolayer in the presence of EGTA was 760 $\pm$ 75 $\mu M$ compared with $<2 \mu M$ for both PLD$_{57}$ and clipped but associated PLD$_{42/20}$ (<50 $\mu M$). In the presence of 5 mM Ba$^{2+}$, the $K_D$ for PLD$_{42}$ binding to PA-coated SDVB beads was 2.0 $\pm$ 0.2 mM; the $K_D$ for PLD$_{42/20}$ was 0.46 $\pm$ 0.02 mM. Thus, removal of the 20-kDa fragment weakened PLD binding to anionic phospholipid surfaces. The 20-kDa fragment exhibited the weakest interaction with PA vesicles (Fig. 3, compare lane 3 (total PLD$_{42/20}$) with lane 7 (PLD$_{42/20}$ in the filtrate)) and PA-coated SDVB beads (no binding could be measured). These differences suggest that part of the PA activation phenomenon involves selective binding to PA in the presence of Ca$^{2+}$ and that an intact C-terminal part of the protein is involved.

**PLD Binding to PC Interfaces**—In contrast to the tight binding to anionic phospholipid surfaces, either monolayers on beads or vesicles, PLD exhibited poor binding to PC surfaces in the presence of EGTA or EDTA. The binding was sufficiently weak that estimates of the PLD $K_D$ for PC-coated SDVB beads were >1 mM for intact PLD$_{57}$ and associated PLD fragments (PLD$_{42/20}$). The interaction of the enzyme with PC surfaces was examined in detail using PC unilamellar vesicles and monitoring the amount of PLD bound to the PC bilayer (Table IV). In the absence of divalent metal ions, isolated PLD fragments

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**Table II**

| Phospholipid | $M^+$ | $E/K_*$ | pH 5 | pH 6 | pH 7 | pH 8 |
|--------------|------|--------|-----|-----|-----|-----|
| PA           | Ba$^{2+}$ | 0.16 | 0.54 | 0.78 | 0.86 |
| PI           | 5     | 0.95  | 0.90 |
|              | 0.24  | 0.24  | 0.55 | 0.57 |
|              | 0.10  | 0.41  | 0.28 |

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**Table III**

| Interface | Ba$^{2+}$ | NaCl | Enzyme bound$^a$ |
|-----------|-----------|-----|------------------|
| POPA (0.5 mM) | 0 | 0.1 | 94 |
| 0 | 0.2 | 92 |
| 0 | 0.4 | 60 |
| 0 | 0.6 | 59 |
| 0 | 0.8 | 36 |
| 0 | 1.0 | 34 |
| POPE (10 mM) | 4 | 0.1 | 56 |
| 4 | 1.0 | 61 |
| POPE (10 mM) | 4 | 0.1 | 48 |
| 4 | 1.0 | 52 |

$^a$ The percentage of enzyme bound to vesicles is calculated as $E/E_T \times 100$.

**Table IV**

| Protein | Ba$^{2+}$ | PC SUVs | PC/PA (9:1$^b$) | PC/PA (7:3$^b$) | PS SUVs | PC/PS (9:1$^b$) |
|---------|-----------|---------|----------------|----------------|---------|----------------|
| PLD$_{57}$ | 0 | 19 $\pm$ 4 | 83 $\pm$ 10 | 91 $\pm$ 11 | 47 $\pm$ 7 | 47 $\pm$ 7 |
| 5 | 42 $\pm$ 7 |
| 10 | 90 $\pm$ 14 |
| PLD$_{42/20}$ | 0 | 27 $\pm$ 5 | 52 $\pm$ 7 | 61 $\pm$ 8 | 79 $\pm$ 10 | 79 $\pm$ 10 |
| 5 | 46 $\pm$ 6 |
| 10 | 79 $\pm$ 12 |
| PLD$_{42}$ | 0 | 12 $\pm$ 3 | 52 $\pm$ 7 | 69 $\pm$ 7 | 81 $\pm$ 9 |
| 5 | 31 $\pm$ 5 |
| PLD$_{20}$ | 0 | 7 $\pm$ 3 | 33 $\pm$ 5 | 46 $\pm$ 6 | 88 $\pm$ 11 | 88 $\pm$ 13 |
| 5 | 13 $\pm$ 4 |

$^a$ The total phospholipid concentration was 10 mM.
(PLD42 and PLD20) exhibited even less binding to the PC surface than intact PLD57 or the clipped dimer (PLD42/20). Inclusion of 5 mM Ba\(^{2+}\) enhanced PLD57 and the clipped PLD complex binding to the PC surface by a factor of two; increasing the Ba\(^{2+}\) to 10 mM led to most of the PLD partitioning onto the PC surface under these conditions. This is quite different from what was observed for PLD binding to PA or other anionic phospholipid interfaces where divalent cations lessened PLD partitioning to the surface. Furthermore, the binding of PLD57 to POPC in the presence of Ba\(^{2+}\) was unaffected by up to 1.0 M NaCl (Table III) or over the pH range from 6 to 8 (Fig. 4). A direct comparison of PLD binding to PC and PS vesicles in the absence or presence of Ba\(^{2+}\) emphasizes this behavior (Table IV). High Ba\(^{2+}\) basically prevented PLD57 from binding to the PS surface, whereas that amount of metal ion enhanced PLD57 binding to PC surfaces about 4-fold. Only PLD57 bound to PS exhibited this sensitivity to Ba\(^{2+}\); clipped PLD or the isolated fragments still bound tightly to the PS surface whether Ba\(^{2+}\) was present or not. Thus, PLD57 has different interactions with anionic lipids and divalent cations than the fragments or even the associated but clipped PLD42/20.

If a small amount (10 mol %) of anionic phospholipid, either PA or PS, was included in the PC vesicles, partitioning of all forms of PLD to PC surfaces was enhanced (Table IV). Increasing the PA content enhanced this partitioning still further and was most effective for PLD57. The proteolytically clipped version of the enzyme did not bind to the mixed PC/PA phospholipid interfaces as well as intact protein. However, PS was more effective than PA with proteolytically clipped or purified fragments (PLD42 and PLD20). This is consistent with the PA activation of PLD involving enhanced binding of the protein to substrate interfaces. However, this surface binding cannot be the only effect of the PA.

The inclusion of either PA or PI at 10 mol % in PC vesicles also altered the pH behavior of PLD57 binding to PC surfaces. In the absence of metal ion, the enzyme partitioning reflected binding of PLD57 to the anionic phospholipid (e.g., binding decreased with decreasing pH) and not the PC (partitioning was invariant between pH 6 and 8). In the presence of 5 mM Ba\(^{2+}\) (Fig. 4), PLD57 binding to the PC/PI surface (Fig. 4C) had the same profile as for PC without PI (Fig. 4A); PLD57 binding in the presence of Ba\(^{2+}\) was strongest to the PC/PA vesicle, particularly at pH 5 (Fig. 4B).

**Monomer PA Effect on PLD Binding and Hydrolysis of PC Surfaces**—The results discussed above indicate that intact PLD57 has a higher affinity for PC surfaces containing PA. Is this change in surface binding the main reason for the interfacial activation by PA? DiC\(_4\)PA has a critical micelle concentration of \(>100 \text{ mM}\) and should not partition into PC bilayers. If it can bind as a monomer to PLD, it may affect partitioning of the enzyme to vesicle surfaces. Therefore, partitioning of intact PLD57 to POPC vesicles was examined in the absence of Ba\(^{2+}\) (which by itself can enhance binding to PC surfaces). As shown in Fig. 5A, under the conditions used, the bulk of the enzyme was free and not bound to PC in the absence of the short chain PA. However, as diC\(_4\)PA was titrated into the system, more of
the enzyme was partitioned onto the vesicle surface (notice the increasing loss in intensity for free PLD$_{57}$ in Fig. 5 for lanes 4–6, which reflect incubation with 1, 2.5, and 5 mM diC$_4$PA, respectively). 5 mM diC$_4$PA was not quite as efficient at driving the enzyme to the POPC surface as a vesicle composed of 0.9 mM POPC and 0.1 mM POPA (lane 7). Thus, a much higher concentration of water-soluble diC$_4$PA than long chain PA (which is already localized in the bilayer) is needed to translocate PLD$_{57}$ to the bilayer surface. The observation that water-soluble diC$_4$PA drives the PLD to the membrane surface is consistent with the PA acting allosterically. In the presence of Ba$^{2+}$, the diC$_4$PA was less effective at partitioning PLD to PC surfaces (similar to what was observed for long chain PA species).

The same PLD$_{57}$ binding experiments were carried out in the presence of water-soluble diC$_4$PMe (Fig. 5A, lanes 9–12). In contrast to diC$_4$PA, high concentrations of water-soluble diC$_4$PMe did not induce any detectable partitioning of PLD$_{57}$ to the PC bilayers. Thus, the enhanced surface binding effect was specific to PA. There must be a discrete binding site on the enzyme that interacts with PA head groups and promotes a change in the PLD that enhances productive binding to zwitterionic interfaces.

If this binding is a key parameter in PA-activation of PLD$_{57}$, then the presence of diC$_4$PA should activate the enzyme toward POPC vesicles. This was monitored by $^3$H NMR spectroscopy (11, 15). The initial rate (first 15 min) of hydrolysis of 10 mM POPC vesicles in 50 mM imidazole with 5 mM Ca$^{2+}$, pH 7.2, was increased ~4-fold when 10 mM diC$_4$PA was present (Fig. 5B), although the soluble diC$_4$PA was not as effective an activator as long chain PA incorporated in the vesicle (11). Furthermore, the hydrolysis of POPC without the short chain PA was non-linear. The rate was quite low for the first 5–10 min and then exhibited a nonlinear increase to where the rates for the two samples were within 30%. At the point where PLD showed an increased hydrolysis rate toward POPC vesicles, 5% of the long chain PA activator was generated in situ. The biphasic behavior for pure POPC vesicles suggests that as long chain PA is generated, the enzyme becomes activated. Because activation by interfacial PA is more effective than soluble PA, the PLD hydrolysis rates become more similar. Clearly, maximum activation by PA requires interfacial PA. What does the surface PA do to PLD$_{57}$ that soluble diC$_4$PA cannot?

**PLD Aggregation State Bound to Vesicles**—One possible explanation for PA activation of PLD$_{57}$ toward PC vesicles is that the enzyme becomes oligomerized on the vesicle surface through its interactions with PA and Ca$^{2+}$. EDC, a heterobifunctional cross-linker that forms amide bonds from closely juxtaposed aspartate/glutamate and lysine side chain amino groups, was used to explore the aggregation state of PLD when bound to different phospholipid vesicle surfaces (Fig. 6A). In the presence of Ba$^{2+}$ (to supply a divalent cation but inhibit PLD hydrolysis of the PC) and absence of any phospholipids, PLD$_{57}$ (typically 30 μg/ml) formed only a small proportion of oligomers (<5%) when treated with EDC (Fig. 6A, lane 1). When PLD$_{57}$ was incubated with PC/PA (9:1) vesicles and 1 mM EGTA (lane 2), there was very little cross-linking of the protein. However, when 5 mM Ba$^{2+}$ was present along with the PC/PA vesicles and EGTA, there was extensive cross-linking of the protein subunits to dimers and tetramers (lane 3). PLD$_{57}$ incubated with PC/PI (9:1) vesicles, and Ba$^{2+}$ also showed cross-linking by EDC (lane 4), but it was not as extensive (dimers were the preferred species formed) as with PA. Oligomerization of the PLD$_{57}$ by EDC absolutely required an interface because incubation of PLD$_{57}$ with the monomeric substrates diC$_4$PC and diC$_4$PA in the absence or presence of Ba$^{2+}$ yielded no cross-linked protein oligomers (data not shown).

The extent of cross-linking was dependent on the identity of the divalent cation as well as the concentration of PLD$_{57}$. The gel in Fig. 6B shows how Ba$^{2+}$ (5 mM) or Ca$^{2+}$ (2 mM) affect cross-linking of the enzyme to pure PA or PI vesicles (in the presence of 1 mM EGTA). Binding of the enzyme to both anionic phospholipid vesicles in the presence of metal ions leads to oligomers trapped by EDC. PA was more effective at inducing PLD$_{57}$ trimers and tetramers than PI (Fig. 6B, compare lanes 1 and 2 with lanes 3 and 4). Furthermore, a larger proportion of higher order aggregates (tetramers and trimers) was formed with Ca$^{2+}$ than with Ba$^{2+}$ for PLD$_{57}$ binding to both PA and PI vesicles (Fig. 6B, lane 1 versus lane 2). The PLD$_{57}$ aggregates formed on Ca$^{2+}$-PA vesicles were still cross-linked by EDC (10 mM) when the enzyme was diluted 5-, 10-, or 20-fold in the presence of 10 mM PA vesicles (lanes 5–7). In fact, at the lowest dilution of the enzyme, the tetramer is the darkest band of PLD$_{57}$ (lane 4). Lanes 5–7 show 10 mM EDC cross-linking of lower concentrations of PLD$_{57}$ incubated with 1 mM PA, 2 mM Ca$^{2+}$, or 5 mM Ba$^{2+}$ (lane 4). Lanes 5–7 also show 10 mM EGTA and the following concentrations of buffer: lane 5, 6 μg/ml; lane 6, 3 μg/ml; lane 7, 1.5 μg/ml.

**FIG. 6.** A. 6.5% SDS-PAGE analysis of cross-linking of PLD$_{57}$ (30 μg/ml) by EDC (10 mM) in 1 mM EGTA, 100 mM MES, pH 6.25, with the following: lane 1, 5 mM Ba$^{2+}$ ions; lane 2, POPC/POPA (9:1 mM) vesicles; lane 3, POPC/POPA (9:1 mM) vesicles with 5 mM Ba$^{2+}$; lane 4, POPC/PI (9:1 mM) vesicles with 5 mM Ba$^{2+}$. Molecular mass markers are shown by the standards. B. 7.5–15% gradient SDS-PAGE showing EDC (10 mM) cross-linking of PLD$_{57}$ (30 μg/ml) in 1 mM EGTA, 100 mM MES, pH 6.5, in the presence of 1 mM PA and 2 mM Ca$^{2+}$ (lane 1) or 5 mM Ba$^{2+}$ (lane 2) or in the presence of 1 mM PI and 2 mM Ca$^{2+}$ (lane 3) or 5 mM Ba$^{2+}$ (lane 4). Lanes 5–7 show 10 mM EDC cross-linking of lower concentrations of PLD$_{57}$ incubated with 1 mM PA, 2 mM Ca$^{2+}$, or 5 mM Ba$^{2+}$.
DISCUSSION

Interfacial substrate hydrolysis by lipolytic enzymes can have two distinct binding modes for the enzyme: an initial interfacial interaction (that can be rather nonspecific and rely on electrostatics or hydrophobic interactions) that anchors the enzyme to the surface and a specific binding of a single substrate molecule to the catalytic site. Either step can be rate-limiting and affect the observed rate of vesicle hydrolysis. Phospholipase D from *S. chromofuscus* is an unusual phospholipase in that it displays neither interfacial activation nor surface dilution kinetics with micellar substrates. However, with monomeric diC₄PC as the substrate, interfaces can enhance the specific activity of the intact protein (but not the proteolytically clipped enzyme whose activity is about four times higher) toward this water-soluble substrate (11). POPC vesicles are also poor substrates for this enzyme; enzyme activity increases if PA is incorporated into the PC bilayers (11). These kinetic observations suggest an allosteric role for PA (or similar interfacial molecules). At least in the case of the PC vesicles, such an allosteric role could involve increasing the amount of enzyme that binds to the vesicle surface, the first binding step in processing substrate at an interface.

PLD₅₇ (or any of the PLD fragments) binding to pure PC vesicles is extremely weak but enhanced by Ba²⁺; enzyme partitioning on PC vesicles with Ba²⁺ present is not pH-dependent over the pH range of 6–8 and is not affected by salt. In contrast, PLD₅₇ binding to anionic phospholipid vesicles in the absence of divalent metal ions is pH-dependent with higher affinity as the pH is increased (Kᵦ<2 µM at pH 8). PLD₅₇ binding to PI vesicles in the absence of divalent cation exhibits the same pH dependence. The binding of PLD₅₇ to PA surfaces can also be inhibited by high NaCl, implying that the PA-PLD interaction has a large electrostatic component. Although PLD₅₇ should have a net negative charge (pI = 5.1 (8)), there must be distinct cationic sites on the enzyme that mediate this binding. When 10 mol % of anionic lipids are incorporated into PC vesicles, PLD binding (in the presence of EGTA) to the predominantly PC vesicle is dramatically enhanced, presumably through binding to the anionic lipid. However, the addition of divalent metal ions decreases binding of the enzyme to PA (alone or mixed with PC in vesicles) at pH 8. Millimolar divalent metal ions enhance PLD binding to PC but not PA interfaces.

PLD₅₇ absolutely requires Ca²⁺ for activity; the Kᵦ for Ca²⁺ is 75 µM (15) with monomeric diC₄PC as the substrate, but higher Ca²⁺ (>1 mM) is required for activity of the enzyme toward PC in vesicles (11). The excess Ca²⁺ will interact with the PA head groups (causing clustering of the PA in the membrane) as well as acidic groups on the protein. At these higher levels of Ca²⁺ (e.g., 10 mM), a large fraction of PLD₅₇ should be bound to PC vesicles (assuming the binding will be similar to that measured with Ba²⁺). Yet the enzyme is not maximally active under these conditions and requires PA in the bilayer for a higher specific activity. This clearly indicates that partitioning of the enzyme to a bilayer surface can be separate from optimal active site binding and catalysis. Furthermore, under low Ca²⁺ conditions, PLD₅₇ would bind very tightly to 10 mol % PA (or other anionic phospholipid) that is incorporated into PC membranes. However, the rate of PLD₅₇ catalyzed hydrolysis of POPC with 10 mol % PA in the bilayer, and 0.5 mM Ca²⁺ was inhibited when compared with pure POPC vesicles (11). Again, the binding studies indicate all the enzyme would be on the PC/PA bilayer surface. Clearly, more than just binding to the interface is involved in the kinetic activation of PLD by PA.

A possible specific role of PA-Ca²⁺ in activating PLD₅₇ is to cause a conformational change in the enzyme that alters its accessibility to substrate. Whatever the change, it occurs with intact PLD₅₇ and not with PLD₄₂ (which is already optimally active toward PC vesicles or diC₄PC) or PLD₄₂. This suggests that the PA-Ca²⁺ interaction occurs with the C-terminal domain of the protein. Lipases have “lids” that are opened in the presence of appropriate substrates or cofactors (22, 23), and perhaps a similar lid is formed by the C-terminal domain of PLD₅₇. Substrate accessibility is enhanced, and, more intriguingly, a surface-induced aggregation of PLD₅₇ is observed. Aggregation of PLD₅₇ is particularly interesting because the crystal of a small nuclease that is a member of the PLD superfamily shows a dimer and shared active site (24). However, such aggregation was not detected with monomeric substrates (EDC did not cross-link PLD₅₇ in the presence of diC₄PC/Ba²⁺ or diC₄PA/Ca²⁺) or with PLD₄₂ where enzyme activity is high. Nonactivating anionic lipids (e.g., PI) also induce PLD₅₇ aggregation in the presence of Ca²⁺. The one striking difference in enzyme aggregation induced by PA-Ca²⁺ and PI-Ca²⁺ is that a larger fraction of tetramers are generated with PA. Perhaps the surface activated form of PLD₅₇ is a tetramer.

The results generated thus far with *S. chromofuscus* PLD suggest a model for PA activation of the enzyme. A key component of the model is that there are two sites on the enzyme: an active site and a surface binding site. Regions near the active site of PLD, if similar to the nuclease, are likely to have significant cationic character; hence anionic lipids (such as PA) would bind with a high affinity (Fig. 7A). Low concentrations of...
Ca\textsuperscript{2+} binding to the catalytic site would have small effects on ligand binding. As Ca\textsuperscript{2+} is increased, secondary Ca\textsuperscript{2+} sites on the protein are saturated, and PA becomes clustered in the membrane. The PA-Ca\textsuperscript{2+} binds to an allosteric site on the enzyme that serves to expose the catalytic site to the bilayer surface for easy diffusion of PC substrate. Protein aggregation also occurs under these conditions and may be a component of the kinetic activation of PLD\textsubscript{57}. That monomeric diC\textsubscript{4}PA enhanced both binding of PLD\textsubscript{57} to PC surfaces and hydrolysis, although not as effectively as long chain PA in the bilayer, confirms the PA-Ca\textsuperscript{2+} interaction as allosteric (Fig. 7B). The lower activation by diC\textsubscript{4}PA could correlate with the lack of protein aggregates cross-linked by EDC. The weaker binding of PLD\textsubscript{42} to anionic vesicles and its higher specific activity suggest that, upon proteolytic cleavage, there is a change in the orientation of the C-terminal domain with respect to the catalytic site such that substrate accessibility is not a problem for PLD\textsubscript{42} (Fig. 7C). That PLD\textsubscript{20} and PLD\textsubscript{42} alone bind to PA surfaces with weaker affinity (Fig. 7C) would be expected if the protein is anchored via two distinct domains (C-terminal portion and catalytic domain). Clearly, a detailed structure of PLD will be needed to understand the relationship of C-terminal portion of the protein to the active site. Also critical to sorting out the spatial relationship of the PA allosteric site to the active site is the number and location of Ca\textsuperscript{2+} sites. Because roughly 10% of all the amino acids in PLD are glutamate, it is likely that multiple Ca\textsuperscript{2+} surface sites exist.

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