Phosphohydrolase and transphosphatidylation reactions of two *Streptomyces* phospholipase D enzymes: Covalent versus noncovalent catalysis

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Abstract

A kinetic comparison of the hydrolase and transferase activities of two bacterial phospholipase D (PLD) enzymes with little sequence homology provides insights into mechanistic differences and also the more general role of Ca^{2+} in modulating PLD reactions. Although the two PLDs exhibit similar substrate specificity (phosphatidylcholine preferred), sensitivity to substrate aggregation or Ca^{2+}, and pH optima are quite distinct. *Streptomyces* sp. PMF PLD, a member of the PLD superfamily, generates both hydrolase and transferase products in parallel, consistent with a mechanism that proceeds through a covalent phosphatidylhistidyl intermediate where the rate-limiting step is formation of the covalent intermediate. For *Streptomyces chromofuscus* PLD, the two reactions exhibit different pH profiles, a result consistent with a mechanism likely to involve direct attack of water or an alcohol on the phosphorus. Ca^{2+}, not required for monomer or micelle hydrolysis, can activate both PLDs for hydrolysis of PC unilamellar vesicles. In the case of *Streptomyces* sp. PMF PLD, Ca^{2+} relieves product inhibition by interactions with the phosphatidic acid (PA). A similar rate enhancement could occur with other $HxKx_4D$-motif PLDs as well. For *S. chromofuscus* PLD, Ca^{2+} is absolutely critical for binding of the enzyme to PC vesicles and for PA activation. That the Ca^{2+}-PA activation involves a discreet site on the protein is suggested by the observation that the identity of the C-terminal residue in *S. chromofuscus* PLD can modulate the extent of product activation.

Keywords: Phospholipase D; ^{31}P NMR; phosphatidylcholine; phosphatidic acid; Ca^{2+} activation

Many phospholipase D (PLD) enzymes can catalyze two reactions: (1) hydrolysis of a phospholipid to produce phosphatidic acid (PA) and a free alcohol, and (2) transphosphatidylation of one phospholipid with an alcohol to form a new phosphatidylalcohol. The hydrolysis product PA is an important second messenger in mammalian signal transduction pathways (English 1996). Although products and derivatives of the transphosphatidylation reaction may also have physiological roles in vivo (Exton 1997), the production of a nonnaturally occurring phosphatidylalcohol by PLD has been used as a specific assay for detecting PLD activity in a variety of cells (Heller 1978). This reaction, often carried out in heterogeneous microemulsion systems (e.g., in ether–water), has also been used to synthesize rare phospholipids, unusual nonnaturally occurring lipids, and isotopically labeled phospholipids from naturally abundant PC (Dawson 1967; Eibl and Kovatchev 1981).

Given the usefulness of PLD in generating novel or labeled phospholipids, there have been a number of studies screening different organisms for PLD activities with high transphosphatidylation specific activity. In particular, many *Streptomyces* PLD enzymes, for example, *Streptomyces* sp. PMF, have higher transphosphatidylation activity than plant or many other documented sources of the enzyme (Juneja et al. 1988; Hagishita et al. 1999). A notable exception to this is *Streptomyces chromofuscus* PLD which has significantly lower transphosphatidylation activity compared to its hydrolysis activity (Juneja et al. 1988; Hagishita et al. 1999).

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Unlike the other *Streptomyces* PLDs, the *S. chromofuscus* enzyme does not contain the characteristic $HxKx_4D$ (HKD) domains (Yang and Roberts 2002), but instead has tightly bound iron that is necessary for catalytic activity (Zambonelli and Roberts 2003), suggesting that this PLD catalyzes the hydrolysis of phospholipids via a different mechanism. *S. chromofuscus* can carry out a transphosphatidylation reaction but requires higher concentrations of primary alcohols (Geng et al. 1999). There are few kinetic studies of the transphosphatidylation reaction, particularly in homogeneous solutions. With this in mind, we have examined both hydrolase and transferase activities of both *S. chromofuscus* and *Streptomyces* sp. PMF PLD enzymes using NMR spectroscopy (for example, Fig. 1 for hydrolysis of PI vesicles). Properties of the two different PLDs, including the pH optimum, Ca^{2+} dependence, substrate specificity, and effect of substrate aggregation state on activity have been determined. A comparison of the two enzymes strongly suggests that they have quite different mechanisms—the *Streptomyces* sp. PMF uses covalent catalysis while *S. chromofuscus* PLD utilizes direct attack of water or alcohol on the phosphorus atom to generate PA or new phospholipid products. However, the effect of Ca^{2+} on the kinetics of both enzymes suggests that caution is needed in implicating Ca^{2+} as necessary for activity of PLD enzymes.

Results

Phosphohydrolase activity of Streptomyces *sp. PMF PLD*

The substrate specificity and sensitivity to interfaces of *Streptomyces* sp. PMF PLD, a member of the PLD superfamily (Ponting and Kerr 1996), were examined. Although a variety of phospholipids could be hydrolyzed by *Streptomyces* sp. PMF PLD, the highest specific activity was for PC with anionic phospholipids relatively poor substrates. This Ca^{2+}-independent PLD (5 mM Ca^{2+} had no effect on diC_4PC hydrolysis) showed good activity toward monomeric diC_4PC (Table 1). The dependence of specific activity on chain length at fixed substrate concentration (Table 1) suggested that the longer the chain length, the lower the specific activity of the enzyme. However, if the concentration dependence of this PLD activity were examined, the enzyme activity toward short-chain micelles would have increased substantially (but not in the discreet two-phase model fashion often seen for phospholipases). For diC_7PC, both the hydrolase and transferase data (Fig. 2A) could be fit by a single apparent K_m of 1.6 ± 0.3 mM, a value rather close to the CMC of that PC (1.5 mM). Pure diC_6PC has a CMC of 14 mM (Bian and Roberts 1992). The activity of this PLD as a function of diC_6PC concentration was not hyperbolic (Fig. 2B). If data $\leq$5 mM PC was used to extract an apparent K_m, the value was ~9 mM, a value not too far off the CMC of diC_6PC. The dramatic increase in an apparent K_m that tracks the substrate CMC strongly suggests that the presence of micelles enhances PLD activity (possibly enhancing product release), and that the enzyme is likely to lower the CMC of the substrate. However, the affinity of the enzyme for the surfaces of micelles is not high because the kinetic effect is gradual (at least with this particular substrate). Thus, the *Streptomyces* sp. PMF PLD appears to exhibit some interfacial behavior.

Table 1. Streptomyces *sp. PMF PLD activity toward phospholipids in monomer, micelles, vesicles, and mixed micelles at 37°C*

Substrate	Conc. (mM)	Ca^{2+} (mM)	Physical state	Specific hydrolase[a]	Activity transferase[a]
diC_4PC	5	0	Monomer	12.9	5.20
diC_6PC	5	0	Monomer	9.94	3.26
diC_7PC	5	0	Micelle	6.92	1.69
POPC	10	0	SUV	0.93[b]	
	10	5	SUV	2.7[b]	
	10	5	LUV	0.78[b]	
POPC/chol	5/5	0	SUV	0.33[b,c]	
	5/5	5	SUV	2.7[b,c]	
POPC/POPA	9/1	0	SUV	0.012[b]	
	9/1	5	SUV	1.93[b]	
POPC/TX-100	5/20	0	Mixed micelle		155[d]
POPC/Zwitt[e]	5/15	0	Mixed micelle	0.23	

[a] Hydrolase (generation of PA) and transferase (generation of PG) activities of *Streptomyces* sp. PMF PLD toward 5 mM diC_nPC with 5 mM glyerol present in 50 mM MES-NaOH buffer, pH 6.5; ^{31}P NMR was used to monitor PA and PG production.
[b] The specific activity of the enzyme was measured by ^1H NMR spectroscopy, which monitored choline production, and hence, is a combination of hydrolase and transphosphatidylation reaction rates. Duplicate assay samples were used to obtain rates; errors in the rates were <15%.
[c] The specific activity is based on the rate over the first 10 min; unlike with pure POPC SUVs, the rate decreased over longer times when cholesterol was present in the vesicles.
[d] With this amount of TX-100 present, the major (>90%) product for *Streptomyces* sp. PMF PLD is the transphosphatidylation product with the Triton molecule.
[e] Zwitt = zwittergent 3–14.

PC vesicles were also substrates for *Streptomyces* sp. PMF PLD with much higher activity toward highly curved SUVs than LUVs (Table 1). In the absence of Ca^{2+}, incorporation of 10 mole % PA into the PC vesicles significantly inhibited PMF PLD (leading to an 78-fold decrease in activity). Although Ca^{2+} was not required for the hydrolysis of monomer and micellar short-chain PC substrates by this PLD, addition of Ca^{2+} could enhance *Streptomyces* sp. PMF PLD activity toward PC packed in vesicles. Ca^{2+} (5 mM) increased the activity of *Streptomyces* sp. PMF PLD 2.9-fold toward 10 mM POPC SUVs. Ca^{2+} added to POPC/POPA (9:1) SUVs gave rise to a PLD specific activity that increased 161-fold but was still less than for PC SUVs with Ca^{2+}. With detergent mixed micelle substrates, *Streptomyces* sp. PMF PLD activity could be either activated or inhibited depending on the type of detergent was used. POPC solubilized in Triton X-100 was a much better substrate than

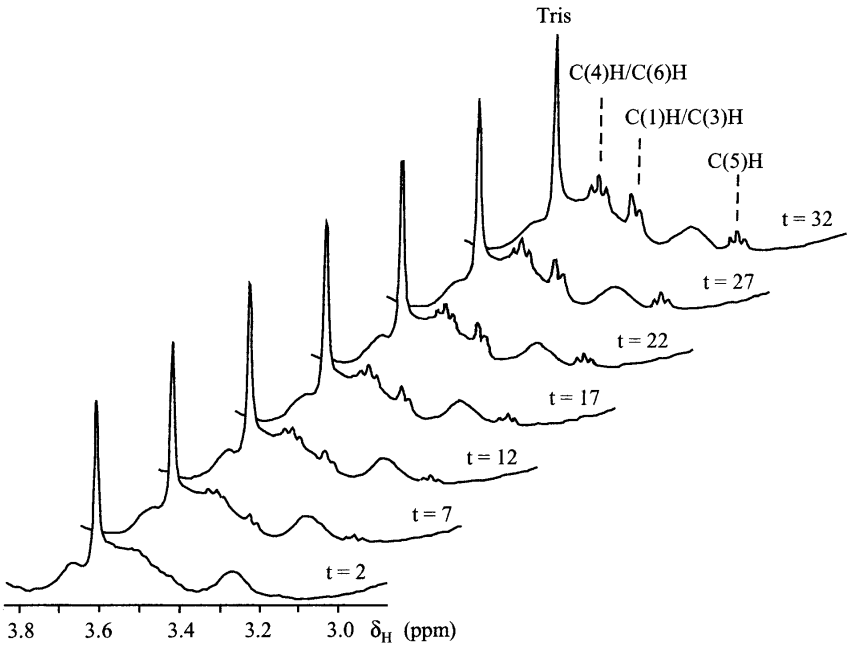

Figure 1. 500 MHz ^{1}H spectra (2.9–3.8 ppm) of 5 mM PI SUVs (in 20 mM imidazole, pH 7.0, with 1 mM Ca^{2+}, 23°C) as a function of incubation time (in minutes) with 135 μg *S. chromofuscus* rPLD. Note the appearance of the sharp myo-inositol peaks (identified by carbon number) in the spectrum.

when presented in SUVs (even with Ca^{2+} added). With PC/Triton X-100 (5 mM/20 mM), the transphosphatidylation reaction led to the formation of a different phosphodiester (the chemical shift of the product peak, shifted slightly downfield of PC, was invariant to pH) that was assumed to be the PA–Triton adduct (Triton X-100 has a hydroxyl group). This new phospholipid represented >90% of the product and was produced at a rate that was significantly activated, 57-fold, above the hydrolysis of PC vesicles with 5 mM Ca^{2+}. The increased specific activity likely represents the high interfacial concentration of Triton. In contrast to Triton X-100, zwittergent 3–14 as a matrix for PC solubilization inhibited hydrolysis of the PC by the enzyme.

Transphosphatidylation activity of Streptomyces *sp. PMF PLD*

Because diC_4PC was a good monomeric substrate for *Streptomyces* sp. PMF PLD, it was used to examine the transphosphatidylation reaction of this PLD. The enzyme is typically stored in 10% (w/w) glycerol. Assay mixtures with 5 mM diC_4PC contained 5 mM glycerol from dilution of enzyme stock into the assay medium. Both diC_4PA and diC_4PG were formed as products with the rate for diC_4PG contributing ~40% of the phospholipid products. Methanol added to the assay mixture competed effectively with water and the glycerol as a nucleophile to generate diC_4PMe from the covalent intermediate (Fig. 3). The mole fraction of diC_4PMe increased with increasing methanol concentration in hyperbolic fashion with an effective K_m for methanol of 0.045 ± 0.013 M (Fig. 3A). The sum of hydrolase and transphosphatidylation reactions of *Streptomyces* sp. PMF was roughly constant as a function of methanol. The ability of water-miscible alcohols to compete effectively with water is a trait similar to that of other HKD-domain PLD enzymes, where low concentrations of 1-butanol or ethanol (e.g., 0.3% to 2% or 0.04 to 0.43 M) have been routinely used to assay PLD activity (Whatmore et al. 1996; Meacci et al. 2002; Xie et al. 2002).

The pH dependence of *Streptomyces* sp. PMF PLD activity toward diC_4PC was examined in the presence of 50 mM methanol, a concentration that allows rates of diC_4PA, diC_4PMe, and diC_4PG formation to be measured. Under these conditions, the activity exhibits a maxima ~ pH 6.5 (Fig. 3B). As might be expected for a mechanism that proceeds through a covalent intermediate, the rates for diC_4PA and diC_4PMe/diC_4PG production behaved in parallel. The ratio of transphosphatidylation product to PA in 50 mM methanol changed very little in this pH range (0.65 at pH 5 to 0.75 at pH 8; Fig. 3B, +). This suggests that the slow step at each pH must be formation of the covalent intermediate with rapid decomposition to either PA or PMe, depending on relative concentrations of the two nucleophiles water and methanol (or glycerol).

Phosphohydrolase activity of S. chromofuscus *rPLD*

In contrast to PLD from *Streptomyces* sp. PMF, PLD from *S. chromofuscus* requires Ca^{2+} for binding to (Stieglitz et al.

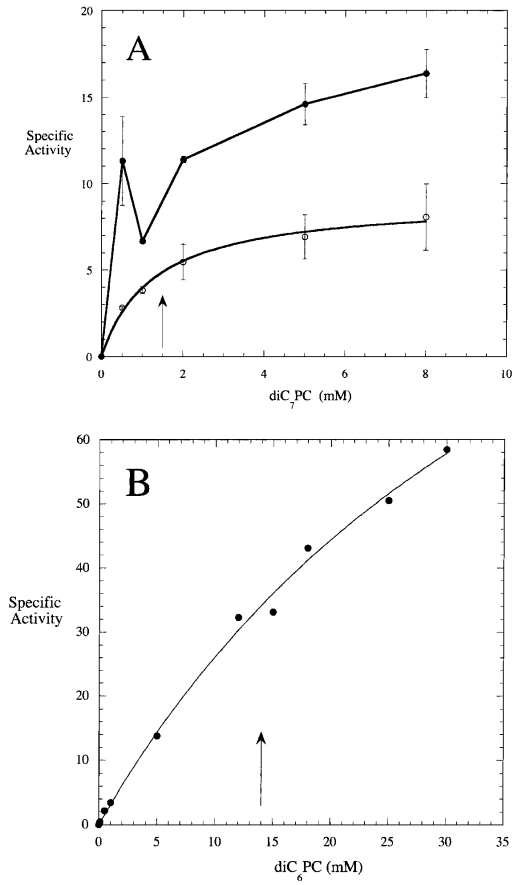

Figure 2. Specific activity (μmole min^{-1} mg^{-1}) of (*A*) *Streptomyces* sp. PMF PLD (open circles) and *S. chromofuscus* rPLD (filled circles) toward diC_7PC in 50 mM Tris HCl, pH 8.0, as a function of substrate concentration. Error bars indicate standard deviations in activity for each PC concentration. (*B*) Specific activity (μmole min^{-1} mg^{-1}) of *Streptomyces* sp. PMF PLD cleavage of diC_6PC as a function of substrate concentration. The arrows indicate the CMC of the pure PC.

1999) and hydrolysis of zwitterionic PC bilayers (Geng et al. 1998), and is activated by its product PA in the presence of Ca^{2+} (Geng et al. 1998). Although it carries out a transphosphatidylation reaction, this PLD has been reported to require significantly higher alcohol concentrations than HKD-type PLD enzymes (Juneja et al. 1988). Recent work has shown that *S. chromofuscus* PLD contains iron as well as zinc or manganese, and that the tightly bound iron ion is critical for catalysis (Zambonelli and Roberts 2003). Addition of EDTA to reaction mixtures interacts with these transition metals as well as any Ca^{2+}, and inactivates the enzyme. These observations suggest that this bacterial PLD carries out PC cleavage with a different mechanism than that of the HKD-type PLD enzymes. With this in mind, we examined the activity of a recombinant *S. chromofuscus* PLD (rPLD) toward a variety of substrates for comparison with the *Streptomyces* sp. PMF enzyme.

As with the rPLD cloned from the ATCC *S. chromofuscus* strain, Ca^{2+} caused a biphasic decrease in the USDA rPLD intrinsic fluorescence. The decrease was characterized by an S_{50} value of 0.51 mM (~5 times higher than the S_{50} of ATCC rPLD). However, even though this rPLD could bind Ca^{2+} ions, they were not necessary for catalytic activity under some circumstances. At pH 8, rPLD cloned from the USDA strain of *S. chromofuscus* was active toward diC_4PC in the absence of Ca^{2+} (the K_m for this monomeric substrate was ~0.3 mM). Addition of Ca^{2+} had no effect on the hydrolase activity under these conditions. The lack of activation by Ca^{2+} is in contrast to the enzyme cloned from the ATCC strain of the organism, which was significantly activated (5.6-fold) by 5 mM Ca^{2+} under the same conditions (Yang and Roberts 2002). With increasing monomeric substrate fatty acyl chain length (Table 2), rPLD activity (in the absence of Ca^{2+} and at pH 8) increased (from 14 to 54 μmole min^{-1} mg^{-1} as chain length increased from four to

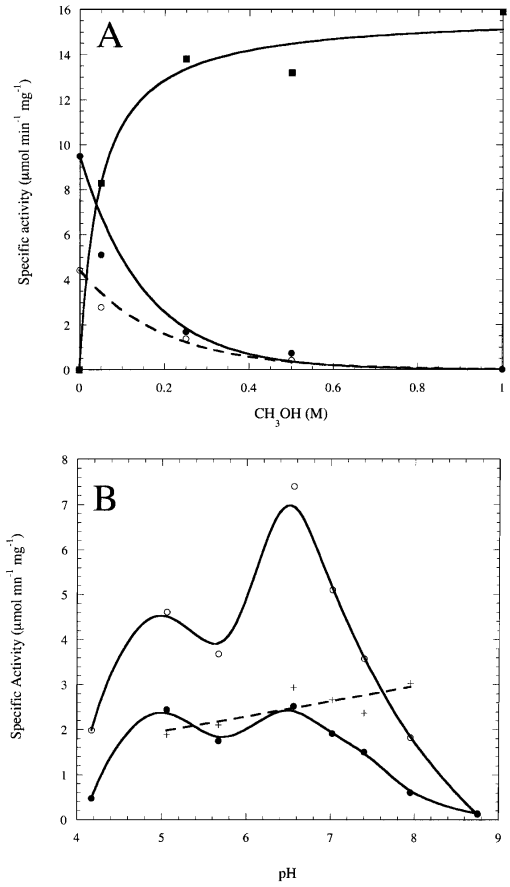

Figure 3. (*A*) Rates of diC_4PA (filled circles), diC_4PMe (filled squares), and diC_4PG (open circles) production (μmole min^{-1} mg^{-1}) by *Streptomyces* sp. PMF PLD (4 μg) as a function of CH_3OH concentration at fixed (5 mM) diC_4PC (assay mixtures also contained 5 mM glycerol from the enzyme stock). (*B*) Rates of diC_4PA (filled circles), diC_4PMe, and diC_4PG production (open circles) from diC_4PC (5 mM) as function of pH with 5 mM glycerol and 50 mM CH_3OH; (+) represents the mole fraction transferase products (diC_4PG and diC_4PMe) generated by *Streptomyces* sp. PMF PLD as a function of pH.

Table 2. S. chromofuscus *rPLD activity toward phosphatidylcholine substrates*

Substrate	Concentration (mM)	Physical state	Specific activity[a] (μmole min^{-1} mg^{-1})
diC_4PC	5	Monomer	14[b]
diC_6PC	5	Monomer	41[b]
diC_7PC	0.5	Monomer	54[b]
	5	Micelle	68[b]
1-C_6PC	5	Monomer	33[b]
POPC	10	SUVs	11.7[c]
	10	LUVs	12.5[c]
POPE	10	SUVs	3.7[c]
	10	LUVs	1.5[c]
DOPMe	10	SUVs	0.44[c]
	10	LUVs	0.80[c]
POPG	10	SUVs	0.013[c]
POPC/DOPMe	5/5	SUVs	11.9 (PC)/0.46 (PMe)[c]
POPC/DOPMe/chol	5/5/5	SUVs	11.5 (PC)/0.93 (PMe)[c]
POPS	10	SUVs	0.0020[c]
PI	5	SUVs	0.058[c]
SM	5	SUVs	0.0021[c]

[a] Errors in specific activities (rates for given assay samples were done in duplicate) are <15%.
[b] Activities determined by pH-stat at pH 8.0 with 5 mM Ca^{2+} present.
[c] Activities determined by ^{31}P NMR at pH 7.0 in 20 mM imidazole and 5 mM Ca^{2+} (except for PI, which had 1 mM Ca^{2+}).

seven). A monomeric lyso-PC (1-C_6-PC) was almost as good a substrate as the diacyl-PC (diC_6PC). The activity of the enzyme toward various diC_7PC concentrations was examined in the absence of Ca^{2+} (Fig. 2A) to check for interfacial activation. In the absence of Ca^{2+}, *S. chromofuscus* rPLD exhibited a small (25%) increase in activity upon micellization of diC_7PC (comparison of 0.5 and 5 mM concentrations). Similar behavior was observed for the PLD purified from *S. chromofuscus* media (Geng et al. 1999). This very small activation was much less than that of other phospholipases (Roberts 1999), and, with the exception of the activity at 1 mM diC_7PC (right around the effective CMC in the assay mixture), could be accounted for by a K_m ~0.3–0.5 mM. The decreased specific activity at 1 mM (near the pure diC_7PC CMC of 1.5 mM; Bian and Roberts 1992) could indicate that when micelles form, they initially lower the number of particles (monomers plus micelles) available to interact with the enzyme (effectively reducing the particle concentration). Such behavior would be consistent with "hopping" mode kinetics (nonprocessive catalysis in the presence of an interface) for this particular phospholipase (Jain and Gelb 1991).

Although no Ca^{2+} was needed for hydrolysis of the short-chain PC substrates by this recombinant *S. chromofuscus* PLD, this divalent cation was absolutely critical for hydrolysis of PC vesicles (Table 2). With 5 mM Ca^{2+} present, *S. chromofuscus* PLD specific activities toward PC vesicles were similar to that for diC_4PC hydrolysis. There was also little difference in enzyme activity toward PC SUVs and LUVs. This latter observation is unusual in that most phospholipases (including *Streptomyces* sp. PMF PLD) show higher activity toward PC substrates packed in small, highly curved vesicles than towards LUVs where the interface is relatively flat (Geng et al. 1998; Gadd and Biltonen 2000). Previous studies have shown that *S. chromofuscus* PLD prefers monomeric diC_6PC over similar chain length anionic phospholipids, although most of these are reasonable substrates for the enzyme (Geng et al. 1999; Martin et al. 2000). As shown in Table 2, zwitterionic phospholipid vesicles (e.g., PC and PE) were much better substrates than anionic phospholipid vesicles (PMe, PS, PI, PG) for this recombinant enzyme. The severely reduced activity of the recombinant *S. chromofuscus* protein toward anionic phospholipid vesicles does not reflect differences in binding of the enzyme to vesicles because in binary vesicles of PC and PMe, the rates of hydrolysis of each substrate were comparable to the rate toward the single component vesicle. Decreasing the flexibility of phospholipids by incorporation of cholesterol (Table 2) did not affect PLD hydrolysis of PC and PMe in the binary vesicle, an observation consistent with a "hopping" mode for this enzyme.

It has been previously reported (Imamura and Horiuti 1979) that sphingomyelin is a substrate for *S. chromofuscus* PLD. rPLD has very low sphingomyelinase activity (~5000 times lower than that toward PC SUVs). This ratio was significantly different from the reported ratio of 22% for sphingomyelinase activity compared to PLD activity in experiments that used PLD purified from culture medium (Imamura and Horiuti 1979), and hence, could have been contaminated with sphingomyelinase.

Effect of the C terminus on rPLD phosphohydrolase activity and vesicle binding

Although substrate physical state has little effect on PLD activity, modification of the C-terminal residue can dramatically affect the enzyme specific activity. The specific activities of rPLD and rPLD-E_{510} toward diC_4PC (5 mM) and POPC SUVs (10 mM) are shown in Table 3. Replacement of the C-terminal valine with glutamate yielded protein with much higher specific activity. Previous studies of the PLD purified from *S. chromofuscus* culture media suggested that the C-terminal region was important for PA activation of the enzyme toward PC vesicles (Stieglitz et al. 1999). Therefore, the recombinant PLDs were screened for PA (10 mole %) activation of PC SUVs (Table 3). rPLD-E_{510}, with higher specific activity toward diC_4PC, also exhibited a larger PA activation (threefold) compared to rPLD with a C-terminal valine (which exhibited 1.8-fold PA activation). As with the authentic *S. chromofuscus* PLD (Geng et al. 1998), other anionic phospholipids either had no effect (PI) or were inhibitors (oleic acid) of PC hydrolysis. Replacement of the C-terminal valine with a lysine residue (rPLD-

Table 3. *Comparison of the specific activities of C-terminal mutants of* S. chromofuscus *PLD toward diC_4PC (5 mM) and POPC (10 mM) vesicles*

	rPLD-V_{510}	rPLD-E_{510}	rPLD-K_{510}
Substrate		Specific activity[a]	
diC_4PC[b]	13.5	56.2	21.0
POPC[c]	11.7	47.4	14.4
POPC/POPA[c]	21.1	142	18.7
Binding[d]		%	
POPC + EDTA	32	4	19
POPC + Ba^{2+}	100	100	100
POPC/POPA (9:1) + EDTA	51	66	10
POPA + EDTA	94	100	70

[a] Typical errors in measuring PLD activity ≤15%.
[b] Specific activities of PLD enzymes toward this monomeric substrate where measured by pH-stat at pH 8.0, with 5 mM Ca^{2+}.
[c] Specific activities of PLD toward SUVs were measured by ^{1}H NMR in 20 mM imidazole, 5 mM Ca^{2+}, pH 7.0.
[d] All binding assays were carried out with small sonicated vesicles containing 2 mM total phospholipids in 10 mM Tris HCl, pH 8.0; the error in integrating the free PLD on SDS-gels was typically 10% of the total PLD intensity.

K_{510}) did not affect the specific activity toward diC_4PC but did further reduce the extent of PA activation of the enzyme (at most the rate increased 20% with PA added).

Previous studies have shown that divalent metal ions and PA can enhance PLD partitioning to the surfaces of POPC vesicles (Stieglitz et al. 1999; Yang and Roberts 2002). Similar experiments were carried out for this recombinant USDA strain PLD, and rPLD-E_{510}, and rPLD-K_{510}. Like ATCC PLD (Yang and Roberts 2002) and authentic *S. chromofuscus* PLD purified from growth media (Stieglitz et al. 1999), rPLD partitioned weakly to mM concentrations of PC vesicles in the presence of EDTA. Addition of 10 mM Ba^{2+} dramatically promoted PLD partitioning to the lipid surfaces. With 1–2 mM PC vesicles the added Ba^{2+} caused all the enzyme to translocate to the vesicle surface (Table 3). In the absence of any divalent metal ion (5 mM EDTA in buffer), rPLD had high binding affinity for PA vesicles. Incorporation of 10% PA with PC led to ~51% rPLD enzyme partitioning to the PC/PA (9:1) vesicles under these conditions. With rPLD-E_{510}, divalent metal ions were still needed for the enzyme to bind to PC vesicles. However, binding to PC/PA (9:1) SUVs in the absence of Ba^{2+} was even stronger than for rPLD with a C-terminal valine. Moreover, the rPLD-K_{510} mutant with the positively charged C-terminal residue had the weakest binding affinity to PC/PA (9:1) and PA SUVs (Table 3). This suggests that a negative charge at the C terminus alters the enzyme conformation in a way that it has stronger interactions with a PA surface. Whatever the interaction of PLD with anionic vesicles, the C terminus cannot be directly involved because a positively charged C terminus leads to weaker interactions with a surface with negative charges.

Transphosphatidylation activity of rPLD

Monomeric diC_4PC was used as a substrate in the presence of methanol and Ca^{2+} to examine the transphosphatidylation specific activity of rPLD under different conditions. This is an excellent substrate to use for this PLD because the product, diC_4PA, does not precipitate with ≤20 mM Ca^{2+} added. Screening for optimal methanol concentration was carried out with 5 mM diC_4PC and 5 mM Ca^{2+} (the latter suggested as necessary for transphosphatidylation activity by this PLD; Geng et al. 1999). Below 1 M (3.2% w/w) methanol, there was no detectable diC_4PMe. Increasing the methanol concentration led to accumulation of diC_4PMe with optimal production of diC_4PMe around 10–12 M methanol under these conditions (Fig. 4). Higher methanol concentrations caused denaturation of the recombinant PLD and decreased the production of both diC_4PA and diC_4PMe. Interestingly, addition of methanol to the assay mixtures also activated the phosphohydrolase activity of rPLD (e.g., with 6 M CH_3OH, diC_4PA production was increased 3.8-fold). DiC_4PA production reached a maximum around 10 M methanol (Fig. 4A). This is in dramatic contrast with HKD-motif PLDs (including the *Streptomyces* sp. PMF PLD examined here) where 1% ethanol or other small alcohols could block the phosphodiesterase activity to yield only transphosphatidylation product (Juneja et al. 1988; Xie et al. 2002).

Because *S. chromofuscus* PLD from the USDA strain does not need Ca^{2+} for diC_4PC hydrolysis, the requirement for Ca^{2+} in the transphosphatidylation reaction was examined. At pH 5.5 and 6 M methanol, no transphosphatidylation product, PMe, was produced from diC_4PC alone. Addition of 5 mM Ca^{2+} activated the hydrolysis reaction around fourfold at this pH but there was still little diC_4PMe generated. At pH 8.0, addition of 5 mM Ca^{2+} had little effect on PLD phosphohydrolase activity toward diC_4PC but significantly enhanced the transphosphatidylation reaction (Fig. 5). Increasing the Ca^{2+} concentration further to 20 mM slightly inhibited the hydrolysis reaction of rPLD at pH 8.0 but had little effect on diC_4PMe production. At pH 9.0, Ca^{2+} was not necessary for either hydrolysis or transphosphatidylation reactions. In fact, addition of Ca^{2+} inhibited both activities with much higher inhibition of PA production. The highest ratio of diC_4PMe to diC_4PA generated by *S. chromofuscus* rPLD was 2.9 at pH 9.0 in the presence of 20 mM Ca^{2+}. By contrast, *Streptomyces* sp. PMF PLD with 1 M methanol yielded exclusively diC_4PMe as a product.

A more careful investigation of the pH dependence of hydrolase and transferase activities was undertaken for comparison to the pH profile for *Streptomyces* sp. PMF PLD. Similar to the pH profile for the hydrolysis reaction measured by pH-stat (Geng et al. 1999), which appeared to have broad peak from pH 6 to 8, the optimum pH for rPLD hydrolase activity measured by ^{31}P NMR in the presence 5 mM Ca^{2+} was around 7.5. In the presence of 2 M CH_3OH,

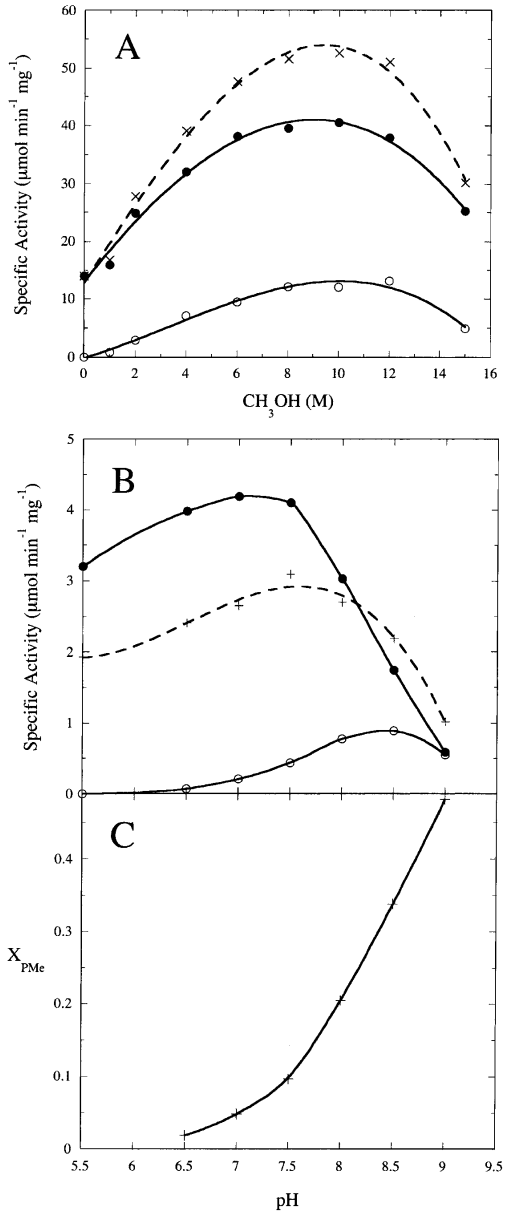

Figure 4. (*A*) Rate of diC_4PA (filled circles) and diC_4PMe (open circles) production by *S. chromofuscus* rPLD from 5 mM diC_4PC in 50 mM Tris HCl, 5 mM Ca^{2+}, pH 7.5, as a function of added CH_3OH. Enzyme specific activity for generating both products is shown as (X). (*B*) The pH dependence of the rate of diC_4PA production in the absence (+) and the presence (filled circles) of 2 M CH_3OH; the rate of diC_4PMe (open circles) production in the presence of 2 M CH_3OH is also shown. (*C*) Mole fraction of PMe produced as a function of pH with 5 mM diC_4PC, 5 mM Ca^{2+} and 2 M methanol, pH 8.0.

which generates some transphosphatidylation product, the optimum pH for the phosphohydrolase activity was slightly more acidic. In contrast, the optimum pH for the transphosphatidylation reaction was 8.5 (Fig. 4B). The mole fraction of transphosphatidylation product as a function of pH increased steadily with increasing pH (Fig. 5C) in contrast to the HKD-motif *Streptomyces* sp. PMF PLD, which exhibited a relatively constant mole fraction of PMe and PG over a comparable pH range.

Discussion

We have examined two *Streptomyces* PLDs—one a member of the PLD superfamily (see Stuckey and Dixon 1999, for the first structure of a PLD family member) whose mechanism involves covalent catalysis and for which a crystal structure exists (Leiros et al. 2000), and the other a very disparate protein with virtually no sequence similarity and whose activity, dependent on the presence of tightly bound iron, is also activated by Ca^{2+} (Zambonelli and Roberts 2003)—for sensitivity to substrate physical state, substrate specificity, pH effects, and whether or not Ca^{2+} can enhance activities. A comparison of these two PLDs provides insights into mechanistic differences and also the more general role of Ca^{2+} in modulating PLD reactions.

Both bacterial PLDs exhibited a preference for PC, although each could catalyze the hydrolysis of a range of phosphodiester bonds. A preference for PC when presented in bilayers has been observed in most eukaryotic HKD-type PLDs identified so far, although there are reports that some

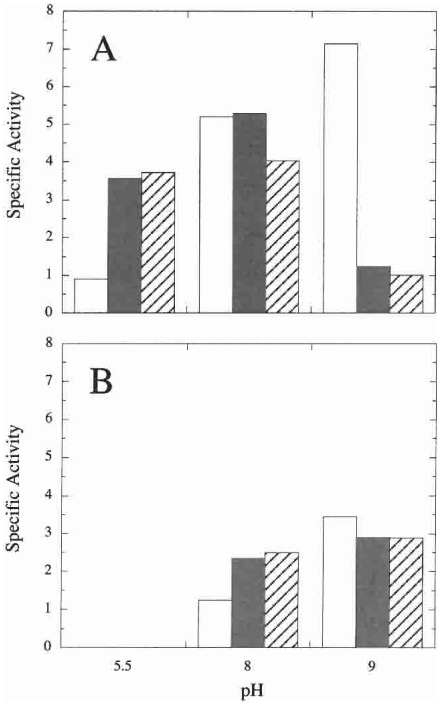

Figure 5. Effect of Ca^{2+} concentration on the (*A*) hydrolysis and (*B*) transphosphatidylation reactions of *S. chromofuscus* rPLD. Assay conditions included 5 mM diC_4PC, 6 M CH_3OH, and 9 μg rPLD. The empty rectangles represent assays in the absence of Ca^{2+}; the filled rectangles represent assays with 5 mM Ca^{2+}; diagonally striped rectangles represent assays with 20 mM Ca^{2+} added.

PLDs prefer phospholipids with other headgroups (e.g., a recently identified intracellular PLD from *Streptoverticillium cinnamoneum* is reported to prefer PE followed by PS and PI, while it is inert for PC and PG; Ogino et al. 2001). When a preference for PC is observed for substrate presented in a bilayer, it may reflect properties of the phospholipid in the bilayer (e.g., accessibility of headgroup, local dielectric constant) rather than an inherent weaker efficiency for the other phospholipids.

Where the two *Streptomyces* PLD enzymes begin to differ is in the sensitivity to substrate physical state. *Streptomyces* sp. PMF PLD, like a more typical phospholipase (Roberts 1999), exhibited moderate interfacial activation (the "K_m" for the enzyme toward diC_7PC correlates with the CMC of that lipid, and a comparison of specific activities for monomeric and micellar diC_6PC clearly showed rate enhancement for micellar substrate) and reduced activity toward PC packed in vesicles compared to PC solubilized in mixed micelles. In contrast, *S. chromofuscus* PLD was insensitive to aggregation of short-chain PC substrates and equally active towards PC packed in both small unilamellar and large unilamellar vesicles. This insensitivity to PC physical state (lack of classical interfacial activation, no increase in PMe or PC cleavage when binary vesicles used, insensitivity to the inclusion of cholesterol in the vesicles) strongly indicates that *S. chromofuscus* PLD works in a hopping mode and is easily able to isolate the phosphodiester bond of an individual PC molecule in an interface.

Effects of Ca^{2+} on PLD activities

Both Ca^{2+}-dependent and Ca^{2+}-independent PLD enzymes have been identified in mammals (Exton 1997, 2000), yeast (Mayr et al. 1996; Hammond et al. 1997; Waksman et al. 1997) and bacteria (Imamura and Horiuti 1979; Juneja et al. 1988; Saito et al. 1990; Hasagawa et al. 1992; Shimbo et al. 1993), and nearly all plant PLDs require micro- to millimolar Ca^{2+} for stimulation (Wang 2000). When a Ca^{2+} requirement exists, the question is whether it reflects a direct binding of that cation to the enzyme (causing a change that enhances substrate binding, optimizes catalysis, promotes product release from the enzyme, etc.), or whether it reflects physical effects on the product PA. The PLD product PA has a moderately high affinity for Ca^{2+}; addition of this ion will cause PA to cluster in bilayers, and in some situations lead to vesicle fusion (Swairjo et al. 1994). PA could also be a competitive inhibitor of substrate and inclusion of Ca^{2+} in assay systems could interact with PA and effect its release from the active site. With *S. chromofuscus* PLD, we have shown that added Ba^{2+} and Ca^{2+} reduce the high affinity of that enzyme for PA surfaces (Stieglitz et al. 1999).

S. chromofuscus PLD purified from growth medium (Geng et al. 1999; Stieglitz et al. 1999; El Kirat et al. 2002) and recombinant PLD from the ATCC strain of *S. chromofuscus* (Yang and Roberts 2002) have been demonstrated to require Ca^{2+} for binding to lipid surfaces and enhancing catalysis (both phosphohydrolase and phosphatidylation reactions). Ca^{2+} is also an activator when other soluble substrates (e.g., *p*-nitrophenylphosphocholine, bis-*p*-nitrophenylphosphocholine) are examed (Zambonelli and Roberts 2003). rPLD cloned from a second *S. chromofuscus* strain also requires Ca^{2+} for binding to PC vesicles. However, with short chain diC_4PC as substrate the dependence of rPLD activity on Ca^{2+} is not absolute. At acidic pH, Ca^{2+} enhanced the hydrolysis reaction, while there was no phosphatidylation product diC_4PMe even in the presence of 6 M methanol. Under basic conditions (most notably pH 9), Ca^{2+} inhibited both phosphohydrolase and transferase reactions. A similar pH-dependent Ca^{2+} stimulation was also observed in plant PLD, although the trend was reversed with protein activated by millimolar range Ca^{2+} at neutral pH but by 50 μM Ca^{2+} at pH 4.5–5.0 (Pappan and Wang 1999). This confirms that Ca^{2+} is not absolutely necessary for *S. chromofuscus* PLD hydrolase activity, although it can enhance catalysis. Similar to *S. chromofuscus*, *Streptomyces* sp. PMF PLD is Ca^{2+}-independent with diC_4PC as a substrate at neutral or more basic pH. The only activation observed with monomeric PC is at acidic pH values.

Where Ca^{2+} has a major role is in the PLD-catalyzed hydrolysis of PC vesicles. *S. chromofuscus* PLD (recombinant protein from both strains of the organism) does not bind well to PC vesicles without that cation, so the observed PLD specific activity is very Ca^{2+}-dependent. In contrast, *Streptomyces* sp. PMF PLD could bind and hydrolyze PC SUVs well without Ca^{2+}, although there was a threefold increase in rate with Ca^{2+} added. Part of the Ca^{2+} activation observed for *S. chromofuscus* PLD (and probably most of the activation seen with *Streptomyces* sp. PMF PLD) is likely due to relief of inhibition of PLD by product PA. Both enzymes were inhibited by PA; for the Ca^{2+}-independent PMF PLD, the rate was ~78-fold lower with 10 mole % PA in the PC vesicle. Addition of Ca^{2+} enhanced the rate to a value approaching that observed for *Streptomyces* sp. PMF PLD action on PC SUVs with Ca^{2+} present. These observations are consistent with Ca^{2+} enhancing *Streptomyces* sp. PMF PLD activity towards vesicles by complexation of PA and preventing it from binding to the enzyme active site. This may be a major factor in explaining the Ca^{2+}-enhancing effects of many other PLDs as well.

For *S. chromofuscus* PLD, PA inhibition depends on the Ca^{2+} concentration. With ~0.2 mM Ca^{2+}, which saturates at least one binding site on the enzyme (Stieglitz et al. 2001) and is more than enough for the maximum activity toward diC_4PC (Geng et al. 1998), *S. chromofuscus* activity toward PC vesicles with 10 mole % PA was inhibited ~10-fold compared to pure PC vesicles. Addition of 5 mM Ca^{2+} to the PC/PA (9:1) vesicles led to a rate enhancement over and above that observed for single-component PC vesicles and

Ca^{2+} (a twofold increase with rPLD), consistent with a specific interaction of Ca^{2+}-PA and the enzyme. For *S. chromofuscus* PLD, the Ca^{2+}-PA activation appears to involve the C terminus of the protein. Interestingly, the charge of the C terminus is key. There was a fourfold difference in specific activity and an enhanced PA activation when the C terminus was changed from neutral and hydrophobic valine to a negatively charged glutamate side chain. In the presence of EDTA, binding of rPLD-E_{510} to PC/PA bilayers was enhanced by this negatively charged side chain at the C terminus (a result that might seem counterintuitive because the membrane surface already has a negative charge due to the PA). Switching the side chain to positively charged lysine reduces PA activation while having little effect on specific activity.

S. chromofuscus PLD appears to have a binuclear metal ion site responsible for phosphodiester bond cleavage with at least Cys123, Asp151, Tyr154, and His391 residues critical for iron content (Zambonelli and Roberts 2003). Only one residue thus far, His226, has been linked to PA activation (Yang and Roberts 2002). H226A, with roughly 0.10 times the activity of rPLD towards PC substrates, exhibited a greatly enhanced PA activation, while binding to vesicles was not significantly affected. The results with C-terminal mutants also suggest that there really is a distinct site on the enzyme for PA binding that leads to kinetic activation. Whether or not either the C terminus or His226 is a direct part of this site is unclear at present, and likely will require detailed structural information.

Covalent versus noncovalent catalysis

The mechanism of HKD-type of PLD enzymes has been proposed to center around production of a phosphatidyl–histidine intermediate (Leiros et al. 2000). Indeed, for other members of the PLD superfamily, a phosphoryl–enzyme intermediate has been detected (Gottlin et al. 1998). Furthermore, it has been proposed that this first step, forming the phosphoryl–histidyl intermediate, should be the rate-limiting step (Leiros et al. 2000). The second nucleophilic attack by a water molecule or methanol should have little significant effect on catalytic rate. Such a mechanism predicts production of PA and the transphosphatidylation product in parallel as long as it is formation of the intermediate and not its decomposition that is rate limiting. Generating a pH profile for the phosphatidylation reaction is difficult to do in a heterogeneous assay system (e.g., a solvent system of ether–water mixed with alcohol is typically used). DiC_4PC is the ideal substrate for activities with mixed water-miscible solvents because it does not form aggregates (Bian and Roberts 1992). The present work with this monomeric substrate shows that the two reactions of *Streptomyces* sp. PMF PLD have the same pH optima. However, the two reactions catalyzed by *S. chromofuscus* PLD exhibit different pH optima with a considerably more basic optimum pH for the transphosphatidylation activity such that the ratio of the two products is not constant but increases dramatically with increasing pH. The requirement for Ca^{2+} for production of PA at acidic pH values is also not consistent with decomposition of a covalent intermediate becoming rate limiting. The shifted pH profiles and differential Ca^{2+} requirement for these two reactions either indicate that *S. chromofuscus* PLD catalyzes PC cleavage in a single step (where either water or the alcohol is the nucleophile and a more basic pH is needed to increase the nucleophilicity of the alcohol) or forming the substrate–enzyme intermediate is not rate limiting and the ionization status of the second nucleophile critically affects the reaction rate.

Although most of the identified PLDs have both phosphodiesterase and transferase activities, the transphosphatidylation reaction is regarded as the hallmark of the enzyme–substrate intermediate (Exton 1997, 2000). There are some PLDs identified, including a bacterial PLD (Ogino et al. 2001), a yeast Ca^{2+}-dependent PLD (Mayr et al. 1996; Waksman et al. 1997), and a mammalian mitochondrial PLD (Madesh and Balasubramanian 1997), that have no transferase activity (or at least none under conditions where other PLDs show efficient transferase actvity). For eukaryotic PLDs that catalyze both reactions, 1% ethanol could block the hydrolysis reaction and produce only the phosphatidylation product, phosphatidylethanol. In marked contrast, the hydrolysis activity of the yeast PLD identified from *Saccharomyces cerevisiae* is stimulated by alcohol (Ella et al. 1995). Similar to this yeast PLD, our recombinant USDA PLD was also activated by alcohol. In the presence of 6 M methanol, the hydrolysis activity of USDA PLD is activated 3.8-fold. In general, addition of methanol will decrease the activity of water molecules and inhibit the hydrolysis reaction. Activation by methanol likely reflects the influence of a more hydrophobic environment at the PLD active site that can enhance substrate binding and catalysis.

Cofactors and activators of a non-HKD-motif PLD

The work presented on the *S. chromofuscus* PLDs strongly supports a direct attack of water (or alcohol) on the phosphorus and regulation of enzyme activity via the interaction of two functionally distinct phospholipid binding sites on the enzyme: (1) the active site where a binuclear metal center is absolutely essential for catalysis (Zambonelli and Roberts 2003), and (2) an activator site that is specific for PA or other amphiphilic phosphomonoesters (Geng et al. 1998; Stieglitz et al. 1999). Ca^{2+} binds to this PLD but under some conditions (e.g., high pH) is not needed for catalysis with monomer or micelle substrates. A major effect of Ca^{2+} binding is to cause a conformational change in the protein that enhances binding of the protein to zwitte-

rionic interfaces. Previous work suggested PLD binding to PC vesicles with Ba^{2+} as the noncatalytic Ca^{2+} substitute was insensitive to pH over the range 5–9. PA-driven binding to vesicles, however, was sensitive to pH with a pKa ~7 (Stieglitz et al. 1999). Deprotonation of a key side chain (possibly a histidine) on the enzyme as the solution pH increases shifts the equilibrium toward a vesicle-bound form. Ca^{2+} may be critical (but not absolutely necessary) at acidic pH values (for even monomeric substrates) by lowering the pK_{a2} of active site residues. In the absence of divalent cations (and the presence of EDTA), product PA can drive the PLD to the membrane surface. This binding mode is complicated because PA can occupy the active site as well as another site. The Ca^{2+} site (or sites) on *S. chromofuscus* PLD could resemble a C2 domain and the activating interaction could involve a Ca^{2+}-PA complex. Indeed, Ca^{2+} binding to rPLD does cause a loss of secondary structure as measured by ellipticity at 222 nm (C. Zambonelli, unpubl.) as has been seen for plant PLD C2 domains (Zheng et al. 2000). Looking for sequence homologies to known C2 sequences does not identify a C2 domain in the *S. chromofuscus* PLD sequence; however, such a β-sandwich structural domain could still occur. The combination of Ca^{2+} and PA could alter the PLD conformation to (1) destabilize active site binding of PA and (2) allow more productive binding to substrate and/or release of product. That PA can bind to the enzyme at other than the active site and enhance catalysis is confirmed with mutants with altered PA activation. Neither His226 nor Val510 are near residues in the sequence known to be critical for iron binding in the protein. However, mutation of either of these positions can affect PLD specific activity and more importantly the extent of PA activation.

Materials and methods

Chemicals

All phospholipids were obtained from Avanti and used without further purification. The long-chain phospholipids used were all 1-palmitoyl-2-oleoyl phospholipids with the exception of dioleoylphosphatidylmethanol (DOPMe). Triton X-100 was obtained from Sigma while Zwittergent 3–14 was obtained from Calbiochem. The *Streptomyces sp*. PMF PLD, 95% pure, was obtained from Biomol. All the other chemicals were of the highest purity available. The DNA extraction kit was obtained from Stratagene. Oligonucleotide primers, for amplification of the *pld* gene from *S. chromofuscus* DNA, were purchased from Operon Technology. The Advantage GC genomic PCR kit was purchased from Clonetech. Restriction enzymes and plasmids pTYB11 and ER2566 *Escherichia coli* strains were purchased from New England BioLabs.

PLD overexpression and purification

PLD cloned from the ATCC *S. chromofuscus* strain 23616 (Yang and Roberts 2002) has a slightly different sequence (87% identity) from the *S. chromofuscus* PLD published previously (Yoshioka et al. 1991) Differences in some kinetic parameters could be related to the sequence changes, so the *S. chromofuscus pld* gene was cloned from the originally sequenced strain of the organism (strain NRRL 11098 from the USDA) using primers based on the published sequence for this enzyme (Yang and Roberts 2002). To optimize purification, the *pld* gene was inserted into pTYB11 to generate an intein-PLD fusion protein. The PLD proteins rPLD, rPLD-E_{510} and rPLD-K_{510} (where the C-terminal residue was valine as in the original sequence, changed to negatively charged glutamate, or to positively charged lysine residue using a QuikChange mutagenesis kit) were purified as described previously (Yang and Roberts 2002). About 4 to 11 mg rPLD protein (and similar amounts of mutants) could be obtained from 1 L cell culture.

Analysis of the secondary structure of rPLD, rPLD-E_{510}, and rPLD-K_{510} by CD spectroscopy revealed the same secondary structure content and T_m (59°C) as the ATCC recombinant PLD (Yang and Roberts 2002). High concentrations of the protein also exhibited a visible absorption band (λ_{max} ~500 nm) similar but not identical to that of the *S. chromofuscus* PLD cloned previously from the ATCC strain of the organism (λ_{max} ~570 nm; Zambonelli and Roberts 2003). This pink color was also correlated with the PLD activity. Dialyzing rPLD solution extensively against 20 mM Tris-HCl buffer with 2 mM EDTA, pH 8.0, caused loss of the pink color and loss of the PLD activity.

^{31}P NMR assays of PLD activity

PLD specific activities toward short-chain diacylphosphatidylcholine (diC_nPC), including diC_4PC, diC_6PC and diC_7PC, and detergent/1-pamitoyl-2-oleoyl-phosphatidylcholine (POPC) mixed micelles were measured by ^{31}P NMR spectroscopy in the absence of Ca^{2+} as described previously (Geng et al. 1999). Samples typically included 5–10 mM phospholipid in 50 mM buffer (variable depending on pH) in a volume of 0.5 mL, of which 0.1 mL is D_2O. The diC_nPA product was easily detectable as a pH-dependent resonance (δ_P = 1.9, 3.2, and 3.8 ppm at pH = 6.4, 7.1, and 8.0, respectively) downfield from the PC resonance (−0.5 ppm). The transphosphatidylation reaction was examined with diC_4PC and CH_3OH as substrates. The chemical shift for dibutyroylphosphatidylmethanol (diC_4PMe) did not vary significantly over the pH range studied (pH 5.5 to 9). Most rates were determined from fixed time point assays (3–20 min) where the *Streptomyces* sp. PMF PLD reaction was stopped by heating at 98°C in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus) and the *S. chromofuscus* rPLD reaction was stopped by the addition of 50 mM EDTA. Amounts of enzymes added varied between 1 and 18 μg. All rates are based on assays run at least in duplicate.

^{1}H NMR assays of PLD activity

^{1}H NMR spectra were acquired to monitor the release of the water-soluble base from various phospholipids presented as small unilamellar vesicles (SUVs). For some of the phospholipids, large unilamellar vesicles (LUVs) prepared by multiple passages of the aqueous lipid solutions through polycarbonate membranes (100 nm pore diameter) using a Lipofast extruder from Avestin were also examined as substrates. The PA generated by PLDs in the presence of Ca^{2+} (needed for *S. chromofuscus* activity toward phospholipid vesicles; Geng et al. 1998) interacts with the divalent cation, and this interaction leads to broadening of the ^{31}P resonance for the PA. The presence of Ca^{2+}-PA also leads to vesicle

fusion, making accurate integrations of substrate and product difficult. However, the water-soluble product choline is not affected by Ca^{2+}, and can be easily detected by sharp resonances in the ^1H spectrum. Although this technique has been used to monitor choline release from POPC SUVs (Dorovska-Taran et al. 1996; Geng et al. 1998, 1999), it is also useful for monitoring the water-soluble hydrolase product generated by PLD from other phospholipids as long as product protons can be easily distinguished from the broader substrate vesicle resonances. Phosphatidylethanolamine (PE) hydrolysis yields ethanolamine whose CH_2N and CH_2OH protons are detected at 3.02 and 3.69 ppm, respectively. Phosphatidylserine (PS) hydrolysis can be measure by the appearance of a sharp resonance (the CH_2O protons) at 3.72 ppm, while phosphatidylmethanol (PMe) hydrolysis yields a singlet for methanol at 3.23 ppm. Hydrolysis of phosphatidylglycerol (PG) can be detected from the glycerol resonance at 3.81 ppm (—CH_2OH), and PI hydrolysis can be measured by integrating the *myo*-inositol reonances (e.g., the C[5]H at 3.15 ppm is well separated from PI peaks). With each phospholipid, the amount of soluble alcohol generated was quantified by comparing the integrals for the sharp product resonances to an internal standard of 20 mM Tris HCl (the —CH_2OH protons) added with PLD enzyme. As an example, Figure 1 shows the time-dependent changes in ^1H spectra upon incubation of PI SUVs with PLD. Intensities of the C(5)H triplet (3.15 ppm), C(3)H/C(1)H doublet (3.42 ppm), and C(4)H/C(6)H triplet (3.50 ppm) increase with time. The C(2)H resonance at 3.95 ppm is not shown in these spectra. Amounts of enzyme added were similar to what was used for the ^{31}P assays.

rPLD intrinsic fluorescence

Ca^{2+} binding to rPLD (~20 μg/mL) was measured by changes in the intrinsic fluorescence of the protein as Ca^{2+} ions were titrated into the solution (25°C) as described previously (Stieglitz et al. 2001; Yang and Roberts 2002). The protein was excited at 290 nm and emission monitored at 337 nm, the wavelength for maximum emission.

Vesicle binding assays

Aliquots of PLD (48 μg/mL final concentration) were mixed with POPC/POPA vesicles (total phospholipid concentration 2 mM with 0, 10, or 100 mole % POPA) in 10 mM Tris-HCl, 5 mM EDTA, pH 8.0. Ba^{2+} (15 mM) was added to this buffer when the effect of Ba^{2+} (a competitive inhibitor of Ca^{2+} binding to PLD; Geng et al. 1998). This yielded a net Ba^{2+} concentration of 10 mM. The mixtures were placed in Amicon centricon-100 filters and centrifuged (14,000 *g*) to separate vesicle-bound from free PLD in the filtrate. The filtrates were lyophilized and the amount of free PLD (PLD_f) was quantified with SDS-PAGE by comparing band intensities for samples incubated with vesicles to controls without phospholipids. The percent of PLD bound to vesicles was estimated as $(PLD_o - PLD_f)/PLD_o$ where PLD_o is the total amount of PLD.

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