Conversion of Lysophospholipids to Cyclic Lysophosphatidic Acid by Phospholipase D*

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Phospholipase D from Streptomyces chromofuscus hydrolyzes lysophosphatidylcholine or lysophosphatidylethanolamine in aqueous 1% Triton X-100 solution. In situ monitoring of this reaction by 31P NMR revealed the formation of cyclic lysophosphatidic acid (1-acyl 2,3-cyclic glycerophosphate) as an intermediate which was hydrolyzed further by the enzyme at a functionally distinct active site to lysophosphatic acid (lyso-PA). Synthetic cyclic lyso-PA (1-octanoyl 2,3-cyclic glycerophosphate) was found to be stable in aqueous neutral solutions at room temperature. It was hydrolyzed by the bacterial phospholipase D to lyso-PA at a rate which was approximately 4-fold slower than the rate of formation of cyclic lyso-PA. The addition of 5–10 mM sodium vanadate could partially inhibit the ring opening reaction and thus increase substantially the cyclic lyso-PA accumulation. Cyclic lyso-PA may act as a dormant configuration of the physiologically active lyso-PA or may even possess specific activities which await verification.

Activation of phospholipases has been implicated in a wide range of signal transduction pathways (1). With regard to phospholipase D (PLase D), the current information as to the molecules generated by PLase D activation and their mode of action is still scarce (reviewed in Ref. 2).

The first step in the lytic activity of phospholipase D is the formation of a phosphoryl enzyme intermediate which is analogous to the acyl enzyme in the action of common esterases (3, 4). This intermediate is generally cleaved by the ambient water molecules with the net hydrolysis of one phosphoester bond. However, alcoholic hydroxyl residues can compete for the cleavage of the phospholipid enzyme intermediate to yield a phosphodiester product (5, 6). If a hydroxyl group is positioned appropriately in the substrate it can also compete for the phosphoryl enzyme yielding a cyclic product. Cyclic products of phospholipase action have been detected in the action of phosphatidylinositol-specific phospholipase C (PLase C) on phosphatidylinositol yielding 1,2-cyclic inositol phosphate as the initial product (7, 8), and in the PLase C hydrolysis of phosphatidylglycerol to 1,3-cyclic glycerophosphate (9). Similar cyclization takes place in the hydrolysis of glycerophosphorylcholine or glycerophosphorylcholine amine by glycerophosphoinositol diesterase, where 1,2-cyclic glycerophosphate is formed (10, 11). All of these cyclic phosphates are relatively stable at neutral pH but can be hydrolyzed by specific phosphodiesterases to form the respective phosphate monoesters (12, 13). It seems, therefore, that when the phosphoryl residue attached to the enzyme includes a free hydroxyl group, the formation of a cyclic phosphodiester by intramolecular transphosphorylation can take place. In analogy to the above reactions with a hydroxyl group, it has been suggested (9) that phospholipids with a free primary amine group (i.e. phosphatidylethanolamine and phosphatidylinerine) may yield a cyclic phosphoramidate intermediate upon the action of PLase C. These putative five-membered cyclic phosphoramidates are expected to be hydrolyzed spontaneously (9).

The action of PLase D on phospholipids forms a phosphatidyl ethanolamine intermediate which can either react with water to yield phosphatic acid (PA) or with an alcohol (5, 6) to yield a new phospholipid (e.g. phosphatidylethanol amine if ethanol is added as the alcohol). This transesterification reaction raises the possibility that the action of PLase D on lysophospholipids may yield cyclic lyso-PA by intramolecular transphosphorylation with the hydroxyl on carbon 2 of the glycerol backbone as schematically presented in Fig. 1. The present work provides evidence that cyclic lyso-PA is formed upon the interaction of bacterial PLase D with lysophosphatidylcholine (lyso-PC) or lysophosphatidylethanolamine (lyso-PE).

MATERIALS AND METHODS

Lysophospholipids—L-α synthetic acyl (hexanoyl-, octanoyl-, decanoyl-, and oleoyl-) glycerid γ-phosphorylcholines (lyso-PC) were obtained from Avanti Polar Lipids. Lyso-PE, L-α-palmitoyl glycerid γ-phospho-}

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§ The abbreviations used are: PLase D, phospholipase D; PLase C, phospholipase C; lyso-PA, lysophosphatic acid; lyso-PE, lysophosphatidylethanolamine; lyso-PC, lysophosphatidylcholine.

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lyso-PC) are characterized by resonances in the range of δ 18.5 ppm due to the formation of cyclic phosphodiester. Five-membered cyclic phosphates have a downfield shift of 0.8 ppm, with or without vanadate, respectively, was consistent with the formation of a five-membered cyclic phosphodiester. Two possible explanations for the unusual downfield shifted resonance were considered: (1) the presence of a cyclic diester, i.e., a cyclic diester-phosphate; (2) the presence of a cyclic diester-phosphate, coincided with the PLase D activity. Therefore, we tentatively conclude these activities. In all of these attempts the phosphatase activity, as monitored by hydrolysis of p-nitrophenyl phosphate (data not shown). To explore this further, we have chromatographed PLase D preparations used in the NMR experiments also exhibited a strong phosphatase activity with the conventional affinity binder of alkaline phosphatase, in an attempt to dissociate these activities. In all of these attempts the phosphatase activity, as monitored by hydrolysis of p-nitrophenyl phosphate, coincided with the PLase D activity. Therefore, we tentatively conclude that the phosphatase activity is inherent in the bacterial PLase D.

The reaction profiles presented above and the fact that cyclization and phosphatase activities always coincided suggest that cyclization and ring opening take place at the same catalytic site.

**RESULTS AND DISCUSSION**

Preliminary screening has indicated that bacterial PLase D, unlike cabbage PLase D, can cleave the headgroup of lyso-PC and lyso-PE in the presence of other or 1% Triton X-100. Fig. 2 presents the comparative degradation profiles of PC and lyso-PC solubilized in Triton X-100 mixed micelles, pH 8.0, as assayed by TLC and determination of the phosphorus content of each lipid spot. Under these conditions lyso-PC is a substrate for bacterial PLase D, but it is not as rapidly hydrolyzed as PC, in qualitative agreement with the findings of Imamura and Horiuti (18). In an analogous assay with dipalmitoyl-PE and 1-palmitoyl-1-oleoyl-PE (lyso-PE), similar profiles and rates of degradation were obtained. It should be noted that the enzyme "lysophospholipase D" (19, 20) acts selectively on L-α-ether-linked lysophospholipids and not on the common ester-linked L-α-lysophospholipids which were studied here.

The aqueous medium consisting of phospholipid (5 mg/ml) dispersed in 1% Triton X-100, 10 mM borate buffer, pH 8.0, with 10 mM calcium chloride, was incubated with bacterial PLase D at pH 5.5 (100 mM acetate buffer containing 10 mM calcium chloride, open symbols). In the presence of 1% Triton X-100 at 25°C. At each time point an aliquot was removed from the reaction mixture and phospholipids were separated by TLC and determined by phosphorus analysis (see "Materials and Methods").

The formation of cyclic lyso-PA by the action of PLase D on lyso-PC or lyso-PE was verified with 31P NMR spectra of synthetic 1-octanoyl 2,3-cyclic glycerophosphate (i.e. cyclic lyso-PA). The phosphorus chemical shift of this material corresponded to 18.5 ppm, essentially the same shift as the resonance observed by 31P NMR in assays of the action of PLase D on lyso-PC or lyso-PE (Fig. 3). The synthetic 1-octanoyl 2,3-cyclic glycerophosphate was examined for its stability under assay conditions and as a substrate for bacterial PLase D, since in the NMR assays eventually all the cyclic product was converted to lyso-PA. The synthetic cyclic lyso-PA was found to be stable at pH 6-8 for at least 10 h as was indicated by invariant intensity at +18.5 ppm (Fig. 4). However, in the presence of PLase D, the intensity of this resonance decreased, while that of lyso-PA increased, indicating phosphatase activity. The addition of egg PC in 1% Triton X-100 or in a 1:1 mixture with lyso-PC did not affect the rate of this reaction which excluded the possibility that the cyclization and the ring opening take place at the same catalytic site.

The cyclization and the ring opening activities could, in principle, be related to separate entities which copurified in the partial enzyme purification used to generate the PLase D. All the PLase D preparations used in the NMR experiments also exhibited a strong phosphatase activity with the conventional substrate p-nitrophenyl phosphate (data not shown). To explore this further, we have chromatographed PLase D preparations by gel filtration using Sphadex G-75, G-100, G-150, and S-300, DEAE-cellulose-52, and red Sepharose, which is an affinity binder of alkaline phosphatase, in an attempt to dissociate these activities. In all of these attempts the phosphatase activity, as monitored by hydrolysis of p-nitrophenyl phosphate, coincided with the PLase D activity. Therefore, we tentatively conclude that the phosphatase activity is inherent in the bacterial PLase D.

**Cyclic Lyso phosphatidic Acid**

**Fig. 2. Degradation profile of egg PC and egg lyso-PC by bacterial PLase D.** Egg PC (○, ●) or lyso-PC (△, ▲), each at an initial concentration of 5 mg/ml, was incubated with bacterial PLase D at pH 5.5 (100 mM acetate buffer containing 10 mM calcium chloride, open symbols) or at pH 8.0 (100 mM borate buffer containing 10 mM calcium chloride, filled symbols) in the presence of 1% Triton X-100 at 25°C. At each time point an aliquot was removed from the reaction mixture and phospholipids were separated by TLC and determined by phosphorus analysis (see "Materials and Methods").
that PLase D carries out two sequential enzymatic reactions with lysophospholipids: (i) intramolecular cyclization to form cyclic lyso-PA, followed by (ii) hydrolysis to lyso-PA, namely a phosphodiesterase type cleavage.

An alternative approach for dissecting the cyclization activity from that of the ring opening was the use of phosphatase competitive inhibitors. In a series of $^3$P NMR experiments which were carried out under the same conditions as in Fig. 3, we have added $\beta$-glycerophosphate (5 mM), $p$-nitrophenyl phosphate (5 mM), and sodium vanadate (2–100 mM). Sodium vanadate at concentrations of 5–10 mM could partially inhibit the phosphatase activity of the PLase D. This was measured by a substantial increase in relative content of cyclic lyso-PA compared with lyso-PA. An example of a reaction profile in the presence of 5 mM sodium vanadate is shown in Fig. 5. Under these reaction conditions there was a clear relative increase in the intensity of the resonance at $\delta = 18.5$ ppm. The presence of sodium vanadate caused a downfield shift and splitting in the resonance of the lyso-PA. One possible explanation for the marked effect of vanadate on the lyso-PA resonances could be the formation of pyrophosphovanadate which is stabilized by the presence of Ca$^{2+}$. $^3$P spectra recorded in the above system (not shown) displayed an upfield shift similar to that recorded in a pyrophosphovanadate formed between adenosine monophosphate (AMP) and sodium vanadate (23). The two resonances at the region of lyso-PA (Fig. 5) might be related to $\alpha$ and $\beta$ isomers.

Table I represents a summary of the $^3$P chemical shifts of the compounds presented in this communication.

Assuming that each of the above PLase D reactions follows a Michaelis-Menten type kinetic scheme with two independent enzyme sites, $E_1$ and $E_2$, then the reaction steps may be presented as follows,

\[
\begin{align*}
A + E_1 &\rightarrow AE_1 \rightarrow B (\text{cyclization}) \\
B + E_2 &\rightarrow BE_2 \rightarrow C (\text{ring opening})
\end{align*}
\]

where $A$ is the starting lysophospholipid (e.g. lyso-PC), $B$ is the putative cyclic lyso-PA, and $C$ is the lyso-PA (in principle in either $\alpha$ or $\beta$ form). $V_A$ and $V_B$ are the $V_{\text{max}}$ values, while $K_A$ and $K_B$ are the corresponding $K_m$ values. Accordingly (24),

\[
\begin{align*}
\frac{dA}{dt} &= -\frac{V_A \cdot A}{K_A + A} \quad (\text{Eq. 1a}) \\
\frac{dB}{dt} &= \frac{V_A \cdot A}{K_A + A} - \frac{V_B \cdot B}{K_B + B} \quad (\text{Eq. 1b}) \\
\frac{dC}{dt} &= \frac{V_B \cdot B}{K_B + B} \quad (\text{Eq. 1c})
\end{align*}
\]

The solution for the reduction of $A$ with time is

\[
\ln \frac{A_t}{A_0} + \frac{V_A}{K_A} (A_0 - A_t) = \frac{V_A}{K_A} \cdot t \quad (\text{Eq. 2})
\]

or

\[
\ln \frac{A_t}{A_0} - \frac{V_A}{K_A} \cdot t - \frac{1}{K_A}
\]

To a first approximation one can assume that along the whole reaction profile $A < K_A$, which upon integration of Equation 1a–1c leads to a simple first order decline in $A$. 

![Fig. 3](image-url) In vitro monitoring by $^3$P($^1$H) NMR of lyso-PC degradation by bacterial PLase D. Synthetic lyso-PC (1-octanoyl 3-glycerol phospho-rerylcholine) at the initial concentration of 100 mM in borate buffer, pD 8.0 (containing 10 mM Ca$^{2+}$) was incubated with bacterial PLase D in the NMR tube at 28°C for 14 h. The recorded $^3$P($^1$H) NMR spectra indicate the formation and the decline of a peak of a five-membered phosphodiester ring at $\delta = 18.5$ ppm (cyclic lyso-PA, c-LPA), decay of the lyso-PC (LPC) resonance at $\delta = 0.5$ ppm, and the subsequent emergence of a lyso-PA (LPA) resonance at $\delta = 0.8$ ppm. Inset, $^3$P spectra of synthetic cyclic lyso-PA. Scanning conditions: 70° pulses using 4-s relaxation delay and composite pulse proton decoupling (see “Materials and Methods”).

![Fig. 4](image-url) Hydrolysis profile of synthetic cyclic lyso-PA. 1-Octanoyl 2,3-cyclic glycerophosphate in the presence (●) and absence (▲) of bacterial PLase D. The emerging lyso-PA is also indicated (○). The experimental conditions are as described in the legend to Fig. 3.
Assuming further that the same approximation also holds for $B$ (i.e., $B < K_B$) then

$$B = \frac{V_A A_0}{K_A} \cdot \frac{V_B}{V_A} \cdot \left( e^{-\frac{V_B}{K_B}} - e^{-\frac{V_A}{K_A}} \right).$$  
(Eq. 4)

The rapid cleavage of the putative cyclic lyso-PA implied, from the leveling off and decline in the 18.5 ppm band (see Fig. 3), hampered our attempts to apply the kinetic analysis described above. In practice, however, this could be carried out only in the presence of sodium vanadate which acted as an inhibitor of the phosphodiesterase component (see Fig. 5). A typical experimental presentation of the change in $A$, $B$, and $C$ with time in the presence of 5 mM sodium vanadate is shown in Fig. 6. The presented values were obtained by pick integration under instrumental setup for quantitative evaluation (see “Materials and Methods”). The relative content of lyso-PC (.), lyso-PA (•), and cyclic lyso-PA (○) were obtained by area integration of the resonances at 0.5, 5.1, and 18.5 ppm, respectively. The calculated profile of the expected change in cyclic lyso-PA according to Equation 4 is also presented (---).

Lyso-PA has been recently cited as an important modulator
of cell functions (25, 26). It is synthesized either by the action of PLase A₂ on PA or by phosphorylation of α-monoglyceride by a specific kinase. Cyclic lyso-PA can in principle be formed in cells by the action of PLase A₂ on membrane phospholipids (27) followed by the action of PLase D which is described here. These consecutive enzyme reactions may impose a serious stringency on the formation of cyclic lyso-PA. Furthermore, the conversion of cyclic lyso-PA to lyso-PA will demand a third enzymatic action. Detection of these yet unexplored reactions will clarify whether cyclic lyso-PA is actually formed in cells and whether it serves as a dormant form of lyso-PA or acts as a modulator of cell functions on its own right.

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REFERENCES

1. Nishizuka, Y. (1992) Science 258, 607–614
2. Liscovitch, M. (1994) in Signal-activated Phospholipases (Liscovitch, M., ed) pp. 31–63, G. Landes Co., Austin, TX
3. Waite, M. (ed) (1987) The Phospholipases, Plenum Press, New York
4. Halbrook, P. G., Pannell, L. K., and Daly, J. W. (1991) Biochim. Biophys. Acta 1084, 155–159
5. Zwall, R. F. A., and Roelefsen, B. (1974) Method Enzymol. 52, 154–161
6. Elbi, A., and Kovatchev, S. (1981) Methods Enzymol. 72, 632–639
7. Griffith, O. H., Volwerk, J. J., and Kuppe, A. (1991) Methods Enzymol. 197, 493–499
8. Dawson, R. M. C., Freinkel, N., Jaisingwalla, F. B., and Clarke, N. (1971) Biochem. J. 122, 605–607
9. Shinitzky, M., Friedman, P., and Haimovitz, R. (1993) J. Biol. Chem. 268, 14109–14115
10. Clarke, N., and Dawson, R. M. C. (1976) Biochem. J. 153, 745–747
11. Clarke, N., and Dawson, R. M. C. (1978) Biochem. J. 173, 579–589
12. Majerus, P. W., Connolly, T. M., Dechmyn, H., Ross, T. S., Brosi, T. E., Ishii, H., Bansal, V. S., and Wilson, D. B. (1986) Science 234, 1519–1526
13. Ross, T. S., and Majerus, P. W. (1986) J. Biol. Chem. 261, 11119–11123
14. DeHaas, G. H., Podoma, N. M., Nieuwenhuizen, W., and VanDeenen, L. M. (1968) Biochim. Biophys. Acta 159, 103–117
15. Reynolds, L., Hughes, L. I., Kramer, R. M., and Dennis, E. A. (1993) Biochim. Biophys. Acta 1167, 272–280
16. Ten Hoeve, W., and Wynberg, H. (1985) J. Org. Chem. 50, 4804–4814
17. Rouser, B., Slakotos, A., and Fleischer, S. (1966) Lipids 1, 85–86
18. Imamura, S., and Horii, Y. (1979) J. Biochem. (Tokyo) 85, 79–95
19. Wykle, R. L., Kraemer, W. F., and Schrenner, J. M. (1980) Biochim. Biophys. Acta 619, 58–67
20. Wykle, R. L., and Strum, J. C. (1991) Methods Enzymol. 197, 583–590
21. Blackburn, G. M., Cohen, J. S., and Weatherall, I. (1971) Tetrahedron 27, 2903–2912
22. Gallagher, G. (1987) in Methods in Stereochemical Analysis (Verkade, J. G., and Quin, L. D., eds) Vol. 8, pp. 297–330, VCH Inc., Deerfield Beach, FL
23. Tracey, A. S., Gresser, M. J., and Lin, S. (1988) J. Am. Chem. Soc. 110, 5869–5874
24. Segal, I. H. (1975) Enzyme Kinetics, Wiley-Interscience, New York
25. Jalink, K., Hordijk, P. L., and E. J. Moolenaar, W. H. (1994) Biochim. Biophys. Acta 1198, 185–196
26. Cook, S. J., Rubinfeld, B., Albert, I., and McCormick, F. (1993) EMBO J. 12, 3475–3485
27. Liscovitch, M. (1992) Trends Biochem. Sci. 17, 393–399