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Regiospecificity and Catalytic Triad of Lysophospholipase I*

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A 25-kDa murine lysophospholipase (LysoPLA I) has been cloned and expressed, and Ser-119 has been shown to be essential for the enzyme activity (Wang, A., Deems, R. A., and Dennis, E. A. (1997) J. Biol. Chem. 272, 12723–12729). In the present study, we show that LysoPLA I represents a new member of the serine hydrolase family with Ser-119, Asp-174, and His-208 composing the catalytic triad. The Asp-174 and His-208 are conserved among several esterases and are demonstrated herein to be essential for LysoPLA I activity as the mutation of either residue to Ala abolished LysoPLA I activity, whereas the global conformation of the mutants remained unchanged. Furthermore, the predicted secondary structure of LysoPLA I resembles that of the αβ-hydrolase fold, with Ser-119, Asp-174, and His-208 occupying the conserved topological location of the catalytic triad in the αβ-hydrolases. Structural modeling of LysoPLA I also indicates that the above three residues orient in such a manner that they would comprise a charge-relay network necessary for catalysis. In addition, the regiospecificity of LysoPLA I was studied using 31P NMR, and the result shows that LysoPLA I has similar LysoPLA1 and LysoPLA2 activity. This finding suggests that LysoPLA I may play an important role in removing lysophospholipids produced by both phospholipase A1 and A2 in vivo.

LysoPLA1 and LysoPLA2 regulate LysoPL levels by further hydrolyzing the LysoPL generated by PLA1 or PLA2. Over the past few years, PLA1 has attracted much attention due to its roles in signal transduction and in the release of arachidonic acid, an important precursor for other lipid messengers such as the prostaglandins and leukotrienes (12–16). Arachidonic acid that occurs predominantly in the sn-2 position of phospholipids, however, could also be released by the sequential actions of PLA2 and LysoPLA1. Therefore, LysoPLA1 may also contribute to arachidonic acid release in vivo.

LysoPLA has been identified in a variety of cells and tissues, and recently a rat and a mouse enzyme have been sequenced, cloned, and expressed in Escherichia coli cells (17, 18). These two enzymes (both of 25 kDa molecular mass) share very high sequence homology as well as similar properties and represent the first characterized mammalian lysophospholipid-specific LysoPLA (referred to as LysoPLA I (18). Both the mouse and the rat enzymes contain a GXSGX motif, and the serine residue in the center of the motif was shown to be essential for enzymatic activity (18). In the present work, we have used site-directed mutagenesis and structural modeling to investigate the mechanism of action of LysoPLA I and to determine if a Ser/His/Asp catalytic triad is involved in catalysis. We have also investigated the substrate regiospecificity of LysoPLA I using 31P NMR spectroscopy.

EXPERIMENTAL PROCEDURES

Subcloning of LysoPLA I from pLEX to pET28a(+).—Previously, we cloned the murine LysoPLA I gene into the pLEX vector (Invitrogen) at the multiple cloning sites of NdeI and EcoRI (18). To subclone LysoPLA into the pET28a(+) vector (Novagen), both the pET28a(+) and the pLEX/LysoPLA I vectors were digested by the same two restriction enzymes, NdeI and EcoRI, and then separated by 1% agarose gel. The bands corresponding to LysoPLA I (~780 base pairs) and pET28a(+) (~5300 base pairs) were purified from the gel using Qiagen PCR Preme (Promega), and ligated together with T4 DNA ligase (Pharmacia Biotech Inc.). The ligation product was used to transform competent E. coli NovaBlue cells (Novagen), and the resulting colonies were screened by restriction enzyme analysis of the isolated plasmids. Finally, the entire LysoPLA I coding region in the pET28a(+) vector was checked by the automated DNA sequencer (Applied Biosystems 373 from Perkin-Elmer) at the University of California at San Diego Center for AIDS Research Molecular Biology Core. It should be noted that this cloned LysoPLA I has an extra 20 amino acids in the N-terminal of the protein, the sequence of which is shown in Sequence 1.

MGSSHHHHHHSSGLVPR—GSH—LysoPLA I
His-Tag—thrombin site

As indicated above, the His-Tag can be removed by thrombin cleavage, leaving only an extra three amino acids at the N terminus of the protein.

Site-directed Mutagenesis—Three pairs of mutagenic primers with H28A, D174A, and H208A mutations were synthesized by Life Technologies, Inc. (H28A, 5’GGGTTAATTTTCCTTGGCAGTGGAATACGAGGCGC 3’ and 5’GCCCTGATTCCTCCCAATTCGCGCAAGAATAACCCG 3’; D174A, 5’GATATGGGAGTTTCGACCTCTAGTCC 3’ and 5’GGGGAATTTAAGGCCCACACTCTCATGCG 3’; H208A, 5’GATGATGGGAGTTTCGACCTCTAGTCC 3’ and 5’GGGGAATTTAAGGCCCACACTCTCATGCG 3’; H208A, 5’GATGATGGGAGTTTCGACCTCTAGTCC 3’ and 5’GGGGAATTTAAGGCCCACACTCTCATGCG 3’)
Expression and Purification of Wild-type and Mutant LysoPLA I Proteins—The pET28a(+) vector harboring either the wild-type or the mutant LysoPLA I insert was used to transform competent _E. coli_ BL21(DE3) (Novagen), and a single colony was inoculated in an overnight culture in LB-kanamycin (50 μg/ml) medium. This overnight culture was then diluted 40-fold into Terrific Broth-kanamycin (50 μg/ml) medium and allowed to grow at 37 °C until the optical density at 600 nm reached 1. Then IPTG (Fisher) was added to a final concentration of 0.4 mm, and the cells were grown at 22 °C for another 4 to 5 h to induce foreign protein expression. Finally, the _E. coli_ cells were centrifuged and the pellet was stored at −20 °C.

The same purification scheme was used to purify both the wild-type and mutant enzymes, and all procedures were carried out at 4 °C. The purification was started by resuspending the _E. coli_ pellet in lysis buffer (25 mm Tris-HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole, and 10 mM β-mercaptoethanol). Lysozyme (Sigma) was added to a final concentration of 0.5 mg/ml, and the mixture was stirred slowly for half an hour. The mixture was then sonicated intermittently 6 times for 10 s each and then centrifuged at 100,000 × g for 45 min. The supernatant was collected and passed through a Ni-NTA column (Qiagen). The column was then washed with 40 ml of the lysis buffer, and the bound proteins were eluted with elution buffer (25 mM Tris-HCl, pH 8.0, 500 mM NaCl, 250 mM imidazole, and 10 mM β-mercaptoethanol). Generally, LysoPLA I was eluted in the first 15 ml of the elution buffer with some minor high molecular weight contaminants. It was then loaded onto a gel filtration column, Sephadex G-75 (2.5 × 90 cm, Pharmacia Biotech Inc.) equilibrated in buffer A (10 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 10 mM β-mercaptoethanol). The LysoPLA I eluted from the G-75 column was essentially free of contaminants and was used for both CD measurements and activity assays. To remove the His-Tag at the N-terminus of the protein, the purified protein was digested with biotinylated thrombin (Novagen) for more than 16 h at 4 °C, and the biotinylated thrombin was removed at the end of the digestion by streptavidin-agarose (Novagen). The His-Tag-removed protein was used for both 31P NMR measurements and activity assays.

Protein Activity and Purity Determination—Lysophospholipase activity was measured at 40 °C in 0.1 M Tris-HCl buffer, pH 8.0, 125 μM 1-[14C]palmitoyl-sn-glycero-3-phosphorylcholine (1.6 μCi/μmol) (obtained from Avanti and NEN Life Science Products) in a total volume of 0.5 ml. The assay was initiated by adding an aliquot of enzyme solution to the substrate mixture and incubating for the desired time. The released fatty acid was extracted by the Dole method and then quantified by scintillation counting (19). The protein concentration of the _E. coli_ homogenate was quantified by the Bio-Rad protein assay using bovine serum albumin as standard, and the purified LysoPLA I was quantified by absorbance at 280 nm using an extinction coefficient of 0.85 (μg/ml)−1 cm−1. This coefficient was calculated based on the absorbance (20) and the numbers of Trp and Tyr residues in the LysoPLA I sequence and was found to give essentially the same result as that obtained by Bio-Rad protein assay. Protein purity was examined using 12% SDS-polyacrylamide gel electrophoresis using the method of Laemmli (21), and the protein bands were visualized by staining with Coomassie Blue.

Analysis of the Enzyme Conformation by CD Spectroscopy—CD spectra were measured using a modified Cary 1 UV spectrophotometer (18). CD spectra were collected at 7 °C using a cylindrical quartz cuvette with path length of 0.5 mm. For each protein sample (purified wild-type or mutant LysoPLA I) and blank solution (10 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 10 mM β-mercaptoethanol) 10 separate spectra were collected and averaged. The final protein spectra were obtained by subtracting the blank spectra from the sample spectra and converting the difference to mean residue ellipticity.

Synthesis of 2-Palmitoyl-1-hydroxy-sn-glycero-3-phosphocholine (2PGPC)—Conversion of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (2PGPC) into 1,2-dipalmitoyl-3-{[2-14C]palmitoyl}glycerophosphoethanolamine (1 Ci/mmol (obtained from Avanti and NEN Life Science Products) in a total volume of 0.5 ml. The assay was initiated by adding an aliquot of enzyme solution to the substrate solution and incubating for the desired time. The released fatty acid was extracted by the Dole method and then quantified by scintillation counting (19). The protein concentration of the _E. coli_ homogenate was quantified by the Bio-Rad protein assay using bovine serum albumin as standard, and the purified LysoPLA I was quantified by absorbance at 280 nm using an extinction coefficient of 0.85 (μg/ml)−1 cm−1. This coefficient was calculated based on the absorbance (20) and the numbers of Trp and Tyr residues in the LysoPLA I sequence and was found to give essentially the same result as that obtained by Bio-Rad protein assay. Protein purity was examined using 12% SDS-polyacrylamide gel electrophoresis using the method of Laemmli (21), and the protein bands were visualized by staining with Coomassie Blue.
(Avanti Polar Lipids) to 2PGPC was achieved using *Rhizopus* lipase (Boehringer Mannheim) following published procedures (22). The enzymatically catalyzed reaction did not proceed to completion. However, 2PGPC could be separated from the starting material by chromatography on Sephadex LH-20 (Sigma). The 2PGPC prepared in this manner was the wild-type LysoPLA I treated with thrombin, and the slight shift of the protein band indicates the removal of the His-Tag from the N terminus of the protein. B, lysophospholipase activity of the purified wild-type (WT) and mutant proteins.

purification, the coding region of LysoPLA I in the pLEX vector was subcloned into pET28a(+) at restriction sites of NdeI and EcoRI, as described under “Experimental Procedures.” This expression system provides a His-Tag at the N-terminal of LysoPLA I, which can be easily removed by thrombin cleavage after the fusion protein is purified by the Ni-NTA column. As shown in Fig. 1, a protein band at an apparent molecular mass of ∼29 kDa was strongly induced by 0.4 mM IPTG in *E. coli* (lane 3 versus lane 2). The lysophospholipase activity in *E. coli* homogenate harboring pET28a(+)LysoPLA I was also increased more than 35-fold compared with the control, demonstrating that such a system expressed an active LysoPLA I at a very high level. With the purification procedures described below, more than 20 mg of pure recombinant protein can be obtained from a liter of *E. coli* culture.

To purify the expressed protein, the homogenate of induced *E. coli* cells was centrifuged at 100,000 × g for 45 min. The LysoPLA I in the supernatant fraction was then purified by a Ni-NTA column, which yielded highly purified LysoPLA I (>96%) in a relatively small volume (15 ml). To remove the minor high molecular weight contaminations and to exchange the enzyme into a low salt buffer in which it is more stable, LysoPLA I isolated from the Ni-NTA column was further purified using a gel filtration column. The LysoPLA I thus obtained was essentially free of contamination on the SDS-polyacrylamide gels and possessed a specific activity of 1.09 ± 0.02 μmol/min·mg (Fig. 2). After the removal of the His-Tag at the N terminus of the protein by thrombin, the specific activity of LysoPLA I remained the same.

**Candidates for the Catalytic Triad**—Previously, the Ser-119 residue in the conserved GXGXG motif of LysoPLA I was found to be essential for protein function (18). This suggests that LysoPLA I may be a new member of the serine hydrolase superfamily, the catalytic mechanism of which generally involves a catalytic triad composed of a nucleophile (Ser), an acid (Asp/Glu), and a base (His). While the serine residue in the catalytic triad can often easily be identified by the conserved GXGXG motif, the sequences around the acid and the base are generally much less conserved. Identification of the acid and the base residues is made even more difficult by the fact that the three catalytic residues often occur far apart in the amino acid sequence, and the order of their appearance in the primary sequence also varies from enzyme to enzyme (25–27). As a

RESULTS

**Subcloning, Protein Expression, and Purification**—To optimize protein expression and to simplify large scale protein
result, the candidates for the acid and the base residues are often identified by comparison with other proteins based on either amino acid sequences or secondary/tertiary structures (13, 25, 27, 28). As shown in Fig. 3, the predicted secondary structure of LysoPLA I resembles those of the $\alpha/\beta$-hydrolase fold, especially in the C-terminal half of the sequence. $\alpha/\beta$-Hydrolases constitute a family of enzymes with different phylogenetic origins and catalytic functions but share a common protein structure (termed $\alpha/\beta$-hydrolase fold) and a conserved catalytic mechanism (the catalytic triad) for activity (26). Both the sequence (namely a nucleophile, followed by an acid and then a base) and the topological position (all on loops formed between a $\beta$-strand and an $\alpha$-helix) of the catalytic triad are highly conserved in the $\alpha/\beta$-hydrolase family.

In addition, when the amino acid sequence of LysoPLA I is compared with several other esterases or putative esterases that share more than 25% homology to LysoPLA I, only one acid residue, Asp-174, is conserved among all of them (Fig. 3B). More importantly, this Asp-174 occurs on a loop between $\beta$-strand 7 and helix E, the site conserved for the acid residue in the $\alpha/\beta$-hydrolases (Fig. 3A). Three His residues, His-28, His-170, and His-208, are also conserved among all the listed
proteins (Fig. 3). However, only His-208 has the features of the previously identified Ser-119 also occupies the conserved site termed the “nucleophile elbow” between β-strand 5 and helix C (Fig. 3). Therefore, LysoPLA I appears to be a new member of the α/β-hydrolases with Ser-119, Asp-174, and His-208 composing its catalytic triad.

Site-directed Mutagenesis—To verify that the Asp-174 and His-208 in LysoPLA I are indeed the components of the catalytic triad, each residue was changed to an Ala by site-directed mutagenesis. In addition, His-28 was also mutated to an Ala to examine how important it is for LysoPLA I activity. E. coli cells transformed with the vectors harboring the mutant genes expressed the mutant proteins (D174A, H208A, and H28A) at about the same level as that of the wild-type protein (Fig. 1). However, lysophospholipase activity in the E. coli homogenate expressing either the D174A or the H208A mutant was at the same level as the control, more than 35-fold lower than that of the wild-type enzyme (Fig. 1). In contrast, the E. coli homogenate expressing the H28A mutant retained more than 40% activity of the wild-type enzyme. Similarly, when the mutant proteins were purified and assayed for activity, it was found that mutation at Asp-174 and His-208 abolished the activity of these two purified proteins (Fig. 2). The H28A mutant, on the other hand, had a specific activity of 0.500 ± 0.007 μmol/min/mg (−50% wild-type enzyme activity), indicating that His-28 is not absolutely required for LysoPLA I activity (Fig. 2).

CD Spectra of Wild-type LysoPLA I and H28A, D174A, and H208A Mutant—To exclude the possibility that the loss of the enzyme activity in the mutant proteins was due to conformational changes in the mutants, CD spectra were taken for each of the purified mutants as well as the wild-type protein. As shown in Fig. 4, the CD spectra of all the proteins were essentially identical, demonstrating that the decreased enzyme activity, whether a 100% loss for D174A and H208A or a 50% loss for H28A, is not the result of misfolding or global conformational changes in the mutants.

Regiospecificity of LysoPLA I—To explore the regiospecificity of LysoPLA I, 31P NMR was used to monitor the hydrolysis of both natural regioisomers (1PGPC and 2PGPC) under conditions in which acyl migration was minimized. As shown in Fig. 5A, LysoPLA I readily processed both isomers at similar rates. As the substrate concentration (10 mM) used in the NMR measurements was much higher than the \(K_M\) value (22 μM) reported previously (19), a linear time course was expected if the reaction was not complicated by substrate/product inhibition or activation. However, examination of the time courses for the consumption of both isomers as well as the production of glycerocephosphocholine revealed that the reaction had two zero-order phases (Fig. 5B), an early slower phase for up to 25 min and a later faster phase for up to 40 min. The data points after 40 min became non-zero order, reflecting the much smaller concentrations of substrates remaining. Similar reaction profiles were observed with secretory PLA\(_2\) (29), and the complex time courses were attributed to changes in the interface resulting from the fatty acid produced initially in those reactions. However, as fatty acids inhibit the LysoPLA I (19), it remains unclear what causes this complex time course. It is apparent, however, that LysoPLA I can function equally well as either a LysoPLA\(_1\) or a LysoPLA\(_2\).

DISCUSSION

LysoPLA\(_s\) have been identified in many mammalian tissues and cells and are considered to be the major route by which lysophospholipids are degraded. The substrate used in lysoPLA I activity assays is known to exist as a 9:1 equilibrium mixture of 1PGPC and 2PGPC, with the fatty acid predominantly at the sn-1 position (22). The Dole extraction, however, is unable to distinguish which isomer is hydrolyzed. To examine whether LysoPLA I has a preference for one isomer over the other, we have followed the hydrolysis of both isomers by 31P NMR. Remarkably, the results show that LysoPLA I processes both regioisomers at almost identical rates. These findings were obtained at 20 °C, and similar results were also obtained at 40 °C (data not shown). These observations are in contrast to what was found for the lysophospholipase activity of the Group IV Ca\(^{2+}\)-dependent cytosolic PLA\(_2\), which exhibited a strong preference for 1PGPC over 2PGPC (30). The ability of LysoPLA I to efficiently hydrolyze both regioisomers suggests that LysoPLA I may function as both a LysoPLA\(_1\) and a LysoPLA\(_2\) in vivo, controlling lysophospholipids produced by both PLA\(_1\) and PLA\(_2\) (Scheme I). In addition, LysoPLA I may also play a role in arachidonic acid release and signal transduction since the arachidonic acid that occurs predominantly in the sn-2 position of phospholipids could also be cleaved by the sequential actions of a PLA\(_1\) and LysoPLA I. Interestingly, the enzyme’s activity does not appear to be affected by the aggregation state of the substrate. Its activity does not vary dramatically as the substrates’ concentration increases above its critical micelle concentration suggesting that interfacial activation does not play the same role in lysophospholipase activity as it does in phospholipase A\(_2\) activity (31).

By structural comparison to other esterases and α/β-hydrolase, we have identified two putative catalytic residues Asp-174 and His-208 that, together with the previously identified Ser-119, may form the catalytic triad in LysoPLA I. This hypothesis is supported by the site-directed mutagenesis studies, which showed that mutations of either residue rendered the enzyme completely inactive, although the D174A and H208A mutants retained the same protein global conformation as that of the wild-type enzyme. These results are in striking contrast to that observed for another mutant, H28A. Even though His-28 is also highly conserved, mutation of it to Ala retained 50% activity of the wild-type enzyme. Taken together with the previous results, we have herein demonstrated that Ser-119, Asp-174, and His-208 are essential for LysoPLA I activity, and LysoPLA I appears to function by a mechanism involving a catalytic triad.

Structural prediction for LysoPLA I was achieved based on...
the predicted secondary structure of LysoPLA I and the known crystal structure of acetylcholinesterase, a member of the \( \alpha/\beta \)-hydrolase family (23). As shown in Fig. 6, the three catalytic residues Ser-119, Asp-174, and His-208, although far apart in the primary sequence, come together and orient in such a way that they could form the charge-relay network. Similar results

**FIG. 5.** Regiospecificity of LysoPLA I. A, \(^{31}\)P NMR spectra of LysoPLA I catalyzed hydrolysis of \( \sim 1:1 \) mixture of 1PGPC and 2PGPC as a function of time. Peak 1 (0.68 ppm) is 1PGPC, peak 2 (0.52 ppm) is 2PGPC, and peak 3 (0.99 ppm) is the product glycerophosphocholine. The external standard pyrophosphate is labeled as peak 4 (−5.46 ppm). B, the time course of the above reaction. The concentrations of 1PGPC (●), 2PGPC (■), and glycerophosphocholine (○) determined by \(^{31}\)P NMR are plotted as a function of reaction time, with lines connecting the data points.
were obtained using another α/β-hydrolase (dielactone hydrolase) as template and agree with the notion that through divergent evolution the three-dimensional configuration of the triad and therefore the catalytic mechanism are highly conserved in many hydrolases (26, 27). The structural model obtained here provides a valuable tool for the design of studies to determine the function and regulation of this enzyme. The validity of this structural model, however, awaits x-ray structural studies for the enzyme, which are currently underway.

In summary, we have established an improved protein expression/purification procedure to quickly purify a large amount of active enzyme and demonstrated that LysoPLA I represents a new member of the serine hydrolase family with the catalytic triad composed of Ser-119, Asp-174, and His-208. LysoPLA I hydrolyzes both 1PGPC and 2PGPC equally well and may play an important role in controlling the level of lysophospholipids produced by both PLA₁ and PLA₂.

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