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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 α/β-hydrolase fold, with Ser-119, Asp-174, and His-208 occupying the conserved topological location of the catalytic triad in the α/β-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 ^{31}P NMR, and the result shows that LysoPLA I has similar LysoPLA$_1$ and LysoPLA$_2$ activity. This finding suggests that LysoPLA I may play an important role in removing lysophospholipids produced by both phospholipase A$_1$ and A$_2$ *in vivo*.

Lysophospholipids (LysoPL)[1] are the detergent-like intermediates in phospholipid metabolism whose *in vivo* levels must be strictly regulated for proper cell function and survival. Accumulation of LysoPL can perturb the activities of many membrane-bound signal-transducing enzymes (1–4), distort cell membrane integrity, and even cause cell lysis (5, 6). Increased LysoPL levels have also been detected in a variety of disease states including lethal dysrhythmias in myocardial ischemia and segmental demyelination of peripheral nerves (7–11). The increased LysoPL levels are believed to be caused by the malfunction of LysoPL-regulating enzymes including lysophospholipases (LysoPLA), phospholipases A$_1$ and A$_2$ (PLA$_1$ and PLA$_2$), transacylases, and acyltransferases (Scheme I).

As shown in Scheme I, lysophospholipases (LysoPLA$_1$ and LysoPLA$_2$) regulate LysoPL levels by further hydrolyzing the LysoPL generated by PLA$_1$ or PLA$_2$. Over the past few years, PLA$_2$ 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 PLA$_1$ and LysoPLA$_2$. Therefore, LysoPLA$_2$ 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 G*X*S*X*G 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 ^{31}P 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 *Nde*I and *Eco*RI (18). To subclone LysoPLA I into the pET28a(+) vector (Novagen), both the pET28a(+) and the pLEX/LysoPLA I vectors were digested by the same two restriction enzymes, *Nde*I and *Eco*RI, and then separated by 1% agarose gel. The bands corresponding to LysoPLA I (~700 base pairs) and pET28a(+) (~5300 base pairs) were purified from the gel using Wizard™ PCR Preps (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 at the N-terminal of the protein, the sequence of which is shown in Sequence 1.

MGSSHHHHHHSSGLVPR↓GSH—LysoPLA I
His · Tag thrombin site

SEQUENCE 1

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′GCGGTTATTTTCCTTGCCGGATTGGGAGATACAGGGC 3′ and 5′GCCCTGTATCTCCCAATCCGGCAAGGAAAATAACCGC 3′; D174A, 5′GCCATGGAGATTGTGCCCCTTTAGTTCCCC 3′ and 5′GGGGAACTAAAGGGGCACAATCTCCATGGC 3′; H208A,

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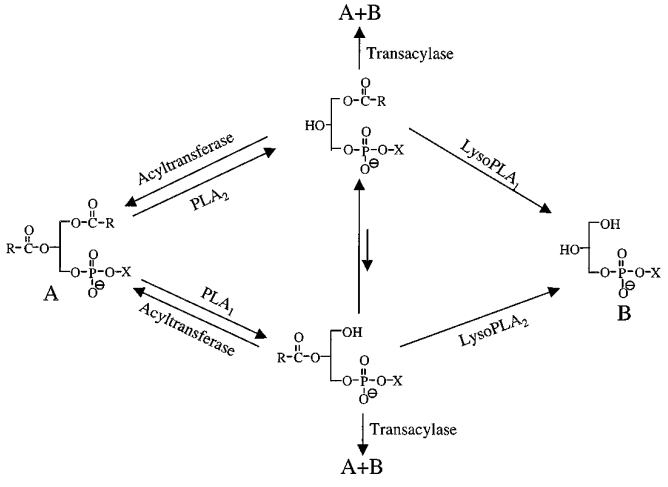
[1] The abbreviations used are: LysoPL, lysophospholipid; LysoPLA$_1$ and LysoPLA$_2$, lysophospholipase A$_1$ and A$_2$, respectively; PLA$_1$, and PLA$_2$, phospholipase A$_1$ and A$_2$, respectively; 1PGPC, 1-palmitoyl-*sn*-glycero-3-phosphocholine; 2PGPC, 2-palmitoyl-*sn*-glycero-3-phosphocholine; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactoside; Ni-NTA, nickel-nitrilotriacetic acid.

5′CTATGAAGGCATGATGGCCAGCTCATGTCAGCAGG 3′ and 5′CCTGCTGACATGAGCTGGCCATCATGCCTTCATAG 3′). These primers were used to generate the mutated proteins by PCR using QuickChange Site-directed Mutagenesis kit from Stratagene. Here, the pET28a(+)/LysoPLA I plasmid isolated from the NovaBlue cells was used as the template for the *Pfu* DNA polymerase (Stratagene). After PCR, the wild-type parent plasmid remaining in the PCR product was selectively digested by the *Dpn*I restriction enzyme (Stratagene), and the resultant mixture was used to transform competent *E. coli* NovaBlue cells. The desired mutations were selected by restriction analysis and DNA sequencing and were confirmed to be the only mutation introduced in each mutant.

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 $A_{600\ nm}$ 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 ^{31}P NMR measurements and activity assays.



SCHEME 1. **Metabolic interconversions involving lysophospholipids.**

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-[^{14}C]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 (mg/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 61 spectropolarimeter (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

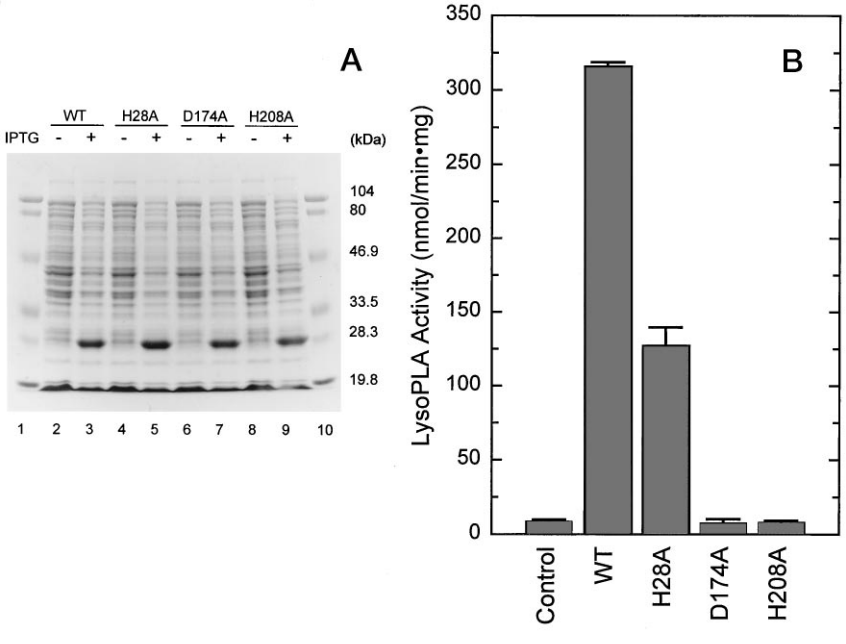


FIG. 1. **Comparison of the wild-type and mutant LysoPLA I in *E. coli* homogenates.** *A*, expression of the wild-type (*WT*) and mutant LysoPLA I in *E. coli* homogenates as examined by SDS-polyacrylamide gel electrophoresis. *E. coli* cells harboring either the wild-type or mutant H28A, D174A, and H208A gene were sampled before (−) and after (+) 4 h IPTG induction. The cells were lysed in SDS sample buffer and boiled for 5 min before being loaded onto a 12% gel. The strongly induced bands at ~29 kDa are the His·Tag-attached LysoPLA I (either wild-type or mutants). *B*, lysophospholipase activities in *E. coli* homogenates expressing wild-type (*WT*) or mutant proteins. The control sample was obtained from *E. coli* cells transformed with only the plasmid itself. The *E. coli* homogenates used for activity assays were obtained by lysozyme digestion and sonication, as described under "Experimental Procedures."

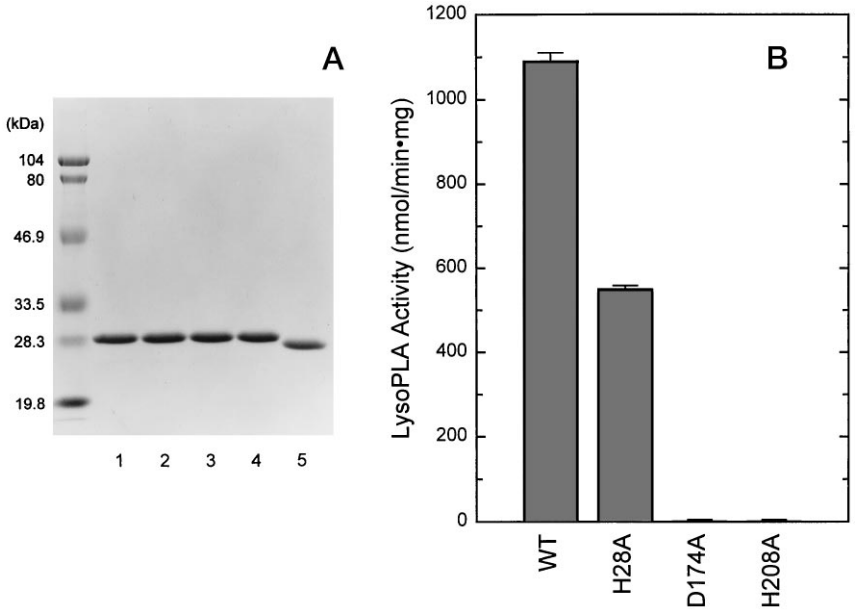


FIG. 2. **Comparison of the purified wild-type and mutant LysoPLA I.** *A*, SDS-polyacrylamide gel electrophoresis of the purified wild-type (*lane 1*) or mutant H28A (*lane 2*), D174A (*lane 3*), and H208A (*lane 4*) proteins (4 μg per lane). The sample in *lane 5* was the wild-type LysoPLA I treated 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.

(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 gave a single spot on analytical TLC but showed two peaks on ^{31}P NMR corresponding to a 4:1 mixture of 2PGPC:1PGPC isomers. Similarly, commercial 1PGPC contains approximately 10% of the corresponding 2PGPC isomer. These cross-contaminations have been documented and are attributed to migration of the fatty acyl chain during the preparation and purification of lysophospholipids (22).

NMR Measurements—NMR samples were made up in a final buffer of 100 mM Tris-HCl, pH 8.0. Appropriate amounts of 1PGPC (Avanti Polar Lipids) and 2PGPC (synthesized above) were combined to give a ~1:1 mixture of isomers. As lysophospholipids were stored as chloroform solutions at −20 °C, the chloroform was removed at the beginning of the experiment by evaporation under a stream of nitrogen. The resulting film was dissolved in the reaction buffer by vortexing and bath sonication to give a clear and colorless solution (14.3 mM total lysophospholipid). From this lysophospholipid solution, 350 μl was transferred to a 5-mm NMR tube. After a reference spectrum was obtained, 150 μl of purified LysoPLA I was added to initiate the reaction. The starting concentrations of reagents were 10 mM substrate and 540 μg/ml enzyme in 500 μl of buffer. The reaction was carried out at 20 °C. In addition, an insert containing 10 mM pyrophosphate in D_2O (Cambridge Isotopes) was used as an external standard.

^{31}P NMR spectra were obtained on a Brucker spectrometer operating at 243 MHz. A 90° pulse with a 2-s delay and a spectral width of 2671 Hz and 8 K data points was used. Broad band proton decoupling was utilized. Spectra were obtained at varying time intervals at 20 °C. The resulting free induction decay was apodized with an exponential multiplication with line broadening of 5 Hz. Peak intensities were taken to represent the relative concentrations of the phosphorus-containing species in solution. Under the same conditions as NMR experiments, nonenzymatic catalyzed hydrolysis was negligible as determined by the Dole assay.

Structure Prediction of LysoPLA I—The LysoPLA I sequence was aligned with that of the acetylcholinesterase (23) or dielelactone hydrolase (24) based on the predicted secondary structure of LysoPLA I (18) and the known crystal structures of the above two proteins. The modeling was performed on a Silicon Graphics workstation using Modeller 3, a program[2] for automated comparative modeling based on the satisfaction of spatial restraints. The resulted protein structure was visualized by the RasMol and Insight II programs.

RESULTS

Subcloning, Protein Expression, and Purification—To optimize protein expression and to simplify large scale protein purification, the coding region of LysoPLA I in the pLEX vector was subcloned into pET28a(+) at restriction sites of *Nde*I and *Eco*RI, 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 G*X*S*X*G 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 G*X*G*X*G 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

[2] R. Sánchez and A. Šali, http://guitar.rockefeller.edu

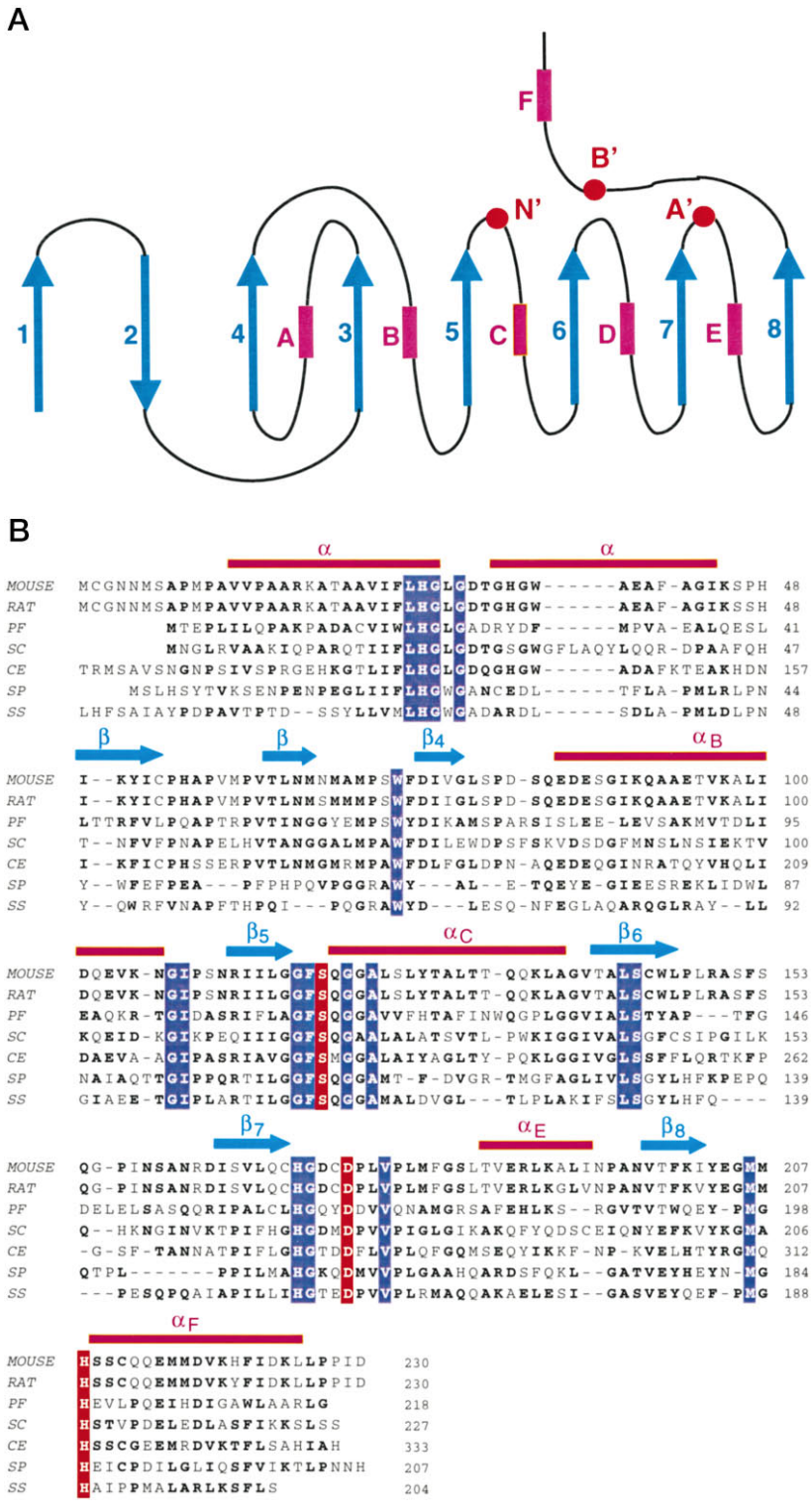


FIG. 3. **Structural comparison of LysoPLA I with α/β-hydrolase and esterases.** *A*, the prototypic α/β-hydrolase fold with β-strands marked and *numbered* by *arrows 1–8* and α-helixes by *boxes A–F*. The catalytic triad residues are colored in *red*, and *N′* indicates the catalytic nucleophile, *A′* indicates the acid, and *B′* indicates the base. *B*, conserved primary and secondary structural elements in LysoPLA I. The amino acid sequence of mouse LysoPLA I (*MOUSE*, GenBank accession number U89352) is compared with all the other proteins that share more than 25% homology as searched by the BLAST program in protein data bases: *RAT* is the rat LysoPLA (GenBank accession number D63885); *PF* is *Pseudomonas fluorescens* carboxylesterase (PIR accession number JU0277); *SP* is an esterase from *Spirulina platensis* (PIR accession number S43880), and *SC*, *CE*, and *SS* are the putative esterases from *Saccharomyces cerevisiae* (GenBank accession number U53877), *Caenorhabditis elegans* (EMBL accession number Z75712), and *Synechocystis* sp. (DDBJ accession number D90904), respectively. Spaces (−) are inserted for the optimum alignment of the proteins. The residues that are conserved in all proteins are highlighted in *purple* with Ser-119, Asp-174, and His-208 highlighted in *red*. Similar residues that occur in at least four proteins are in *bold*. The predicted secondary structure elements of LysoPLA I are indicated *above* the MOUSE sequence by the same symbols used in *A* for the α/β hydrolyase fold.

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 α/β-hydrolase fold, especially in the C-terminal half of the sequence. α/β-Hydrolases constitute a family of enzymes with different phylogenetic origins and catalytic functions but share a common protein structure (termed α/β-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 β-strand and an α-helix) of the catalytic triad are highly conserved in the α/β-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. 3*B*). More importantly, this Asp-174 occurs on a loop between β-strand 7 and helix E, the site conserved for the acid residue in the α/β-hydrolases (Fig. 3*A*). Three His residues, His-28, His-170, and His-208, are also conserved among all the listed

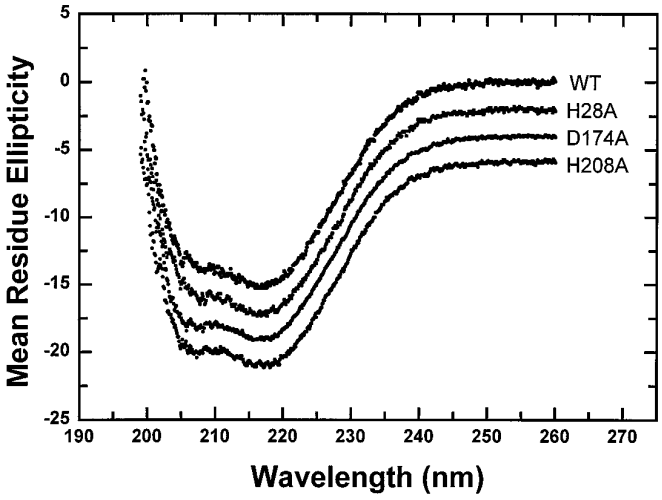


FIG. 4. **CD spectra of the wild-type and mutant LysoPLA I proteins.** The mean residue ellipticity is reported in units of millidegree × cm^2dmol^{-1}. For clarity, the CD spectra of the mutants are frameshifted down relative to that of the wild-type enzyme (*WT*) by 2 units for H28A, 4 units for D174A, and 8 units for H208A. Without such frameshifts, all four spectra overlapped with one another.

proteins (Fig. 3). However, only His-208 has the features of the catalytic base residue in the α/β-hydrolases; it occurs downstream of Asp-174 and is on a loop between β-strand 8 and helix F. 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, ^{31}P 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. 5*A*, 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 glycerophosphocholine revealed that the reaction had two zero-order phases (Fig. 5*B*), 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 secretary PLA_2s (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

LysoPLAs 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 ^{31}P 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 strong 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

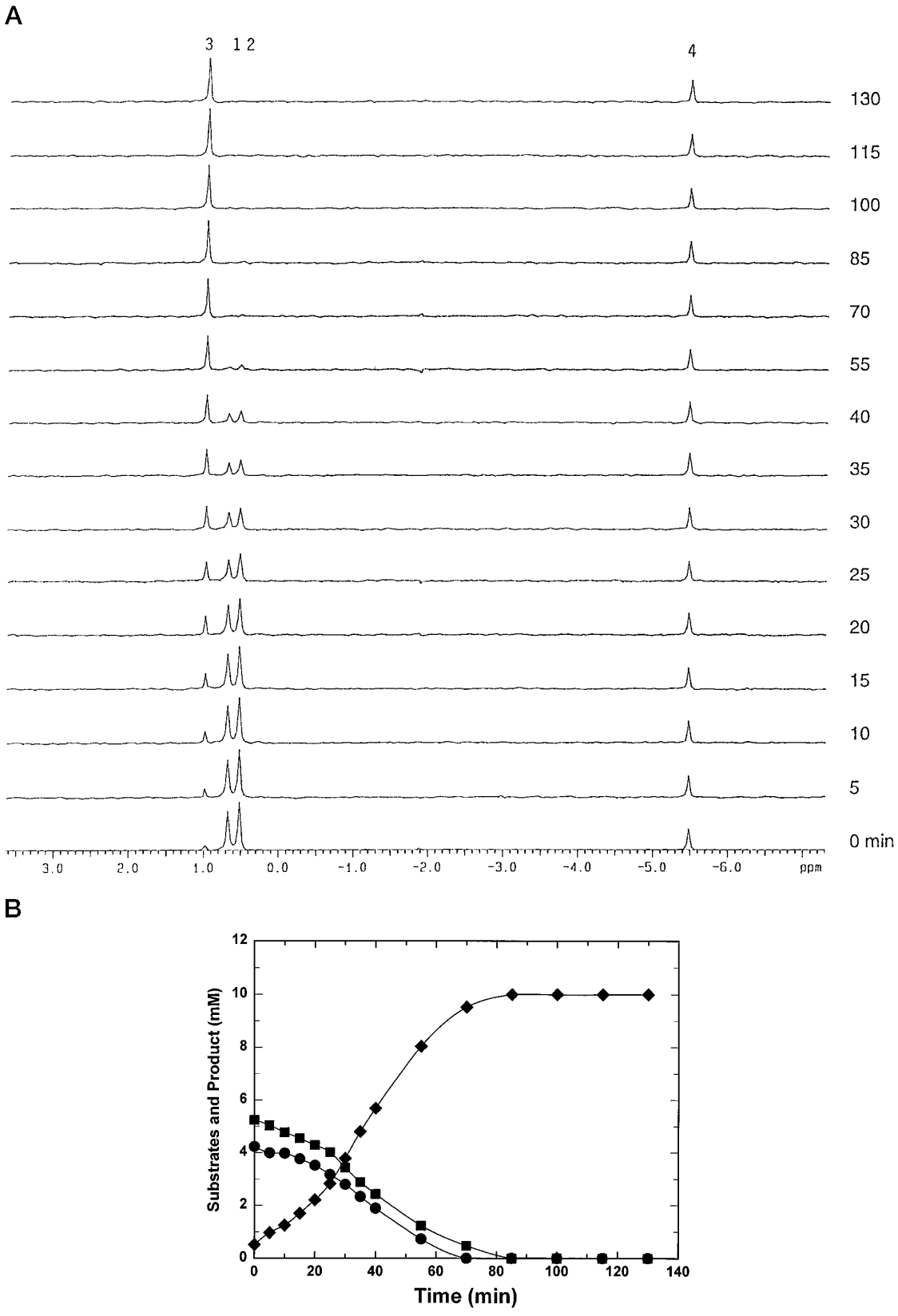


FIG. 5. **Regiospecificity of LysoPLA I.** *A*, ^{31}P NMR spectra of LysoPLA I catalyzed hydrolysis of ~1:1 mixture of 1PGPC and 2PGPC as a function of time. *Peak 1* (0.68 ppm) is 1PGPC, *peak 2* (0.52 ppm) is 2PGPC, and *peak 3* (0.99 ppm) is the product glycerophosphocholine. The external standard 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.

the predicted secondary structure of LysoPLA I and the known crystal structure of acetylcholinesterase, a member of the α/β-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

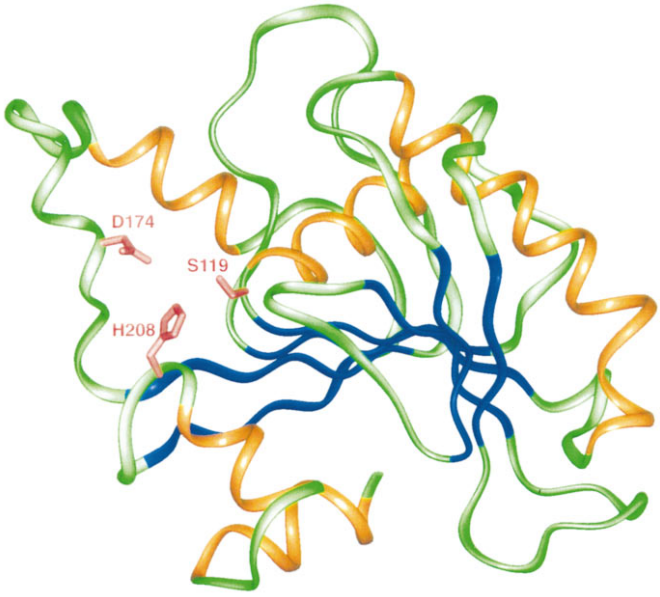


FIG. 6. **Predicted structure of LysoPLA I.** Ribbon schematic of LysoPLA I (44–203 residues) was obtained by structural modeling using the acetylcholinesterase as template. The α-helixes are *brown*, β-strands *blue*, and the catalytic residues *red*.

were obtained using another α/β-hydrolase (dielelactone 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 PLA_1 and PLA_2.

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