Structure and function of lysosomal phospholipase A_2: identification of the catalytic triad and the role of cysteine residues

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Abstract **Lysosomal phospholipase A_2 ($LPLA_2$) is an acidic phospholipase that is highly expressed in alveolar macrophages and that may play a role in the catabolism of pulmonary surfactant. The primary structure found in LCAT is conserved in $LPLA_2$, including three amino acid residues potentially required for catalytic activity and four cysteine residues. $LPLA_2$ activity was measured in COS-7 cells transfected with *c-myc*-conjugated mouse $LPLA_2$ ($mLPLA_2$) or mutated $LPLA_2$. Single alanine substitutions in the catalytic triad resulted in the elimination of $LPLA_2$ activity. Four cysteine residues (C65, C89, C330, and C371), conserved between $LPLA_2$ and LCAT, were replaced with alanine. Quadruple mutations at C65, C89, C330, and C371, double mutations at C65 and C89, and a single mutation at C65 or C89 resulted in the elimination of activity. Double mutations at C330 and C371 and a single mutation at C330 or C371 resulted in a partial reduction of activity. Thus, the presence of a disulfide bond between C330 and C371 is not required for $LPLA_2$ activity. We propose that one disulfide bond between C65 and C89 and free cysteine residues at C330 and C371 and the triad, serine-198, aspartic acid-360, and histidine-392, are required for the full expression of $mLPLA_2$ activity.**—Hiraoka, M., A. Abe, and J. A. Shayman. **Structure and function of lysosomal phospholipase A_2: identification of the catalytic triad and the role of cysteine residues. *J. Lipid Res.* 2005.** 46: **2441–2447.**

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Recently, a novel lysosomal phospholipase A_2 ($LPLA_2$) was purified from bovine brain (1), and the bovine, mouse, and human genes encoding $LPLA_2$ were cloned (2). $LPLA_2$ is highly expressed in the alveolar macrophages of rats and mice (3). Granulocyte-macrophage colony-stimulating factor-deficient mice, a model of pulmonary alveolar proteinosis (4), exhibited significantly lower $LPLA_2$ activity in alveolar macrophages compared with wild-type mice, consistent with a role for $LPLA_2$ in the phospholipid catabolism of pulmonary surfactant (3).

$LPLA_2$ has both transacylase and phospholipase A_2 activities under acidic conditions (1, 5). Divalent cations are not required for $LPLA_2$ activity, although calcium slightly enhances enzyme activity (1). The purified $LPLA_2$ is a water-soluble glycoprotein consisting of a single peptide chain with a molecular mass of 45 kDa (1). The primary structure of $LPLA_2$, deduced from DNA sequences encoding $LPLA_2$, is highly conserved between mammals, including mouse, rat, human, and bovine. The amino acid sequence of $LPLA_2$ is 49% identical to that of LCAT (**Fig. 1**). Based on their primary structures, both enzymes are members of the αβ-hydrolase superfamily and have the amino acid residues forming a catalytic triad (2, 6). In general, the triad consists of three amino acid residues, serine, aspartic acid/glutamic acid, and histidine, a structure that is essential for the hydrolysis reaction. We previously demonstrated that the serine-198 (S198) residue within a putative lipase motif sequence and within the catalytic triad is required for phospholipase A_2 activity (2).

In addition, there are four cysteine residues that are conserved between $LPLA_2$ and LCAT (2, 6). Two of the cysteines are proximate to the N terminus, and the two other cysteines are proximate to the C terminus. Two intramolecular disulfide bonds found in LCAT are formed at the conserved cysteine residues (7), and the loop spanned by the disulfide bridge between cysteine-50 (C50) and C74 is essential for LCAT binding to the lipoprotein surface (8, 9). $LPLA_2$ activity is unaffected by treatment with the thiol compound DTT and the thiol reagent *N*-ethylmaleimide (1).

Independent of the structural similarities between LCAT and $LPLA_2$, there exist significant differences in the acceptor specificity for the transacylase activity. For example, $LPLA_2$ is unable to use cholesterol as an acceptor in

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Fig. 1. Primary structures of lysosomal phospholipase A_2 (mLPLA$_2$) and mLCAT. The amino acid sequence of mLPLA$_2$ was deduced from the cDNA sequence of mLPLA$_2$ (2). S, D, and H indicate the amino acid residues of the catalytic triad. Cs indicate the conserved cysteine residues in both enzymes. The underline and asterisk indicate the lipase motif and one of the oxyanion holes, respectively.

the transacylase reaction (2). In addition, LPLA$_2$ and LCAT exhibit different pH optima for their enzymatic activities.

With the exception of S198, the functional importance of these other amino acid residues for LPLA$_2$ activity was unknown. In the present study, we evaluated the role of the catalytic triad and cysteine residues in LPLA$_2$ by measuring the enzyme activities of site-directed mutated mouse LPLA$_2$s expressed in COS-7 cells.

MATERIALS AND METHODS

Reagents

Phosphatidylethanolamine and 1,2-dioleloyl-*sn*-glycero-3-phosphorylcholine were obtained from Avanti Polar Lipids (Alabaster, AL). *N*-Acetyl-D-*erythro*-sphingosine (NAS) was from Matreya LLC (Pleasant Gap, PA). MJ33 was from EMD Biosciences (San Diego, CA). BCA protein assay reagent was obtained from Pierce Chemical Co. (Rockford, IL). Monoclonal anti-c-Myc clone 9E10 mouse ascites fluid, anti-mouse IgG HRP conjugate goat antibody, diaminobenzidine, *p*-nitro-phenyl acetate, and *p*-nitro-phenyl butylate were from Sigma Chemical Co. (St. Louis, MO). The polyvinylidene difluoride membrane (Westran) was from Schleicher and Schuell Bioscience (Keene, NH). HPTLC silica gel plates, 10 × 20 cm, were purchased from Merck.

Construction of LPLA$_2$ expression plasmids

The entire open reading frame of mouse LPLA$_2$ (mLPLA$_2$) was obtained by PCR from mouse kidney cDNA as described previously (2). The construct was then subcloned into the *Hind*III and *Xho*I sites of pcDNA3-c-Myc to generate C-terminal tagged LPLA$_2$ proteins.

Site-directed mutations of the amino acid residues in the putative catalytic triad and conserved cysteine residues of mLPLA$_2$ were generated by the overlap extension method (10). The amino acid residues in the triad and the conserved cysteine residues in mLPLA$_2$ were replaced with alanines. In brief, two PCRs were used to amplify the overlapping fragments, and another PCR was used to fuse the fragments. To obtain the overlapping fragments, amplification primers (**Table 1**) and the template DNA consisting of the plasmid containing the mLPLA$_2$ gene between the *Hind*III and *Xho*I sites (TOPO-mLPLA$_2$) were applied to the PCR. For the generation of single mutations, including A196G, S198A, D351G, D351A, D360A, H392A, C65A (C1A), C89A (C2A), C330A (C3A), and C371A (C4A), a primer pair consisting of one mutagenic primer (forward) and the mLPLA$_2$-*Xho*I primer or the same mutagenic primer (reverse) and the mLPLA$_2$-*Hind*III primer was used with TOPO-mLPLA$_2$ in the first PCR. The single mutated mLPLA$_2$ was obtained by the second PCR using two overlapping fragments and mLPLA$_2$-*Xho*I and mLPLA$_2$-*Hind*III primers. The double mutations of the cysteine residues in mLPLA$_2$, C12A (C65A and C89A), were generated by PCR of three fragments, being obtained from the first PCR of mLPLA$_2$-*Hind*III and the C1A (reverse) primer pair, those obtained from the C1A (forward) and C2A (reverse) primer pair and C2A (forward), and those from the mLPLA$_2$-*Xho*I primer pair with TOPO-mLPLA$_2$ and the mLPLA$_2$-*Xho*I and mLPLA$_2$-*Hind*III primers. Another double mutation, C34A (C330 and C371 replaced with alanines), was generated similarly by PCR of three fragments, being obtained from the first PCR of mLPLA$_2$-*Hind*III and the C3A (reverse) primer pair, the C3A (forward) and C4A (reverse) primer pair, and the C4A (forward) and mLPLA$_2$-*Xho*I primer pair with TOPO-mLPLA$_2$, and the

TABLE 1. Oligonucleotides used for the site-directed mutagenesis of lysosomal phospholipase A_2

Oligonucleotide	Sequence
A196G	(F) 5′-TGCTGGTCGGACACAGCATG-3′
	(R) 5′-ATGCTGTGTCCGACCAGCACCA-3′
S198A	(F) 5′-GCCCACGCTATGGGCAAC-3′
	(R) 5′-TTGCCCATAGCGTGGGCGACCA-3′
D351G	(F) 5′-GATCGGGGTCCCAAAATCTGCTTC-3′
	(R) 5′-TTTGGGACCCCGATCAGGAAAGCT-3′
D351A	(F) 5′- GATCGGGCACCCAAAATCTGCTTC-3′
	(R) 5′-TTTGGGTGCCCGATCAGGAAAGCT-3′
D360A	(F) 5′-CGATGGTGCAGGCACGGTGAAC-3′
	(R) 5′-ACCGTGCCTGCACCATCGCCGAA-3′
H392A	(F) 5′-AGCGAGGCTATTGAGATGCTAGCC-3′
	(R) 5′-CTCAATAGCCTCGCTTCCCGGCAG-3′
C65A (C1A)	(F) 5′-CACTACCTTGCGTCCAAGAAGACG-3′
	(R) 5′-CTTCTTGGACGCAAGGTAGTGTAC-3′
C89A (C2A)	(F) 5′-ATCATTGACGCCTGGATTGACATT-3′
	(R) 5′-GTCAATCCAGGCGTCAATGATAAC-3′
C330A (C3A)	(F) 5′-GAGCTGCACGCATTGTATGGCACT-3′
	(R) 5′-GCCATACAATGCGTGCAGCTCCA-3′
C371A (C4A)	(F) 5′-TCCTGCAGGCTCAAGCCTGGCA-3′
	(R) 5′-AGGCTTGAGCCTGCAGGACGCT-3′
mLPLA$_2$-*Hin*dIII	(F) 5′-CCC*AAGCTT*GGGATGGATCGCCATCTC-3′
mLPLA$_2$-*Xho*I	(R) 5′-AAACGTGTGCTTCTGGAACCTCCG*CTCGAG*CGG-3′

F and R indicate forward and reverse, respectively. Underlined sequences mismatch with the template. Italic sequences indicate the restriction endonuclease cleavage sites.

mLPLA$_2$-*Xho*I and mLPLA$_2$-*Hin*dIII primers. The quadruple mutations, C1234A (C65, C89, C330, and C372 replaced with alanines), were generated by PCR of five fragments, being obtained from the first PCR of mLPLA$_2$-*Hin*dIII and the C1A (reverse) primer pair, the C1A (forward) and C2A (reverse) primer pair, the C2A (forward) and C3A (reverse) primer pair, the C3A (forward) and C4A (reverse) primer pair and the C4A (forward) and mLPLA$_2$-*Xho*I primer pair with TOPO-mLPLA$_2$, and the mLPLA$_2$-*Xho*I and mLPLA$_2$-*Hin*dIII primers. The PCR product of the second PCR was ligated into pcDNA3-c-Myc. All mutant construct sequences were confirmed by sequencing in both directions.

Cell culture and transfection

COS-7 cells were transiently transfected with pcDNA3 containing the entire open reading frame of mouse LPLA$_2$ to obtain LPLA$_2$ overexpressed cells, as described previously (2). COS-7 cells were grown in DMEM (Gibco BRL) supplemented with 10% fetal bovine serum. For transient expression, COS-7 cells were cultured in 35 mm dishes. Upon reaching 80% confluence, the cells were transfected with 1 μg/ml purified plasmid using LipofectAMINE Plus™ (Invitrogen, Carlsbad, CA) in 1 ml of opti MEM medium (Gibco BRL). One milliliter of DMEM containing 20% fetal bovine serum was added after incubation for 3 h at 37°C in 5% CO_2. Twenty-four hours after transfection, the cells were washed three times with 2 ml of phosphate-buffered saline, incubated with 2 ml of 1 mM EDTA in PBS for 20 min at 37°C, and transferred into a centrifuge tube. The following procedures were carried out at 4°C. The cells were collected by centrifugation at 800 *g* for 10 min. Each cell pellet was dispersed into 0.5 ml of 0.25 M sucrose, 10 mM HEPES, and 1 mM EDTA (pH 7.4) by sonication. Sonication was carried out for 10 s four times at 0°C with a probe sonicator. The suspension was centrifuged for 1 h at 100,000 *g*. The resulting supernatant was passed through a 0.2 μm filter and used as a soluble fraction.

Immunoblotting

The soluble fraction was precipitated by the method of Bensadoun and Weinstein (11). The resulting pellet was dissolved with 30 μl of loading buffer plus 1.5 μl of 2 M Tris for SDS-polyacrylamide gel electrophoresis. Proteins were separated using a 10% or 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane using transfer buffer (20 mM Tris and 150 mM glycine in 20% methanol) at a constant voltage (100 V for 3 h at 4°C). The membrane was incubated with an anti-mouse LPLA$_2$ peptide (^{100}RTSRATQFPD), rabbit serum, or monoclonal anti-c-Myc mouse ascites fluid. The antigen-antibody complex on the membrane was visualized with an anti-rabbit IgG horseradish peroxidase-conjugated goat antibody or an anti-mouse IgG horseradish peroxidase-conjugated goat antibody using diaminobenzidine and hydrogen peroxide.

Transacylase activity of LPLA$_2$

NAS was used as an acyl group acceptor. The reaction mixture consisted of 40 mM sodium citrate (pH 4.5), 10 μg/ml BSA, 40 μM NAS incorporated into phospholipid liposomes [phosphatidylcholine (PC)/phosphatidylethanolamine/dicetyl phosphate/NAS (5:2:1:2 in molar ratio)], and the soluble fraction (2 μg) in a total volume of 500 μl. The reaction was initiated by adding the soluble fraction, maintained for 10, 20, and 30 min at 37°C, and terminated by the addition of 3 ml of chloroform-methanol (2:1) plus 0.3 ml of 0.9% (w/v) NaCl. The mixture was centrifuged for 5 min at room temperature. The resulting lower layer was transferred into another glass tube and dried down under a stream of nitrogen gas. The dried lipid was dissolved in 40 μl of chloroform-methanol (2:1), half of which was applied on a high-performance thin-layer chromatography plate and developed in a solvent system consisting of chloroform-acetic acid (9:1). The plate was dried down and soaked in 8% (w/v) $CuSO_4 \cdot 5H_2O$, 6.8% (v/v) H_3PO_4, and 32% (v/v) methanol. The uniformly wet plate was briefly dried down with a hair dryer and charred for 15 min in a 150°C oven. The plate was scanned, and the content of the product (1-*O*-acyl-NAS) was estimated by NIH Image 1.63.

RESULTS

Site-directed mutagenesis of LPLA$_2$

Substitution with alanine of the amino acid residues of the catalytic triad and of the cysteines in mLPLA$_2$ was carried

out by the overlap extension method using the primers listed in Table 1. COS-7 cells were transiently transfected with c-Myc peptide-conjugated $mLPLA_2$ or mutated $mLPLA_2$ gene. $LPLA_2$ expression in COS-7 cells was detected by Western blot using anti-c-Myc antibody and was demonstrated to be comparable between the transfectants (**Figs. 2**, **3**). In a previous study, we had demonstrated that the specific activity of $LPLA_2$ expressed in COS-7 cells was identical to that of the native enzyme expressed in alveolar macrophages (3).

The catalytic triad in $LPLA_2$

The catalytic triad in the αβ-hydrolase superfamily of enzymes consists of three amino acid residues, serine, aspartic acid/glutamic acid, and histidine. The triad is an essential structure to allow the enzyme to catalyze a hydrolytic reaction by the catalytic nucleophile. Previously, S198 in $mLPLA_2$, part of the putative lipase motif AXSXG (where X represents any amino residue), was shown to be essential for enzyme activity (2). The active site serine residue was proposed to form an acyl-$LPLA_2$ intermediate during the hydrolysis reaction. In the present study, the amino acid residues of the putative triad in $mLPLA_2$, S198, D360, and H392, were replaced with alanine. $mLPLA_2$ and single mutated $mLPLA_2$s were equally expressed in the soluble fraction of COS-7 cells (Fig. 2). The transacylase activity of $LPLA_2$ was measured to ascertain the effects of amino acid substitutions. Site-directed mutations of the triad eliminated the transacylase activity (Fig. 2). On the other hand, the replacement of aspartic acid at position 351 with alanine or glycine in $mLPLA_2$ did not affect the enzyme activity (data not shown) in spite of its proximate location to D360. These results indicate that each amino acid residue in the triad is crucial for $LPLA_2$ activity. Interestingly, $mLPLA_2$ activity was reduced to 10% of its original activity when A196 was substituted with glycine found in another lipase motif, GXSXG, which is also preserved in LCAT (data not shown).

Cysteine residues in $LPLA_2$

$LPLA_2$ is highly homologous with LCAT (Fig. 1). LCAT has six cysteine residues, four of which are involved in the formation of intramolecular disulfide bonds (7). The primary amino acid sequence of $LPLA_2$ reveals that the cysteine residues involved in disulfide linkages in LCAT are conserved in $LPLA_2$. The disulfide bonds in LCAT not only contribute stabilization of the protein but also play a crucial role in the interaction between LCAT and lipoprotein surfaces (8, 9). Unlike LCAT, $LPLA_2$ is not able to catalyze the transacylation of an acyl group from the *sn*-2 position in PC to cholesterol (2), even though $LPLA_2$ has both phospholipase A_2 and transacylase activities, as does LCAT. The pH optima for $LPLA_2$ and LCAT activities are acidic and neutral, respectively. Previously, it was shown that $LPLA_2$ activity is not sensitive to either thiol compounds or thiol reagents (1). On this basis, we hypothesized that there might be a difference between LCAT and $LPLA_2$ with respect to the formation of disulfide linkages. However, a disulfide linkage in an intact protein is not always accessible by thiol compounds or thiol reagents. To further evaluate the role of cysteine residues in $LPLA_2$ activity, the four cysteine residues in $mLPLA_2$ that are preserved in LCAT were substituted with alanines.

$mLPLA_2$ and mutated $mLPLA_2$s were equally expressed in the soluble fraction of COS-7 cells (Fig. 3). The transacylase activity of $LPLA_2$ in each soluble fraction was measured to ascertain the effect of amino acid substitutions of each cysteine residue on $LPLA_2$ activity. Quadruple mutations at C65, C89, C330, and C371, double mutations at C65 and C89, and a single mutation at C65 or C89 resulted in the elimination of $LPLA_2$ activity (Fig. 3). On the other hand, double mutations at C330 and C371 and a single mutation at C330 or C371 resulted in a partial reduction of the activity (Fig. 3). The remaining $LPLA_2$ activity in each mutated $mLPLA_2$ was 29 ± 5.7% for the double mutations at C330 and C371, 4.8 ± 0.4% for the single mutation at C330, and 36 ± 5.7% for the single mutation at C371. Thus, the formation of a disulfide bond between C330 and C371 is not necessarily required for $LPLA_2$ activity, suggesting that both of these cysteine residues in intact $LPLA_2$ contain a free sulfhydryl group.

Although mouse, rat, and human $LPLA_2$s have an additional cysteine residue located between the cysteine resi-

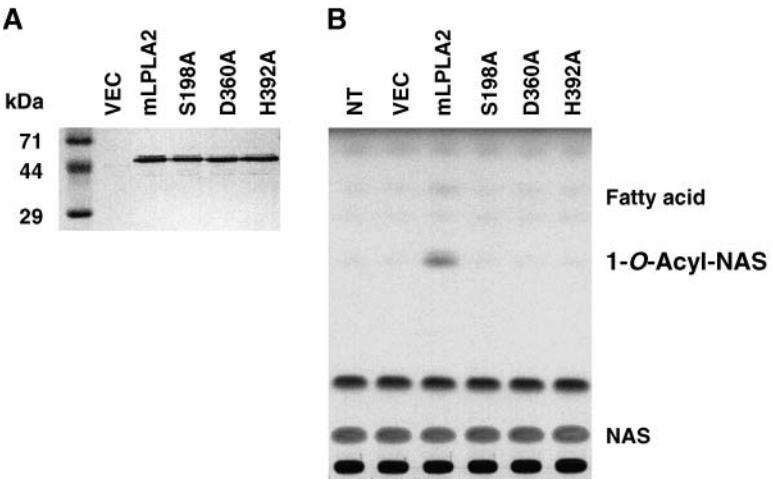

Fig. 2. Expression of $mLPLA_2$ and the triad mutated $mLPLA_2$ in COS-7 cells. COS-7 cells were transiently transfected with pcDNA3-c-Myc (VEC), pcDNA3-c-Myc-tagged $mLPLA_2$ ($mLPLA_2$), or pcDNA3-c-Myc-tagged mutated $mLPLA_2$ (S198A, S198 replaced with A; D360A, D360 replaced with A; H392A, H392 replaced with A). Each soluble fraction of proteins obtained from nontransfected (NT) or transfected cells was used for Western blotting and the $LPLA_2$ activity assay. A: In Western blotting, 20 μg of protein in each soluble fraction was separated by SDS-polyacrylamide gel electrophoresis and subjected to immunoblotting with a monoclonal anti-c-Myc mouse ascites fluid, and c-Myc-tagged $LPLA_2$s were visualized as described in Materials and Methods. B: In the $LPLA_2$ assay, 3 μg of each soluble fraction was incubated for 10 min at 37°C with liposomes containing *N*-acetyl-D-*erythro*-sphingosine (NAS) as described in Materials and Methods.

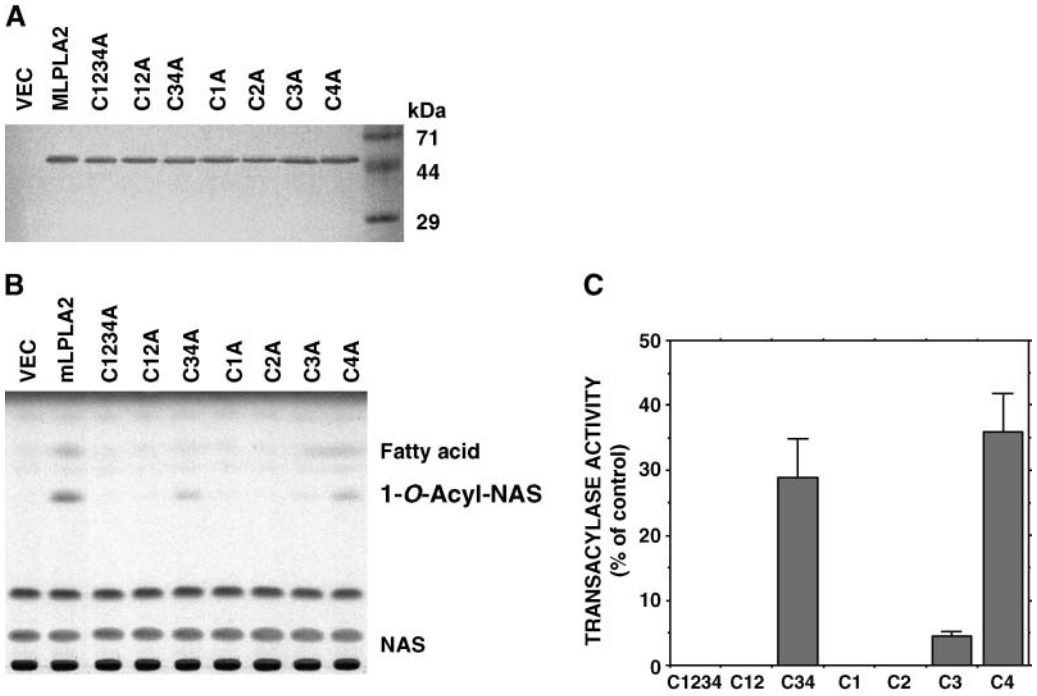

Fig. 3. Expression of $mLPLA_2$ and the conserved cysteine mutated $mLPLA_2$ in COS-7 cells. COS-7 cells were transiently transfected with pcDNA3-c-Myc (VEC), pcDNA3-c-Myc-tagged $mLPLA_2$ ($mLPLA_2$), or pcDNA3-c-Myc-tagged mutated $mLPLA_2$ (C1A, C65 replaced with A; C2A, C89 replaced with A; C3A, C330 replaced with A; C4A, C371 replaced with A; C12A, C65, and C89 replaced with As; C34A, C330, and C371 replaced with As; C1234A, C65, C89, C330, and C371 replaced with As). Each soluble fraction of proteins obtained from transfected cells was used for Western blotting and the $LPLA_2$ activity assay. A: In Western blotting, 20 μg of protein in each soluble fraction was separated by SDS-polyacrylamide gel electrophoresis and subjected to immunoblotting with a monoclonal anti-c-Myc mouse ascites fluid, and c-Myc-tagged $LPLA_2$s were visualized as described in Materials and Methods. B: In the $LPLA_2$ assay, 3 μg of each soluble fraction was incubated for 10 min at 37°C with liposomes containing NAS as described in Materials and Methods. The transacylase activity of the control was measured using the soluble fraction obtained from the $mLPLA_2$ transfectant. C: The percent transacylase activities in the mutant $mLPLA_2$ compared to control $mLPLA_2$. Error bars indicate SD (n = 3).

dues in the C-terminal region, bovine $LPLA_2$ lacks the additional cysteine. Therefore, partially purified bovine $LPLA_2$ was used to demonstrate the existence of the free cysteine residues in conserved cysteine residues of $LPLA_2$ by application to an organomercury agarose column. $LPLA_2$ activity was completely absorbed to the organomercury agarose column and eluted from the column with a buffer containing excess 2-mercaptoethanol (**Fig. 4**). The organomercury compound forms a covalent bond with a free sulfhydryl group that is cleaved in the presence of excess thiol compounds. These results indicate that at least two cysteine residues of the conserved cysteine residues in $LPLA_2$ are free cysteine residues.

Generally, a nonreduced single protein molecule with an intramolecular disulfide bond or bonds is known to have faster mobility compared with the reduced protein in SDS-PAGE under nonreducing conditions. This change in mobility is likely attributable to the compact molecular size of the protein with one or more disulfide bonds. When nonreduced $LPLA_2$ was subjected to SDS-PAGE under nonreducing conditions, nonreduced $LPLA_2$ was able to move slightly faster than the reduced $LPLA_2$ (**Fig. 5**). This result is consistent with the presence of an intramolecular disulfide bond. Interestingly, the color development of nonreduced $LPLA_2$ by diaminobenzidine was considerably slower than that of reduced $LPLA_2$ (Fig. 5). This observation suggests that the interaction between the antibody against $LPLA_2$ and $LPLA_2$ on the blot membrane is weakened by the remaining disulfide bond in $LPLA_2$. Cysteines C65 and C89 are located in the proximity of the region of the sequence of $LPLA_2$ (^{100}RTSRATQFPD) recognized by the antibody. Therefore, the disulfide linkage between C65 and C89 may physically interfere with the binding of the antibody to $LPLA_2$ on the membrane.

These results indicate that one disulfide linkage between C65 and C89 and two free cysteine groups (C330 and C371) of the four conserved cysteine residues in $mLPLA_2$ may be essential for full $LPLA_2$ activity.

DISCUSSION

The catalytic triad and four cysteine residues in LCAT are well preserved in $LPLA_2$. Both enzymes belong to the lipase family that is part of the αβ-hydrolase superfamily and that catalyzes the transfer of the acyl group at the *sn*-2

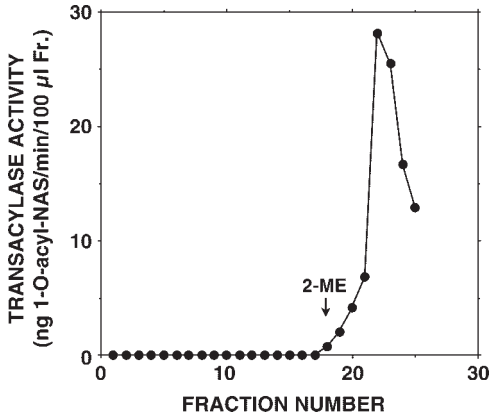

Fig. 4. Organomercury agarose column chromatography of $LPLA_2$. Partially purified bovine brain $LPLA_2$ (48 μg of protein) dialyzed against 25 mM potassium phosphate (pH 7.4) was applied to an organomercury column (0.5 ml bed volume) equilibrated with 25 mM potassium phosphate (pH 7.4). After applying the protein, the column was washed extensively with the same buffer and then washed with 25 mM potassium phosphate (pH 7.4) containing 200 mM 2-mercaptoethanol (2-ME), as indicated by the arrow. Eluates consisting of 2 ml/tube and 4 ml/tube were collected in fractions 1–10 and fractions 11–26, respectively. The entire process was carried out at 4°C. One hundred microliters of each fraction (Fr.) was used for the transacylase activity assay as described in Materials and Methods.

position of PC to an acceptor. As confirmed in the present study, the catalytic triad of $LPLA_2$ is essential for enzymatic activity. The serine residue present in both the LCAT and the LCAT triads is contained within lipase motifs, ^{181}GXSXG and ^{196}AXSXG, respectively. Based on the secondary structure deduced from the primary structure, the serine of the active site is the nucleophile located in a keen turn structure in both cases (12). This strand-turn-helix structure is a conserved feature in the αβ-hydrolase proteins (13) and may allow the nucleophile group the ease of access not only to the substrate but also to the acceptor molecule.

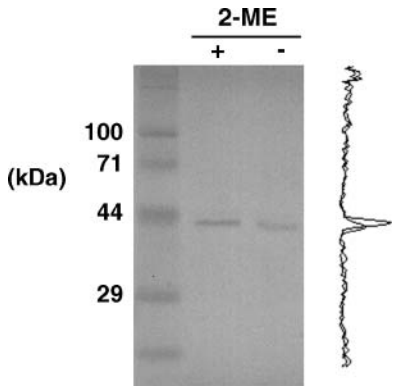

Fig. 5. Profile of $mLPLA_2$ in SDS-PAGE. The soluble fraction (15 μg of protein) obtained from mouse alveolar macrophages was prepared as an SDS-PAGE sample with or without 100 mM 2-mercaptoethanol (2-ME). Each sample was separated on a 10% polyacrylamide gel in the presence of 1% SDS under nonreduced conditions and transferred to a polyvinylidene difluoride membrane. $LPLA_2$ on the membrane was detected by anti-$LPLA_2$ peptide serum as described in Materials and Methods. Each protein band was scanned using NIH Image 1.63.

The phenylalanine at residue 103 in LCAT is one of two amino acid residues involved in the formation of the oxyanion hole (14) and is conserved in $mLPLA_2$ and other $LPLA_2$s (Fig. 1). This homology suggests that the coordination of the enzyme-substrate intermediate at the catalytic site in the transition state is similar between LCAT and $LPLA_2$. However, the difference in the lipase motif between LCAT and $LPLA_2$ also suggests that the enzyme specificity and activity might be affected by such a small difference. Thus, when alanine-196 (A196) in $mLPLA_2$ was substituted with glycine (G196), the mutated $mLPLA_2$ showed both phospholipid-NAS 1-*O*-transferase and PLA_2 activities, but the substitution resulted in a 10-fold reduction of the enzyme activity. Thus, the substitution of A196 with G196 of $mLPLA_2$ changes the enzyme activity but not the enzyme specificity. A characteristic helical wheel alignment of homologous regions of mLCAT (residues 153–170) and $mLPLA_2$ (residues 171–188) is predicted from their amino acid sequences (12) and is located in the proximity of the catalytic serine residue. Their helical wheels are amphipathic helices that may be involved in the binding of phospholipid at the active site (15). Mutagenesis studies have shown that the helical domain in LCAT plays a crucial role in the phospholipid substrate specificity as well as in phospholipid binding (16).

The four conserved cysteine residues in $mLPLA_2$ are located at C65, C89, C330, and C371. The present study supports a model in which one disulfide bridge is formed between conserved cysteine residues C65 and C89 and two free cysteine residues are located at C330 and C371. In both $LPLA_2$ and LCAT, the region between the cysteine residues forming the disulfide bridge in the proximity of the N-terminal region is predicted to be the most hydrophobic region that is spanned as a loop containing an amphipathic helix (8, 9). The loop formed in LCAT is essential for the interaction between LCAT and lipoprotein surfaces and is thought to be indispensable for the interfacial activation of LCAT (17). By analogy, the loop in $LPLA_2$ may also play a crucial role in the interaction of $LPLA_2$ with hydrophobic structures such as phospholipid membranes. The substitution of C65 and/or C89 with alanine resulted in the elimination of $LPLA_2$ activity, suggesting that a proper interaction between mutated $LPLA_2$ and lipid membrane is not accomplished by disruption of the structural loop of this region when the disulfide bridge is absent. As proposed in LCAT and shown in other lipases of the αβ-hydrolase superfamily, the loop in $LPLA_2$ may act as a lid to regulate the access of substrate to the active site (6, 18, 19). Therefore, a degree of hydrophobicity may be required for substrates to access the active site. In support of this interpretation is the observation that the degradation rate of the artificial substrate *p*-nitro-phenyl butylate by $LPLA_2$ under acidic conditions is 7-fold higher than that of *p*-nitro-phenyl acetate (data not shown). $LPLA_2$ mutated at S198 does not hydrolyze these artificial substrates. This finding indicates that the triad in $LPLA_2$ is essential for the hydrolysis reaction of the artificial substrates as well as for naturally occurring phospholipids. Similar properties have already been reported for LCAT (20).

Unlike LCAT, mLPLA$_2$ may have two conserved free cysteine groups, C330 and C371, in the proximity of the C-terminal region. C330 and C371 are adjacent to the triad amino acids, D360 and H392. The catalytic triad structure in LPLA$_2$ may be similar to that observed in other lipases, in which hydrogen bonds formed between the amino acid residues in the triad play a key role in enzyme activation and reaction (21). Although a single or double substitution of each cysteine residue (C330, C371) with alanine reduces the original enzymatic activity, each mutated LPLA$_2$ still retains some enzyme activity. Thus, the free sulfhydryl groups at C330 and C371 are likely to be crucial for providing a proper tertiary structure for the catalytic triad. Although the tertiary structure of the active site in LPLA$_2$ is thought to be similar to that of LCAT, differences in disulfide bridge formation between LCAT and LPLA$_2$ may contribute to different substrate specificity and enzyme activity. For example, LPLA$_2$ catalyzes the transacylation of the acyl group at *sn*-2 of PC to an acceptor with a hydroxyl group but is not able to use cholesterol as an acceptor (2). Our preliminary studies have shown that, in addition to NAS, LPLA$_2$ prefers to use neutral and relatively hydrophobic compounds with a primary alcohol group rather than secondary and tertiary alcohol groups as the acceptor (unpublished data).

The presumed importance of the free sulfhydryl groups at C330 and C371 for enzyme activity could be questioned based on the failure of thiol reagents to decrease activity. When LPLA$_2$ was initially purified from bovine brain and its activity characterized, *N*-ethylmaleimide only decreased the transacylase activity by 5%. This observation was initially interpreted as suggesting that the free sulfhydryl groups in LPLA$_2$ were unimportant for activity. In the context of the present studies, another interpretation should be considered. The free sulfhydryl groups of the intact protein may not be readily accessible to thiol reagents. A similar result was observed in studies of the glycolipid transfer protein (22).

Further studies comparing the structure and function of LCAT and LPLA$_2$ are likely to be informative. Such studies should include the construction of chimeric enzymes to confirm the importance of specific domains of each protein in determining substrate specificity. Such studies may also be informative regarding the evolutionary biology of both LPLA$_2$ and LCAT. The genes encoding both proteins are members of an extensive and evolutionarily ancient gene family. Homologs of these genes are present in many ancient species that lack lungs (as might be predicted for LPLA$_2$) and lipoproteins (as would be predicted for LCAT). *LCAT* appears to be the more rapidly diverging gene, and it is restricted to vertebrates. LPLA$_2$ is more closely aligned to invertebrate homologs in the gene family. Thus, further studies on the protein structures of LCAT and LPLA$_2$, including the catalytic domains and disulfide bonds, and the roles of their protein domains in determining the substrate specificity and activity of these closely related enzymes will be important in understanding the biology of these enzymes and their family members.

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