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An Acyl-covalent Enzyme Intermediate of Lecithin:Retinol Acyltransferase*[S]

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Synthesis of fatty acid retinyl esters determines systemic vitamin A levels and provides substrate for production of visual chromophore (11-*cis*-retinal) in vertebrates. Lecithin:retinol acyltransferase (LRAT), the main enzyme responsible for retinyl ester formation, catalyzes the transfer of an acyl group from the *sn*-1 position of phosphatidylcholine to retinol. To delineate the catalytic mechanism of this reaction, we expressed and purified a fully active, soluble form of this enzyme and used it to examine the possible formation of a transient acyl-enzyme intermediate. Detailed mass spectrometry analyses revealed that LRAT undergoes spontaneous, covalent modification upon incubation with a variety of phosphatidylcholine substrates. The addition of an acyl chain occurs at the Cys^{161} residue, indicating formation of a thioester intermediate. This observation provides the first direct experimental evidence of thioester intermediate formation that constitutes the initial step in the proposed LRAT catalytic reaction. Additionally, we examined the effect of increasing fatty acyl side chain length in phosphatidylcholine on substrate accessibility in this reaction, which provided insights into the function of the single membrane-spanning domain of LRAT. These observations are critical to understanding the catalytic mechanism of LRAT protein family members as well as other lecithin:acyltransferases wherein Cys residues are required for catalysis.

A representative member of the NlpC/P60 superfamily (1), lecithin:retinol acyltransferase (LRAT),[2] the main enzyme responsible for retinyl ester formation, catalyzes the transfer of an acyl group from the *sn*-1 position of phosphatidylcholine (PC) to a retinoid acceptor (2–4). LRAT plays a fundamental role in the retention and storage of vitamin A and controls the generation of all-*trans*-retinoic acid, a potent expression regulator of many genes via retinoic acid nuclear receptors (5, 6). Importantly, LRAT also plays a key role in the retinoid cycle by providing substrate for 11-*cis*-retinal production (7, 8), the chromophore required for regeneration of mammalian visual pigments (9–11). Unlike many other acyltransferases, this single membrane-spanning protein does not require an activated fatty acyl group attached by a thioester bond to coenzyme A as a donor (12). Instead, this enzyme uses a variety of phosphatidylcholines as substrates (13, 14). The proposed mechanism of LRAT catalysis involves formation of a transient covalent acyl-enzyme intermediate accompanied by the release of lyso-phosphatidylcholine (15, 16). A number of biochemical studies involving site-directed mutagenesis and protein labeling suggest that the LRAT reaction mechanism may involve a Cys side chain that serves as an active site nucleophile (17). However, despite the above observations, self-acylation of a protein constituting part of the catalytic acyl transfer mechanism, even though postulated for several other enzymatic reactions including lysophosphatidylcholine:lysophosphatidylcholine acyltransferase and class II tumor suppressor H-Rev107, has yet to be directly documented for this class of enzymes (18, 19). In this study, we employed mass spectrometry to investigate formation of a covalent acyl-enzyme intermediate and determine the site of putative modification. Additionally, we examined the influence of changes in the acyl chain length at the *sn*-1 position of phosphatidylcholine and the surrounding lipid phase on LRAT enzymatic activity and formation of the enzyme-bound intermediate.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Mouse LRAT cDNA encoding amino acids ^{30}GGG…TVK^{186} was subcloned into a pGEX_2T vector (GE Healthcare) using BamHI and EcoRI restriction sites to generate the glutathione *S*-transferase fusion protein GST-tLRAT. This fusion protein then was expressed in the Bl21(DE3) *Escherichia coli* strain. Bacteria were grown in the presence of 50 μM ampicillin and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside. Protein expression was carried out at 22 °C for 5 h. The bacteria were harvested, treated with lysozyme, and disrupted by osmotic shock (20). Bacterial crude lysate was centrifuged at 36,000 × *g* at 4 °C for 30 min. The soluble fraction, after adjustment of its salt composition with 67 mM phosphate buffer, pH 7.4, and 50 mM NaCl, was incubated with glutathione-Sepharose (GE Healthcare) for 3 h at 4 °C. The resin then was placed in a chromatography column and washed with 10 column volumes of 67 mM phosphate buffer, pH 7.4, and 50 mM NaCl. GST-tLRAT fusion protein was eluted with 10 mM reduced glutathione in 10 mM Tris/HCl buffer, pH 8.0. Fractions containing the protein were pooled together, concentrated at a Centricon (Amicon) cutoff of 30 kDa, and loaded onto a SRT SEC-300 size exclusion column (Sepax Technologies) equilibrated with 10 mM Tris/HCl buffer, pH 8.0. Eluted protein was concentrated again to ~5 mg/ml and stored at −80 °C. Thrombin digestion of GST-tLRAT was

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.
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[2] The abbreviations used are: LRAT, lecithin:retinol acyltransferase; PC, phosphatidylcholine.

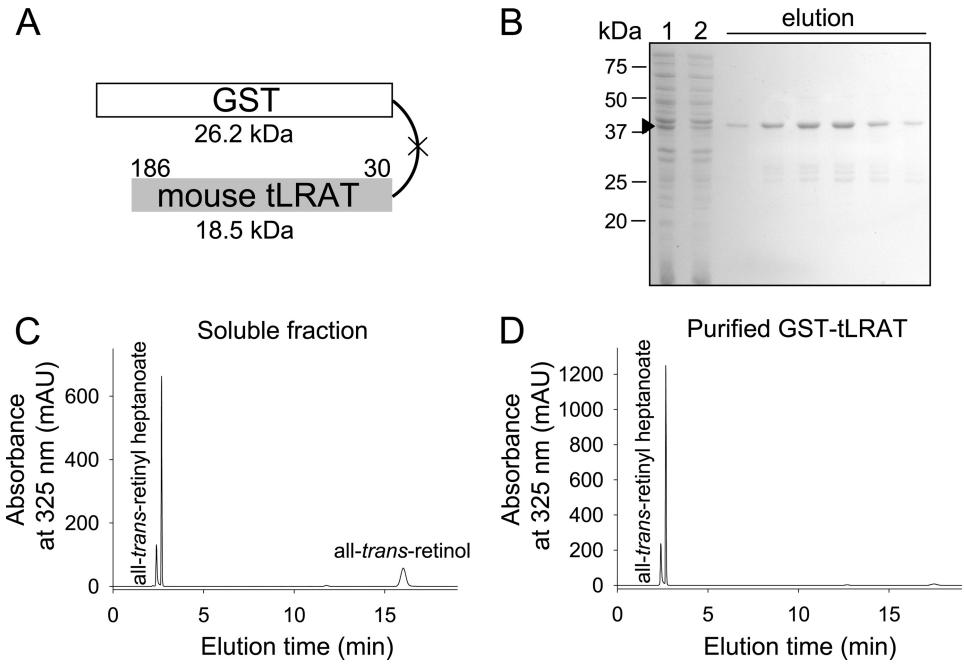

FIGURE 1. **Purification and acyltransferase activity of GST-tLRAT.** *A*, graphic representation of GST-tLRAT fusion protein. Molecular masses shown in the picture represent protein fragments liberated upon digestion with thrombin. *B*, protein purification on a glutathione affinity column. The *black arrowhead* indicates the position of expressed protein in a 12% polyacrylamide gel. *C* and *D*, HPLC separation of retinoids extracted from the acyltransferase activity assay performed with crude bacterial extract (*left*) and purified fusion protein (*right*) in the presence of 7:0 PC and all-*trans*-retinol as substrates. Formation of retinyl heptanoate indicates robust enzymatic activity of soluble GST-tLRAT fusion protein.

performed in 10 mM Tris/HCl buffer, pH 8.0, at room temperature for 1 h by using 2 units of thrombin activity/1 mg of protein.

LRAT Enzymatic and Self-acylation Assays—Acyltransferase activity was assayed in 10 mM Tris/HCl buffer, pH 8.0, 1 mM DTT, and 1% BSA with 10 μg of purified GST-tLRAT protein or UV-treated bovine retinal pigment epithelium microsomes (300 μg of total protein) isolated as described previously (21). The reaction was initiated by the addition of variable amounts of PC along with 10 μM all-*trans*-retinol delivered in 1 μl of dimethylformamide to a total volume of 0.2 ml. PCs containing eight or more carbon acyls were delivered in liposomes formed by sonication of a lipid film in 10 mM Tris/HCl buffer, pH 8.0. The reaction mixture was vigorously vortexed and incubated at 37 °C for 20 min and then quenched with 0.3 ml of methanol and extracted with 0.3 ml of hexane. Retinoid composition in the organic fraction was analyzed on a normal phase silica column (Zorbax Sil 5 μm, 4.6 × 250 mm) (Agilent Technology) by using a mobile phase composed of 10% ethyl acetate in hexane at a flow rate of 1.4 ml/min. Alternatively, to ensure separation of retinyl esters containing acyl chains of different length, a step gradient of ethyl acetate in hexane was used (0.5% for the initial 15 min and then 20% up to 25 min.). Individual retinoids were detected and quantified based on their UV-visible spectra. Self-acylation of tLRAT was detected after incubation of the protein sample with various PC substrates in 5 mM Tris/HCl, pH 8.0, and 1 mM DTT.

Mass Spectrometry Analysis—All of the experiments were performed with a LXQ linear ion trap mass spectrometer equipped with an electrospray ionization source (Thermo Scientific). An Agilent Technologies 1100 Series HPLC system (Agilent Technology) was interfaced with the mass spectrometer for online protein/peptide separation. To record spectra of intact proteins, ~2 μg of the protein was loaded onto a C8 cartridge column (Luna 5 μm, 20 × 2.0 mm; Fenomenex) and eluted with a linear 10–100% gradient of acetonitrile/water over 20 min at a flow rate of 0.2 ml/min. Both solvents contained 0.1% formic acid. Because the chromatographic separation served as a desalting step, the initial 2 min of each run were directed to waste. Intact protein masses were deconvoluted either by use of ProMass for Xcaliber software (Thermo Scientific) or manually for selected peaks. The same column and solvents were used to separate products of trypsin and pepsin digestion except the acetonitrile gradient was developed within 40 min. Because the thioester bond might be chemically unstable under acidic conditions, pepsin digestion in solution was performed at 4 °C, pH 2.0, for 15 min followed by immediate injection onto the column. Trypsin digestion in solution was performed in 5 mM Tris/HCl, pH 8.0, for 16 h at 37 °C.

RESULTS

Expression and Purification of LRAT-GST Fusion Protein—To establish a source of LRAT suitable for MS experiments, we expressed a truncated mouse enzyme (tLRAT) containing residues 30–186 in the form of a GST fusion protein in *E. coli* (Fig. 1, *A* and *B*). Importantly, affinity-purified GST-tLRAT protein did not require detergent to remain soluble, and it also exhibited robust enzymatic activity when assayed in the presence of short acyl chain PC and all-*trans*-retinol (Fig. 1, *C* and *D*).

Covalent LRAT Modification upon Incubation with PC—The first step of the proposed catalytic reaction involves acyl-group transfer from PC and concomitant formation of an acyl-enzyme intermediate (supplemental Fig. S1). To detect putative enzyme modification, electrospray ionization-MS analyses were carried out first with intact protein where the charge state distribution of the protein ions provided information about the polypeptide mass. The purified protein spectrum recorded under acidic conditions revealed a broad distribution of charged states, which correspond to a protein with a calculated mass of 44,672 Da, identical to the theoretical mass of GST-tLRAT (Fig. 2*A* and supplemental Fig. S2). Incubation of GST-tLRAT with PC led to rapid modification of the protein indicated by the appearance of an additional series of peaks in the mass spectra. Two representative

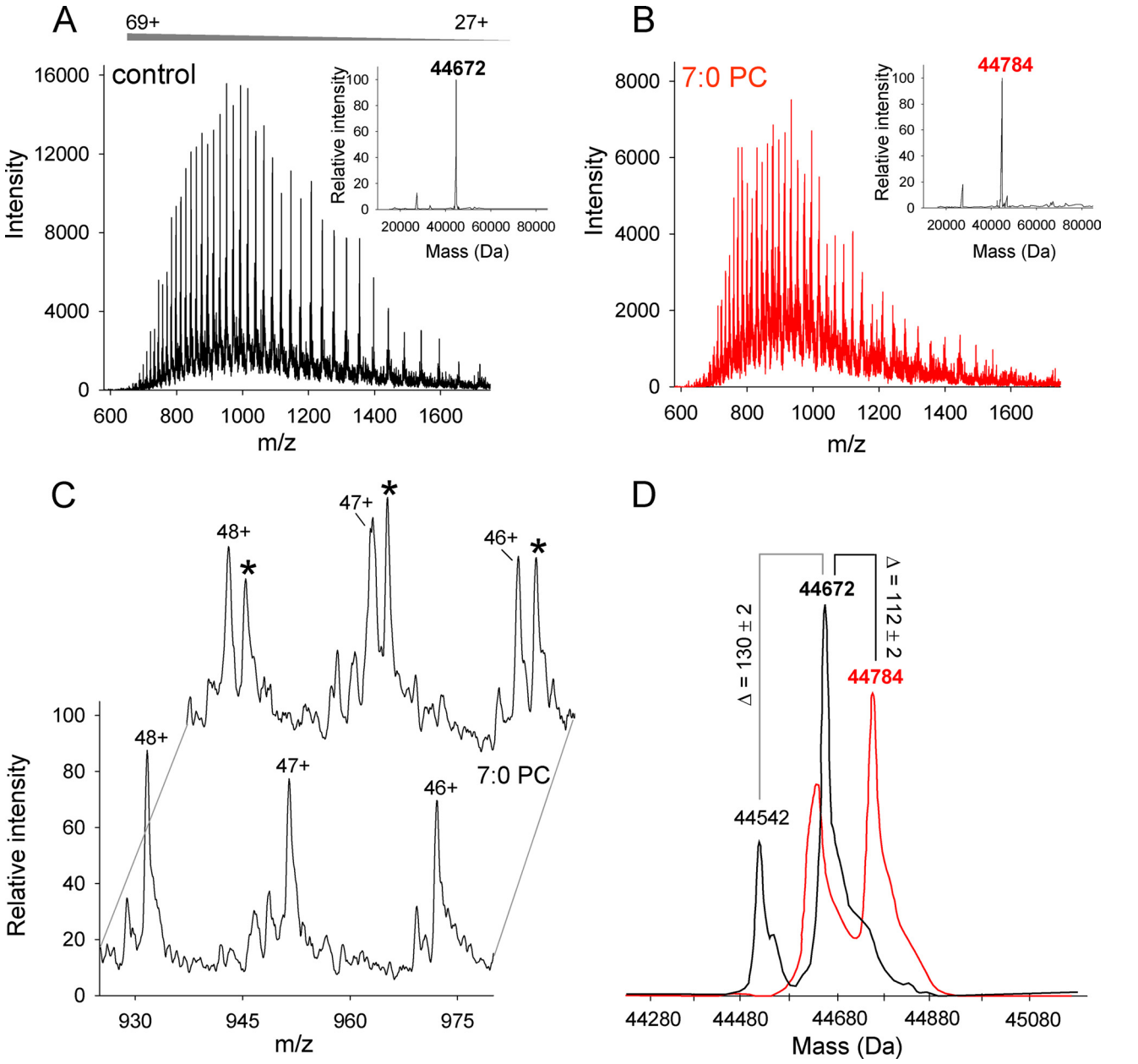

FIGURE 2. **Electrospray ionization mass spectra of intact GST-tLRAT.** *A*, purified protein spectrum shows a broad distribution of charged states (27+ to 69+). Deconvolution of GST-tLRAT spectrum reveals a protein mass of 44,672 Da (*inset*), which is identical to the theoretical mass of GST-tLRAT. *B*, incubation of the protein with 1 mM of 7:0 PC led to a 112-Da shift in experimentally obtained protein mass (*inset*). *C*, zoomed spectrum displays a group of selected multiply charged protein ions for the unmodified and acylated protein. Upon incubation with 0.3 mM of 7:0 PC, the protein became covalently modified as indicated by an additional peak at the higher *m/z* values marked by *asterisks*. *D*, deconvoluted mass spectrum of purified GST-tLRAT (*black trace*) reveals an additional peak at 44,542 Da corresponding to the N-terminal Met cleavage product (Δ = 130 ± 2 Da). The *red trace* represents protein mass upon incubation with the lipid substrate. The 112-Da shift in mass indicates covalent protein modification with a C7 acyl chain.

examples are shown in Fig. 2 and supplemental Fig. S3. The presence of 7:0 PC caused a 112-Da shift in the deconvoluted mass of this protein, suggesting covalent modification of GST-tLRAT with the C7 fatty acid moiety (Fig. 2, *B* and *D*). There was excellent agreement between the experimentally obtained and calculated mass with an increase in mass adequate to accommodate the length of an acyl chain in a variety of tested substrates (Table 1). The additional mass peak at 44,542 Da seen in the spectrum of unmodified protein corresponds to the N-terminal Met cleavage product (mass difference = 130 ± 2 Da) (Fig. 2*D*). Incomplete removal of

TABLE 1
Determination of protein masses by electrospray ionization MS
The MS analysis was preformed as described under "Experimental Procedures."

Protein	Deconvoluted mass	Calculated average mass
	Da	*Da*
GST-tLRAT	44,672.4 ± 2	44,672
GST-tLRAT (6:0)	44,769.2 ± 2	44,770
GST-tLRAT (7:0)	44,783.6 ± 2	44,784
GST-tLRAT (8:0)	44,796.0 ± 2	44,798
GST-tLRAT (9:0)	44,811.0 ± 1	44,812
tLRAT	18,522.4 ± 1	18,523
tLRAT (6:0)	18,621.0 ± 2	18,621
tLRAT (7:0)	18,635.6 ± 2	18,635

N-terminal Met is caused by a Pro residue present in the P2′ position within the GST amino acid sequence (22). To confirm that acylation occurs within the tLRAT portion of the fusion protein after incubation with PC, GST-tLRAT was treated with thrombin, and liberated peptides were separated chromatographically. Cleavage of the GST tag revealed that the site of modification was located within the tLRAT sequence (supplemental Fig. S4).

Identification of Acylated Amino Acid Side Chain—The relatively stable nature of LRAT self-acylation permitted identification of the acylated residue. Combined sequence coverage for tLRAT upon trypsin and pepsin digestion reached 90% and included all Cys and Ser residues present in the sequence. Detailed analysis of MS data revealed the addition of an acyl group to Cys^{161} in a peptide containing the conserved NCEHF sequence characteristic for the LRAT-like protein family (^{158}WNNCEHFVTY^{167}) upon incubation with lipid substrates. To gain more confidence in this result, the same experiment was repeated for a number of PCs with acyl chains of differing length. Fig. 3 shows a comparison of tandem MS spectra for unmodified peptide and peptides with C6 and C8 fatty acyls. Differences in acyl chain length between lipid substrates were documented by 28-Da shifts in a series of corresponding b and y ions. Comparison of fragmentation patterns of differently modified peptides is especially important because thioester modifications often do not produce a marker or neutral loss ions; therefore backbone fragmentation appears to be the primary way of peptide decomposition (23). In conclusion, we directly demonstrated that LRAT forms transient thioester bonds during enzymatic activity, providing proof for previous hypotheses.

Effect of Lipid Phase on tLRAT Enzymatic Activity—By studying the relationship between LRAT activity and the length of the *sn*-1 fatty acyl moiety of its phosphatidylcholine substrates, we found that the soluble form of tLRAT catalyzed the most efficient retinyl ester formation in the presence of PCs with short chain fatty acids, whereas increasing the acyl chain length resulted in gradual loss of enzymatic activity accompanied by inefficient formation of the thioester intermediate (Fig. 4, *A* and *B*). In contrast to the soluble form of the protein, enzymatic assays performed in the presence of full-length, membrane-anchored protein revealed that LRAT preferentially utilized lipid substrates with long acyl chains such as externally added C12 and endogenous C16 rather than those with shorter chains (Fig. 4*C*). Moreover, LRAT is known to efficiently transfer C14, C16, and C18 fatty acyls onto retinoids *in vivo* (14). Thus, this discrepancy has the potential to reveal other interesting mechanistic properties of this enzyme. We found a simple correlation between aqueous solubility of lipid in monomeric form and its ability to be utilized by LRAT. Aggregation of PC monomers above their critical micelle concentrations inhibited acyltransferase activity and protein acylation, indicating that the lipid-water interface of the micellar aggregates is not accessible or does not bind to the truncated enzyme (supplemental Fig. S5).

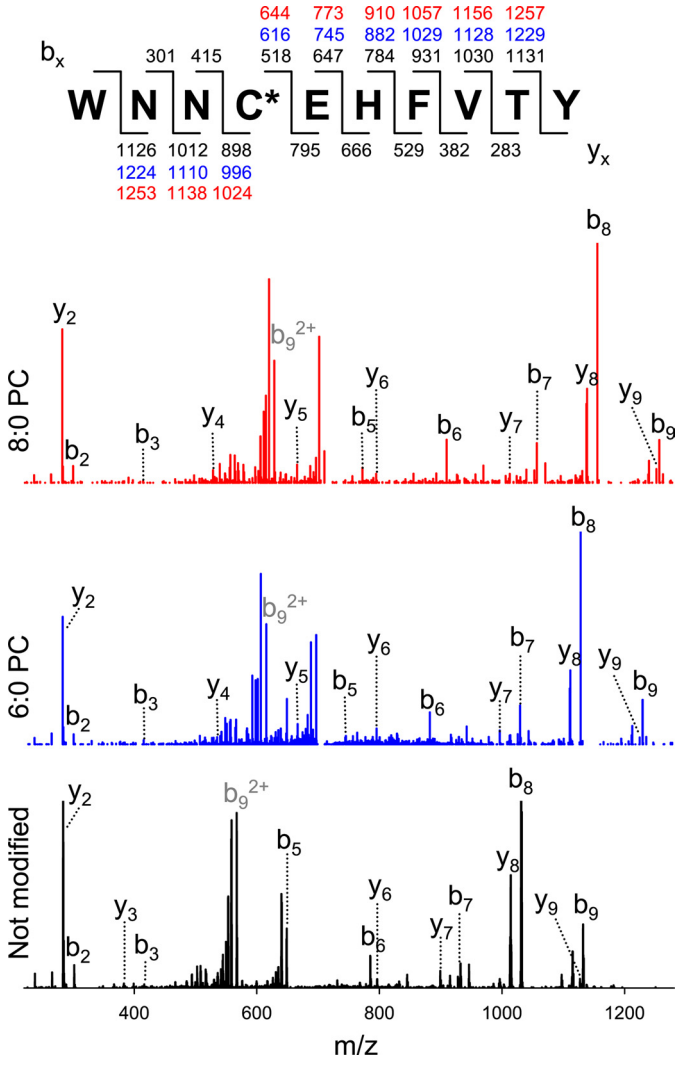

FIGURE 3. **Determination of the acylation site by tandem MS.** The spectra were recorded for the 2+ precursor ion of a peptide containing Cys^{161}. The protein modification upon incubation with PC caused a shift in masses corresponding to a series of y and b ions shown in *blue* and *red* for 6:0 PC and 8:0 PC, respectively.

DISCUSSION

The mass spectrometry approach used in this study was designed to directly test the catalytic mechanism of LRAT and avoid the obvious disadvantages of site-directed mutagenesis, especially in loss-of-function experiments. LRAT was proposed to be mechanically related to thiol proteases. However, instead of hydrolysis of an acyl-enzyme intermediate by water, the fatty acyl moiety is transferred to retinol (17). Thus, water needs to be excluded from the active site of the protein, allowing stabilization of the thioester intermediate in the absence of retinol. Consequently, the catalytic mechanism can be tested directly without using acetylating suicide inhibitors. Identification of an acylation site on highly conserved Cys^{161} suggests its importance in enzymatic activities of diverse LRAT protein family members. Recently, three other members of the family, tazarotene-induced protein 3 and H-Ras-like suppressor family 2 and 3, were shown to exhibit phospholipase $A_{1/2}$ activity (19, 24). Thus, the experimental strategy used here could be

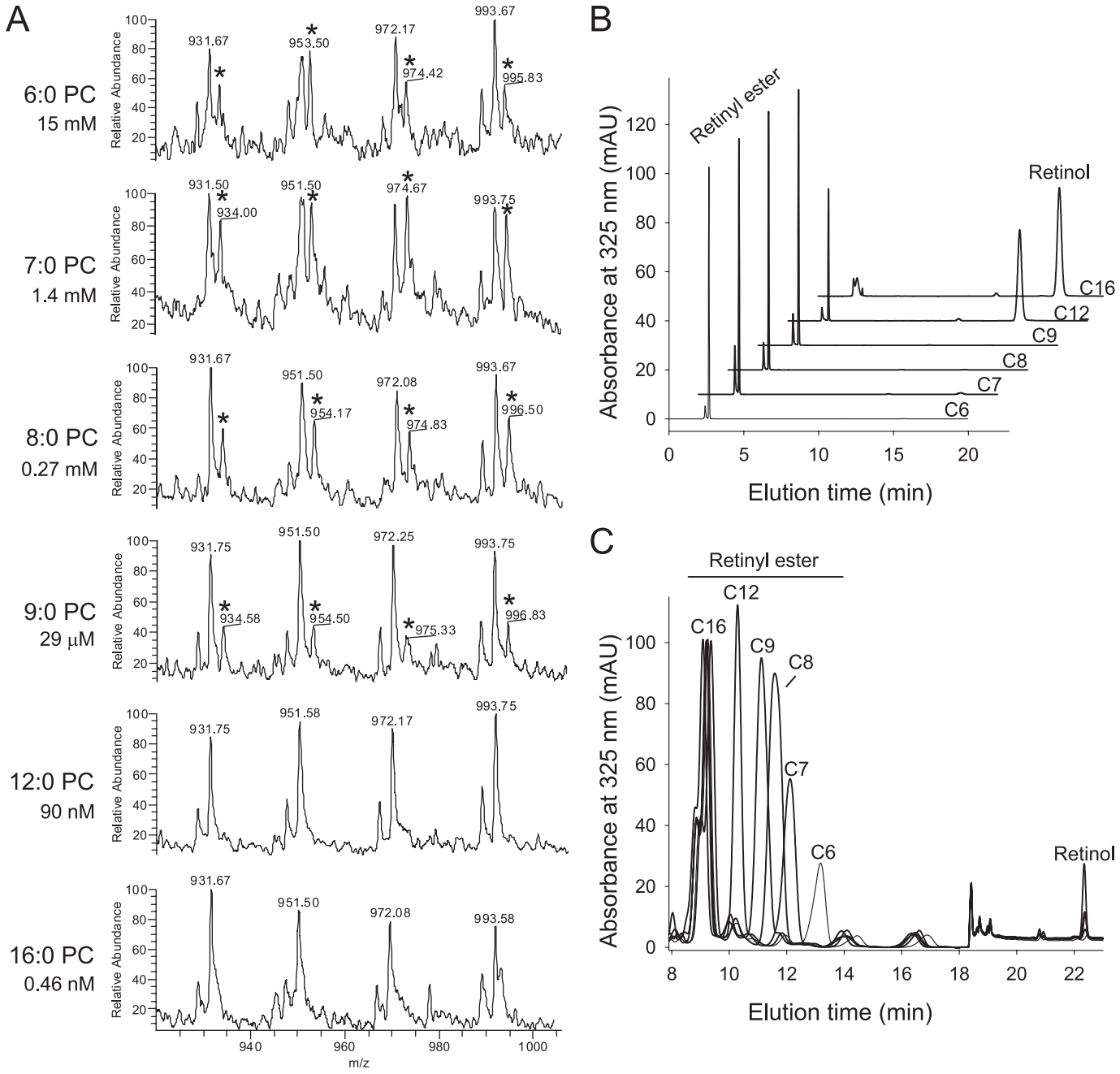

FIGURE 4. **Correlation between enzyme acylation, its enzymatic activity and critical micelle concentration values of tested lipid substrates.** *A* and *B*, formation of acyl-intermediates in GST-tLRAT (marked with *asterisk* in *A*) correlates with transfer of the acyl group to retinol observed in enzymatic assays (*B*). An increase in fatty acyl chain length markedly reduced the catalytic activity of soluble tLRAT. This decrease in enzymatic activity correlated with the declining concentration of monomeric lipid substrate as determined by critical micelle concentration values. The critical micelle concentration values for each of the tested PCs are cited from the Avanti Lipids Polar database available on the company website. The lipid concentration in each experimental set was fixed at 1 mM. *C*, enzymatic activity of the full-length, membrane-bound LRAT in the presence of lipid substrates with fatty acyl chains of different lengths. UV-treated bovine retinal pigment epithelium microsomes were incubated with 200 μM of tested PCs and 10 μM all-*trans*-retinol for 20 min at 37 °C, and retinoid composition was subsequently examined by HPLC. Chromatogram signals were normalized with respect to the intensity of a retinyl palmitate peak.

employed for rapid identification of putative acyltransferases in the remaining members of the LRAT-like-containing domain protein family.

By studying the relationship between PC fatty acyl chain length and tLRAT enzymatic activity, we found that monomeric lipid substrates are preferred by this enzyme. Therefore, LRAT, unlike many other lipolytic enzymes including phospholipase A_2, is not activated by phospholipid interfaces but instead exhibits features characteristic of esterases rather than lipases. This observation in turn suggests that in the case of LRAT, the C-terminal membrane-anchoring domain is important for targeting the protein to the endoplasmic reticulum and also determines the accessibility of long chain fatty acyl substrates for catalysis by this membrane-bound enzyme. The latter conclusion is further supported by the experiment in which LRAT embedded in reti-

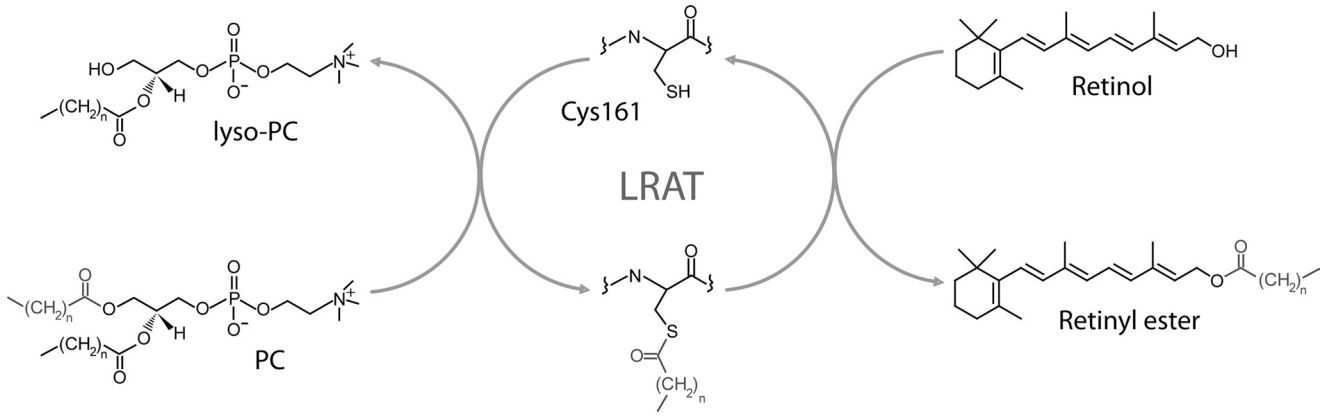

FIGURE 5. **Schematic representation of LRAT enzymatic activity mechanism.**

nal pigment epithelium microsomes preferentially accepted long acyl chain PCs that more readily partition into the membrane phase (Fig. 4*C*).

In conclusion, this study provides insights into the catalytic mechanism of LRAT by providing the first direct evidence for an enzyme-bound thioester intermediate (Fig. 5). Moreover, it presents an important mechanistic observation about the role of the surrounding lipid phase in the substrate availability and enzymatic activity of LRAT. This information may help elucidate and verify the largely unknown biological functions of the LRAT-like protein family as well as serve as a model for studies of lipolytic enzymes that might employ a similar catalytic mechanism.

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