Lecithin Retinol Acyltransferase Contains Cysteine Residues Essential for Catalysis†

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ABSTRACT: Lecithin retinol acyltransferase (LRAT) is an essential enzyme in vitamin A metabolism and mobilization. The membrane-bound enzyme catalyzes the transfer of an acyl group from the *sn*-1 position of lecithin to vitamin A to generate retinyl esters. The sequence of LRAT is novel and hence does not suggest a mechanistic class to which the enzyme belongs. However, the activity of the enzyme is exceedingly sensitive to affinity labeling and group-specific reagents directed toward thiol groups. LRAT from human retinal pigment epithelium has cysteine residues at positions 161, 168, 182, and 208. Site-specific mutagenic studies show that C182 and C208 can be converted to alanines with little affect on activity. The activities of the C161A and C168A mutants are virtually nil. Moreover, while C168S is substantially active, C161S possesses only a few percent of the activity of wild-type (WT) LRAT. Also, pH-rate profiles show that C168S has virtually the same profile as WT LRAT, while C161S shows an aberrant profile quite unlike that of WT LRAT. Therefore, LRAT is a thiol acyltransferase and C161 may be the essential nucleophilic residue critical for catalysis.

Integral membrane-bound proteins continue to present substantial barriers to their purification and eventual cloning and sequencing. This is particularly true of the minor membrane proteins, which often suffer irreversible denaturation before full purification is achieved. Thus, a developed literature on the similarities and differences between membrane-bound enzymes and their soluble cognates is unavailable. That membrane-bound enzymes may function by mechanisms distinct from their soluble counterparts, which catalyze similar chemical reactions, is illustrated here in studies on lecithin retinol acyltransferase.

Lecithin retinol acyltransferase (LRAT)[1] is a membrane-bound enzyme, which transfers an acyl group from the *sn*-1 position of phosphatidylcholine to vitamin A to produce retinyl esters (*1*−*3*). The enzyme is found in those tissues known to be involved in the processing and mobilization of vitamin A, including the retinal pigment epithelium, the liver, and the intestine (*4*). LRAT plays a critical role in vision because it provides the *all-trans*-retinyl ester substrates for the isomerohydrolase; an enzyme that processes retinyl esters into 11-*cis*-retinol, the precursor of the visual chromophore of rhodopsin (*5*, *6*). While LRAT has been solubilized and partially purified, it has resisted full purification as a consequence of its relative instability in the solubilized form (*1*, *7*). Nevertheless, even without full purification, a considerable amount is known about LRAT.

The substrate specificity for LRAT is known, as is its sensitivity to inhibitors (*7*, *8*). The enzyme is particularly sensitive to thiol reagents, suggesting that a cysteine residue(s) of the enzyme may be essential for catalysis (*7*, *8*). The kinetic mechanism of LRAT is known and is of the form of an ordered ping-pong bi-bi mechanism, with lecithin binding first and retinyl esters departing last (*9*). This kinetic mechanism strongly suggests the presence of an acyl enzyme intermediate. Given the sensitivity of LRAT toward thiol reagents, a thiol ester intermediate is considered a mechanistic possibility.

The full characterization of LRAT was complicated by many of the typical problems associated with working with integral membrane proteins that are present as minor constituents. Most importantly, as the purification proceeded, the stability of LRAT decreased, preventing its full purification. The eventual identification of the protein depended on the chemical labeling of LRAT by specific affinity labeling reagents. As mentioned above, LRAT activity is quite sensitive to thiol reagents, such as the organomercurials, suggesting that the enzyme possesses catalytically essential, nucleophilic cysteine residues (*1*, *7*). Indeed, LRAT requires added reduced thiols to maintain substantial activity after solubilization (*1*, *7*). LRAT was specifically labeled by electrophilic reagents, such as RBA and BACMK (Chart 1) (*10*, *11*). The labeling of LRAT allowed for its identification

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[1] Abbreviations: LRAT, lecithin retinol acyltransferase; DPPC, L-α-dipalmitoylphosphatidylcholine; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; BACMK, *N*-Boc-L-biocytinyl-11-aminoundecane chloromethyl ketone; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; RPE, retinal pigment epithelium; RBA, *all-trans*-retinyl-α-bromoacetate; WT, wild type; UTR, untranslated region.

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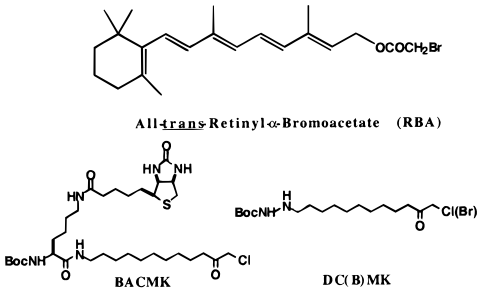
Chart 1



as an approximately 25 kDa protein with novel peptide sequences (*11*). On the basis of the peptide sequence information, the human RPE enzyme was cloned and fully sequenced (*11*). The sequence is unique, and as such provides little in the way of insight with respect to the mechanistic class of enzymes to which LRAT belongs. Interestingly, LRAT shows no sequence homology with lecithin cholesterol acyltransferase (LCAT), an important soluble enzyme central to cholesterol mobilization (*12*). LCAT catalyzes a reaction very similar to the reaction catalyzed by LRAT: LCAT transfers an acyl group from the *sn*-2 position of lecithin to cholesterol (*12*). This enzyme possesses an essential active-site serine residue, which probably acts as an acyl-group acceptor with lecithin (*13*, *14*). As expected, LCAT contains the G-X-S-X-G serine protease signature at its active site (*15*). No such signature is found in the case of LRAT, suggesting that it is not serine protease-like. In fact, LRAT also does not possess any of the other local sequences that define serine proteases (*16*). A central question with respect to LRAT, then, is to define the mechanistic class to which the enzyme belongs. LRAT clearly does not belong to the standard serine protease/lipase families of enzymes.

Given the sensitivity of LRAT to thiol reagents, the possibility of a catalytically essential cysteine residue(s) needs to be considered. Human RPE LRAT contains four cysteine residues at positions 161, 168, 182, and 208 (*11*). In the study reported here, the results of site-specific mutagenic experiments are provided which demonstrate that cystein residues 161 and 168 are important for catalysis, while cysteine residues 182 and 208 are not. Moreover, cysteine 161 seems absolutely essential for catalysis and is a good candidate for an important active-site nucleophile central to the catalytic mechanism of LRAT.

MATERIALS AND METHODS

Materials

Frozen bovine eye cups were obtained from W. L. Lawson Co. (Lincoln, NE). [11,12-$^3H_2$]*All-trans*-retinol (31.4 Ci/mmol) was obtained from NEN Life Sciences, Inc. L-α-Dipalmitoylphosphatidylcholine (DPPC), BSA, dithiothreitol (DTT), *all-trans*-retinyl palmitate, and protein A were from Sigma. Triton X-100 was from Calbiochem, Inc. HPLC-grade solvents were obtained from J. T. Baker. The ECL western blotting kit was from Amersham Inc.. All other reagents were of analytical grade.

Methods

LRAT Constructs and Generation of Mutants. A ~1.0 kb *Eco*RI LRAT fragment was subcloned into the mammalian pcDNA 3 expression vector (Invitrogen). The LRAT fragment contained sequence encoding 92 bp of 5′ UTR, 690 bp of coding sequence, and 254 bp of the 3′ UTR as described before (*11*). The presence of the DNA insert in the vector was confirmed by *Eco*RI digestion and the correct orientation was determined by sequencing using the dideoxy chain-termination system (U.S. Biochemical Corp.). Several LRAT mutants were generated by overlap extension using the polymerase chain reaction (PCR) procedure described by Ho et al. (*17*). Briefly, a set of forward and reverse primers flanking the coding region of LRAT were designed and designated 5′ UTR (F) and 3′ UTR (R). Forward and reverse primers of the same sequence surrounding the Cys residue to be mutated were designed and the nucleotide triplet encoding the Cys was changed to Ala or Ser as shown in Table 1. A first PCR amplification of the LRAT DNA template was generated by using the 5′ UTR primer (F) with the reverse primer of the mutated Cys. Separately, a PCR amplification was performed with the 3′ UTR (R) primer and the forward primer of the mutated Cys. Subsequently, a mixture of the two PCR products, which now contained overlapping sequences, were fused by PCR with primers 5′ UTR (F) and 3′ UTR (R). The final product containing the mutation was confirmed by sequencing and was subcloned into the expression vector pcDNA 3 (Invitrogen) for subsequent transfection experiments in HEK-293 T cells. All PCR experiments were performed according to the vendor's recommendations provided in the GeneAmp PCR core reagents (Perkin-Elmer) kit. The reactions were carried out in a Robocycler 40 apparatus (Stratagene).

Table 1: LRAT Primers Used

LRAT primers	primer sequences	position
5′ UTR	F5′tttaccttcctctctcctcagcggc3′	−59 to −35
mut C/A 161	F5′ctgtggaacaac***gcc***gagcacttcgtg3′	469−495
	R5′cacgaagtgctc***gct***gttgttccacag3′	469−495
mut C/S 161	F5′ctgtggaacaac***agc***gagcacttcgtg3′	469−495
	R5′cacgaagtgctc***gct***gttgttccacag3′	469−495
mut C/A 168	F5′cgtgacctac***gcc***agatatggcacc3′	492−516
	R5′ggtgccatatct***ggc***gtaggtcacg3′	492−516
mut C/S 168	F5′cgtgacctac***agc***agatatggcacc3′	492−516
	R5′ggtgccatatct***gct***gtaggtcacc3′	492−516
mut C/A 182	F5′cagtccgacaagttt***gct***gagactgtgaagata3′	529−561
	R5′tatcttcacagtctc***agc***aaacttgtcggactg3′	529−561
mut C/A 208	F5′ttggcgtctatagtc***gct***acgggcttggtatca3′	607−639
	R5′tgataccaagcccgt***agc***gactatagacgccaa3′	607−639
3′ UTR	R5′tacagaatacacacactgacatgggg3′	702−727

Transfection of HEK-293T Cells with LRAT and LRAT Mutants. A human embryonic kidney cell line (HEK-293T) was transfected with LRAT or LRAT mutant DNA to study the induction of LRAT activity. Maintenance and processing of the HEK-293T cells has been previously described in detail (*18*). HEK-293T cells grown to ~80% confluency in 100 mm culture plates were transfected with 30 μg/plate of plasmid DNA by use of either LipofectAMINE (2 μg/mL; Gibco−BRL, Gaithersburg, MD) following the manufacturer's instructions or calcium phosphate, according to the published method (*19*). After 24 h of incubation, transfected cells were collected in Hanks' balanced salt solution (Irvine Scientific, Irvine, CA) and pelleted in 15 mL tubes at 1000*g* for 5 min at 4 °C. Cell pellets were frozen at −80 °C until further analysis. A plasmid with no LRAT insert was used as a negative control. Western blot analysis and a peptide polyclonal antibody against LRAT described previously (*11*) were used for the evaluation of the expression of LRAT protein in transfected cells compared to extracts from RPE cells as a positive control.

Steady-State Kinetics. The steady-state kinetic experiments for LRAT activity were performed by monitoring the LRAT-catalyzed formation of retinyl esters from *all-trans*-retinol and DPPC. For all experiments, the initial rates for the formation of retinyl esters were measured by varying the concentration of retinol, while the concentration of DPPC was kept constant at a saturation level. To maintain a saturating level of DPPC, experiments were performed by the addition of excess DPPC to the reaction mixture. As expected, the experimental data under the above conditions were

observed to follow typical Michaelis−Menten-type kinetics. This analysis allows determination of apparent Michaelis constant (K_M) and maximum velocity (V_{max}) of processing *all-trans*-retinol for LRAT activity. All procedures for the kinetic experiments were performed following the previously reported method (*9*, *10*). Briefly, the incubation and reactions were carried out under dim red light at room temperature. [11,12-$^3H_2$]*All-trans*-retinol was dissolved in DMSO to make a stock solution. *All-trans*-retinol (1 μL) in DMSO was added to a 100 μL total buffer volume containing membrane suspension, DPPC, BSA, EDTA, and DTT. The final concentrations of the components in the reaction mixtures are 20 mM Tris-HCl, 250 μM DPPC, 0.6% BSA, 1 mM EDTA, 2 mM DTT, varying concentrations of *all-trans*-retinol, and desired amount of protein. The reactions were performed at room temperature for varying lengths of time depending on the specific activity of WT and mutant LRATs. However, in all cases, the conditions were carefully maintained to measure only the initial reaction rates. The reactions were quenched by addition of 500 μL of methanol, 100 μL of H_2O was added, and 500 μL of hexane (containing butylated hydroxy toluene at 1 mg/mL) was used to extract the retinoids. The retinoids were analyzed on a PVA-Sil (250 × 4.0, YMC) HPLC column with 7% dioxane in hexane as eluant at a flow rate of 1.5 mL/min. Radioactivity was counted with an online Berthold LB 506-C HPLC radioactivity monitor interfaced with IBM computer. Most experiments were performed in triplicate, although measurements in duplicate were done for some experiments. The average values from these measurements were used for analysis.

The units for steady-state rate of formation of retinyl ester products (catalyzed by WT and mutant proteins) were normalized with respect to the relative expression amounts of LRAT proteins as observed from the Western blotting analysis of WT and mutants. This method provides the advantage of using normalized reaction rates that can be directly used to assess the relative activity of WT and other mutant proteins. Protein concentrations were determined by the Bradford method with a Bio-Rad protein assay kit.

pH Dependence of the Steady-State Kinetics. The pH-dependent kinetic experiments were performed in either Tris-HCl or phosphate buffer (20 mM). The pH values of all samples were further checked after making the final reaction mixture. The samples were allowed to equilibrate at room temperature for sufficient time before the reaction was started by the addition of protein. All conditions were maintained as described above. V_{max} and K_M values at various pH were determined from the methods described above. The experimental data for V_{max} vs pH were fitted to a bell-shaped curve with limiting values for V_{max} at low and high pH. The data of K_M vs pH were fitted to a single pK_A curve with a saturating value of K_M at low pH.

RESULTS

Kinetic Studies on LRAT and C161S(A) and C168S(A) Mutants. Initial experiments were performed on WT LRAT and mutants expressed in HEK-293T cells. HEK cell membrane fractions were used in the assays without further processing (*11*). Kinetic analyses were carried out by measuring the rates of ^{3}H-*all-trans*-retinyl ester formation under conditions where ^{3}H-*all-trans*-retinol was varied at saturating lecithin concentrations (*9*, *10*). In Figure 1A are shown data for wild-type LRAT and in Figure 1B are shown data for empty vector. While we had previously reported that single point assays of empty vector show some LRAT-like activity (*11*), the kinetic experiments reported here show that this activity is feeble and unrelated to WT LRAT activity. In Figure 2 A, B are shown data for the 168S and the 161S mutants. As indicated in Figure 2A, the 168S mutant is nearly as active as WT LRAT, strongly suggesting that 168C is not a critically important residue in catalysis. On the other hand, 161S (Figure 2B) proved to be barely

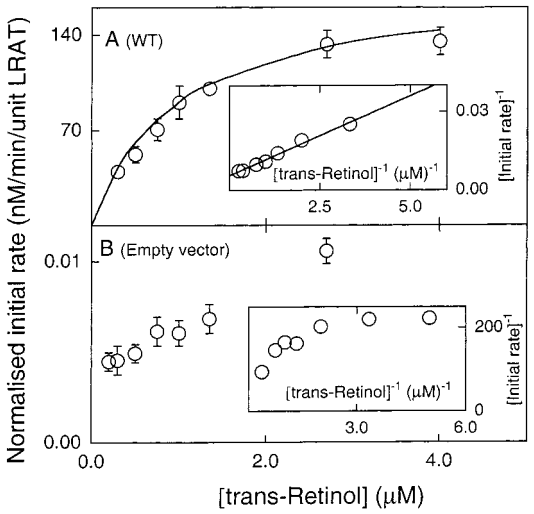


FIGURE 1: Steady-state kinetic plots for the formation of retinyl ester products by WT LRAT (A) and empty vector (B). The initial rates of the formation of ester products were measured at increasing concentrations of *all-trans*-retinol. The DPPC concentration was 250 μM. The symbols represents the mean value of duplicate or triplicate determinations, and error bars give the standard deviation from the mean. The insets to the figure show Lineweaver−Burk plots of the initial rate measurements.

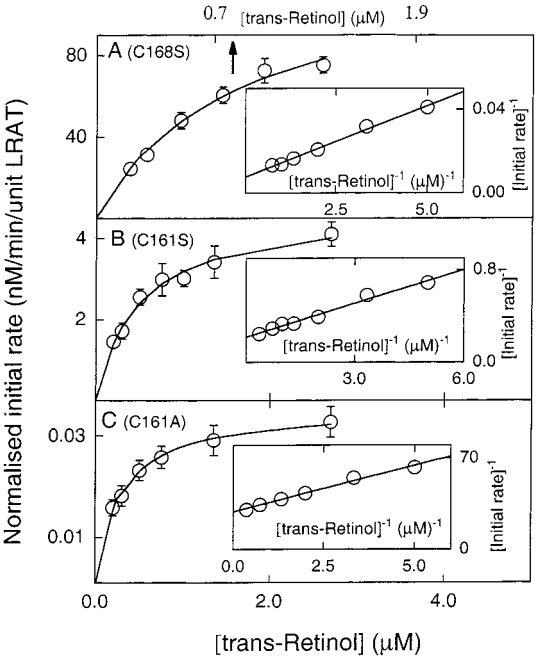


FIGURE 2: Steady-state kinetic plots for the formation of retinyl ester products by C168S (A), C161S (B), and C161A (C). The initial rates of the formation of ester products were measured at increasing concentrations of *all-trans*-retinol. The DPPC concentration was 250 μM. The symbols represent the mean value of duplicate or triplicate determinations, and error bars give the standard deviation from the mean. The insets to the figure show Lineweaver−Burk plots of the initial rate measurements.

active, suggesting an important role for 161C in catalysis. This idea is consistent with the studies on 161A mutant, which showed virtually no activity as shown in Figure 2C. Interestingly, though, 168A also showed no measurable activity (data not shown). Not surprisingly, the double mutant 161A/168A proved to be inert (data not shown). The various kinetically determined constants are summarized in Table 2.

Table 2: Parameters Obtained from the Steady-State Kinetic Analysis for WT and Mutant LRAT Proteins at Room Temperature

protein	K_M (μM)	normalized V_{max} [nM min^{-1} (unit of LRAT)$^{-1}$]	V_{max} [nM min^{-1} (mg of LRAT)$^{-1}$]
WT	0.94 ± 0.05	177 ± 4	644 ± 15
C182A	0.34 ± 0.06	257 ± 14	374 ± 21
C208A	0.51 ± 0.09	159 ± 11	415 ± 29
C168S	0.82 ± 0.13	125 ± 10	582 ± 36
C161S	0.47 ± 0.06	4.7 ± 0.19	20 ± 0.8
C161A	0.27 ± 0.02	0.035 ± 0.006	0.32 ± 0.01
empty vector			

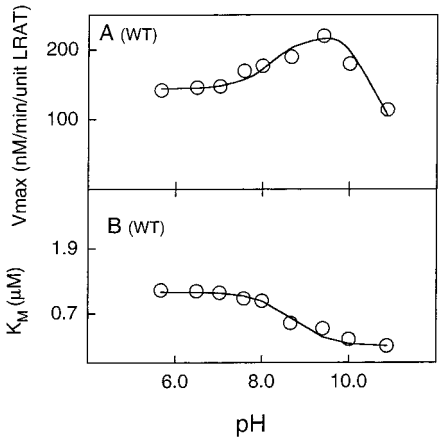


FIGURE 3: Plots of V_{max} (A) and K_M (B) vs pH for WT LRAT protein. K_M and V_{max} values were determined from the initial rate measurements at various concentrations of *all-trans*-retinol. The solid lines through experimental data points are from a computer-generated fit as described under Methods.

pH versus Rate Profiles for the C161S and C168S Mutants. Measurements were made on WT and the 168S and 161S mutants. As shown in Figure 3A, a V_{max} vs pH profile for WT LRAT shows two pK_A values, at 8.30 and 10.8, respectively. The K_M versus rate profile is given in Figure 3B and shows a pK at 8.7. The same profiles for the 168S mutant are not much different, as shown in Figure 4A,B. As shown here, the V_{max} vs pH profile for 168S mutant LRAT shows two pK_A values, at 8.21 and 10.46, respectively. The K_M versus rate profile is given in Figure 3B and shows a pK of 9.28. On the other hand, the 161S mutant did not show pK_A values (Figure 4C,D) that are readily interpretable. They are certainly much different than those of WT LRAT and the 168S mutant. These experiments also point to the importance of 161C in normal LRAT catalysis.

Kinetic Studies on LRAT and C182A and C208A Mutants. As mentioned above, there are two additional cysteine residues in LRAT beside C161 and 168. The two mutants C182A and C208A were prepared and analyzed for activity. As shown in Figure 5 and Table 2, the mutants were at least as active as WT LRAT. In fact, the C182A is substantially more active than WT LRAT. These experiments demonstrate that neither C182 nor C208 is in any way important for catalysis in LRAT. Moreover, neither cysteine residue can be importantly involved in disulfide bond formation in LRAT.

DISCUSSION

Although membrane-bound LRAT has resisted purification, it nevertheless has been cloned and sequenced (*11*).

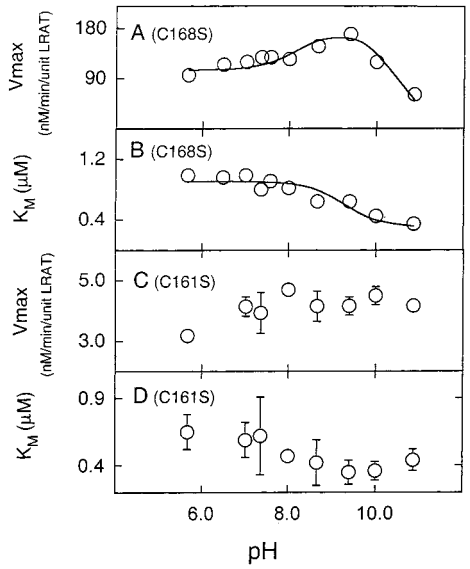


FIGURE 4: Plots of V_{max} (A) and K_M (B) vs pH for C168S. The similar plots for V_{max} (C) and K_M (D) for C161S are also shown in the figure. K_M and V_{max} values were determined from the initial rate measurements at various concentrations of *all-trans*-retinol. The solid lines through experimental data points are from a computer-generated fit as described under Methods.

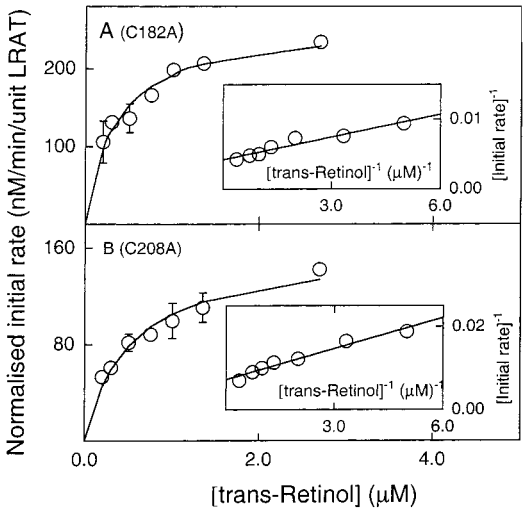


FIGURE 5: Steady-state kinetic plots for the formation of retinyl ester products by C182A (A) and C208A (B) mutants. The initial rates of the formation of ester products were measured at increasing concentrations of *all-trans*-retinol. The DPPC concentration was 250 μM. The symbol represents the mean value of duplicate or triplicate determinations, and error bars give the standard deviation from the mean. The insets to the figure show Lineweaver−Burk plots of the initial rate measurements.

Partial sequence information on LRAT was obtained through affinity labeling studies with RBA and similar reagents (*9*−*11*). The labeling procedure identified an approximately 25 kDa protein, which yielded unique sequence information (*11*). The cloning and expression of this protein in HEK-293T cells, chosen because these cells do not process retinoids, demonstrated that the membrane-bound 25 kDa protein was necessary and sufficient for expressing LRAT activity (*11*). Kinetic studies on empty vector-transfected HEK-293T cells show that no LRAT activity is manifest in the absence of the putative LRAT gene. That the 25 kDa protein is also sufficient for LRAT activity is implied by

the fact that the HEK-293T cells would be unlikely to possess a membrane-bound protein that complements the 25 kDa protein, given that HEK-293T cells are not known to process retinoids.

Any discussion of the work reported here should begin with the fact that LRAT is a unique protein, with very little in the way of sequence homology to other proteins (*11*). Thus, its mechanism of action is likely to be relatively novel. It might have been suggested that LRAT and LCAT would overlap mechanistically, given the similarity in the reactions that the two enzymes catalyze. However, LCAT has the typical serine protease/lipase active-site signature and does not possess catalytically essential cysteine residues (*14*). The data reported here demonstrate that two of the LRAT cysteine residues (161 and 168) are important, if not essential, for enzyme catalysis. Therefore, LRAT and LCAT are mechanistically distinct. The finding that these two cysteine residues are essential for catalysis is consistent with previous observations showing that reduced thiols are important for maintaining LRAT activity and that LRAT is inactivated by cysteine-directed reagents (*1*, *9*, *10*).

The kinetic mechanism already determined for LRAT is consistent with an acyl enzyme intermediate (*9*). A thiol ester intermediate would be a reasonable possibility, given the results reported here. On the surface, however, it is somewhat difficult to decide whether C161 or C168 is the best candidate. Both C161A and C168A are essentially inert with respect to possessing LRAT activity. However, the alanine for cysteine substitution is not a highly conservative change. It is likely that analysis of the C161S and C168S mutations are more telling in this regard. Here a clear-cut difference is found; only C168S shows substantial LRAT activity and, therefore, C168 is unlikely to be a critical active-site nucleophile. The C161S mutant is only weakly active, suggesting that C161 is the critical active-site cysteine residue involved in catalysis. Recent observations on the C161S and C168S mutants with BACMK, a specific affinity labeling agent of LRAT (*11*), are consistent with the catalytic importance of 161C residue (unpublished results). C168S was readily inactivated by BACMK, but no significant inactivation was observed with the C161S mutant. Finally, would one expect the C161S mutant to show any activity at all as a lipase-like enzyme, given the differences in nucleophilicity between cysteine and serine? Certainly cysteine substituted for serine mutants in serine proteases are known to retain some enzymatic activity (*20*, *21*). This is reasonably explained by the enhanced nucleophilicity of C versus S. However, in at least one cysteine protease, the less nucleophilic serine substitution is also tolerated, meaning that under certain conditions a C for S substitution still supports enzymatic activity (*22*).

It is also instructive to compare pH versus rate and K_M profiles for the mutants as compared to WT LRAT. The rate versus pH profile for WT LRAT shows two pK_A values at 8.3 and 10.8. The lower pK_A value is consistent with a cysteine residue being important for catalysis. The higher pK_A is in a range where a lysine residue might be considered. The K_M profile shows a pK at 8.7. Mutant C168S is virtually identical to WT LRAT with respect to these profiles. Therefore, the catalytic machinery required for substrate processing is left intact in this mutant. On the other hand, C161S certainly did not provide pH profiles consistent with WT LRAT. The profiles are, in fact, nondescript and suggest the possibility that the C161S mutant may function by providing a binding site to bring the substrates together, rather than by a chemical catalysis. In other words, catalysis could be effected by a solely entropic mechanism. Catalysis by this kind of mechanism has been previously observed in proteases where the active-site nucleophile has been mutated to a nonnucleophilic residue (*23*). Finally, it should be noted that the fact that C168S is substantially active removes obligate disulfide bond formation between C161 and C168 from consideration in the mechanism of LRAT action.

While C161 and, to a lesser extent, C168 are important for LRAT activity, it is clear that C182 and C208 are unimportant because even the alanine substitution mutations are without effect on enzymatic activity. It is also clear from the data that disulfide bond formation involving these cysteine residues must also be unimportant for catalytic activity. It is possible that these cysteine residues could be important for the stability of LRAT, or possibly even in mediating the interactions of LRAT with other proteins in the membranes of RPE membranes. It is interesting to note that hydropathy plot analysis of LRAT structure indicates that the C-terminal fragment, where C182 and C208 are located, is almost certainly buried in the membrane and, therefore, unlikely to be involved in catalysis.

The studies reported here indicate that C161 and C168, to a lesser extent, are important for LRAT catalysis. On the basis of the aggregate evidence, C161 appears to be especially important for catalysis. Our working hypothesis is that C161 is the nucleophilic residue in LRAT that reacts with lecithin and is acetylated to generate a thioacyl enzyme intermediate. This fatty acyl moiety is then transferred to the vitamin A by the kinetic scheme already established, generating retinyl esters (*9*). The role of C168 in this scheme is left undefined, although it could be involved in a second fatty acyl transfer reaction not absolutely essential for catalysis. Alternatively, C168 could be important for maintaining structure. It is unlikely that a cysteine moiety would be involved in base catalysis. In any case, ongoing active-site mapping studies will enhance our understanding of the mechanism of action of this unusual enzyme. In particular, sequencing studies on active-site affinity-labeled LRAT should be quite informative with regard to defining the amino acid residues involved in catalysis. Further site-specific mutagenic studies are also anticipated. Here, we will be interested in the possible role of histidine, aspartate, and asparagine residues as essential residues for catalysis. The possible involvement of lysine residues in catalysis will also be investigated. The pK_a = 10.8 revealed in the pH vs rate profile suggests the possible role of a lysine residue in catalysis.

Finally, it is important to reiterate that LRAT appears to be mechanistically unusual and not in the class of typical serine lipases and acyltransferases. Whether this mechanism will define a new class of membrane-bound acyltransferases is unknown. Since so much of what is known about enzyme mechanisms is derived from studies on soluble proteins, it is perhaps not too surprising to find unusual mechanisms in the area of catalysis by membrane-bound enzymes where structural constraints are likely to be unique to this class of enzymes.

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