Effects of carboxy-terminal truncation on human lecithin:cholesterol acyltransferase activity

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Abstract Mutagenesis was carried out in human lecithin:cholesterol acyltransferase (LCAT) to generate mutants with stop codons at positions corresponding to amino acids 315, 341, 359, 375, 388, 394, and 398 of the 416-amino acid sequence of the mature enzyme protein. Deletion of the 18 terminal amino acids of the protein was without effect on LCAT phospholipase or acyltransferase activity, or the stability of the protein to denaturation at 37°C. Further deletion led to loss of most of the activity, associated with a 10-fold increase in the rate of denaturation at 37°C. These data indicate that the proline-rich C-terminus of LCAT is not required for effective enzyme activity. The loss of activity that accompanied deletion of residues 394–398 suggests a structural role for these residues, part of a series of predicted beta-sheet sequences in the C-terminal third of the LCAT primary sequence.—**Francone, O. L., L. Evangelista, and C. J. Fielding.** Effects of carboxy-terminal truncation on human lecithin:cholesterol acyltransferase activity. *J. Lipid Res.* 1996. **37:** 1609–1615.

Supplementary key words mutagenesis • apolipoprotein A-I • cholesteryl esters • phospholipase

Lecithin:cholesterol acyltransferase (LCAT; phosphatidyl-choline: sterol O-acyltransferase; EC 2.3.1.43) catalyzes the hydrolysis and subsequent transfer of the 2-acyl group of phosphatidylcholine and other plasma glycerophospholipids to cholesterol. LCAT is synthesized mainly in the liver, and the secreted 416-amino acid protein is present in plasma as a complex of lipids and apolipoproteins in the high density lipoprotein (HDL) fraction (1). The activity of LCAT is responsible for a large part of the cholesteryl esters (CE) present in human plasma. Because of its ability to convert unesterified cholesterol to insoluble CE on HDL, LCAT can catalyze the net transfer to plasma of cholesterol from cell membranes. Because of this activity. LCAT is considered a key protein in the reverse cholesterol transport (RCT) pathway by which peripheral cell cholesterol can be returned through the plasma to the liver. LCAT also promotes the net transfer of cholesterol from other lipoprotein surfaces to HDL (2). In the absence of cholesterol or other fatty alcohols, LCAT functions as a phospholipase, producing unesterified fatty acid from its lecithin substrate rather than CE (3). Both the phospholipase and acyltransferase activities of LCAT are promoted by apolipoprotein A-I, the major protein of HDL (4).

Like several other enzymes, including bile salt-activated lipase (5), LCAT contains a hydrophilic, proline-rich C-terminus (6). The LCAT C-terminus shows some sequence similarity to that of the salivary, Ca^{2+}-binding proline-rich proteins (7) even though LCAT, unlike most other phospholipases, is Ca^{2+}-independent (3). Other functions ascribed to proline-rich C-terminal sequences include resistance to cellular or plasma proteases, stabilization against thermal or chemical degradation, and promotion of protein–protein interactions (8).

Natural mutations at positions 321, 347, 376, and 391 produced LCAT species with impaired acyltransferase activity (9–12). These observations suggested a possible functional role for the C-terminal part of LCAT. To further define the contribution of the C-terminus to LCAT structure and function, cDNAs coding for a series of carboxy-terminal truncated LCAT proteins have been constructed by site-directed mutagenesis of wild-type human cDNA. These were then stably expressed in CHO cells.

Abbreviations: HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; CE, cholesteryl esters; RCT, reverse cholesterol transport; PCR, polymerase chain reaction; dhfr, dihydrofolate reductase; MTX, methotrexate.

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EXPERIMENTAL METHODS

Site-directed mutagenesis

A full-length human LCAT cDNA was synthesized by reverse transcription polymerase chain reaction (PCR) from mRNA isolated from human hepatoblastoma (HepG2) cells (13). LCAT cDNA extending from 8 bases before the ATG start codon to 5 bases after the TAA stop codon was ligated into a pTZ19 phagemid plasmid. MV1190 *E. coli* cells were infected with the pTZ19LCAT construct. Single-strand DNA was obtained after infection with M13K07 helper phage (Bethesda Research Laboratories, Bethesda, MD). Site-directed mutagenesis was modified from the method of Taylor, Ott, and Eckstein (14). Briefly, 5 μg of single-stranded pTZ19LCAT DNA was annealed to a synthetic oligonucleotide (Operon, Alameda, CA) including one mismatched base designed to generate a stop codon at this point (**Table 1**). The mutant oligonucleotides annealed to the single-stranded template were then extended by Klenow DNA polymerase (Amersham, Arlington Heights, IL) in the presence of T4 DNA ligase, and thionucleotides to generate the corresponding heteroduplex. Selective removal of the nonmutant strand was achieved by selective restriction enzyme digestion with Nci I, followed by digestion with exonuclease III. The Nci I does not cut a DNA strand where a thionucleotide is incorporated, only the unmutated strand is nicked. The mutant strand was then used as a template to reconstruct homoduplex mutant double-stranded closed circular DNA. This was transfected into TG1 competent cells and plated on LB ampicillin plates. The predicted sequence of all LCAT cDNAs was confirmed by the dideoxy method (15).

Successful generation of cDNAs coding from truncated LCAT species was confirmed by cell-free protein synthesis. pTZ19 plasmids containing wild-type or truncated LCAT cDNAs were grown in MV1190 *E. coli* cells and then isolated by alkaline hydrolysis (16). This was followed by two rounds of ultracentrifugation in a cesium chloride–ethidium bromide gradient. Plasmid DNA was linearized and transcribed by T7 RNA polymerase according to the manufacturer's protocol (Promega Biotec, Madison, WI). RNAs were then translated into a cell-free system which included a lysate of rabbit reticulocytes, [^{35}S]methionine, and RNAs in as previously described (13). This system contains the cellular components for protein synthesis but lacks the enzyme responsible for signal peptide cleavage. Transcripts (including the leader sequence) were capped by addition of m^{7}G(5′)ppp(5′)G (Pharmacia LKB, Piscataway, NJ). Translation products were analyzed by 8% w/v polyacrylamide-SDS gels. After electrophoresis the gels were fixed in isopropanol–water–acetic acid 25:65:10 (v/v) for 30 min, vacuum-dried, and autoradiographed using Kodak X-Omat AR film.

TABLE 1. Nucleotides used to generate truncated LCAT species

Mutant	C-Terminus/Primer DNA Sequence
$LCAT_{398 \rightarrow stop}$	-leu-leu-gly-ala-COOH 5-CTGCTGGGTGCCTAACGCCAGCAG-3
$LCAT_{394 \rightarrow stop}$	-asn-ala-ile-COOH 5-AATGCCATCTAGCTGGGTGCC-3
$LCAT_{388 \rightarrow stop}$	-thr-leu-COOH 5-GACCCTGTAGCAGATCAATG-3
$LCAT_{375 \rightarrow stop}$	-his-gly-ile-COOH 5-CACGGGATATAGCATCTCAAC-3
$LCAT_{359 \rightarrow stop}$	-cys-gly-leu-COOH 5-TGTGGCCTGTGACAGGGCCGCCAG-3
$LCAT_{341 \rightarrow stop}$	-val-gly-val-leu-COOH 5-GTGGGTGTGCTCTAAGAGGATGGTGAT-3
$LCAT_{315 \rightarrow stop}$	-val-tyr-cys-leu-COOH 5-GTATACTGTCTTTAAGGCGTGGGCCTG-3

Expression of LCAT cDNA

To generate cell lines stably transfected with wild-type or truncated LCAT cDNAs, expression plasmid pSV2dhfr (American Type Culture Collection, ATCC 37146), containing the sequence of the mouse dihydrofolate reductase (dhfr) gene, was digested with Hind III and Bgl II restriction nucleases to remove the dhfr sequence. Wild-type and mutant pTZ19LCAT species were digested with Hind III and Bgl II restriction endonucleases and ligated to the same site in the pSV2 plasmid. The pSV2LCAT constructs were transfected into MV1190 *E. coli* and grown in LB-ampicillin medium until stationary phase. Plasmid DNA was then purified by cesium chloride–ethidium bromide gradient as described above. Stably transfected clones in mammalian cells were obtained by co-transfecting pSV2LCAT and pSV2dhfr plasmids in a 20:1 ratio into CHO cells deficient in the dhfr gene (DXB 11 line) as previously reported (13). Clones expressing the mouse dhfr gene were selected by growing the cells in MEM medium deficient in nucleosides, supplemented with 10% v/v dialyzed fetal calf serum, gentamycin (5 μg ml^{-1}) and 5 nM methotrexate (MTX). Positive clones were isolated and grown up. PCR- and immuno-assays were carried out to confirm the integration and expression of LCAT cDNA. To increase the amount of LCAT protein produced by transfected cells, these were then grown in the same medium supplemented with 100 nM MTX. MTX is a potent competitive inhibitor of dhfr, so increasing MTX concentration selects for clones that express increased levels of dhfr. At confluency, cell monolayers

were washed (×4) with MEM medium and then cultured for 24 h in MEM medium supplemented with gentamicin and 100 nM MTX.

Assay of LCAT activity

Acyltransferase activity in culture medium from transfected cells was determined as the rate of synthesis of ^{3}H-labeled CE in assay medium containing egg lecithin (40 μg ml^{-1}) (Sigma, St. Louis, MO), [1,2-^{3}H]cholesterol (5 μg ml^{-1}, specific activity 5×10^4 cpm μg^{-1}) (New England Nuclear, Boston, MA), apoA-I (5 μg ml^{-1}) (Sigma) and recrystallized human serum albumin (2.5% w/v in buffer containing 0.15 M NaCl, 0.01 M phosphate, pH 7.4 (3). This substrate was shown previously to consist of discoidal recombinant particles (17) made up of a lipid bilayer whose circumference is probably stabilized by the repeat units of apoA-I (18). The production of labeled CE was determined by extracting portions of assay medium with chloroform–methanol 1:1 (v/v). After separation of the phases, the content of [^{3}H]CE radioactivity in the chloroform phase was determined by thin-layer chromatography on silica gel plates developed in hexane–diethyl ether–acetic acid 83:16:1 (v/v). CE radioactivity (R_f 0.9–1.0 in this system) was determined by liquid scintillation spectrometry. LCAT activity was linear over a 6 h incubation at 37°C. Nontransfected cells secreted no detectable LCAT under the same conditions.

LCAT phospholipase activity was determined in the same medium, except that no cholesterol was present, and the egg lecithin was labeled to a final specific activity of 4×10^3 cpm μg^{-1} with [^{3}H]dipalmitoyl phosphatidyl choline (New England Nuclear) (3). In some experiments the apolipoprotein-dependence of LCAT acyltransferase and phospholipase activities was determined by excluding apoA-I from the assay medium.

To determine the thermal stability of the LCAT activity secreted from transfected cells, culture medium was incubated at 37°C for different periods of time, prior to the measurement of enzyme activity. In some experiments, confluent cell monolayers were washed as described above and then incubated for 40 h at 37°C in the presence or absence of LCAT substrate (apoA-I-activated phosphatidylcholine-cholesterol vesicles) in MEM medium containing 1.5 mM dithiobis(2-nitrobenzoic acid) to inhibit LCAT activity (19). At the end of the incubation, inhibition was reversed with 2-mercaptoethanol (20 mM). Enzyme activity was then determined over 6 h at 37°C.

LCAT mass in medium from cultures of transfected cells was determined by solid phase immunoassay as previously described (13). Briefly, pure LCAT standard purified from plasma (3), medium from nontransfected control CHO cells, and medium from transfected cells were transferred to nitrocellulose membrane (Sartorius; West Coast Scientific, Hayward, CA) and then incubated with ^{125}I-labeled rabbit anti-human LCAT antibody raised against residues 165–181 of the sequence of mature human LCAT (13). Individual screens were then analyzed in a Searle 1185 gamma spectrometer. Nontransfected CHO cells released no immunologically detectable LCAT into the culture medium.

Secondary structure prediction

Analysis of predicted secondary structure in the LCAT C-terminus was carried out by the Chou-Fasman (20) and Rose (21) methods using PROSIS software (Hitachi America Inc., Brisbane, CA) and an IBM PS-70 computer.

RESULTS

Secondary structure analysis of the carboxy-terminal quarter of normal LCAT indicates mainly beta-sheet between val_{309} and gly_{396}, stabilized by the disulfide bridge between cys_{313} and cys_{356} (22). Three sheets of beta-structure separated by one turn and one coil are predicted (**Fig. 1**). These extend from val_{309} to leu_{340}, from thr_{347} to gln_{360}, and from pro_{366} to gly_{396}. Between gly_{396} and the C-terminus (glu_{416}) extends a hydrophilic sequence of predicted coil structure. This contains eight prolines in a total of 18 amino acids.

The LCAT species synthesized had C-termini corresponding to residues 397, 393, 387, 374, 358, 340, and 314 (as the following codon had been mutated to stop in each case). The molecular weights observed for wild-type and mutant LCAT species after the cell-free translation of synthetic mRNAs corresponded to those predicted (**Fig. 2**).

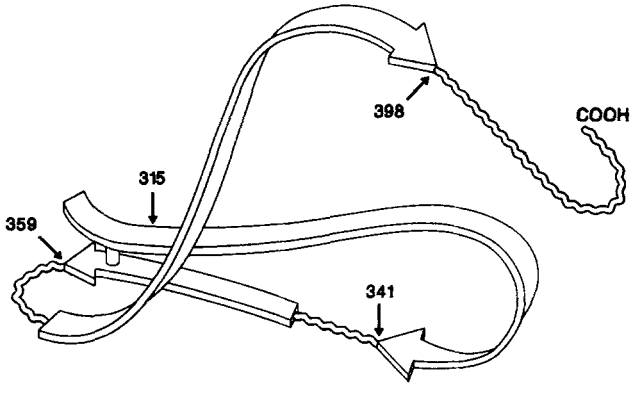

Fig. 1. Predicted secondary structure of the C-terminus of LCAT. Beta-sheet is shown as ribbon while random coil is shown as wavy thread. Truncation sites are also indicated.

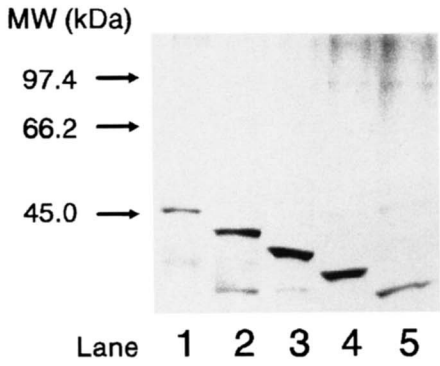

Fig. 2. Translation products of representative wild-type and truncated LCAT cDNAs. cDNAs were prepared as described under Experimental Methods. Transcription was with T7 RNA polymerase, and translation of the mRNA product was carried out in vitro with rabbit reticulocyte lysate in the presence of [^{35}S]methionine. After SDS-gel electrophoresis of the translation products, labeled proteins were detected by autoradiography. The predicted protein molecular mass of the wild-type protein is 47.1 kDa. Apparent molecular weights are expressed relative to the migration rates of pure protein standards. Lane 1, wild-type; lane 2, $LCAT_{398 \rightarrow stop}$; lane 3, $LCAT_{359 \rightarrow stop}$; lane 4, $LCAT_{341 \rightarrow stop}$; lane 5, $LCAT_{315 \rightarrow stop}$.

Wild-type and all mutant LCAT proteins were secreted into the culture medium. All species were secreted at similar rates (0.15–0.18 μg ml^{-1} per 24 h) based on the accumulation of LCAT protein in the culture media, except in the case of the $LCAT_{398}$ truncation, where the secretion rate (0.06 μg ml^{-1} per 24 h) was about one third of the other values. The specific activity of wild-type and mutant LCAT proteins, determined with [^{3}H]cholesterol-lecithin liposomes, differed significantly (**Table 2**). The protein with a stop codon at position 315 was almost completely deficient in LCAT activity. Inclusion of the first sequence of beta-sheet (the mutant with a stop codon at position 341) was associated with no increase in LCAT activity. The same result was obtained when the C-terminus was extended to residue 358. However, when the whole of the third and final beta-sheet was included (the mutant whose C-terminus was codon 397) LCAT specific activity was at least as great as that of the wild-type protein (Table 2). These data clearly indicate that the proline-rich 'tail' of LCAT is not required for activity for cholesteryl ester synthesis.

TABLE 2. Specific activities of wild-type and truncated LCAT species

Enzyme Species	Specific Activity[a]
Wild type	468.5 ± 28.3
$LCAT_{398 \rightarrow stop}$	593.3 ± 65.8
$LCAT_{394 \rightarrow stop}$	25.6 ± 6.7
$LCAT_{388 \rightarrow stop}$	8.1 ± 5.7
$LCAT_{375 \rightarrow stop}$	17.3 ± 10.0
$LCAT_{359 \rightarrow stop}$	7.8 ± 4.2
$LCAT_{341 \rightarrow stop}$	12.2 ± 4.1
$LCAT_{315 \rightarrow stop}$	27.0 ± 9.0

[a]Specific activity is expressed as pmol CE esterified μg^{-1} h^{-1} with liposome substrate containing lecithin, cholesterol, and apoA-I in the weight ratio 8:1:1 as described under Experimental Procedures. Values shown are means ± 1 SEM for 3–6 different experiments.

Because of the abrupt difference in the properties of $LCAT_{358}$ and $LCAT_{397}$, additional mutants were engineered whose C-termini were ile_{374}, leu_{387}, and ile_{393}. All activities were close to baseline (~5% of wild type) (Table 2). These data indicate that a sudden loss of activity occurs as residues 393–398 are deleted from the primary sequence.

The phospholipase activity of wild-type and mutant LCAT species was also determined. As shown in **Fig. 3,** phospholipase and transferase activities were modified in parallel. The acyltransferase and phospholipase activities of the same proteins were determined in the presence and absence of apoA-I. No change in apoA-I-induced activation was observed in different LCAT species. These data suggest that the loss of activity observed when truncation of the primary sequence extends from residue 398 to residue 393 was not the result of a change in response to apoA-I.

A possible role for the LCAT C-terminus in stability was investigated. Wild-type LCAT, the fully active mutant $LCAT_{398 \rightarrow stop}$, and the poorly active mutant $LCAT_{315 \rightarrow stop}$ were incubated at 37°C in the presence or absence of substrate (apoA-I-activated lecithin-cholesterol liposomes); the other mutants had too little activity to permit these measurements. As shown in **Fig. 4,** loss

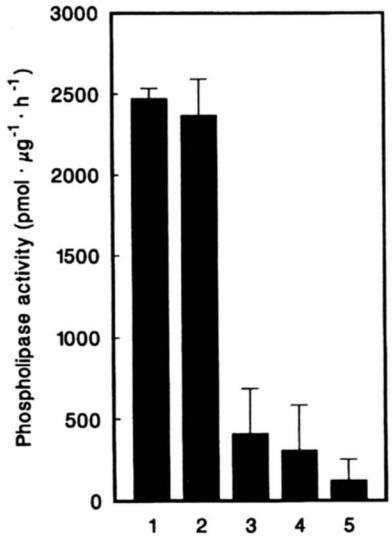

Fig. 3. Phospholipase specific activity of wild-type LCAT and truncation mutants. LCAT from cell culture medium was assayed with [^{3}H]dipalmitoyl phosphatidyl choline vesicles activated by apoA-I, as described under Experimental Methods. Specific activity was determined as in Table 2. The identity of samples 1–5 is the same as in Fig. 2. Each shows the mean ± 1 SD of three experiments.

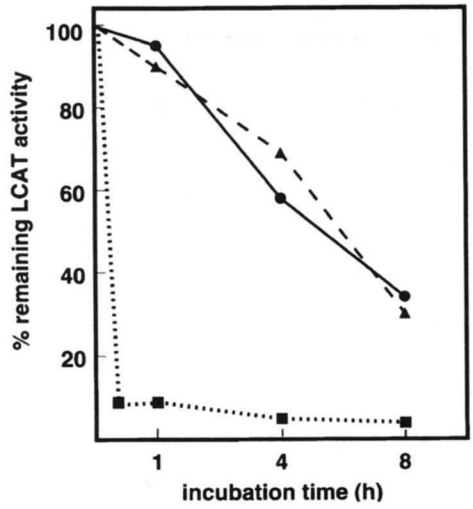

Fig. 4. Thermal stability of wild-type LCAT (circles), $LCAT_{398 \rightarrow stop}$ (triangles), and $LCAT_{315 \rightarrow stop}$ (squares). Incubation was carried out in the absence of substrate at 37°C for the time indicated. Initial activity was set to 100% in each case.

of the proline-rich C-terminus was without effect on the stability. Both wild-type and $LCAT_{398 \rightarrow stop}$ mutants lost activity at the same rate in the absence of substrate, with a $t_{1/2}$ of about 4.5 h. The $LCAT_{315 \rightarrow stop}$ mutant lost activity much more rapidly: >90% of activity was lost during the first 30 min of incubation at 37°C (from 4.6 pmol CE ml^{-1} h^{-1} to 0.38 pmol CE ml^{-1} h^{-1}). This finding suggests that most or all of the decreased specific activity of $LCAT_{315 \rightarrow stop}$ in the culture medium might be the result of changes occurring after the initial secretion of the mutant LCAT protein into the medium. With wild-type LCAT, activity recovered in the presence of substrate was 2-fold higher than in its absence (297.0 ± 49.7 vs. 142.0 ± 6.0 pmol CE ml^{-1} h^{-1}) (**Fig. 5**). On the other hand, there was no increase in the specific activity of the $LCAT_{315 \rightarrow stop}$ species when substrate was present in the culture medium. These findings indicate that the major influence of residues 393–398 was on enzyme activity, not stability.

DISCUSSION

LCAT is the source of a major part of plasma cholesteryl esters. The role of LCAT in cholesterol transport out of peripheral cells and in the orderly metabolism of free cholesterol from triglyceride-rich lipoproteins makes its structure of particular interest. LCAT has several unusual properties including its selectivity for cholesterol over water as acyl acceptor, its preference for discoidal HDL, and the dependence of activity on an apolipoprotein cofactor, most effectively apoA-I. Structurally, LCAT is distinguished by an apolipoprotein-like 21-aa amphipathic helical repeat immediately prior to the active site serine residue (ser_{181}) and the presence of a proline-rich, negatively charged C-terminus.

Three-dimensional analysis from X-ray crystallography or solution nuclear magnetic resonance is not yet available. As a result, information on structure–function relationships of LCAT has been obtained by studies of the activity of mutant enzyme species generated by site-directed mutagenesis. Additional data have come from studies of spontaneous mutants of human LCAT, expressed in transfected cells, and by comparison of the LCAT primary sequence in different species.

The results of these experiments indicate that the proline-rich C-terminus of wild-type LCAT is not required for full enzyme activity with the discoidal HDL that represent the most effective substrate of this enzyme. Proline-rich regions have been reported to bind efficiently but nonspecifically to other proteins (8). In native plasma, LCAT is present almost exclusively as a complex with apoA-I. Nevertheless, the reaction involving apoA-I, which is essential for LCAT activity, is not affected by the loss of the proline-rich region. The terminal region of LCAT contains several short O-linked carbohydrate chains (23). Unlike the larger and more complex N-linked chains, which have a significant influence on both specific activity and substrate specificity in LCAT (24–26) these O-linked residues did not contribute to function, at least in terms of the assays used in this study.

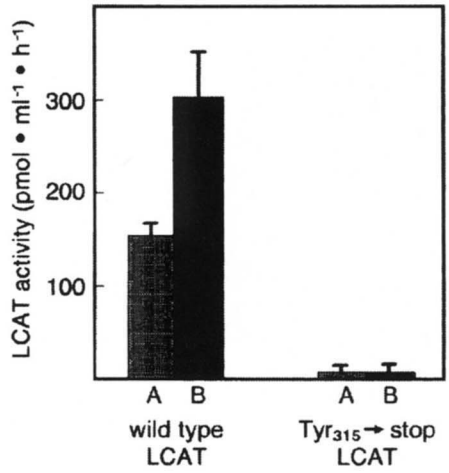

Fig. 5. Thermal stability of wild-type LCAT and $LCAT_{315 \rightarrow stop}$ secreted from transfected cultures cell monolayers in the absence (A) or the presence (B) of substrate, as described under Experimental Methods. LCAT activity was measured for 6 h at 37°C. Values shown are means ± 1 SD for three experiments.

LCAT has been linked to several other lipases on the basis of several short homologous amino acid sequences. These include the amino acids surrounding the active site serine (ser_{181}) (27, 28). Such lipases are believed to share a catalytic triad of serine, histidine, and aspartate residues. Where these residues have been definitively identified from three-dimensional structural studies, all three are in the anterior half or two thirds of the primary sequence (29, 30). Bile salt-stimulated lipase (cholesterol ester hydrolase), one of these lipases, terminates like LCAT in a proline-rich region. Deletion of this C-terminus was, as with LCAT, without effect on catalytic activity (31). Deletion of further short segments led to almost complete loss of enzyme activity. Human pancreatic lipase lacks the proline-rich region of the other two enzymes but, as in LCAT, its C-terminus is required for enzyme stability (32).

Several pieces of evidence argue against another possibility, that the C-terminus of LCAT contains or is adjacent to an essential residue. The sequence between residues 393 and 398 (-ile_{393}-leu-leu-gly-ala-tyr) is required for activity but itself contains no appropriate amino acid contributor to the LCAT active site. Such evidence makes it more likely that the highly hydrophobic sequence between residues 393 and 398 mainly serves a stabilizing function, perhaps by making up one strand of a hydrophobic cleft or basket accommodating the elongated acyl chains of phosphatidyl choline. Three-dimensional analysis using crystallography or NMR will be required to definitively establish this prediction. Because residues 393–398 are required equally for the phospholipase and acyltransferase activities of LCAT, these are probably not involved in sterol binding.

While this report was being prepared, Hengstschlager-Ottnad, Kuchler, and Schneider (33) showed that the chicken LCAT cDNA encoded a mature catalytically active protein in which the proline-rich C-terminus common to described mammalian sequences was absent. This observation is consistent with the present finding, that in human LCAT the C-terminus is not required for activity. The chicken LCAT protein terminates at residue 391. Residues 377–388 are identical between species, despite an overall homology that reaches only 73%. This sequence includes (at residue 384) one of four conserved N-linked polysaccharide chains. However, the chicken enzyme does lack the sequence corresponding to residues 393–398 in the human enzyme. Comparative studies of the turnover and thermal stability of human and avian LCAT could provide useful information on the role of this region in the human protein.

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