Roles of Cysteines in Human Lecithin:Cholesterol Acyltransferase†

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ABSTRACT: Human lecithin:cholesterol acyltransferase (LCAT, E.C. 2.3.1.43) is a serine-type esterase that contains six cysteines, two of which, Cys^{31} and Cys^{184}, are free. The remaining cysteines form disulfide links. One of these is between Cys^{50} and Cys^{74} and the other is between Cys^{313} and Cys^{356}. The cDNA of LCAT and mutants in which one or two of the six cysteines were replaced by glycine was expressed in COS-6 cells. Polymerase chain reactions and Northern blot analysis indicated that LCAT mRNA was produced by all transfectants. Western blots of all transfected cells probed with a polyclonal antibody revealed intracellular LCAT. Substitution of glycine for either Cys^{50}, Cys^{74}, Cys^{313}, or Cys^{356} was associated with a nearly total absence of activity in the medium. No protein was secreted when glycine replaced either of the amino acid residues that link Cys^{313} and Cys^{356}. The small amounts of the $Cys^{50} \rightarrow$ Gly and $Cys^{74} \rightarrow$ Gly mutants found in the medium had specific activities that were much lower than that of the wild-type LCAT. All other transfectants secreted immunologically measurable amounts of active enzyme. Mutants in which one or both free cysteines, Cys^{31} and Cys^{184}, were replaced with glycine were less active than the wild type and only partially inhibited by a sulfhydryl blocking reagent. The substrate specificities of the $Cys^{31} \rightarrow$ Gly and $Cys^{184} \rightarrow$ Gly mutants differed from that of the wild type. The specific activity of the wild-type LCAT against a substrate composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine was 8 times higher than that measured against a substrate containing diarachidonoylphosphatidylcholine. The specific activities of mutants were nearly the same with both substrates. These data support a model of LCAT in which (1) Cys^{31} and Cys^{184} are not required for catalysis but sterically block access to the active-site region and (2) the disulfide bonds in native LCAT are required for activity and secretion-competent LCAT.

Lecithin:cholesterol acyltransferase (LCAT,[1] E.C. 2.3.1.43), the major cholesterol-esterifying activity of human plasma, is a glycoprotein containing 416 amino acids in a single polypeptide chain (Fielding, 1990; Jonas, 1991). In model systems, human LCAT transfers small fatty acyl chains such as myristate, palmitate, oleate, and linoleate more efficiently than arachidonate and other fatty acyl groups that are bulky due to side chains or a large number of double bonds (Subbaiah et al., 1992; Pownall et al., 1985a,b, 1987; Ueno et al., 1986; Grove & Pownall, 1991). In contrast, rat LCAT appears to transfer bulky acyl groups with greater efficiency than human LCAT. Both human and rat LCAT are more reactive with phosphatidylcholines (PCs) that are in a fluid environment (Pownall et al., 1985a,b).

Spectroscopic studies (Chong et al., 1983) and predictive algorithms (Yang et al., 1987) suggest that LCAT contains about 55% random coil and nearly equal amounts of β-sheet and α-helix. On the basis of homology with other serine-dependent esterases, the active site is thought to include a serine at residue 181 (McLean et al., 1986). The primary substrates for LCAT are the high-density lipoproteins (HDL). LCAT activity is stimulated by apolipoprotein A-I (apoA-I), the major protein of HDL, and its acyltransferase activity is inhibited by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), which binds to one or both free sulfhydryl groups on Cys^{31} and Cys^{184} (Aron et al., 1978; Yang et al., 1987). LCAT contains four other cyteines that are involved in disulfide links. These are Cys^{50}–Cys^{74} and Cys^{313}–Cys^{356} (Yang et al., 1987). In the absence of cholesterol, LCAT exhibits a phospholipase activity that is also stimulated by apoA-I but is not inhibitied by DTNB (Jauhiahanen & Dolphin, 1986). The role of cysteine residues in the structure and function of LCAT has not been fully elucidated. The specific activities of LCAT mutants in which one or both of the free cysteines were replaced by glycine were similar to that of the wild-type enzyme; DTNB had little effect on the double mutant but reduced the activity of the single mutants by half (Francone & Fielding, 1991). On the other hand, the essential nature of the disulfide links is not known. In this study, LCAT mutants in which one or more of the cysteines were replaced by glycine were prepared by site-directed mutagenesis. The roles of each of the cysteine residues on LCAT synthesis, secretion, and activity were examined after transient expression of the mutants in COS-6 cells.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from Promega. Sequenase version 2.0 DNA sequencing kit from U.S. Biochemical Corp. was used for sequencing. [α-^{35}S]α-thio-dATP (1000 Ci/mmol), [α-^{32}P]ATP(5000 Ci/mmol), and Na^{125}I were obtained from Amersham Corp. All other chemicals were of analytical or molecular biological grade. Rabbit anti-goat IgG was from Bio-Rad, [^{3}H]cholesterol was

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[1] Abbreviations: LCAT, lecithin:cholesterol acyltransferase; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; R-HDL, model reassembled high-density lipoproteins; apoA-I, apolipoprotein A-I; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DAPC, 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine; PCR, polymerase chain reaction; DNA, deoxyribonucleic acid; cDNA, complementary DNA; RNA, ribonucleic acid; DEAE, diethylaminoethyl; DMEM, Dulbecco's modified Eagle's medium; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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Table I: Synthetic Oligonucleotides Used for LCAT Mutants[a]

Oligonucleotides for Site-Directed Mutagenesis	
$Cys^{31} \rightarrow Gly$	ATT-CCC-CAG-GCC-GCC-GGG-CAC
$Cys^{184} \rightarrow Gly$	CAA-GTG-TAG-ACC-GCC-GAG-GTC
$Cys^{50} \rightarrow Gly$	TGT-CTT-GCG-GAT-GCC-CTA-CCA-GTT-CAC
$Cys^{356} \rightarrow Gly$	CCA-CAG-GCC-ACC-GAG-CT-CGT
$Cys^{74} \rightarrow Gly$	ATC-GAT-CCA-GDC-GTC-TAC-CCC
$Cys^{313} \rightarrow Gly$	GCC-GTA-AAG-ACC-GTA-TAC-TTC
PCR Primers for LCAT cDNA Amplification	
downstream	TTC-TGG-CTC-CTC-AAT-GTG-CTC
upstream	CAA-GTG-TAG-ACC-GCC-GAG-GTC

[a] All the oligonucleotides are listed from 5′ to 3′; the mutagenesis primers are antisense strand. The single base shifted is underlined.

from Dupont, and the phospholipids were from Avanti Polar Lipids.

A full-length cDNA clone of human LCAT (pUCL-CAT.10), covering the entire coding region, cloned into the *Eco*RI/*Bam*HI sites of pUC19, was kindly provided by Richard Lawn of Genentech Inc. (McLean et al., 1986). The insert contained 102 nucleotides in the 5′-untranslated region, the entire coding region, and 68 nucleotides in the 3′-untranslated region. Both ends were bounded by multiple linkers.

Construction of the Mutant Clones. Oligonucleotides (Table I) were synthesized on a Cyclone Plus DNA Synthesizer. After digestion with *Eco*RI and *Bam*HI, the pUCL-CAT.10 was subcloned into M13mp18 and M13mp19. Uracil-containing single-stranded DNA was prepared by using *Escherichia coli* host strain CJ236 (*ung*$^-$) to culture the M13mp18 or M13mp19 LCAT clones (Kunkel et al., 1985). These ssDNA were used as the template and hybridized to the oligonucleotides which contained the mismatched nucleotides that coded for the desired mutant amino acid and then 5′-phosphorylated. Mutagenesis was carried out according to Taylor et al. (1985). After hybridization, the second strand was synthesized using T7 DNA polymerase and T4 ligase. The double-stranded DNA was transformed into an *ung*$^+$ *E. coli* strain, DH-5α'. The plaques of mutant cDNA were identified by the dideoxynucleotide method; to confirm the desired mutation, the entire LCAT coding domain was sequenced (Sanger et al., 1977). After digestion with *Eco*RI and *Bam*HI, the wild-type and mutant cDNAs were subcloned into a eukaryotic expression vector, pSG5 (Green et al., 1988), and transformed into *E. coli* AG1. The positive clones were checked by restriction enzyme mapping and direct sequencing. The selected mutant cDNAs wre prepared in larger quantities for expression in mammalian cells.

In Vitro Expression of LCAT. A modified DEAE–dextran method (Selden et al., 1986) was used to transfect wild-type and mutant cDNAs of LCAT into COS-6 cells. Cells were grown in high-glucose DMEM with 10% heat-inactivated fetal bovine serum and 1× antibiotic–antimycotic liquid (Gibco–BRL) in 75-cm^2 flasks. After reaching >90% confluence, the cells were subcultured at a 1:4–1:6 ratio and incubated for 24–48 h. The cells were transfected with 20 μg of plasmid/75-cm^2 flask. The cells were incubated with the DNA–DEAE–medium stock and chloroquine for 3 h and shocked with 10% dimethyl sulfoxide for 2 min. In each experiment, two flasks containing only COS-6 cells and COS-6 cells plus pSG5 plasmids were used as controls. After a 72-h incubation, the medium was collected, centrifuged, and immediately tested for LCAT activity using model reassembled HDL (R-HDL) as the substrate. If necessary, the cells were washed with DMEM and incubated another 48 h before the medium was tested for activity.

RNA Preparation and Analysis. After the medium was collected for LCAT assays, the transfected COS-6 cells were used to prepare total RNA. Extraction of RNA with guanidinium thiocyanate (Chirgwin et al., 1979) was followed by centrifugation in cesium trifluoroacetate (Okayama, 1987) and precipitation in ethanol. Before use, the RNA precipitations were stored in 70% ethanol at −70 °C. For Northern blot analysis, RNA was fractionated on a 1.2% agarose gel and 10 mM sodium phosphate buffer, pH 7.0, after denaturation with glyoxal/dimethyl sulfoxide (McMaster et al., 1977). RNA bands were directly transferred to nitrocellulose and prehybridized at 42 °C in a pH 6.8 phosphate buffer containing 50% formamide, 0.1% sodium dodecyl sulfate, and 0.1 mg/mL denatured salmon sperm DNA for 3 h. A full-length LCAT cDNA was cut from wild-type pSG5 using *Eco*RI and *Bam*HI and purified by electroelution. The cDNA was labeled with [^{32}P]dATP using random primers (DNA labeling system, BRL) giving a specific activity of $>2 \times 10^8$ cpm/μg. The ^{32}P-labeled cDNA probe (coding region only) was added to the hybridization mixture and incubated at 42 °C overnight. The polymerase chain reaction (PCR) was performed on RNA using the GeneAmp RNA PCR kit from Perkin-Elmer Cetus.

Substrate Preparation and LCAT Assays. Model reassembled high-density lipoproteins (R-HDL) were prepared by a detergent removal technique (Matz & Jonas, 1982). 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) or diarachidonoylphosphatidylcholine (DAPC) and 2 mol % cholesterol and traces of [^{3}H]cholesterol (7500 dpm/pmol) were mixed, and the organic solvent was removed under a stream of N_2. The lipids were suspended in Tris-buffered saline, pH 7.4, by vortexing, and the suspension was combined with human apoA-I at a lipid-to-protein ratio of 100:1 (M/M). Sodium cholate (10%) was added until the turbidity of the solution disappeared and then exhaustively dialyzed against the same buffer.

The 30-min LCAT assays were performed at 37 °C in a final volume of 0.2 mL containing 2% human serum albumin, 0.5 mM dithiothreitol, 100 mM NaCl, 10 mM Tris, pH 7.4, and 0.15 mM PC as R-HDL. For each assay, 10 μL of medium was mixed with the substrate solution. After incubation, the reaction was quenched with 0.5 mL of methanol and 1.0 mL of hexane. The upper phase was applied to a small silica column and the cholesteryl esters were eluted with ether/hexane (1:6) and quantified by liquid scintillation counting. Experiments for the substrate saturation curves were conducted similarly, except that the substrate concentration was varied as needed.

Quantification of LCAT and Western Blot Analysis. A solid-phase enzyme-linked immunoassay was used to determine the mass of secreted LCAT in the medium using purified human plasma LCAT as the standard. The polyclonal antibody used was raised in goat against pure human plasma LCAT. Media from transfected COS-6 cells and the controls (untransfected COS-6 cells and the same cells transfected with expression vector pSG5) were concentrated 10-fold with a Centriprep-30 from Amicon. The samples were applied to nitrocellulose membranes in a Bio-Rad dot-blot apparatus. After blotting with 5% milk in PBS, LCAT was reacted with goat anti-human LCAT serum and then with rabbit anti-goat [^{125}I]IgG. After extensive washing with 5% milk in PBS, autoradiography was performed by exposing the membrane to X-ray film overnight. For quantification, the dots were cut out and the radioactivity was measured by γ counting.

Table II: Triplet Codons Changed in Mutant LCAT cDNA[a]

substitution	codon changed
$Cys^{31} \rightarrow Gly$	TGC → GGC
$Cys^{184} \rightarrow Gly$	TGT → GGT
$Cys^{31,184} \rightarrow Gly$	TGC (TGT) → GGC (GGT)
$Cys^{50} \rightarrow Gly$	TGC → GGC
$Cys^{356} \rightarrow Gly$	TGT → GGT
$Cys^{50,365} \rightarrow Gly$	TGC (TGT) → GGC (GGT)
$Cys^{74} \rightarrow Gly$	TGC → GGC
$Cys^{313} \rightarrow Gly$	TGT → GGT

[a] All the codons correspond to the sense strand.

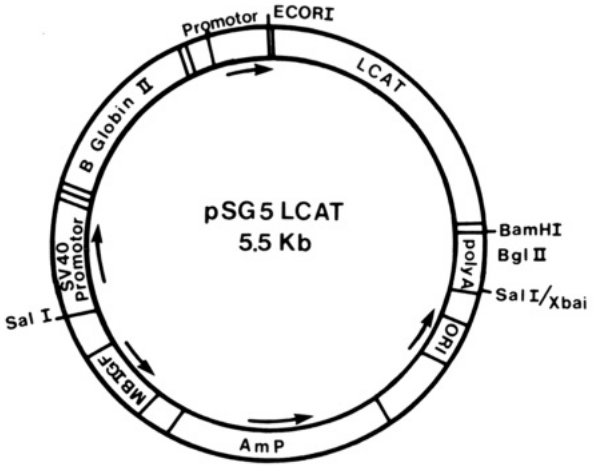

FIGURE 1: Structure of the expression vector for wild-type and mutant cDNA of LCAT. Plasmid pSG5 LCAT and its mutants were constructed as described under Materials and Methods. The components of the plasmid are as indicated.

Western blot analysis was performed using the same set of 10-fold-concentrated media. Electrophoresis was conducted in an SDS–12% polyacrylamide gel. Following electrophoresis, the protein was transferred to a nitrocellulose membrane and incubated with goat anti-LCAT serum. The bands were visualized by horseradish peroxidase-conjugated rabbit anti-goat IgG (Bio-Rad).

RESULTS

Mutagenesis Strategy and Construction of Expression Vectors. Within the primary structure of LCAT there are six cysteines, which are located at residues 31, 50, 74, 184, 313, and 356. Two of them, Cys^{31} and Cys^{184}, contain free sulfhydryl groups that are required for maximum catalytic activity. Disulfide bonds link Cys^{50} to Cys^{74} and Cys^{313} to Cys^{356} (Yang et al., 1987). Six single point mutants in which each of the cysteines were replaced by glycine were prepared. Two double mutants were also prepared. In one of these, both Cys^{31} and Cys^{184} were changed to glycine. In the other, Cys^{50} and Cys^{365} were replaced by glycines. Table 1 contains the identities of the oligonucleotides that were used to make the LCAT mutations as well as the primers that were used for the cDNA amplification. In each case, either TGC or TGT was changed to GGC or GGT (Table II). The configuration of the constructed vector is shown in Figure 1. Finally, the whole region of the cDNA coding for the LCAT was sequenced to confirm that the desired mutation was the only change. The conparison of DNA sequences of LCAT cDNA from wild-type and mutants in regions surrounding the mutation sites is shown in Figure 2.

Northern Blot and PCR Analysis. Total RNA of the COS-6 cells, which were transfected by pSG5 (inserted LCAT cDNA) was used in both analyses. For PCR analysis, the primers are

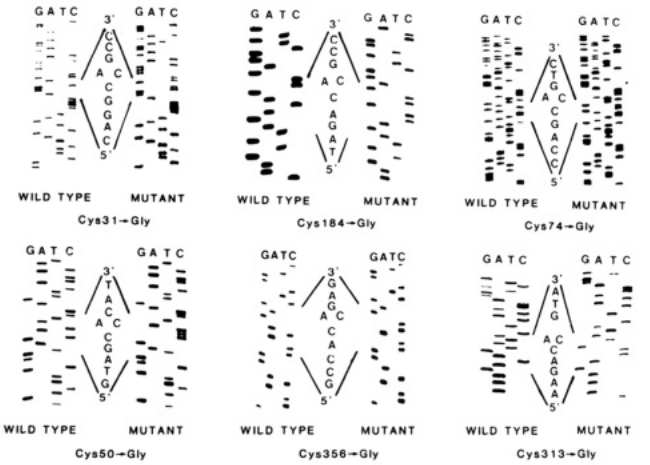

FIGURE 2: Comparison of the DNA sequences of LCAT cDNA from wild-type and cysteine mutants. Each pair of autoradiograms represents wild-type and the site-directed mutant sequence in the region surrounding the mutation. All the sequences are antisense strand.

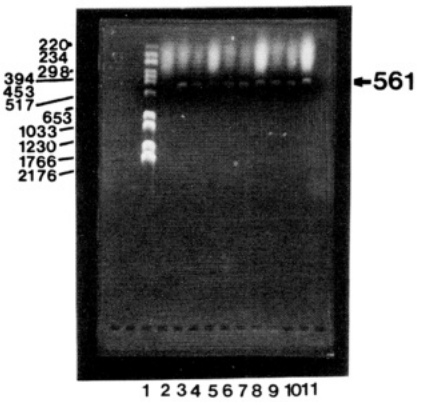

FIGURE 3: Polymerase chain reaction amplification of cDNA from total RNA. PCR was performed with the total RNA expressed by COS-6 cells using the GeneAmp RNA PCR kit from Perkin-Elmer Cetus. Electrophoresis was performed on 1.2% agarose gels. From the total 100 μL of resulting PCR mixture, 5 μL was applied to each lane. Lane 1, standards; lane 2, cells only; lane 3, wild-type LCAT; lane 4, $Cys^{31} \rightarrow Gly$; lane 5, $Cys^{184} \rightarrow Gly$; lane 6, $Cys^{31,184} \rightarrow Gly$; lane 7, $Cys^{50} \rightarrow Gly$; lane 8, $Cys^{356} \rightarrow Gly$; lane 9, $Cys^{50,356} \rightarrow Gly$; lane 10, $Cys^{74} \rightarrow Gly$; lane 11, $Cys^{313} \rightarrow Gly$.

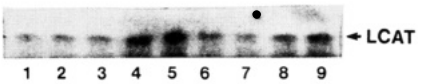

FIGURE 4: Northern blot analysis using LCAT cDNA as the probe. Each lane contained 10 μg of total RNA from the COS-6 cells transfected by wild-type and mutant LCAT cDNA expression vectors. Lane 1, wild type; lane 2, $Cys^{31} \rightarrow Gly$; lane 3, $Cys^{184} \rightarrow Gly$; lane 4, $Cys^{31,184} \rightarrow Gly$; lane 5, $Cys^{50} \rightarrow Gly$; lane 6, $Cys^{356} \rightarrow Gly$; lane 7, $Cys^{50,356} \rightarrow Gly$; lane 8, $Cys^{74} \rightarrow Gly$, lane 9, $Cys^{313} \rightarrow Gly$.

listed in Table I. If the LCAT-specific mRNA is present after PCR, a fragment of 561 bp should be found. As shown in Figure 3, no specific band was found for the cell plus pSG5 vector control. In contrast, wild-type and all the mutants had positive bands that corresponded to fragments of 561 bp.

Northern blot analysis was performed using a full-length wild-type cDNA of human LCAT as the probe. The results from COS-6 cells transfected with the wild-type and mutant cDNA of LCAT are shown in Figure 4. No LCAT-positive RNA was detected in the control cells (data not shown). For the remainder, including the wild type and all of the mutants, a specific band contained mRNA that was positive when probed with the LCAT cDNA. From the results of the PCR and the Northern blot analysis, we concluded that LCAT-

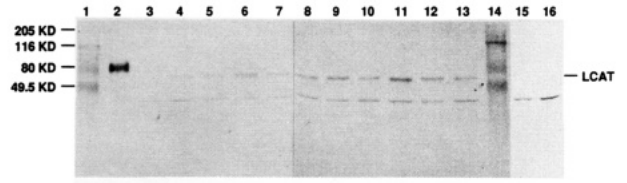

FIGURE 5: Western blot analysis of transfected COS-6 cell lysates from control, wild type, and mutants. After incubation for 72 h, cells were washed with PBS and trypsinized. Cells from each 75-cm^2 flask were homogenized using solubilizing buffer (1.6% Triton X-100, 0.3 mM leupeptin, 5.0 M urea and 1.5 mM phenylmethanesulfonyl fluoride) and centrifuged at 25 000 rpm for 30 min. The supernatants were concentrated 10-fold, and 10-μL aliquots of each were used to perform the Western blots as described above. Lanes 1 and 14, standards; lane 2, human plasma LCAT; lane 3, cells; lanes 4 and 13, wild-type LCAT; lane 5, $Cys^{31} \rightarrow Gly$; lane 6, $Cys^{184} \rightarrow Gly$; lane 7, $Cys^{31,184} \rightarrow Gly$; lane 8, $Cys^{50} \rightarrow Gly$; lane 9, $Cys^{356} \rightarrow Gly$; lane 10, $Cys^{50,356} \rightarrow Gly$; lane 11, $Cys^{74} \rightarrow Gly$; lane 12, $Cys^{313} \rightarrow Gly$; lane 15, cells using preimmune goat serum as first antibody; lane 16, wild-type LCAT using preimmune serum as first antibody. Note the nonspecific band in lanes 15 and 16.

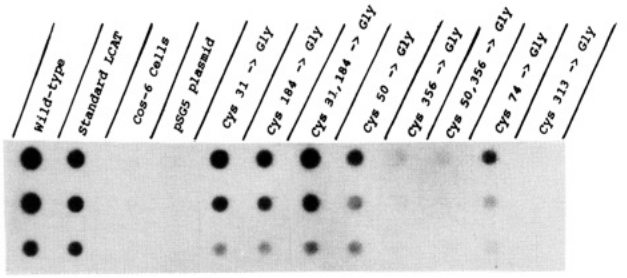

FIGURE 6: Quantitative dot-blot analysis of human LCAT and cell culture media from control, wild-type LCAT cDNA, and its mutants. Controls were untransfected COS-6 cells and the same cells transfected by expression vector pSG5. For each sample, 20, 40, or 80 μL of 4 μg/mL standard LCAT or 2-fold concentrated media were applied. LCAT was detected by successive reactions with goat anti-human LCAT serum and ^{125}I-labeled rabbit anti-goat IgG.

specific mRNA accumulated in all of the transfected COS-6 cells.

Intracellular LCAT Mass. The intracellular contents of COS-6 that had been transfected with the various mutants were tested for the presence of LCAT by Western blot analysis (Figure 5). These data showed that all of the cells containing the mutant and wild-type inserts exhibited a positive response when probed with an LCAT-specific antibody. Control tests with preimmune goat serum demonstrated that the additional band corresponding to a molecular mass about 40 kDa was due to nonspecific binding.

Secreted LCAT Mass. After a 48-h incubation, the media were collected from the transfected cells and analyzed by a solid-phase immunoassay using ^{125}I-labeled rabbit anti-goat IgG as the probe (Figure 6). Western blot analysis of the media demonstrated that the immunoreactivity was due only to a protein with a migration pattern identical to that of LCAT (Figure 7). The data on LCAT mass (Table III) showed that the cells that were transfected with the wild-type LCAT cDNA secreted the greatest amount of LCAT activity. The LCAT concentrations of the media from the transfectants in which one or both of the free cysteines were replaced by glycines were about 40% lower. In contrast, the concentrations of the media from cells in which one or more of the cysteines involved in disulfide bonds were replaced were 70% lower than that from the wild-type transfectant. LCAT was not found in the media from any of the transfections in which either Cys^{365} or Cys^{313} was replaced by glycine.

Activities of Wild-Type and Mutant LCAT. Wild-type and mutant cDNA of LCAT cloned into the expression vector

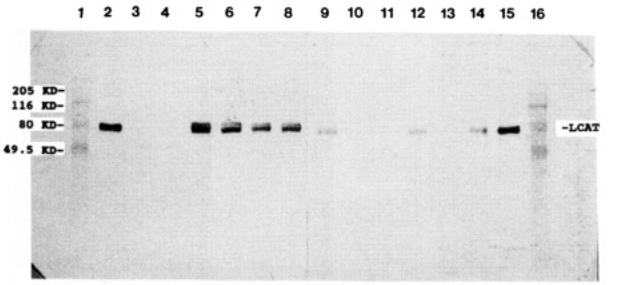

FIGURE 7: Western blot analysis of human LCAT and transfected COS-6 cell culture media. The media cells were concentrated 10-fold, and 5-μL aliquots were applied to each lane. After SDS–polyacrylamide gel electrophoresis, the protein was transferred to nitrocellulose membrane and reacted with goat anti-LCAT serum and then horseradish peroxidase-conjugated rabbit anti-goat IgG. Lanes 1 and 16, protein standards; lanes 2 and 15, human plasma LCAT (2 pg); lane 3, untransfected COS-6 cells; lane 4, COS-6 cells transfected with pSG5 plasmid; lane 5, wild-type LCAT pSG5; lane 6, $Cys^{31} \rightarrow Gly$; lane 7, $Cys^{184} \rightarrow Gly$; lane 8, $Cys^{31,184} \rightarrow Gly$; lanes 9 and 14, $Cys^{50} \rightarrow Gly$; lane 10, $Cys^{356} \rightarrow Gly$; lane 11, $Cys^{50,356} \rightarrow Gly$; lane 12, $Cys^{74} \rightarrow Gly$; lane 13, $Cys^{313} \rightarrow Gly$.

Table III: Specific Activities of Mutant and Wild-Type LCAT[a]

mutant	activity[b] (nmol mL^{-1}h^{-1})	concn (μg/mL)	sp act. (nmol/μg)	% inhibition by DTNB
wild type	10.5 ± 0.4	2.4 ± 0.1	4.4 ± 0.2	98
$Cys^{31} \rightarrow Gly$	3.7 ± 0.7	1.6 ± 0.1	2.7 ± 0.4	50
$Cys^{184} \rightarrow Gly$	5.5 ± 0.6	1.4 ± 0.1	3.0 ± 0.4	51
$Cys^{31,184} \rightarrow Gly$	4.3 ± 0.4	1.5 ± 0.3	2.3 ± 0.3	43
$Cys^{50} \rightarrow Gly$	0.24 ± 0.02	0.7 ± 0.01	0.34 ± 0.001	nd[c]
$Cys^{74} \rightarrow Gly$	0.11 ± 0.02	0.62 ± 0.03	0.18 ± 0.01	nd
$Cys^{313} \rightarrow Gly$	0.16 ± 0.06	0.0	0.0	nd
$Cys^{356} \rightarrow Gly$	0.13 ± 0.02	0.0	0.0	nd
$Cys^{50,365} \rightarrow Gly$	0.17 ± 0.03	0.0	0.0	nd

[a] Triplicate experiments based on direct counting; control tests showed that the amount of pSG5 sequences were nearly the same in all transfectants. Values are reported as means ± SD. [b] Nanomoles of cholesteryl ester formed per milliliter of medium per hour. [c] nd, not determined.

pSG5 were transfected into COS-6 cells. After incubation for 48–72 h, the LCAT in the media were screened for activity using R-HDL as the substrate. No measurable LCAT activity was found in the media collected from the COS-6 cells that were not transfected nor from COS-6 cells containing the pSG5 plasmids lacking the LCAT insert. The media from the cells transfected with the wild-type DNA exhibited the highest specific activity, while the specific activity of the media from the transfections in which single and double substitution of glycine for the free cysteines (Cys^{31}, Cys^{184}) was slightly lower (Table III). Addition of the sulfhydryl reagent DTNB to the test media totally inhibited the activity of the wild-type LCAT, whereas the activity of the the mutants in which one or both of the free cysteines were replaced by glycine was reduced by about 50%. In contrast, when glycine was substituted for Cys^{50} or Cys^{74}, which are linked together via a disulfide bond in wild-type LCAT, the specific activity was nil.

Other experiments compared the specificty of wild-type LCAT with those of the mutants in which one or both of the free cysteines were replaced by glycine. On the basis of substrate saturation curves (Figure 8) the apparent K_m and V_{max} were calculated (Table IV). With both POPC and DAPC, the apparent K_m was higher with the wild-type enzyme than with any of the mutants. These data showed that wild-type LCAT was much more active against substrates that contain POPC than against those composed of DAPC. As a consequence, the ratio of V_{max} found with POPC to that

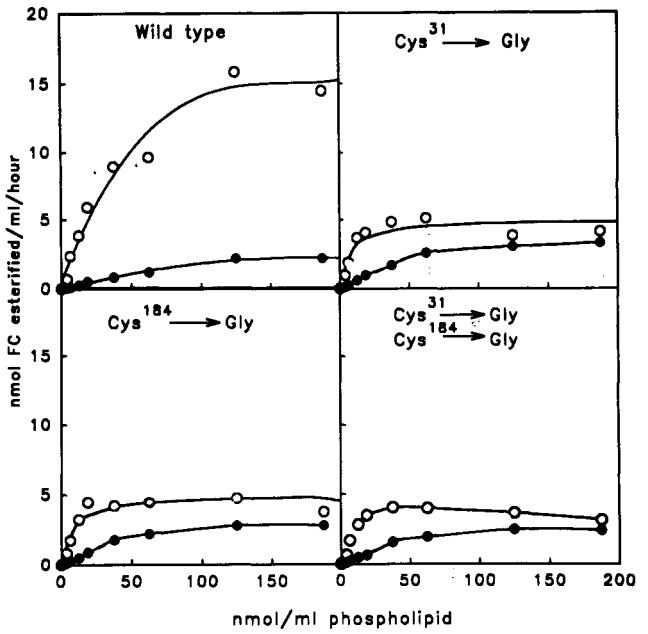

FIGURE 8: Substrate saturation curves for LCAT. A, wild type; B, $Cys^{31} \rightarrow Gly$; C, $Cys^{184} \rightarrow Gly$; D, $Cys^{31,184} \rightarrow Gly$. PC/cholesterol/apoA-I ratios used were 100:2:1 (M/M/M). The PCs used as substrates were POPC (○) and DAPC (●).

Table IV: Kinetic Parameters[a] for LCAT Mutants

	POPC		DAPC		
mutant	$K_m \times 10^6$ (M)	V_{max} (nmol mL^{-1} h^{-1})	$K_m \times 10^5$ (M)	V_{max} (nmol mL^{-1} h^{-1})	R[b]
wild type	40	16	13	2	8.0
$Cys^{31} \rightarrow Gly$	7	4	6	3	1.3
$Cys^{184} \rightarrow Gly$	8	4	5	3	1.3
$Cys^{31,184} \rightarrow Gly$	7	4	6	2.5	1.6

[a] K_m is expressed in molarity of POPC or DAPC; V_{max} is in nanomoles of cholesteryl ester formed per milliliter per hour. Error in V_{max} is ±15% or less and in K_m ±30%. [b] R represents the ratio of the V_{max} for LCAT-catalyzed cholesteryl ester production with POPC to that found with DAPC.

measured with DAPC was 8. The kinetic parameters for all three mutants were very similar, and R, the ratios of V_{max} measured using POPC to that using DAPC as the substrate, likewise were very similar. Substrates were also prepared in the same way without the addition of apoA-I. This gave rise to large vesicles (Rhoden & Goldin, 1979), which were used without further purification or characterization. When the assays were conducted without the addition of apoA-I, the activities were nearly an order of magnitude lower but the value of R remained nearly the same (data not shown).

DISCUSSION

LCAT Synthesis and Secretion by COS-6 Cells. Wild-type LCAT and eight different mutants in which one or two cysteine residues have been replaced by glycine were transfected into COS-6 cells. According to PCR and Northern blot analyses, all nine transfectants were positive for LCAT mRNA. Moreover, according to Western blot analysis, all nine transfectants contained intracellular LCAT mass. However, when the media were analyzed for mass and activity, there were some major differences among the transfectants. Little or no enzyme was secreted from those in which one or more of the cysteines that form disulfide bonds in LCAT ($Cys^{50,74,313,356}$) were replaced by glycines. A small amount of LCAT mass (30% of wild type) was found in the media of the cells containing the $Cys^{50} \rightarrow Gly$ or $Cys^{74} \rightarrow Gly$ mutation; no detectable mass was found in the media of cells that contained the $Cys^{313} \rightarrow Gly$ or $Cys^{356} \rightarrow Gly$ mutations. Polypeptide conformation is thought to be one determinant of whether or not a protein is secretion-competent (Braakman et al., 1992). Within the endoplasmic reticulum there are proteins that remodel proteins to the correct conformation, and enzymes can degrade proteins that do not have the correct conformation (Hurtley et al., 1989). In general, proteins are conformationally flexible and various parts of the polypeptide chain can undergo small translational movements with respect to the rest of the protein at relatively low energy expense. Disulfide bonds between cysteines immobilize a part of the protein and thereby prevent certain translational movements. In this way, disulfide bonds can stabilize protein structures that are secretion-competent. The decreases in LCAT secretion are the same irrespective of which cysteine in a given pair that composes a given disulfide link is replaced. Replacement of either of the cysteines in the Cys^{50}–Cys^{74} link results in comparable reductions in secretion. Similarly, replacement of either Cys^{313} or Cys^{356} with glycine results in a total inhibition of LCAT secretion. These results suggest that the decreases in secretion are due to a loss of the disulfide link and not to some other effect that is a function of the differences in the structures of glycine and cysteine. Since the greatest inhibition of secretion was found with mutations involving Cys^{313} and Cys^{356}, we conclude that the disulfide link at this site is required for secretion.

Structure–Activity Correlations. Early studies have shown that DTNB, a sulfhydryl-reactive reagent, inhibits LCAT activity (Aron et al., 1978). This could occur through a steric effect in which the binding of DTNB decreases the accessibility of substrate to the active-site region. If this were the case, substitution of another amino acid for the Cys^{31} or Cys^{184} might relieve the DTNB effect. Alternatively, these two amino acids may be involved in the actual acyltransferase reaction and may transfer an acyl group from the donor to cholesterol through a thioester intermediate that is formed with one or both free cysteines (Jauhianan & Dolphin, 1986). Our data support the former mechanism. The most compelling argument is provided by the data on the double mutant. Replacement of both cysteines with glycine reduces the activity to about half of that of the wild type. Therefore, the free cysteines at residues 31 or 184 may enhance LCAT activity against some substrates but are not necessary for activity; moreover, an acyl-cysteine intermediate is not required for the acyltransferase activity that characterizes LCAT. These data confirm the report of Francone and Fielding (1991), who found similar results in LCAT mutants that were expressed in Chinese hamster ovary cells. However, we also found that DTNB inhibited the activity of the LCAT mutant in which both Cys^{31} and Cys^{184} were replaced by glycine. This finding does not alter our conclusion that these two residues are not directly involved in the catalytic step but rather that the accessibility of the active site is sterically hindered when Cys^{31} and Cys^{184} are covalently modified by DTNB.

Substrate Specificity. Enzymatic reactions that occur at interfaces can be characterized by a K_m and a V_{max}. However, the mechanistic interpretation of these kinetic constants has been a matter of some controversy; for example, it is currently not possible to determine whether the K_m of a reaction with an insoluble substrate represents binding to a single substrate molecule or binding to an interface (Verger & de Hass, 1977). However, it is still possible to compare the effects of various mutations on the measured kinetic constants of LCAT without

assigning them to a specific kinetic step. Human plasma LCAT is typically less reactive against highly unsaturated acyl donors than against the saturated straight-chained analogs. A higher specificity of plasma LCAT for POPC relative to DAPC is observed both in mixtures of natural lipids (Subbaiah et al., 1992; Ueno et al., 1986) and in inert matrices of ether phosphatidylcholines (Pownall et al., 1985a,b; Grove & Pownall, 1991). Therefore, the differences, if any, in the interfacial properties of POPC and DAPC do not appear to affect the relative values of V_{max} for POPC and DAPC. As expected, the molecular specificity and specific activity of wild-type LCAT secreted by COS-6 cells is similar to that reported for isolated human plasma LCAT; the activity with DAPC is lower than that found for POPC and the specific activity against POPC is lower than that found by Chen and Albers (1985) but higher than that expressed by CHO cells (Francone & Fielding, 1991), both of whom used substrates that were different from those used in this study.

In contrast, with mutants in which one or both of the free cysteines are replaced by glycine, the magnitudes of V_{max} against POPC and DAPC are similar. Two factors might explain these differences. These are (1) intrinsic differences in the chemical nature of the active-site region and (2) differences in accessibility of the active site. The small difference (small R) between the reactivities of the mutants against DAPC suggests that much of the selectivity of the enzyme due to differences in the bulkiness of the substrates has been relieved by replacement of the cysteines with glycine. However, the similarity of V_{max} of the free cysteine mutants against POPC and DAPC is not due to the increase of the activity of these toward the bulkier substrate. Rather, it is due to the decrease of the activity of the mutants against POPC. Thus, the presence of free cysteines is not responsible for the low activity of the wild type against DAPC. In support of this, rat LCAT, which contains two free cysteines, catalyzes the transfer of bulky acyl chains relatively efficiently (Pownall et al., 1985b). By contrast, the high reactivity of wild-type LCAT, versus the mutants, against POPC, which is not sterically excluded from the active-site region, must be due to some effects of both free cysteines on the catalytic step. One might speculate that the free cysteines are aligned in such a way that a polarization and eventual cleavage of the acyl group is facilitated. Thus, the molecular specificity of LCAT may be regulated by the bulkiness of both the substrates and the amino acids that are within or adjacent to the active site. However, there are probably other amino acids that can affect the insertion of substrate molecules into the active site.

The K_m's for the reaction of LCAT mutants are lower than those of the wild type. The effects of mutations on K_m are more difficult to interpret because it is not known what step in the reaction pathway the binding constant represents. One explanation is that K_m represents the affinity of the enzyme for the lipid interface. If this is the case, one would conclude that the Cys → Gly mutants have a greater affinity for the substrate. Alternatively, the K_m may represent the association of the enzyme with a single acyl donor molecule after the enzyme binds to the surface. In this instance, the lower K_m would represent a higher affinity of the enzyme for individual phospholipid molecules. Additional kinetic and binding studies with purified LCAT and LCAT mutants will be required to identify the molecular determinants of specificity and the mechanistic meaning of the observed kinetic constants.

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