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Accelerated Publications

Structure–Function Relationships in Human Lecithin:Cholesterol Acyltransferase. Site-Directed Mutagenesis at Serine Residues 181 and 216†

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ABSTRACT: The functions of serine residues at positions 181 and 216 of human plasma lecithin:cholesterol acyltransferase have been studied by site-directed mutagenesis. The serine residue at either site was replaced by alanine, glycine, or threonine in LCAT secreted from stably transfected CHO cells. All substitutions at position 181 gave rise to an enzyme product that was normally secreted but had no detectable catalytic activity. On the other hand, all substitutions at position 216 gave active products, whose activity was fully inhibitable by the serine esterase inhibitor diisopropyl fluorophosphate (DFP). A secondary (although not direct) role for serine-216 was indicated by a 14-fold increase in catalytic rate when this residue was substituted by alanine. Sequence comparison with other lipases suggests that serine-216 may be at or near the hinge of a helical flap displaced following substrate binding. These data strengthen the structural–functional relationship between LCAT and other lipases.

Lecithin:cholesterol acyltransferase (LCAT;[1] phosphatidylcholine–sterol *O*-acyltransferase, EC 2.3.1.43) catalyzes the synthesis of cholesteryl esters from free cholesterol originating in cell membranes or plasma lipoproteins. LCAT drives the "reverse transport" of cholesterol from cell membranes to plasma and may play an important role in regulating cellular cholesterol accumulation (Fielding & Fielding, 1981; Davis et al., 1982). In the absence of cholesterol, LCAT acts as a phospholipase, generating free fatty acids in place of cholesteryl esters (Aron et al., 1978). The enzyme has been cloned and sequenced (McLean et al., 1986) and shares with other lipases several regions of local sequence similarity.

Both acyltransferase and phospholipase activities of LCAT are blocked by classical inhibitors of serine esterases such as diisopropyl fluorophosphate (DFP) and diethyl *p*-nitrophenyl phosphate (E-600) (Glomset, 1968; Aron et al., 1978; Jauhiainen & Dolphin, 1986). However, as in the case of other lipases, attempts to assign the active-site serine in the primary sequence by chemical modification have given inconclusive results. In pancreatic lipase, E-600 inhibited lipase activity with long-chain glycerides but not esterase activity, while DFP reacted with a tyrosine rather than a serine residue (Maylie et al., 1972). These data were interpreted to indicate the

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[1] Abbreviations: LCAT, lecithin:cholesterol acyltransferase; DFP, diisopropyl fluorophosphate. Amino acid sequences are described using the single-letter code. Subscripts refer to amino acid position within the primary sequence of the mature protein.

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presence in lipases of distinct interfacial binding and active sites, the latter containing the sequence -GGS- (Maranganore & Heinrickson, 1986). On the other hand, recent crystallographic data suggested that the active-site serine of pancreatic lipase lay within the interfacial binding site, even though this residue is buried in the crystal structure (Winkler et al., 1990).

In LCAT, similarly conflicting data have been reported. Reaction of human LCAT with [^{3}H]DFP led to the recovery of label at serine-181 (Farooqui et al., 1988), but a similar study with porcine LCAT identified the major part of the same label at a residue corresponding to human serine-216 (Park et al., 1987). Both these residues fall within short sequences that are well conserved among serine hydrolases including LCAT (Fielding, 1990) and have the same sequence as the two alternative active-site sequences proposed for pancreatic lipase (Maranganore & Heinrickson, 1986; Winkler et al., 1990).

Site-directed mutagenesis offers an alternative approach to the identification of essential residues in the primary sequence of enzymes. It has been used previously to demonstrate that the free cysteine residues of LCAT are not required for cholesteryl ester synthesis (Francone & Fielding, 1991). In the present investigation, human LCAT expressed in transfected mammalian cells has been modified by replacing the serine residues at either position 181 or 216 by residues which do not form acylated intermediates in catalysis.

Experimental Procedures

Site-Directed Mutagenesis. A full-length human LCAT cDNA extending from 8 bases before the ATG start codon to 5 bases after the TAA stop codon was isolated from human hepatoblastoma (Hep G2) cells (Francone & Fielding, 1991) and cloned in a pTZ18 phagemid vector. MV1190 *Escherichia coli* cells were transfected and single-stranded DNA was obtained by infection with M13K07 helper phage (Bio-Rad, Richmond, CA). Synthetic oligonucleotides 30 bases long (Operon, Alameda, CA) were 5′-phosphorylated and annealed with single-stranded substrate. To mutagenize serine-181 to alanine, the primer used was 5′-GTG TAG ACA GCC GAG *GGC* GTG GCC AAT GAG-3′, complementary to GCC (alanine) instead of AGC (serine) as in the LCAT wild-type cDNA sequence. To mutagenize serine-181 to glycine or threonine, the same primer was used except that the triplet corresponding to serine-181 was replaced by GCC (complementary to glycine, GGC) or GGT (complementary to threonine, ACC). The serine-216 to alanine mutant was obtained using the primer 5′-CAG CAT GGG CTT GAT *GGC* GCC ACC CCA GGG AGC-3′, complementary to GCC instead of TCC (serine) as in the wild type cDNA sequence. Serine-216 to glycine or threonine mutants were synthesized with the same primer except that the triplet corresponding to serine-216 was *GCC* (complementary to glycine, GGC) or *GGT* (complementary to threonine, ACC). Oligonucleotide-directed mutagenesis reactions were carried out as described by Taylor et al. (1985). Mutant and wild-type LCAT cDNA were used to transform *E. coli* TG1 cells. Clones containing the desired mutations were identified by dideoxynucleotide sequencing (Sanger et al., 1977).

Transfection and Expression of Human LCAT cDNA. Wild-type and mutant full-length LCAT cDNA species were isolated from pTZ18 LCAT cDNA by restriction enzyme digestion and cloned in the unique *Hin*dIII/*Bgl*II sites of the pSV2 *dhfr* plasmid replacing the coding sequence for the mouse dihydrofolate reductase (*dhfr*) (American Type Culture Collection ATCC 37146). The resulting wild-type and mutant pSV2hLCAT were purified by alkaline hydrolysis (Birnboim, 1983) and by $CsCl_2$ gradient centrifugation and were used to transfect Chinese hamster ovary cells (CHO) (DXB11 line) deficient in the *dhfr* gene. The cells were grown in F-12 medium supplemented with 10% fetal calf serum and gentamycin. The pSV2hLCAT plasmids were cotransfected together with the pSV2 *dhfr* plasmid into CHO cells in a ratio of 20:1 by calcium phosphate mediated transfection (Graham & van der Eb, 1973; Chen & Okayama, 1987). Clones expressing the *dhfr* gene were selected by growing the cells for 10-15 days in modified Eagle's medium (MEM) without nucleosides and containing 10% dialyzed fetal calf serum and gentamycin. Between 15 and 20 clones were then screened for the production of human LCAT by solid-phase immunoassay (Francone & Fielding, 1991) and functional activity (Aron et al., 1978). As reported previously, about 30% of the clones expressed the LCAT protein.

Determination of LCAT Mass and Activity. Clones secreting LCAT protein were grown in T25 flasks in MEM without nucleosides and containing 10% dialyzed fetal calf serum and gentamycin. When the cells reached 90–95% confluency, the flasks were washed with MEM medium + gentamycin without nucleosides or serum and then incubated for 24 h at 37 °C in the same medium containing 4% (v/v) serum substitute (Ultroser G, Gibco). LCAT mass was determined by solid-phase immunoassay on nitrocellulose membranes (Sartorius, West Coast Scientific, Hayward, CA) using affinity-purified rabbit anti-LCAT antibody (Francone et al., 1989) and ^{125}I-labeled goat anti-rabbit IgG second antibody as previously described (Francone & Fielding, 1991). Membrane-bound radioactivity was measured by γ scintillation spectrometry and was proportional to LCAT mass up to 150 ng.

LCAT activity was determined by the rate of production of labeled cholesteryl esters from unilamellar vesicles (Hamilton et al., 1980) containing [1,2-^{3}H]cholesterol (New England Nuclear, Boston, MA) [(1–1.2) × 10^5 cpm/μg] and egg lecithin (1/8 w/w) activated to form discoidal synthetic lipoprotein recombinants with human apolipoprotein A-I (Fielding et al., 1972). Labeled cholesteryl ester was separated from free cholesterol by thin-layer chromatography on silica-gel layers developed in hexane/diethyl ether/acetic acid (83:16:1 v/v/v), followed by liquid scintillation spectrometry. The production of labeled cholesteryl ester was linear for at least 6 h at 37 °C. In some experiments LCAT activity was inhibited with DFP (Aldrich, Milwaukee, WI) (0.5 or 2.0 mM final concentration). After preincubation of inhibitor and cell culture medium at 25 °C for 30 min, LCAT activity was assayed as described above. LCAT rate as a function of substrate concentration was determined over the range 8–90 μM free cholesterol. The proportions of free cholesterol, egg lecithin, and apo A-I were 1:8:1 w/w/w (Aron et al., 1978).

Structure Predictions. Secondary structure prediction was carried out using protein analysis software (Wolf et al., 1988) based on the algorithms of Garnier et al. (1978) using a VAX 8650 mainframe computer.

Results

Wild-type LCAT secreted by CHO cells was indistinguishable from the native plasma enzyme in molecular weight (Francone & Fielding, 1991). The specific activity of the wild-type secreted enzyme (675 ± 87 pmol μg^{-1} h^{-1}) (n = 6) was comparable to that reported previously by this laboratory and others (Taramelli et al., 1990; Francone & Fielding, 1991; Collet & Fielding, 1991). Specific activities obtained from LCAT secreted from transfected cells are generally lower than those reported for LCAT protein isolated from plasma [e.g.,

Table I: Enzyme Activity, Immunoreactive Mass, and Specific Activity of Wild-Type and Mutant LCAT Species

species	LCAT activity[a] (pmol mL^{-1} h^{-1})	LCAT mass (μg mL^{-1})	sp act. (pmol μg^{-1} h^{-1})
wild type	29.7 ± 4.5	0.044 ± 0.003	675 ± 87
alanine-181	0	0.048 ± 0.009	0
glycine-181	0	0.041 ± 0.007	0
threonine-181	0	0.043 ± 0.014	0
alanine-216	241.7 ± 16.7[b]	0.043 ± 0.007	5675 ± 601[b]
glycine-216	1.9 ± 0.3[b]	0.038 ± 0.018	52 ± 0.3[b]
threonine-216	18.3 ± 1.6[c]	0.037 ± 0.006	490 ● 8.0

[a] Enzyme activity was determined in the presence of apo A-I as described under Experimental Procedures. Values shown are means ± 1 SEM for 5–7 different experiments. [b] $p < 0.001$. [c] $p < 0.05$.

Aron et al. (1978)] because of the lower stability of dilute enzyme preparations, differences in the carbohydrate moiety, or other factors. However, the transfected cell system used here is clearly suited for the comparison of the activities of expressed LCAT species of wild-type and mutant sequence.

As shown in Table I, none of the modifications made in the LCAT sequence had a significant effect on the rate of secretion of LCAT protein. These data indicate that neither of these regions is likely to be involved in the recognition of glycosylated LCAT by the secretory pathway (Collet & Fielding, 1991). LCAT activities in the medium of cells transfected with wild-type or mutant LCAT cDNA are shown in Table I. When serine-181 was substituted by alanine, glycine, or threonine, the secreted LCAT had no detectable catalytic activity, under conditions where 1% of wild-type activity would have been detected. As also shown in Table I, when serine-216 was replaced by alanine, glycine, or threonine, all the mutants retained significant LCAT activity. This finding excludes the possibility that serine-216 has an active-site function in human LCAT. However, significantly different specific activities were obtained with the different mutants at this position (Table I). Substitution of serine by threonine had little effect on activity. Substitution of serine-216 by glycine reduced activity by >90%. On the other hand, substitution of serine-216 by alanine significantly increased LCAT specific activity above wild-type levels.

To investigate the mechanism of this effect further, the kinetic properties of the recombinant serine-216 to alanine mutant and wild-type enzymes were compared. The apparent Michaelis constants (K_m) were 95.3 ± 23.3 μM ($n = 4$) and 155.1 ± 25.2 μM free cholesterol for the wild type and the serine-216 to alanine mutant, respectively ($n = 4$, $p > 0.05$). However, the maximal reaction velocity (V_{max}) was increased about 14-fold (from 47.8 ± 13.5 to 677.2 ± 51.9 pmol of cholesteryl ester mL^{-1} h^{-1} for wild-type and serine-216 to alanine mutant LCAT, respectively) ($n = 4$, $p < 0.001$).

When the mutants at serine-216 and the wild-type LCAT were assayed in the presence of DFP, activity was in all cases almost completely inhibited. This indicates that the reaction of DFP, at least under the conditions used in this study, did not occur at position 216 (Table II).

Table II: Effect of DFP on Wild-Type and Serine-216 Mutant LCAT Species[a]

DFP (mM)	LCAT activity (pmol mL^{-1} h^{-1}) wild type	alanine-216	glycine-216	threonine-216
0	26.4 ± 4.4	283.6 ± 36.4	1.6 ± 0.3	18.5 ± 3.2
0.5	0.7 ± 0.5	4.5 ± 0.1	0.1 ± 0.1	5.1 ± 0.1
2.0	0.4 ± 0.3	0.3 ± 0.3	0.1 ± 0.1	0.1 ± 0.1

[a] Cell culture medium from clones expressing either wild-type or serine-216 mutant LCAT were preincubated at 25 °C for 30 min.

Discussion

LCAT has little overall sequence homology with other lipases, although it contains both the -GXSXG- and -GGS- motifs characteristic of many other lipases. Each has been identified as the serine component of an active-site triad (serine, histidine, aspartate) in LCAT and other lipases in different laboratories [review: Fielding (1990)]. The present study has used site-directed mutagenesis to demonstrate that substitution of position 216 with several different amino acids (alanine, glycine, threonine) leads to a mutant enzyme which retains LCAT activity. Serine-216 therefore cannot form part of the active site of LCAT, covalently modified during catalysis. It is not clear why under some conditions residue 216 was covalently modified by DFP (Park et al., 1987), although anomalous derivatization with this agent has been previously reported with other lipases.

The present study provides strong evidence that serine-181 of LCAT does form part of the active site of LCAT. None of the three mutants at this position had any detectable residual activity. While this might be a secondary effect of steric hindrance, several lines of evidence argue against this. First, mutagenesis at a neighboring residue (cysteine-184) was without effect on catalytic rate (Francone & Fielding, 1991). Second, the substitutions made at position 181 generated LCAT species secreted normally but without detectable activity, although these do not involve significant changes in side-chain volume or charge or predicted secondary structure. Finally, the sequence -GHS$_{181}$LG- is identical to the sequence containing an active-site serine as predicted by crystallography in pancreatic lipase (Winkler et al., 1990) and to closely related sequences in lipoprotein lipase and hepatic triglyceride lipase (Wion et al., 1987; Komaromy et al., 1987), in which mutagenesis of the serine residue leads to loss of activity (Davis et al., 1990; Faustinella et al., 1991). In summary, the results of this study strongly suggest that the serine residue at position 181 forms part of the active site of human LCAT. This data, together with previous mutagenesis data arguing against a unique catalytic role for free cysteine residues in LCAT (Francone & Fielding, 1991), greatly strengthens the concept that LCAT has a structure and mechanism closely related to that of other lipases.

This study provides new evidence that the sequence surrounding serine-216 has nevertheless an important although indirect role in the catalysis mediated by LCAT. Substitution of this residue by alanine increased LCAT catalytic rate at saturation by 14-fold. [A comparable but smaller (3-fold) effect can be seen in hepatic triglyceride lipase (Davis et al., 1990).] Substitution of serine-216 by glycine in LCAT results in a greater than 90% inhibition of activity. This region therefore shows extraordinary sensitivity to modification, and addition of a single methylene group at this position results in a >100-fold increase in catalytic rate.

Each of the lipases discussed shows a common sequence in this region, whose consensus is -P$^{W}_{N}$GG$^{S}_{T}$XXP-. In LCAT, the substitution of an alanine residue at position 216 has a significant effect. In particular, the predicted turn preceding the sequence -PWGGS$_{216}$IKP- is lost. X-ray crystallography in the case of pancreatic lipase predicts that a similar sequence represents the beginning of a flexible helical "flap" which covers the active-site triad (including its serine residue) in the absence of substrate (Winkler et al., 1990). The increased catalytic rate of the alanine-216 mutant LCAT may indicate that this modification reduces the energy required to transform LCAT from a "closed" to an "open" structure involving the adjacent helical loop, if this structure is similarly present in

LCAT. The region around residue 216 may therefore play an important role in orienting substrate for effective catalysis in the LCAT reaction with individual substrate particles.

This study indicates that LCAT shows marked local structural and functional resemblance to other lipases. Such comparisons should assist in determining the detailed mechanism of this important enzyme of plasma cholesterol metabolism.

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