Structural and Functional Properties of Two Mutants of Lecithin-Cholesterol Acyltransferase (T123I and N228K)*

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Two naturally occurring mutants of human lecithin-cholesterol acyltransferase (LCAT), T123I and N228K, were expressed in COS-1 and Chinese hamster ovary cells, overproduced, and purified to homogeneity in order to study the structural and functional defects that lead to the LCAT deficiency phenotypes of these mutations. The mutants were expressed and secreted by transfected cells normally and had molecular weights and levels of glycosylation similar to wild type LCAT. The purified proteins (>98% purity) had almost indistinguishable structures and stabilities as determined by CD and fluorescence spectroscopy. Enzymatic activities and kinetic analysis of the pure enzyme forms showed that wild type LCAT and both mutants were reactive with the water-soluble substrate, p-nitrophenyl butyrate, indicating the presence of an intact core active site and catalytic triad. Both the T123I and N228K mutants had markedly depressed reactivity with reconstituted HDL (rHDL), but T123I retained activity with low density lipoprotein. To determine whether defective binding to rHDL was responsible for the low activity of both mutants with rHDL, the equilibrium binding constants were measured directly with isothermal titration calorimetry and surface plasmon resonance (SPR) methods. The results indicated that the affinities of the mutants for rHDL were only about 2-fold lower than the affinity of wild type LCAT ($K_d = 2.3 \times 10^{-7}$ M). Together, the activity and equilibrium binding results suggest that the T123I mutant is defective in activation by apolipoprotein A-I, and the N228K mutant has impaired binding of lipid substrate to the active site. In addition, the kinetic binding rate constants determined by the SPR method indicate that normal LCAT dissociates from rHDL, on average, after one catalytic cycle.

Lecithin-cholesterol acyltransferase (LCAT) plays a key role in the extracellular transport and metabolism of cholesterol. By esterifying cholesterol on lipoproteins, particularly on high density lipoproteins (HDL), LCAT promotes cholesterol efflux from peripheral cells, storage of cholesterol esters in lipoproteins, and transport of cholesterol to the liver and steroidogenic tissues for excretion or re-utilization.

Disruptions in the human LCAT gene result in one of two rare genetic disorders, familial LCAT deficiency (FLD) or fish-eye disease (FED). The biochemical and clinical phenotypes of carriers of FLD or FED are different. The first naturally occurring variant of LCAT associated with FLD was discovered by Norum and Gjone (1) in 1967, although the specific molecular defect in these patients was identified only in 1992 (2). To date, around 40 different mutations have been described that result in either one of these disorders (3). The mutations associated with FED are localized to exons 1, 4, and 6, whereas the mutations resulting in FLD are dispersed throughout the LCAT gene (4).

FLD patients exhibit a wide variety of clinical symptoms that include massive corneal opacification, anemia, proteinuria, and renal disease (5). These clinical manifestations are due to elevated cholesterol and phospholipid levels in the affected cells and tissues. FLD is generally not accompanied by atherosclerosis, although a few cases have been reported (4). The biochemical characteristics of FLD include the virtual absence of plasma LCAT activity and severely reduced cholesterol ester and plasma LCAT concentrations (3). In addition, all classes of lipoproteins exhibit abnormal profiles, have altered morphologies, and contain high proportions of unesterified cholesterol and phosphatidylycholine (PC). The concentration of HDL cholesterol is markedly reduced and is accompanied by an abnormal distribution of HDL subclasses (4).

In FED patients, the only clinical signs are the dense age-dependent corneal opacification (3). No other clinical symptoms are evident in FED. A distinguishing feature of this disorder is the presence of an apparently normal ability of LCAT to esterify cholesterol in plasma, resulting in a normal plasma cholesterol ester content. Other characteristics of FED include HDL deficiency and partial reduction in LCAT concentration (6). In vitro, LCAT from FED patients does not esterify cholesterol in HDL (α-migrating lipoproteins) but retains its ability to esterify cholesterol in LDL (β-migrating lipoproteins). In contrast, LCAT from FLD patients does not esterify cholesterol in any lipoproteins (7). Although not observed previously, Kuivenhoven et al. (3) have recently reported that 7 out of 19 FED probands they followed suffered from premature coronary artery disease.

The fact that two distinct biochemical phenotypes arise from mutations within the same gene strongly suggests the presence of multiple functional domains in LCAT. In order to assess the molecular basis of these naturally occurring mutants, several of these mutants have been reconstructed and expressed in...
mammalian cells (8–10). Santamarina-Fojo and co-workers (9) reconstructed and expressed nine LCAT mutants associated with either FLD or FED in human embryonic kidney cells. Their results suggested that the functional domain mediating the phospholipase activity of LCAT was intact in these FLD and FED mutants but that possibly there was an alteration of structural regions involved in lipoprotein binding or in the trans-esterification reaction. Pownall and colleagues (8) expressed 11 FED or FLD constructs in COS-6 cells and found that FED mutants were more active with DLH than with HDL, whereas FLD mutants were, in general, less secretion-competent.

These in vitro expression studies of the natural mutants of LCAT (2, 8–10, 11) have addressed the changes in enzymatic activity associated with the gene defects. They confirm the functional abnormalities observed for the natural mutants but do not provide additional insights into the structural domains of the enzyme that determine the functions nor the molecular mechanisms involved. In this study we describe the reconstruction, in vitro expression, purification, and characterization of two natural mutants, Thr-123 → Ile (T123I) and Asn-228 → Lys (N228K). These two variants were chosen based on their high expression levels and reported substrate specificities (9). The T123I mutant, which results in the FED phenotype, has been shown to esterify cholesterol on LDL but not on HDL (10, 11). In contrast, the N228K is an FLD mutant that lacks activity with both HDL and LDL substrates (9, 12). These functional differences between the two mutants could be due to differences in binding to lipoprotein surfaces, altered activation by apolipoprotein A-I in HDL, or impaired binding of lipid substrate molecules at the active site of LCAT. In order to delineate the specific underlying causes of the defects, these mutants have been transiently expressed in COS-1 cells and, permanently, in CHO cells, and their enzymatic activity, structure, stability, and binding to lipoprotein surfaces have been investigated in detail. Two new methods to study directly the binding of LCAT to lipoproteins are introduced, isothermal titration calorimetry and the surface plasmon resonance method.

**EXPERIMENTAL PROCEDURES**

**Primer Design and PCR Mutagenesis**—The T123I and N228K mutants were constructed using the overlap extension method (13) and the pGEMLCAT (Promega, WI) vector as template. The oligonucleotides used to engineer the mutations were constructed by the Genetic Engineering Facility, University of Illinois (Urbana, IL). The sequences of the forward and reverse primers are as follows: for the T123I mutant: forward primer, 5′ AGG AGC AAC CAT GCT TCA GCC GGC TAC ACT GTC TGG CAG 3′; reverse primer, 5′ CTC CAG CAC TAT GAG CGA TTC GCA CCT GTC AAG CTT GCT CTT 3′. For the N228K mutant: forward primer, 5′ GTG GTC TGG GCT AGC GGT GAC AAA CAG GGC ATC 3′; reverse primer, 5′ GAT GGC CTG TTT GTC ACC GCT AGC CAA GAC CAG CAT 3′. The regions underlined represent the codon changes used to introduce the respective mutations. The thermal cycling conditions were used as described previously (14). After verifying the presence of the mutation, the mutant LCAT was subcloned into the expression vector (16). The additional subcloning step was performed to minimize the region to be sequenced. The PCR was carried out using the full-length LCAT cDNA, but by subcloning only the region around the mutation, scanning of the PCR was carried out using the full-length LCAT cDNA, after subcloning into the expression vector (16). The expression vectors, pSVDNA1 and pSVLCAT (Kozak) (15), were used as negative and positive controls, respectively. For transfection, 2.5 μg of DNA were introduced into triple 100-mm dishes containing 106 cells each. After 24 h, the transfected cells were placed in serum-free Opti-MEM I medium (Life Technologies, Inc.), and the cells and medium were harvested 48 h later. The media containing the secreted LCAT were collected and stored at −70 °C until analysis. The cell lysates and supernatant were obtained by freeze-thawing in lysis buffer. The composition of the lysis buffer was 50 mM Tris-HCl, pH 7.6, 1% Nonidet, 5 mM EDTA, 0.5% sodium deoxycholate. The lysates were also stored at −70 °C.

**Western Blot Analysis**—The expression of the T123I and N228K mutants was analyzed by Western blotting using the enhanced chemiluminescence kit (ECL) (Amersham Pharmacia Biotech) for detection and quantitation of LCAT mass as described previously (14, 17). The proteins in the concentrated cell media and cell lysates were separated on 10% SDS-PAGE gels and transferred onto a nitrocellulose membrane, and the LCAT bands were visualized using a modification of the ECL Western blotting procedure.

**Enzyme Activity Assays and Kinetics**—The enzymatic activities of the negative control, wild type, and the mutants of LCAT in the cell media were measured using reconstituted HDL (rHDL), LDL, and p-nitrophe- nyl butyrate (PNPB) as substrates. LDL and rHDL were prepared and radiolaabeled with [14C]cholesterol as described previously (18–20). The percent conversion of [14C]cholesterol to cholesterol esters was used to calculate the acyltransferase reaction velocities of LCAT with rHDL and LDL substrates. The esterase activity of the cell media with PNPB was measured by monitoring the formation of p-nitrophenoxy ion (PNP−) as detected by an increase in absorbance at 400 nm. The initial velocity in nmol PNP− formed per min was calculated from a standard curve of A400 versus [PNP−] (21).

In order to obtain apparent kinetic parameters for those enzymatic reactions that gave significant reaction rates, the assays were also performed at varying substrate concentrations using purified LCAT mutants and wild type LCAT. For rHDL, final substrate concentrations of 1.4 × 10−7 to 2.6 × 10−6 M apoA-I were used, and the activity at each substrate concentration was measured in duplicate; 12.8 ng of LCAT (wild type or mutants) was used for each reaction in a total volume of 0.5 ml. For LDL, the assay mixtures were set up using increasing free cholesterol concentrations of 2.2 × 10−5 to 3.96 × 10−5 M, and 640 ng of LCAT were used for each reaction.

For obtaining kinetic parameters using the water-soluble substrate, PNPB, the hydrolysis rate of PNPB was monitored at substrate concentrations of 1.0 × 10−5 to 8.0 × 10−4 M, following the procedure of Bonelli and Jonas (21). The reaction mixtures contained 3 μg/ml LCAT (wild type or mutant), the appropriate concentration of PNPB prepared in acetonitrile (5% acetonitrile, v/v), and buffer (10 mM Tris·HCl, 0.15 mM NaCl, 0.1 mM EDTA, and 1 mM NaN3, pH 7.4) in a total volume of 1 ml. The kinetics of the reactions were monitored at 37 °C by measuring absorbance at 400 nm.

The phospholipase reaction of LCAT and the mutant LCAT forms was measured using rHDL particles, containing egg phosphatidylcholine (egg-PC), [3H]dipalmitoyl-phosphatidylcholine (DPNPC), and apoA-I in a 80:20:1 ratio of 100:PC:DPPC. The reaction mixtures were identical to those used in the acyltransferase reactions with rHDL, but 256 ng of enzyme were used in each sample. The range of apoA-I concentrations in rHDL was from 3.0 × 10−7 to 2.8 × 10−6 M. After the usual reaction, the [3H]-fatty acid product of the reaction was separated from the [3H]DPNPC reactant by instant thin layer chromatography (Gelman Sciences) developed in 80:20:1, petroleum ether/ethyl ether/acetic acid (v/v). The apparent kinetic parameters were obtained from Lineweaver-Burk plots of the data.

**Stable Expression and Purification of LCAT Wild Type and Mutants**—Stable dhfr CHO cell lines expressing wild type and mutant LCATs were prepared using the CaPO4 method (16) as described previously (22). The stable clones obtained after selection were amplified using methotrexate (23) in order to obtain cell lines producing high levels of the wild type and mutant LCATs. After the stable transfectants were established, they were grown in roller bottles (Corning) in order to increase the surface area for cell attachment. The harvested media containing the secreted proteins (wild type or mutant) were filtered and passed over a phenyl-Sepharose CL-4B column (1 × 20 cm) (Amersham Pharmacia Biotech), followed by elution through an acetonitrile gradient column (1 × 10 cm) (80:20:1, petroleum ether/ethyl ether/acetic acid). The fractions containing LCAT were collected and stored at concentrations of approximately 0.2 mg/ml under N2 at 4 °C to prevent self-association and oxidation of the proteins (22).

**N-Terminal Sequencing of Wild type and Mutants**—250 picomoles of the wild type LCAT and the T123I and N228K mutants were submitted to the Sequencing Department of the Departamento de Biologia, Istituto di Biologia Molecolare, Pisa, Italy. The sequencing reaction mixtures containing the secreted LCAT were collected and stored at 37 °C by measuring absorbance at 400 nm.
for N-terminal sequencing to the Biotechnology Center, University of Illinois (Urbana, IL) to verify whether the N termini of the proteins were intact. Sequence analysis was carried out by Edman degradation of the first 10 residues on an Applied Biosystems model 477A instrument.

**Circular Dichroism (CD)—** Spectra were recorded at 25 °C for the wild type and mutant proteins (0.06–0.08 mg/ml) using a water-jacketed CD cuvette of a 1-mm path length (Helmer) on a Jasco J-720 spectropolarimeter (Laboratory for Fluorescence Dynamics, University of Illinois, Urbana, IL). The secondary structure content of the proteins was estimated using the K2d program (24).

**Fluorescence Spectroscopy—** The intrinsic tryptophan fluorescence emission spectra for the wild type and mutants were recorded using the Fluoromax-2 spectrofluorometer (Laboratory for Fluorescence Dynamics, University of Illinois, Urbana, IL). Protein concentrations of 0.05 mg/ml were used for the measurements.

Denaturation reactions were set up with 0.05 mg/ml LCAT (wild type or mutant) and stepwise increasing concentrations of guanidine hydrochloride (GdnHCl) (0.5 M increments) up to 6.0 M. It was determined that equilibrium was attained in 30 min at each GdnHCl concentration. The wavelength of maximum fluorescence obtained at each concentration of GdnHCl was used to calculate the apparent fraction unfolded. Values of ΔGΔHΔm, the free energy change of denaturation in the absence of GdnHCl, m, the cooperativity of the transition, and the midpoint of denaturation (GdnHCl)m were obtained as described previously (22).

**Isothermal Titration Calorimetry—** Titrations of the wild type and LCAT mutants with rHDL along with the appropriate controls were performed at 25 °C using an MCS isothermal titration calorimeter from Microcal Inc. (Northampton, MA). The reactant (1.5 μM wild type or mutant LCAT) was placed in the cell (1.4 ml), and the injectant (65 μM rHDL) was introduced into the calorimeter in 10-μl increments spaced 4 min apart. The injection syringe rotated at a speed of 600 rpm throughout the experiment to facilitate mixing of the reaction components. The peaks generated were integrated using the ORIGIN software (Microcal Inc.), and the values in microcalories were plotted against the molar ratio of injectant to reactant in the cell. The binding curve was obtained by nonlinear least squares fit for one set of sites carried out by the ORIGIN program. The fit gave values for the equilibrium association constant, K, from which values of the dissociation constant, Kd, were obtained. The secondary structure content of the proteins was estimated using the K2d program (24).

**Surface Plasmon Resonance Method—** The binding constants of wild type LCAT and the mutants to rHDL were also measured by the surface plasmon resonance method (25) using a BIAcore 2000 instrument (Biacore, Inc., Piscataway, NJ). The rHDL particles were biotinylated by incorporating N-biotinyl phosphatidylethanolamine along with the egg-PC and cholesterol matrix lipids during the reconstitution of the rHDL particles which contained 100:5:1, PC/cholesterol/ apoA-I (mol/mol). On average, 1.4 biotin groups were incorporated per particle; they had no significant effect on the enzymatic reaction of LCAT with the rHDL particles which contained 100:5:1, PC/cholesterol/ apoA-I (mol/mol). The concentration of rHDL along with the appropriate controls were performed at 25 °C using an MCS isothermal titration calorimeter from Microcal Inc. (Northampton, MA). The reactant (1.5 μM wild type or mutant LCAT) was placed in the cell (1.4 ml), and the injectant (65 μM rHDL) was introduced into the calorimeter in 10-μl increments spaced 4 min apart. The injection syringe rotated at a speed of 600 rpm throughout the experiment to facilitate mixing of the reaction components. The peaks generated were integrated using the ORIGIN software (Microcal Inc.), and the values in microcalories were plotted against the molar ratio of injectant to reactant in the cell. The binding curve was obtained by nonlinear least squares fit for one set of sites carried out by the ORIGIN program. The fit gave values for the equilibrium association constant, K, from which values of the dissociation constant, Kd, were obtained. The secondary structure content of the proteins was estimated using the K2d program (24).

**Results**

Immunoblots of concentrated cell media and intracellular contents of COS-1 cells, transfected with the pSVLCAT plasmid for wild type and the T123I and N228K mutants, are shown in Fig. 1. Clearly the levels of expression and secretion of the three forms of LCAT are very similar. Within cells, partially glycosylated proteins predominated, whereas in cell media the fully glycosylated proteins along with smaller amounts of unglycosylated forms are detected.

The cell media were used to examine the enzymatic activities of the expressed wild type and mutant forms of LCAT (Fig. 2). Both mutants have very low (5–10%) activity with rHDL but normal activity with PNPB. The T123I mutant has almost normal activity with LDL (87%), whereas the N228K mutant exhibits very low activity with LDL. These observations are entirely consistent with the previous reports on the enzymatic properties of these mutants (8–10) and the typical phenotypes observed in FED and FLD (3, 6).

In order to elucidate further the structural and functional defects in LCAT associated with these point mutations, they were permanently expressed, together with wild type LCAT, in dhfr CHO cells. All three forms were secreted at levels of up to 15 μg/ml and gave yields of 6 mg of 98% pure proteins per liter of culture media. Fig. 3 shows the SDS-PAGE gel of the purified proteins. N-terminal sequencing results showed that the wild type LCAT and the T123I and N228K mutants all had the same N-terminal sequences as follows: about 60% starting at the normal LCAT start site (FWLL . . .), with the remainder starting at residue 6 of the full sequence (VLFP . . .). This was observed previously in our laboratory.

![Fig. 1. Western blots of LCAT expression in COS-1 cells. A, intracellular proteins; B, secreted proteins in cell media. The lanes in each panel correspond to the following: lane 1, plasma LCAT control; lane 2, cells transfected with pSVDNA- as the negative control; lane 3, wild type LCAT; lane 4, T123I mutant; lane 5, N228K mutant.](http://www.jbc.org/content/journal/jbc/273/33/32563.full)

![Fig. 2. Enzymatic activities of COS-1 cell media (at equivalent enzyme concentrations) for wild type (WT) LCAT (▪), T123I mutant (○), N228K mutant (□), and negative control (●). The enzymatic activities with rHDL, LDL, and PNPB (in nanomoles of cholesterol ester formed per h or nanomoles of PNP· formed per min) are expressed as a percent of wild type LCAT activity. The high background in measurements with PNPB is subtracted out.](http://www.jbc.org/content/journal/jbc/273/33/32563.full)

![Fig. 3. SDS-PAGE (12.5%) of purified wild type LCAT (lane 2), T123I mutant LCAT (lane 3), and N228K mutant LCAT (lane 4).](http://www.jbc.org/content/journal/jbc/273/33/32563.full)
The origin of this micro-heterogeneity is not known; however, our previous studies demonstrated that it has no structural nor functional effects when compared with human plasma LCAT (22).

The enzyme kinetics of the purified LCAT forms were studied with the two interfacial substrates, rHDL and LDL, and the water-soluble substrate, PNPB. In addition to the acyltransferase reaction rates, the phospholipase reaction rates were measured with rHDL substrates. The results, expressed as apparent \( k_m \) and \( k_{cat} \) values, are summarized in Table I. Although the parameters for the different reactions cannot be easily compared because of the different substrates that were used, comparison of the mutant reaction with the corresponding reaction of wild type LCAT provides useful information. The kinetic parameters for the acyltransferase reaction of wild type LCAT with rHDL agree reasonably well with published results (22). Both mutants had low activities with rHDL that precluded measurement of the acyltransferase reaction kinetics. The phospholipase reactions with rHDL were measured using much higher enzyme concentrations (20-fold higher than for the acyltransferase reaction). In this case, the N228K mutant gave measurable results, which indicated a decrease in \( k_{cat} \) and an increase in apparent \( K_m \) relative to wild type LCAT, giving rise to an overall reactivity (\( k_{cat}/K_m \)) of 12%. The reaction with LDL required 50-fold higher enzyme levels than the acyltransferase reaction with rHDL. The T123I mutant had slightly decreased \( k_{cat} \) and a slightly increased apparent \( K_m \) relative to the wild type, resulting in a 55% relative reactivity (\( k_{cat}/K_m \)). If the \( K_m \) values are considered to be proportional to the equilibrium dissociation constants for the binding of the enzyme to the lipoprotein surfaces, then the increases in measured apparent \( K_m \) values for mutants suggest somewhat decreased binding affinities for the interfacial substrates. The esterase reaction with PNPB is comparable for the mutants of LCAT and both mutants; they correspond to only marginally decreased binding affinities for the interfacial substrates. The \( K_m \) values are identical which indicates equal binding affinity of the PNPB molecule for the active site of all three enzyme forms, whereas the small decreases in the \( k_{cat} \) for the mutants suggest a slightly decreased catalytic efficiency. The relative reactivities (\( k_{cat}/K_m \)) for the T123I and the N228K mutants are 81 and 57%, respectively, of the wild type enzyme. Nevertheless, it is evident that the active site architecture is preserved, to a large extent, in the LCAT mutants. Qualitatively, the relative activities of the mutants with respect to the wild type LCAT, calculated from the \( k_{cat}/K_m \) values in Table I, are similar to the relative activities shown in Fig. 2. The quantitative differences are due to the use of pure enzymes and to the precise determination of enzyme concentrations in the experiments reported in Table I.

Circular dichroism and intrinsic Trp fluorescence spectra of the enzyme forms are shown in Fig. 4. The overall shapes and amplitudes of the spectra of the mutants are similar to the spectra of wild type LCAT. They indicate that the content and nature of the secondary structures are similar, and the overall 3-dimensional organization of the proteins in the vicinity of their Trp residues is comparable for all three forms of LCAT. Deconvolution of the CD spectra using the method of Andrade et al. (24) gave the contents of secondary structure reported in Table II. The wavelength of maximum fluorescence of 340 nm, obtained for all three LCAT forms, indicates a relatively polar average environment for the Trp residues.

The structural similarity of the three forms of LCAT is further demonstrated by the identical denaturation profiles, shown in Fig. 5. Free energy changes of denaturation (\( \Delta G_{D_{H2O}} \)), cooperativities (\( m \)), and concentrations of GdnHCl at half-denaturation, calculated from the data in Fig. 5, are recorded in Table II. These values are essentially the same for wild type LCAT and both mutants; they correspond to only marginally

### Table I

| Mutants of LCAT | rHDL (acyltransferase reaction) | rHDL (phospholipase reaction) | LDL (acyltransferase reaction) | PNPB (esterase reaction) |
|----------------|--------------------------------|--------------------------------|--------------------------------|---------------------------|
|                | \( K_m \) (nmol CE/h/µg) | \( k_{cat} \) (nmol FA/h/µg) | \( K_m \) (nmol CE/h/µg) | \( k_{cat} \) (nmol PNP⁻/min/µg) |
| Wild type      | 6.1 × 10⁻⁷               | 11                            | 4.0 × 10⁻⁷              | 3.4 × 10⁻⁶               | 8.3 × 10⁻⁵           | 0.26 |
| N228K          | ND                        | ND                            | ND                        | ND                        | 7.5 × 10⁻⁷           | 13   |
| T123I          | 6.1 × 10⁻⁷               | 11                            | 4.0 × 10⁻⁷              | 3.4 × 10⁻⁶               | 8.3 × 10⁻⁵           | 0.26 |
| N228K          | ND                        | ND                            | ND                        | ND                        | 7.5 × 10⁻⁷           | 13   |

a With rHDL substrates the measurements were made as a function of apoA-I concentrations.
b With LDL substrates the measurements were made as a function of cholesterol concentration.

\( \Delta G_{D_{H2O}} \) and \( m \): measured with rHDL substrates. The results, expressed as \( \Delta G_{D_{H2O}} \) and \( m \), are due to the use of pure enzymes and to the precise determination of enzyme concentrations in the experiments reported in Table I.
stable proteins that have low cooperativity in their unfolding behavior.

A critical series of experiments in this study was the measurement of the binding constants of the three LCAT forms to the surface of rHDL particles by isothermal titration calorimetry (Fig. 6) and the surface plasmon resonance method (Fig. 7). Fig. 6 shows the heat changes upon addition of rHDL to the three LCAT solutions and the resulting titration curves. Albeit incomplete, because of experimental limitations (due to low maximum attainable LCAT concentration and small heat changes), the ITC results show binding of wild type LCAT and the N228K mutant to rHDL and give the $K_d$ values recorded in Table III. Apparently, for the T123I mutant there is no binding under the conditions of the experiment. However, it should be noted that the enthalpy changes observed with this mutant are even smaller than those detected with the other two LCAT forms, thus binding of the T123I mutant may occur but is undetectable by the ITC method.

In contrast to the ITC results, the SPR sensorgram, shown in Fig. 7, indicates binding of the T123I mutant, as well as the wild type LCAT and the N228K mutant, to the immobilized rHDL particles. Clearly, all three enzyme forms are capable of binding to lipoprotein surfaces, i.e. rHDL. The equilibrium $K_d$ values are listed in Table III and agree well with the values measured by ITC. Both mutants bind with about half the affinity of wild type LCAT to rHDL. As indicated above, the likely explanation for the discrepancy of the results for the T123I mutant between the two methods is that the small heat changes for this mutant do not reach the detection level required in the ITC measurements.

Finally, the SPR method provides the first experimental measure of the association and dissociation rate constants for the binding of LCAT and its mutants to a lipoprotein particle (rHDL) (see Table III). Although the association rate constants are the same for the three forms of LCAT, the dissociation rate constants are 0.05 s$^{-1}$ for wild type LCAT and increase to 0.08 and 0.12 s$^{-1}$ for the T123I and N228K mutants, respectively. By comparison, the catalytic turnover constant ($k_{cat}$) for wild type LCAT, reported in Table I, is 11 nmol of cholesterol ester/h/µg, which expressed in s$^{-1}$ and nmol of enzyme is 0.18 s$^{-1}$. Furthermore, this value has to be corrected for the temperature difference between the enzymatic activity measurements (at 37 °C) and the SPR measurements (at 25 °C). Using the temperature dependence of the reaction rates of LCAT with rHDL containing palmitoyloleoyl-PC, from our previous work ($E_a = 18.8$ kcal/mol) (26), the catalytic turnover constant becomes 0.049 s$^{-1}$. Thus the dissociation rate constant and the turnover constant are the same for wild type LCAT! This is the first indication that LCAT may dissociate after each catalytic cycle. Also, this result has important implications for the mechanism of action of LCAT. Compared with wild type LCAT, the mutant LCAT forms have even faster dissociation rates from rHDL.

| Table II  | Biophysical properties of wild type and mutant LCAT forms |
|-----------|-----------------------------------------------------------|
|           | Wild type LCAT | T123I mutant | N228K mutant |
| Fluorescence $\lambda_{max}$ (nm)$^a$ | 340 | 340 | 340 |
| Secondary structure $\%$ (%) ($\alpha$-helix, $\beta$-sheet, random coil) | 19, 32, 49 | 18, 33, 49 | 28, 22, 50 |
| Free energy of denaturation ($\Delta G_{H2O}$) (kcal/mol) | $1.92 \pm 0.10$ | $2.00 \pm 0.13$ | $1.91 \pm 0.11$ |
| Cooperativity of denaturation, $m$ | $-0.76 \pm 0.04$ | $-0.75 \pm 0.03$ | $-0.77 \pm 0.04$ |
| Midpoint of denaturation ($[\text{GdnHCl}]_{1/2}$ (M)) | 2.52 | 2.61 | 2.57 |

$^a$ From uncorrected fluorescence spectra.  
$^b$ From CD measurements using the method of Andrade et al. (24).

Fig. 5. Denaturation profiles for wild type LCAT (○), T123I mutant LCAT (△), and N228K mutant LCAT (□). The fraction unfolded was obtained from the wavelength of maximum fluorescence as a function of guanidine hydrochloride concentration ($[\text{GdnHCl}]$). The curves are theoretical fits to an equation relating the “fraction unfolded” to $\Delta G_{H2O}$ and $m$ (cooperativity) (22).

Fig. 6. Isothermal titration calorimetry experiments for the binding of wild type LCAT (A), T123I mutant LCAT (B), and N228K (C) mutant LCAT to reconstituted HDL (rHDL) particles. The I panels show the raw data of heat changes upon addition of titrant (rHDL) aliquots. The II panels show the processed data after subtraction of heat changes due to dilution and the theoretical fits generated by the ORIGIN program.
and N228K mutant LCAT (LCAT), the T123I mutant LCAT (a), and dissociation kinetics of wild type LCAT. The SPR measurements were performed twice at 25 °C and were reproducible within 5 and 15%.

**DISCUSSION**

It is important to note that the two natural mutations of LCAT studied here occur at residues Thr-123 and Asn-228 that are conserved across the six animal species whose LCATs have been fully sequenced to date. The 20 amino acids surrounding each of these residues are also highly conserved across species; they retain 100% sequence similarity and 73 and 87% sequence identity. This strongly suggests an important role of these regions in determining the structure and/or function of LCAT.

The results of this study indicate that these two point mutations have minimal effects on the structure of LCAT. First, both LCAT mutants are secreted in comparable levels to wild type LCAT from two types of transfected animal cells (COS-1 and CHO cells). The secreted proteins have the same molecular weight as wild type LCAT suggesting normal glycosylation. The purified proteins have very similar CD and fluorescence spectral properties, denaturation curves, and free energy changes of denaturation, indicating very similar, if not identical, secondary and tertiary structures for these proteins, and equivalent low stabilities in aqueous solution.

The enzymatic reaction of LCAT on lipoprotein particles consists of a series of steps (33). The first step is the binding of the enzyme to the lipoprotein surface. This step is followed by activation with apoA-I, binding of PC to the active site, formation of the acyl-enzyme intermediate and lyso-PC, binding of cholesterol to the active site, production of cholesterol ester, removal of products, and either a repeat of the catalytic cycle on the lipoprotein surface or release of the enzyme.

The first step, the binding step, does not depend on the apolipoprotein but is regulated by the nature of the lipid surface (phospholipid head group, phospholipid packing and hydration) (34, 35). The lid region of LCAT, like that of many lipases (36, 37), is essential for this interaction (17). We recently confirmed that deletion of residues 53–71 of LCAT produces an enzyme form that retains most of the structural properties of wild type LCAT but does not bind to rHDL as determined by ITC and SPR methods. Although this region of LCAT appears to be critical for interfacial binding, other regions of LCAT may also be involved.

TABLE III

| Mutants of LCAT | Wild type LCAT | T123I mutant | N228K mutant |
|-----------------|----------------|--------------|--------------|
| $K_a$ (equilibrium dissociation constant) (m (ITC)) | $2.3 \times 10^{-7}$ | ND<sup>a</sup> | $3.2 \times 10^{-7}$ |
| $K_a$ (equilibrium dissociation constant) (m (ITC)) | $2.0 \times 10^{-7}$ | $4.1 \times 10^{-7}$ | $4.8 \times 10^{-7}$ |
| $k_a$ (association rate constant) (s<sup>-1</sup>, m<sup>-1</sup>) (SPR) | $2.5 \times 10^{5}$ | $1.9 \times 10^{5}$ | $2.8 \times 10^{5}$ |
| $k_d$ (dissociation rate constant) (s<sup>-1</sup>) (SPR) | $4.9 \times 10^{-2}$ | $7.8 \times 10^{-2}$ | $12 \times 10^{-2}$ |

<sup>a</sup>Binding not detected by the ITC method.

<sup>b</sup>The SPR measurements were performed twice at 25 °C and were reproducible within 5 and 15%.

S. Adimoolam, unpublished results.
the binding of cholesterol to the active site and trans-esterification of the acyl chain lead to the formation of cholesterol ester. The binding site for cholesterol appears to be distinct from that of PC, as reagents that modify the free Cys residues, Cys-31 and Cys-184, interfere more with the acyltransferase reaction of LCAT than with the phospholipase or esterase reaction (21, 41). Thus the Cys residues appear to be located near the sterol-binding site.

In this study we demonstrated that the T123I and N228K mutants of LCAT are both capable of binding to rHDL, albeit with about half the affinity of wild type LCAT. Both mutants have almost full activity with the PNPB substrate, indicating that the catalytic triad and its immediate three-dimensional environment have not been affected by the mutations. The N228K mutant has low activity (5–12% of wild type LCAT) with both rHDL and LDL. Since the interfacial binding does not account for the low enzymatic activity with the interfacial substrates, the binding of the lipid substrates to the active site may be impaired. Because the acyltransferase and phospholipase reactions are both impaired to a similar extent, it appears that the N228K mutation interferes with the binding of PC molecules. The mild effect of the N228K mutation on the equilibrium dissociation constant for rHDL suggests that the PC substrate contributes to the affinity of the enzyme for the lipoprotein surface. Perhaps the substrate PC is not completely extracted from the lipoprotein surface during normal catalysis, thus contributing to hydrophobic interactions with the surface.

In contrast to the N228K mutant, the T123I mutant is unreactive with rHDL but substantially reactive with LDL. Because the binding affinity of the T123I mutant to rHDL is only slightly decreased compared with wild type LCAT, the defect in this LCAT variant is also in a step downstream from the interfacial binding to the rHDL particles. The affected step is most likely the activation of LCAT by apoA-I, since the catalytic steps are normal as indicated by the reactions with LDL lipids and with PNPB. Thus the region of LCAT including Thr-123 is a strong candidate for a surface site involved in the recognition of apoA-I. Furthermore, the small decrease in affinity for the rHDL, observed with the T123I mutant, suggests that interaction of LCAT with apoA-I normally may contribute to the overall affinity of the interfacial interaction.

Finally, in addition to examining the structural and functional properties of the T123I and N228K mutants of LCAT, we measured the rate constants for the interaction of LCAT and the mutants with rHDL. The fact that all the LCAT forms have equal association rate constants suggests that their approach to and recognition of the rHDL surface is identical. The increased dissociation rate constants for the mutants (0.08 and 0.12 s⁻¹, for the T123I and N228K, respectively), compared with 0.05 s⁻¹ for wild type LCAT, support the hypothesis that the interactions with apoA-I and with the PC substrate make some contributions to the overall interaction of normal LCAT with the rHDL surface.

Comparison of the catalytic rate constant (0.049 s⁻¹ at 25 °C) and dissociation rate constant (0.05 s⁻¹) for wild type LCAT shows for the first time that, on average, each catalytic cycle is followed by LCAT dissociation from the rHDL surface. This suggests that product release may coincide and perhaps may trigger the enzyme dissociation event. Possibly the diffusion of cholesterol ester product from the active site of LCAT to the core of the rHDL weakens the interaction of LCAT and rHDL and causes release of the enzyme. In any event, the observation that LCAT dissociates from the lipoprotein surface after one catalytic cycle sets it apart from some phospholipases that operate in the “scotting” mode. In the scotting mode these enzymes remain bound to the lipid surface until all substrate lipids are consumed (42).
Structural and Functional Properties of Two Mutants of Lecithin-Cholesterol Acyltransferase (T123I and N228K)
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