THE MOLECULAR PATHOLOGY OF LECITHIN:CHOLESTEROL
ACYLTRANSFERASE DEFICIENCY

by

JOHN STUART HILL

B.M.L.Sc., The University of British Columbia, 1988
M.Sc., The University of British Columbia, 1990

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Department of Pathology

The University of British Columbia
Vancouver, Canada

Date March 4, 1994
ABSTRACT

Epidemiological studies have indicated that decreased levels of plasma HDL are associated with an increased risk of coronary artery disease. However, there are several rare familial disorders of HDL metabolism in which, despite very low levels of HDL cholesterol, affected individuals do not appear to be at an increased risk for premature atherosclerosis. Examples of such disorders include familial LCAT deficiency and fish eye disease which are rare autosomal recessive diseases associated with inherited defects within the gene coding for lecithin:cholesterol acyltransferase (LCAT). In both cases, corneal opacities and a severe HDL deficiency are characteristic features of the disease. However, unlike fish eye disease, familial LCAT deficiency is associated with severe lipoprotein abnormalities and additional clinical symptoms including hemolytic anemia, proteinuria and a progressive renal insufficiency. The basis for this segregation is believed to be a result of functional differences associated with the LCAT enzyme.

The main purpose of this thesis was to define the molecular basis for the clinical and biochemical heterogeneity observed for different genetic defects of LCAT. To achieve this, the expression of recombinant LCAT (rLCAT) in mammalian cell culture was established as a model to analyse the properties of human plasma LCAT. Subsequently, a series of natural mutations associated with familial LCAT deficiency and fish eye disease were re-created and expressed in both monkey kidney COS-1 and baby hamster kidney cell lines. To determine the functional significance of each mutation, the specific activity of different mutant enzymes was analysed using both synthetic and natural substrates.

A wide range of functional abnormalities were identified: (i) defects of
secretion, (ii) loss of activity against HDL, (iii) loss of activity against all lipoproteins and (iv) variable reactivities for all lipoproteins. For defects which occur in the homozygous form, the properties of most mutant rLCATs were consistent with the biochemical phenotype observed in the plasma of affected probands. The recreation and analysis of single mutations associated with compound heterozygous genotypes revealed that the products of each allele pair had different characteristics. In addition, the results suggested that the presence of only a single allele coding for a partially active LCAT is sufficient to maintain normal rates of cholesterol esterification. From these biochemical analyses, it appears that LCAT has at least two functionally important domains, the catalytic center and a recognition site for HDL substrates.

In conclusion, it seems that the original families identified with familial LCAT deficiency and fish eye disease represent the clinical and biochemical extremes of a wide range of defects of LCAT function. Consequently, these disorders should not be classified as separate diseases but thought of as part of a larger group of LCAT deficiency syndromes.
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<td>apo</td>
<td>apolipoprotein</td>
</tr>
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<td>BHK</td>
<td>baby hamster kidney</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CE</td>
<td>cholesteryl ester</td>
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<td>CER</td>
<td>cholesterol esterification rate</td>
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<td>CETP</td>
<td>cholesteryl ester transfer protein</td>
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<td>CHO</td>
<td>Chinese hamster ovary</td>
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<td>COS</td>
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<td>dATP</td>
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<td>FBS</td>
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<td>IDL</td>
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<td>lecithin:cholesterol acyltransferase</td>
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<td>LDL</td>
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<td>Lp</td>
<td>lipoprotein</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>phosphatidylcholine</td>
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<td>PCR</td>
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<td>PNP</td>
<td>p-nitrophenol</td>
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<td>POPC</td>
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<td>rHDL</td>
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<td>SDS</td>
<td>sodium dodecylsulfate</td>
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<td>TG</td>
<td>triglyceride</td>
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<td>unesterified cholesterol</td>
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<td>very high density lipoprotein</td>
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<td>VLDL</td>
<td>very low density lipoprotein</td>
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<td>Amino Acid</td>
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INTRODUCTION

Atherosclerosis is a slowly progressive disease characterized by the accumulation of lipid within the intima of large elastic and muscular arteries. For many years, it has been recognized as the primary cause of myocardial and cerebral infarction. One of the major determinants for the development of atherosclerosis is the concentration of lipid, particularly cholesterol and its esters, circulating in plasma. Much effort has been devoted to the study of the specific mechanisms of lipid and lipoprotein metabolism. Lipoproteins are complexes of lipid and protein which function as carriers of lipid in the blood, interstitial fluid and lymph. These particles consist of a neutral lipid core surrounded by a surface monolayer containing phospholipids, unesterified cholesterol and various apolipoproteins. The protein components of these particles have both polar and nonpolar properties (amphipathic) and to a large extent determine the structure and metabolism of different lipoproteins. A variety of functional roles have been attributed to apolipoproteins such as cofactors for enzymes of lipid metabolism, facilitators of lipid transfer reactions, and as ligands for specific cell membrane receptors.

Traditionally, plasma lipoproteins are characterized according to their relative density: very low density lipoprotein (VLDL, d<1.006 g/ml), low density lipoprotein (LDL, d=1.006-1.063 g/ml) and high density lipoprotein (HDL, d=1.063-1.21 g/ml). The synthesis and secretion of VLDL by the liver is recognized as the initial event of a complex pathway describing the transport and metabolism of endogenous lipids. The primary function of LDL, generated by the metabolism of VLDL, involves the "forward" delivery of cholesterol from the liver to peripheral tissues. Apolipoprotein B (apo B), located on the surface of LDL, is recognized by specific receptors which
mediate the uptake and distribution of LDL cholesterol to peripheral cells (Brown and Goldstein, 1986). The association of elevated concentrations of LDL with an accelerated development of atherosclerosis has been well established by clinical and experimental observations (Goldstein and Brown, 1987). By contrast, high levels of plasma HDL appear to be protective against coronary artery disease (Gordon et al., 1977). Additional evidence supporting a link between HDL and atherogenesis has been shown in studies associating low or absent HDL with premature atherosclerosis in families with inherited defects of HDL metabolism (Schaefer, 1984). A large body of evidence has been accumulated which indicate that HDL can stimulate the efflux of cholesterol from peripheral cells (Badimon et al., 1992). This "reverse" delivery of cholesterol out of cells into the plasma is considered to be the first step in the transport of cholesterol from peripheral tissues back to the liver for excretion. Unlike the forward transport of cholesterol, the specific mechanisms of reverse cholesterol transport are not well understood. The complexity of these reactions is due in part to the considerable heterogeneity associated with HDL particles. Consequently, a detailed understanding of the distinct structural, functional, and metabolic properties of different subfractions of HDL is required.

1.1 HDL Structure and Composition

By definition, HDLs are composed of lipoproteins with densities between 1.063 and 1.21 g/ml (Havel et al., 1955). Within this density range, particles with differences in size (7.0-12.0 nm), hydrated density, apolipoprotein and lipid composition, and the presence of additional protein constituents have been identified (Fruchart et al., 1993). A variety of techniques including ultracentrifugation,
polyanion precipitation, polyacrylamide gradient gel electrophoresis, agarose electrophoresis, and immunoaffinity chromatography have been used to analyse different subpopulations of HDL.

Ultracentrifugation enables the separation of two major density classes, HDL$_2$ (1.063-1.125 g/ml) and HDL$_3$ (1.125-1.21 g/ml) (Patsch et al., 1980) while non-denaturing polyacrylamide gradient gel electrophoresis differentiates at least five subclasses, two of lower density (HDL$_{2a}$ and HDL$_{2b}$) and three of higher density (HDL$_{3a}$, HDL$_{3b}$, and HDL$_{3c}$) (Blanche et al., 1981). Differential dextran sulfate-magnesium chloride precipitation of serum has also been used to isolate HDL$_2$ and HDL$_3$ subfractions similar to those obtained by ultracentrifugation (Barstein et al., 1989). After agarose gel electrophoresis, the majority of HDL particles migrate with α-mobility (α-HDL) whereas only a small fraction (approximately 5% of total HDL) has preβ-mobility (preβ-HDL) (Kunitake et al., 1985). Further analysis of these particles by polyacrylamide gradient electrophoresis in a second dimension separates preβ-HDL into three subfractions (preβ$_{1-}$, preβ$_{2-}$, and preβ$_{3-}$-HDL) and α-HDL into two (α-HDL$_2$ and α-HDL$_3$) (Castro and Fielding, 1988; Fielding et al., 1991).

More recently, the classification of HDLs based on their apolipoprotein composition has found favour since these particles appear to have distinct metabolic, functional and clinical significance. Sequential immunoaffinity chromatography has been used to isolate four types of particles: Lp A-I, Lp A-I/A-II, Lp A-I/A-IV, and Lp A-IV (Fruchart et al., 1993). Particles were defined by their major apolipoprotein content such that Lp A-I and Lp A-IV contain a single major apolipoprotein. Despite a more complex protein composition, Lp A-I/A-II and Lp A-I/A-IV have apo A-I as the major apolipoprotein. Table 1 describes the lipid and
apolipoprotein composition for each lipoprotein particle. All particles contained a similar proportion of protein but showed differences in cholesterol and triglyceride (TG) content. Lp A-I and Lp A-I/A-II had higher cholesterol but lower triglyceride levels compared to Lp A-I/A-IV and Lp A-IV.


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<tr>
<td>Proteins</td>
<td>62.0±7.4</td>
<td>65.2±7.3</td>
<td>70.2±20.1</td>
<td>61.3±9.4</td>
</tr>
<tr>
<td>Lipids</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TC</td>
<td>11.8±4.1</td>
<td>11.5±3.9</td>
<td>4.2±3.5</td>
<td>8.0±3.8</td>
</tr>
<tr>
<td>UC</td>
<td>2.8±1.1</td>
<td>1.6±1.1</td>
<td>0.4±0.2</td>
<td>1.2±1.1</td>
</tr>
<tr>
<td>TG</td>
<td>5.0±2.2</td>
<td>3.8±1.8</td>
<td>9.4±6.8</td>
<td>9.6±5.7</td>
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<tr>
<td>PL</td>
<td>21.2±4.4</td>
<td>19.5±4.3</td>
<td>16.2±10.5</td>
<td>21.1±6.9</td>
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<tr>
<td>Apolipoproteins</td>
<td></td>
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<tr>
<td>A-I</td>
<td>97.6±2.5</td>
<td>67.4±13.3</td>
<td>-</td>
<td>57.8±14.4</td>
</tr>
<tr>
<td>A-II</td>
<td>-</td>
<td>31.4±13.5</td>
<td>0.4±0.7</td>
<td>10.2±2.7</td>
</tr>
<tr>
<td>A-IV</td>
<td>2.1±2.4</td>
<td>1.0±1.9</td>
<td>99.6±0.8</td>
<td>31.5±21.0</td>
</tr>
<tr>
<td>C-III</td>
<td>0.3±0.3</td>
<td>1.2±0.2</td>
<td>0.1±0.1</td>
<td>0.4±0.1</td>
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Lp, lipoprotein; TC, total cholesterol; UC, unesterified cholesterol; TG, triglycerides; PL, phospholipids. All values are given as mean ± standard deviation.

Table 2 indicates the percentage of total recovered weight for apolipoproteins A-I, A-II, and A-IV for each lipoprotein particle. The majority of apo A-I and apo A-II are found in Lp A-I/A-II particles and greater than 80% of apo A-IV is contained in Lp A-IV particles (Fruchart et al., 1993).
Table 2. Percentage of total recovered weight for apolipoproteins A-I, A-II, and A-IV. Compiled data from Duverger et al. (1993) and Fruchart et al. (1993).

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<tr>
<td>A-I</td>
<td>25%</td>
<td>65%</td>
<td>0%</td>
<td>2%</td>
</tr>
<tr>
<td>A-II</td>
<td>0%</td>
<td>98%</td>
<td>0%</td>
<td>2%</td>
</tr>
<tr>
<td>A-IV</td>
<td>3%</td>
<td>5%</td>
<td>83%</td>
<td>9%</td>
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When isolated particles were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting, several additional proteins including lecithin:cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP), and apo D were identified with each type of isolated particle. In the presence of apolipoproteins, LCAT utilizes HDL phospholipid and unesterified cholesterol to generate cholesteryl ester whereas CETP facilitates the exchange of HDL cholesteryl ester for triglyceride contained within VLDL and LDL particles. The measurement of LCAT activity associated with each subfraction of HDL indicated that Lp A-IV particles had the highest activity, followed by Lp A-I/A-IV and Lp A-I whereas Lp A-I/A-II particles had the lowest levels of activity (Fruchart et al., 1993). In addition, CETP was mainly associated with Lp A-I, Lp A-I/A-IV, and Lp A-IV but not with Lp A-I/A-II.

1.2 HDL Metabolism

Current evidence indicates that the synthesis of plasma HDL is derived from the liver and intestine. These newly formed or "nascent" HDL appear as both small spherical and discoidal particles which are protein-rich but lipid-poor and exhibit
preß-electrophoretic mobility on agarose gels. The lipid composition of these particles consists mainly of phospholipid and small amounts of unesterified cholesterol and triglyceride. The intestine produces nascent HDL which contain apo A-I and apo A-IV but not apo A-II (Brewer et al., 1990). By contrast, hepatic nascent HDL consist of both Lp A-I and Lp A-I/A-II particles (Forte et al., 1989). In addition to the direct secretion of HDL particles by tissues, discoidal HDL particles rich in phospholipid, apo A-I and apo Cs can be generated extracellularly from the production of excess apolipoprotein and phospholipid surface components created by the action of lipoprotein lipase (LPL) on triglyceride-rich lipoproteins (Tall and Small, 1978).

1.2.1 Reverse Cholesterol Transport

Although all cells are capable of cholesterol synthesis, only the hepatocyte has the ability to degrade cholesterol. Thus, a mechanism(s) is required for the transport of cholesterol from peripheral cells to the liver where cholesterol is further metabolized or excreted from the body in the form of bile and bile acids. The process of "reverse cholesterol transport" was first proposed by Glomset (1968) and since then has been studied by numerous investigators. Despite intensive analysis, the characteristics of this pathway, particularly its putative anti-atherogenic role, remain poorly understood. Figure 1 reflects the most current concepts of this hypothetical pathway which is composed of several distinct elements.

1) Cholesterol efflux from cells

Several different models of cholesterol efflux have been proposed: 1) The passive diffusion of cholesterol molecules through the aqueous phase surrounding
the cell (Phillips et al., 1980); 2) cholesterol translocation and efflux promoted by the binding of HDL to cell surface receptors (Oram, 1990); 3) acceptor retroendocytosis (Schmitz et al., 1985); 4) apolipoprotein-mediated cholesterol efflux (Rothblat et al., 1992). The present data suggest that cholesterol efflux from different cell types cannot be explained by one mechanism, but rather several mechanisms may operate simultaneously depending on the existing conditions.

Figure 1. Schematic diagram of the process of reverse cholesterol transport. The dark arrows indicate the flux of unesterified cholesterol (UC) and the light grey arrows indicate the transport of cholesteryl ester (CE). CETP, Cholesteryl ester transfer protein; LCAT, lecithin:cholesterol acyltransferase.

One of the most efficient acceptors of membrane cholesterol has been shown to be small HDL enriched in phospholipid and protein and relatively depleted of
cholesterol (Castro and Fielding, 1988). The composition and structure of these HDL, termed preß-HDL, are very similar to nascent HDL synthesized by the liver or intestine or formed by the lipolysis of triglyceride-rich lipoproteins. In most cases, apo A-I is the only apolipoprotein present on these particles. Studies by Segrest et al. (1992) predict that the conformation of the apo A-I molecule contained within small discs would enable a hinged region of the molecule to extend into the aqueous phase. It has been postulated that the extended portion of the apo A-I molecule would interact with specific lipid domains and anchor the lipoprotein to the plasma membrane thus facilitating the diffusion of cholesterol into the acceptor lipoprotein (Rothblat et al., 1992). The accumulation of unesterified cholesterol within the disc may modify the conformation of the apo A-I to regulate its dissociation from the membrane. Such a phenomenon would not necessarily be restricted to apo A-I, but could be mediated to differing extents by amphipathic helices of other apolipoproteins. A recent study has demonstrated that the size of discoidal reconstituted HDL (rHDL), determined by the number of apo A-I molecules present, correlates positively with cholesterol efflux but negatively with cholesterol transfer (Agnani and Marcel, 1993).

A second model describes an HDL receptor-mediated cholesterol translocation and efflux (Oram, 1990). In this model, specific HDL apolipoproteins (A-I and A-IV) are recognized by a cell surface receptor, which initiates a signal-transduction pathway stimulating the translocation of intracellular cholesterol to the membrane (Steinmetz et al., 1990; Mendez et al., 1991). However, the low affinity and high capacity of these receptors demonstrated in some systems are not indicative of classic receptor metabolism. Although the balance of evidence would
indicate that this pathway is not responsible for the bulk of cholesterol efflux, its specific quantitative role in cholesterol homeostasis remains to be determined.

2) *Creation of a cholesterol concentration gradient*

Pulse-chase studies using $[^{3}H]$cholesterol-loaded fibroblasts incubated with human plasma have examined the transfer and esterification of cell-derived unesterified cholesterol. As mentioned above, cellular unesterified cholesterol is first taken up by small discoidal, apo A-I containing HDL with preβ-mobility. From these preβ$_1$-HDL, unesterified cholesterol is rapidly transferred to larger preβ$_2$-HDL and subsequently to preβ$_3$-HDL to be esterified in the latter (Castró and Fielding, 1988; Francone *et al.*, 1989). Immunochemical studies have indicated that LCAT and CETP comigrate with preβ$_3$-HDL but were absent in the two smaller components (Francone *et al.*, 1989). The rapid and specific transfer of unesterified cholesterol among different HDLs could not be the result of simple diffusion but may result from a precursor-product relationship.

More recently, the transfer of cellular unesterified cholesterol has been shown to continue through α-HDL and subsequently to LDL (Huang *et al.*, 1993). Most of the LDL-derived unesterified cholesterol is redistributed to α-HDL to be esterified by LCAT (Miida *et al.*, 1990; Fielding *et al.*, 1991). In addition, the transfer of unesterified cholesterol from LDL to HDL is LCAT dependent since inhibition of the enzyme decreased not only the production of cholesteryl ester but also the removal of unesterified cholesterol from LDL (Huang *et al.*, 1993). As a result, cell membranes appear to be only a minor donor of unesterified cholesterol to the LCAT reaction. In fact, most unesterified cholesterol esterified by LCAT has been shown to originate from apo B-containing particles (Fielding and Fielding, 1981; Park *et al.*, 1990).
These results indicate that a cholesterol gradient between cell membranes and HDL can be established through at least two mechanisms: (i) The esterification of unesterified cholesterol by LCAT in HDL and (ii) the transfer of unesterified cholesterol to LDL via HDL followed by its uptake by hepatic receptors. These proposed mechanisms would provide an effective means of reverse cholesterol transport by maintaining an LCAT-independent mechanism of cholesterol transport to the liver and secondly, through the redistribution of unesterified cholesterol to a larger HDL pool (α-HDL), cholesterol esterification is maximized.

3) **Transport of cholesterol to the liver**

Once cholesterol is esterified in HDL it can be transferred to LDL and VLDL in exchange for triglyceride through the action of CETP. VLDL-remnants and LDL can then be cleared from the plasma by hepatic apo B/E receptors. This pathway is particularly prominent in species with elevated CETP activity. However, for species which lack CETP, alternative pathways may be utilized. For example, HDL containing apo E may be recognized and internalized directly by apo E receptors in the rat liver (Glass *et al.*, 1985). Additional evidence has demonstrated that HDL can interact with cell membranes of hepatocytes such that cholesteryl esters can be transferred into the cell through the action of a specific acceptor protein (Rinninger *et al.*, 1988). In addition, it has recently been demonstrated in perfused rat livers that hepatic lipase (HL) is required for the hepatic uptake of HDL triglycerides and cholesteryl esters and that the uptake of cholesteryl esters was not dependent on the hydrolysis of HDL triglyceride but on the hydrolysis of HDL phospholipid (Kadowki *et al.*, 1992).
1.2.2 HDL Remodelling

The interconversion of HDL particles consists of a complex cycling of lipids and apolipoproteins associated with different sizes and shapes of HDL particles (Figure 2). HDL-like particles and free apolipoproteins (apo A-I, apo A-II, and apo A-IV) have been identified in interstitial fluid (Lefevre et al., 1988). Since apo A-I, apo A-II and apo A-IV are not commonly synthesized by peripheral tissues, their presence in interstitial fluid probably reflects diffusion of free apolipoproteins and/or the filtration of plasma HDL across the endothelium. In addition, the observation that plasma levels of preß1-HDL were maintained only if plasma was incubated with various cell types suggests that preß1-HDL can be formed by the interaction of HDL components and cell membranes (Miida et al., 1992). Indeed, HDL in interstitial fluid have preß-mobility and appear to be effective acceptors of cellular cholesterol. However, in the same manner as preß1 and 2-HDL, they do not appear to be good substrates for cholesterol esterification (Wong et al., 1992). The conversion of plasma HDL discs into spherical HDL is initially mediated by the esterification of surface unesterified cholesterol by LCAT and the migration of cholesteryl ester to the core forming HDL3. The release of unesterified cholesterol and phospholipid as a consequence of the action of LPL on triglyceride-rich lipoproteins and the continuous action of LCAT results in the transformation of HDL3 to larger HDL2 particles (Patsch et al., 1978). In contrast, in vitro studies have shown that through the synergistic effects of CETP, HL, and VLDL, spherical HDL lose cholesteryl ester, triglyceride, and phospholipid, and become progressively smaller resulting in the dissociation of apo A-I and the formation of discoidal, preß-HDL (Newnham and Barter, 1990; Clay et al., 1991, 1992; Kunitake et al., 1992). The observation that
the concentration of plasma HDL₂ correlates positively with the postheparin activity of LPL (Patsch et al., 1987) but negatively with HL (Kuusi et al., 1980; Patsch et al., 1987) is evidence that these transformations also occur in vivo. Thus, HDL appear to cycle from discs→HDL₃→HDL₂→HDL₃→discs enabling the continuous movement of lipids to and from different lipoproteins.

**Figure 2. Schematic diagram of HDL remodelling.** UC, unesterified cholesterol; PL, phospholipid; TG, triglyceride; CETP, cholesteryl ester transfer protein; HL, hepatic lipase; LPL, lipoprotein lipase; LCAT, lecithin:cholesterol acyltransferase; FA, fatty acid.
Yet another plasma protein has been shown to modify HDL size. The putative HDL conversion factor, now identified as the phospholipid transfer protein (PLTP), can convert HDL of homogeneous size into two subpopulations of smaller and larger particles (Jauhiainen et al., 1993). The mechanisms involved or the specific physiological role of PLTP has not been determined but its action in plasma may contribute to the supply of phospholipid to HDL for the LCAT reaction.

Therefore, changes in the size, shape, lipid and apoprotein composition of HDL particles are modified by several plasma factors including LCAT, CETP, LPL, HL and PLTP. The specific influence of these different components on the structure and function of different HDLs is regulated by other lipoproteins and possibly additional plasma constituents. Consequently, the metabolism of HDL consists of a highly complex series of events involving the transfer and exchange of apolipoproteins and lipids among cell membranes and plasma lipoproteins.

1.2.3 Relationship Between Apolipoprotein Composition and the Metabolism of HDL

As described above, HDL can be divided into different subpopulations on the basis of their apolipoprotein composition. In most cases, studies have focused on two major HDL populations: those containing apo A-I but not apo A-II (Lp A-I) and those containing both apo A-I and apo A-II (Lp A-I/A-II). Specific interest in these two types of particles has been emphasized by a clinical study indicating that levels of Lp A-I, but not Lp A-I/A-II, were predictive for coronary artery disease (Puchois et al., 1987). Also, plasma Lp A-I levels were higher and Lp A-I/A-II lower in octogenarians (Luc et al., 1991). These findings have led many investigators to determine if metabolic differences exist between these two particles. There has
been conflicting data regarding the ability of these particles to stimulate efflux of cholesterol from cultured cells (Barkia et al., 1991; Johnson et al., 1991). It is likely that different metabolic characteristics may exist for different cell types which could contribute to these discrepancies. In a recent report by Fruchart et al. (1993), both Lp A-I and Lp A-I/A-II promoted the efflux of cholesterol from the membrane of bovine aortic endothelial cells to a similar extent. However, only Lp A-I could stimulate the efflux of intracellular cholesterol.

Analysis of the in vivo kinetics of Lp A-I and Lp A-I/A-II particles has revealed that Lp A-I is catabolized more rapidly than Lp A-I/A-II (Rader et al., 1991). Thus, both the increased ability to mobilize intracellular cholesterol and its faster rate of catabolism may indicate an increased anti-atherogenic potential for Lp A-I.

In several instances, the metabolism of apo A-II-containing particles in vitro has been shown to be distinct from Lp A-I. For example, the preferential conversion of Lp A-I/A-II-HDL₂, rather than Lp A-I-HDL₂, to HDL₃ was associated with the presence of apo A-II, which enhanced the HL-mediated lipolysis of these particles (Mowri et al., 1992). However, the presence of apo A-II in either discoidal rHDL or spherical native HDL₃ inhibited their conversion into smaller particles mediated by CETP (Rye et al., 1992; Lagrost, 1992). Despite this observation, it has been demonstrated that both Lp A-I and Lp A-I/A-II particles are capable of generating preß-HDL (Hennessy et al., 1993).

There is now significant evidence that these two HDL subfractions differ with respect to a number of biological activities including cholesterol efflux, membrane binding, and metabolism. However, given the variability of results obtained so far, further investigation of the specific roles of these different particles is required.
1.3 LCAT Biochemistry

Lecithin:cholesterol acyltransferase (LCAT) is the enzyme responsible for the esterification of cholesterol in plasma. It is unique in that a single polypeptide sequence catalyzes sequential reactions. Thus, by combining both phospholipase A\textsubscript{2} and acyltransferase activities, LCAT catalyzes the transfer of an \textit{sn}-2 fatty acid from phosphatidylcholine to the 3-hydroxyl group of cholesterol forming lysophosphatidylcholine and cholesteryl ester (Figure 3). LCAT is synthesized in the liver and is secreted into the plasma where it associates primarily with HDL, the major site of cholesterol esterification (Fielding and Fielding 1971; Chen and Albers, 1982). As described above, LCAT creates a cholesterol concentration gradient between peripheral cells and HDL and thereby influences the concentration of unesterified cholesterol and cholesteryl ester associated with plasma lipoproteins and tissues. The significance of its role in lipid metabolism is demonstrated by the clinical and biochemical pathology observed in inherited defects of the LCAT gene (Norum \textit{et al.}, 1989). The physiological importance of LCAT, combined with the general interest for enzymes of lipoprotein metabolism, led to work on the purification, and its subsequent physical and chemical characterization.
LECITHIN

\[
\begin{align*}
\text{CH}_2 - & \text{saturated fatty acid} \\
\text{CHO} & \text{unsaturated fatty acid} \\
\text{CHO} - & \text{P} - \text{choline} \\
\text{OH} & 
\end{align*}
\]

LYSOLECITHIN

\[
\begin{align*}
\text{CH}_2 - & \text{saturated fatty acid} \\
\text{CHOH} & \\
\text{OH} & 
\end{align*}
\]

Phospholipase A2

\[\text{LCAT} \rightarrow\]

Acylationtransferase

CHOLESTEROL

\[
\begin{align*}
\text{HO} & 
\end{align*}
\]

CHOLESTERYL ESTER

\[
\begin{align*}
\text{unsaturated fatty acid} & 
\end{align*}
\]

Figure 3. Schematic diagram of the LCAT reaction.

1.3.1 Physical properties of LCAT

The primary structure of human LCAT has been determined by the cloning and sequencing of the LCAT cDNA (McLean et al., 1986a). A 24 amino acid hydrophobic leader sequence precedes the mature protein, which consists of a single polypeptide of 416 amino acids with a calculated molecular weight of 47,090. LCAT is glycosylated and contains about 25% carbohydrate increasing its molecular weight to about 65,000 to 69,000 (Marcel, 1982). Chemical analysis of LCAT peptides have identified two disulfide bridges between Cys-50-Cys-75 and Cys-313-Cys-356 while the two remaining cysteine residues (Cys-31 and Cys-184) have free sulfhydryl groups (Yang et al., 1987) (Figure 4). Analysis of the amino acid sequence predicts four potential N-linked glycosylation sites at Asn residues 20, 84, 272 and 384. Based on the Chou-Fasman structural algorithm, the proportion of \textit{a}-
helix, β-sheet and random coil structures in LCAT are 21, 24, and 55%, respectively (Yang et al., 1987) and are similar to those determined by circular dichroism (Chong et al., 1983). Analysis of the helical hydrophobic moments indicates several regions of possible amphipathic structure but only the sequence between Glu154-Lys173 could potentially form an α-helix (McLean et al., 1986b). Thus, the lack of α-helical structures and increased hydrophobicity associated with LCAT distinguish it from the lipid binding properties of the soluble apolipoproteins.

Figure 4. Structural features of human LCAT. The relative positions of the four N-linked glycosylation sites are indicated above the line whereas the bridged and free cysteine residues are indicated below the line. The catalytic Ser-181 is marked by the vertical box.

LCAT is known to have multiple isoelectric points ranging from pI 3.9 to 4.4. After the removal of sialic acids by neuraminidase digestion, these multiple bands shift toward higher pH regions and converge into a single band after extensive treatment (pI 5.2) (Doi and Nishida, 1983). By counting the number of bands present in the isoelectric focusing gel, there appear to be a total of 16 sialic acids/molecule of LCAT. Desialylation of LCAT reduces the apparent molecular weight by 3,000 and is associated with an increase in LCAT activity, suggesting that the removal of additional negative charges enhances the LCAT-substrate interaction (Doi and
Analysis of the distribution of LCAT in plasma has indicated that about 90% is associated with HDL but small amounts remain bound to LDL (Chen and Albers, 1982). Separation of HDL particles by gel filtration indicates that LCAT is more commonly associated with larger particles (Chen and Albers, 1982; Park et al., 1987). After the isolation of Lp A-I and Lp A-I/A-II particles, most of plasma LCAT protein (70±15%) was detected in Lp A-I whereas a smaller portion (16±7%) was detected in Lp A-I/A-II (Cheung et al., 1986).

1.3.2 The Catalytic Mechanism of LCAT

Comparative amino acid sequence analyses have indicated that although LCAT has little overall sequence identity with other lipases, it does contain a Gly-X-Ser-X-Gly motif characteristic of the active site of several other lipases (Brenner, 1988). Francone and Fielding (1991b) have used site-directed mutagenesis and in vitro expression to investigate the role of Ser-181 and Ser-216 contained within different lipase consensus sequences of human LCAT. This study provided strong evidence that Ser-181 forms part of the active site of LCAT as the exchange of Ser-181 to either threonine, glycine or alanine resulted in a complete loss of LCAT activity. By contrast, the substitution of Ser-216 with these same amino acids did not abolish LCAT activity but resulted in significantly different specific activities observed for each mutant. As a result, it appears that Ser-216 does not form part of the active site but retains an important, albeit indirect role, in the catalytic reaction of LCAT. The three-dimensional structure of pancreatic lipase indicates that the active Ser contained within this consensus sequence is part of a catalytic Asp-His-Ser triad.
(Winkler et al., 1990). It is likely that a similar conformation exists for LCAT since a monoclonal antibody raised against LCAT inhibits the enzyme activity of several phospholipases (Khalil et al., 1986).

Jauhiainen and Dolphin (1986) have demonstrated that the chemical modification of Ser, His and Cys residues inhibited LCAT activity. Specifically, the modification of one serine residue by phenylmethanesulfonyl fluoride or one histidine residue by diethyl pyrocarbonate inhibited both the phospholipase and acyltransferase activities of LCAT. From the results obtained with sulfhydryl inhibitors, they proposed that the two free cysteines of LCAT (Cys-31 and Cys-184) were essential for activity through their participation in the formation of thioester bonds prior to transfer of the acyl group to cholesterol. In a subsequent study, Dolphin and co-workers demonstrated that the free cysteine residues of LCAT are vicinal to one another and within the catalytic site (Jauhiainen et al., 1988).

However, the results of in vitro site-directed mutagenesis studies demonstrated that the substitution of free Cys residues did not affect LCAT reactivity and that the action of sulfhydryl inhibitors was attributed to steric inhibition (Francone and Fielding, 1991a; Qu et al., 1993).

Site-directed mutagenesis has also been applied to produce LCAT species in which the four potential individual attachment sites for N-linked glycosylation chains are destroyed (Francone et al., 1993). From these studies, it was concluded that elimination of the glycosylation consensus sequence at positions 20, 84 or 384 had only minor effects on either the phospholipase or acyltransferase activities of LCAT, whereas the oligosaccharide moiety at position 272 had an essential role in acyltransferase activity. Studies in our laboratory, conducted in parallel with those
reported in this thesis, provide a more detailed analysis of the role of N-linked carbohydrate in LCAT function (O et al., 1993). We definitively identified that all four potential N-glycosylation sites in LCAT are occupied and that the loss of carbohydrate at each site has diverse effects on enzyme function. Interestingly, the loss of carbohydrate at position 384 was associated with a two-fold increase in enzyme specific activity suggesting that the oligosaccharide at this position has an inhibitory effect on LCAT activity. In addition, a quadruple mutant in which all four sites were eliminated was secreted poorly (10% of wild type) and its specific activity was decreased to 5% of wild type. Additional studies will be necessary to define further the specific role of carbohydrate in the reactivity of LCAT for native substrates.

Based on the current knowledge obtained from the physical and biochemical analysis of LCAT, the catalytic steps of the enzyme reaction can be divided into six separate steps: (i) reversible binding of the enzyme to the lipoprotein surface (ii) apolipoprotein activation (optimally apo A-I) facilitating access of lipid to the active site (iii) deacylation of the phospholipid substrate and release of lysophosphatidylcholine (iv) formation of a Ser-O-acyl intermediate (v) subsequent transfer of the acyl chain to cholesterol (vi) release of cholesteryl ester product into the core of the lipoprotein. The inhibition of the LCAT reaction by lysophosphatidylcholine has been reported by several investigators and is thought to be mediated either by an end-product inhibition or its detergent-like properties (Fielding et al., 1972; Smith and Kuksis, 1980). However, the majority of lysolecithin is believed to exist as a complex with albumin and, as a result, it may not have a profound physiological role.

20
Although cholesterol is the preferred acyl acceptor, LCAT has also been shown to carry out the acylation of lysophosphatidylcholine to reform phosphatidylcholine (Subbaiah et al., 1982). The catalytic mechanism for the lysolecithin acyltransferase reaction (LAT) appears to be the reversal of the phospholipase A\(_2\) action of LCAT. In vivo, the increased affinity of LDL for lysophosphatidylcholine confines LAT activity almost exclusively to the LDL fraction. In fact, the acylation of lysophosphatidylcholine has been shown to be dependent on the presence of LDL but not apo A-I (Subbaiah et al., 1982). Specifically, apo B present in LDL was essential for enzyme activation since trypsin treatment of LDL abolished its ability to activate LAT (Subbaiah et al., 1985). Further studies indicated that the specific conformation of apo B was affected by the concentration of LDL triglyceride which, in turn, influenced the magnitude of LAT activity (Liu et al., 1992). The LAT activity of LCAT was also demonstrated in proteoliposomes containing egg phosphatidylcholine and radiolabeled lysophosphatidylcholine. Apolipoproteins C-I and E were respectively, 70% and 40% as effective as apo A-I in the activation of LAT (Liu and Subbaiah, 1993). These results suggest that in addition to end-product inhibition, lysophosphatidylcholine may compete with unesterified cholesterol as an acyl acceptor. The physiological significance of the LAT reaction, however, remains to be determined.

It has also been reported that LCAT is capable of hydrolysis of cholesteryl ester to produce unesterified cholesterol (Sorci-Thomas et al., 1990). However, the rate of release of unesterified cholesterol was only about 10% of the forward rate of esterification. In addition, there was no evidence that the fatty acid obtained from the cholesteryl ester could be transferred to lysophosphatidylcholine to form
phosphatidylcholine. Thus, the two "forward" activities of the LCAT reaction are each independently reversible but not in succession.

1.3.3 Substrates of LCAT

Due to the heterogenous nature of plasma HDL, it is difficult to study the different binding and enzyme reactivity of LCAT for different HDL subfractions. As a result, it has been necessary to synthesize chemically and physically defined substrates to investigate the properties of the LCAT reaction.

The first synthetic substrates to be used were unilamellar vesicles containing phosphatidylcholine, cholesterol and exogenous apolipoproteins which formed particles termed "proteoliposomes" (Ho and Nichols, 1971). Subsequently, the sodium cholate dialysis method was developed to create discoidal complexes of similar composition (Matz and Jonas, 1982). The stability and uniformity of these complexes has enabled the investigation of the substrate specificity, apolipoprotein activation and kinetic properties of LCAT.

The known molecular substrates of LCAT and their physical state in aqueous solutions are listed in Table 3. Although non-physiological, the monomeric acyl donors can be helpful in the analysis of the enzyme kinetics and mechanism in the absence of a lipid/water interface. The physiological substrates of LCAT exist in aggregated form either in native lipoproteins, synthetic lipid vesicles or reconstituted HDL (rHDL) (Jonas, 1986). The chemical and physical properties of these different substrates determine their relative reactivity for LCAT.
1.3.3.1 Monomeric Substrates

Bonelli and Jonas (1989) have used p-nitrophenol (PNP) esters to investigate the effects of substrate chain length, ion concentration and apolipoprotein composition on the enzyme kinetics of LCAT in solution. However, it should be noted that these studies assess only the phospholipase activity of LCAT. They reported that with increasing chain length, both \( K_m \) and \( V_{\text{max}} \) decreased, but the ratio of \( V_{\text{max}}/K_m \) increased. On the one hand, from these studies, it was concluded that the binding of molecules with long acyl chains to the active site of LCAT is determined by hydrophobic interactions such that their affinity increases with increasing chain length. On the other hand, the decreasing \( V_{\text{max}} \) values may arise from a restricted mobility of the long chain substrates in the active site that could reduce the frequency of successful binding.
Table 3. Molecular substrates of LCAT. Adapted from Jonas (1991).

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Physical State</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acyl donors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipids</td>
<td>Aggregate</td>
<td>Aron et al., 1978</td>
</tr>
<tr>
<td>Cholesteryl esters</td>
<td>Aggregate</td>
<td>Sorci-Thomas <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>Arachidonyl CoA</td>
<td>Micelle</td>
<td>Jauhiainen and Dolphin, 1986</td>
</tr>
<tr>
<td>PNP acyl esters</td>
<td>Monomer</td>
<td>Bonelli and Jonas, 1989</td>
</tr>
<tr>
<td>DPycbPC</td>
<td>Monomer</td>
<td>Bonelli and Jonas, 1992</td>
</tr>
<tr>
<td><strong>Acyl acceptors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sterols (cholesterol)</td>
<td>Aggregate</td>
<td>Kitabatake <em>et al.</em>, 1979</td>
</tr>
<tr>
<td>Lysophospholipids</td>
<td>Aggregate</td>
<td>Subbaiah <em>et al.</em>, 1982</td>
</tr>
<tr>
<td>Alcohols (long chain)</td>
<td>Aggregate</td>
<td>Kitabatake <em>et al.</em>, 1979</td>
</tr>
<tr>
<td>Water</td>
<td>Monomer</td>
<td>Aron <em>et al.</em>, 1978</td>
</tr>
</tbody>
</table>

PNP, p-nitrophenol; DPycbPC, 1,2-bis[4-(1-pyreno)-butanoyl]-sn-glycero-3-phosphocholine

The influence of surfactants on LCAT reactivity with water soluble substrates has also been studied. Ionic detergents in the concentration range of $10^{-4}$ to $10^{-3}$M inhibited LCAT, whereas two nonionic detergents activated the enzyme in a similar concentration range (Bonelli and Jonas, 1993). From the analysis of enzyme kinetics and the patterns of inhibition, it was proposed that SDS binds cooperatively to LCAT.
and causes inhibitory structural changes, whereas laurate and cholate appear to bind to specific sites either at the active site or negative effector sites elsewhere. By contrast, nonionic detergents may create an interfacial activation of the phospholipase reaction near their critical micellar concentrations. Also, in contrast to aggregated substrates, the presence of anions and free apolipoproteins had no specific effect on LCAT activity in solution (Bonelli and Jonas, 1992). Therefore, these studies would seem to indicate that the activation of LCAT by apolipoproteins and its modulation by anions are associated with interactions at the lipid/water interface, and are not a result of direct effects on the enzyme.

1.3.3.2 Aggregated Substrates

As mentioned above, the first steps of the LCAT reaction for aggregated substrates include interaction of the enzyme with a lipid interface, followed by binding of a lipid substrate to the active site. The characteristics of these initial events are influenced to varying degrees by several interrelated components including lipid and apolipoprotein composition, apolipoprotein conformation, and the size and shape of the lipoprotein substrate.

Apo A-I is recognized as the most potent activator of the LCAT reaction (Fielding et al., 1972). However, other apolipoproteins and even synthetic amphipathic peptides are known to activate LCAT to varying degrees (Jonas, 1991). This topic will be discussed in detail in a later section.

The composition of phospholipids have multiple effects on LCAT reactivity which include: 1) the relative fluidity, lipid packing, and hydration of the lipid interface to which LCAT binds; 2) the conformation of apolipoproteins in the
membrane; and 3) their interaction with the active site.

It has been established that phospholipids which increase membrane fluidity also enhance cholesterol esterification by LCAT (Pownall et al., 1985). For synthetic dispersions of phosphatidylcholine and cholesterol, maximal enzyme activity is observed at a phosphatidylcholine/cholesterol ratio of 4:1 in the presence of apo A-I (Fielding et al., 1972). The creation of rHDL particles containing either palmitoyloleoylphosphatidylcholine (POPC) or dipalmitoylphosphatidylcholine (DPPC) exhibit distinct and characteristic size distributions. Consequently, differences in both the number and conformation of apo A-I molecules bound to the surface will affect activation of the LCAT reaction. When using rHDL substrates, phosphatidylcholines with two saturated chains containing 16 carbons or less were excellent substrates for LCAT but poor LCAT reactivity was observed when both chains were longer (Pownall et al., 1987). However, the composition of surrounding phosphatidylcholines can influence the reactivity for a single particular phosphatidylcholine type, and thus, the acyl chain specificity of LCAT observed for artificial substrates is difficult to relate to the function of LCAT in vivo (Jonas et al., 1986). In plasma, the phosphatidylcholine preference is largely determined by the availability of the phosphatidylcholine species in the plasma lipoproteins (Subbaiah and Monshizadegan, 1988). These studies indicated that in native plasma, LCAT prefers 16:0>18:1>18:0 acyl groups at the 1-position and 18:2>18:1>22:6>20:4 acyl groups at the 2-position. Analysis of the positional specificity of human LCAT has indicated that greater than 90% of the acyl groups used for cholesteryl ester synthesis are derived from the sn-2 position of most of the naturally occurring phosphatidylcholine substrates (Subbaiah et al., 1992). However, about 75% of the
cholesteryl ester species were derived from the sn-1 position when phosphatidylcholines containing 20:4 or 22:6 acyl chains at the sn-2 position were used as substrates. This observation was not influenced by membrane fluidity, apolipoprotein activator, or by the unesterified cholesterol/phosphatidylcholine ratio in the substrate. It is possible that the bulky 20:4 and 22:6 groups in the phosphatidylcholine do not fit efficiently into the active site and, as a result, the sn-1 acyl group which is not usually a preferred substrate, is more favourably oriented toward the active site. However, a finding that is inconsistent with this hypothesis is that, while the positional specificity is altered in the presence of 14:0-20:4 phosphatidylcholine, 16:0-20:4 phosphatidylcholine, and 16:0-22:6 phosphatidylcholine, it is altered to a much lesser extent in the presence of 18:0-20:4 phosphatidylcholine. The reason for this exception is not clear, but it appears that 18:0 acyl chains are even less preferred than 20:4 groups by the enzyme (Subbaiah et al., 1992).

Particles of different size have been shown to have significantly different reactivities with LCAT. In general, smaller HDL (HDL$_3$) are better substrates for LCAT compared to larger HDL$_2$ particles (Fielding and Fielding, 1971; Jahani and Lacko, 1982). For synthetic rHDL discs containing POPC, these differences have been attributed to changes in the conformation of apo A-I (Jonas et al., 1989). However, in rHDL particles prepared with apo A-I and DPPC, the conformation of apo A-I was quite similar in particles with diameters ranging from 9.7 to 18.6 nm, but a 20-fold difference in LCAT reactivity was observed between the smallest and largest particles (Hefele Wald et al., 1990). Thus, in certain conditions, the size or surface curvature of the particle may independently influence its ability to interact
with LCAT.

In general, discoidal HDL are more reactive with LCAT when compared to native spherical HDL (Marcel et al., 1980). As mentioned above, changes in apolipoprotein conformation and/or lipid packing may be responsible for these differences. Studies performed with synthetic discs indicate that LCAT reacts best with large discs containing 4 molecules of apo A-I, followed by progressively smaller discs containing 3 and 2 molecules of apo A-I, respectively (Meng et al., 1993). In addition, LCAT activity is also proportional to disc circumference which may reflect the binding of more amphipathic α-helices/particle increasing the stability of apo A-I and thus LCAT reactivity (Meng et al., 1993). In addition, the increased ability of synthetic discs to store cholesteryl ester compared to vesicles of a similar composition may explain the higher reactivity of these particles compared to spherical substrates. (Matz and Jonas, 1982). However, Barter et al. (1985), reported an inverse relationship between plasma HDL₃ particles size and substrate reactivity that was independent of cholesteryl ester content. The preparation of spherical rHDL with different cholesteryl ester to triglyceride ratios in their cores indicated that triglyceride correlated negatively and cholesteryl ester positively with the $V_{\text{max}}$ of LCAT, whereas the apparent $K_m$ remained unchanged (Sparks and Pritchard, 1989). However, the surface composition of the rHDL influenced enzyme kinetics such that the $K_m$ was positively related to protein content and particle size but negatively with phospholipid and unesterified cholesterol content. From these studies, it is apparent that the surface and core lipid composition, and the proportion of lipid to apolipoprotein affect particle size and shape. In turn, the size and shape of the particle influences the structure of the apolipoprotein. As a result, the
interdependence of these parameters must be considered when determining the properties of substrates for optimal LCAT reactivity.

It has been reported that LCAT can also interact directly with LDL to esterify cholesterol (Barter, 1983; Barter et al., 1984; Knipping et al., 1986). When the formation of cholesteryl ester was analysed in incubated plasma devoid of CETP activity, 73% of the esterified cholesterol was in HDL, 25% in LDL and only 1% in VLDL (Rajaram and Barter, 1985). Additional studies using purified LCAT and isolated lipoprotein fractions from both human and pig plasma confirmed the ability of LCAT to esterify cholesterol in LDL (Knipping et al., 1986). The removal of non-apo B proteins from LDL reduced but did not eliminate this activity. Since pig plasma lacks CETP activity, the appearance of cholesteryl ester in specific lipoproteins can be attributed to the direct action of LCAT. After incubation of pig plasma at 37°C, the greatest amount of cholesteryl ester (60-70%) was consistently found in the LDL fraction suggesting that pig LCAT may have a preference for LDL, even in the presence of physiological concentrations of HDL (Knipping et al., 1987).

The substrate specificity of LCAT from a number of different species have been compared indicating differing abilities to transfer acyl groups depending on their length and degree of a saturation (Grove and Pownall, 1991). As a result, the authors suggested that the conformation of the active sites of these enzymes are not the same. However, the use of synthetic substrates in this study may not be representative of their action in vivo. On the other hand, there is in vivo evidence that rat LCAT has a greater specificity for arachidonate (20:4) acyl groups compared to human LCAT (Subbaiah et al., 1992). The purification of rat LCAT has enabled the comparison of its characteristics with its human counterpart (Furkawa et al.,
The affinity of the rat enzyme to rat apo A-I-containing vesicles was stronger when compared to the interaction of the human enzyme with human apo A-I (Furkawa et al., 1992). Rat apo A-I was able to activate human LCAT at only 18% of the rate obtained with human apo A-I. By contrast, the rates of esterification were more than two-fold higher for the human apo A-I activation of rat LCAT compared to human LCAT.

1.3.5 Activation of the LCAT Reaction

Initially, Fielding et al. (1972) demonstrated that egg-phosphatidylcholine and cholesterol vesicles required apo A-I to activate the LCAT reaction. Since then, a number of investigators have shown other exchangeable apolipoproteins also activate LCAT but to a lesser extent (Soutar et al., 1975; Steinmetz and Utermann, 1985). Several studies have attempted to localize the domain(s) within apo A-I responsible for its LCAT activating properties by using synthetic peptide analogues (Pownall et al., 1984; Anantharamaiah et al., 1990) and monoclonal antibodies (Banka et al., 1991). In most cases, the activating efficiency of peptides as well as other apolipoproteins has an intrinsic upper limit, approximately 30% of apo A-I (Anantharamaiah et al., 1990). Therefore, the disruption of the lipid/water interface by amphipathic helixes may be necessary for LCAT activation but it is clearly not sufficient. There is evidence indicating that a specific region of apo A-I (residues 66-120) is associated with a predominant LCAT activating domain of apo A-I. The identification of apo A-I-specific monoclonal antibodies which inhibit LCAT activity and the use of synthetic peptides has defined the region spanning residues 95-121 as critical for activation of the LCAT reaction (Banka et al., 1991). Segrest and
colleagues (1992) have proposed that a unique positioning of Glu residues on the non-polar face of two helixes, not present in the other apolipoproteins, is responsible for the unique activating abilities of apo A-I. However, point mutations in this region do not significantly affect activation (Jonas et al., 1991; Minnich et al., 1992). Therefore, a series of amino acids rather than individual residues appears to be responsible for the apo A-I activation of LCAT. In addition, it is technically very difficult to assess the relative reactivity of synthetic peptides and apolipoproteins. Thus, the definition of specific activating domains of apo A-I or the mechanism by which such a region could enhance LCAT reactivity remains elusive. Hopefully, site-directed mutagenesis and in vitro expression of recombinant A-I will be helpful in this regard.

1.3.5 Measurement of LCAT Activity and Cholesterol Esterification Rate

Two methods are routinely used to assess the ability of a patient’s plasma to esterify cholesterol. Firstly, the measurement of LCAT activity is determined in the absence of plasma constituents in order to reflect the amount of active enzyme in the plasma. Therefore, in the absence of any defects, LCAT activity should be proportional to plasma LCAT protein levels. In this assay, an excess of artificially prepared liposomes containing known amounts of $[^3]H$cholesterol, phospholipid and apo A-I act as the substrate for LCAT present in a small sample of plasma. The activity is determined by measuring the rate of conversion of $[^3]H$cholesterol into $[^3]H$cholesteryl ester and the result is expressed as nmoles of cholesterol converted to cholesteryl ester per hour per millilitre of plasma. The endogenous cholesterol esterification rate (CER) measures the rate at which cholesteryl esters are
synthesized in whole plasma in the absence of any exogenous substrate. In this assay, the endogenous pool of unesterified cholesterol in the patient’s plasma is radiolabeled by equilibration with $[^3]$Hcholesterol at 4°C. Subsequent incubation of the patient’s plasma at 37°C and determination of the rate of synthesis of $[^3]$Hcholesteryl ester provides an estimate of the net (or endogenous) synthesis rate. As a result, the measurement of CER is influenced by both the amount of enzyme present and the nature of plasma lipoprotein substrates as well as cofactors and other plasma constituents. Thus, the CER is not necessarily proportional to the concentration of LCAT protein. The units of activity are identical to those stated for LCAT activity (nmoles of cholesterol esterified per hour per millilitre) but the two assays are not equivalent.

1.4 Clinical Findings in LCAT Deficiency and Fish Eye Disease

It has been nearly 30 years since the first patient with familial lecithin:cholesterol acyltransferase (LCAT) deficiency was described by Kare Norum and collaborators (Norum and Gjone, 1967; Gjone and Norum, 1968; Torsvik et al., 1968). Since that time, the study of LCAT deficiency has received a great deal of attention from clinical and basic scientists throughout the world. These research efforts have provided information on the clinical presentation and laboratory findings in affected individuals which have led to a better understanding of the pathological changes that are common in this rare yet instructive disease process.

As expected, a functional or absolute deficiency of this enzyme in plasma leads to the failure to synthesize normal amounts of cholesteryl esters. Thus, it is not surprising that such defects lead to the formation of abnormal lipoproteins that
are enriched in unesterified cholesterol and phosphatidylcholine. The data presented in this section are derived from summaries from over 50 patients with familial LCAT deficiency and 11 patients with fish eye disease (FED) who have been reported in the literature. The major clinical and biochemical features of LCAT deficiency and FED are summarized in Table 4.

Table 4. A summary of the major clinical and biochemical features of homozygotes of familial LCAT deficiency and FED

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>Biochemical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCAT deficiency</td>
<td></td>
</tr>
<tr>
<td>Corneal opacities</td>
<td>↓ LCAT activity</td>
</tr>
<tr>
<td>Mild hemolytic anemia</td>
<td>↑ unesterified cholesterol</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>↑ phosphatidylcholine</td>
</tr>
<tr>
<td>Progressive renal insufficiency</td>
<td>↓ cholesteryl ester</td>
</tr>
<tr>
<td>Renal failure</td>
<td>HDL deficiency and ↓ apo A-I</td>
</tr>
<tr>
<td>Fish eye disease</td>
<td></td>
</tr>
<tr>
<td>Corneal opacities</td>
<td>↓ LCAT activity</td>
</tr>
<tr>
<td></td>
<td>HDL deficiency and ↓ apo A-I</td>
</tr>
<tr>
<td></td>
<td>Normal ratio of unesterified to esterified cholesterol</td>
</tr>
</tbody>
</table>

1.4.1 LCAT Deficiency

1.4.1.1 Corneal Changes

Corneal opacities develop in all patients with familial LCAT deficiency in early childhood and are easily detectable in the second decade of life. The entire corneal stroma contains numerous greyish dots giving the appearance of a diffuse clouding of the cornea. A much denser accumulation of these dots exists in the corneal
periphery resulting in an arcus-like appearance; its border, however, is less sharply defined than classical arcus lipoides senilis. Initial ultrastructural examination of sections obtained by superficial keratotomy (Bron et al., 1975; Bethell et al., 1975) revealed numerous vacuoles in both Bowman's layer and the anterior stroma, some of which contained electron dense or "membranous" deposits. Chemical analysis demonstrated high concentrations of phospholipid and unesterified cholesterol compared to the normal cornea (Winder et al., 1978). In subsequent studies, the staining of cryostat sections of the cornea confirmed the presence of lipid infiltration (Winder et al., 1985) and unesterified cholesterol (Cogan et al., 1992) throughout the stroma while electron microscopy indicated multilaminar inclusion bodies.

Corneal opacities have also been identified in other genetic disorders associated with HDL deficiency. For example, similar findings have been observed in patients with Tangier disease (Chu et al., 1979), HDL deficiency with planar xanthomas (Gustafson et al., 1979), combined apo A-I/C-III deficiency (Norum et al., 1982), apo A-I/C-III/A-IV deficiency (Schaefer et al., 1985) and in a frameshift mutation of the apo A-I gene (Funke et al., 1991a).

1.4.1.2 Hematological Abnormalities

Most patients with homozygous familial LCAT deficiency present with a mild normochromic anemia which from radioisotope kinetic data and bone marrow studies appears to be due to a moderate hemolysis combined with a reduced compensatory erythropoiesis (Gjone et al., 1968; Chevet et al., 1978). In addition, abnormalities in the appearance, lipid composition and function of erythrocytes have been observed (Table 5). Examination of peripheral blood smears often indicates an
increased proportion of target cells and occasional anisocytosis, poikilocytosis and stomatocytes. Measurement of the erythrocyte lipid composition has indicated increased concentrations of unesterified cholesterol and phosphatidylcholine, but decreased amounts of phosphatidylethanolamine and sphingomyelin. Total phospholipid concentrations, however, remain normal (Norum et al., 1970; Godin et al., 1978; Chevet et al., 1978). In some cases, a marked decrease in osmotic fragility of erythrocytes has been reported (Godin et al., 1978; Shojania et al., 1983). Additional functional abnormalities observed include a decreased sodium influx and reduced acetylcholinesterase activity (Murayama et al., 1984a). It is unclear if abnormalities also exist for other blood cells. However, platelet function and lipid composition appear normal (Nordoy and Gjone, 1971).

Analysis of bone marrow often reveals target cells and a small number of foam cells (Table 6). Giemsa stain has identified "sea-blue histiocytes" in both the bone marrow and spleen of many patients (Jacobsen et al., 1972). Ultrastructural studies have demonstrated that the histiocyte granules are composed of membranes in a lamellar arrangement suggestive of high concentrations of unesterified cholesterol and phosphatidylcholine.
Table 5. Abnormalities of composition and function of erythrocytes obtained from patients with familial LCAT deficiency.

<table>
<thead>
<tr>
<th>Erythrocyte Abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑ unesterified cholesterol</td>
</tr>
<tr>
<td>↑ phosphatidylcholine</td>
</tr>
<tr>
<td>↓ phosphatidylethanolamine</td>
</tr>
<tr>
<td>↓ sphingomyelin</td>
</tr>
<tr>
<td>↑ mechanical fragility</td>
</tr>
<tr>
<td>↓ osmotic fragility</td>
</tr>
<tr>
<td>↓ sodium influx</td>
</tr>
<tr>
<td>↓ acetylcholinesterase activity</td>
</tr>
</tbody>
</table>

1.4.1.3 Renal Disease

Proteinuria is a common finding in these patients and is frequently detected in the second and third decade of life. In addition, erythrocytes and hyaline casts are present in the urine of many patients. Electrophoresis of urinary protein indicates that albumin is the predominant protein although $\alpha_1$- and $\alpha_2$-migrating proteins are also present. The quantity of protein in the urine of the patient remains moderate (0.5 to 1.5 mg/ml) and persists for many years. Indices of renal function such as serum urea, serum creatinine and creatinine clearance usually remain normal in the first three decades of life. However, later in life, deterioration of renal function may occur suddenly and develop into a rapid progression of renal insufficiency with increasing proteinuria and hypertension. It is interesting to note that there are several patients with complete LCAT deficiency who, despite increasing age, have not developed proteinuria or impaired renal function (Gjone et al., 1974; Borysiewicz et al., 1982; Murayama et al., 1984b).

Light microscopy of kidney biopsies (Hovig and Gjone, 1973) have identified
foam cells in the glomeruli (Table 6). These kidney lesions are characterized by the deposition of phospholipid membranes and membrane-bound particles in the glomeruli, mesangium and arterioles. Lipid material has been found in the subendothelial space as well as in the basement membrane of renal arteries and arterioles. Analysis of the lipid composition of renal tissues has indicated elevated concentrations of unesterified cholesterol and phospholipid (Stokke et al., 1974). Electron microscopy has demonstrated the narrowing of capillary lumens, the detachment of endothelial cells from the basement membrane, fusion of endothelial foot processes and the presence of membrane-surrounded particles in both the subendothelial and subepithelial regions (Hovig and Gjone, 1973). Immunohistology has revealed that immunoglobulins and complement-components were only weakly positive. However, indirect immunofluorescence for apo B showed strong staining along the glomerular capillary walls as well as in mesangial regions (Ohta et al., 1986).

Table 6. Tissue localization of abnormal cell types found in familial LCAT deficiency

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target cells</td>
<td>peripheral blood, bone marrow</td>
</tr>
<tr>
<td>Foam cells</td>
<td>bone marrow, spleen, kidney</td>
</tr>
<tr>
<td>Sea-blue histiocytes</td>
<td>bone marrow, spleen</td>
</tr>
<tr>
<td>Multilaminar inclusion</td>
<td>peripheral blood, bone marrow, cornea, spleen, kidney</td>
</tr>
<tr>
<td>bodies</td>
<td></td>
</tr>
</tbody>
</table>
1.4.2 Fish Eye Disease

1.4.2.1 Corneal Changes

FED was initially reported in two Swedish families and was characterized by a severe HDL deficiency and extensive corneal opacities (Carlson and Philipson, 1979; Carlson, 1982). The latter was responsible for the unusual name of the disease, since the appearance of the eyes of affected individuals was said to be similar to that of boiled fish. In contrast to LCAT deficiency, there have been much fewer reports of patients with FED with a total number of 11 published patients from 6 families originating from Sweden, Germany, Algeria and the Netherlands. There is also one case from Canada whose biochemical description closely resembles FED (Frohlich et al., 1987).

Corneal opacities have been observed as early as the second decade and are similar in appearance to those described in familial LCAT deficiency. They consist of minute greyish dots throughout the entire corneal stroma with increasing density at the periphery revealing a diffuse bilateral arcus. However, the degree of opacification in FED appears to be greater than familial LCAT deficiency as visual acuity is often impaired at an earlier age. Analysis of the corneal disc of one patient obtained at keratoplasty revealed high concentrations of unesterified cholesterol and the presence of vacuoles in the stroma containing membrane-like material in the periphery (Carlson, 1982).

Other than the corneal opacities, there are no other clinical symptoms such as anemia, proteinuria or renal insufficiency commonly found in familial LCAT deficiency.
1.5 Laboratory Findings in LCAT Deficiency and Fish Eye Disease

1.5.1 Standard Lipid Profile

The standard lipid profile for both familial LCAT deficiency and FED is presented in Table 7. In each case, there is a significant reduction of HDL cholesterol and apo A-I, but total cholesterol levels are usually within the normal range. A moderate hypertriglyceridemia is common in both disorders. However, levels can be significantly elevated in LCAT deficiency after the onset of renal insufficiency. Since the lipid profiles of these disorders as well as other HDL deficiencies may be similar, additional special tests are required for a differential diagnosis.

1.5.2 Specialized Laboratory Tests

The determination of the cholesterol esterification rate (CER) and the measurement of LCAT enzyme activity as described previously using both synthetic and endogenous lipoprotein substrates is necessary to distinguish LCAT deficiency from FED. As indicated in Table 8, LCAT deficiency is associated with very low concentrations of plasma cholesteryl ester and a virtual absence of activity for both synthetic and endogenous substrates. In contrast, there is only a mild reduction in the percentage of esterified cholesterol in the plasma of FED patients and the endogenous cholesterol esterification rate may be within normal limits or only slightly reduced. However, when LCAT activity is measured using a synthetic proteoliposome substrate, the ability to esterify cholesterol is usually less than 10% of normal. Although there is considerable variation in plasma LCAT protein levels in LCAT deficiency (Albers et al., 1981), they are usually lower when compared to those concentrations observed in FED.
Table 7. Laboratory findings in representative homozygotes for familial LCAT deficiency and FED.

<table>
<thead>
<tr>
<th></th>
<th>Total Cholesterol (mmol/L)</th>
<th>HDL Cholesterol (mmol/L)</th>
<th>Triglycerides (mmol/L)</th>
<th>Apo A-I (g/L)</th>
<th>Apo B (g/L)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Familial LCAT deficiency (n=5)</td>
<td>3.56±1.14</td>
<td>0.14±0.11</td>
<td>2.74±1.30</td>
<td>0.44±0.06</td>
<td>0.35±0.01</td>
<td>Frohlich et al., 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Funke et al., 1993</td>
</tr>
<tr>
<td>Fish eye disease (n=4)</td>
<td>5.11±0.40</td>
<td>0.05±0.06</td>
<td>2.80±0.71</td>
<td>0.28±0.09</td>
<td>0.83±0.11</td>
<td>Funke et al., 1991b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Kastelein et al., 1992</td>
</tr>
<tr>
<td>Normals (n=8)</td>
<td>5.39±0.85</td>
<td>1.48±0.31</td>
<td>0.97±0.27</td>
<td>1.44±0.2</td>
<td>1.00±0.2</td>
<td>Frohlich et al., 1988</td>
</tr>
</tbody>
</table>
Table 8. Laboratory findings in familial LCAT deficiency and FED: specialized tests.

<table>
<thead>
<tr>
<th></th>
<th>% Cholesteryl esters</th>
<th>LCAT Activity (nmol/h/ml)</th>
<th>Cholesterol esterification rate (CER) in plasma (nmol/h/ml)</th>
<th>LCAT Concentration (µg/ml)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Familial LCAT deficiency (n=5)</td>
<td>12±2</td>
<td>0.6±0.8</td>
<td>0</td>
<td>0.09±0.13</td>
<td>Frohlich et al., 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Funke et al., 1993</td>
</tr>
<tr>
<td>Fish eye disease (n=4)</td>
<td>56±3</td>
<td>0.6±0.3</td>
<td>58±13</td>
<td>2.30±0.21</td>
<td>Funke et al., 1991b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Kastelein et al., 1992</td>
</tr>
<tr>
<td>Normals (n=7)</td>
<td>68±7</td>
<td>30±2.6</td>
<td>94±27</td>
<td>4.83±0.2</td>
<td>Funke et al., 1991b</td>
</tr>
</tbody>
</table>
1.5.3 Lipoprotein Abnormalities in Familial LCAT Deficiency

Analysis of the plasma from LCAT deficient patients reveals multiple lipoprotein abnormalities and a considerable heterogeneity of plasma lipid concentrations. However, the most consistent biochemical findings are high plasma concentrations of unesterified cholesterol and phosphatidylcholine and low concentrations of plasma cholesteryl ester and lysolecithin. The very low amounts of plasma cholesteryl ester that are present are likely to originate from the intestine as a result of the action of the intracellular enzyme acyl coenzyme A:cholesterol acyltransferase (ACAT). The higher proportions of palmitic and oleic acids in comparison with linoleic acid present in cholesteryl esters are consistent with the pattern normally produced by ACAT activity.

Plasma lipoproteins have been isolated and analysed by a number of different methodologies including ultracentrifugation, gel filtration, electrophoresis and electron microscopy. Very low density lipoproteins (VLDL) of d<1.006 g/ml are usually elevated in concentration and contain higher concentrations of unesterified cholesterol and phosphatidylcholine relative to triglyceride and protein (Glomset et al., 1973). Apoprotein analysis has indicated increased amounts of apo C-I and apo E, but decreased levels of apo C-II and apo C-III (Glomset et al., 1980). After fasting overnight, the VLDL fraction still contains a large number of particles that are greater than 90 nm in diameter. However, dietary studies (Glomset et al., 1975) have demonstrated that the proportion of these large lipoproteins can be markedly reduced when the patients consume fat-free diets, suggesting that these particles are in fact chylomicrons.

Abnormalities are also present in intermediate density lipoproteins (IDL,
d=1.006-1.019 g/ml). These particles have been shown to be heterogeneous in nature and rich in unesterified cholesterol and triglyceride (Glomset et al., 1973). One possible cause of the triglyceride enrichment of IDL may arise from the decreased ability of hepatic lipase to hydrolyse triglyceride in these particles (Murano et al., 1987).

Gel filtration of the low density lipoprotein fraction (LDL, d=1.019-1.063 g/ml) usually yields three relatively well defined subfractions, whereas only one fraction is obtained from normal LDL. The relative proportion of each fraction differs among individuals and in a similar manner to the VLDL fraction, the quantity of the larger molecular weight LDL particles appears to be related to the fat content in the diet (Glomset et al., 1975; Borysiewicz et al., 1982). The largest subfraction contains multilaminar particles which are 90 nm in diameter consisting mostly of lipid with a high molar ratio of unesterified cholesterol to phospholipid (2:1). The small amount of protein present in these particles is mostly albumin.

A second subfraction of patient LDL is made up of particles which range in diameter from 30 to 80 nm and like the larger particles, have no obvious counterparts in normal plasma. However, the characteristics of these particles are similar to the abnormal lipoprotein LP-X present in cholestasis (Hamilton et al., 1971). These particles are rich in unesterified cholesterol and phosphatidylcholine and have a disc-shaped appearance when examined by electron microscopy. Apo C, mainly C-I, makes up the primary protein component of these particles. Spherical particles resembling normal remnants of VLDL and chylomicrons are also present in this fraction.

The third subfraction of patient LDL are spherical and have a similar size...
compared to normal LDL (20-22 nm). The major protein is apo B\textsubscript{100} and the proportion of core lipid to surface lipid and protein is normal. However, the lipid composition is markedly different with 1.5 to 3 times as much unesterified cholesterol and phosphatidylcholine and 13-times as much triglyceride per mg of protein (Norum \textit{et al.}, 1971).

Like the other lipoproteins associated with familial LCAT deficiency, high density lipoproteins (HDL, d=1.063-1.21 g/ml) are heterogeneous in nature and consist of abnormal shapes and lipid composition. Many of the particles are disc-shaped while others remain spherical, but unusually small. Disc-shaped particles mainly consist of unesterified cholesterol and phosphatidylcholine. Some contain mixtures of apo A-I, apo A-II, apo A-IV and the C apolipoproteins (Torsvik, 1972; Soutar \textit{et al.}, 1982) while other discs remain rich in apo E (Mitchell \textit{et al.}, 1980). The small spherical HDL are about 6 nm in diameter and contain unesterified cholesterol, phosphatidylcholine, a small amount of core lipid and 2 molecules of apo A-I (Chen \textit{et al.}, 1984).

1.5.4 Lipoprotein Abnormalities in Fish Eye Disease

Results from the analysis of the composition and structure of the plasma lipoproteins in FED were obtained in large part from the original Swedish patients (Carlson, 1982; Carlson and Holmquist, 1983; Forte and Carlson, 1984). The concentrations of VLDL cholesterol and triglyceride are increased five-fold although the triglyceride to cholesteryl ester ratio remains normal. VLDL particles have a larger mean diameter due to a much broader range of particle size not present in controls.
Both the IDL and LDL fractions are enriched in triglyceride, particularly LDL, in which the triglyceride to cholesteryl ester ratio is ten-fold greater than normal. The LDL from FED patients are mostly homogeneous round structures with a smaller mean diameter than control LDL. Occasionally, a few large structures (50 to 85 nm diameter) are seen in the patient LDL fraction, but are absent from normal LDL.

The HDL fraction of these patients demonstrate the greatest abnormalities. The total mass of HDL is only 10% that of controls and the percentage of unesterified cholesterol (60%) is about three-fold greater compared to control HDL. There is an enrichment of apo E and a reduction of the A apolipoproteins by 90% with a relatively greater reduction of apo A-II compared to apo A-I (Carlson and Holmquist, 1983). The patient HDL contained several particle morphologies. The two major components are small round particles with a diameter of 7.6 nm and discoidal particles with a thickness of 4.4 nm and an average diameter of 17.4 nm (Forte and Carlson, 1984). There are also larger particles present which range from 40 to 90 nm in diameter.

1.6 Heterozygotes of LCAT Deficiency and Fish Eye Disease

1.6.1 Laboratory Findings in LCAT Deficiency

The measurement of the endogenous cholesterol esterification rate does not differentiate heterozygotes from unaffected family members or normal subjects. However, LCAT activity is reduced to 50% of normal and can be used to assign genotype in non-obligate heterozygotes. Despite these changes in LCAT activity, no major abnormalities of plasma lipoproteins have been observed in heterozygous LCAT deficiency. However, abnormalities in erythrocyte composition and function
have been described in several heterozygotes (Godin et al., 1978; Jain et al., 1982). In addition, there have been reports of hyperlipidemia, especially hypercholesterolemia, in several different kindreds (Torsvik et al., 1968; Chevet et al., 1978; Borysiewicz et al., 1982).

A more detailed report of one family indicated that heterozygotes had significantly higher fasting plasma triglycerides, apo B, and lower HDL cholesterol and apo A-I than the unaffected family members (Frohlich et al., 1988). In addition, HDL cholesteryl ester was decreased by approximately 50%. Differences in these values were intermediate between those for normal and homozygous family members. Therefore, it seems likely that most of these abnormalities occur as a result of reduced LCAT activity.

1.6.2 Laboratory Findings in Fish Eye Disease

As with LCAT deficiency, heterozygotes of FED can only be distinguished by the measurement of LCAT activity and not the CER. In FED, LCAT activity is reduced to 50% of normal. Although not necessarily a consistent finding, HDL cholesterol levels are often decreased in heterozygotes. In contrast, LCAT protein levels and endogenous cholesterol esterification rates are usually within the normal range.

1.7 Pathophysiology

1.7.1 LCAT Deficiency

1.7.1.1 Lipoprotein Abnormalities

From the analysis of the lipoprotein abnormalities in LCAT deficient plasma, it appears that the failure to esterify cholesterol causes a chain reaction of events
which affects the normal metabolism of all lipoprotein classes. The changes in the composition, structure and metabolism of these lipoproteins give rise to the tissue pathology that ultimately lead to clinical complications. To determine the mechanisms associated with these different abnormalities, several studies have conducted in vitro experiments utilizing patient lipoproteins and normal LCAT.

Incubation experiments with partially purified LCAT and patient lipoproteins (Glomset et al., 1970) have indicated that the enzyme reacts primarily with HDL and to a much lesser extent with lipoproteins of lower density. These results indicated that the lipoprotein abnormalities present in familial LCAT deficiency were not caused by the inability of these particles to react with LCAT, but were directly related to the LCAT deficiency. Later studies demonstrated that LCAT converted patient HDL from a highly heterogeneous mixture of disc-shaped particles and small spherical particles to material that resembled normal HDL$_2$ and HDL$_3$ (Glomset et al., 1980). The time course of this reaction implied that the small spherical HDL were converted into HDL$_3$ and the discs into HDL$_2$. These results may indicate that HDL$_3$ and HDL$_2$ originate from different nascent particles and that the role of LCAT is not simply a conversion of HDL$_2$ to HDL$_3$. There is considerable evidence that disc-shaped HDL represent newly formed or nascent HDL. Structures of similar size and shape have been identified in perfused rat livers (Hamilton et al., 1976), human hepatic venous blood (Turner et al., 1979) and from the human hepatoblastoma cell line, HepG2 (Thrift et al., 1986).

In vitro experiments have demonstrated that the action of LCAT on patient HDL indirectly affects the characteristics of both LDL and VLDL (Glomset et al., 1970). Several studies have indicated that the source of unesterified cholesterol and
phosphatidylcholine consumed by the LCAT-HDL complex is derived mostly from LDL and VLDL (Fielding and Fielding, 1981; Park et al., 1987; Cheung and Wolfe, 1989; Huang et al., 1993) and that the majority of cholesteryl esters synthesized are transferred to these lipoproteins in exchange for triglyceride by plasma cholesteryl ester transfer protein (CETP) (Chajek and Fielding, 1978). The combined effect of these lipid transfer reactions in patient plasma incubated with normal LCAT results in the normalization of LDL and VLDL with a particular reduction in the large sized particles and an increase in cholesteryl ester in the normal sized LDL and VLDL.

When patients with familial LCAT deficiency consume fat-free diets for several days, the concentration of the large VLDL, the large and intermediate sized LDL and in one case the small spherical HDL all decreased (Glomset et al., 1975). The possibility that these particles are derived from chylomicrons is supported by in vitro studies in which lipoprotein lipase (LPL) incubated with triglyceride-rich lipoproteins produced particles which were rich in unesterified cholesterol and phosphatidylcholine (Deckelbaum et al., 1979). Thus, these lipoproteins might be formed from surface remnant unesterified cholesterol, phosphatidylcholine and protein which would otherwise be normally metabolized. There are conflicting reports of the ability of these particles, such as LP-X, to act directly as substrates for LCAT (Wengeler and Seidel, 1973; Patsch et al., 1977), but there is evidence that LP-X can provide a source of unesterified cholesterol for the LCAT reaction (Ritland and Gjone, 1975). Thus, it may be that the lipids from these surface remnants are transported to LDL and VLDL particles which in turn supply the substrate for the LCAT reaction in the HDL fraction.

In familial LCAT deficiency, the relationship between tissue abnormalities and
the presence of abnormal lipoprotein particles remains unclear. Results of incubation studies with normal erythrocytes and patient plasma suggest that the abnormal lipid composition of erythrocytes is related to the high concentrations of unesterified cholesterol in patient plasma (Norum and Gjone, 1968). It is likely that the altered membrane composition and function of erythrocytes observed in these patients contributes to the hemolytic anemia normally present in LCAT deficiency.

1.7.1.2 Renal disease

Multilaminar structures have been consistently found in the plasma, bone marrow, spleen, cornea and kidneys of patients with familial LCAT deficiency and appear to be responsible in part for the pathology recognized in these different tissues. It has been suggested that the accumulation of these membranes and membrane-bound particles in the glomerular capillaries may cause damage to the endothelium and underlying basement membrane (Hovig and Gjone, 1973). These changes could be responsible for the proteinuria, reduced filtration efficiency and increasing renal insufficiency observed in these patients. Initially, it was proposed by Gjone et al. (1974) that the large molecular weight LDL may be responsible for the impaired kidney function. From the initial eight patients studied, this lipoprotein was identified in seven subjects with renal involvement, but was not present in the only patient without proteinuria and normal renal function (Gjone et al., 1974). However, in a subsequent report, these particles were found in large quantity in two cases of familial LCAT deficiency which had no proteinuria and normal renal function (Borysiewicz et al., 1982). Kidney biopsies taken from these asymptomatic patients contained widespread subendothelial, subepithelial and intramembranous glomerular
lipid deposits as well as occasional foam cells. In addition, a similar lipid accumulation has been observed in renal biopsies taken six months after transplantation from LCAT deficient patients with normally functioning grafts (Flatmark et al., 1977). Therefore, it would seem that the infiltration of lipid alone is not sufficient to initiate the onset of reduced renal function. It has been suggested by Borysiewicz et al. (1982) that this condition could cause a reduced clearance of immune complexes predisposing to the development of glomerulonephritis, which has been reported in several patients with LCAT deficiency.

From a review of the literature, there are total of 10 reported cases of familial LCAT deficiency in which the patient had no proteinuria and normal renal function. Three cases presented with partial LCAT deficiency in which the percentage of plasma cholesteryl ester was greater than 40% (Sakuma et al., 1982; Gylling and Miettinen, 1992). In two other cases, the patients were very young at examination and could represent early stages of the disease (Frohlich et al., 1978; Winder and Bron, 1978). There have been four reported cases of females, all of which were over 50 years of age at examination (Gjone et al., 1974; Borysiewicz et al., 1982; Murayama et al., 1984) which had no symptoms but had male siblings which demonstrated typical signs of renal involvement. Also, in three cases, the asymptomatic patient was a vegetarian (Murayama et al., 1984b; Takata et al., 1989; Frohlich, unpublished results). As a result, the influence of diet as well as sex hormones should also be considered as factors which may affect the development of renal disease.
1.7.1.3 Corneal changes

The pathophysiological mechanism responsible for the development of corneal opacities remains to be determined. As mentioned earlier, there are a number of different disorders associated with HDL deficiency and corneal lipid deposits, but the degree of opacification does not correlate with the extent of HDL deficiency or the risk of premature atherosclerosis. From the analysis of the different characteristics of the lipoproteins in these disorders, it is more likely that the specific composition and structure of the abnormal HDL particles will reflect the extent and rate at which they accumulate in the corneal tissue.

1.7.2 Fish Eye Disease

1.7.2.1 Lipoprotein Abnormalities

Initial study of the activity of LCAT from FED plasma indicated near normal rates of esterification when the patients lipoproteins acted as substrate but was only 10-15% of normal when proteoliposomes were used as substrate (Carlson and Holmquist, 1985a). This "paradoxical esterification" of plasma cholesterol was further investigated by performing experiments with mixtures of isolated lipoproteins and plasma fractions from patients with FED and normal lipoproteins and plasma fractions from controls. HDL isolated from FED patients were found to be excellent substrates when incubated with normal LCAT as present in lipoprotein depleted plasma from healthy individuals (Carlson and Holmquist, 1985b). Most of the unesterified cholesterol in these HDL fractions became esterified and shifted the size of these particles in the range of that of normal HDL. However, lipoprotein depleted FED plasma was unable to esterify cholesterol in HDL isolated from either
control subjects or from the plasma of FED patients. In addition, the unesterified cholesterol contained within the combined fraction of LDL and VLDL was esterified in the presence of lipoprotein depleted plasma from a control subject or a patient with FED (Carlson and Holmquist, 1985c). These results led to the formulation of a hypothesis put forth by Carlson and Holmquist that two different LCAT activities exist in normal plasma. One of these activities, was denoted α-LCAT which esterifies cholesterol present in HDL particles and the other, was denoted β-LCAT which esterifies cholesterol present in LDL and VLDL particles. Further evidence to support this hypothesis was demonstrated when HDL cholesteryl ester content and its particle size were normalized after small amounts of highly purified LCAT were added to FED plasma (Holmquist and Carlson, 1988). According to this new concept, FED would be classified as an α-LCAT deficiency, in contrast to familial LCAT deficiency, which lacks both α- and β-LCAT activities.

In normal plasma, the majority of LCAT is associated with HDL and probably continuously cycles from mature HDL to newly formed nascent HDL while only a small portion of LCAT remains associated with LDL (Chung et al., 1982). In FED, the binding of LCAT to HDL may be impaired resulting in a shift in the distribution of LCAT towards LDL enabling the maintenance of normal endogenous plasma cholesterol esterification rates. Despite the normal esterification of cholesterol in the LDL fraction, abnormalities persist in the lower density lipoproteins. The accumulation of triglycerides in LDL is probably a reflection of the inability of CETP to carry out its normal lipid exchange of cholesteryl ester for triglyceride in HDL.
1.8 Coronary Artery Disease in HDL Deficiency Syndromes

A causal relationship between decreased HDL levels and coronary artery disease is now well established. There are a wide variety of HDL deficiency syndromes which consist of disorders of different etiology and clinical phenotypic expression. Disorders such as HDL deficiency with planar xanthomas (Gustafson et al., 1979), apo A-I/C-III deficiency (Norum et al., 1982) and apo A-I/C-II/A-IV deficiency (Schaefer et al., 1985) have been associated with premature coronary heart disease. In contrast, such an association has not been reported for Tangier disease (Chu et al., 1979), HDL deficiencies secondary to LPL deficiency (Brunzell, 1989) or mutations of the apo A-I gene (Franceschini et al., 1980; von Eckardstein et al., 1989; Funke et al., 1991a). In addition, although some patients affected with either LCAT deficiency or FED have developed coronary artery disease, the clinical data does not indicate that they are at an increased risk. One possible explanation of this apparent paradox may be related to the presence of small pools of HDL in these disorders unlike HDL deficiencies caused by defects of the A-I/C-III/A-IV gene cluster. These small nascent HDL particles may still be able to induce an efflux of unesterified cholesterol from peripheral tissues and transport cholesterol to other lipoproteins or directly to the liver in order to maintain reverse cholesterol transport. This notion is supported by the description of an LCAT-independent mechanism of reverse cholesterol transport discussed previously (Huang et al., 1993).

1.9 Treatment of Familial LCAT Deficiency

Dietary treatment in the form of fat restriction has been implemented as a conservative approach in several patients in order to reduce the levels of the
potentially pathogenic large molecular weight lipoprotein particles found in the LDL and VLDL fractions. Presently, there is no information of the effectiveness of such a treatment in either the prevention or amelioration of reduced renal function.

The first reported case of whole plasma or blood transfusions in familial LCAT deficiency consisted of the administration of 450 ml blood and 500 ml plasma in a single transfusion (Norum and Gjone, 1968). This resulted in an immediate increase in plasma cholesteryl ester which continued at a slower rate reaching a peak at 6 days, followed by a decrease to pre-treatment levels in 2 weeks. The in vivo half-life of LCAT in plasma was calculated to be 4.6 days. In a second report, the plasma transfusion consisted of 2200 ml of plasma over a period of ten days (Murayama et al., 1984b). LCAT activity only increased from 9.4% of the normal value to 17.4%. Despite this small increase in LCAT activity, there were significant increases in cholesteryl ester in all lipoprotein fractions and electron microscopy demonstrated that both VLDL and LDL fractions converted to near normal size particles. However, there appeared to be no effect on the size or shape of the HDL particles as the characteristic disc-shaped particles still remained. The effectiveness of the treatment continued for 7 days after the cessation of the infusion. These reports indicate that plasma infusion is a relatively inefficient form of treatment as the administration of large amounts of plasma provided only a modest improvement of the biochemical findings for a short duration of time.

Renal transplantation has been performed in several patients with familial LCAT deficiency. Although the plasma lipid and lipoprotein abnormalities persist, a significant reduction in triglyceride levels was observed in one patient after transplantation (Norum et al., 1989). As mentioned previously, there is evidence of
early lipid deposition in the transplanted kidneys; however, normal renal function can still be maintained (Flatmark et al., 1977). From a biochemical point of view, a liver transplant would correct the underlying defect of LCAT deficiency; however, it would be difficult to justify such a high risk procedure for what would otherwise be considered a normal functioning liver.

1.10 LCAT Gene Structure and Expression

The LCAT gene is found on the long arm of chromosome 16 and is divided into six exons spanning 4.2kb of genomic DNA (McLean et al., 1986b). Southern blot hybridization data suggest that there is only one LCAT gene in humans. An unusual feature of the LCAT mRNA is that the poly(A) signal overlaps the carboxyl-terminal glutamic acid and stop codons, and the 3' untranslated region contains only 23 bases (McLean et al., 1986a). The first 59 nucleotides of exon 5 share a 66% DNA sequence homology with the 3' terminal coding region of the apo E gene. This appears to be the only region which could potentially form an α-helix.

Functional analysis of the 2.9 kb of the 5' flanking sequences of the LCAT gene indicated that there was no change in promoter activity for deletions from -2900 to -300, a 50% reduction after deleting to position -71, and a 100% reduction after deleting to position -42 (Meroni et al., 1991). Site-directed mutagenesis within the 71 bp region indicated that the activity of the promoter was dependent on the integrity of two putative Sp1 binding sites. In addition, since the promoter was active in both hepatic and non-hepatic cell lines, the presence of hepato-specific regulatory elements were not indicated.

The human LCAT mRNA has been detected in the liver and HepG2
(hepatocyte) cells, but not in small intestine, spleen pancreas, placenta or adrenal tissue (McLean et al., 1986b). Analysis of the tissue expression in the rat also indicated the presence of LCAT mRNA in the brain and testes (Warden et al., 1989). More detailed analysis of the cellular localization of LCAT mRNA in rhesus monkeys demonstrated that in addition to hepatocytes and brain cells, LCAT mRNA was present in the basal cell layer of the dermis (Smith et al., 1990). In the brain, LCAT mRNA was synthesized by scattered neurons, neuroglial cells, ependymal cells, as well as a discrete cell layer in the cerebellum. In a recent study, the expression of LCAT in baboon tissues was analysed using an RNase protection assay (Hixson et al., 1993). The highest levels of LCAT mRNA were found in the cerebellum and liver (33% compared to cerebellum), whereas only trace amounts were identified in the ileum, spleen and cerebral cortex. From these results, it would seem that LCAT also participates in lipid transport in the brain, particularly the cerebellum.

Comparison of the percent homology of human LCAT nucleotide and amino acid sequences with the LCAT sequences reported for mouse (Warden et al., 1990), rat (Meroni et al., 1990), pig (Pritchard et al., unpublished observations) and baboon (Hixson et al., 1993) is depicted in Table 9. These data demonstrate a high degree of homology of LCAT sequence between all species. The rat and mouse sequences have a higher degree of homology when compared to one another, 92% and 90% at the amino acid and nucleotide levels, respectively (Meroni et al., 1990). All of the major structural features of LCAT such as the catalytic Ser, glycosylation sites and Cys residues are conserved among all species.
Table 9. Homology of human LCAT with different species of LCAT.

<table>
<thead>
<tr>
<th>Species</th>
<th>Nucleotide</th>
<th>Amino Acid</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>85%</td>
<td>85%</td>
<td>Warden et al., 1990</td>
</tr>
<tr>
<td>Rat</td>
<td>84%</td>
<td>86%</td>
<td>Meroni et al., 1990</td>
</tr>
<tr>
<td>Pig</td>
<td>90%</td>
<td>92%</td>
<td>Pritchard et al.,</td>
</tr>
<tr>
<td>Baboon</td>
<td>97%</td>
<td>98%</td>
<td>unpublished observations</td>
</tr>
</tbody>
</table>

1.11 Gene Defects Associated with LCAT Deficiency and Fish Eye Disease

Analysis of the clinical and biochemical phenotypes of relatives within different families affected with either familial LCAT deficiency or FED clearly indicate that these diseases are inherited as an autosomal recessive trait. Over the last 5 years, several laboratories have reported gene defects that appear to be causative for LCAT deficiency and FED. This has been done by cloning and sequencing of the entire LCAT gene or by amplification and sequencing of the exon and intron/exon boundaries. The sequence analysis of the LCAT gene in patients with familial LCAT deficiency and FED has revealed a large variety of mutations spread throughout all six exons (Table 10). Only one of the mutations (Gly183→Ser) appears to affect the catalytic site directly by disrupting the highly conserved structural motif Gly-X-Ser-X-Gly associated with the catalytic domain of many lipases (Brenner, 1988). The causative nature of these mutations has been established from the fact that none of these defects have been identified in a normal population and, in most cases, the observed mutation was the only one found on each allele analysed. The assignment of these mutations to either LCAT deficiency or FED is often initially based on the presence or absence of specific clinical symptoms. However, more detailed
biochemical analyses have indicated that such a segregation should be used with caution. The variety of LCAT concentrations and activities observed in patient plasma indicate a wide range of functional defects which often consist of characteristics of both LCAT deficiency and FED. The recreation and expression of selected mutants of LCAT has been used to investigate the functional significance of different inherited defects of the LCAT gene.

1.12 Mutagenesis and Expression of Recombinant LCAT

The first genetic defect of LCAT to be reported was also the first to be expressed in mammalian cell culture (Taramelli et al., 1990). Expression vectors containing either the wild type or mutant LCAT (Arg147→Trp) genomic clones were transiently transfected into monkey kidney COS-1 cells. Despite comparable levels of mRNA transcribed from normal and mutated alleles, LCAT protein and activity in the culture medium could only be detected for the wild type construct. It was suggested that this mutation may cause a structural change resulting in a decreased stability of the enzyme inside or outside the cell.
### Table 10. Natural mutations of the LCAT protein.

<table>
<thead>
<tr>
<th>Codon</th>
<th>Structural Change</th>
<th>Zygosity</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Pro→Leu</td>
<td>Homozygous</td>
<td>FED</td>
<td>Skretting and Pyrdz, 1992</td>
</tr>
<tr>
<td>10</td>
<td>Pro→Frameshift</td>
<td>Homozygous</td>
<td>LCAT Def.</td>
<td>Bujo et al., 1991</td>
</tr>
<tr>
<td>32</td>
<td>Leu→Pro</td>
<td>Heterozygous</td>
<td>LCAT Def.</td>
<td>McLean, 1992</td>
</tr>
<tr>
<td>83</td>
<td>Tyr→Stop</td>
<td>Heterozygous</td>
<td>LCAT Def.</td>
<td>Klein et al., 1993a</td>
</tr>
<tr>
<td>93</td>
<td>Ala→Thr</td>
<td>Homozygous</td>
<td>LCAT Def.</td>
<td>Funke et al., 1993</td>
</tr>
<tr>
<td>119</td>
<td>Gly→Frameshift</td>
<td>Heterozygous</td>
<td>LCAT Def.</td>
<td>Gotoda et al., 1991</td>
</tr>
<tr>
<td>123</td>
<td>Thr→Ile</td>
<td>Homozygous</td>
<td>FED</td>
<td>Funke et al., 1991b</td>
</tr>
<tr>
<td>135</td>
<td>Arg→Trp</td>
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<td>Funke et al., 1993</td>
</tr>
<tr>
<td>141</td>
<td>Gly Insertion</td>
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<td>Gotoda et al., 1991</td>
</tr>
<tr>
<td>144</td>
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<td>FED</td>
<td>Contacos et al., 1993</td>
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<tr>
<td>147</td>
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<td>Taramelli et al., 1990</td>
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<td>156</td>
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<td>Klein et al., 1993a</td>
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<td>158</td>
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<td>183</td>
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<td>McLean, 1992</td>
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</tr>
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<td>Gotoda et al., 1991</td>
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<tr>
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<td>McLean, 1992</td>
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<td>Maeda et al., 1991</td>
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<tr>
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<td>McLean, 1992</td>
</tr>
<tr>
<td>347</td>
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<td>FED</td>
<td>Klein et al., 1992</td>
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<td>375</td>
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<td>LCAT Def.</td>
<td>Funke et al., 1993</td>
</tr>
<tr>
<td>391</td>
<td>Asn→Ser</td>
<td>Heterozygous</td>
<td>FED</td>
<td>Pritchard et al., unpublished observations</td>
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LCAT Def., familial LCAT deficiency; FED, Fish eye disease
The application of site-directed mutagenesis and the in vitro expression of recombinant LCAT (rLCAT) has provided new insights into the catalytic function of LCAT (Francone and Fielding, 1991a; Francone and Fielding, 1991b; Francone et al., 1993; Qu et al., 1993). These same tools have been used to recreate natural mutations of the LCAT gene in order to establish their functional significance. The in vitro expression of rLCAT containing a Tyr156→Asn substitution in human embryonic kidney-293 cells indicated that this mutant enzyme was secreted inefficiently and was only partially active (Klein et al., 1993a). It was suggested that this mutation may cause irregular folding of the protein resulting in reduced secretion and/or rapid degradation of the enzyme. In addition, it was speculated that the Tyr156→Asn mutation may disrupt the function of an α-helix conformation predicted to span residues 156-169 of human LCAT.

In a recent report by Klein et al. (1993b), the expression of rLCAT containing a Leu300 deletion revealed normal mRNA and intracellular LCAT concentrations, but only very low amounts of secreted rLCAT which had a normal specific activity. Thus, the authors suggest that the FED-like symptoms present in the patient with this defect arise from a quantitative rather than a functional deficiency of the enzyme.

The continuing analysis of mutant recombinant enzymes should increase our understanding of the structure-function relationships of LCAT and provide a basis to investigate the specific mechanisms responsible for the biochemical and clinical abnormalities associated with this disease.
1.13 Rationale

The relationship between decreased levels of plasma HDL and an increased risk of coronary artery disease is well established. However, there are several rare familial disorders of HDL metabolism in which, despite very low levels of HDL cholesterol, affected individuals do not appear to be at an increased risk for premature atherosclerosis. Familial LCAT deficiency and fish eye disease are two examples of such inherited disorders. Thus, to determine the basis of this paradox, a more detailed understanding of lipoprotein metabolism in LCAT deficiency syndromes is necessary.

The application of site-directed mutagenesis and in vitro expression enables the recreation, expression and functional analysis of a variety inherited defects of the LCAT gene. Through the description of the molecular basis of the phenotypic heterogeneity which exists for LCAT deficiency syndromes, we can identify relationships between specific structural changes and the functional properties of the enzyme.

According to the hypothesis of reverse cholesterol transport, LCAT plays a key role in the delivery of excess cellular cholesterol from peripheral tissues to the liver for excretion. As a result, if we are to fully understand the role of LCAT in reverse cholesterol transport, we must determine the factors that govern its ability to esterify cholesterol in different lipoprotein classes. Therefore, a complete understanding of the relationship between LCAT function and HDL metabolism is essential for the characterization of the anti-atherogenic properties of HDL.
1.14 Specific Aims

(i) To establish the expression of recombinant LCAT in mammalian cell culture as a model to analyse the structure and function of human plasma LCAT.

(ii) To create a series of natural mutations associated with familial LCAT deficiency and fish eye disease through PCR site-directed mutagenesis.

(iii) To express these mutant cDNA constructs by transient transfection into monkey kidney COS-1 cells.

(iv) To establish the functional significance of each mutation through the measurement of the specific activity using synthetic HDL-like substrates and heat-inactivated plasma.

(v) To create a series of stably transfected baby hamster kidney (BHK) cell lines which secrete greater quantities of recombinant protein allowing the analysis of the lipoprotein substrate specificity for each mutant enzyme using synthetic HDL-like substrates, LDL and VLDL.
2 MATERIALS AND METHODS

2.1 Materials

A full length LCAT cDNA contained within pUC19 was kindly supplied by John McLean, Genentech, Inc., San Francisco. Restriction and modification enzymes for the manipulation of DNA sequences were purchased from Bethesda Research Laboratories (BRL, Burlington, Ont.), Pharmacia-LKB Biotechnology (Baie d’Urfe Que.), and Boehringer Mannheim Corporation (BMC, Laval, Que.). Enzymes and reagents for DNA sequencing were from United States Biochemical (USB, Cleveland, Ohio). Geneclean and MERmaid kits (Bio101, La Jolla, Ca.) were used for the purification of double stranded DNA fragments. Radiolabeled products such as $\text{[}^{35}\text{S}\text{]}$-dATP, $\text{[}^{35}\text{S}\text{]}$methionine, $\text{[}^{3}\text{H}\text{]}$cholesterol and $\text{[}^{14}\text{C}\text{]}$methylated protein molecular weight markers were obtained either from New England Nuclear or Amersham Canada Ltd. Before autoradiography, fixed gels were equilibrated with Amplify (Amersham Canada Ltd.) and exposed on X-Omat AR film from Eastman-Kodak.

Oligonucleotides were prepared in the Oligonucleotide Synthesis Laboratory, Department of Biochemistry, UBC. The pNUT vector (Palmiter et al., 1987; Funk et al., 1990) and BHK cells were kindly provided by Dr. Ross MacGillivray, Department of Biochemistry, UBC. COS-1, an SV40-transformed African Green Monkey kidney cell line, was obtained from the American Type Culture Collection (Rockville, Md.). All tissue culture reagents including fetal bovine serum (FBS), Dulbecco’s Modified Eagle Medium (DMEM) and reduced serum medium (Opti-MEM) were supplied by Gibco-BRL (Mississauga, Ont.) Methotrexate (Cyanamid Canada, Inc., Montreal, Que.) for selection medium was obtained in sterile saline from the University Hospital Pharmacy.
Endoglycosidase H, N-glycanase and tunicamycin were supplied by Boehringer Mannheim Corporation whereas neuraminidase (Clostridium perfringens, type V) was obtained from the Sigma Chemical Co. (St. Louis, Mo.)

Polyclonal goat anti-human LCAT antibodies were kindly provided by Dr. Andras Lacko, Texas College of Osteopathic Medicine, University of North Texas, Fort Worth. Agarose-immobilized Protein G used for the immunoadsorption of recombinant LCAT was supplied by Pharmacia-LKB Biotechnology. Electrophoresis grade reagents, protein G conjugated to horseradish peroxidase (Protein G-HRP) and a protein assay kit were obtained from BioRad (Richmond, Ca.).

The concentration of unesterified cholesterol in plasma and isolated lipoproteins was determined enzymatically by a reagent kit (Boehringer Mannheim). All other chemicals were of reagent grade or better and were purchased either from Sigma Chemical Co. or from BDH Inc. (Vancouver, B.C.)

2.2 Molecular Biology Methods

2.2.1 Growth and Transformation of *E. Coli*

*E. Coli* strains DH5α or MC1061 were maintained in LB (10g/L tryptone, 5g/L yeast extract, 10g/L NaCl). Frozen bacterial stocks were prepared in 20% glycerol from late log-phase broth cultures and were stored at -70°C.

Competent *E. Coli* were prepared by inoculating a single colony into a 20 ml TYM broth (20 g/L tryptone, 5 g/L yeast extract, 5.84 g/L NaCl, 1.20 g/L MgSO₄). After the growth of the cells had achieved midlog phase (OD₆₀₀ = 0.2-0.8), they were added to a 100 ml of TYM. After an OD₆₀₀ of 0.5-0.9 was reached, the culture was diluted with TYM to 500 ml. When an OD of 0.6 was reached, the culture was
rapidly cooled on ice, pelleted at 5000 x g for 15 min, and resuspended on ice in
100 ml of cold 30 mM KAcetate, 50 mM MnCl₂, 100 mM KCl, 10 mM CaCl₂ and 15%
glycerol. The mixture was pelleted at 5000 x g for 10 min and resuspended in 20 ml
of cold 10 mM NaMOPS, 75 mM CaCl₂, 10 mM KCl and 15% glycerol. Aliquots of 0.1
and 0.5 ml were frozen in liquid nitrogen and stored at -70°C.

Transformation was accomplished by gently mixing competent cells with
plasmid DNA in an ice bath. After 30 min, the mixture was heat shocked at 37°C for
5 min and then diluted 1:10 in LB and incubated with gentle agitation at 37°C for 90
min. Mixtures were plated onto LB-agar plates containing 100 μg/ml ampicillin and
incubated inverted at 37°C for 16 h. All plasmids used in these studies contained the
β-lactamase gene allowing growth of the transformants in the presence of ampicillin.

2.2.2 Purification of DNA

2.2.2.1 Small Scale Plasmid Preparation

Aliquots of 1.5 ml of LB broth containing ampicillin (100 μg/ml) were
inoculated with a single bacterial colony and were incubated at 37°C for 16 h with
vigorous agitation. Bacteria were pelleted by microcentrifugation and resuspended in
100 μl of 25 mM Tris-HCl pH 8.0, 50 mM glucose, 10 mM EDTA. Subsequently, the
cells were lysed with 200 μl 0.2M NaOH, 1% SDS and neutralized with 150 μl of 5M
KAcetate pH 4.8. Cellular debris was removed by microcentrifugation and the
supernatant was extracted with an equal volume of phenol:chloroform (1:1). After
centrifugation to separate phases, the upper aqueous layer is collected and the
plasmid DNA precipitated by the addition of 2 volumes of absolute ethanol. After
microcentrifugation, the pellet is washed with 70% ethanol to remove coprecipitating
salts. Finally, the pellet is air-dried and dissolved in 50 μl of sterile water or TE (10mM Tris-HCl pH 8.0, 0.1mM EDTA).

2.2.2.2 Large Scale Plasmid Preparation

To obtain larger quantities of plasmid DNA suitable for eucaryotic cell transfection a 5 ml overnight culture was used to inoculate 250 ml of selective LB broth. After a 16h incubation with vigorous agitation, cells were pelleted at 2800 x g and resuspended on ice in 5 ml of 25mM Tris-HCl pH 8.0, 50 mM glucose, 10 mM EDTA. After 20 min, the mixture was transferred to sterile 30 ml polycarbonate screw cap tubes before cells were lysed with 10 ml of 0.2M NaOH, 1% SDS by gentle inversion. The lysate was then treated with 7.5 ml of 5M KAacetate pH 4.8. After 10 min, the mixture is centrifuged at 25,000 x g for 15 min and the supernatant transferred to a 50 ml polypropylene screw cap tube. This mixture is digested with 50μg of ribonuclease for 20 min at 37°C. The DNA solution is precipitated with 2 volumes of ice-cold 95% ethanol at -20°C for 20 min. The DNA is pelleted by centrifugation and washed with 70% ethanol before it is resuspended in 2 ml of sterile water. The mixture is transferred to a 15 ml polypropylene screw cap tube and extracted twice with phenol:chloroform (1:1) and once with chloroform before transferring 1.6 ml of DNA solution to a 15 ml silanized glass tube (Corex). After the addition of 0.4 ml of 5M NaCl and 2.0 ml of 13% polyethyleneglycol (PEG-8000), the plasmid DNA is precipitated on ice for 60 min. Following centrifugation (12,000 x g, 15 min), the pellet is redissolved in 200-500 μl of TE and quantitated by ultraviolet absorbance at 260 nm.
2.2.3 Oligonucleotide-directed Mutagenesis

A series of mutagenic oligonucleotides were synthesized which correspond to natural mutations identified within the LCAT gene (Table 11). Using a mutagenic oligonucleotide as primer, each mutation was introduced into the LCAT cDNA contained within the pUC19 vector by the polymerase chain reaction (PCR). First round amplification with PCR was carried out by using 10 pg of template DNA, 100 pmole of mutagenic oligonucleotide as a forward primer, and a 100 pmole of the reverse pUC primer which hybridizes to the vector downstream from the LCAT cDNA sequence. PCR was carried out for 30 cycles, with step cycles of 95°C for 30 sec, 55°C for 30 sec, and 74°C for 60 sec. After the thirtieth cycle, the reaction was extended at 74°C for 5 min. After purification of the PCR product, a second round of PCR was performed which contained 20 pg of the LCAT cDNA template in pUC19, 10 pmole of universal primer, and the purified first round amplification product as the other primer. PCR conditions were the same as those outlined in the first round, except that the extension time for each cycle was increased to 90 sec.
Table 11. Oligonucleotides used for site-directed mutagenesis of an LCAT cDNA.

<table>
<thead>
<tr>
<th>Structural Change</th>
<th>Mutagenic Primer</th>
</tr>
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<tbody>
<tr>
<td>Pro10→Leu</td>
<td>5'-CTTCCCCCCTGCACACCACGC-3'</td>
</tr>
<tr>
<td>Ala93→Thr</td>
<td>5'-TGTCACACACCCCTGGT-3'</td>
</tr>
<tr>
<td>Thr123→Ile</td>
<td>5'-CCTGCACATACTGGTGTC-3'</td>
</tr>
<tr>
<td>Arg135→Trp</td>
<td>5'-GCTACGTGGGGACGAGG-3'</td>
</tr>
<tr>
<td>Arg158→Cys</td>
<td>5'-AGTACTACCTGAAAGCTC-3'</td>
</tr>
<tr>
<td>Leu209→Pro</td>
<td>5'-CATCTCTCTGGGGCTC-3'</td>
</tr>
<tr>
<td>Met252→Lys</td>
<td>5'-CCCCTGGAAGTTTCCT-3'</td>
</tr>
<tr>
<td>Leu300 deletion</td>
<td>5'-GTCACGTGACCTGGCAGGAC-3'</td>
</tr>
<tr>
<td>Thr347→Met</td>
<td>5'-TGATGACATGGTGGCGA-3'</td>
</tr>
<tr>
<td>Ile375→FS</td>
<td>5'-CACGGAATACACAGCATC-3'</td>
</tr>
<tr>
<td>Asn391→Ser</td>
<td>5'-GCACATCAGTGCCATCC-3'</td>
</tr>
</tbody>
</table>

The sequence modifications of the mutagenic primers are printed in italic. The altered codon is underlined. FS, frameshift.

2.2.4 Isolation of cDNA Fragments

PCR products or fragments obtained from the restriction enzyme digestion of plasmids were purified by agarose gel electrophoresis. Specific bands were excised from the gel and DNA was recovered according to the protocols outlined for Geneclean or MERmaid (Bio101). The quantity of product was estimated by visualization of the DNA in ethidium-stained agarose gels.

2.2.5 DNA Sequence Analysis

The sequencing of the different mutant LCAT cDNAs was carried out to
confirm the presence of the mutation and the absence of any other anomalous mutations. Double stranded sequencing was performed by the enzymatic chain termination method (Sanger et al., 1977) using a modified T7 polymerase (Sequenase, United States Biochemical). Only those fragments generated by the PCR were subjected to sequencing. Reactions were performed according to the manufacturers recommendations. Approximately 2 μg of plasmid DNA was incubated in 20 μl of 0.2M NaOH and 0.2mM EDTA for 15 min at room temperature. The mixture was neutralized by the addition of 3μl of 3M NaAcetate and then precipitated with 75μl of absolute ethanol. After the pellet was washed and dried, it was resuspended with 5 pmol of sequencing primer in Sequenase reaction buffer. The mixture was incubated at 37°C for 20 min and subsequently at room temperature for 10 min. The annealed primer was then simultaneously extended and labeled with Sequenase in the presence of 1.5μM dGTP, dTTP, dCTP and 2.5μCi of α-[35S]-dATP (5 min at room temperature). Chain termination was then achieved by removing 2.75μl of the labeling reaction mixture into 1.25μl of a prewarmed solution containing dideoxynucleotides, four reactions corresponding to the four types of bases. Termination reactions were performed at 37°C for 5 min and stopped by the addition of Stop Solution (95% formamide, 20mM EDTA, 0.05% Bromphenol blue, 0.05% xylene cyanole FF).

Labeled products were separated on 6% polyacrylamide gels containing 1 x TBE (89mM Tris, 89mM boric acid, 2mM EDTA) and 8M urea. At the completion of separation, the gel was dried onto Whatman 3MM chromatography paper and exposed to autoradiographic film for 16-72 hours.
2.2.6 Construction of Expression vector

The amplified DNA was purified and digested with specific restriction enzymes to generate DNA fragments which encompassed the desired mutation. These fragments were inserted as a cassette in the wild type LCAT cDNA contained within the pUC19 vector. The mutant cDNAs were excised from positive clones and then transferred to the mammalian expression vector pNUT (Palmiter et al., 1987). In one case, a construct containing two mutations at codons 93 and 158 was made through the selection of restriction enzymes to create two fragments each containing a different mutation which were subsequently ligated into the pNUT expression vector. All final constructs were sequenced to confirm the presence of the desired mutation. The pNUT vector contains SV40 and mouse metallothionein promoters as well as a mutant form of the dihydrofolate reductase (DHFR) gene permitting the selection of cells stably transfected with the plasmid DNA by their survival in high concentrations of methotrexate (Funk et al., 1990).

2.3 Eucaryotic Cell Culture

COS-1 and BHK cells were maintained in Dulbecco’s Modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). FBS was heat-inactivated at 56°C for 30 min prior to use as a growth supplement. Cell cultures were maintained in a humidified incubator with a 5% CO₂ atmosphere.

2.3.1 Transient Transfection of COS-1 Cells

The pNUT expression vector containing either wild type or a mutant LCAT cDNA was transiently transfected into COS-1 cells by DEAE-dextran transfection
(Kriegler, 1990). Subconfluent COS-1 cell monolayers were washed twice with transfection buffer (25mM Tris-HCl, pH 7.4, 140mM NaCl, 1mM CaCl$_2$, 3mM KCl, 0.5mM MgCl$_2$, 0.9mM Na$_2$HPO$_4$) before incubation of the cells with the DNA solution at 37°C for 30 min. Subsequently, the DNA solution was removed and replaced with DMEM containing 10% FBS and 80µM chloroquine. After a 3h incubation, transfection was completed by 3 min incubation with DMEM containing 10% DMSO at room temperature. After washing with transfection buffer, the cells were incubated in DMEM containing 10% FBS for 12h and subsequently in serum-free medium (Opti-MEM)) for 48h.

2.3.2 Stable Transfection of BHK Cells

To mediate the transfection of BHK cells, coprecipitates of plasmid DNA and CaPO$_4$ were prepared (Kriegler, 1990). Plasmid DNA (20-30 µg) in 0.50 ml of 0.25M CaCl$_2$ was mixed with 2X HEPES-buffered saline (2X HBS) containing 40mM HBS, pH 6.96, 280nM NaCl, 10mM KCl, 1.5mM Na$_2$HPO$_4$ and 10mM glucose. The calcium phosphate-DNA mixture was incubated at room temperature for 30 min before it was added dropwise to a 100 mm culture dish containing a 50% confluent BHK monolayer. Following an overnight incubation at 37°C under 5% CO$_2$, the transfection medium was replaced with DMEM/10% FBS for 24 hours before transfected cells were selected over a period of 10-14 days in DMEM/10% FBS containing 500 µM methotrexate. Surviving colonies were transferred to 20mm culture wells and grown to confluency under selected conditions. Clones expressing maximal quantities of LCAT were identified by solid-phase LCAT immunoassay.
2.3.2.1 Tunicamycin Treatment of BHK Cells

Tunicamycin was dissolved in 95% ethanol to produce a stock solution of 200 ug/ml. Confluent BHK cell monolayers were incubated in serum-free Opti-MEM containing 10-250 ng/ml tunicamycin for 24h. The culture medium was collected and the protein mass and enzyme activity were determined. Cells were removed from the culture dish with a rubber policeman and LCAT activity was measured in the cellular lysate.

2.3.3 Endogenous Radiolabeling of Recombinant LCAT

BHK or COS-1 cells were incubated in methionine-free DMEM (DMEM-Met) for 20 minutes at 37°C to deplete the methionine pool. The endogenous methionine pool was then labelled for 30 minutes in DMEM-Met supplemented with 100-200 μCi/ml [35S]methionine. Subsequently, the labelling medium was removed and the cells were incubated with DMEM/10% FBS. After a specified incubation period, the medium was collected and the cellular protein harvested in lysis buffer (50mM Tris-HCl, pH8.0, 62.5mM EDTA, 1% Nonidet P40, 0.4% sodium deoxycholate, 1mM phenylmethylsulfonylfluoride) for subsequent analysis.

2.3.3.1 Immunoadsorption of Recombinant LCAT

Solid phase immunoadsorption was used to detect the presence of LCAT in the culture medium and the cellular lysate. Polyclonal goat anti-human LCAT antibodies were pre-adsorbed onto agarose-immobilized protein G for 30 minutes at 4°C. An aliquot of medium or cellular lysate was added to the antibody-protein G-agarose suspension and the mixture was rotated overnight at 4°C. After
centrifugation and washing, the adsorbed material was then eluted from the agarose beads by heating at 90°C in the presence of 2X sodium dodecyl sulfate (SDS) sample buffer (0.1M Tris-Hcl, pH6.8, 2% SDS, 40% glycerol). The beads were removed by centrifugation and the supernatant recovered for electrophoretic analysis.

2.4 Protein Analysis

2.4.1 Enzymatic Deglycosylation of LCAT

Neuraminidase (Clostridium perfringens, type V, Sigma Chemical Co.) was used to remove the sialic acid residues from LCAT by adding 3 µl of neuraminidase (1U/ml) to 7 µl of culture medium or purified human LCAT. Samples were incubated at 37°C for 1h. N-glycanase digestion was performed by adding 10 µl of 0.9M sodium phosphate, pH 8.7 and 4 µl of 10% Nonidet P-40 to 15 µl of sample. The mixture was boiled for 10 min, cooled and 1 µl of N-glycanase (250U/ml) was added before incubation at 37°C overnight. Endoglycosidase H digestion was carried out by adding 10 µl of 0.15M sodium acetate buffer, pH5.8 and 4 µl of 10% Nonidet P-40 to 15 µl of sample. The mixture was boiled for 10 min, cooled and 2 µl of endoglycosidase H (1U/ml) was added before incubating at 37°C for 16h.

2.4.2 SDS-Polyacrylamide Gel Electrophoresis

Samples containing LCAT were mixed 1:1 with 2X SDS sample buffer containing 10% β-mercaptoethanol and 0.1% Bromphenol Blue. The mixture was boiled for 5 min prior to loading onto a 10% polyacrylamide gel run at a constant current of 15mA/gel for 45 min. [14C]methylated protein molecular weight markers
were used as standards. Before autoradiography, the fixed gels were equilibrated with Amplify and dried onto Whatman 3MM chromatography paper. Autoradiograms were exposed on X-Omat AR film for 16-72h.

2.4.3 Immunoblot Analysis

Polyacrylamide gels were electroblotted onto nitrocellulose paper (0.45µM) as described by Towbin et al. (1979). Transfer was achieved at 100V for 1h in a cooled chamber containing blot buffer (25mM Tris, 192mM glycine, 20% methanol). Unoccupied nitrocellulose sites were blocked with 5% non-fat powdered milk in PBS (30 min). Membranes were then incubated successively with polyclonal goat anti-human LCAT antibodies and Protein G-HRP to detect the LCAT protein.

2.4.4 Purification of Recombinant LCAT

Approximately 300 ml of culture medium containing rLCAT was loaded onto a phenyl-Sepharose column (10x15cm) which was previously equilibrated with 0.005M sodium phosphate, 0.3M sodium chloride, pH 7.4. The column was washed with the same buffer until the absorbance (280nm) fell below 0.01. Subsequently, the rLCAT was eluted with deionized water.

2.4.5 Quantitation of LCAT Protein

The immunoassay of LCAT was carried out by using nitrocellulose membranes (0.45µM pore size) as a solid-phase support. Samples containing either culture medium or purified human recombinant LCAT standard were bound to the membrane in a BioRad slot blot apparatus. The membrane was subsequently
blocked with 5% non-fat dry milk in PBS for 30 min at 37°C. Polyclonal goat anti-
human LCAT antibodies were incubated with the membrane overnight at room
temperature in PBS containing 0.5% non-fat dry milk. The membrane was then
washed three times (5min) in PBS containing 0.02% Tween before it was reacted
with Protein G conjugated to horseradish peroxidase for 60 min. The membrane was
washed again three times and then developed in 50 ml of PBS containing 25 mg
diaminobenzidine (Sigma Chemical Co.), 15 mg CoCl₂ and 0.010 ml of 30% H₂O₂ to
visualize the protein. The blot was scanned using a BioRad Video Densitometer
(Model 620). The interassay coefficient of variation was 7.3% for a single protein
measurement in nine separate assays. Total protein determinations were carried out
with a BioRad protein assay kit using bovine immunoglobulin as the standard.

2.4.6 Preparation of Plasma and Lipoproteins

Blood was collected from normal volunteers after 12h fasting and plasma was
prepared by low speed centrifugation (1,200g, 20 min). The different lipoproteins
were isolated by sequential ultracentrifugation using lipoprotein fractions defined by
their densities: d<1.006 g/ml for VLDL and 1.006<d<1.063 g/ml for LDL. Total
lipoprotein-depleted plasma was prepared by ultracentrifugation at density 1.21 g/ml.
All lipoprotein fractions were dialyzed extensively at 4°C against 0.01M Tris-HCl (pH
7.4) containing 0.15M NaCl and 0.005M EDTA. Plasma was heat inactivated at
56°C for 30 min to eliminate endogenous activity. The concentration of unesterified
cholesterol in these different fractions was determined enzymatically by a reagent
kit.
2.4.7 Measurement of LCAT Activity

2.4.7.1 Exogenous Substrates

The enzyme activities of wild type and mutant LCAT gene products were determined using HDL-like proteoliposomes containing \[^3\text{H}\]cholesterol, phosphatidylcholine, and apolipoprotein A-I. The egg yolk phosphatidylcholine:cholesterol liposome was prepared by ethanol injection according to Batzri and Korn (1973). The substrate mixture containing 4.66 nmol \[^3\text{H}\]cholesterol (0.03\text{μCi/nmol}), 18.46 nmol phosphatidylcholine, and 7.5 \text{μg} purified human apo A-I in 10mM Tris-HCl (pH 7.4)-150mM NaCl-5mM EDTA was preincubated at 37°C for 30 min. Subsequently, 5mM β-mercaptoethanol and 1.5% bovine serum albumin (essentially fatty acid free) were added to the substrate mixture. The reaction was initiated by the addition of either 100 \text{μl} of cell culture medium from transfected COS-1 cells or 50 \text{μl} of medium from transfected BHK cells. The reaction was carried out at 37°C for 1 h and was terminated by the addition of 4 ml of chloroform:methanol (2:1) and incubated for two hours at room temperature to extract lipids. Labeled cholesterol and cholesteryl ester were separated by thin-layer chromatography on silica gel layers incubated in petroleum ether:diethyl ether:acetic acid (70:12:1) and radioactivity was determined by liquid scintillation spectrometry.

2.4.7.2 Endogenous Substrates

The ability of rLCAT to esterify cholesterol was also determined by using \[^3\text{H}\]cholesterol labelled plasma or lipoprotein fractions as a source of substrate. After heat-inactivation, radiolabeled plasma or lipoproteins were prepared by equilibration
with $[^3]$H]cholesterol at 4°C as described by Dobiasova et al. (1992). An aliquot of culture medium containing rLCAT (100 µl for transfected COS-1 cells or 50 µl for transfected BHK cells) was added to the radiolabeled substrate (100-150 nmol of unesterified cholesterol/ml), 5mM β-mercaptoethanol and 1.5% bovine serum albumin (essentially fatty acid-free). The reaction mixture was incubated at 37°C for 4h. The reaction was terminated by adding 2 ml of ethanol. Cholesterol and cholesteryl ester were separated by thin-layer chromatography and radioactivity determined by liquid scintillation counting. The results were expressed as nmoles of cholesteryl ester formed per h per µg of rLCAT protein.
3 RESULTS

3.1 Expression of Recombinant LCAT in BHK Cells

The autoradiogram in Figure 5 indicates that BHK cells transfected with pNUTLCAT secreted a predominant protein that was precipitable by antibodies specific for human plasma LCAT. This protein migrated as a broad band typical of glycosylated proteins in SDS-polyacrylamide gels and spanned a molecular weight of 64,000-67,000. This is consistent with previous determinations for purified human LCAT (Marcel, 1982). No LCAT protein was detected in the medium from those cells transfected with the pNUT vector containing no LCAT cDNA insert.

3.1.1 Properties of Recombinant LCAT

Aliquots of serum-free medium were collected at several time intervals from 35mm culture dishes containing BHK-LCAT cells and assayed for the presence of LCAT protein and activity. As shown in Table 12, the levels of both LCAT protein and activity continued to increase during a 48h incubation period. The average specific activity of the LCAT enzyme calculated from these values of protein and activity was 3.75±0.39 nmol/h/μg. The specific activity of rLCAT was similar to plasma LCAT (5.64±0.32 nmol/h/μg) (Funke et al., 1991) and LCAT purified from plasma (0.75±0.08 nmol/h/μg) using the same substrate. In addition, the ability of apo A-I to activate both rLCAT and human plasma LCAT were compared. Both enzymes demonstrated a typical saturation curve with a maximal activation between 5 and 7.5 μg of apo A-I/assay (Figure 6). After numerous cell passages, both the activity and protein concentration of rLCAT secreted from this stable BHK cell line have remained consistent.
Figure 5. Expression of rLCAT. BHK cells transfected with pNUT (control) or pNUTLCAT (wt) plasmids were subsequently labelled with $[^{35}\text{S}]$methionine for a 30 min pulse. After 6h of incubation, medium was collected and rLCAT was immunoadsorbed as described in Materials and Methods. The immunoadsorbed protein was reduced and electrophoresed in 10% SDS-polyacrylamide gels. After gel drying, radioactivity was detected by autoradiography. Molecular weight standards are indicated in kilodaltons.

Table 12. Secretion of rLCAT protein and activity from BHK-LCAT cells into serum-free medium.

<table>
<thead>
<tr>
<th>Hours of Incubation</th>
<th>Protein (µg/ml)</th>
<th>Activity (nmol/h/ml)</th>
<th>Specific Activity (nmol/h/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>4.36</td>
<td>17.90</td>
<td>4.11</td>
</tr>
<tr>
<td>30</td>
<td>6.91</td>
<td>26.39</td>
<td>3.82</td>
</tr>
<tr>
<td>48</td>
<td>10.30</td>
<td>34.30</td>
<td>3.33</td>
</tr>
</tbody>
</table>

Confluent BHK-LCAT cells were incubated in serum-free Opti-MEM for the indicated time intervals. LCAT activity and protein were determined as described in Materials and Methods. Activity units are expressed as nmol of cholesterol esterified per hour per ml. The values depicted are the mean of two separate experiments, each done in duplicate.
Figure 6. Activation of plasma LCAT and rLCAT as a function of apo A-I concentration. Assays were performed by using single bilayer cholesterol:lecithin (1:4) vesicles. LCAT activity was measured using 0.015 ml of plasma (●) or culture medium containing rLCAT (■) and expressed as nmol of cholesteryl ester formed per hour per ml. Data points are means of duplicate assays.

3.1.2 Production and Purification of Recombinant LCAT

The LCAT cDNA is under the control of the mouse metallothionein promoter which can be stimulated by the presence of divalent cations (Stuart et al., 1984). Due to its lower cell toxicity, the effect of Zn$^{2+}$ ion concentration on the secretion of rLCAT from the BHK-LCAT cells was investigated. As shown in Figure 7, a Zn$^{2+}$ ion concentration of 20μM produced the highest secretion rate of rLCAT.
Figure 7. Effect of Zn$^{2+}$ ion concentration on the secretion of rLCAT. Different concentrations of ZnSO$_4$ were added to a suspended culture of BHK-LCAT cells after the initiation of incubation in serum-free medium. Culture medium was analysed for LCAT activity expressed as a percentage of cholesterol esterified per hour.

After a 72 hour incubation in serum-free Opti-MEM, BHK cells attached to microcarrier beads secreted rLCAT at levels exceeding 10 $\mu$g/ml. The secreted rLCAT was purified by phenyl-Sepharose chromatography with the elution profile depicted in Figure 8. Following elution with deionized water, 97% of the original activity was recovered resulting in a 29-fold purification (Table 13). A homogeneous
LCAT protein was eluted from the column as observed by a single band following SDS polyacrylamide electrophoresis (data not shown).

**Figure 8. Elution profile of Phenyl Sepharose CL-4B chromatography.** An aliquot (300 ml) of culture medium containing rLCAT secreted from BHK cells was applied to a phenyl sepharose CL-4B column (10x15 cm) equilibrated with 0.005 M potassium phosphate buffer (pH 7.4) containing 0.3 M sodium chloride. The column was washed with the same buffer until the absorbance (280nm) fell below 0.01 at which point the rLCAT was eluted with deionized water (arrow). The protein absorbance and LCAT activity expressed as a percentage of cholesterol esterified per hour were determined in each fraction.
Table 13. Purification of rLCAT secreted by transfected BHK cells.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Specific Activity (units/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture Medium Phenyl-Sepharose eluent</td>
<td>350</td>
<td>149</td>
<td>105</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Phenyl-Sepharose eluent</td>
<td>110</td>
<td>4.9</td>
<td>3,090</td>
<td>29</td>
<td>97</td>
</tr>
</tbody>
</table>

3.1.3 Enzymatic Deglycosylation of Plasma LCAT and Recombinant LCAT

To compare the carbohydrate structure of purified human LCAT and rLCAT, each was selectively deglycosylated with either neuraminidase to remove sialic acid residues or N-glycanase to digest N-linked carbohydrate chains. Figure 9 shows a Western blot of an SDS polyacrylamide gel which demonstrates that both enzymes have equivalent molecular weights before and after enzymatic deglycosylation. However, it is apparent that rLCAT migrates as a broader band compared to plasma LCAT. Digestion with neuraminidase was accompanied by a reduction in molecular weight which was consistent with previous data (Chung et al., 1979; Collett and Fielding 1991) indicating that sialic acid makes up a significant portion of the total carbohydrate mass. In addition, reaction with N-glycanase reduced the apparent molecular weight of LCAT to about 48,000, which was comparable to the calculated molecular weight for the mature protein of 47,090 (McLean et al., 1986a). In addition, the removal of the N-linked carbohydrate significantly decreased the size heterogeneity of rLCAT. However, both plasma LCAT and rLCAT were resistant to digestion with endoglycosidase H (data not shown) which only removes N-linked
oligosaccharides that terminate with mannose residues. These results indicate the assembly of either complex or hybrid oligosaccharide chains in the Golgi apparatus prior to the secretion of LCAT from the cell (Tarentino et al., 1974).

Figure 9. Comparison of the effects of enzymatic deglycosylation of human plasma LCAT and rLCAT. The molecular weight of human plasma LCAT and rLCAT were determined by SDS polyacrylamide gel electrophoresis. LCAT protein was blotted onto nitrocellulose membranes and detected immunologically as described in Materials and Methods. Lane 1, untreated rLCAT; Lane 2, untreated human plasma LCAT; Lane 3, neuraminidase-treated rLCAT; Lane 4, neuraminidase-treated human plasma LCAT; Lane 5, N-glycanase-treated rLCAT; Lane 6, N-glycanase-treated human LCAT.

3.1.4 Tunicamycin Treatment of BHK Cells

3.1.4.1 Quantitation of Secreted LCAT Protein and Activity

The effect of tunicamycin treatment on the secretion and enzyme activity of rLCAT is shown in Table 14. Tunicamycin inhibits the synthesis of the oligosaccharide chain on the lipid carrier (dolicholpyrophosphate) thereby preventing
the addition of oligosaccharides to asparagine residues. Tunicamycin concentrations of 10-250 ng/ml were added to the culture medium. After 24h of incubation, untreated cells secreted 8.57 μg/ml of LCAT protein but less than 1.0 μg/ml was observed in tunicamycin-treated cells. Similarly, LCAT activity from the culture medium of treated cells was 5-12% of the activity observed from the medium of untreated cells. Higher concentrations of tunicamycin (5 μg/ml) did not further reduce the levels of either LCAT protein or activity (data not shown). There was no activity associated with the cellular fraction of tunicamycin-treated cells. However, low levels of LCAT activity (0.29 nmol/h/ml) could be detected within untreated cells.

Table 14. Effect of tunicamycin on the secretion of rLCAT.

<table>
<thead>
<tr>
<th>Tunicamycin Concentration (ng/ml)</th>
<th>LCAT Protein (μg/ml)</th>
<th>LCAT Activity (nmol/h/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.57</td>
<td>2.50</td>
</tr>
<tr>
<td>10</td>
<td>1.31</td>
<td>0.30</td>
</tr>
<tr>
<td>25</td>
<td>0.84</td>
<td>0.27</td>
</tr>
<tr>
<td>50</td>
<td>0.71</td>
<td>0.22</td>
</tr>
<tr>
<td>100</td>
<td>0.93</td>
<td>0.23</td>
</tr>
<tr>
<td>250</td>
<td>0.50</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Confluent BHK-LCAT cells were incubated for 24h in Opti-MEM containing tunicamycin. Esterification rates were measured over a period of 30 min using 0.050 ml of culture medium in the presence of apo A-I. LCAT protein was measured immunologically as described in Materials and Methods.

3.1.4.2 Immunoabsorption and Western Blotting of Recombinant LCAT

Immunoadsorption of the cellular lysate and culture medium (Figure 10)
indicated that unglycosylated LCAT with an approximate molecular weight of 46,000 was accumulating within tunicamycin-treated cells. In contrast, a partially glycosylated intermediate form of LCAT with an estimated molecular weight of 55,000 was detected within untreated cells. Only fully glycosylated LCAT, however, appeared in the culture medium regardless of tunicamycin treatment. However, pretreatment of the cells with tunicamycin for 2h prior to a 24 hour incubation eliminated the activity and the protein in the culture medium (data not shown).

Figure 10. Effect of tunicamycin on the processing of rLCAT. Immunoadsorbed protein from the cellular lysate and the culture medium was reduced and electrophoresed in 10% SDS-polyacrylamide gels. After blotting, LCAT protein was detected immunologically as described in Materials and Methods. The concentration of tunicamycin in ng/ml is indicated above each lane.
3.2 Biochemical Analysis of Selected Families with Inherited Disorders of LCAT Deficiency

The genetic and biochemical analysis of published reports of patients with familial LCAT deficiency and FED is summarized in Table 15. In this group of families, higher concentrations of plasma LCAT protein and an increased cholesterol esterification rate (CER) were used to distinguish the FED phenotype from familial LCAT deficiency. However, the range of LCAT activities and concentrations reported for LCAT deficiency syndromes suggests that neither of these parameters can necessarily provide a definitive diagnosis. In several instances, two different genetic defects have been inherited simultaneously. For such cases, the association between each mutation and the corresponding biochemical phenotype remains unclear. In attempt to resolve these ambiguities, the structural changes indicated in Table 15 were re-created individually by site-directed mutagenesis of an LCAT cDNA. The initial assessment of the functional significance of these mutations was achieved by the transient transfection of COS-1 cells.
Table 15. Structural changes and biochemical analyses for a group of molecular defects associated with LCAT deficiency syndromes.

<table>
<thead>
<tr>
<th>Structural Change</th>
<th>LCAT Protein</th>
<th>LCAT Activity</th>
<th>CER</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygosity: Pro10→Leu</td>
<td>50%</td>
<td>10%</td>
<td>100%</td>
<td>FED</td>
<td>Skretting and Prydz, 1992, Frohlich et al., 1987</td>
</tr>
<tr>
<td>Homozygosity: Ala93→Thr and Arg158→Cys</td>
<td>ND</td>
<td>3%</td>
<td>0%</td>
<td>LCAT Def.</td>
<td>Funke et al., 1993</td>
</tr>
<tr>
<td>Homozygosity: Thr123→Ile</td>
<td>50%</td>
<td>3%</td>
<td>57%</td>
<td>FED</td>
<td>Funke et al., 1991</td>
</tr>
<tr>
<td>Heterozygosity: Arg135→Trp and Ile375→FS</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>LCAT Def.</td>
<td>Funke et al., 1993</td>
</tr>
<tr>
<td>Homozygosity: Leu209→Pro</td>
<td>3%</td>
<td>0%</td>
<td>0%</td>
<td>LCAT Def.</td>
<td>Funke et al., 1993</td>
</tr>
<tr>
<td>Heterozygosity: Met252→Lys and Asn391→Ser</td>
<td>30%</td>
<td>10%</td>
<td>64%</td>
<td>FED*</td>
<td>Frohlich et al., 1987</td>
</tr>
<tr>
<td>Heterozygosity: Thr123→Ile and Thr347→Met</td>
<td>50%</td>
<td>3%</td>
<td>54%</td>
<td>FED</td>
<td>Klein et al., 1992</td>
</tr>
<tr>
<td>Homozygosity: Leu300 Del</td>
<td>20%</td>
<td>14%</td>
<td>42%</td>
<td>FED*</td>
<td>Klein et al., 1993b</td>
</tr>
</tbody>
</table>

LCAT protein, LCAT Activity and cholesterol esterification rate (CER) are expressed as a percentage of normal. FED, fish eye disease; LCAT Def., LCAT deficiency; FS, frameshift; ND, not determined; Del, deletion. FED*, these cases were described as ones which resembled fish eye disease.
3.3 Transient Transfection of COS-1 Cells

3.3.1 Endogenous Radiolabeling of Wild Type and Mutant LCAT Species

Site-directed mutagenesis was used to recreate several mutations in the human LCAT cDNA corresponding to the structural changes listed in Table 15. After the transfection of each mutant LCAT cDNA construct, the synthesis and secretion of recombinant LCAT (rLCAT) were studied by a pulse-chase experiment with \(^{35}\)S-methionine. A representative autoradiogram depicting the expression of (Thr123→Ile)rLCAT is indicated in Figure 11. COS cells transiently transfected with wild type or mutant LCAT cDNA secreted a major protein with an apparent molecular weight of 67,000 which was comparable to fully glycosylated plasma LCAT. This LCAT protein was not seen in the culture medium isolated from the cells transfected with vector DNA which did not contain the LCAT cDNA insert (control). In some experiments, a faint protein band with a lower molecular weight (47,000) was observed in the media. However, this band also appeared in the medium from the cells transfected with the empty expression vector. Furthermore, an equivalent band was observed when irrelevant antibodies were used for the immunoadsorption. Thus, it appears that the 47,000-molecular weight protein does not originate from the LCAT cDNA. Immunoadsorption of the cellular lysate revealed a specific protein band with a molecular weight lower than the mature LCAT (52,000) in all experiments except the cells transfected with the expression vector DNA alone. The extent of glycosylation of this intermediate form of rLCAT was investigated and is described in the next section. In addition, a specific protein band of molecular weight 32,000 was observed in the cells transfected with the vector containing the
Thr123→Ile substitution. We speculate that this may be a product of degraded mutant LCAT protein.

![Image of immunoadsorption of wild type and mutant LCAT](image)

**Figure 11. Immunoadsorption of wild type and mutant LCAT.** The transfected COS-1 cells were pulse-labeled with [³⁵S]methionine (200 μCi/ml) for 30 min followed by a chase incubation with nonradiolabeled methionine for 4h. Subsequently, proteins in the culture medium and cells were immunoadsorbed with polyclonal anti-human LCAT antibodies and electrophoresed in 10% SDS polyacrylamide gels. The radiolabeled proteins of wild type and mutant LCAT were visualized by autoradiography. Migration position and size (in kilodalton) of protein standards are indicated.

Figure 12 compares the secretion and intracellular forms for (Arg135→Trp)rLCAT and (Ile375→FS)rLCAT. The LCAT protein containing the Arg135→Trp mutation was secreted into the medium and had a molecular weight identical to that of the fully glycosylated wild type rLCAT. In contrast, no secreted
rLCAT could be detected for the protein containing the frameshift mutation following codon 375. However, analysis of the cellular lysate indicated a lower molecular weight partially processed form of LCAT for both mutant enzymes. The intracellular form of LCAT corresponding to the frameshift mutant consistently had a lower molecular weight when compared to either the wild type or the Arg135→Trp mutant LCAT. All other mutant rLCAT proteins expressed in COS cells were indistinguishable from the wild type.

Figure 12. Immunoadsorption of two mutant LCAT species. The transfected COS-1 cells were pulse-labelled with \([^{35}S]\)methionine (200\(\mu\)Ci/ml) for 30 min followed by a chase incubation with non-radiolabelled methionine for 4h. Subsequently, proteins in the culture medium and cells were immunoadsorbed with polyclonal anti-human LCAT antibodies and electrophoresed in 10% SDS polyacrylamide gels. The radiolabelled proteins were visualized by autoradiography. Migration position and size (in kilodaltons) of protein standards are indicated.
3.3.1.1 Endoglycosidase H Digestion of Intracellular Recombinant LCAT

Digestion of the intracellular form of wild type rLCAT with endoglycosidase H (endo H) (Figure 13) reduced the molecular weight to about 47,000 which was comparable to the calculated molecular weight of the mature unglycosylated protein (McLean et al., 1986a). This result indicates the presence of high-mannose chains normally associated with intracellular processing in the endoplasmic reticulum (Tarentino et al., 1974). The secreted form of wild type rLCAT remained endo H resistant (data not shown).

Figure 13. Endoglycosidase H treatment of intracellular wild type recombinant LCAT. Transfected COS-1 cells were pulse-labelled with [\(^{35}\)S]methionine for 30 min followed by a chase incubation with non-radiolabelled methionine for 4h. Subsequently, proteins in the cellular fraction were immunoabsorbed with LCAT antibodies and incubated in either the presence (+) or absence (-) of endo H. After polyacrylamide gel electrophoresis, the radiolabeled proteins were visualized by autoradiography. Migration position and size of protein standards are indicated.
3.3.2 Establishment of Enzyme Assay Conditions for Wild Type Recombinant LCAT Secreted by Transfected COS-1 Cells

The conditions for enzyme assays containing proteoliposomes were not altered when compared to those described for human plasma (Funke et al., 1991b). Nevertheless, linearity of the assay for different timed intervals was confirmed with rLCAT. However, specific assay conditions were assessed for heat-inactivated plasma and LDL substrates. The influence of incubation time and unesterified cholesterol concentration on LCAT activity for heat-inactivated plasma is depicted in Figure 14. The cholesterol esterification rate was linear during 1-6 h of incubation at an unesterified cholesterol concentration of 40 nmol/ml. When the rate of cholesterol esterification was determined with various amounts of plasma cholesterol (15-80 nmol/ml plasma), the activity of wild type rLCAT increased and then plateaued after reaching concentrations of unesterified cholesterol greater than 40 nmol/ml. Since this assay involves the addition of exogenous rLCAT to a heat-inactivated plasma substrate, it is important to note that the measured activity is not directly comparable to those values reported previously for the cholesterol esterification rate (CER) in patient plasma.
Figure 14. Enzyme activity of wild type recombinant LCAT secreted by COS-1 cells for heat-inactivated plasma. A: An aliquot of cell culture medium containing wild type rLCAT was incubated with heat-inactivated plasma (40 nmol/ml) pre-equilibrated with [3H]cholesterol. The incubation was carried out at 37°C for various time periods. Enzyme activity was determined as described in Materials and Methods and was expressed as nmol of cholesterol esterified per μg LCAT protein. B: An aliquot of cell culture medium containing wild type rLCAT was incubated with various amounts of heat-inactivated [3H]cholesterol plasma for 5h at 37°C. Enzyme activity was determined and expressed as nmol of cholesterol esterified per h per μg LCAT protein.

The ability of the secreted rLCAT protein to esterify cholesterol in the LDL fraction was also assessed (Figure 15). [3H]cholesterol was pre-equilibrated with LDL and the esterification of cholesterol catalyzed by rLCAT was determined. The [3H]cholesterol esterification rates in LDL catalyzed by the wild type enzyme was very low but still measurable after a 3h incubation. When increasing amounts of LDL cholesterol (20-100 nmol) were added to the assay mixture, the enzyme activity increased but began to plateau after unesterified cholesterol concentrations of 50
nmol/ml were reached. However, due to the limited activity observed in this assay, it was not possible to accurately assess the specific activity of different recombinant mutant enzymes.

![Graph A](image1.png)

**Figure 15. Enzyme activity of wild type recombinant LCAT secreted by COS-1 cells for LDL.** A: An aliquot of cell culture medium containing wild type rLCAT was incubated with [³H]cholesterol LDL (80 nmol/ml) at 37°C for various time periods. Enzyme activity was determined as described in Materials and Methods and was expressed as nmol of cholesterol esterified per μg LCAT protein. B: An aliquot of cell culture medium containing wild type rLCAT was incubated with various amounts of labeled LDL for 5h at 37°C. The enzyme activity was determined and expressed as nmol of cholesterol esterified per h per μg LCAT protein.

### 3.3.3 Quantitation of Secreted LCAT Protein and Activity for a Series of Mutant Recombinant Enzymes

The data obtained from the analysis of the secretion of rLCAT from transiently transfected COS-1 cells is summarized in Table 16. Abnormalities in the secretion of rLCAT from COS-1 cells were detected for only three mutations. There was approximately a 50% reduction of the secreted protein for the (Thr123→Ile)rLCAT
compared to wild type, a 75% reduction for (Leu209→Pro)rLCAT and a complete absence of secreted LCAT protein for the frameshift mutation following codon 375. Analysis of the specific activity of these mutant enzymes for HDL analogues revealed that all mutants, with the exception of the Asn391→Ser and Arg158→Cys substitutions, had specific activities of less than 10% of wild type. LCAT species containing the Ala93→Thr mutation alone or in combination with the Arg158→Cys substitution had specific activities of less than 10% of wild type using either HDL-like proteoliposomes or heat inactivated plasma as substrate. In contrast, the mutant LCAT containing the single amino acid exchange Arg158→Cys had a specific activity of greater than 80% of wild type for both proteoliposomes and heat inactivated plasma. Four of the mutations associated with FED (Pro10→Leu, Thr 123→Ile, Thr347→Met, Asn391→Ser) demonstrated a moderate increase in the proportion of cholesterol esterified when radiolabeled heat-inactivated plasma was used as substrate, while the rest of the mutants remained inactive.
Table 16. Measurement of LCAT protein and specific activity for different mutant rLCAT species secreted from transfected COS-1 cells.

<table>
<thead>
<tr>
<th>Structural Change</th>
<th>Secreted Protein</th>
<th>LCAT Activity (HDL Analogue)</th>
<th>LCAT Activity (Plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Pro10→Leu</td>
<td>94±6</td>
<td>6±3</td>
<td>24±6</td>
</tr>
<tr>
<td>Ala93→Thr</td>
<td>88±20</td>
<td>7±3</td>
<td>9±5</td>
</tr>
<tr>
<td>Ala93→Thr/Arg158→Cys</td>
<td>91±15</td>
<td>3±1</td>
<td>8±8</td>
</tr>
<tr>
<td>Thr123→Ile</td>
<td>42±5</td>
<td>0.2±0.1</td>
<td>35±7</td>
</tr>
<tr>
<td>Arg135→Trp</td>
<td>145±21</td>
<td>0</td>
<td>0.5±0.9</td>
</tr>
<tr>
<td>Arg158→Cys</td>
<td>75±10</td>
<td>86±11</td>
<td>84±29</td>
</tr>
<tr>
<td>Leu209→Pro</td>
<td>24±6</td>
<td>1±1</td>
<td>2±2</td>
</tr>
<tr>
<td>Met252→Lys</td>
<td>118±17</td>
<td>0.3±0.3</td>
<td>3±4</td>
</tr>
<tr>
<td>Leu300 Deletion</td>
<td>107±16</td>
<td>1±1</td>
<td>4±4</td>
</tr>
<tr>
<td>Thr347→Met</td>
<td>101±20</td>
<td>3±1</td>
<td>19±12</td>
</tr>
<tr>
<td>Ile375→FS</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Asn391→Ser</td>
<td>92±23</td>
<td>15±3</td>
<td>25±5</td>
</tr>
</tbody>
</table>

Culture medium collected from transfected COS-1 cells was analysed for LCAT protein and LCAT activity as described in Materials and Methods. Specific LCAT activity using either an HDL analogue or heat inactivated plasma was determined as nmol of cholesterol esterified per hour per μg of rLCAT protein. The data is expressed as a percentage of wild type: LCAT protein, 1.14±0.26 μg/ml (n=6); Specific LCAT activity: HDL analogue, 2.08±0.49 nmol/h/μg (n=6); Heat-inactivated plasma, 0.39±0.06 nmol/h/μg (n=5). Values shown are mean ± standard deviation for 3-6 different experiments. Statistical comparison of enzyme activity values of the wild type LCAT and each mutant enzyme for both substrates indicated that each mutant protein was significantly different from the wild type (p<0.001) with the single exception of the (Arg158→Cys)rLCAT.

3.4 Stable Transfection of BHK Cells

3.4.1 Endogenous Radiolabeling of Wild Type and Mutant LCAT Species

With the exception of the frameshift mutation which failed to be secreted by
COS-1 cells, all of the mutant LCAT cDNAs were stably transfected into BHK cells. The synthesis and secretion of these different mutant rLCATs were studied by pulse-chase experiments using $[^{35}\text{S}]$methionine. As depicted in Figure 16, analysis of the cellular lysate indicated that all of the mutant proteins were synthesized and were indistinguishable from the wild type construct. A partially glycosylated LCAT with an approximate molecular weight of 55,000 accumulated intracellularly. In addition, a protein of about 46,000 molecular weight was present in all lanes but appeared to be a non-specific immunoadsorbed protein as it was also associated with cells transfected with the empty expression vector (control). Analysis of the culture medium revealed that all but two of the mutant constructs were normally secreted and had an apparent molecular weight of 66,000, comparable to fully glycosylated plasma LCAT. The LCAT immunoassay was unable to detect any LCAT protein in the medium from BHK cells transfected with the Leu209→Pro mutant cDNA. However, low levels could be consistently detected for the double mutant Ala93→Thr/Arg158→Cys. The quantity of rLCAT secreted after a 48h incubation differed among the various mutant BHK cell clones and ranged from 2-12 $\mu\text{g/ml}$ of culture medium.
Figure 16. Immunoadsorption of intracellular and secreted forms of a series of mutant LCAT species. The transfected BHK cells were pulse-labelled with \[^{35}\text{S}]\text{methionine}\ (200\mu\text{Ci/ml})\ for\ 30\ \text{min}\ followed\ by\ a\ chase\ incubation\ with\ non-radiolabeled\ methionine\ for\ 4h.\ Subsequently,\ proteins\ in\ the\ culture\ medium\ and\ cells\ were\ immunoadsorbed\ with\ polyclonal\ anti-human\ LCAT\ antibodies\ and\ electrophoresed\ in\ 10\%\ SDS\ polyacrylamide\ gels.\ The\ radiolabelled\ proteins\ were\ visualized\ by\ autoradiography.\ Migration\ position\ and\ size\ (in\ kilodaltons)\ of\ protein\ standards\ are\ indicated.

3.4.2 Establishment of Enzyme Assay Conditions for Wild Type Recombinant LCAT Secreted by Transfected BHK Cells

In the same manner as previously described for COS-1 cells, the influence of incubation time and unesterified cholesterol concentration on LCAT activity for LDL and VLDL substrates was analysed. Figures 17 and 18 indicate that a 4h incubation with an unesterified cholesterol concentration greater than 125 nmol/ml were sufficient to provide linear cholesterol esterification rates and to maintain an excess concentration of LDL and VLDL substrate. A similar kinetic analysis was performed
for those mutant recombinant enzymes which either had normal or partial reactivity with LDL. The relative specific activities of these mutants remained consistent throughout the indicated concentration range of unesterified cholesterol (0-150 nmol/ml).

Figure 17. Enzyme activity of wild type recombinant LCAT secreted by BHK cells for LDL. A: An aliquot of cell culture medium containing wild type rLCAT was incubated with [³H]cholesterol LDL (125 nmol/ml) at 37°C for various time periods. Enzyme activity was determined as described in Materials and Methods and was expressed as nmol of cholesterol esterified per µg LCAT protein. B: An aliquot of cell culture medium containing wild type rLCAT was incubated with various amounts of labeled LDL for 4h at 37°C. The enzyme activity was determined and expressed as nmol of cholesterol esterified per h per µg LCAT protein.
Figure 18. Enzyme activity of wild type recombinant LCAT secreted by BHK cells for VLDL. A: An aliquot of cell culture medium containing wild type rLCAT was incubated with \[^{3}H\]cholesterol VLDL (125 nmol/ml) at 37°C for various time periods. Enzyme activity was determined as described in Materials and Methods and was expressed as nmol of cholesterol esterified per \( \mu \)g LCAT protein. B: An aliquot of cell culture medium containing wild type rLCAT was incubated with various amounts of labeled VLDL for 4h at 37°C. The enzyme activity was determined and expressed as nmol of cholesterol esterified per h per \( \mu \)g LCAT protein.

3.4.3 Lipoprotein Substrate Specificity of Wild Type and Mutant LCAT

The enzymic activity of the secreted rLCAT protein was determined with either proteoliposomes containing \[^{3}H\]cholesterol (HDL analogues) or with isolated lipoproteins radiolabeled with \[^{3}H\]cholesterol. For each activity assay, the substrate was present in excess as determined by the measurement of maximal esterification rates for increasing concentrations of unesterified cholesterol. The specific activities of wild type and mutant enzymes were calculated within these defined parameters. As shown in Table 17, the specific activity of wild type rLCAT secreted from BHK
cells was nearly 10-fold lower for LDL compared to HDL analogues and 100-fold lower when VLDL was used as substrate.

**Table 17. Specific activity of wild type rLCAT secreted from BHK cells for selected substrates.**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific Activity (nmol/h/μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL Analogue</td>
<td>3.42±0.45 (n=5)</td>
</tr>
<tr>
<td>LDL</td>
<td>0.52±0.07 (n=5)</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.056±0.005 (n=5)</td>
</tr>
</tbody>
</table>

After a 48h incubation, culture medium from BHK cells stably transfected with the wild type LCAT cDNA was collected and analysed for LCAT activity and protein as described in Materials and Methods. Specific LCAT activity using either HDL analogues, LDL or VLDL as substrate was expressed as nmol of cholesterol esterified per hour per μg of rLCAT protein. The results are depicted as mean ± standard deviation (number of experiments).

Table 18 compares the specific LCAT activity for the different mutant rLCAT species using these different substrates. With the exception of a higher reactivity for the Thr123→Ile mutant, the results obtained for the specific activity for HDL analogues with transfected BHK cells were very similar to those reported for transiently transfected COS-1 cells (Table 16). Three of the mutants associated with FED had average specific activities for HDL analogues ranging from 11-31% of wild type (Pro10→Leu, Thr123→Ile, Asn391→Ser) while the remaining mutations had activities ranging from 0-6% of wild type. Differences in the specific activity among the mutant rLCAT species were more striking when LDL acted as the substrate. Activities comparable to wild type were obtained for both homozygous mutants of FED (Pro10→Leu, Thr123→Ile). Intermediate activities were observed for the
Asn391→Ser defect while all other mutants had activities of less than 10% of wild type. In contrast, specific activities obtained for VLDL substrates did not exceed 50% of wild type for any of the mutations. In some cases, the relative activity for VLDL substrates was higher when compared to LDL while for others it decreased. However, it should be noted that the specific activity of wild type rLCAT for VLDL substrates was considerably lower when compared to LDL (Table 17).

Table 18. Specific LCAT activity of a series of mutant rLCAT species for selected substrates.

<table>
<thead>
<tr>
<th>Structural Change</th>
<th>HDL Analogue</th>
<th>LDL</th>
<th>VLDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Pro10→Leu</td>
<td>11±3</td>
<td>118±20</td>
<td>33±6</td>
</tr>
<tr>
<td>Ala93→Thr/Arg158→Cys</td>
<td>6±3</td>
<td>5±2</td>
<td>13±2</td>
</tr>
<tr>
<td>Thr123→Ile</td>
<td>31±9</td>
<td>104±10</td>
<td>47±14</td>
</tr>
<tr>
<td>Arg135→Trp</td>
<td>0.1±0.1</td>
<td>0.8±0.3</td>
<td>4±3</td>
</tr>
<tr>
<td>Leu209→Pro</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Met252→Lys</td>
<td>0.3±0.3</td>
<td>3±3</td>
<td>9±2</td>
</tr>
<tr>
<td>Leu300 Deletion</td>
<td>4±1</td>
<td>5±1</td>
<td>10±2</td>
</tr>
<tr>
<td>Thr347→Met</td>
<td>3±1</td>
<td>8±2</td>
<td>34±9</td>
</tr>
<tr>
<td>Asn391→Ser</td>
<td>18±4</td>
<td>53±7</td>
<td>22±7</td>
</tr>
</tbody>
</table>

Culture medium collected from transfected BHK cells was analyzed for LCAT protein and LCAT activity as described in Materials and Methods. Specific LCAT activity using either HDL analogues, LDL or VLDL as substrate was expressed as nmol of cholesterol esterified per hour per μg of rLCAT protein. The data is expressed as a percentage of wild type (specific activities indicated in Table 17). Values shown are mean ± standard deviation for 4-5 different experiments. Statistical comparison of enzyme activity values of the wild type LCAT and each mutant enzyme for all substrates indicated that each mutant protein was significantly different from the wild type (p<0.001) with the exception of the activity of (Pro10→Leu)rLCAT and (Thr123→Ile)rLCAT for LDL substrates.
4.0 DISCUSSION

4.1 Expression of Recombinant LCAT in Mammalian Cell Culture

The transient transfection of COS-1 cells and the stable transfection of BHK cells with an expression vector containing an LCAT cDNA has enabled the production of recombinant LCAT (rLCAT) whose properties closely resemble those of plasma LCAT with respect to molecular weight, activation by apo A-I, specific activity and carbohydrate content. However, for both cell types, there was a greater size heterogeneity associated with rLCAT in SDS polyacrylamide electrophoresis when compared to LCAT purified from plasma. Similar observations have been reported for recombinant antithrombin secreted from BHK and CHO cells (Bjork et al., 1992). In each case, removal of the N-linked carbohydrate resulted in the migration of a single sharp band with no apparent difference in mobility compared to the plasma protein suggesting a greater degree of heterogeneity in the glycosylation of the recombinant protein. It is possible that differences in the specific activity of LCAT reported for different cell lines such as COS-1 cells (3-12 nmol/h/μg), CHO cells (1-2 nmol/h/μg), and human embryonic kidney-293 cells (46 nmol/h/μg) could be attributed in part to differences in the glycosylation patterns present in each mammalian cell type. However, it is likely that a much greater source of variability is associated with the different methods used for substrate preparation and the measurement of LCAT activity as well as the variety of polyclonal antibodies employed for LCAT protein analysis.

Tunicamycin markedly reduced the secretion of rLCAT by BHK-LCAT cells even at concentrations as low as 10 ng/ml. Immunoadsorption of the cellular lysate and culture medium revealed that unglycosylated LCAT was not secreted but
accumulated within the cellular fraction. Additional studies in our laboratory have
demonstrated that the removal of all four N-linked glycosylation sites of LCAT by
site-directed mutagenesis significantly impairs the secretion of rLCAT by both COS-
1 and BHK cells. However, the secretion of unglycosylated rLCAT protein by
transfected Chinese hamster ovary cells (CHO) was not affected by tunicamycin
(Collet and Fielding, 1991). It is possible that these different cell types may vary with
respect to their mechanisms of glycosylation and protein transport within the cell.
However, in COS-1, BHK, and CHO cells, N-linked glycosylation of LCAT is
necessary for normal LCAT activity. The influence of carbohydrates on protein
expression and function of plasma LCAT still remains unclear. A comparative
analysis of the specific composition and structure of the carbohydrate chains for
both rLCAT and plasma LCAT will be necessary to determine the basis for these
functional differences.

The transient transfection of COS-1 cells has enabled the functional
assessment of a large number of LCAT mutations. Defects in both the secretion and
LCAT activity of different recombinant enzymes are easily detected. However, the
assessment of the ability of these different mutant enzymes to use endogenous
substrates is technically more demanding. The presence of endogenous LCAT, the
necessity for heat-inactivation and the presence of a heterogeneous mixture of
lipoproteins associated with human plasma limits the extent to which the results of
specific activity for this substrate can be interpreted. In addition, often the quantity of
rLCAT protein obtained from transfected COS-1 cells does not permit more detailed
structural and functional analyses. To overcome these difficulties larger quantities of
rLCAT were obtained from stably transfected BHK cells. This permitted the
measurement of the reactivity of rLCAT for isolated lipoprotein substrates. As expected, the ability of rLCAT to esterify cholesterol in lower density lipoproteins was considerably reduced, approximately ten-fold lower for LDL and 100-fold lower for VLDL when compared to HDL analogues.

The culture of stably transfected BHK cells in conjunction with the use of serum-free medium has also facilitated the purification of larger quantities of rLCAT by a single step chromatography procedure. The simplification of the production and purification of rLCAT will pave the way for a more in depth analysis of the structure and function of this enzyme. However, although the expression of rLCAT in mammalian cell culture has been established as a suitable model for the study of human LCAT, the extent to which the extrapolation of its properties, especially its cellular processing, to the characteristics of human LCAT in vivo remains to be determined.

4.2 Comparison of the In Vivo and In Vitro Biochemical Characteristics for a Series of LCAT Mutations.

4.2.1 Pro10→Leu

Homozgyosity for a Pro10→Leu exchange in LCAT was reported for the original FED patients in Sweden (Skretting and Pyrdz, 1992). These patients had about 50% of normal plasma LCAT protein and 10% of normal LCAT activity, but completely normal cholesterol esterification rates (CER) (Table 15). There was no apparent abnormality in either the synthesis or the secretion of this mutant rLCAT from transfected COS-1 or BHK cells. The enzyme activity for HDL analogues of (Pro10→Leu)rLCAT secreted from both cell types was significantly reduced and
similar to those values previously reported in patient plasma (Carlson and Holmquist, 1985b). In addition, in the same manner as LCAT in patient plasma, the ability of this recombinant enzyme to esterify cholesterol in LDL was equal to wild type. In contrast, the specific activity for VLDL substrates was reduced to only one-third of wild type. Thus, the reactivity of (Pro10→Leu)rlCAT for LDL and VLDL substrates was not equivalent suggesting that the previously termed β-LCAT activity may be restricted to LDL particles only. From these results, it is apparent that the biochemical characteristics of (Pro10→Leu)rlCAT closely correspond to the properties of this mutant form in human plasma.

4.2.2 Ala93→Thr and Arg158→Cys

Site-directed mutagenesis and in vitro expression is particularly useful for cases in which two different genetic defects have been inherited simultaneously. In most cases, this would occur in instances of compound heterozygosity; however, there has been one report of a patient with familial LCAT deficiency in which two different mutations are present on each allele (Ala93→Thr, Arg158→Cys) (Funke et al., 1993). To determine the causative nature of these defects and to define the extent to which each contributes to the biochemical phenotype, each mutation was re-created and expressed individually and in combination in COS-1 cells. LCAT species containing the Ala93→Thr mutation alone or in combination with the Arg158→Cys substitution had specific activities of less than 10% of wild type for both HDL analogues and heat-inactivated plasma. By contrast, the mutant LCAT containing the single amino acid exchange Arg158→Cys had a specific activity of greater than 80% of wild type for both substrate types.
Thus, the characteristics of those mutant recombinant enzymes containing the Ala93→Thr mutation were consistent with the biochemical phenotype of LCAT deficiency reported from the analysis of this patient's plasma (Funke et al., 1993) whereas the rLCAT containing only the Arg158→Cys substitution had a specific activity similar to the wild type enzyme regardless of the substrate utilized. As a result, it appears that this second mutation (Arg158→Cys) is a natural polymorphism of LCAT which has little effect on enzyme function. Since there are no known DNA polymorphisms within the LCAT gene, this mutation represents the first reported protein polymorphism of LCAT. However, the frequency of this mutation in the normal population is presently unknown.

As recently described by Klein et al. (1993a), the prediction of the secondary structure of LCAT would place Arg158 as one of the residues of the hydrophillic face within a segment of \( \alpha \)-helix conformation spanning residues 156 to 169. Comparison of the complete amino acid sequence of human, baboon, pig, rabbit, mouse, and rat LCAT protein indicated that codon 158 was the only residue for which there was a different amino acid present for each species (Figure 19). It should be noted that all other codons containing mutants reported in this study are conserved among all six species of LCAT. There are a variety of amino acids at codon 158 among the different species which include hydrophobic, hydrophillic and positively charged residues. In addition, the in vitro expression of the rLCAT containing an Arg158→Cys exchange had similar properties when compared to the wild type LCAT. As a result, it would seem unlikely that Arg158 plays a critical role in maintaining a hydrophillic face of an amphipathic helix. However, another mutation associated with LCAT deficiency just two residues from codon 158 (Tyr156→Asn) was shown to have a
significant effect on both the secretion and specific activity of rLCAT secreted by transfected human embryonic kidney-293 cells (Klein et al., 1993a).

In keeping with the findings in COS-1 cells, the (Ala93→Thr/Arg158→Cys)rLCAT secreted by transfected BHK cells was relatively inactive for all substrates. However, unlike transfected COS-1 cells, this double mutant was poorly secreted. It should be noted that the quantity of rLCAT protein secreted from BHK cells may not only be affected by the nature of the mutation, but also by the number of plasmid copies transfected per cell. As a result, caution should be used when interpreting the significance of the properties of secretion of rLCAT from cell culture and its relationship to what might occur for hepatocytes in vivo.

![Codon Alignment Table]

<table>
<thead>
<tr>
<th>Codon</th>
<th>153</th>
<th>154</th>
<th>155</th>
<th>156</th>
<th>157</th>
<th>158</th>
<th>159</th>
<th>160</th>
<th>161</th>
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<th>163</th>
</tr>
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<tbody>
<tr>
<td>Human LCAT</td>
<td>Gln</td>
<td>Glu</td>
<td>Glu</td>
<td>Tyr</td>
<td>Tyr</td>
<td>Arg</td>
<td>Lys</td>
<td>Leu</td>
<td>Ala</td>
<td>Gly</td>
<td>Leu</td>
</tr>
<tr>
<td>Baboon LCAT</td>
<td>Gln</td>
<td>Glu</td>
<td>Glu</td>
<td>Tyr</td>
<td>Tyr</td>
<td>His</td>
<td>Lys</td>
<td>Leu</td>
<td>Ala</td>
<td>Gly</td>
<td>Leu</td>
</tr>
<tr>
<td>Pig LCAT</td>
<td>Gln</td>
<td>Glu</td>
<td>Glu</td>
<td>Tyr</td>
<td>Tyr</td>
<td>Leu</td>
<td>Lys</td>
<td>Leu</td>
<td>Ala</td>
<td>Gly</td>
<td>Leu</td>
</tr>
<tr>
<td>Rabbit LCAT</td>
<td>Gln</td>
<td>Glu</td>
<td>Glu</td>
<td>Tyr</td>
<td>Tyr</td>
<td>Gly</td>
<td>Lys</td>
<td>Leu</td>
<td>Ala</td>
<td>Gly</td>
<td>Leu</td>
</tr>
<tr>
<td>Mouse LCAT</td>
<td>Gln</td>
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<td>Glu</td>
<td>Tyr</td>
<td>Tyr</td>
<td>Lys</td>
<td>Lys</td>
<td>Leu</td>
<td>Ala</td>
<td>Gly</td>
<td>Leu</td>
</tr>
<tr>
<td>Rat LCAT</td>
<td>Gln</td>
<td>Asp</td>
<td>Glu</td>
<td>Tyr</td>
<td>Tyr</td>
<td>Gln</td>
<td>Lys</td>
<td>Leu</td>
<td>Ala</td>
<td>Gly</td>
<td>Leu</td>
</tr>
</tbody>
</table>

**Figure 19.** Comparison of the amino acid sequence of a segment of LCAT among six different species. The amino acid sequence of residues 153-163 is compared between human (McLean et al., 1986a), baboon (Hixson et al., 1993), pig (Pritchard et al., unpublished observations), rabbit (Shaw and Pritchard, unpublished observations), mouse (Warden et al., 1990), and rat (Meroni et al., 1990) LCAT protein sequences. The residues at codon 158 (boxed) are non-conserved among all six species.
4.2.3 **Thr123→Ile**

Homozygosity for a Thr123→Ile mutation has now been identified in two families of different ancestry affected with FED (Funke *et al.*, 1991; Kastelein *et al.*, 1992). Homozygotes of these families have 50% of normal LCAT protein levels, less than 5% of normal LCAT activity and about 60% of normal CER. The (Thr123→Ile)rLCAT secreted from transfected COS-1 cells displayed the same molecular weight as the wild type protein, but its protein concentration in culture medium was about 50% of the wild type enzyme. The occurrence of a unique intracellular immunoabsorbed protein band of approximately 32,000 molecular weight associated with this mutation suggested that increased cellular degradation of this mutant protein may occur. However, observations of this kind were not observed in transfected BHK cells.

The fact that the (Thr123→Ile)rLCAT secreted by BHK cells had a higher reactivity for HDL analogues compared to COS-1 cells indicates that variability of the characteristics of rLCAT secreted from different mammalian cell types may exist. Nevertheless, a significant reduction of the reactivity of (Thr123→Ile)rLCAT for HDL analogues was observed in both cell types. Also, in the same manner as the original FED defect (Pro10→Leu), the Thr123→Ile mutation did not impair the ability of rLCAT to esterify cholesterol in the LDL fraction. Thus, these results are consistent with the hypothesis of Carlson and Holmquist (1985b) suggesting that LCAT in FED plasma esterifies cholesterol in lower density lipoprotein (β-LCAT activity) and that its ability to esterify cholesterol in HDL (α-LCAT activity) is significantly reduced.
4.2.4 Arg135→Trp and Ile375→Frameshift

The plasma analysis of both homozygotes in this family with familial LCAT deficiency revealed an absence of LCAT protein, LCAT activity and CER. They also had typical corneal opacities and normochromic anemia. However, only one proband developed kidney disease. The mutant LCAT containing the Arg135→Trp substitution was secreted normally from the transfected COS-1 cells while the frameshift mutation resulted in the synthesis and partial intracellular processing of rLCAT, but not its secretion. The insertion of an adenine following codon 375 causes a shift in the reading frame such that the final 40 amino acids of the protein are of different composition compared to the native sequence. As a result, an N-linked glycosylation site normally present at Asn384 is eliminated and the stop codon normally present at position 417 now occurs prematurely at codon 416. The reduction in the molecular weight of the intracellular form of this mutant would be consistent with the loss of a glycosylation attachment site. This is supported by additional studies reported by our laboratory which demonstrate that the loss of a single glycosylation site causes a reduction in protein size, but does not impair the secretion of rLCAT from transfected COS cells. Therefore, the inability of this mutant to be secreted may be related to a marked conformational change in the protein induced by the altered amino acid sequence contained within the final 40 residues. The accumulation of an endoglycosidase H sensitive intracellular form of rLCAT is consistent with a rate-limiting step determined by the processing of secreted proteins within the endoplasmic reticulum (Rose and Doms, 1988). In the absence of any higher molecular weight forms of mutant LCAT or degradation products within the cells or culture medium, it is possible that the 375 frameshift mutation causes
this protein to be retained within the endoplasmic reticulum.

Analysis of the enzyme activity of the (Arg135→Trp)rLCAT from both transfected COS-1 and BHK cells indicated a virtual absence of activity for either HDL-like proteoliposomes, heat-inactivated plasma, LDL, or VLDL. Thus, both of these re-created mutations were associated with a complete loss of LCAT activity, but for different reasons. The frameshift mutation after codon 375 resulted in the inability of the LCAT protein to be secreted while the Arg135→Trp substitution was associated with the normal secretion of a dysfunctional enzyme. This is consistent with the analysis of heterozygote carriers of these two mutations among whom no significant differences in the biochemical phenotype could be detected (Frohlich et al., 1988). However, the absence of LCAT protein from the plasma of homozygotes (Frohlich et al., 1982, 1988) suggests that if the Arg135→Trp LCAT protein is normally secreted, it is probably rapidly catabolized. From these analyses, it is not possible to determine if the cause of such a catabolism could arise from a lipid binding defect and/or inactivation of the catalytic site of the LCAT enzyme.

4.2.5 Leu209→Pro

Analysis of the plasma of the proband in this family affected with familial LCAT deficiency indicated only 3% of normal LCAT protein levels and no detectable LCAT activity or CER (Funke et al., 1993). In a similar manner to the (Ile375→frameshift)rLCAT, the (Leu209→Pro)rLCAT was synthesized and partially processed intracellularly, but its secretion was impaired. It is possible that the introduction of a proline residue in the middle of the primary amino acid sequence of the LCAT protein would result in a conformational change associated with defective
secretion and/or an increased susceptibility of intracellular degradation. Therefore, the very low levels of plasma LCAT protein reported in this family may be a result of impaired secretion of mutant LCAT. However, analysis of the low levels of (Leu209→Pro)rLCAT obtained from COS cells suggested that this enzyme is also functionally defective.

4.2.6 Met252→Lys and Asn391→Ser

The proband of this family was initially reported to have hypoalphalipoproteinemia resembling FED (Frohlich et al., 1987). Plasma LCAT protein was about 30% of normal and plasma CER was 64% of normal but LCAT activity was approximately 10% of normal suggestive of a higher specific activity compared to previous reports of FED. The proband has a bilateral dense corneal arcus but not the characteristic cloudy corneal opacities identified with either FED or familial LCAT deficiency. In addition, an increased number of target cells and decreased osmotic fragility of the proband's erythrocytes were similar to those reported for homozygotes of familial LCAT deficiency.

The Met252→Lys substitution was initially identified as a homozygous defect in three of the original Norwegian LCAT deficiency families (Skretting et al., 1992). Although normally secreted, the (Met252→Lys)rLCAT obtained from either transfected COS-1 or BHK cells was virtually unreactive for all substrate types. This confirms the causative nature of this mutation and is consistent with the biochemical changes reported in the Norwegian LCAT deficient patients. In contrast, the (Asn391→Ser)rLCAT appeared to have unique characteristics which were intermediate compared to those observed in FED or familial LCAT deficiency. This
mutation was associated with a partial reactivity for both HDL-like and LDL substrates. As a result, it is not surprising that a compound heterozygous defect containing these mutations is associated with the characteristics of both FED and familial LCAT deficiency. Thus, the nature of the clinical and biochemical characteristics observed for this patient are reflected by the different properties of each inherited defect.

4.2.7 Thr123→Ile and Thr347→Met

The clinical and biochemical description of the proband with this compound heterozygous defect was almost identical to the reports of patients who are homozygous for the Thr123→Ile defect. Plasma LCAT protein is about 50% of normal, LCAT activity, 3% of normal and CER, 54% of normal. Despite efficient secretion, the (Thr347→Met)rLCAT was associated with a poor reactivity for all substrates and was similar to the characteristics of rLCAT containing the substitutions Met252→Lys or Arg135→Trp.

Thus, in a similar manner to the Met252→Lys and Asn391→Ser compound heterozygote, the product of one allele was associated with a complete loss of LCAT activity while the second mutation demonstrated either normal (Thr123→Ile) or partial (Asn391→Ser) β-LCAT activity. As a result, these data demonstrate that the mutations at codons 123 and 391 present on a single allele govern the biochemical and clinical phenotype observed in these compound heterozygotes. In addition, these studies suggest that the presence of only a single allele coding for a partially active LCAT is sufficient to maintain near normal levels of plasma cholesteryl ester.
4.2.8 Leu300 Deletion

The classification of 'FED' in this family was initially based on the presence of corneal opacities and the absence of hematologic and renal abnormalities (Clerc et al., 1991). However, in a subsequent more detailed report, the analysis of the properties of the mutant LCAT revealed several unique characteristics. The proband in this family has low levels of plasma LCAT protein (20% of normal) and a reduced CER (42% of normal) but interestingly had an LCAT activity of 14% of normal. These results suggest that this mutant LCAT has a higher specific activity compared to other FED mutants (Klein et al., 1993b). In addition, supportive evidence was provided by the expression of (Leu300 deletion)rLCAT in human embryonic kidney-293 cells which revealed a poorly secreted but functionally normal enzyme.

However, the expression and analysis of (Leu300 deletion)rLCAT from transfected COS-1 and BHK cells does not concur with these results. In both of these cell types, the (Leu300 deletion)rLCAT was secreted normally but had a poor reactivity for all substrate types used. As previously mentioned, differences in the secretion and activity of rLCAT from different mammalian cell types for species containing the same mutation has been observed. Thus, differences in cell type as well as the methods of measurement of LCAT activity and protein for both the in vivo and in vitro studies reported by Klein et al. (1993b) may contribute to the discrepancy between these results. It should be noted that the measurement of LCAT activity for the other reported family studies was performed with Batzri and Korn (1973) proteoliposomes (vesicular structures), the same substrate used for the analysis of rLCAT secreted by transfected COS-1 and BHK cells. In contrast, discoidal structures were used by Klein et al. (1993b) in their studies. Therefore, it is possible...
that some mutations of rLCAT may differentiate their ability to utilize these different substrates. To resolve this issue it will be necessary to analyse the plasma LCAT activity of this proband using vesicular substrates.

4.3 Genetic and Biochemical Heterogeneity of LCAT Deficiency Syndromes

Familial LCAT deficiency and fish eye disease are rare autosomal recessive disorders associated with inherited defects within the gene encoding for lecithin:cholesterol acyltransferase. In both cases, corneal opacities and a severe HDL deficiency are characteristic features of the disease. However, unlike FED, familial LCAT deficiency is associated with severe lipoprotein abnormalities and additional clinical symptoms including a mild hemolytic anemia, proteinuria, progressive renal insufficiency and renal failure. The basis for this segregation is believed to be a result of functional differences associated with the LCAT enzyme. In familial LCAT deficiency, there are very low levels of plasma cholesteryl ester and a virtual absence of LCAT activity for both synthetic and endogenous substrates. In contrast, the CER and the concentration of cholesteryl ester in FED plasma are near normal. Therefore, the ability of some LCAT mutants to utilize lipoproteins other than HDL to synthesize cholesteryl esters is directly related to the clinical phenotype. However, the ability of FED LCAT to esterify cholesterol in HDL-like substrates is significantly reduced and is similar to the properties of LCAT observed in the plasma of patients with familial LCAT deficiency.

My current hypothesis regarding the explanation of the different biochemical and clinical abnormalities observed in LCAT deficiency syndromes is depicted in Figure 20. In both complete and partial LCAT deficiency, the ability of LCAT to act
upon preß-HDL is lost preventing the formation of mature HDL causing HDL deficiency. As a result, the small spherical and discoidal HDL accumulate and penetrate the vasculature of the cornea where over time they form corneal opacities. To maintain a cholesterol gradient between peripheral cells and the plasma, unesterified cholesterol is redistributed to LDL and triglyceride-rich particles. However, if LCAT is unable to esterify cholesterol in the LDL fraction, the flux of lipids associated with the lipolysis of chylomicrons and VLDL particles to LDL is impaired. Consequently, abnormal lipoproteins rich in triglyceride, unesterified cholesterol and phospholipid accumulate, deposit in the kidney and contribute to renal disease. On the other hand, if LCAT is able to esterify cholesterol in LDL, a more efficient cholesterol gradient can be established through the generation and redistribution of cholesteryl esters. As a result, the severe lipoprotein abnormalities normally associated with familial LCAT deficiency do not occur and renal disease does not develop. However, it is important to note that in both scenarios, cholesterol can still be transported from peripheral tissues to the liver for degradation. This may explain the paradoxical absence of premature atherosclerosis in LCAT deficiency syndromes despite a deficiency of the mature HDL pool.
The original hypothesis that two types of LCAT activity exist in plasma, $\alpha$-LCAT (acting on HDL) and $\beta$-LCAT (acting on LDL and VLDL) is supported by the results from the analysis of recombinant enzymes containing different FED mutations. However, the relatively low reactivity of rLCAT for VLDL substrates and the decreased activity of rLCAT containing FED mutants for VLDL suggest that LDL is the primary substrate associated with $\beta$-LCAT activity. In addition, the results indicate that the relative magnitude of $\alpha$- and $\beta$-LCAT activity may differ among the

Figure 20. The pathophysiology of LCAT deficiency syndromes. UC, unesterified cholesterol; LCAT, lecithin:cholesterolacyltransferase; TG, triglyceride.
various mutants. As depicted in Figure 21, in the presence of a poor LCAT activity for HDL analogues, the ability of mutant rLCATs to esterify cholesterol in LDL determines the biochemical and ultimately the clinical phenotype observed in the patients with these inherited defects.

Figure 21. The functional heterogeneity of LCAT mutations. Individual LCAT mutations were plotted according to their relative ability to esterify cholesterol in LDL and HDL analogue substrates.

The number of different molecular defects identified in the LCAT gene so far is consistent with the heterogeneity of the phenotypic expression seen in these disorders. In addition, the frequency of compound heterozygous defects of LCAT will contribute to the variability of the characteristics of LCAT observed in vivo. However, given the variability of the clinical expression reported within some families, it is
likely that the additional effects of the environment and other genes will also influence the expressivity of the clinical and biochemical phenotypes. From these analyses, it is apparent that the absolute assignment of these mutations to either familial LCAT deficiency or FED is not straightforward and caution should be used in such a segregation. This is especially true in cases where the clinical phenotype is caused by a compound heterozygosity. It appears that the original families identified with familial LCAT deficiency and FED represent the clinical and biochemical extremes of a wide range of defects of LCAT function. As a result, these disorders should not be classified as separate diseases but thought of as part of a larger group of LCAT deficiency syndromes. From a biochemical perspective, it is likely that even milder forms of LCAT deficiency exist, but in the absence of any clinical signs, these defects would remain undetected. However, it is possible that the measurement of HDL cholesterol levels in large population studies may result in the discovery of a greater number of cases.

4.4 Proposed Structure-Function Relationships of LCAT

Following the crystallization of pancreatic lipase and the elucidation of its three-dimensional structure (Winkler et al., 1990), many investigators have used this protein as model to predict and investigate the structure-function relationships for lipases with similar amino acid sequence homology (Derewenda and Cambillau, 1991). Although LCAT does not share a high degree of overall sequence homology with lipases in general, it does contain specific homologous regions which correspond to important functional domains such as the consensus sequence containing the catalytic Ser. Using pancreatic lipase as a model, the Asp-His-Ser
catalytic triads of lipoprotein lipase (LPL) and hepatic lipase (HL) have been proposed and subsequently confirmed by site-directed mutagenesis and in vitro expression (Emmerich et al., 1992; Davis et al., 1990). A comparison of the primary amino acid sequence of these lipases with LCAT indicates that Asp204, His286, and Ser181 of human LCAT could form a similar catalytic triad. There is much evidence to justify the assignment of Ser181 to the catalytic site of LCAT but further studies will be necessary to investigate the specific roles of Asp204 and His286.

Lipase sequence comparison also indicates a second homologous region near Ser216 of LCAT which may be at or near the hinge of a helical flap covering the catalytic site. Similar movable segments have been found to be essential for the activity of several enzymes that function at lipid/water interfaces. For example, it has been demonstrated that a phospholipase A$_2$ contains a mobile, hydrophobic flap which opens upon interfacial activation, allowing the formation of a hydrophobic channel in which the phospholipid substrate is translocated into the protein and then hydrolysed at the active site inside the channel (Scott et al., 1990). In addition, there is evidence that the carboxyl terminus of CETP may form a "flexible tail" which moves in relation to the rest of the molecule, creating a hydrophobic surface for interaction with lipids (Wang et al., 1993). The substitution of Ser to Ala within a homologous region of HL, LPL, and LCAT has increased the specific activity of these lipolytic enzymes (Davis et al., 1990; Emmerich et al., 1992; Francone and Fielding, 1991b). It has been speculated that such a change in structure may favour an "open" conformation of the loop covering the active site of LCAT (Francone and Fielding, 1991b). In addition, the enhanced LCAT reactivity associated with the elimination of the carbohydrate moiety at Asn384 suggests that this region of the
molecule may also influence either the conformation or access to the catalytic site.

It is apparent that the further analysis of the functional abnormalities of the LCAT mutations described in this thesis will be necessary to define the structure-function relationships of LCAT in more detail. However, some general relationships between these structural defects and LCAT function can be proposed. For example, mutations which are likely to have a major affect on the structure of LCAT such as frameshifts, premature stop codons or the introduction of a proline residue appear to be associated with a near absence of LCAT enzyme protein in plasma and, consequently, LCAT activity is absent. Evidence to support this was observed in the secretion defects associated with (Ile375→frameshift)rLCAT and (Leu209→Pro)rLCAT. By contrast, single amino acid changes are more often associated with higher levels of LCAT protein and activity. Once again, this is consistent with the characteristics of rLCAT in vitro. Most mutations did not affect the synthesis or secretion of rLCAT but rather the activity of the enzyme was most sensitive to structural changes. FED mutations such as Pro10→Leu and Thr123→Ile which do not affect the ability of LCAT to esterify cholesterol in LDL indicate that the structural integrity of the catalytic site remains intact. As a result, the ability of LCAT to react with HDL in these patients may stem from a lipid binding defect of LCAT for HDL. To maintain normal endogenous plasma cholesterol esterification rates, the distribution of LCAT would have to shift from HDL toward LDL. Thus, it is possible that this class of mutation may prevent LCAT from making a conformational change which is necessary for normal binding to HDL, but not LDL. However, for those mutations which exhibited only a partial or an absence of reactivity for different substrates, it is difficult to distinguish between lipid binding and/or abnormalities in
catalytic function.

Without the knowledge of the three dimensional structure of LCAT, it remains difficult to resolve how mutations seemingly distant to one another (e.g., Pro10→Leu and Thr123→Ile) have almost identical biochemical characteristics yet mutations closely spaced to one another (e.g., Thr123→Ile and Arg135→Trp) are associated with dramatic functional differences. The presence of widely spaced mutations in human LCAT and its high degree of sequence homology with other species of LCAT suggest that its normal function is very sensitive to changes throughout its primary amino acid sequence. From the biochemical analysis of these different recombinant enzymes, it appears that LCAT has at least two functionally important domains, the catalytic center and a recognition site for HDL substrates. Additional studies will be required to determine the specific mechanisms responsible for the abnormalities of enzyme function identified for these different LCAT mutations.

4.5 Perspectives for Future Study

The recent history of the investigation of LCAT deficiency syndromes would indicate that many new LCAT mutations will continue to be discovered with a corresponding increase in the number of reported families affected with LCAT deficiency. To increase our understanding of plasma lipoprotein metabolism in these individuals, it will be necessary to investigate the specific compositional and metabolic heterogeneity of the HDL pool in these different disorders. Specifically, the quantitation of Lp A-I and Lp A-I/A-II particles and their functional capacity to induce cholesterol efflux from cells should be a priority. In addition, analysis of the in vivo kinetics of these apolipoproteins in affected homozygotes could be helpful to
determine the basis for the absence of increased atherosclerotic risk despite very low plasma HDL levels. From a clinical perspective, much remains to be learned regarding the pathogenesis of the progressive renal insufficiency observed in some of these patients. Hopefully, the creation of suitable animal models discussed below will provide some opportunities for study in this regard. With respect to treatment, the production and purification of large quantities of rLCAT described in this thesis may allow for an efficient means of enzyme replacement therapy for those patients at risk for developing renal abnormalities. Also, a more detailed biochemical and clinical analysis of the heterozygote members of different kindreds should provide more information on the influence of specific genetic defects of LCAT in vivo. Results from these studies will not only provide additional information on the potential pathological mechanisms of LCAT deficiency but will also be essential for the understanding of HDL metabolism and reverse cholesterol transport in normal individuals.

As new LCAT mutations are identified, the application of site-directed mutagenesis and in vitro expression of rLCAT should continue to provide new information on the functional significance of these different defects. However, further studies will be required to investigate the specific lipid binding and catalytic properties of these mutant enzymes. In the first instance, endogenous radiolabeling and purification of rLCAT could be used to assess the relative association of mutant enzymes for different lipoproteins. Also, the use of water-soluble substrates should enable the investigation of the catalytic potential of different enzymes in the absence of a lipid/water interface. Hopefully, these studies will be able to identify specific amino acids or structural domains within the LCAT protein which distinguish the lipid
binding and catalytic properties of the enzyme.

It is likely that site-directed mutagenesis will also be used to establish the functional significance of specific amino acids or structural domains proposed to have an important role in enzyme function. For example, the determination of the Asp-His-Ser catalytic triad as well as the properties of the putative loop domain of human LCAT shielding the catalytic site. In addition, the specific role of carbohydrates in substrate specificity and conformation of LCAT remains to be clearly defined. Also, differences in the molecular substrate specificity of rat and mouse LCAT compared to human LCAT may be related to differences in their primary amino acid sequence. Through the substitution of specific amino acids in human and mouse rLCAT and the analysis of their reactivity with artificial substrates of defined lipid composition, potential changes in their molecular substrate specificity can be determined. The description of the properties of LCAT from different species and their interaction with endogenous lipoproteins should provide useful information regarding the factors which influence the interaction of LCAT with various substrates. Specifically, the knowledge of mouse LCAT and its interactions with lipoproteins will be helpful for the analysis of transgenic human LCAT mice and mice genetically modified to eliminate LCAT expression. These animal models could provide for the first time the means for detailed in vivo studies of the metabolism of LCAT and its influence on lipoprotein metabolism in both deficient and over-expressed states.

Finally, the preparation of large quantities of homogenous recombinant enzyme should enable the investigation of some of the physical properties of this protein. For example, analysis of carbohydrate structure by fast atom bombardment
mass spectrometry, conformational analysis by circular dichroism and NMR spectroscopy and, quantitative and qualitative analysis of the interaction of LCAT with surface lipid monolayers. Ultimately, the crystallization of LCAT and X-ray diffraction analysis should reveal its three-dimensional structure. With this knowledge, specific structure-function relationships of LCAT can be proposed and investigated in more detail.

From the above discussion, it is apparent that the availability of new tools will advance our knowledge of the structure and function of LCAT including its relationship with HDL metabolism. Consequently, this will benefit our understanding of the mechanisms of reverse cholesterol transport and the putative anti-atherogenic properties of HDL.


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