Analysis of Lecithin: Cholesterol Acyltransferase (LCAT) Protein Structure and Its Influence on Binding to Plasma Lipoproteins

by

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B.Sc.(Honours), University of Toronto, 1993

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN THE FACULTY OF GRADUATE STUDIES
(Department of Pathology and Laboratory Medicine)

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

August, 2000

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Date Sept. 01/2000

Abstract

Lecithin: cholesterol acyltransferase (EC 2.3.1.43) (LCAT) is synthesized by the liver in mammals and it is responsible for esterification of unesterified cholesterol (UC) in plasma. This plasma glycoprotein plays an important role in reverse cholesterol transport (RCT) and High density lipoprotein (HDL) metabolism.

In this thesis, functional mutants of LCAT were utilized to study the factors influencing interaction of this protein with the plasma lipoproteins. McArdle 7777 cells were used to produce LCAT from a hepatic derived expression system. The expressed enzyme was uniformly glycosylated with biantennary oligosaccharide residues, which was significantly different from the glycosylation pattern observed in the plasma LCAT and the baby hamster kidney (BHK) expressed LCAT. Despite the differences in the glycosylation architecture, the new recombinant LCAT (rLCAT) displays similar kinetic properties to those of the plasma and BHK LCATs, suggesting that the differences in glycosylation architecture may not influence activity of the enzyme.

Subsequently, interaction of rLCAT with lipoprotein substrates was studied to determine the binding characteristics of wild type (WT) and fish-eye disease (FED) plasma LCAT *in vivo*, and rLCAT *in vitro*. Endogenous LCAT was shown to remain bound to low-density lipoprotein (LDL) as well as HDL and that beta and alpha LCAT activity co-eluted with LDL and HDL particles, respectively. *In vitro* binding studies with whole plasma, total lipoproteins, and individual lipoproteins showed no differential association of rLCAT, either WT or FED (T123I), with HDL and LDL particles and that the majority of rLCAT did not bind to the plasma lipoproteins. BIAcore experiments using native plasma lipoproteins and proteoliposmes also showed little or no association of rLCAT with plasma lipoproteins, but a strong association with the synthetic HDL analogue particles.

The final aspect of this thesis was concerned with the relationship of LCAT mutations with its structural and functional characteristics involving the study of two very unique mutations, which result in an increase in the LCAT activity. The kinetic data obtained from this study showed that the combination of the two mutations did not have an additive effect, but in fact resulted in reduction of LCAT activity. Although this effect may be due to lack of independence in mechanism of action or inability of the protein to tolerate sequence alterations, it suggests that the fourth oligosaccharide chain may influence LCAT function in more ways than just inhibiting substrate accessibility.

In conclusion, although the glycosylation architecture of LCAT does not significantly alter the kinetic properties of the recombinant protein, it may influence binding/association of rLCAT to the plasma lipoproteins. In addition, the fourth oligosaccharide moiety of LCAT may play an important role (other than inhibitory) in LCAT activity.

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Amino Acid Designations

Amino Acid	Single Letter	Three Letter
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamic acid	E	Glu
Glutamine	Q	Gln
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

Abbreviations

[^{3}H] cholesterol	Cholesterol radiolabeled with tritium
ACAT	acyl coenzyme A: cholesterol acyltransferase
ACTH	Adrenocorticotropic hormone
apo	Apolipoprotein
BHK	baby hamster kidney
BSA	bovine serum albumin
CAD	Coronary artery disease
CE	Cholesterol ester
CER	Cholesterol esterification rate
CETP	Cholesteryl ester transfer protein
cDNA	complementary DNA
CHO	Chinese hamster ovary
CLD	classic LCAT deficiency
DE	delayed extraction
DMEM	Dulbelco's modified Eagle medium
DMSO	Dimethylsulfoxide
DNA	deoxyribonucleic acid
DPPC	Dipalmitoylphosphatidylcholine
EDTA	ethylenediamine tetra-acetic acid
FAB	fast atom bombardment
FBS	fetal bovine serum
FC	Free cholesterol
FED	fish-eye disease
FER	Fractional esterification rate
FLD	Familial LCAT deficiency
GC-MS	gas chromatograph/mass spectrophotometry
G-HRP	horse radish peroxidase
HDL	High density lipoprotein
HDL-C	HDL cholesterol
HL	hepatic lipase
HS	horse serum
hu AI	human apolipoprotein AI
hu AII	human apolipoprotein AII
IDL	Intermediate density lipoprotein
LAT	lysolecithin acyltransferase
LCAT	Lecithin: cholesterol acyltransferase
LDL	Low density lipoprotein
Lp	Lipoprotein
mRNA	messenger RNA
MW	molecular weight
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PAPC	1-Palmitoly-2-arachodonyl phosphatidylcholine
PBS	phosphate buffered saline
PC	Phosphatidylcholine
PCR	polymerase chain reaction

PSF	antimycotic reagent
PNGaseF	peptide N-glycosidase F
PNP	p-nitrophenol
PNPB	p-nitrophenol benzoate
POPC	Palmitoyloleoylphosphatidylcholine
PUFA	Polyunsaturated fatty acids
RCT	Reverse cholesterol transport
rHDL	Reconstituted high density lipoprotein
rLCAT	Recombinant LCAT
RNA	Ribonuclease
RT-PCR	reverse transcripatse PCR
SDS	sodium dodecylsulfate
SPR	surface plasmon resonance
SR-BI	scavenger receptor class B type I
TG	Triglyceride
TMS	Trimethylsilylation
TLC	thin layer chromatography
TPCK	N-Tosyl-L-phenylalanine Chloromethyl Ketone
UC	unesterified cholesterol
VLDL	very low density lipoprotein
WT	wild type

Acknowledgements

I would like to recognize my academic mentor Dr. Haydn Pritchard for teaching me the ropes and for his invaluable guidance, patience, and enthusiasm. I also would like to thank Dr. Jiri Frohlich for giving me the opportunity to better understand the clinical aspects of my work. Lastly, and by no means least, I would like to thank all those from the Atherosclerosis Specialty Laboratory and individuals who have contributed to my work over the last five years.

Dedication

I dedicate this thesis to my parents for their faith, support, guidance, and love. Above all, I thank my *mother*

for her sense of passion and keeping my spirits up when the muses failed me.

'To Mina'

Chapter 1
Introduction

Information presented in this chapter has contributed to the following publication:

Pritchard P.H., **Ayyobi A.F.**, and Hill J.S. Lecithin: Cholesterol Acyltransferase (LCAT). In *Plasma Lipids and Their Role in Disease,* edited by P.J. Barter and K.A. Rye, Amsterdam, pp 233-256, 1999.

1.1 Lecithin: cholesterol acyltransferase

Lecithin: cholesterol acyltransferase (E.C. 2.3.1.43)(LCAT) is the enzyme responsible for the synthesis of the majority of cholesteryl esters (CE) in plasma. The transfer of a sn-2-fatty acid from phosphatidylcholine (PC) to the 3-hydroxyl group of the cholesterol results in formation of lysolecithin and cholesterol ester [1]. This single polypeptide enzyme catalyses two distinct reactions: I) Phospholipase A2, II) Transacylase (Figure 1-1) [2].

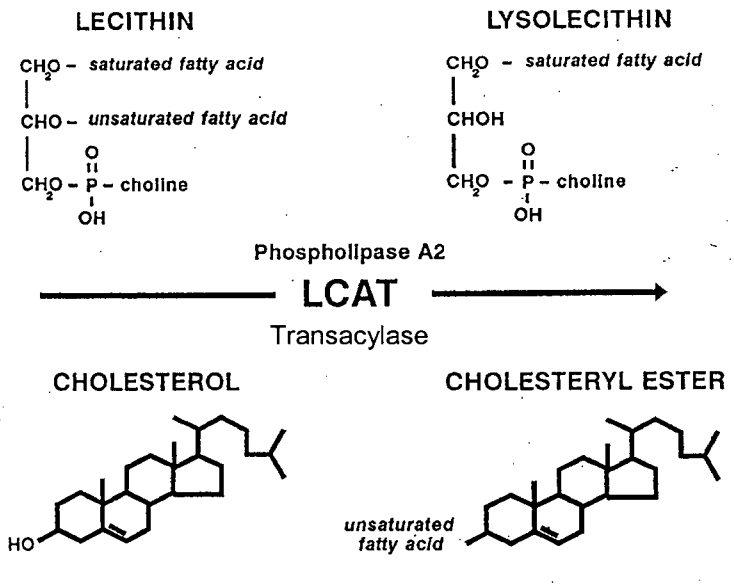


Figure 1-1. Schematic outline of LCAT activity

It is generally believed that LCAT regulates the transport of cholesterol between extravascular and intravascular pools, which implicates the central role of this important enzyme in the early steps of the process known as "reverse cholesterol transport" (RCT). (Figure 1-2)

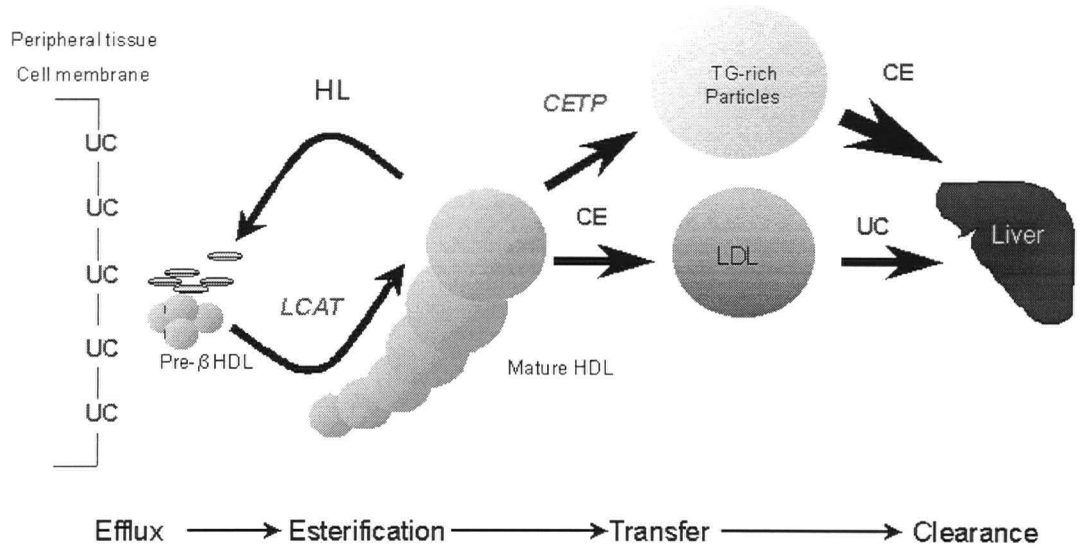


Figure 1-2. Schematic diagram of the "reverse cholesterol transport" system.

In this theoretical pathway, LCAT reaction generates and maintains a net concentration gradient of unesterified cholesterol between peripheral cells, plasma and the liver [3]. In plasma, the preferred substrates of LCAT are high density lipoproteins (HDL). Thus, LCAT plays an important role in the metabolism of HDL. Since plasma HDL concentrations are inversely correlated with the risk of coronary heart disease, LCAT may be important in modulating the risk of coronary heart disease. The significance of the role of this enzyme in lipid metabolism is demonstrated by the clinical and biochemical pathology observed in genetic disorders caused by LCAT gene defects.

1.1.1 Physical properties of LCAT

The gene coding for human LCAT is localised in the q21-22 region of chromosome 16. It consists of 6 exons separated by 5 introns and spans a 4.2 kilobase region. The enzyme is primarily expressed in the liver as a 1550 base mRNA strand

which in turn encodes for a protein, 416 amino acids in length, including a hydrophobic leader sequence of 24 residues. Mature LCAT has a calculated molecular weight of 47,090 and an apparent molecular weight of 67,000 Dalton on SDS-PAGE gels. The 25% increase in molecular weight is primarily attributed to extensive glycosylation of the enzyme. This observation may help to explain its water solubility despite having a higher hydrophobic index than the other plasma apolipoproteins. Analysis of the amino acid sequence of LCAT has revealed four potential N-linked glycosylation sites: Asn-20, Asn-84, Asn-271, and Asn-384. All four of the sites have been shown to contain carbohydrate residues. Further sequence analysis of LCAT peptides has identified 6 Cysteine residues, four of which form disulfide bonds: Cys-50 with Cys-75 and Cys-313 with Cys-356. The remaining two, Cys-31 and Cys-184 retain free sulfhydryl groups [4] (Figure 1-3).

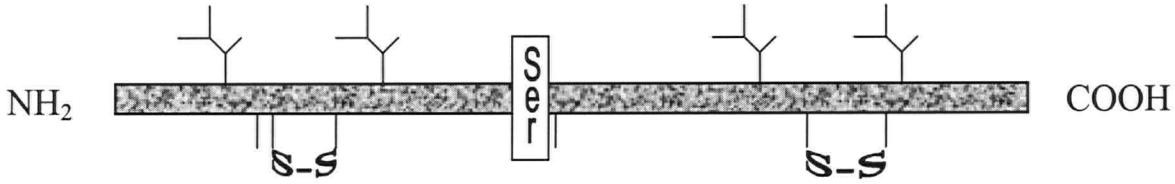


Figure 1-3. Chemical characteristics of LCAT. This is a linear representation of LCAT, indicating the major structural components of the enzyme. The branched structures represent the N-glycosylation sites. S-S and the vertical lines below the horizontal bar represent the Cys residues, 4 of which generate disulfide bonds. The Ser in the vertical box represents Ser-181 of the proposed active site.

There are multiple isoelectric points of LCAT ranging from pI 3.9 to 4.4. Following enzymatic digestion with Neuraminidase, a convergence of these bands occurs at pI 5.2, with a 3000 Dalton reduction in apparent molecular weight [5]. Removal of the negatively charged sialic acid residues through desialilation appears to increase LCAT activity that may be due to enhanced interaction of LCAT with its substrates [5].

LCAT is unique among apoproteins, not only in its function, but also in its structure. The predicted secondary structure, supported by circular dichroism data, suggests that LCAT contains no long or medium length α-helices or β-sheets in the amino terminus [6, 7]. The predicted secondary structure shows some variation between species [7].

Peelman et al have recently reported a proposed 3-D structure of LCAT. This model was predicted by molecular modelling using a threading strategy, based on alignment of the sequence against the library of previously solved 3-D protein structures [8]. Based on its homology with lipases, LCAT was proposed to belong to a/b hydrolase fold family of proteins and its conserved core domain consist of 7 parallel beta strands, sandwiched between two layers of helices which connect the beta strands (Figure 1-4).

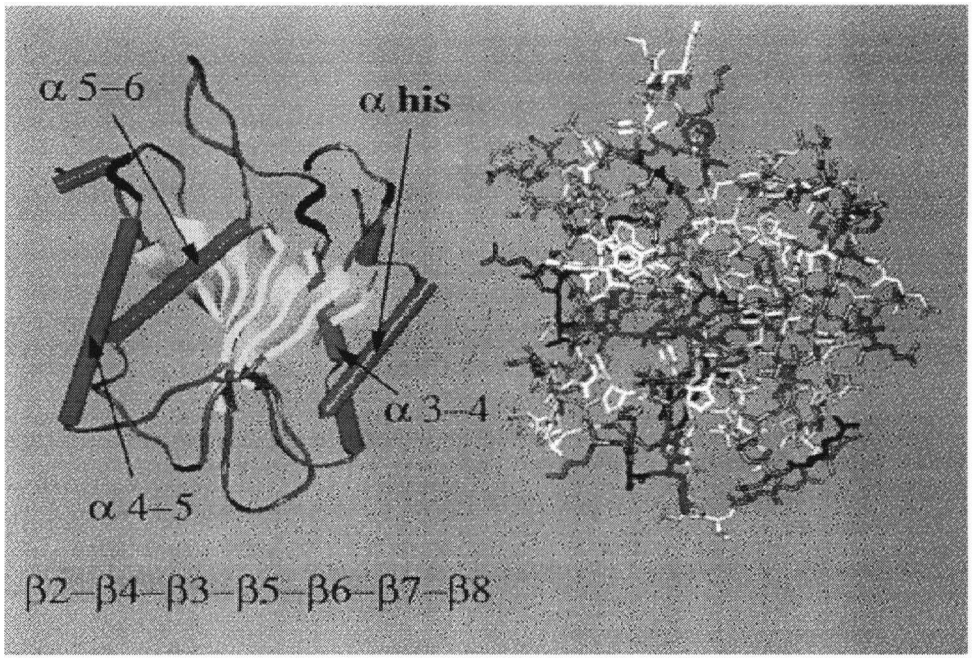


Figure 1-4. Proposed three-dimentional model of LCAT. Working model of LCAT based on pancreatic and *Candida antarctica* lipases. Left, strands are displayed in yellow, helices in red and loops/unaccounted sequences are in green. Right, the same figure displaying the hydrophobic (red) and hydrophilic (blue) residues. Adapted from Peelman et al, Protein Science (1998), 7:587-599.

Using the 3-D model of LCAT structure, a site directed mutagenesis strategy was devised to identify the catalytic residues of Asp-345 and His-377 as well as Phe-103

and Leu-182 as the oxyanion hole residues [8].. Consistent with the lipase structure, a potential "lid" domain was identified at residues 50-74, which has been shown to play an important role in the interfacial recognition and interaction LCAT with its substrate molecules [8-10]. Although this is the most detailed model for the 3-D structure of LCAT currently described, until such time the 3-D LCAT structure is resolved by X-ray crystallography, the applicability of the proposed structure is limited to the regions of LCAT that are homologous with the known structures.

1.1.2 The catalytic mechanism of LCAT

Until the recent report of proposed 3-D model of LCAT, there had been no definitive information on the three-dimensional structure of LCAT. Thus, a great deal of research has been concentrated on functional mapping of LCAT. For these studies, there are a number of techniques that have been used, including site-directed mutagenesis, monoclonal antibodies, chemical and physical interactions, and structural comparison with related, well-characterized proteins such as pancreatic lipase [11].

Although LCAT shares little sequence identity with other lipases, it contains a Gly-X-Ser-X-Gly motif which is characteristic of the active site of many other members of this enzyme family [12]. Using site directed mutagenesis and *in vitro* expression, the role of Ser-181 and Ser-216 were studied [13]. Although the proposed structural model can account for the role of Ser-181, Ser-216 could not be included in the modelling approach as there was not an appropriate template [8]. The exchange of Ser-216 for Gly or Thr reduced but did not eliminate LCAT activity. However, substitution of Ser-216 with Ala resulted in a 14-fold increase in activity. There have been problems in reproducing these experiments in other laboratories including our own. Sequence comparison with other lipases suggests that the Ser-216 residue may be at or near the

hinge of a helical flap domain, which is displaced by substrate binding [13]. However, there is a growing body of evidence that the "lid" domain may correspond to residues 50-74 [8-10]. Thus, Ser-216 may not play a role in the regulatory region for LCAT activity, but it may interact with the lid domain in an alternate manner. This is highly speculative as there is not enough information on the 3-D structural contribution of residues 50-74 due to lack of appropriate structural template used for computer modelling [8]. On the other hand, any substitution of Ser-181 results in inactivation of the LCAT protein. This suggests that Ser-181 plays a role in the LCAT active site. [13]. Using the site-directed mutagenesis strategy, Peelman et al have identified Asp-345 and His-377 as the active site residues.

The catalytic site findings are consistent with those observed in the pancreatic lipase, for which the 3-D structure has been delineated, and the active Ser residue contained within this consensus sequence is part of a catalytic Asp-His-Ser triad [11]. Using an affinity purified monoclonal antibody, raised against LCAT, the esterolytic and cholesterol esterifying activity of LCAT could be inhibited. The same antibody inhibits pancreatic lipase and a number of snake venom Phospholipase A2 species. Furthermore, phenyl bromide (Phospholipase A2 inhibitor) treatment of LCAT resulted in inhibition both of esterolytic activity of LCAT and binding capacity of the monoclonal antibody to LCAT. This suggests that LCAT and this family of phospholipases have a similar active site conformation most likely located near or within the esterolytic active site [14]. The active site (catalytic triad) has been illustrated on the proposed LCAT structure (Figure1-5)

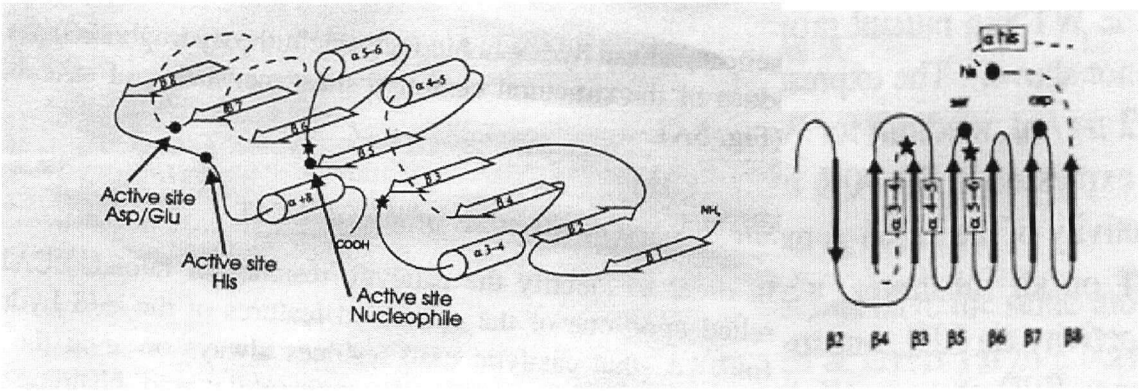


Figure 1-5. Topology diagram of LCAT. *Left Panel* represents a typical structure of α/β hydrolase. Right panel represents the predicted topology diagram of LCAT. The Asp-Ser-His triad is illustrated on both diagrams. Adapted from Peelman et al, Protein Science (1998), 7:587-599.

Loss of N-linked oligosaccharides in any of the four predicted sites resulted in retention of significant phospholipase and acyltransferase activity in three of the four sites (Asn-20, Asn-84, and Asn-384). Mutation at residue Asn-272 produced an enzyme with phospholipase activity that generated fatty acids but no cholesterol ester suggesting this residue was essential for acyl transferase activity [15]. Site directed mutagenesis in our laboratory has shown that all four sites are filled with oligosaccharide moieties [16]. Loss of carbohydrate at each site produced varying effects on enzyme activity. Of particular interest was the finding that the loss of the N-linked site at Asn-384 resulted in a two-fold increase in enzyme specific activity. These experiments also showed that loss of all four N-linked glycosylation sites severely decreased the enzyme activity and secretion to 5% and 10% of the WT respectively.

In addition, Parks and co-workers [17] demonstrated that a single point mutation in residue 149 (Glu→Ala) is sufficient to change the specificity of LCAT from palmitoyloleoylphosphatidylcholine (POPC) to 1-palmitoyl-2-arachodonyl phosphatidylcholine (PAPC). This, in fact, is the first point mutation in an enzyme that

results in an increased Phospholipase A2 activity towards arachidonic acid. This mutation also fits with the proposed 3-D structure of LCAT. Residue 149 is located on the hydrophilic loop at the N-terminal of the α4-5 helix. Glu$\rightarrow$Ala mutation of this site can alter the ability of the enzyme to accommodate larger acyl chains [8].

Work by Adimoolam and Jonas [18] showed that LCAT contains a 25 amino acid surface region linked by a disulfide bond (Cys 50-Cys 74) that is involved in the binding of LCAT. Deletion of most of this region (residues 53-71) abolished LCAT activity using rHDL and LDL substrates, but 30% activity was retained with water-soluble substrate PNPB.

LCAT activity is sensitive to chemical modifications of Cys and Ser residues [19]. Both phospholipase A2 and transferase activity can be inhibited by chemical alteration of one His residue (using diethyl pyrocarbonate) or one Ser residue (using phenylmethane sulfonyl fluoride). Also, Cys-31 and Cys-184 have been shown to be essential for thioester bonds and are vicinal to each other in the catalytic site [20]. Further study of these residues however, showed that substitution of the free Cys residues with Ala did not affect LCAT activity. The mechanism of action for sulfhydryl groups has been attributed to steric hindrance rather than a direct effect on thioester bond formation [21, 22]. In addition, cloned chicken fully functional LCAT does not possess the codon sequence for Cys residues at position 31 and 184 as in man [23].

To date, the accumulated structural and functional data on LCAT activity favors a sequence of steps outlined by Hill (1994):

- Binding to the lipoprotein surface in a reversible process.
- Activation by an apolipoprotein (usually apo A-I) to facilitate access of lipid to active site.
- Phospholipid substrate deacylation to release lysophosphatidylcholine

- Formation of an intermediate Ser-O-acyl moiety.
- Transfer of acyl chain to cholesterol to produce CE.

Release of CE into the core of the lipoprotein.

1.1.2.1 LAT activity

There is evidence that LCAT can partially reverse the cholesterol esterification reaction through the hydrolysis of cholesteryl esters to produce unesterified cholesterol [24]. It has been shown that LCAT reaction can generate PC through lysophosphatidylcholine acyltransferase, (LAT activity) [25]. This activity of LCAT has been shown to be 10% of the forward reaction and confined to the LDL particle, which exhibits higher affinity for lysophosphatidylcholine [26]. This LCAT activity does not require A-I. Instead, apo B is required for the LAT activity [27]. Apo B conformation, on LDL, is a function of the TG content of the particle and can alter the LAT activity [28]. Other apoproteins such as apo A-I, C-I and E may substitute for apo B activation of LAT [29].

1.1.2.2 LCAT as an antioxidant

Recent studies have shown that LCAT can have antioxidant properties. A number of groups have shown that LCAT can metabolise oxidised PC [30, 31]. While the work by Itabe *et al* [30] suggests that LCAT can metabolise a variety of oxidised PC's and prevent accumulation of these molecules on LDL particles, Subbaiah and co-workers [31]have shown that LCAT hydrolyses and transesterifies the longer oxidised PC molecules. Esterification of estrogen molecules by LCAT has also been shown to

result in a potent antioxidant effect of esteradiol esters generated by LCAT [32]. The antioxidant properties of LCAT may not be limited to its ability to metabolise oxidised PC's or generate antioxidant esters. A recent study by Vohl *et al* provides a strong argument for the antioxidant activity of LCAT, which appears to be independent of its traditional transacylase and phospholipase A2 activities [33].

1.1.2.3 Inhibition of LCAT activity

Lysophosphatidylcholine, a product of the LCAT reaction, can inhibit LCAT activity. This property of lysophosphatidylcholine may be due to its detergent effect rather than the end product inhibition proposed by Fielding and co-workers [34, 35].

Lipid peroxidation products have been shown to inhibit LCAT [36-39]. This effect has been proposed to be through modification of apo A-I, the major activator of LCAT. Aldehydes have been shown to inhibit LCAT independent of modifications of apolipoprotein. It was therefore suggested that peroxidation products of LDL inhibit LCAT in one of two ways: I) direct inhibition, or II) through cross-linking of apo A-I [37, 40].

Gas-phase cigarette smoke has also been reported to inhibit LCAT activity. This effect, which did not appear to be due to products of lipid peroxidation, may be due to chemical modification of Cys-31 and Cys-184 residues [36, 41]. It has also been shown that nicotine treated rats display a marked reduction in plasma LCAT activity [42]. Various aldehydes present in the cigarette smoke extract have been shown to be potent inhibitor of LCAT [39, 43].

1.1.3 LCAT substrates

The LCAT esterification reaction occurs preferentially on the surface of HDL, [35, 44, 45], despite some evidence that suggests apo B containing lipoproteins may also serve as a substrate of LCAT [46-48]. Since HDL particles are heterogeneous, studies of binding and LCAT reactivity have not been readily possible. Hence, synthetic substrates with well-defined physical and chemical characteristics have been used in place of native HDL.

Synthetic substrates, unilaminar vesicles [49], or discoidal complexes [50] for LCAT activity have proved to be useful tools in the study of its kinetic properties, substrate specificity, and apoprotein activity. However, LCAT substrates with physiological significance are in aggregate form which only exist in native lipoproteins, reconstituted HDL (rHDL), or synthetic lipid vesicles [51]. On the other hand, monomeric acyl-substrates, despite being non-physiological, are useful for activity measurements and the study of enzyme kinetics and mechanics. (Table 1-1).

Table 1-1. Physical and Chemical properties of LCAT substrates.
(Adapted partially from Jonas, 1991[52])

Substrates	**Role in LCAT reaction**	**Physical conformation**	*Reference*
Alcohols (long chain)	acyl acceptor	aggregate	[53]
Lysophospholipids	acyl acceptor	aggregate	[54]
Sterols	acyl acceptor	aggregate	[53]
Steroids	acyl acceptor	aggregate	[55]
Thiosterols	acyl acceptor	aggregate	[56]
Water	acyl acceptor	monomer	[2]
Phospholipids	acyl donor	aggregate	[2]
Cholesteryl esters	acyl donor	aggregate	[24]
Arachidonyl CoA	acyl donor	micelle	[19]
PNP* acyl esters	acyl donor	monomer	[57]
C6-NBD-PC	acyl donor	micelle	[58]
D-Py-PC**	acyl donor	monomer	[59]

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

1.1.3.1 High density lipoprotein (HDL)

Reconstituted HDL (rHDL) displays a large size distribution depending on its PC content (DPPC or POPC). The difference in size has been proposed to affect the number of A-I molecules and their conformation on the surface of rHDL which in turn influences LCAT activity [60].

As discussed earlier, it is generally believed that HDL participates in the first steps of RCT. Therefore, studies concerned with cholesterol efflux and RCT have focused on utilising isolated and/or reconstituted HDL particles. Furthermore, the ability to synthesise HDL-like particles of various sizes and compositions by altering apolipoprotein and PL composition has made studies of HDL metabolism and cholesterol efflux possible [61-64]. Reconstituted HDL offers other advantages such as setup of well-controlled environment (particle size, PL composition, apoprotein composition, charge and cholesterol content) and extreme variations in cholesterol/lipid content for specific studies targeting the above parameters [52].

Optimal activity of LCAT is obtained when the PC in rHDL substrate contains two saturated chains with 16 or fewer carbons [65]. Since the type of PC in plasma can be influenced by the composition of surrounding PC's, interpretation of data from *in vitro* systems with respect to LCAT may not be appropriate [51].

Differently sized HDL particles in plasma display different rates of esterification by LCAT. The small HDL_3 has been shown to be a preferred substrate for LCAT (as an acyl source) compared to HDL_2, which is a larger particle [35, 66]. As previously mentioned, size of rHDL was shown to influence LCAT activity which was suggested to be a result of altered apo A-I conformation [60]. However, Wald and co-workers [67] reported no conformational changes in A-I, whilst the rHDL with a 9.7 nm diameter

resulted in 20 fold higher LCAT activity than the rHDL with 18.6 nm in diameter. This suggests that the interaction of a HDL particle with LCAT may be independently influenced by the physical characteristics of the particle itself, such as size and surface curvature.

The effect of substrate particle structure extends to differences between discoidal and spherical particles. Discoidal particles are preferentially utilised over spherical HDL [68]. This difference in optimal reactivity may also be attributed to lipid packing properties or apo A-I conformation. In addition, Meng *et al* [69] demonstrated that large discs containing four apo A-I molecules were the optimal substrate for LCAT and smaller discs with fewer apo A-I molecules result in lower LCAT activity. This may be attributed to an increased storage capacity for CE.

On the other hand, Barter and colleagues [45] have proposed that there may be an inverse relationship between LCAT substrate reactivity and substrate particle size that is independent of CE content. Further studies of various core CE:TG ratio indicated that high TG content relative to CE resulted in reduction in LCAT Vmax with an unaffected Km. Km could only be increased by increasing particle size and protein content. Increasing FC or PC content resulted in reduced Km [70]. Hence, HDL particle size may be affected by the proportion of lipid to apolipoproteins as well as surface and core lipid composition. In turn, size and shape have an impact on the conformation of lipoproteins and these independent factors influence the LCAT esterification rate.

1.1.3.2 Low density lipoproteins (LDL)

Utilization of LDL as a source of UC for LCAT activity has been investigated by a

number of studies [44, 71-73]. In human plasma deficient in CETP, (the enzyme responsible for transfer of CE generated by LCAT on HDL particles to the apo-B containing lower density lipoproteins), 73% of CE was found on HDL, 25% in LDL and 1% in VLDL [46]. In pig plasma, which does not contain CETP, removal of apo-B from LDL did not effect the activity of LCAT on these particles and 60-70% of CE generated in pig plasma was found in the LDL fraction. This suggests that porcine LCAT may prefer LDL as a substrate despite the presence of HDL.

1.1.3.3 Effect of phosphatidylcholine species on LCAT activity

Phospholipids are a major component of cell membranes and contribute to membrane fluidity. In addition, they increase cholesterol esterification by LCAT [74]. The optimal ratio of PC:cholesterol for LCAT activity, in the presence of apo A-I, has been shown to be 4:1 [35]. Extensive studies suggest that in humans, native LCAT preferably utilises acyl groups 16:1>18:1>18:0 in the sn-1 position, and 18:2>18:1>22:6>20:4 acyl groups in the sn-2 position for CE synthesis. CE derived from the acyl groups of sn-2 position constitutes over 90% of total CE generated. However, if the sn-2 position is occupied with an acyl chain 20:4 or 22:6, that latter being preferred, then 75% of CE would be derived from the sn-2 acyl chains. This preferential reactivity of LCAT is independent of its cofactor apo A-I, cholesterol: PC ratio, and membrane fluidity. It has been proposed that the preferential specificity of LCAT for shorter acyl chains may be due to a difficulty in accommodating larger acyl chains of PC in the active site. Thus, LCAT uses the acyl chains at the sn-1 position [75]. This does not explain why a longer chain 22:6 is utilised in preference to 20:4. In addition, the reduction in CE generation with larger poly unsaturated fatty acyl (PUFA)

in the sn-2 position has been suggested to be mediated through the reduction of catalytic affinity of the LCAT activation energy and apo A-I stability in the recombinant HDL [76].

1.1.4 Activation of LCAT

Apo A-I is required for the activation of LCAT using PC and UC vesicles [35]. There are conflicting reports on whether "homologous" apo A-I is always the best activator of "homologous" LCAT (both apo A-I and LCAT are from the same species). Data published by Sparrow *et al* [77] suggested that homologous apo A-I is in fact the best activator of the LCAT, whereas, data by Golder-Novoselsky *et al* [78] showed that human A-I is a better activator of mouse LCAT than the mouse A-I by 1.6 fold.

How does apo A-I activate LCAT? There have been numerous attempts to elucidate the type and site of interaction of LCAT with A-I using monoclonal antibodies [79] and A-I synthetic analogues [80-82]. There are also a number of other apolipoproteins that have been reported to activate LCAT [83-85]. These exchangeable apolipoproteins share some structural similarities that may explain their ability to activate LCAT. These apolipoproteins usually contain internal 11-residue amino acid repeats. In apo A-I, A-IV and E, the 11-mer repeats have revealed a 22-mer tandem repeat, the sequence of which has been shown to be critical for LCAT activation [86]. These 11-mer repeats have the characteristics of an amphipathic α-helix. Amphipathic α-helices are a common component of the secondary structure of biologically active proteins and peptides which have the capacity to penetrate the water-phospholipid interface, exposing the buried substrate to LCAT, hence activating the LCAT activity [57, 87]. This is supported by the observation from a study utilising synthetic peptides

[81], which showed that a synthetic peptide with no sequence homology to apo A-I can activate LCAT. It was also shown that this peptide could in fact assume an amphipathic α-helical conformation similar to A-I in an environment similar to a lipoprotein. It was suggested that A-I is stabilised on the lipoprotein particles by hydrogen bonds and hydrophobic interactions, which may play a crucial role in LCAT activation[81].

Epitope mapping of apo A-I using monoclonal antibodies has revealed a specific region (residues 95-121) that is crucial for LCAT activity [79]. Computer analysis of apo A-I structure suggests that specific positions of Glu residues (66-87, 91-120) of A-I may be responsible for higher activation of LCAT by A-I [87] However, Jonas *et al* demonstrated contradictory evidence where point mutations of this region did not affect apo A-I activation of LCAT [52]. In addition, the structural analysis of apo A-I analogues has revealed that the Glu residues are located on the hydrophobic surface and it is unlikely that the negative Glu residues are directly involved in LCAT activation while bound to the lipid [81].

There is currently no consensus on how apo A-I activates LCAT. This fact is due to the complexity of data obtained from experiment with synthetic peptides and their relationship to native apo A-I in terms of lipid binding and interaction with LCAT.

1.1.5 Inter-species variations in LCAT

LCAT in different species exhibits distinctly different specificity toward acyl chains [88]. This may imply that the active site conformation in different species is slightly variable. However, there is little physiological relevance to these experiments since they used synthetic substrates to estimate the LCAT activity. In a study of LCAT from 14 species, Subbaiah and co-workers showed that LCAT in different species has a different preferred PC substrate. Fourteen vertebrate species fell into two distinct

categories of high and low atherogenic risk. The LCAT from the susceptible animals showed lower specificity to sn-2-20:4 and sn-2-22:6 PC's and higher specificity towards sn-2-18:2 PC's than the less susceptible animals. It was further proposed that the presence of one type of LCAT that is not efficient in transfer action of long chain polyunsaturated acyl group will result in generation of saturated CE instead. The saturated CE may contribute to the risk of atherogenesis [89].

1.1.6 *In vitro* expression of rLCAT

Recombinant LCAT has been expressed in a number of systems to study its function and structure [90-93]. Hill *et al* [90] showed that recombinant LCAT (rLCAT) could be expressed in large quantities (10 mg/L) in BHK cells. This recombinant protein displayed very similar physical (i.e. molecular weight (MW), and glycosylation pattern), and chemical, (i.e. specific activity) characteristics when compared to plasma LCAT. However, attempts to crystallise this protein have been unsuccessful due to a number of complicating factors such as self-aggregation in consequence of increasing concentration [93], heterogeneity of carbohydrate chains, and difficulties in protein purification (unpublished data). Turnover studies of rLCAT from the BHK expression system in rabbits have suggested that the majority of rLCAT fails to bind or associate with native plasma lipoproteins [94]. This may be due to the lack of interaction between rLCAT and plasma lipoproteins, resulting in its rapid clearance.

A recent study by Jin *et al* [93] showed that rLCAT can be expressed in significant quantities (5mg/L) and purified to greater than 96% in CHO cells. The rLCAT from this expression system displayed slightly higher molecular weight compared to plasma LCAT [93]. This difference in size was attributed to the extent of glycosylation of the enzyme, since the protein amino acid sequence was based on wild-

type LCAT. While changes in Vmax indicated a change in the catalytic mechanism, unaltered Km suggested that the binding of rLCAT to substrate is unlikely to be affected by the variations in oligosaccharide structure.

Studies of LCAT glycosylation in separate expression systems further emphasise the importance of glycosylation in its enzymatic activity [91]. Kinetic data obtained from HepG2 (hepatocyte derived cell line), SF21 (baculovirus infected cell line), and CHO (Chinese hamster ovary) cell lines suggest that the differences in LCAT enzymatic function are due to altered Vmax of cholesteryl ester (CE) generation rather than the Km and acyl substrate specificity, both of which present minor variations [91]. Interestingly, despite the physical and functional differences between the plasma LCAT and CHO-secreted rLCAT, intrinsic Tryptophan fluorescence emission spectrum and far UV CD spectrum suggest that secondary and tertiary structures of both proteins are very similar [93].

Quantitative analyses of carbohydrate structure and composition of BHK LCAT in comparison to plasma LCAT have revealed two major differences between the two proteins [95]. First, the plasma LCAT contains only bi and tri-antinary structures, while the BHK LCAT displays an additional tetra-antinary structure. Second, the sugar composition of these oligosaccharide chains has been shown to differ in the extent of core fucosylation. Despite these marked differences, both glycoproteins were shown to have very similar activities and monosaccharide compositions [95].

1.2 Measurement of LCAT activity

LCAT activity is quantified by measuring the amount of CE generated over time. Cholesterol esterification by plasma LCAT is routinely measured using two methods

which differ with respect to the nature of the substrate. This has created a source of confusion in the literature regarding interpretation of LCAT activity in plasma.

1.2.1 Exogenous substrate

This method utilises reconstituted proteoliposomes or isolated HDL to measure LCAT activity. This method, which is termed "LCAT activity assay," estimates the activity of the enzyme while minimising the impact of plasma (endogenous lipoprotein substrate) constituents. LCAT activity obtained in this manner is proportional to the amount of LCAT protein present in the system. The substrate used in this reaction can be spherical proteoliposomes consisting of apo A-I, [^{3}H] cholesterol and phospholipids. The ratio of A-I to cholesterol and phospholipid is optimised for maximal activation of LCAT. The proteoliposomes are present in an excess quantity for LCAT utilisation. LCAT activity is estimated by determining [^{3}H] cholesterol to [^{3}H] cholesterol ester conversion and it is expressed as nmol cholesterol esterified per hour per millilitre of plasma. This method is best suited for determining the initial rate of LCAT activity under controlled conditions and excess substrate. However, this does not reflect the physiological conditions and the findings cannot easily be extrapolated to the physiological state.

1.2.2 Endogenous substrate

LCAT activity can also be measured by quantifying the cholesterol esters formed in the plasma lipoprotein pool. This method is known as endogenous cholesterol esterification rate (CER). In order to determine the rate of conversion for cholesterol to cholesterol ester, trace amounts of [^{3}H] cholesterol are equilibrated with the pool of

endogenous unesterified cholesterol in plasma. Equilibration takes place at 4°C in order to minimise LCAT activity during equilibration. LCAT activity is determined by subsequent incubation of the plasma sample at 37°C. The CER is calculated as the fractional rate of [^{3}H] cholesterol converted to cholesterol ester (fractional esterification rate, FER) in the context of plasma cholesterol (moles [^{3}H] cholesterol esterified per hour per millilitre plasma) [96]. This assay assumes that all molecules of cholesterol are equally accessible by LCAT during its reaction. Clearly, this is not the case. LCAT substrates in any reaction are not uniform which result in complications in true assessment of LCAT activity and the respective kinetic parameters such as Vmax and Km.

CER and LCAT activity assays provide different information of LCAT activity. The LCAT activity assay attempts to estimate the enzymatic activity under optimal condition by minimising all the confounding factors present in plasma. CER, however, provides an estimate of synthesis rate of cholesterol esters and is influenced by not only the quantity of LCAT present but also by the substrate lipoprotein composition, cofactors, and other plasma constituents, all of which influence substrate availability.

Furthermore, CER does not provide the full picture of the "net" endogenous conversion rate of cholesterol. Nouri-sorkhubi *et al.,* [97] have shown that CE synthesis rate in whole blood is greater than the rate of CE synthesis in plasma. This implicates other components of blood, absent in plasma, that may also have an impact on the net cholesterol esterification rate in vivo.

1.3 LCAT Transgenics

1.3.1 Overexpression of human LCAT

In recent years, human LCAT has been expressed in both mice [98-100] and rabbits [101]. Mehlum *et al.,* [98], developed a transgenic mouse strain to over-express human LCAT to study the effect of an increase in plasma LCAT activity. This mouse strain expressed 70 times more LCAT than normal human plasma resulting in a 50% decrease in plasma TG. LDL and VLDL concentrations were also reduced to 50% of normal plasma. As expected, HDL-C levels were increased by 20% while accommodating the increase in A-I and A-II. Another study by Vaisman *et al.,* [100] generated strains of mice that expressed 109-fold more LCAT than the control mice. The persistent effect of these high levels of plasma LCAT was indicative of hyperalphalipoproteinaemia which has been proposed to be anti-atherogenic.

Since the rabbit lipoprotein metabolism is the most similar model (in terms of lipoprotein profile and changes in enzymes in lipid metabolism, i.e. CETP) to that of human, a rabbit transgenic strain was developed as a more accurate model for lipoprotein metabolism in human [101, 102]. In these animals, LCAT activity was increased up to 3.1-fold above the background strain. Total cholesterol and HDL-C were increased by 1.5 and 2.5-fold, respectively. This alteration in lipoprotein profile was attributed to the increased apo E rich HDL_1 particles. It was further suggested that overexpression of LCAT in the presence of CETP led to hyperalphalipoproteinaemia and reduction in atherogenic lipoproteins. However, when these transgenic rabbits were fed a high cholesterol diet (0.3% cholesterol) after 11 weeks, the degree of atherosclerosis remained the same for both the transgenic and control groups despite an elevated HDL in the transgenic group. This study concluded that even on a high

cholesterol diet elevated levels of HDL in the LCAT transgenic group was maintained, while there was no assessment of the progression of atherosclerosis[103]. However, the same group in a later report indicated that the rabbit models with a 15 fold increase in activity was protected against atherosclerosis while on a high-fat diet [102]. A natural question would be: "What happened in the mice with only 3.1 fold increase in LCAT activity?"

Co-expression of human A-I and A-II (hu AI and hu AII) with LCAT has also shed some light on the in vivo relationship between LCAT and these plasma apoproteins. A moderate increase in LCAT activity (1.6-fold) in hu AI and hu AI/AII mice resulted in elevation of total cholesterol by 4 and 2-fold, respectively. In hu AI/LCAT and hu AI/AII/LCAT animals, HDL particles were reported to be larger in size and richer in cholesteryl esters (up to 2-fold). In addition, increased LCAT activity in hu AI/LCAT and hu AI/AII/LCAT was closely associated with reduction in the apo A-I ratio in the preβ-HDL fraction. The study concluded that expression of LCAT had a significant impact on plasma lipid metabolism and that human LCAT has a significant preference for HDL particles containing human apo A-I [99].

Although mouse and rabbit transgenic models have improved our knowledge of in vivo LCAT function, there is caveat to the interpretation of these data. Although all transgenic models have achieved overexpression of human LCAT, the physiological relevance of these results is highly questionable. The major concern is due to a large body of evidence on differences in substrate specificity and enzyme activation by apo A-I, among human, mouse, and rabbit species. Furthermore, extremely large quantities of LCAT expressed in these models render the data obtained uninterpretable as they represent extreme, not physiological states. Future studies should address and control for more physiologically relevant variables, such as expression of homologous enzyme

in the animal models. It would also be beneficial to study the role of small changes in the plasma levels of LCAT as this would be more relevant to the physiological conditions and potentially more clinical applicability.

1.3.2 LCAT Knock-out Model

Targeted disruption of the mouse LCAT gene has allowed the generation of LCAT deficient mice [104, 105]. Despite the similarities in most biochemical parameters with human FLD (see section 1.5.1), mouse models do not develop the severe clinical complications present in FLD (i.e. corneal opacity, renal insufficiency). The only remarkable characteristics are elevated TG levels [104, 105] and upregulation of the adrenal SR-BI mRNA due to severe depletion of adrenal lipid stores. These observations suggest that LCAT may play a role in catabolism of TG rich apo-B containing apolipoproteins and that the reduction in plasma HDL-C may have an impact on the cholesterol flux to the adrenal glands via a selective uptake pathway [104]

1.4 LCAT gene structure

The LCAT gene is localised in the q21-22 region of chromosome 16. It has 6 exons divided by 5 introns and spans 4.2 kilobase (kb) of genomic DNA [106]. Functional analysis of the 2.9 kb indicated that deletions made to position -71 and -42 resulted in 50% and 100% reduction in promoter activity respectively [107]. Site directed mutagenesis of the 71 base pair region indicated that activity of the promoter depends on integrity of two putative Sp1 binding sites in this region [107].

Data obtained from southern blot analysis suggests that there is only a single copy of the LCAT gene in humans. The LCAT gene is predominantly expressed in the liver

and has been detected in Hep-G2 cells [106]. Analysis of LCAT mRNA indicates that the 3' untranslated region contains only 23 bases and the poly (A) signal overlaps with the carboxyl-terminal Glu and stop codons [106]. Tissue specific expression of LCAT has been studied in human [106], rat [108], rhesus monkey [109], baboon [110], chicken [23], and rabbit [111]. The current data suggest that LCAT also participates in lipid metabolism in the brain, specifically in the cerebellum [23]. There is a high degree of sequence identity among all species to date. The corresponding data are depicted in Table 1-2.

All the major structural features of LCAT described to date, including the catalytic Ser site, Cys residues, and glycosylation sites, are conserved among all species except chicken. Chicken LCAT amino acid sequence lacks the two Cys residues which have been thought to be important in LCAT function, (Cys-31 and Cys-184 in human LCAT). These data suggest that LCAT activity may not require these two residues [23].

Table 1-2. LCAT sequence homology in various species to human

Species	Nucleotide identity (%)	Amino acid identity (%)	***Reference***
Baboon	97	98	[110]
Rabbit	91	98	[111]
Pig	89	92	[94]
Rat	84	87	[112]
Mouse	85	85	[108]
Chicken	74	73	[23]

1.5 Genetic abnormalities affecting LCAT activity

1.5.1 Historical Perspective

The first case of familial LCAT deficiency (FLD) was described over three decades ago by Kare Norum and collaborators [113-115]. The three probands from this family were characterised by a complete absence of LCAT activity accompanied by

greatly reduced cholesteryl ester and plasma LCAT concentration. There were remarkable lipoprotein abnormalities: HDL deficiency, lipid changes in LDL and VLDL particles, as well as corneal opacification, anaemia, proteinurea, and renal disease [116]. These findings have prompted a great deal of research by both clinical and basic scientists that has led to a better understanding of the pathophysiology of this disorder. Traditionally, the phenotypic and laboratory findings have provided the basis of classification for the variants of this disorder. These are familial LCAT deficiency and fish-eye disease (FED).

It was not until 1992 that Skretting and co-workers described the genetic defect underlying the disorder in this first case of familial LCAT deficiency [117]. DNA sequence analysis of the LCAT gene in these probands revealed a homozygous single point mutation (nucleotide substitution) in the LCAT gene resulting in the substitution of a Met by a Lys residue at position 252 of the secreted mature protein. *In vitro* expression and analyses of this protein has revealed an inactive but normally secreted protein [118].

The first case of fish-eye disease was described by Carlson and co-workers in two Swedish families, in 1979 [119, 120]. Affected individuals presented with severe hypoalphalipoproteinaemia and elevated TG levels. These patients also suffer from corneal opacity, a condition that gave rise to the unusual name for this disorder. These individuals had no other medical complications and appeared to be protected against premature coronary artery disease (CAD). However, recent findings provide some evidence to the contrary [121, 122].

1.5.2 Familial LCAT Deficiency

As described earlier, this disorder was first reported in a Norwegian family [123, 124]. Since then, a number of unrelated families diagnosed with FLD have been identified. There are several clinical and biochemical findings that are common to this disorder [122].

1.5.2.1 Ocular changes

The most striking physical characteristic in these patients is the unusual appearance of their corneas. All patients present with corneal opacities in childhood. They are best described as several minute, greyish dots spread throughout the entire stroma resulting in the cloudy appearance of the cornea. Histological analysis of the corneal sections has indicated the presence of electron dense membranous deposits and increased concentrations of unesterified cholesterol and phosphoplipids [116].

Corneal opacities are not unique to this condition. They have been noted in other genetic disorders affecting HDL metabolism such as apo A-1 deficiency [125], Tangier disease [126], fish-eye disease [120], and a combined deficiency of apo A-I and apo C-III [127].

1.5.2.2 Haematological findings

FLD patients present with normochromic anaemia, which has been suggested to be due to moderate anaemia with weak compensatory erythropoiesis [128]. Furthermore, erythrocytes display abnormalities in appearance and lipid composition [129]. A compositional analysis of red blood cells revealed nearly double the normal

levels of unesterified cholesterol and phosphatidylcholine with a marked reduction in sphingomyelin and phosphatidylethanolamine levels [128, 130, 131]. In addition, the erythrocytes in LCAT deficiency are associated with a significant reduction in sodium influx and decreased activity of acetylcholinesterase [72]. Although it is unclear whether other cells are affected, platelet function and lipid composition appears to be normal [132]. Cellular analysis of bone marrow has revealed target cells and a small number of foam cells. In addition, sea-blue histocytes have been identified in bone marrow and spleen using Giemsa stain [133]. Compositional analysis of histocyte granules has revealed membranes in a lamellar arrangement [134]. It has been suggested that these membranes contain unesterified cholesterol and phosphatidylcholine, since the levels of both lipids are elevated in spleen and liver [135].

1.5.2.3 Renal complications

While in most patients proteinuria is detected early in life, a small number do not develop renal complications [136-138]. Serum albumin concentrations are slightly reduced, but can rapidly decrease due to an increase in proteinuria and subsequent renal failure. Although renal function and blood pressure may remain normal for many years, hypertension and renal insufficiency may develop unexpectedly [136]. Morphological analysis of the kidney has revealed various structural changes assessed by light and electron microscopy [134, 135, 139-141].

1.5.2.4 Plasma lipoprotein abnormalities

Plasma lipoprotein analyses of LCAT deficient patients show a multitude of lipoprotein abnormalities with a great deal of variability in plasma lipoprotein levels. The

common characteristics, however, are high plasma levels of unesterified cholesterol and phosphatidylcholine, and residual levels of cholesteryl esters and lysophosphatidylcholine [142]. The small quantities of cholesteryl esters may be explained by the action of the intracellular enzyme, acyl coenzyme A: cholesterol acyltransferase (ACAT) in the intestine. This is consistent with abnormally high proportion of palmitic and oleic acids instead of linoleic acid in the detected cholesteryl esters.

A number of methods have been employed to isolate and study various lipoprotein fractions. These techniques include ultracentrifugation, gel filtration, electron microscopy and gel electrophoresis [143]. Studies of very low density lipoproteins (VLDL, $d<1.006$ g/ml) showed abnormal mobility of particles, which had a higher concentration of unesterified cholesterol and phosphatidylcholine relative to TG and protein. In addition, protein analysis showed an increase in apo C-I and apo E, but a reduction in apo C-II and apo C-III resulting in the reduction of electronegative proteins, which can explain the unusual electrophoretic mobility [144]. Particle size distribution analysis revealed that a large number of particles in the fraction had a diameter greater than 90 nm in overnight fasting samples. However, these particles were significantly reduced when the patients consumed low fat diets the night before sample collection, which suggested that they were chylomicrons [145].

Intermediate density lipoproteins (IDL, $1.006<d<1.019$ g/ml) show an abnormal size heterogeneity on gel filtration. These particles have been shown to be rich in TG and unesterified cholesterol, and the composition of the normal sized particles was shown to be intermediate between VLDL and LDL particles [143]. It has been suggested that reduced hepatic lipase (HL) activity may contribute to an increase in TG

content of these particles due to decreased ability of HL to hydrolyse TG in these particles[146].

Significant changes were also detected in the low density lipoprotein (LDL, 1.019<d<1.063 g/ml) fraction [143]. The elution profile from the gel filtration of the LDL particles resulted in three well-defined components, while there is only one in normal individuals. The first subfraction, which eluted in the void volume, was of 90 nm diameter size which does not appear in normal LDL fractions. This particle was composed of multilamellar structures containing an unusually high ratio (1:2) of phosphatidylcholine to unesterified cholesterol, both of which contribute to the lamellar structure. The protein content of this subfraction was mainly limited to small quantities of albumin. The total concentrations of these particles were proportional to large VLDL particles (chylomicrons), yet they varied among patients. Furthermore, similar to larger VLDL particles, levels of the larger LDL particles could be altered by the fat content of the patient's diet [145].

The second relatively distinct subfraction was eluted just after the void volume and consisted of abnormal particles 30-80 nm in diameter. Similar to the previous fractions these particle were not observed in normal plasma. The structure of some of these particles closely resembled Lp-X , an abnormal lipoprotein found in cholestasis [147]. Electron microscopy revealed a discoidal structure that form stacks. These particles were composed of phosphatidylcholine, unesterified cholesterol, and apo C-I. This fraction also contained some spherical particles resembling normal remnants of VLDL and chylomicrons [148].

The third subfraction of patient LDL had normal physical characteristics. These particles had a diameter ranging between 20 and 22 nm. Although these spherical particles resemble normal LDL, their TG content is much higher than normal (13 fold)

while the CE level is lower (1.5-3 fold). Nonetheless, the protein-apo B100-to core lipid ratio has been shown to be similar to the normal particles. In addition, the apo B concentration was shown to be low suggesting that normal LDL particles comprise a small fraction of the total LDL pool in these patients [149].

High-density lipoproteins (HDL, 1.063<d<1.21 g/ml) also displayed a very heterogeneous distribution of size and shape, consistent with the other lipoprotein classes. These particles are predominantly disc shaped with a minor component of spherical particles of very small size (6 nm). Phosphatidylcholine and unesterified cholesterol were the major components of these discs, which may also contain a mixture of apo A-I, apo A-IV and C apolipoproteins [150, 151]. Some discs were found to contain apo E [152]. The small spherical HDL particles were composed of unesterified cholesterol, phosphatidylcholine, a small amount of core lipid, and two molecules of apo A-I. The concentration of these particles was also dependent on the fat content of each individual's diet.

1.5.3 Fish-Eye Disease

Two patients from Swedish families were the first cases of FED reported [119, 120]. They were characterised by severe HDL deficiency and extensive corneal opacities that gave the name of the disorder, since the eyes of the affected individuals resembled that of boiled fish. The number of reported cases with FED is much smaller than that of FLD. There have only been 11 cases of FED reported in 6 families from Algeria, Germany, the Netherlands and Sweden. Furthermore, there have been a number of cases, which share some biochemical characteristics with FED [153].

1.5.3.1 Ocular changes

Corneal opacities have been reported as early as the second decade of life [119, 120]. The appearance of corneal changes is similar to that described in FLD, which consist of minute greyish dots throughout the entire stroma while increasing in density at the periphery. Compositional analysis of the vacuoles in the stroma revealed a high concentration of unesterified cholesterol and membrane like material in the periphery [120].

1.5.3.2 Plasma lipoprotein abnormalities

The original Swedish families have been the major source of information on the composition and structure of the plasma lipoproteins in FED [120, 154]. Unlike normal plasma, the VLDL fraction of these patients (subjects) had a great deal of size heterogeneity. The concentration of these particles increased five-fold despite the normal TG to CE ratio. The TG content of IDL and LDL was also increased, especially in the LDL fraction, in which the TG to CE ratio was 10 times greater than normal. These particles were characterised as very homogeneous round structures with a smaller mean diameter than normal LDL. However, there were occasional occurrences of particles with diameters ranging from 50 to 85 nm. These large particles were absent from the normal LDL fraction.

In FED patients, HDL fractions show the greatest abnormalities. This fraction was reduced to 10% of the normal while the unesterified cholesterol level was about three times greater than that of control HDL. Furthermore, these particles were enriched in apo E while the A apolipoprotein levels were drastically reduced (90%) [120]. Structurally, the HDL fraction presented two major subclasses. The small, round

particles had a diameter of 7.6 nm, while discoidal particles had a thickness of 4.4 nm and an average diameter of 17.4 nm [154].

1.5.4 Atherosclerosis in LCAT Deficiency Syndromes

The evidence on the role of LCAT in atherosclerosis is controversial. The role of LCAT in the reverse cholesterol transport (RCT) pathway has been clearly established, and it is well accepted that high esterification rates promote the efflux of cholesterol from the periphery. Therefore, LCAT appears to play an important role in maturation of HDL [116]. However, patients who lack mature HDL, due to dysfunctional LCAT protein, do not present with premature atherosclerotic vascular disease [116, 155, 156]. In FED patients, a partially functional RCT pathway has been suggested to protect against premature atherosclerosis. However, this does not account for the absence of CAD in FLD patients in whom RCT is completely impaired. This issue has been addressed by another hypothesis suggesting that the residual HDL in FLD may be an excellent acceptor for peripheral cholesterol [157]. Findings by Ohta et al [158], on the other hand, indicated that these particles are less efficient acceptors of unesterified cholesterol as originally suggested.

Kinetic analysis of HDL subfractions in both FLD and FED patients has provided further insight into the metabolic basis of the absence of CAD in LCAT deficiency syndromes [159]. It was shown that HDL particles containing both apo A-I and apo A-II (Lp A-I:A-II) were cleared much faster than the particles containing apo A-I alone (Lp A-I). Thus, it was concluded that low plasma HDL level promoted accelerated catabolism of apo A-II and Lp A-I:A-II. The remaining Lp A-I fraction may have a protective potential against atherogenesis [160-162]. The investigators proposed that the remaining Lp A-I could maintain an effective RCT pathway. Furthermore, it was hypothesised that

hypercatabolism of Lp A-I:A-II and the presence of apo A-I and Lp A-I contributed to a relative absence of premature CAD risk in both FED and FLD. However, the subsequent studies with macrophage foam cells showed that Lp A-I and Lp A-I:A-II of FLD patients were poor acceptors of intercellular cholesterol and it appeared that LCAT activity was required to remove cholesterol from the foam cells by Lp A-I [158, 163]. Thus, it was concluded that factors other than apo A-I mediated RCT should be considered in order to explain the apparent protection of FLD patients from premature CAD. The next hypothesis postulated that a reduction in CE-rich LDL could explain the reduced number of CAD cases described.

Absence of CAD in patients with the LCAT deficiency syndromes, may be attributed to a recently described lipoprotein particle termed gamma-LpE (γ-LpE) [164]. Gamma-LpE particles are sphingomyelin-rich lipoproteins with apo E as their sole structural protein [165]. These spherical particles exhibit gamma mobility on agarose gel electrophoresis and their size range between 12 and 16 nm [165]. Efflux studies have shown that γ-LpE is a strong acceptor of cell-derived cholesterol. The potent effect of γ-LpE in mobilisation of cell-derived cholesterol has been shown to be independent of apo A-I containing lipoproteins, although the apo E genotype is an important determinant of cholesterol uptake [166-169]. Patients with LCAT deficiency syndromes, although due to the absence of their HDL pool exhibit significantly reduced net efflux of unesterified cholesterol from the periphery to the plasma compartment, it is proposed that the γ-LpE dependent efflux capacity of the plasma remains intact [170].

Although the incidence of premature CAD in FLD is very limited, there is some evidence of early coronary atherosclerosis in a few cases and first family members [171, 172]. However, 6 out of 19 known FED patients from 5 families have been reported to suffer from premature atherosclerosis [121, 173, 174]. It is noteworthy that

all the patients with premature CAD are male. The difference in risk of premature CAD between FED and FLD requires further investigation. In addition, this difference may be related to the presence of LCAT activity associated with LDL particles in FED (Figure 1-2). It is our contention that the referral biases and small sample size prevent any meaningful conclusion. However, it must be stressed that despite a general absence of premature CAD in LCAT deficiency syndromes, there is growing evidence for the higher risk of premature atherosclerosis in patients with FED.

1.5.5 Classification of LCAT gene defects

In a recent review, our laboratory proposed a new scheme for the classification of natural mutations of the LCAT gene [122]. This classification is based on the specific nature of each mutation and avoids the major complications with classification of these defects based on their biochemical phenotype alone. First, biochemical analysis of many gene defects in vitro has resulted in discrepancies in characterisation of the gene defects. This has been partly attributed to dissimilarities in methodologies for LCAT activity and mass assessments [118, 175-177]. Furthermore, use of multiple expression systems has been shown to result in variations in protein processing due to the differences in the nature and source of the cells and their protein processing machinery [73, 90, 121, 171, 174-182]. Third, not all defects can be identified in the homozygous state. Some LCAT gene defects have manifested in combination with another defect - a compound heterozygous state. This has posed a difficulty in establishing a clear classification for some gene defects. Lastly, some defective gene products display an intermediate biochemical phenotype, which shares specific characteristics with both FED and FLD [90, 118, 174, 183].

Tables 1-3 and 1-4 summarise our classification of the natural mutants of the LCAT gene using a combination (where possible) of chemical, biochemical, and genetic data). The phenotypic expression of LCAT deficiency syndromes is illustrated in Figure 1-6.

Table 1-3. Mutations that underlie LCAT deficiency syndromes.

Defect		Exon	Clinical Phenotype FLD	FED	References[a]
Homozygous mutations					
1. C-insertion (codons 9,10)[b]		1	✓		[146, 184-186]
2. P10L		1		✓	[119, 120, 187]
3. G30S		2	✓		[188-190]
4. Y83-stop		3	✓		[156]
5. A93T		3	✓		[155, 156, 175]
6. R99C		3		✓	[191]
6. T123I		4		✓	[73, 173, 192]
7. N131D		4		✓	[174]
8. R140H		4	✓		[181]
9. G141-insertion		4	✓		[193]
10. TG deletion -Stop(Codon 144)		4	✓		[194]
11. L209P		5	✓		[155, 156]
12. N228K		6	✓		[193]
13. G230R		6	✓		[195]
14. R244G		6	✓		[185, 196]
15. M252I		6	✓		[117, 123, 197, 198]
16. M293		6	✓		[185, 193, 199-202]
17. L300-deletion		6	✓		[183, 203]
18. T321M		6	✓		[156, 204, 205]
19. G344S		6	✓		[182]
20. G-deletion (codon 264)[b]		6	✓		[182]
Heterozygous mutations					
1. P10Q	R135Q	1, 4		✓	[121]
2. L32P	T321M	2, 6	✓		[185]
3. G33R	30bp Insertion (codon 4)	1, 2	✓		[206]
4. Y83-stop	Y156N	3, 5	✓		[179]
5. T123I	Y144C	4, 4		✓	[207]
6. T123I	Intron 4 defect (IVS4:T-22C)	4, intron 4		✓	[178]
7. T123I	T347M	4, 6		✓	[208]
8. T123I	Unknown	4		✓	[209]
9. R135W	A-insertion (codon 376)[b]	4, 6	✓		[155, 156, 210, 211]
10. R147W	Unknown	4	✓		[155, 172, 212]
11. R147W	Y171-stop	4,5	✓		[213]
12. G183S	A-T substitution/C-deletion (codon 120)[b]	6, 5	✓		[185, 214]
13. M252K	N391S	6, 6		✓	[90]
14. T321M	C-deletion (codon 168)[b]	5, 6	✓		[171]
15. R399C	C-insertion (codon 9, 10)[b]	6, 1	✓		[180]

[a] References include biochemical, clinical and genetic reports on the LCAT gene defects presented.
[b] Premature truncation as a result of a frameshift.

Table 1-4. Summary table of recently proposed classes of LCAT deficiency syndromes.

Class/Definition	Defect	*References*[a]
1. Null mutations causing FLD		
Total loss of catalytic activity. LCAT mass: (virtually) absent.	30 bp Insertion (codon 4)	[206]
	C-insertion (codon 9, 10)	[180, 184, 185]
	Y83-stop	[156, 179]
	G141-insertion	[193]
	Intron 4 defect (IVS4:T-22C)	[178]
	A-T substitution/C-deletion (codon 120)	[185]
	Dinucleotide deletion, frame shift Stop codon (144)	[194]
	C-deletion (codon 168)	[171]
	Y171-stop	[213]
	G-deletion (codon 264)	[182]
	A-insertion (codon 376)	[155, 156]
2. Missense mutations causing FLD		
Total loss of catalytic activity. LCAT mass: normal / reduced / absent.	G30S	[188, 190]
	L32P	[176, 185]
	G33R	[206]
	A93T	[155, 156, 175, 176]
	R135W	[118, 155, 156, 176]
	R135Q	[121]
	R140H	[181]
	R147W	[155, 176, 177, 181]
	Y156N	[177, 179]
	G183S	[185]
	L209P	[118, 155, 156, 176]
	N228K	(52,64)
	G230R	[215]
	R244G	[185]
	M252K	[117]; [90]; [118]
	T321M	[156, 171, 176, 185]
	G344S	[182]
	T347M	[118, 176, 179, 208]
	R399C	[180]
3. Missense mutations and minor deletions causing FED		
Partial loss of activity against a) LDL, or b) both HDL and LDL. LCAT mass: reduced.	R99C	[191]
	N131D	[174]
	L300 deletion	[118, 179, 183]
	N391S	[118]; [90]]
4. Missense mutations causing FED		
Partial loss of activity against HDL only. LCAT mass: reduced.	P10L	[118, 176, 177, 187]
	P10Q	[121]
	T123I	[73, 118, 173, 176-178, 207, 208]
5. Unclassified mutations		
	Y144C	[207]
	M293I	[177, 193, 199, 200]
	R158C	[155, 175-177, 216, 217]

[a] The references are reports describing the identification and functional assessment (when available) of the LCAT gene defects.

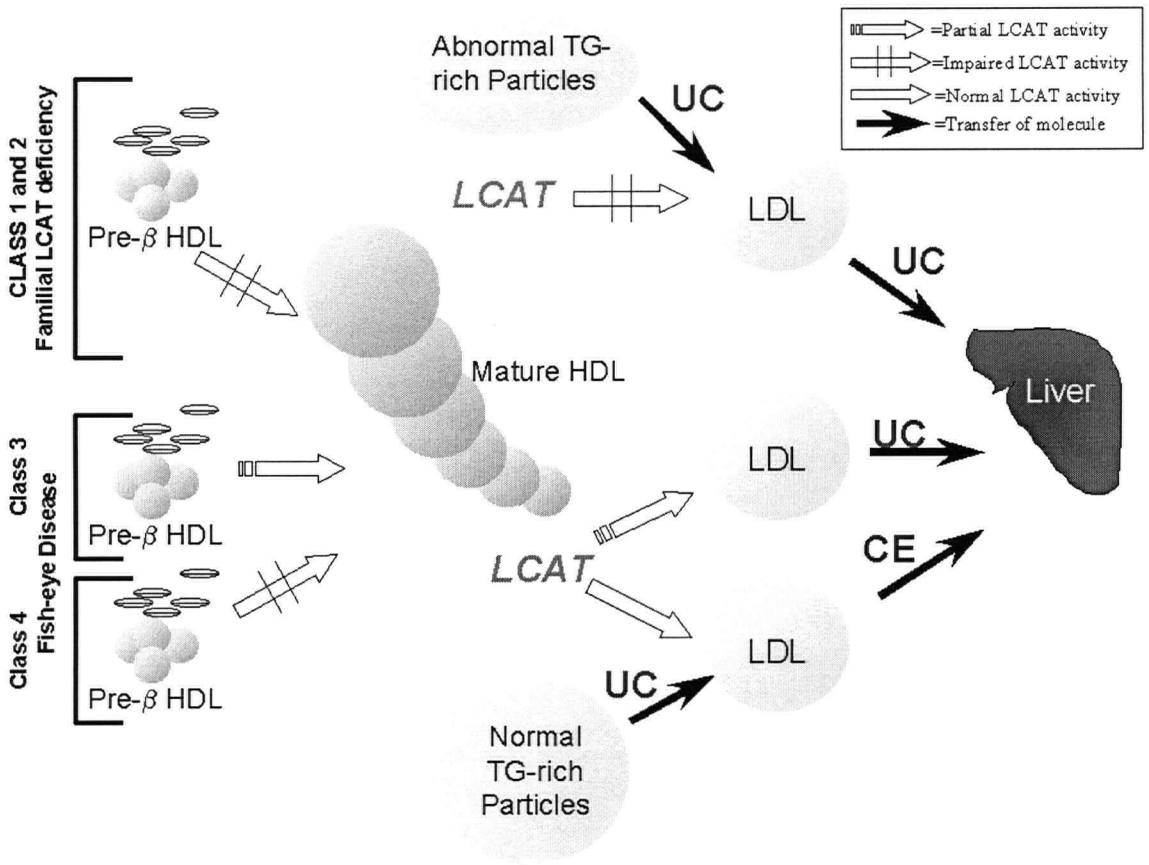


Figure 1-6. Influence of LCAT deficiency syndromes on lipoprotein metabolism. This figure illustrates the biochemical presentation of various classes of LCAT gene defects. **Class 1**- Null mutations causing FLD; there is a total loss of LCAT activity and LCAT mass is virtually absent. **Class 2**- Missense mutation causing FLD; there is a total loss of LCAT activity and LCAT mass can vary from normal to absent. **Class 3**- Missense mutations and minor deletions causing FED; there is a partial loss of activity against HDL or both LDL and HDL; LCAT mass is reduced. **Class 4-** Missense mutations causing FED. There is a partial loss of LCAT activity against HDL only and LCAT mass is reduced. UC, unesterified cholesterol. CE, cholesteryl esters.

1.6 Rationale

Our current understanding of interaction of LCAT with plasma lipoproteins is largely based on well-controlled *in vitro* experiments using LCAT from plasma or various expression systems, and synthetic lipoproteins and their analogues. Although there have been studies investigating effects of over-expression of LCAT in transgenic animal models, metabolism of rLCAT *in vivo* has received little attention and the preliminary work by Pritchard *et al* [94] has been primarily hypothesis generating.

Studies of functional mutants of LCAT have identified specific structural components of the enzyme, which may independently influence its activity such as substrate binding and accessibility as well as lipoprotein specificity and binding. Function and structure studies of LCAT have primarily been concerned with functional changes due to alteration in the amino acid sequence of the enzyme, although the carbohydrate component of LCAT has been shown to alter its activity. The biochemical basis of the contribution of the carbohydrate moieties to the enzymatic activity of LCAT is yet to be defined.

Delineation of the 3-D structure of LCAT will ultimately give us the full picture on LCAT structure providing a new dimension for the researchers to approach the study of LCAT function, however, current efforts are hampered by difficulties in purification and crystallisation of partially purified material. In addition, a recent publication by Peelman et al, has provided a predicted 3-D structure of LCAT based on a computer modelling strategy [8]. Although this information is and will be valuable in design of future studies, it has only become available in the latter part of the research project, and unfortunately unable to contribute to the early conceptualisation stages of the current work.

Therefore, our original interest in investigating LCAT function with respect to HDL metabolism is focused on the use of various functional and structural mutants of LCAT. Findings from this body of work will contribute to the overall understanding of LCAT structure and function and will be complemented with future findings on 3-D structure of the protein.

1.7 Hypothesis

Functional and structural defects of LCAT influence its interaction to plasma lipoproteins

Note: This is the general hypothesis that provides a global view of the research work presented in this thesis. Specific hypotheses are stated in the appropriate chapters.

1.8 Objectives

1: To generate/identify a source of recombinant LCAT that would functionally and structurally be identical to plasma LCAT.

2: To characterise the interaction of LCAT with lipoprotein substrates.

3: To determine the relationship of LCAT mutations with its structural and functional characteristics.

Chapter 2

Production of recombinant LCAT in "McArdle 7777" and "Baby Hamster Kidney" cells.

Data presented in this chapter has contributed to the following manuscript:

Ayyobi A.F., Lacko A.G., Murray K., Nair M., Li M., Molhuizen H.O.F., and Pritchard P.H. *Biochemical and Compositional Analyses of Recombinant Lecithin: Cholesterol Acyltransferase (LCAT) Obtained from a Hepatic Source*. Acta Biochimica et Biophysica, 1484 (2000), 1-13

I would like to acknowledge the work by M-Scan who performed the carbohydrate composition analyses.

2.1 Introduction

Recombinant LCAT has been expressed in a number of expression systems to study its function and structure [90-93]. Hill *et al*[90] showed that recombinant LCAT (rLCAT) could be expressed in large quantities (10 mg/L) in BHK cells. This recombinant protein displayed very similar physical (i.e. molecular weight (MW) and glycosylation pattern) and chemical (i.e. specific activity) characteristics when compared to plasma LCAT. However, attempts to crystallise this protein have been impeded due to a number of complicating factors such as self-aggregation due to increasing concentration [93] and heterogeneity of carbohydrate chains and difficulties in protein purification (unpublished data). Turnover studies of rLCAT from the BHK expression system in rabbits have demonstrated that the majority of rLCAT failed to bind or associate with native plasma lipoproteins, resulting in rapid clearance of the unbound protein [94].

A recent study by Jin *et al* [93] showed that rLCAT can be expressed in significant quantities (5mg/L) and purified to greater than 96% in CHO cells. The rLCAT from this expression system displayed slightly higher molecular weight compared to plasma LCAT [93]. This difference in size was attributed to the extent of glycosylation of the enzyme, since the protein amino acid sequence was based on wild-type LCAT. While changes in Vmax indicated a change in the catalytic rate, unaltered Km suggested that the binding of rLCAT to substrate is unlikely to be affected by the variations in oligosaccharide structure.

Studies of the glycosylation pattern in separate expression systems further emphasizes its importance in the enzymatic function of LCAT [91]. Accordingly, kinetic

data obtained from HepG2 (hepatocyte derived cell line), SF21 (baculovirus infected cell line), and CHO (Chinese hamster ovary) cell lines suggest that the differences in enzymatic function of LCAT are due to altered Vmax of cholesteryl ester (CE) generation rather than the Km and acyl substrate specificity, both of which present minor variations [91]. Interestingly, despite the physical and functional differences between the plasma LCAT and CHO secreted rLCAT, intrinsic tryptophan fluorescence emission spectrum and far UV CD spectrum suggest that secondary and tertiary structures of both proteins are very similar [93].

Quantitative analyses of carbohydrate structure and composition of BHK LCAT in comparison to plasma LCAT have revealed two major differences between the two proteins [95]. First, the plasma LCAT contains only bi and tri-antennary structures, while the BHK LCAT displays an additional tetra-antennary structure. Second, the sugar composition of these oligosaccharide chains has been shown to differ in the extent of core fucosylation. Despite these marked differences, both glycoproteins were shown to have very similar activities and monosaccharide composition [95].

It is therefore crucial to generate a recombinant protein with a very similar, if not identical, glycosylation pattern and composition compared to the native plasma LCAT in order to better understand the biochemistry of the multi-faceted function of this glycoprotein. Thus, the purpose of these studies was to generate and characterize a new expression system using Mc-7777 rLCAT. The recombinant LCAT from Mc-7777 and a BHK expression system was purified and biochemical parameters were determined. Carbohydrate structure and composition was determined by enzymatic degradation of the oligosaccharide moiety and NMR.

2.1.1 Rationale

With the exception of a recent report of HepG2 cells, rLCAT has been expressed in a variety of cell lines derived from organs that are not the native site of expression (liver) for LCAT. Considering the very sensitive nature of this protein to small variations in post-translational processing, physiological relevance of these expression systems with alterations in glycosylation architecture and their apparent Vmax is questionable. It was crucial to generate a recombinant protein functionally and structurally identical to the native plasma LCAT for our biochemical studies.

McArdle RH-7777 cell line (Mc-7777), a rat hepatoma derived line, was used to express rLCAT. The Mc-7777 rLCAT was compared to the BHK rLCAT, which has been extensively studied and characterised, including the architecture and composition of its carbohydrate moieties [95]. Thus, BHK rLCAT was used as the surrogate control instead of plasma LCAT.

2.1.2 Hypothesis

A rat hepatoma based cell line (Mc-7777) can express a glycoprotein (LCAT) that contains an identical carbohydrate composition to that of plasma LCAT

2.1.3 Objectives

- To express LCAT in a hepatoma cell line, Mc-7777 cells.
- To compare the physical and chemical characteristics of rLCAT from Mc-7777 and BHK expression systems.

2.1.4 Specific Aims:

1. To transfect and express rLCAT in Mc-7777 cells.

2. To characterise the expression system.
3. To compare the structural and functional properties of rLCAT from transformed Mc-7777 and BHK cell lines.

2.2 Methods

2.2.1 Tissue Culture for Mc-7777 cells and BHK

Mc-7777 cells were maintained in Dubbelco's Modified eagle medium (D-MEM) supplemented with 10% heat inactivated horse serum (HS), 10% heat inactivated fetal bovine serum (FBS), and 1% antibiotic and antimycotic reagent (PSF). Sera were heat-inactivated at 56°C for 30 min prior to use as a growth supplement only. BHK cells were maintained in D-MEM supplemented with 10% FBS and 1% PSF. All cells were monitored daily and media was changed on alternating days for maintenance.

2.2.2 Stable Transfection of Mc-7777 cells with pNUT-LCAT and pSV2neo plasmids

Mc-7777 cells were co-transfected with pNUT [218, 219], which contained the human LCAT cDNA, and pSV2neo [220] construct that provided resistance to Geneticin for selection purposes. Stable clones in Mc-7777 cells were produced by the calcium precipitation method [90]. Subsequently, the transfected cells were selected using 500μM Geneticin (G418-GibcoBRL) supplemented growth medium. Selection was carried out over a period of 20-30 days, after which individual colonies were macroscopically detectable. Single colonies were transferred to 20-mm culture dishes and grown to confluence under selective conditions. Conditioned medium from each colony was assessed for LCAT activity to identify the colonies that were successfully

double transfected. LCAT positive clones with maximal expression of LCAT were identified by solid-phase LCAT immunoassay as previously described [90].

2.2.3 Production and Purification of rLCAT from Mc-7777 and BHK cells

Mc-7777 cells were seeded at equal density in three 100-mm culture dishes and grown to 80% confluence. Growth medium was replaced with serum-free Opti-MEM (Gibco-BRL) and purged for 6 hrs. Subsequently, fresh media (Opti-MEM) was placed in each dish and samples of media were collected at time 0, 12, 24, and 48 hours. LCAT concentration and activity were determined in duplicate for each time point. All calculations accounted for the reduction of media with each sampling. Recombinant LCAT was purified as previously described [90, 95]. Briefly, 200 ml of conditioned media from BHK and Mc-7777 cells were collected, filtered to remove solid material, and adjusted to 0.3 M NaCl. Medium was loaded onto a phenyl Sepharose column (Pharmacia Bio-Tech) and washed with two volumes of phosphate buffered 0.3 M NaCl, pH 7.4. Recombinant LCAT was eluted by deionized distilled water and further purified through heparin Sepharose (Sigma) and Affi-gel Blue (Bio-Rad) columns. The single peak of LCAT was pooled and concentrated. LCAT concentration was determined by solid-phase immunoadsorption, as previously described [90]. Purified LCAT was stored at –70°C. LCAT enzyme activity was determined using proteoliposomes (HDL analogue particles) as previously described [221].

2.2.4 Structural Analysis of rLCAT

2.2.4.1 Enzymatic Deglycosylation of rLCAT

Prior to N-glycosidase F digestion, 15 μl of purified protein sample was combined with 10 μl of 0.9 M sodium phosphate, pH 8.7 and 4 μl of 10% Nonidet P-40, boiled for

10 min and cooled on ice. 1 μl of N-glycosidase F (250 U/ml) was added and the sample was incubated at 37°C overnight. Endoglycosidase H digestion was performed by adding 10 μl of 0.15 M sodium acetate buffer, pH 5.8 and 4 μl of 10% Nonidet P-40 to 15 μl of sample. The mixture was boiled for 10 min, cooled on ice and 2 μl of Endoglycosidase H (1 U/ml) was added prior to incubation at 37°C overnight. Sialic acid residues of LCAT were removed using Neuraminidase. Three μl of Neuraminidase (1U/ml) was added to 7 μl of culture medium and incubated at 37°C for 1 hr.

2.2.4.2 SDS-PAGE

Samples were mixed 1:1 with 2X SDS sample buffer containing 10% β-mercaptoethanol and 0.1% Bromophenol Blue. The sample mixture was boiled for 5 min before loading onto a 10% polyacrylamide gel. The gel was run at 15 mA/gel (constant current) for 45-min. High molecular weight markers (from BioRad) were used as standards. Gels were stained using Coomassie G250 (0.1% w/v), in ammonium sulphate (8% w/v), phosphoric acid (1.6% w/v), and 20% methanol or silver stain method.

2.2.4.3 Western Blot Analysis

Western blotting was performed as previously described [90]. Subsequent to electrophoresis, the gels were electroblotted onto nitrocellulose (0.45 μm). The membranes were then incubated with polyclonal goat anti-human LCAT antibodies (obtained from Dr. A. Lacko, Fort Worth, TX) and Protein G-HRP to detect the LCAT protein.

2.2.5 Monosaccharide linkage and oligosaccharide composition analysis

This set of analyses was performed by M-Scan Inc., West Chester, Pennsylvania. The following methods and relevant results are based on the information provided by this company. The following material is to illustrate the methodology utilized by M-Scan Inc. to determine the carbohydrate composition and sequence.

2.2.5.1 *Monosaccharide Composition analysis*

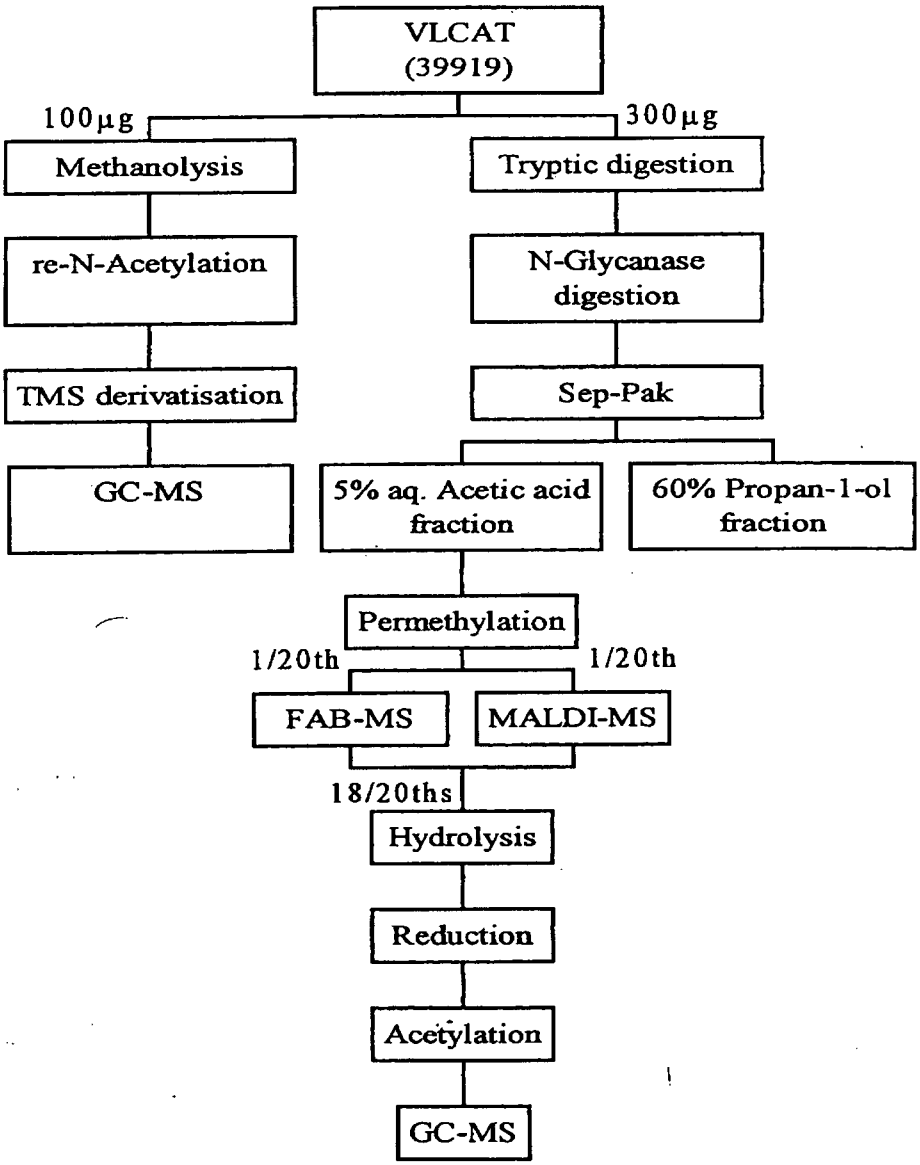


Figure 2-1. Schematic outline of analysis performed for carbohydrate structure and sequence determination of oligosaccharide moieties of Mc-7777 rLCAT.

2.2.5.2 Sample preparation

An aliquot (100 μg) of the sample, containing 40nmoles of internal standard (Arabitol) was lyophilised. The aliquot was hydrolysed at 80°C for 16 hours with methanolic/HCl (IN), re-N-acetylated and derivatised. A standard mixture of monosaccharides with 40nmoles of internal standard (Arabitol), and a tube/reagent blank also containing 40nmoles of internal standard (Arabitol) were also hydrolysed, re-N-acetylated and derivatised.

2.2.5.2.1 GC-MS analysis

An aliquot (1ul) of the derivatised carbohydrate sample, tube/reagent blank and standard mixture dissolved in hexane (100 μl) were analysed by GC/MS using a VG MassLab Trio-1 quadrupole mass spectrometer with integrated Hewlett Packard 5890 capillary gas chromatograph and an INTEL 386 PC data system under the following conditions:

Gas chromatography

Column:	DB5
Injection:	On-column
Injector Temperature:	90°C
Programme:	2 minutes at 90°C then 50°C/minute to 140°C, then 5°C/minute to 220°C, and 10°C/minute to 300°C, finally held at 300°C for 5minutes.
Carrier Gas:	Helium

Mass Spectrometry

Ionisation Voltage:	70eV

Acquisition Mode:	Scanning
Mass Range:	50-650 Daltons
MS Resolution:	Unit

2.2.5.3 Enzymatic digestion of the intact glycoprotein

An aliquot (300ug) of intact glycoprotein was digested with TPCK treated Trypsin. The lyophilised peptide/glycopeptide mixture was then digested with PNGaseF (3.2.8.3) for carbohydrate studies.

2.2.5.4 Peptide N-Glycosidase F Digestion

The tryptically cleaved peptide/glycopeptide mixture obtained from the glycoprotein was treated with the enzyme peptide N-glycosidase F in ammonium bicarbonate buffer, pH 8.4 at 37°C, for 16 hours. The reaction was stopped by freeze-drying. The resulting product was purified using a C_{18} Sep-pak cartridge. N-linked carbohydrates eluted in the 5% aq. acetic acid fraction were permethylated using the sodium hydroxide (NaOH)/methyl iodide (MeI) procedure. (Peptides and potential O-linked glycopeptides were eluted with 60% propan-1-ol in 5% aq. acetic acid and retained). Portions of the permethylated glycan mixture were analysed by FAB- and MALDI-MS and the remainder was subjected to Linkage Analysis.

2.2.5.5 Mass Spectrometry

Positive Fast Atom Bombardment mass spectrometric analyses were carried out on M-Scan's VG AutoSpecE mass spectrometer operating at Vacc = 8kV for 45ll mass range at full sensitivity with a resolution of approximately 2000-3000. A Caesium Ion

Gun was used to generate spectra operating at 30kV. Spectra were recorded on a VAX data system 3100 M76 using Opus software.

Permethylated carbohydrates were dissolved in methanol and loaded onto a target previously smeared with 2-4μl of thioglycerol.

MALDI-TOF mass spectrometry was performed using a Voyager Elite Bio-spectrometry Research Station laser-desorption mass spectrometer coupled with Delayed Extraction (DE). The dried permethylated glycans were re-dissolved in methanol:water (80:20) and analysed using a matrix of 2,5-Dihydroxybenzoic acid. Angiotensin and ACTH were used as external calibrants. The set up of the mass spectrometer used to obtain the data is shown below each mass spectrum.

2.2.5.6 Linkage Analysis

2.2.5.6.1 Sample Preparation

The remainder of the permethylated M-Glycan sample (following FAB-and MALDI-MS) was subjected to Linkage Analysis as follows.

2.2.5.6.2 Derivatisation

The permethylated samples were hydrolysed (2M, Trifluoroacetic acid, 2 hours at 121°C) and reduced (Sodium borodeuteride ($NaBD_4$) in 2M NH_4OH, 2 hours at room temperature). The hydrolysis products were then dried down under a stream of nitrogen. The borate produced on the decomposition of the borodeuteride was removed by 4 additions of a mixture of methanol in glacial acetic acid (90:10) followed by evaporation to dryness under a stream of nitrogen. The samples were then acetylated using acetic anhydride (1 hour at 100°C). The acetylated samples were purified by

extraction into chloroform. The partially methylated alditol acetates were then examined by gas chromatography/mass spectrometry (GC/MS, 3.2.8.5.3). A standard mixture of partially methylated alditol acetates was also analysed under the same conditions.

2.2.5.6.3 GC-MS Analysis

An aliquot (1μl) of the derivatised carbohydrate sample dissolved in hexane was analysed by GC/MS using a VG MassLab Trio-1 quadrupole mass spectrometer with integrated Hewlett Packard 5890 capillary gas chromatograph and an INTEL 386 PC data system under the following conditions:

Gas Chromatography

Column:	DB5
Injection:	On-column
Injector Temperature:	40°C
Programme:	0.5 minutes at 40°C then 70°C/minute to 100°C for 1 minute, then 8°C/minute to 290°C, finally held at 290°C for 5minutes.
Carrier Gas:	Helium

Mass Spectrometry

Ionisation Voltage:	70eV
Acquisition Mode:	Scanning
Mass Range:	35-450 Daltons
MS Resolution:	Unit

2.3 Results

2.3.1 Expression and characterization of LCAT in Mc-7777and BHK cells

Mc-7777 cells were transfected with pSV2neo in addition to LCAT cDNA and forty colonies resistant to G418 were isolated and screened for LCAT activity. Two colonies were strongly positive for LCAT activity while the remaining 38 colonies presented either very low or no activity. Of the two positive colonies, the highest expresser of LCAT was selected and expanded for future studies. The isolated protein from the conditioned media of positive colonies resulted in a protein, approximately 67 kD in size, which was similar to that produced by BHK cells (Figure 2-2)

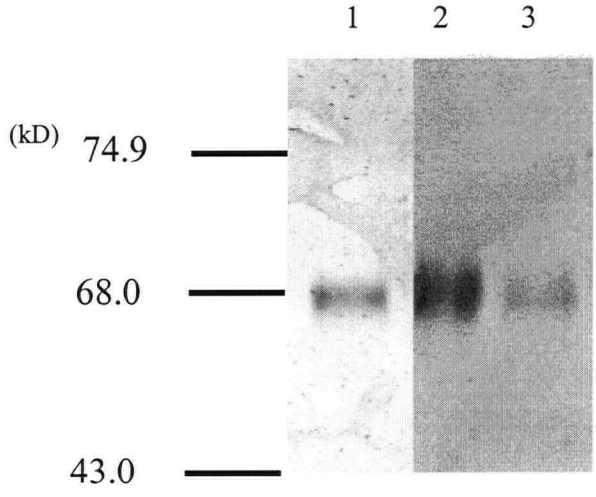


Figure 2-2. Molecular weight of purified rLCAT. rLCAT was purified using a Phenyl sepharose column. Purified rLCAT was reduced and loaded onto a 10% polyacrylamide gel. Lane 1 represents the SDS-PAGE gel stained using silver stain. Lane 2 is the western blot analysis of BHK rLCAT and lane 3 contains Mc-7777 rLCAT.

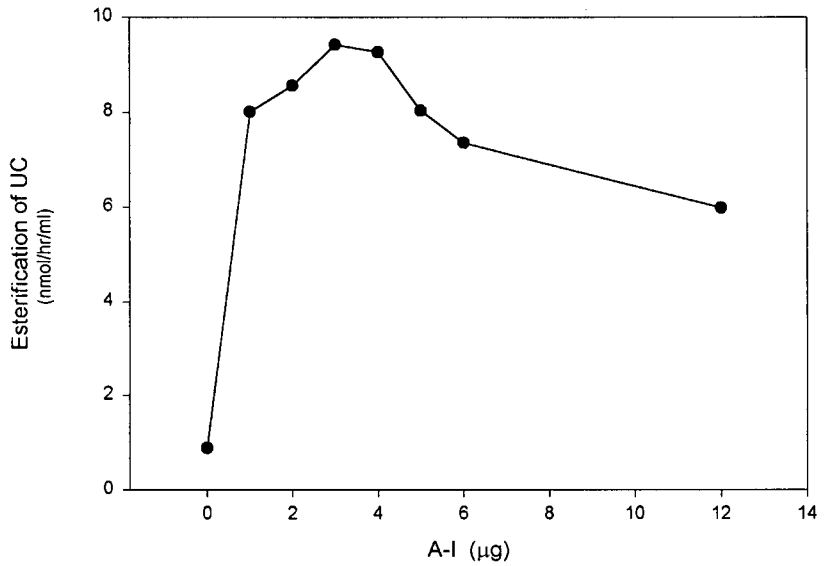


Figure 2-3. A-I activation of Mc-7777 expressed rLCAT. Recombinant LCAT from Mc-7777 was assessed for A-I requirement for peak activation of the enzyme. Various amounts of A-I were used (0-14 μg/ml) while the liposome concentration was maintained the same. Mc-7777 rLCAT was activated at 4 μg/ml of A-I, which is consistent with the results from BHK and human LCAT. Each point on the graph represents mean of duplicate measurements from a single experiment.

2.3.2 Activation by apo A-I

The apo A-I activation curve obtained from increasing apo A-I:UC ratio indicates that 4 μg:4.66 nmol of UC is optimal for Mc-7777 rLCAT activity. This ratio also is the optimal ratio for BHK rLCAT and plasma LCAT (Figure 2-3)

2.3.3 Enzyme Kinetics

Vmax and Km of rLCAT from BHK and Mc-7777 cells were determined (Table 2-1). Vmax values (duplicate measurements with an average variation <5%) of rLCAT from BHK and Mc-7777 cells were 27.55 and 25.91, respectively. Km values (duplicate measurements with an average variation <5%) also were similar for both the BHK and Mc-7777 cell-lines (Table 2-1). These data suggest that kinetic properties of Mc-7777

rLCAT and BHK rLCAT are very similar to those of plasma LCAT (Table 2-1).

Table 2-1. Kinetic properties of rLCAT from BHK and Mc-7777 expression systems. Data obtained from enzyme kinetic experiments were used to determine Km and Vmax for rLCAT from each expression system. Each value represents the mean of duplicate measurements from duplicate experiment.

	Km (mM)	Vmax (nmol/h/ml)	Catalytic efficiency Vmax/Km
BHK	0.57	27.6	48.4
Mc-7777	0.83	25.9	31.2

2.3.4 Enzymatic deglycosylation of rLCAT

The carbohydrate structures of rLCAT expressed in BHK and Mc-7777 cell lines

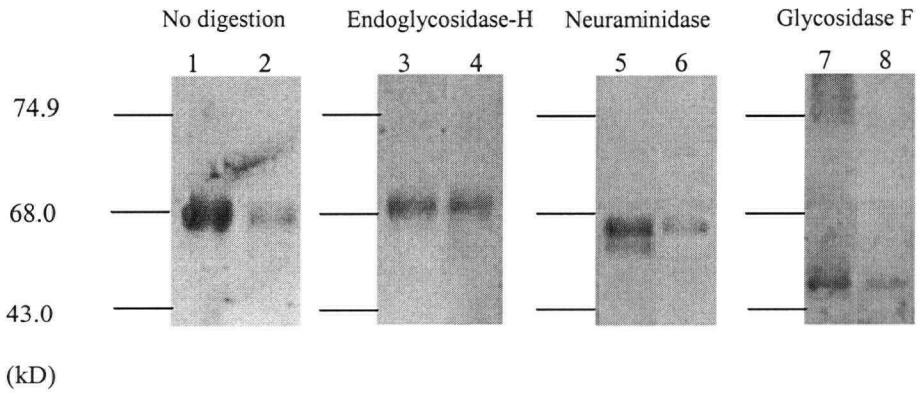


Figure 2-4. Glycosylation pattern of Mc-7777 expressed rLCAT. Purified rLCAT from BHK and Mc-7777 cell were digested using Endoglycosidase H, Neuraminidase, and Glycosidase F. (a) lanes correspond to BHK cell in each group. Lane 1-2: non-digested proteins, lane 3-4: Endoglycosidase H had no digestive effect, lane 5-6 digested with Neuramindase which resulted in approximately 3000 kD loss of MW, and lane 7-8 Glycosidase F treatment resulted in extensive degradation of both proteins to 47 kD, similar in both rLCATs.

were determined by enzymatic deglycosylation. Selective deglycosylation yielded similar patterns of glycosylation for BHK and Mc-7777 rLCAT (Figure 2-4).

Recombinant LCAT, expressed in either cell line, was resistant to digestion by Endoglycosidase H, (lanes 2-4), which specifically hydrolyses the N-linked carbohydrate chain terminated with a Mannose group. Upon digestion with Glycosidase F (lane 7-8), which hydrolyses all N-linked oligosaccharides, rLCAT from both cell lines lost approximately 20 kD in the apparent MW to 48,000 daltons. The apparent MW was consistent with previous findings[90, 175, 222]. In addition, digestion with Neuraminidase (lanes 5-6), which removes Sialic acid residues, resulted in about 3000 dalton MW reduction in rLCAT expressed by both cell lines. This reduction in MW is consistent with the previously reported findings [223-225]. Therefore, Figure 2-4 clearly shows that rLCAT produced by Mc-7777 has a similar carbohydrate digestion pattern to rLCAT expressed in BHK cells that has been reported to be identical to plasma [90].

2.3.5 Analysis of monosaccharide composition and sequence analysis:

The data obtained from GC-MS analysis of the TMS derivatised sugars cleaved from rLCAT are represented in Figure 2-5B. Upon comparison with GC-MS analysis of a standard mixture of TMS derivatised monosaccharides (Figure 2-5A), and GC-MS analysis of TMS derivatised sugars present in blanks (Figure 2-5C), the data from GC-MS analysis of TMS derivatised monosaccharides from rLCAT show the presence of Fucose, Xylose, Mannose, Galactose, Glucose, N-Acetylglucosamine and N-Acetylneuraminc acid (Sialic acid). This data is presented in a tabular form for better illustration and ease of comparison (Table 2-2).

These sugars were also quantified from TMS derivatised standard mixture, blank, and sample obtained from rLCAT. The summary of these results is also presented in Table 2-2.

A)

B)

C)

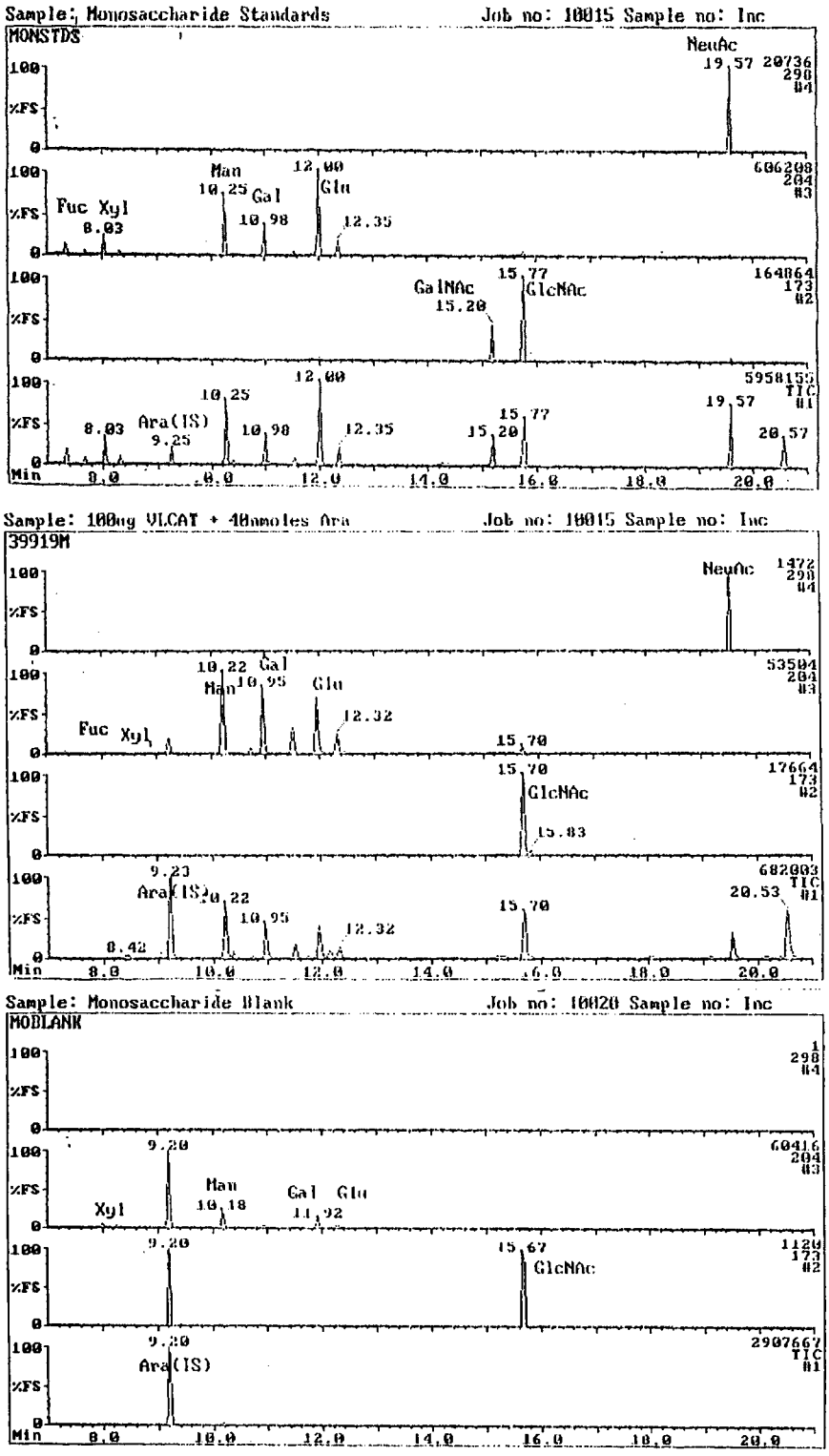


Figure 2-5. GC-MS monosaccharide analysis of rLCAT N-glycans. GC-MS analysis of TMS derivatised sugars was performed in A)monosaccharide standard, B) cleaved from rLCAT and C) the monosaccharide blank. The predominant peaks of cleaved sugars from rLCAT are compared to those of sugar standards and corrected for the blank.

Table 2-2. Quantitation of monosaccharides hydrolysed from rLCAT

Monosaccharide	50 nmoles standard	Blank	Relative content in rLCAT sample (100 ng of Mc-7777 rLCAT)
Fucose	50	-	0.9
Xylose	50	0.3	0.07
Mannose	50	0.3	5.3
Galactose	50	0.1	10.3
Glucose	50	0.1	2.7
N-Acetylgalactosamine	50	-	-
N-Acetylglucosamine	50	0.06	4.4
N-Acetylneuraminic acid	50	-	2.7

2.3.6 N-Linked oligosaccharide screening

Although there were 20 predominant ion fragments present in the FAB spectra and molecular ions present in MALDI spectra of the sample obtained from rLCAT (Table 2-3), only three predominant signals were unique to our sample when compared to tube/reagent blank. The molecular ion region contains predominant signals at m/z 2793 (consistent with an $[M+Na]^+$ quasi-molecular ion for a structure having the composition $NeuAc_2.Hex_5.HexNAc_4$), m/z 2968 (consistent with an $[M+Na]^+$ quasi-molecular ion for a structure having the composition $NeuAc_2.(Fuc).Hex_5.HexNAc_4$), and m/z 3213 (consistent with an $[M+Na]^+$ quasi-molecular ion for a structure having the composition $NeuAc_2.(Fuc).Hex_5.HexNAc_5$) (Figure 2-6). There is no evidence suggesting a tri or tetra-antennary structure. Thus, presence of tri and tetra- antennary structures can be ruled out. There are also no data that could suggest fucosylation of the antennae as shown for BHK secreted rLCAT, because the A-type fragmentation at the reducing end of the HexNAc residues in the antennae produced a signal at m/z 464 ($Hex.Hex.NAc^+$) but not at m/z 638 ($Fuc.Hex.Hex.NAc^+$)[226].

2.3.7 Linkage Analysis

GC-MS analysis of the Mc-7777 rLCAT resulted in a complex chromatogram (Figure 2-7). Provisional assignment of the peaks of interest was possible through comparison of retention times from the test sample and either standard mixture (Table 2-4), or partially methylated alditol acetate standard mixture (Figure 2-7).

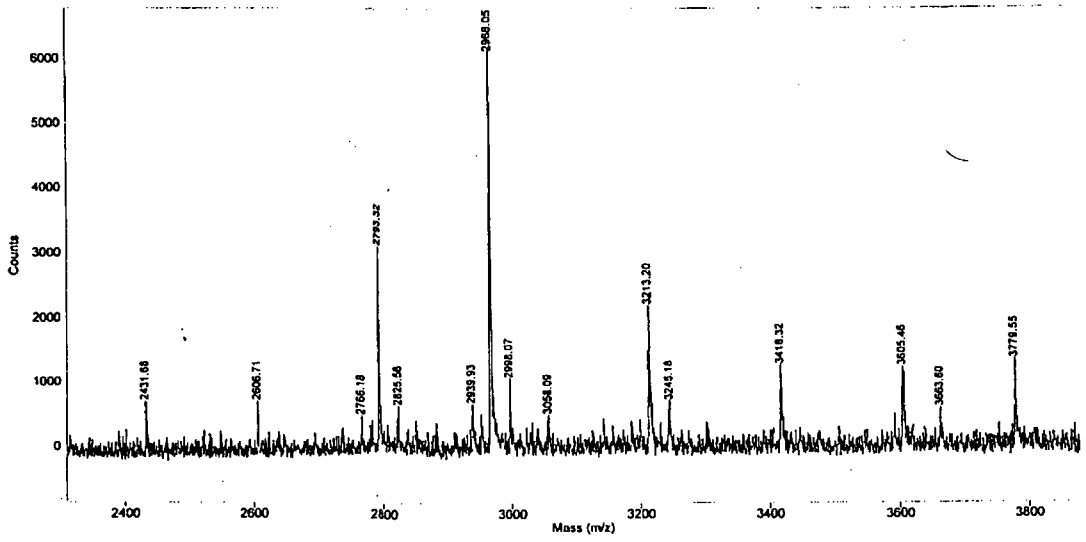
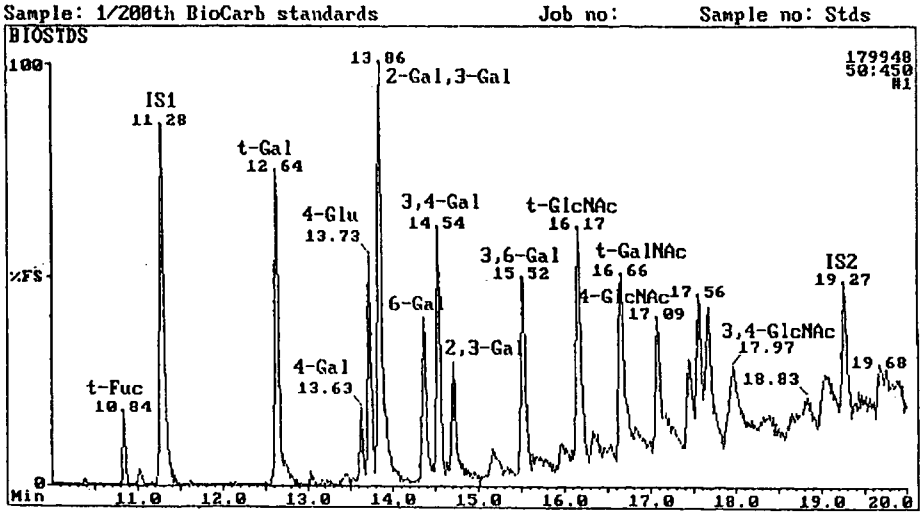


Figure 2-6. N-linked oligosaccharide screening by FAB-MS. Partial FAB mass spectra of fragment ion region of permethylated N-glycans released from recombinant LCAT

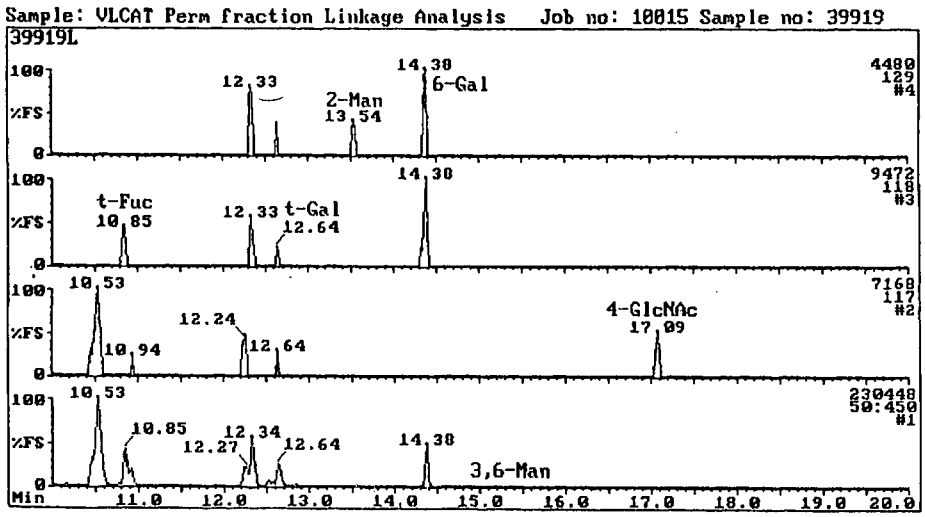
Table 2-3. The list of signals observed in the spectra obtained from the N-linked glycans released from rLCAT.

Signal m/z	Possible Assignment
	Low Mass
228.1	$HexNAc^+$ (- methanol)
260.1	$HexNAc^+$
344.2	$NeuAc^+$ (- methanol)
376.2	$NeuAc^+$
432.2	$Hex.HexNAc^+$ (- methanol)
464.2	$Hex.HexNAc^+$
636.3	$Hex_2.HexNAc^+$ (- methanol)
668.3	$Hex_2.HexNAc^+$
793.4	$NeuAc.Hex.HexNAc^+$ (- methanol)
825.4	$NeuAc.Hex.HexNAc^+$
2493	$NeuAc_2.Hex_5.HexNAc_3^+$
	High Mass
2432	$NeuAc.Hex_5.HexNAc_4OMe + Na^+$
2607	$NeuAc.(Fuc).Hex_5.HexNAc_4OMe + Na^+$
2793	$\mathbf{NeuAc_2.Hex_5.HexNAc_4OMe + Na^+}$
2968	$\mathbf{NeuAc_2.(Fuc).Hex_5.HexNAc_4OMe + Na^+}$
3213	$\mathbf{NeuAc_3.(Fuc).Hex_5.HexNAc_5OMe + Na^+}$
3418	$NeuAc_2.(Fuc).Hex_6.HexNAc_5OMe + Na^+$
3605	$NeuAc_3.Hex_6.HexNAc_5OMe + Na^+$
3664	$NeuAc_2.(Fuc).Hex_6.HexNAc_6OMe + Na^+$
3780	$NeuAc_3.(Fuc).Hex_6.HexNAc_5OMe + Na^+$

A)



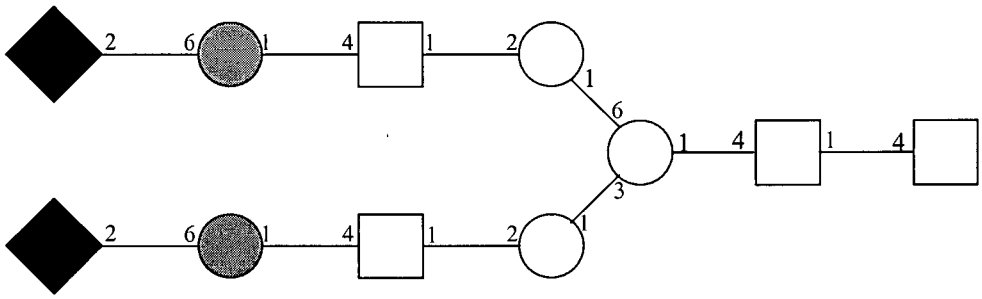
B)



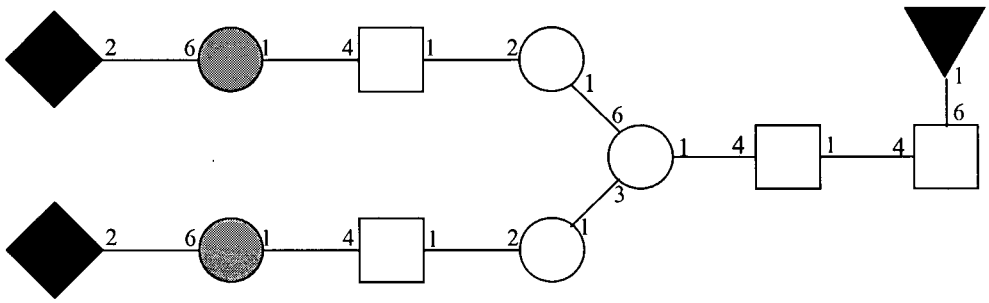
Figures 2-7. GC-MS Linkage analysis of rLCAT N-glycans. GC-MS analysis of partially methylated alditol acetates A) in a standard sugar mixture, B) generated from permethylated N-glycan of rLCAT.

Table 2-4. GC-MS analysis of the partially methylated alditol acetates for oligosaccharide linkage analysis.

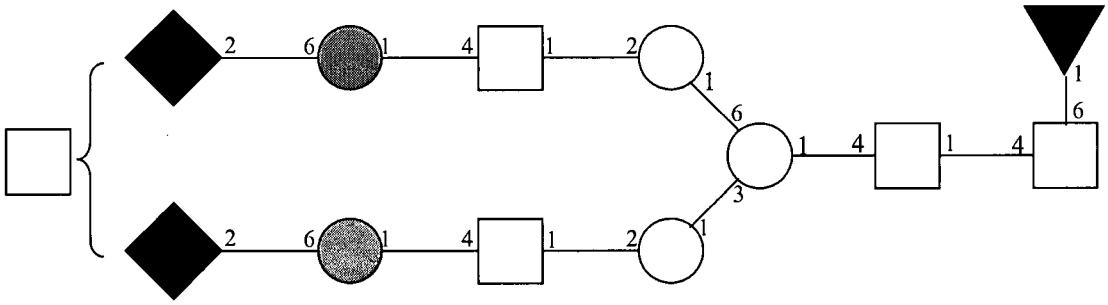
Compounds Observed	Retention Time (minutes)
4-GlcNAc	17.09
3,6-Man	15.35
6-Gal	14.38
2-Man	13.54
t-Gal	12.64
t-Fuc	10.85



Consistent with m/z2793



Consistent with m/z2968



Consistent with m/z3213

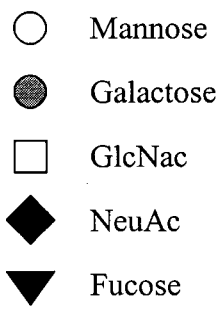


Figure 2-8. Proposed structure of oligosaccharide moieties on Mc-7777 rLCAT.

2.4 Discussion

To date, recombinant LCAT has been generated in a number of cell lines and expression systems. Most of the current knowledge on the catalytic properties of LCAT has been obtained from studies of these recombinant enzyme forms. However, almost none of the cell lines used to generate LCAT for kinetic studies are of hepatic origin, the normal site of LCAT expression in vivo. We have used a hepatoma cell line, Mc-7777, to provide a suitable environment capable of intracellular processing most likely to be similar to that of native plasma LCAT. Accordingly, we have generated a stable cell line of Mc-7777 by co-transfection of pNUT LCAT and pSV2neo. These cells are capable of producing significant quantities of rLCAT suitable for structural studies.

Comparison of rLCAT activity data presented in this study with those from other groups is difficult since the estimation of LCAT activity is highly variable among the major laboratories around the world [90]. LCAT protein secreted into the serum free media was very sensitive to the residence time in the medium. Over a five-day continuous expression, LCAT activity in the media was almost undetectable despite the continuous increase in LCAT mass (data not shown). This observation may be explained by the presence of proteases, either via *de novo* protein synthesis and secretion from the Mc-7777 cells, or release of intracellular proteases of dying cells. This effect was completely inhibited in the presence of serum supplementation. Because serum is rich in protease inhibitors, degradation by protease(s) may be one of the likely explanations for the loss of activity in serum-free media. Furthermore, changes in carbohydrate content of rLCAT may alter its susceptibility to oxidative stress, supported by increasing evidence of oxidation of sulfhydryl groups of the protein resulting in reduction of enzyme activity [227].

Protein production continued linearly over the 48-hr experiment. Therefore, this system, unlike any other mammalian system, is capable of continuous production over long periods of time without the need of replenishing with serum. LCAT production reached approximately 10 mg/L, which may also be enhanced by cation induction since the mouse metallothionine promoter is responsive to Zn^{2+} concentration [228].

Our results from the kinetic studies suggest that rLCAT from Mc-7777 and BHK cells share similarities in apo A-I activation, Km, and Vmax . The capacity of Mc-7777 rLCAT to be activated by apo-AI was very similar when compared to the BHK and plasma LCATs [118]. Furthermore, while Vmax was slightly higher for Mc-7777 than the BHK rLCAT, the Km for Mc-7777 was slightly lower than BHK rLCAT. Comparison of catalytic efficiency of two enzymes revealed only a modest difference between Mc-7777 and BHK rLCATs. This modest difference may be explained by alterations in glycosylation pattern of the secreted protein. Further structural studies of rLCAT with different glycosylation patterns will result in a better understanding of the role of carbohydrate chains of LCAT.

Although, glycosylation of LCAT has been shown to play an important role in processing and secretion of the protein [15, 16], little is known about the exact role of these carbohydrate moieties regarding the catalytic mechanism of the enzyme. Studies of oligosaccharide structure of plasma LCAT [229] and recombinant LCAT [95] have shown a great deal of heterogeneity in both carbohydrate sequence and structure. Therefore, it has been suggested that the oligosaccharides on the protein contribute to structure and conformational stability of the protein in an aqueous environment [4]. Thus, these carbohydrate structures may be important for the conformational integrity of the active site to provide accessibility for the substrate. Macro-structural comparison of the Mc-7777 and BHK rLCAT showed identical patterns of glycosylation when subjected

to Glycosidase F, Endoglycosidase-H and Neuraminidase, while the compositional and sequence analysis of these carbohydrate moieties revealed significant differences.

Plasma LCAT has been shown to consist of primarily triantennary structures with some bi- and tetraantennary structures [229]. Similarly, rLCAT from BHK expression system was characterised as "complex-type" predominantly tri- and tetraantennary oligosaccharide rich, with a minor biantennary component [95]. Interestingly our analysis of rLCAT from the Mc-7777 cell line has shown no evidence of tri- and tetraantennary structures. Consequently, we have looked at the carbohydrate architecture of carbohydrate moieties on Mc-7777 rLCAT. In light of current data [predominant signals at m/z 2793 ($NeuAc_2.Hex_5.HexNAc_4$), m/z 2968 ($NeuAc_2.(Fuc).Hex_5.HexNAc_4$), and m/z 3213 ($NeuAc_2.(Fuc).Hex_5.HexNAc_5$)] all of the oligosaccharide moieties of this protein are of biantennary structure (Figure 2-6, Table 2-3). This homogeneous-type carbohydrate structure may serve advantageous in purification of the protein (Figure 2-8). The homogeneous state of the protein will possibly result in an LCAT preparation with higher purity that would favour generation and growth of protein crystals for X-ray analysis. The variation from plasma LCAT may reduce its applicability for functional studies of the Mc-7777 protein, although the variations in other expression systems have been suggested to be inconsequential in "functional analyses" of the protein and its mutants [91]. In agreement with previous findings, our preliminary assessment of the Mc-7777 secreted rLCAT displays similar kinetic properties to rLCAT from BHK cells. In addition, differences in carbohydrate structure may render the protein secreted by Mc-7777 susceptible to oxidation and proteolysis in the conditioned media. This may explain loss of activity of rLCAT in the media over time.

Most of the oligosaccharide structures on plasma LCAT were sialylated (5.5% w/w) with approximately 30% of the fucose content, exclusive to the triantennary moieties [229]. On the other hand, in excess of 70% of the carbohydrate moieties of BHK-rLCAT were fucosylated, while their sialic acid content was less than 1%. Mc-7777 rLCAT, however, presented approximately 70% fucosylation of carbohydrate structure [estimated from fragment ion abundance data (Figure 2-7)]. In addition the sialic acid content was in agreement with the data for plasma LCAT. Therefore, Mc-7777 LCAT may provide an intermediate protein to study the role of fucosylation and sialylation on protein function. Figure 2-8 represents a schematic view of the architecture of the carbohydrate moieties of Mc-7777 rLCAT.

In conclusion, the kinetic characteristics of the enzyme do not show distinct variations from BHK rLCAT, or in turn from those of plasma LCAT. The changes in carbohydrate structure with stable kinetic properties may be the key for success of future studies involving protein biochemistry and ultimately X-ray crystallography for its 3-D structure.

The rLCAT from this hepatic-based expression system using Mc-7777 is less similar to plasma LCAT when compared to BHK LCAT (Figure 2-9). This suggests that cells originating from different species despite their similar functions can behave differently and utilize alternate pathways in their normal function. This potential difference among different species underlines the importance of extensive knowledge of various expression systems both in vivo (transgenics) and in vitro (expression systems). However, this new and relatively homogeneously glycosylated protein (Figures 2-8 and 2-9) may enable us to advance in 3-D structural analysis, studying the role of carbohydrate structures on enzyme-substrate interaction, and structural stability of LCAT. Future studies can take advantage of a whole range of oligosaccharide

structures of LCAT from plasma, BHK, CHO and Mc-7777 cells now available; to study the role of various structures and saccharide sequences on the interaction of LCAT with lipid surfaces. Homogeneity of the oligosaccharide moiety of LCAT will be advantageous in generation of LCAT crystals in significant sizes for X-ray crystallography. Thus, further attempts will be made to purify rLCAT to its maximum purity, and generate crystals of this protein for complete delineation of the 3-D structure of LCAT and its mutants. However, the BHK rLCAT was utilized for the subsequent biochemical studies due to the greatest similarity shared with plasma LCAT,

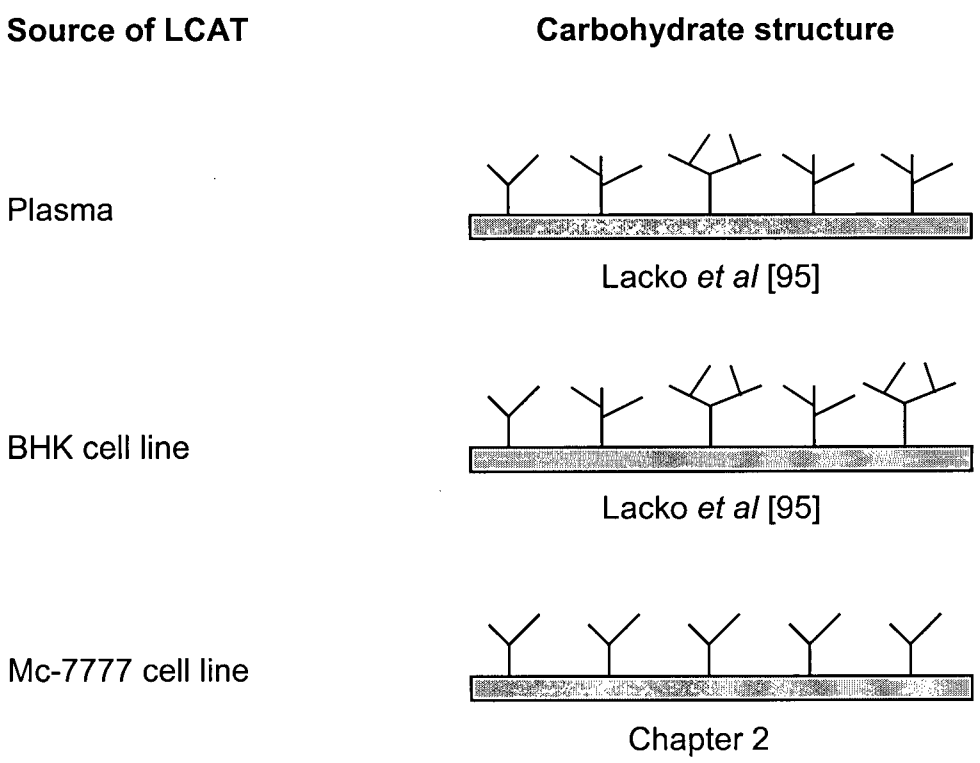


Figure 2-9. Comparison of carbohydrate architecture of human LCAT obtained from human plasma, BHK and Mc-7777 expression systems

Chapter 3
Binding of LCAT to its substrate particles

I would like to thank Ms. Lida Adler and Ms. Sandra McGladdery for helpful discussions and their technical contribution to determination of LCAT activity .

3.1 Introduction

Recent work by Jonas and co-workers [10] has shown that rLCAT reversibly binds to rHDL and LDL. These studies have also determined the binding kinetics of rLCAT to rHDL and chemically modified (biotinylated) LDL particles. Considering that this body of work involves synthetic HDL analogues and chemically altered LDL particles, and there is little known about the association pattern of LCAT, both endogenous and exogenous (rLCAT), in plasma.

As reviewed in the previous chapter, in addition to lipoprotein abnormalities, patients with familial LCAT deficiency (in whom the cholesterol esterification in plasma is completely absent) have haematological and renal complications leading to anaemia and renal failure, respectively.

Previous experiments with plasma infusion in patients with FLD have shown positive, but transient, changes and normalisation of plasma lipid profile and haematological factors [138]. Thus, enzyme replacement therapy may be a viable mode of treatment for FLD patients. Although there has been a number of recent reports on the interaction of recombinant LCAT (rLCAT) with lipoprotein analogues, the current information on metabolism of rLCAT *in vivo* is limited [18, 230]. Studies investigating the metabolism of rLCAT in a rabbit model, to elucidate the nature of interaction of rLCAT with LDL and HDL in the plasma compartment, have shown that rLCAT is rapidly cleared from the circulation. We have hypothesised that the short half-life of rLCAT is due to the inability of the protein to bind to plasma lipoproteins. Thus, the unbound LCAT in circulation is rapidly cleared. Studies outlined in this chapter

investigate the factors that may influence rapid clearance of the enzyme. We will utilise various methods to study wild type (WT) and functional mutants (FED) of LCAT.

3.1.1 Objective

To characterise the binding of LCAT to its substrate particles.

This will allow us to understand the underlying mechanisms/conditions by which LCAT can bind to its natural substrates, HDL and LDL.

3.1.2 Specific Aims:

1. To setup a FPLC-gel filtration system for separation of plasma lipoproteins.
2. To establish the site of LCAT activity in normal and FED plasma.
3. To examine the binding pattern of recombinant LCAT ex-vivo.

3.2 Methods

3.2.1 Production of rLCAT

Previously transfected baby hamster kidney (BHK) cells were seeded into 100 mm culture dishes and grown to 80% confluence under standard conditions (DMEM supplemented with 10% fetal bovine serum and 1% antibiotics, 5% CO_2) [90]. Media was removed at 24 hrs. Cells were washed twice with 3 ml of Opti-MEM (1% antibiotics). Washed cells were purged for 2-3 hours. Media were replaced with 10 ml of fresh media and the cells were incubated for 48 hr.

3.2.1.1 Endogenous radiolabeling of rLCAT

BHK-rLCAT cells were incubated in methionine-free DMEM (DMEM-Met) for 20 min at 37°C to deplete the Methionine pool. The endogenous methionine pool was then labelled for 30 min in DMEM-Met supplemented with 100-200 μCi/ml ^{35}S-methionine (700 Ci/mmol. New England Nuclear). 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 was harvested in lysis buffer (50 mM Tris-HCl, pH 8.0, 62.5 mM EDTA, 1% Nonidet P40, 0.4% sodium deoxycholate, and 1 mM phenylmethylsulfonylfluoride) for subsequent analysis.

3.2.1.2 Purification of rLCAT

The culture medium containing the rLCAT was collected, and spun down or filtered to remove the cell debris. Salt concentration of the media was adjusted to 0.3 M salt (NaCl). Adjusted media was loaded, at 0.5 ml/min, onto a phenyl-sepharose column, which had previously been equilibrated with "High Buffer" (0.3 M NaCl, 5mM PO_4, pH 7.4). The column was washed with "High Buffer" until the absorbance @280 nm reached the base line (below 0.01). Recombinant LCAT was eluted using deionized distilled water (dd H_2O). Fractions were collected and the peak fractions were run on SDS-PAGE to assess homogeneity of the preparation. The peak and pure fraction were combined and concentrated using Amcion concentrators (YM 30).

3.2.2 LDL and HDL Isolation

Isolation of lipoprotein fractions was accomplished by Density Gradient Ultracentrifugation (DGUC), according to earlier published methods (16). Each fraction

was washed once after purification and purity of each fraction was determined by lipoprotein electrophoresis.

3.2.3 Fast Protein Liquid Chromatography (FPLC)

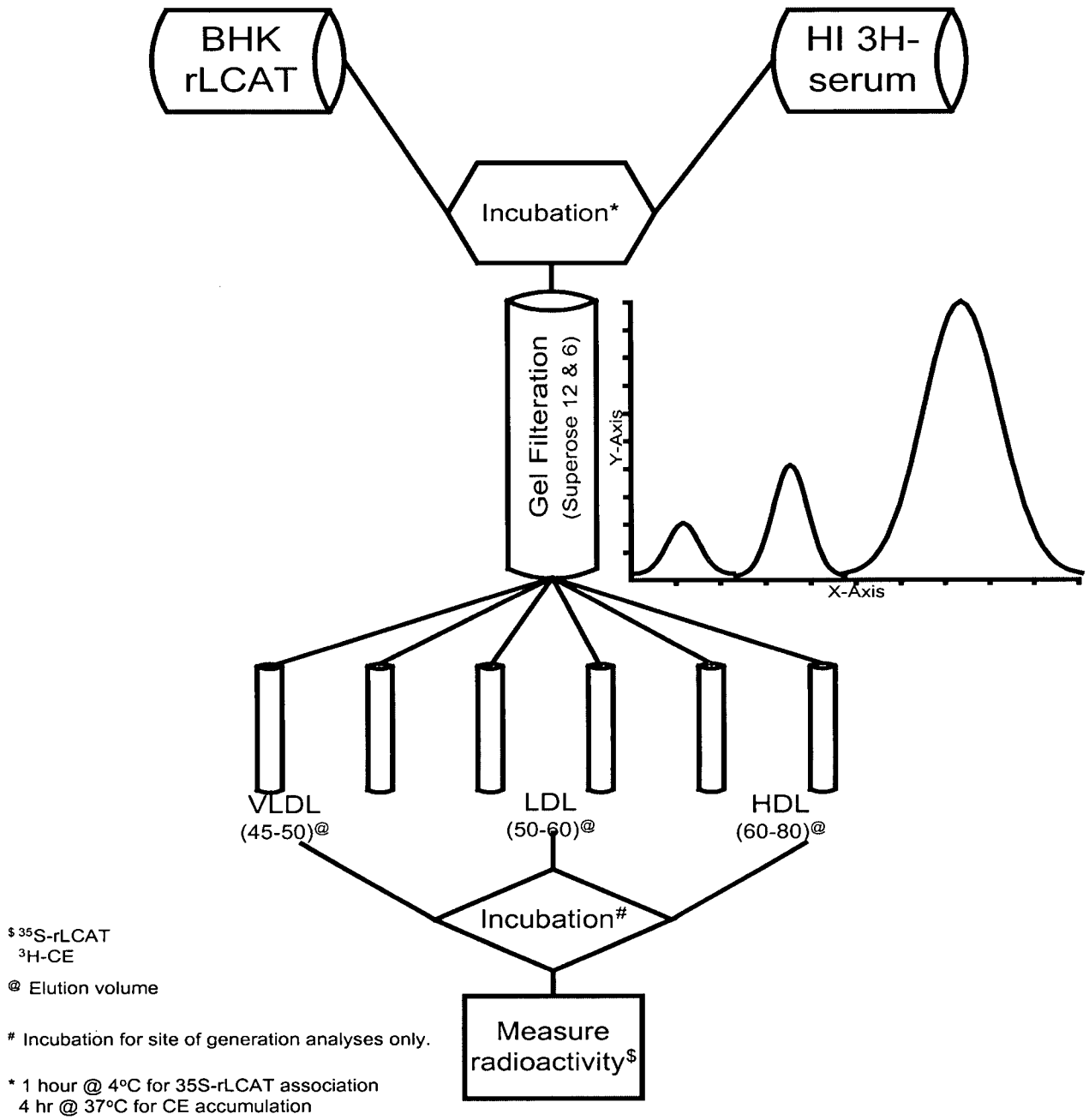


Figure 3-1: Schematic overview of the gel filtration system used for separation of different classes of plasma lipoproteins. Superose 12 and Superose 6 were connected in series to provide the maximum separation of HDL from LDL and VLDL fractions. For assessment of sites of generation, 200 μL of ^{3}H-cholesterol labeled plasma was loaded onto the column and collected fractions were incubated for 16hr at 37°C. Generated ^{3}H-CE was separated by TLC and CE rate was determined. Accumulation of CE required 37°C incubation for 4 hrs prior to fractionation. Binding studies all required 1 hr incubation of lipoprotein/s with rLCAT at 4°C prior to fractionation.

3.2.3.1 Instrumentation

The FPLC instrument consisted of a LKB brommer pump (Germany) linked to a BioRad Econo System Controller running a Pharmacia Superose 6 HR (10mm diameter X 30cm length) column and a Pharmacia Superose 12 prep grade (16mm diameter X 50cm length) column in series. The flow rate was set at 0.5ml/min at 3 bar pressure run on a pump for a total run time of 240 minutes. (Conversions: 1 bar = 0.069 p.s.i. = 1 atmosphere = 0.1MPa = 760 mm Hg). The BioRad Econo System Controller (BioRad, Canada) was set up to collect 60 fractions of 1mL each. The eluting fractions were also monitored by a spectrophotometer attached in series to measure the absorbance of the eluant at 280nm. The running buffer (mobile phase) was 150mM NaCl, 10mM Tris, 0.03% NaN_3 at pH = 7.4 (Figure 3-1).

3.2.4 Detection of radiolabels

^{3}H-Cholesterol radiolabel (Amersham) was quantified using in a liquid scintillation cocktail (4g of Omniflour/ L of Toluene). ^{35}S-Methionine was detected using ACS (aqueous counting scintillant, Amersham). Readings were obtained by a RackBeta liquid scintillation counter, LKB.

3.2.5 Calculation and Curve Area Determination

Sigma Plot was used for determination of the area under the curve utilising the trapezoid rule.

3.3 Results

3.3.1 Characterization of FPLC Gel Filtration System

Isolated lipoprotein components of human plasma (by ultracentrifugation) were used as references to calibrate the gel filtration system. Individual fractions were loaded onto the columns and an optical density (OD), @ 280 nm, trace of eluting particles from the column was recorded. The data were plotted against the elution volume of particles to generate an elution profile of different classes of plasma lipoproteins (Figure 3-1). The elution profiles from individual lipoproteins were overlaid on one another to determine the correct lipoprotein elution volumes corresponding to each lipoprotein fraction.

Each lipoprotein fraction isolated using density gradient ultracentrifugation contained a heterogeneous pool of lipoproteins throughout each class. This resulted in overlapping of each fraction. Each class of plasma lipoproteins was identified from the lowest point of the valley between lipoprotein fraction to the next (Figure 3-2).

The primary objective of this experiment was to identify the HDL fractions. Thus, the presence of apo A-I containing lipoproteins was confirmed by SDS-PAGE. Fractions enriched for HDL were run on the gel along with pure apo A-I fractions (Figure 3-3). This experiment confirmed the presence of apo A-I containing lipoproteins.

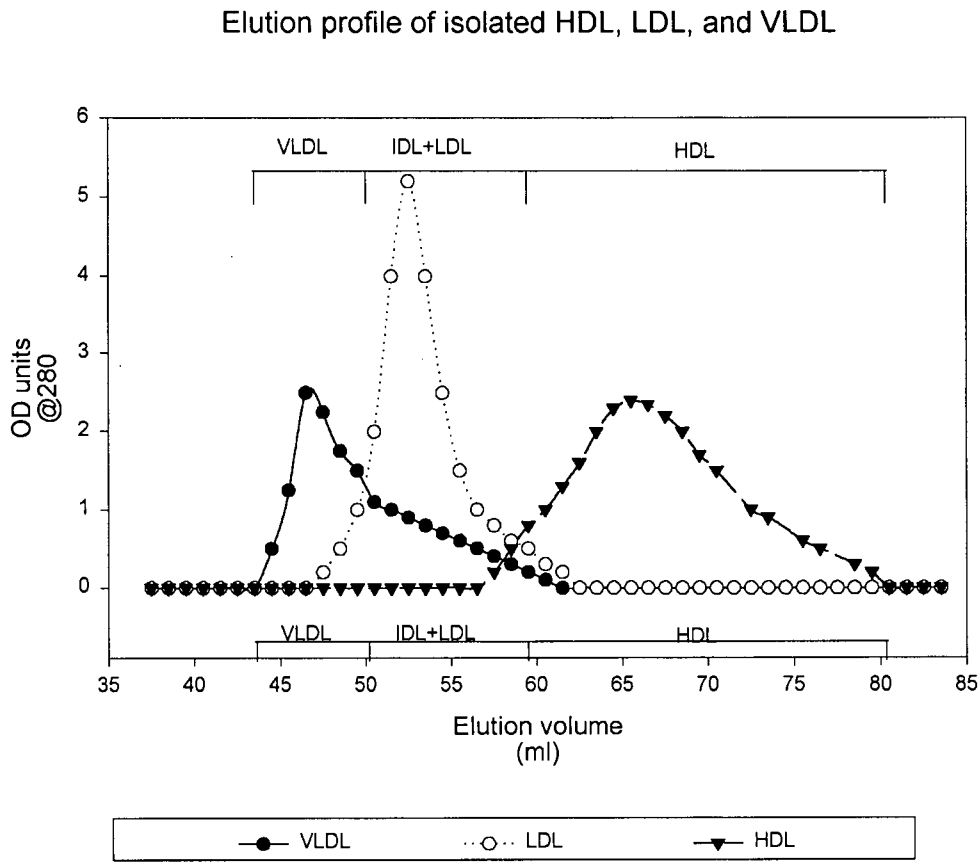


Figure 3-2. Characterization of FPLC set up for separation of plasma lipoproteins. Suparose 6 and 12 were connected in series and samples of individual lipoproteins were loaded on to the system. Optical density readings @280 nm were recorded and elution profiles of proteins corresponding to each fraction were collated and plotted on the same axis.

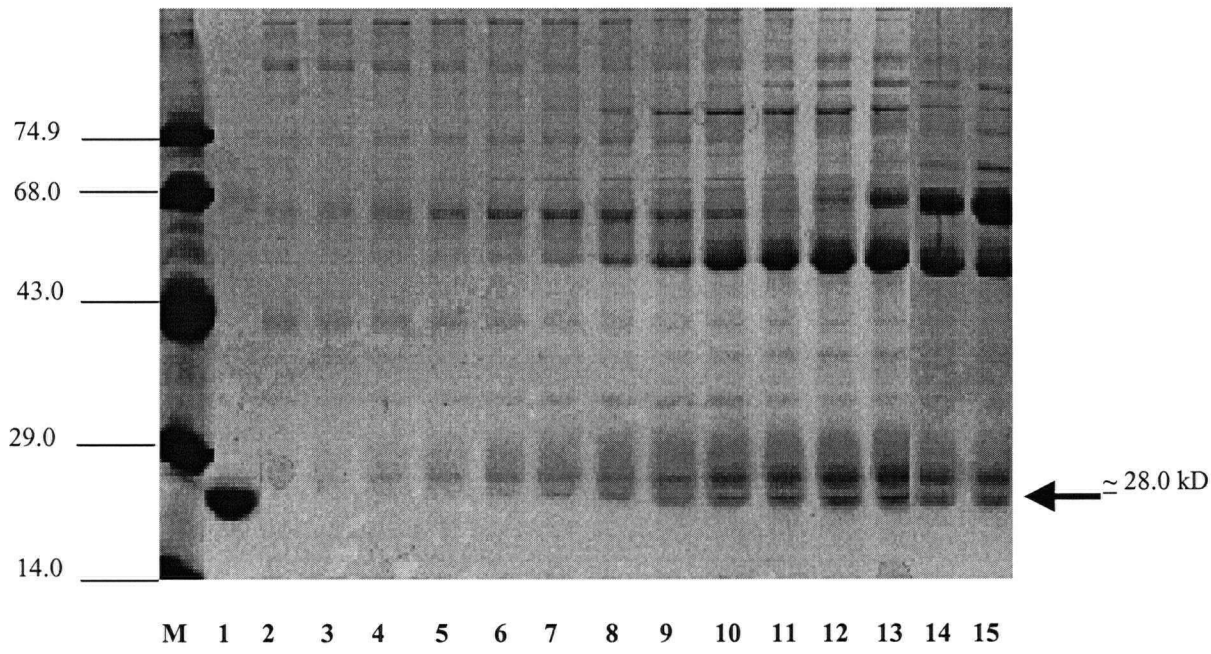


Figure 3-3. Presence of A-I in fractions corresponding to HDL. 200 μl of human serum was fractionated using Superose 6 and Suparose 12 columns. 5 μl of each fraction corresponding to HDL particles (as previously calibrated, figure. 3) was reduced and loaded onto 12.5 % SDS-PAGE gel. In addition, purified human A-I was run as a standard (Lane 1). Lane 2-13 correspond to elution volume (EV) 54-65 in 1 ml fractions. Lanes 14 and 15 correspond to EV 68 and 70.

3.3.2 Biochemical analyses of FED and normal plasma

Plasma samples from two patients identified with FED and normal control were analyzed for various LCAT activity parameters (Table 3-1). FED patients were diagnosed based on the biochemical presentation, in the absence of the genetic information.

Table 3-1. Biochemical analyses of plasma from one normal and two FED patients. Plasma samples from these individuals were assessed for LCAT activity using exogenous and endogenous substrate pools. Plasma unesterified cholesterol was measured using the enzymatic method.

Sample	LCAT activity (Exogenous substrate) nmol FC est'd/ml/hr	LCAT activity (Endogenous substrate) nmol FC est'd/ml/hr	Plasma FC mmol/L
Control	35.0	152.0	1.40
"B"	2.7	68.3	1.89
"C"	2.7	60.2	1.72

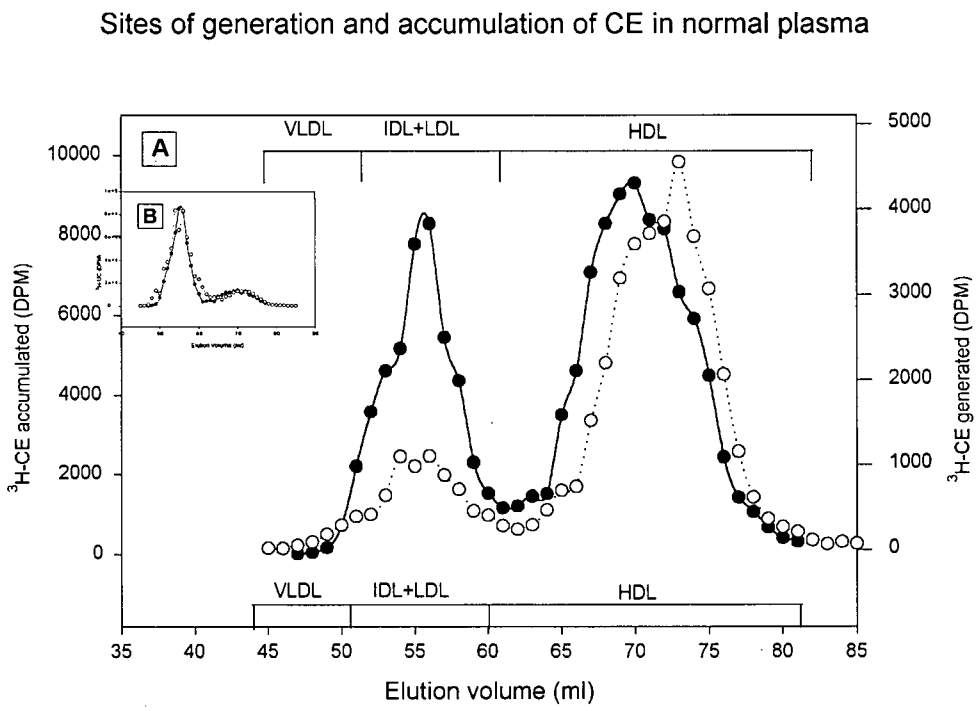
Both FED patients, "B" and "C", show classical FED phenotype with respect to plasma LCAT activity. Using the exogenous substrate assay (HDL analogue) LCAT activity was about 5% of the control (WT), whereas LCAT activity using the endogenous substrate, (total plasma lipoprotein pool) was at about 40% of control. These data are consistent with previous biochemical observations in FED cases [73, 118, 122]

3.3.3 FED Plasma Activity Profile vs. Normal Plasma Activity Profile

Sites of CE generation and accumulation in normal and FED plasma were determined using the gel filtration system. In normal plasma, CE was generated

predominantly in fractions corresponding to HDL with approximately 15 % of the activity present in the LDL fractions. Cholesterol ester accumulation, on the other hand, was shared between LDL and HDL fractions, respectively (Figure. 3-4). These observations were expected since activity of CETP in plasma is unaffected and the generated CE is transferred to lower density lipoproteins. In FED plasma, CE was generated predominantly in LDL fractions, the preferred substrate particle, consistent with previously published work [73](Figure 3-4). Accumulation of CE in FED plasma was different from normal, in that 80-90% of generated CE was found in the LDL fraction. Interestingly, the accumulation pattern did not predict the site of generation. There was considerable CE generation, approximately 35% in the fractions corresponding to HDL, which was indicative of the presence of LCAT activity in these fractions (Table 3-2). However, CE was accumulated only in the LDL particles.

(I)



(II)

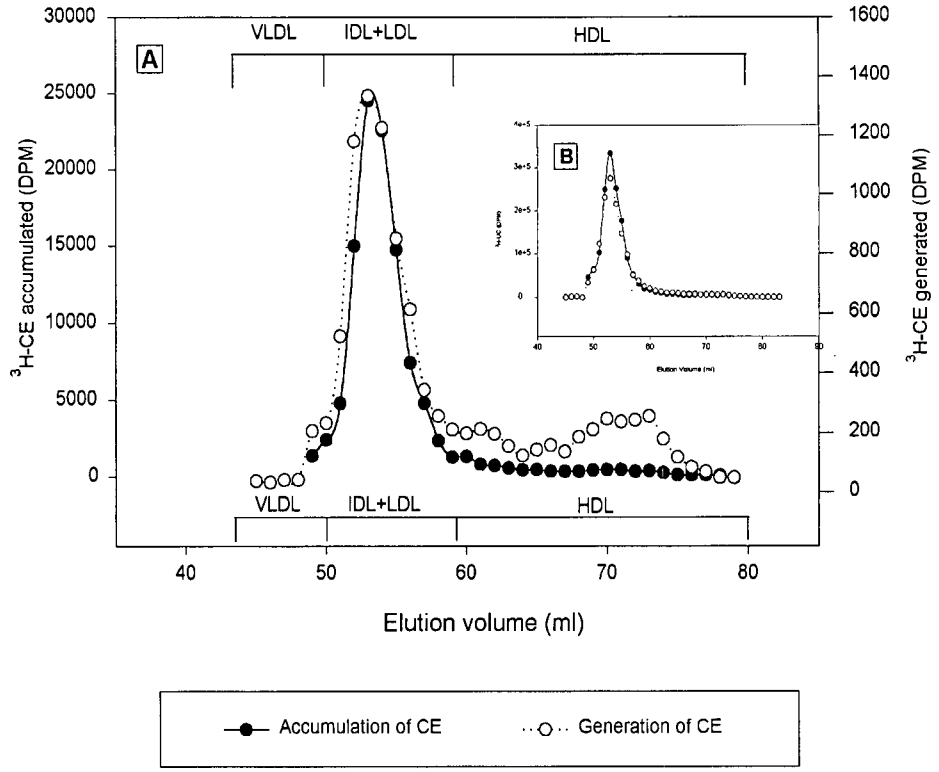


Figure. 3-4- Sites of generation and accumulation of CE in plasma lipoproteins. Whole plasma from a normal individual (I) and Patient "C" (II) were labeled with 3H-cholesterol using the paper disc method. Sites of accumulation and generation of CE were determined according to the outline described above (Figure 3-1). The main panel of each plot (A) represents the elution pattern of 3H-CE, which was generated by the function of LCAT. Plot B is used as the internal control, to follow the elution pattern with ^{3}H-UC.

Table 3-2. Compiled results for determination of sites of accumulation and generation of CE in normal and individuals with FED. Total areas from each plot were calculated using Sigma Plot. Areas corresponding to individual lipoprotein fractions are expressed as a fraction of total area.

	Accumulation of CE		Generation of CE	
Sample	LDL(%)	HDL(%)	LDL(%)	HDL(%)
Wild type	33.71	65.54	15.70	81.62
Patient "B"	81.98	12.57	50.56	38.49
Patient "C"	91.15	7.09	65.13	30.39

3.3.4 Radiolabelled rLCAT

The radiolabeled rLCATs (WT and FED [T123I]) were purified using a phenyl-sepharose column. LCAT was bound to the column under high salt condition and was eluted with ddH_20. The subsequent elution profile was constructed based on elution of radioactivity from the column (Figure 3-5). Autoradiograms of each fraction were used to determine the highest concentration and purity of LCAT for our studies (Figure 3-6). Wild type LCAT formed a broad band at approximately 67 kD, whereas T123I formed a sharper band at the same molecular weight.

3.3.5 Purification of rLCAT

Purified FED (T123I) and WT rLCAT from transfected BHK cells were purified separately. The resultant elution profile was identical (Figure 3-5). T123I and WT constituted the major bands.

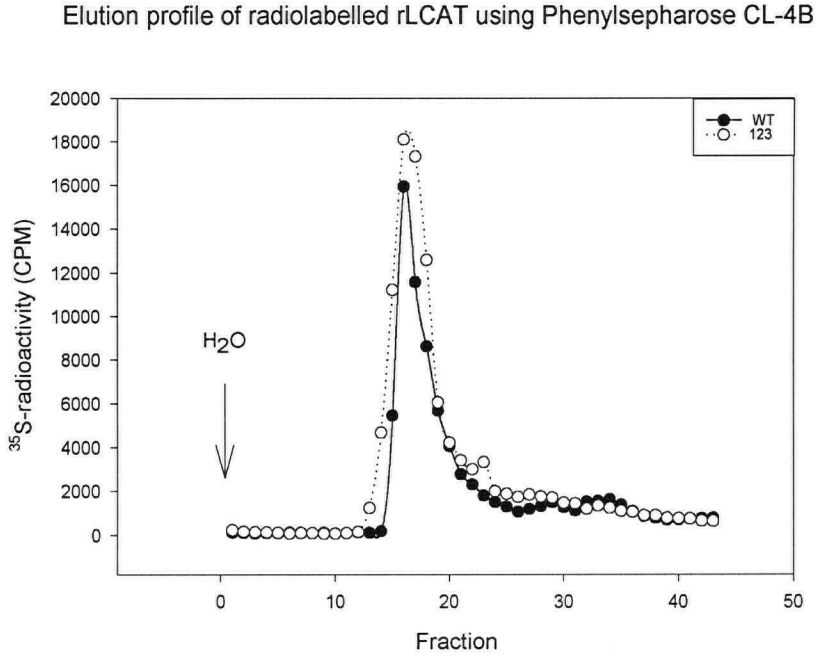


Figure 3-5. Recombinant LCAT purification using Phenyl Sepharose CL-4B. Subsequent to collection of labeled rLCAT in the conditioned media, 10 ml of conditioned media was applied to the column (10X110 mm) equilibrated with High Buffer. Column was washed with High buffer until an absorbance < 0.01 (280 nm) was reached. Then, LCAT was eluted from the column as the it was subjected to deionized water (arrow). Separate columns were used for purification of each specie of rLCAT. Each point on the graph represents the mean of duplicate measurements from a single experiment.

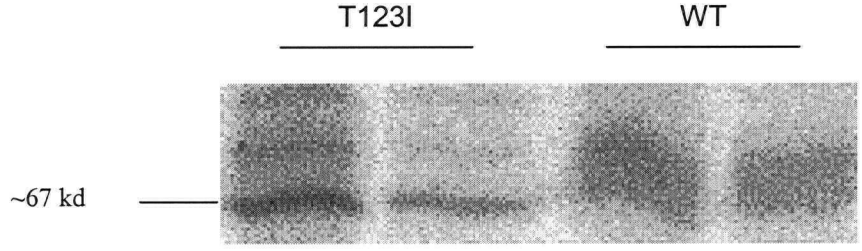


Figure 3-6. Radio-labeled T123I and WT rLCAT. Aliquots (5 μl) of peak fractions eluted from the Phenyl Sepharose column of each sample were reduced and run on an SDS-PAGE. Gel was dried and exposed to film for 48 hrs.

3.3.6 Association of wild type and FED rLCAT with individual lipoproteins

Radiolabeled rLCAT and individual plasma lipoproteins were mixed in equimolar ratio, incubated and applied to the gel filtration system. All fractions were examined for radioactivity to determine the elution profile of radiolabeled rLCAT. This elution profile was corrected for background by subtraction of the elution profile for the protein in absence of lipoproteins (Figure 3-7). Eighteen percent of radiolabeled LCAT eluted with both LDL and HDL fractions (Table 3-3). A very similar pattern of association was observed for the FED related mutant rLCAT. Specifically, 18% of the radiolabeled material eluted from the column along with the LDL fraction and 12% was eluted with the HDL fractions. The majority of the radiolabelled LCAT (98%) was recovered from the GF column, indicating that there was minimal non-specific binding of rLCAT to the Suparose matrix.

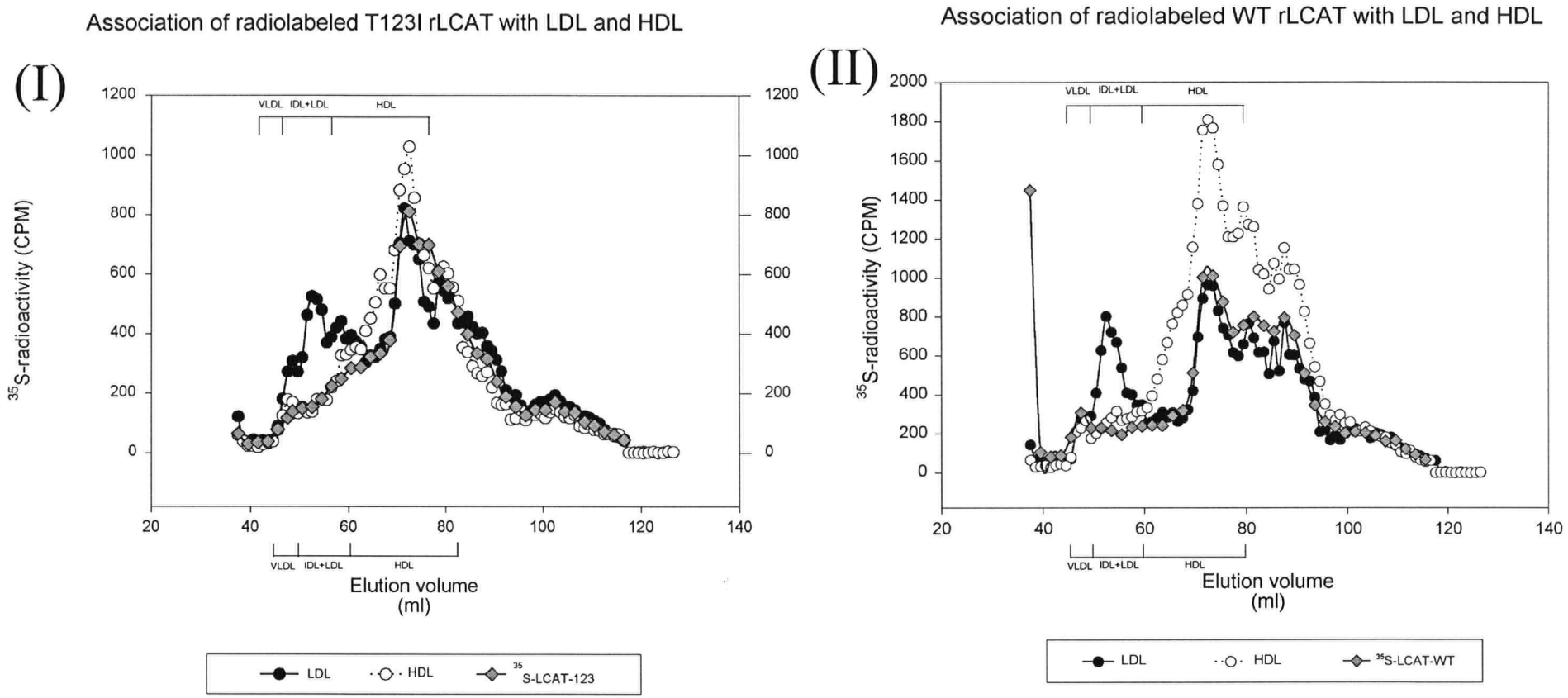


Figure 3-7 . Association of rLCAT with plasma lipoproteins. 200-300 μl of either HDL or LDL were mixed with 150-250 μl of WT rLCAT (I) or T123I rLCAT (II) (1:1 molar ratio was maintained). Mixture was incubated on ice for 1hr to allow for interaction of various molecules. 400 μl of the mixture was loaded onto the FPLC column system. Elution of ^{35}S protein was monitored using the scintillation counter. Corresponding areas to each fraction were tabulated (Table 3-3). Each point on the graph represents the mean of duplicate measurements from a single experiment.

3.3.7 Association of rLCAT with Total Lipoprotein Fraction.

Radiolabeled rLCAT was incubated with the total lipoprotein fraction to allow for presence of all plasma lipoproteins. Further analysis of the elution profile revealed a strikingly similar elution profile for both WT and T123I LCATs (Figure 3-8). Sixteen percent and 14% of the labelled protein was associated with the fractions corresponding to LDL for WT and T123I, respectively. HDL fractions however, carried 32% of the total radioactivity for both WT and T123I rLCAT (Table 3-3).

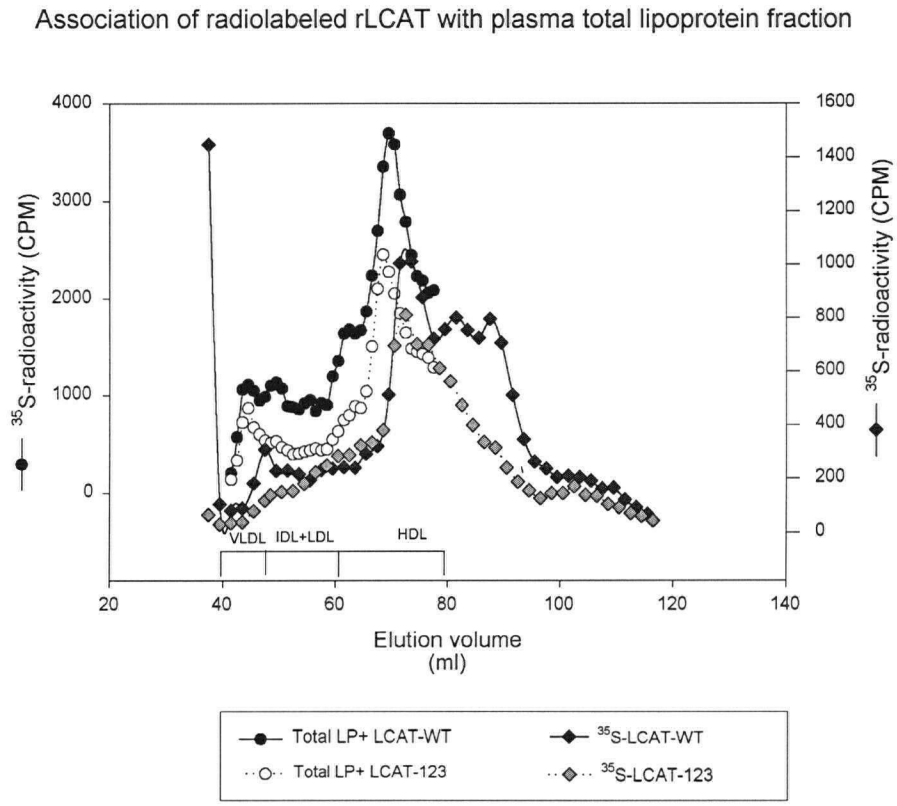


Figure 3-8. Association of rLCAT with total plasma lipoproteins. 200 μl of total plasma lipoprotein was mixed with 200 μl of WT rLCAT or T123I rLCAT. Mixture was incubated on ice for 1hr to allow for interaction of various molecules. 400 μl of the mixture was loaded on to the FPLC column system. Elution of ^{35}S protein was monitored using the scintillation counter. Corresponding areas to each fraction were tabulated (Table 3-3). Each point on the graph represents the mean of duplicate measurements from a single experiment.

3.3.8 Association of rLCAT with Lipoproteins in Whole Plasma

In order to examine the effect of all components of plasma on rLCAT association with lipoprotein fractions, identical conditions as the two previous experiments were set up while replacing the lipoprotein fractions with whole plasma. The observed elution pattern was consistent with the previous experiment using the total lipoprotein fraction, yielding 10-20 % association of radioactivity with LDL fractions and 30-35% association with the HDL fractions (Table 3-3).

Table 3-3. Binding of rLCAT with plasma lipoproteins. Tabulated data was compiled from the Figures (5 and 7). Areas of the curves were assessed according to the protocols outlined in Table 2. Each point on the graph represents the mean of duplicate measurements from duplicate experiments.

	Individual Lp (Equimolar)			Total Lp			Whole plasma		
LCAT	LDL (%)	HDL (%)	Total bound (%)	LDL (%)	HDL (%)	Total bound (%)	LDL (%)	HDL (%)	Total bound (%)
WT	17.7	12.2	30	16.5	31.5	48	9.3	33.0	42
T123I	17.7	17.5	35	13.5	32.2	46	20.0	36.3	56

3.3.9 Effect of concentration on LCAT activity using LDL and HDL

As the concentration of lipoprotein particles was increased by 3.75 fold, the LCAT activity corresponding to the HDL fraction was also increased at a magnitude of 4-fold. However, the same increase in LDL particle concentration resulted in 5.4 fold increase in the amount of cholesterol generated. This 35% increase in the amount CE generated may suggest another mechanism of action for LCAT on LDL particles (Figure 3-9, Table 3-4).

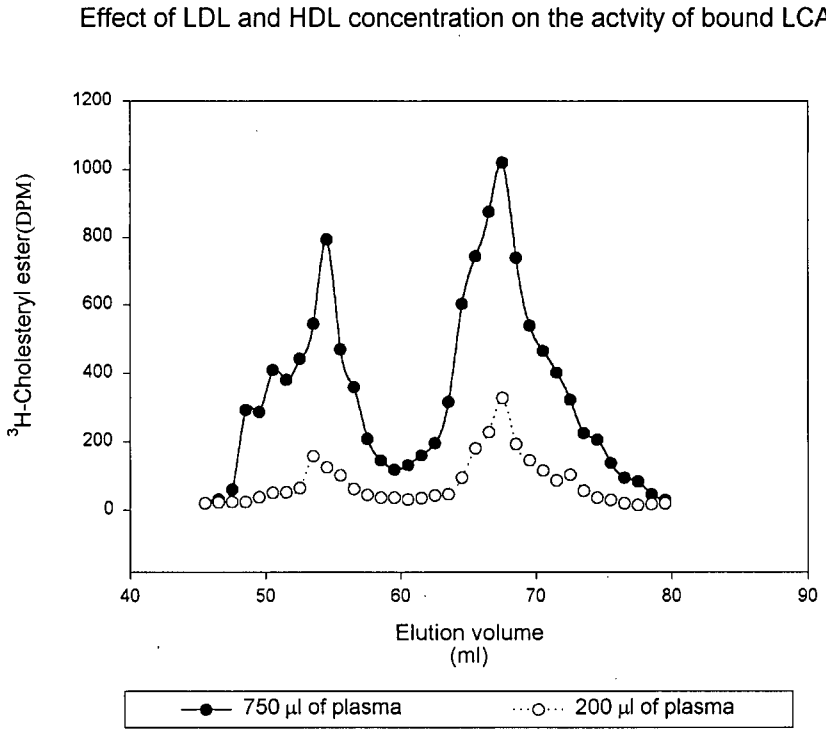


Figure 3-9. Effect of particle concentration on LCAT activity on LDL and HDL. Equivalent quantities of rLCAT were added to 200 and 750 µl of whole radiolabled (^{3}H-cholesterol), heat inactivated human plasma. Each mixture was incubated for 1 hr at 4 °C prior to fractionation by the FPLC system. Each fraction was collected and incubated for 16 hr at 37 °C. Lipids were extracted and separated using TLC. Each point on the graph represents the mean of duplicate measurements from a single experiment.

Table 3-4. Effect of particle concentration on LCAT activity on HDL and LDL. Area under the curve for each component of the lipoprotein fraction, LDL and HDL, was determined. The expected outcome was set as the increase in the amount of plasma loaded onto the column. This value was subtracted from the experimentally obtained outcome. The variation was expressed as a percentage of the expected outcome. Each value represents the mean of duplicate measurements from a single experiment.

	Trial 1	Trial 2	Expected increase	Increase (Fold)	% Variation from the expected outcome (%)
Plasma (μl)	200	750	3.75	3.75	0
LDL (area under the curve)	732	3964	3.75	5.4	+44
HDL (area under the curve)	1836	7390	3.75	4.0	+7

3.4 Discussion

We have established and characterised an efficient method for separation of various fractions of the plasma lipoprotein pool. Using Suparose 6 and Suparose 12 columns in series, LDL fractions can routinely be separated from HDL. In addition, it can be used to resolve a separate VLDL peak before the LDL fractions (Figure 3-1). This robust method of separation was very rapid and convenient and has been used in a number of current studies of lipoprotein fraction analyses. The metabolism of liposomes used in drug delivery systems [231], lipoprotein profile of apo E knockout mice [232], hydrophobic drug interaction [233, 234], activity of human rLCAT in CD1 mouse plasma, and association of rLCAT with LDL and HDL fractions are a few examples. Studies of sites of generation and accumulation of CE in normal and FED plasma provided interesting results. Identification of these sites using the FPLC system was not only in agreement with the previous observations [16, 235], it also shed some light on the nature of binding of LCAT to plasma lipoproteins. These findings suggest that LCAT is bound to the surface of its substrate particle and this association remains for extended periods.

These series of experiments re-illustrated that the LDL fraction is the major site of cholesterol esterification in FED while HDL cholesterol remains the preferred substrate particle for wild-type LCAT.

In FED plasma, although about 35% of CE was generated in the HDL fraction, approximately 10% of the total plasma CE was accumulated in this fraction (Table 3-2). This may be due to the fact that very small, cholesterol-poor HDL provides a substrate for LCAT. However, the generated cholesterol on these particles is transferred to lower density lipoproteins by the action of CETP. This continuous efflux of CE from a particle

with poor LCAT activity will constantly deplete the CE reservoir of these particles, hindering their maturation. On the other hand, this hypothetical continuous efflux of CE may result in an enhanced RCT pathway.

Another explanation can be based on the comparison of data obtained for accumulation and generation of CE in LDL fractions. The peak value for generation of CE in HDL of normal plasma is 50% of the peak value for CE accumulation in the same fraction. However, the peak value of CE generation in LDL fraction of FED plasma is 5 and 12% of the peak value of CE accumulation for FED patients "B" and "C", respectively. This observation was made in light of the fact that equal quantities of labelled plasma from the same batch were used under identical conditions. However, this statement may be influenced by the fact that the reaction on HDL particles may reach saturation by either substrate depletion or dilution, much faster than the reaction on LDL particles.

Another experiment using 200 and 750 μl of labelled normal plasma for FPLC-GF fractionation showed that increasing the amount of plasma 3.75 times results in a 4-fold increase in HDL CE generation. However, the amount of CE generated in the LDL fraction of this sample was increased 5.4 fold, which is 35% higher than the HDL fraction (Figure 3-6, Table 3-3). This experiment made the following assumptions: synthesis of CE in each fraction is due to the LCAT, which co-eluted with the particles; and co-elution of LCAT with the particle must be due to binding of LCAT to each particle.

Therefore, the activity of LCAT in the HDL fraction appears to be independent of particle concentration in the reaction mixture, whereas LDL particle concentration in a reaction mixture is a factor in the fractional rate of esterification (FER). Although this has not been conclusively proven, one likely explanation may be that LCAT, while bound to HDL, utilises the substrates from the very same particle that it is bound to for cholesterol esterification. Thus, the FER_{HDL} is not affected by dilution of the plasma or HDL fractions of plasma. On the other hand, LCAT bound to LDL utilises the substrate from neighbouring LDL particles, hence the dilution sensitivity. This means that the FER_{LDL} reaction and plasma CER are dilution sensitive (affected by dilution of the LDL fraction, or of whole plasma). This proposed mechanism is outlined in Figure3-10.

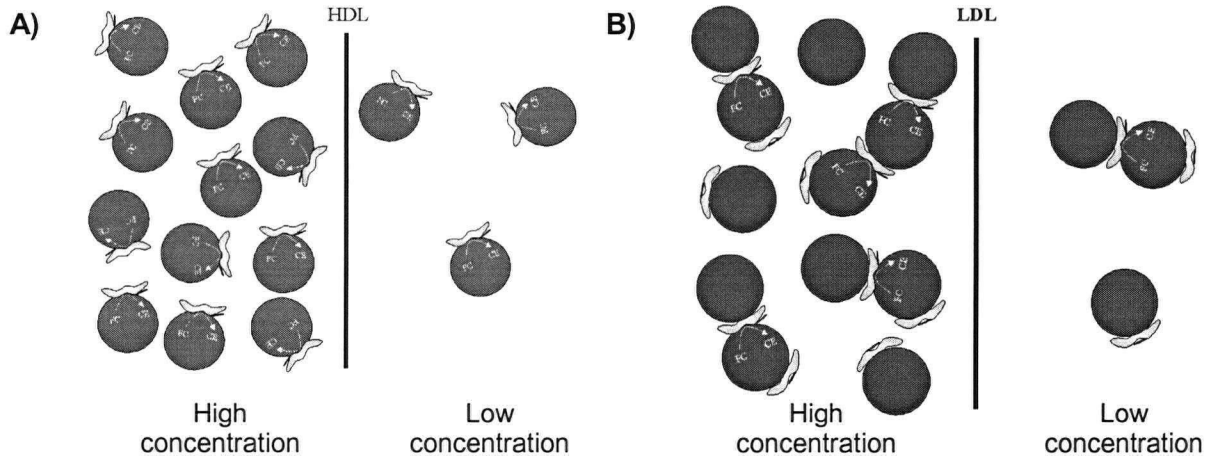


Figure 3-10. Alternative mechanism of action for LCAT in utilization of LDL particles. LCAT bound to LDL particles is capable of esterification of FC. Unlike HDL, in LDL this reaction is sensitive to the concentration of LDL particles. This observation may be explained by an alternate mechanism of action of LCAT on LDL particle. A). **HDL:** LCAT is bound to the surface of the particle where it esterifies the FC from the same particle. Hence, the LCAT activity per HDL particle remains constant, regardless of the particle concentration. B) **LDL:** LCAT bound to the surface of LDL is located such that the active site can not access the FC on the surface of the particle. Instead, the active site is presented to the neighbouring particles, which become associated long enough for esterification of FC on the surface of the particle. Therefore, the increase in particle concentration favours increased chance of interaction between LDL particles resulting in LCAT activity per LDL particle.

This, in fact, may be substantiated by the proposed 3D structure of LCAT [8]. The mutations associated with FED, which results in the loss LCAT activity on HDL particles, have been proposed to form three clusters on the hydrophilic surface of the helices neighbouring the active site (Figure 3-11) [8, 236]. These mutations may alter the interaction of the active site of the enzyme with the substrate molecules and particles due to less than optimal surface interaction. On the other hand, the FED mutations will not influence the interaction with the LDL substrate particles as the opposite side to the active site may be in contact with the LDL particles. Hence, the LCAT activity remains intact with the LDL particles and abolished on the HDL particles.

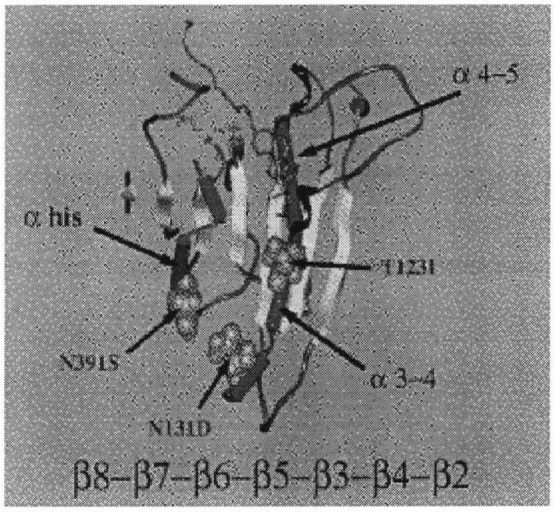


Figure 3-11. Three-dimentional view of the mutations associated with FED. Mutations associated with the FED phenotype are illustrated on the 3-D proposed structure of LCAT. Almost all the mutation described to date fit the model and interestingly all the mutations are clustered on the same side as the catalytic site. (Photo adapted from Peelman et al, Protein Science (1998), 7:587-599)

Although the above FPLC experiment made the assumption that LCAT must bind and co-elute with the particle of interest, there may be other mechanisms involved. It has been shown that rLCAT exists in aggregate form [237], we have observed the same large aggregates of LCAT in the purified protein preparation. These LCAT aggregates

individually or in association with smaller particles may be large enough to co-elute with the larger particles.

Although the free plasma proteins, including the elution of the unbound LCAT, overlapped with the latter part of HDL fractions, binding of ^{35}S-rLCAT was assessed by measuring the whole area corresponding to each lipoprotein.

In the binding studies of rLCAT, FED LCAT and WT LCATs, there was no preference for LCAT to bind either lipoprotein class. This observation may be explained in two ways. First, the FED mutation does not alter the binding capacity of LCAT towards LDL and HDL. If true, this would suggest that the LCAT distribution pattern in plasma does not change. But FED LCAT was only active towards LDL particles whilst the WT LCAT in normal plasma is preferentially active on HDL particles. This suggests that FED related mutations cause a change in the three dimensional configuration of LCAT, which results in alteration of catalytic activity of the enzyme. This explanation supports the proposed mechanism of action for LCAT on LDL and HDL (Figure 3-10). Alternatively, differences in post-translational modification from each expression system may affect the carbohydrate content of rLCAT, resulting in poor protein-lipoprotein interactions. Some supporting data has come from the rabbit rLCAT turnover studies in our laboratory that have indicated that the majority of rLCAT was cleared from the circulation. Recombinant LCAT was present in two distinct pools, fast clearance and slow clearance components. It was proposed that the fast clearing pool was the unbound protein whereas the slow clearing pool consisted of lipoprotein bound rLCAT [94].

The inability of rLCAT to bind mature lipoproteins may be due to the lack of binding/association sites. These sites may be occupied by native LCAT, which only associates itself with nascent HDL particles [238].

In a recent work, rLCAT from COS cells was shown to bind synthetic HDL with different phospholipid compositions [18]. This suggests that rLCAT can become associated with liposomes/lipoproteins as long as there is a physical space to accommodate it.

Chapter 4
Determination of binding kinetics of LCAT to plasma lipoproteins using surface plasmon resonance

I would like to thank Drs. John Kastelein, Hans Panakoek and Henri Molhuizen for the use of their BIACore facilities and technical support provided by their research groups. I would also like to acknowledge Ms. Lissette Klaaijsen and Ms. Marieke Eikenboom for the technical support for the LCAT purification.

4.1 Introduction

Techniques in molecular biology and genetic engineering have provided the tools for researchers to study the function of a gene product and potentially identify the associate factors. One such system, yeast two-hybrid systems have enabled researchers to selectively study enzyme-substrate interactions in an eukaryotic experimental environment. However, complicated biological pathways contain extensive redundancies that would complicate the findings from this system. In vitro techniques have been able to determine binding constants of various molecules in a crude fashion and to a limited extent.

A recently developed in vitro technique, called surface plasmon resonance (SPR) is capable of providing detailed information on properties of interaction between two unmodified particles/molecules [239]. This technique is based on the principle of quantum mechanics, which detects changes in the refractive index close to the surface of thin gold film on a biosensor chip (Figure 4-1). One of the reactants is linked (i.e. covalent bond, biotin-avidin interaction, and hydrophobic interaction for membrane models) to the surface by a caboxymethylated dextran polymer used to coat the chip.

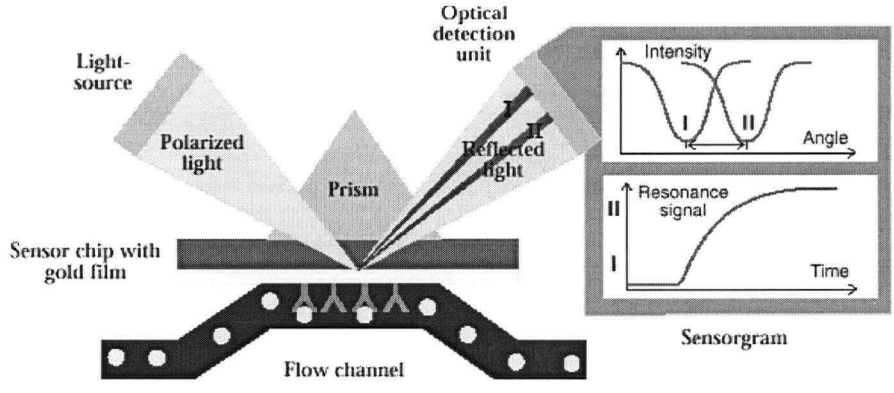


Figure 4-1. Theory of surface plasmon resonance in binding assays.

The soluble component is then injected to flow over the surface where binding of the soluble component to the immobilised one results in an increase in the refractive index altering the SPR. This change is detected optically and the binding is measured in arbitrary units (RU). This powerful technique is capable of determining the rate constants for association (K_{ass}) and dissociation (K_{diss}), and the Equilibrium Dissociation constant (K_d) (Figure 4-2). Machines capable of SPR studies have gained popularity over the past few years and the most popular model is offered by BIAcore Inc. For the purpose of clarity BIAcore and SPR will be used interchangeably throughout this thesis.

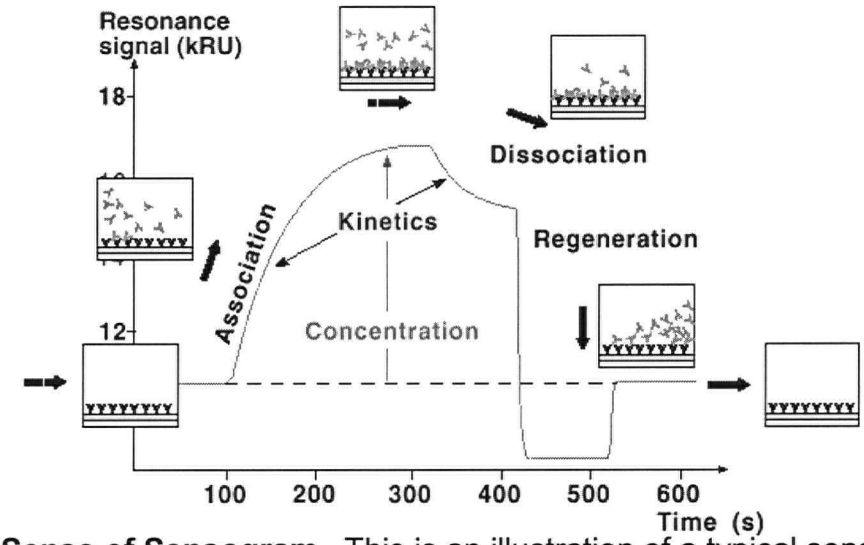


Figure 4-2. Sense of Sensogram. This is an illustration of a typical sensogram from an SPR experiment representing all stages of a binding study.

BIAcore has been extensively used in the study of lipoprotein metabolism [10, 240-242]. Early studies have been concerned with the interaction of lipoprotein lipase with VLDL and recently there have been studies on LDL receptor related protein (LRP) [243-245]. SPR has also been used to study the interaction of LCAT with lipoproteins [10, 246]. However, the published data have only been able to show binding to reconstituted HDL (rHDL), liposomes and biotinylated LDL particles.

Adimmolam *et al* were the first to study the binding constant of rLCAT with lipoproteins [10]. This study investigated the role of two mutants of LCAT causing FED (T123I) and FLD (N228K). SPR was used to determine the binding constant of these functional mutants. The findings showed that binding affinity of the mutants are only 2-fold less than that of WT LCAT. In addition, it was suggested that normal LCAT dissociates from its substrate particles once every catalytic cycle [10].

Another study using SPR, in addition to the above mutants, studied a lid domain mutant (Δ53- 71) [246]. It was suggested that the binding to LDL was normal for T123I and N228K mutants of LCAT, but that the lid domain mutant did not bind the LDL particle. Furthermore, it was shown that increasing ionic strength would reduce binding of LCAT to A-I rHDL particles. This study concluded that apo A-I results in decreased dissociation of LCAT from rHDL and the lid domain (residues 53-71) is essential for interfacial binding [246].

These studies have concentrated on binding of rLCAT to synthetic and/or modified macromolecules. Although this has provided a valuable set of data to improve the current understanding on binding kinetics of LCAT to lipoprotein analogues, little is known on the interaction of rLCAT with the native plasma lipoprotein. In contrast to the conclusions by Adimoolam *et al*, the findings from chapter 3 clearly suggest that the endogenous LCAT is bound to plasma lipoproteins and the LCAT activity co-elutes with the lipoprotein fractions. This discrepancy in findings may be due to the differences between rHDL and HDL. It is therefore crucial to study the interaction of rLCAT and its functional mutants using native plasma lipoproteins with minimal modification to avoid alteration of the structural apoproteins.

4.1.1 Objective;

To determine the binding characteristics of rLCAT to the native plasma lipoproteins.

4.1.2 Specific aims:

- To identify the appropriate sensor chips and conditions to immobilise native lipoproteins with minimal modification.
- To optimise the BIAcore system for study of rLCAT.
- To study binding of rLCAT to the native plasma lipoproteins.

4.2 Methods

4.2.1 Isolation of plasma lipoproteins

Plasma lipoproteins were isolated using sequential density ultracentrifugation, as described in Chapter 3. Isolated lipoproteins were checked for purity using lipoprotein electrophoresis. If not pure, isolated lipoproteins were washed by ultracentrifugation, using appropriate density salt solution.

4.2.2 Purification and quantitation of rLCAT

Recombinant LCAT from conditioned media was purified according to the methods described in Chapter 3.

4.2.3 Generation of biotinylated-PL labeled plasma lipoproteins

1 μg of EZ-Link Biotin DPPE or Biotin-LC-DPPE was applied to a filter paper disk, added to the plasma or the lipoprotein of interest and incubated on ice for 18h to allow for passive transfer to occur. After incubation, the paper discs were taken out and

the labeled lipoproteins were tested for PL incorporation. The labeled material was dialyzed against PBS over night.

4.2.4 Generation of coated gold chips

BIAcore chips are coated with various surface chemistries coupling for covalent immobilisation of ligands (Sensor chip CM5). These sensor chips are also available with streptavidin coated surfaces (Sensor chip SA) as well as hydrophobic interaction surface chemistry (Sensor chip HPA and Pioneer L1).

4.2.4.1 Experimental design

Using chips with various surface chemistries, under conditions suggested by the supplier, rLCAT and plasma lipoprotein were attached to the surface of the sensor chips. Successfully coated chips were characterised using either rLCAT or lipoproteins, depending on the immobilised ligand. For example, in the case of the Pioneer L1 chip, the following protocol was utilised.

Analyses of LCAT binding to HDL, LCAT and SUV were performed on a BIAcore 3000 surface plasmon resonance instrument. All the binding experiments were conducted at 25°C. Buffers were filtered and degassed daily prior to use. All analytes were dialysed against the running buffer. Analyte concentration was 0.03-0.3 μM. Lipoprotein and liposome particles were immobilised on the surface of Pioneer L1 chips. This chip is coated with a gold surface that has been modified with lipophilic substances (alkyl chains, proprietary information), thus anchoring the lipoproteins and liposomes non-covalently. This provides a unique opportunity to immobilise native lipoproteins without any modification or processing. In addition, the limited number of hydrophobic

anchors creates a controlled environment for anchoring equal number of particles on the surface of the chip. The surface of the L1 chip was prepared according to the manufacturer's specification. Flow cells 2, 3, and 4 were coated with HDL, LDL, and SUV, respectively and flow cell 1 remained uncoated. Analytes were injected into individual or all the wells at 5-20 μl/min.

4.3 Results

4.3.1 Characterization of biotinylated liposomes and lipoproteins

Use of bioyinylated particles in coating the gold chip surfaces is well-established. Thus, this strategy was used to coat the gold chips with biotynilated lipoproteins and liposomes. Biotinylated liposomes and lipoproteins were mixed with FITC conjugated streptavidin and run on the GF system. Fluorescence and cholesterol detectors were used to detect eluting particles. Labeling of plasma lipoproteins and production of liposomes using EZ Link Biotin-(LC) DPPE were successful in generating particles which incorporated biotinylated phospholipid and retained their structural integrity as assessed by GF (data not shown).

4.3.2 Immobilization of biotinylated particles

Labelled particles were immobilised on the surface of the sensor chip SA. The resulting response units were significantly lower than the previously published work and this approach provided no reasonable means for regeneration of the sensor chips (data not shown).

4.3.3 Immobilization of liposomes and lipoproteins using Pioneer L1 chip

Isolated lipoproteins and liposomes were successfully immobilised to the gold surface via hydrophobic surface anchors. Assuming uniform distribution of anchors, a

comparable number of each particle (HDL, LDL, or liposome) was anchored to the chip surface. This was reflected in the response units (RU) obtained from each immobilisation trial (Figure 4-3). This observation was repeated reproducibly on three separate sensor chips.

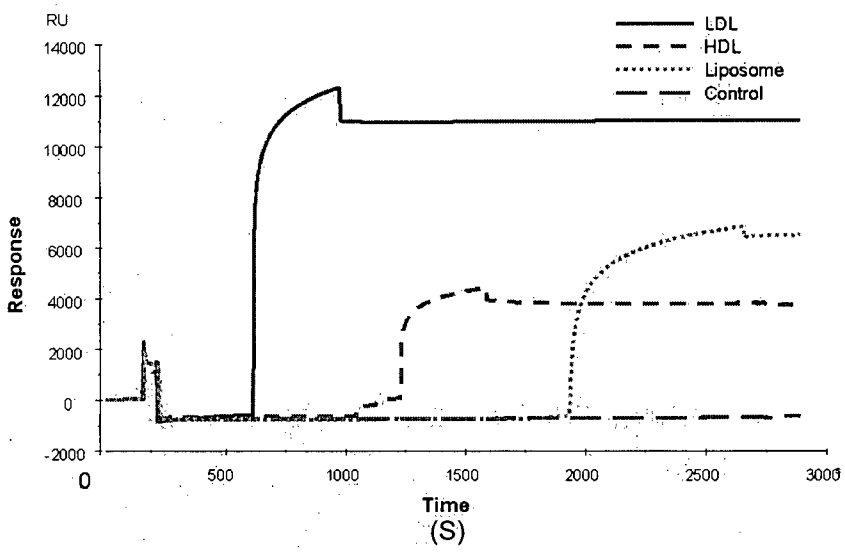


Figure 4-3. Coating of Pioneer L1 sensor chip. Lipoproteins isolated by ultracentrifugation were injected to individual flow cells to generate a chip with four distinct ligands attached to the chip surface. The lipoprotein or liposome particles were attached to the surface through hydrophobic anchors.

4.3.4 Binding studies of LCAT to immobilized liposomes and lipoproteins

The recent publications on SPR studies of LCAT have utilised rHDL and modified LDL particles as well as liposomes. Thus, as our positive control we used proteoliposomes anchored to the surface of sensor chips (Figure 4-3). Purified rLCAT did not react with native lipoproteins while there was a clear association with the anchored liposomes (Figure 4-4). This association was not followed by a complete dissociation from the particles and the analytes accumulated on the surface of the chip.

This was evident from the observed classical saturation binding curve. This observation is consistent with the findings from the study of sites of LCAT activity and CE accumulation. As our positive control for lipoprotein binding, we used various formulations of lipopolysaccharides. LPS particles showed strong association to LDL particles as well as to the hydrophobic surface (Figure 4-5). LPS showed no affinity to proteoliposomes and HDL particles. This observation was repeated on three different chips, as there was no effective method of regenerating the chips. Results from the subsequent experiments were in agreement with the original findings.

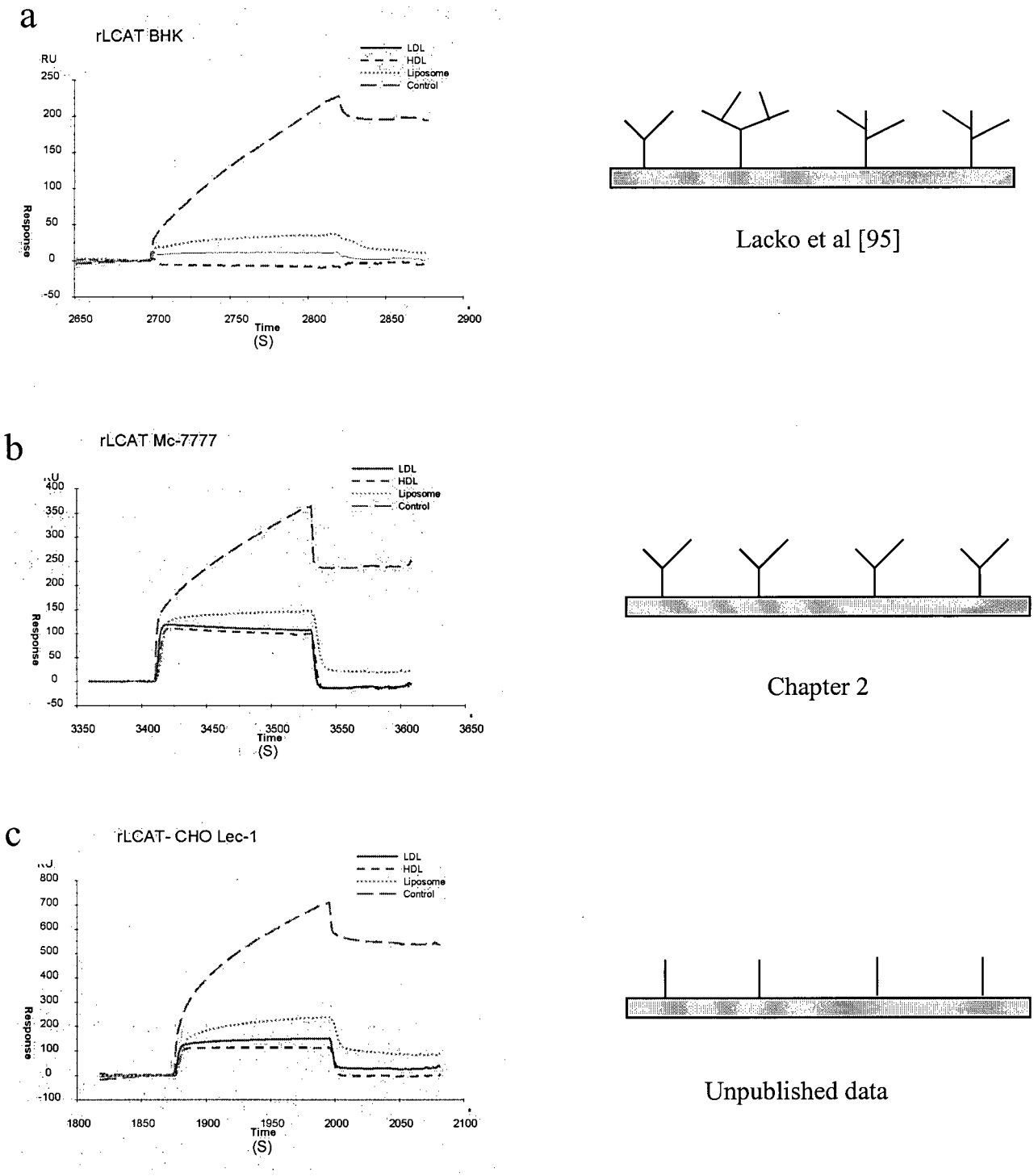


Figure 4-4. Surface plasmon resonance studies of rLCAT. Various concentrations of rLCAT (0.03-0.3 μM) from three different expression systems (a) BHK, (b) Mc-7777 and (c) CHO lec-1, were injected onto the sensor chip coated with flow cell 1: empty control,; flow cell 2: LDL;.flow cell 3: HDL and flow cell 4: liposomes. Binding of rLCAT to the chip surface (via the immobilized ligands) is quantified in response units. Each experiment was repeated three times. On the right panel, the corresponding LCAT glycosylation architecture is depicted.

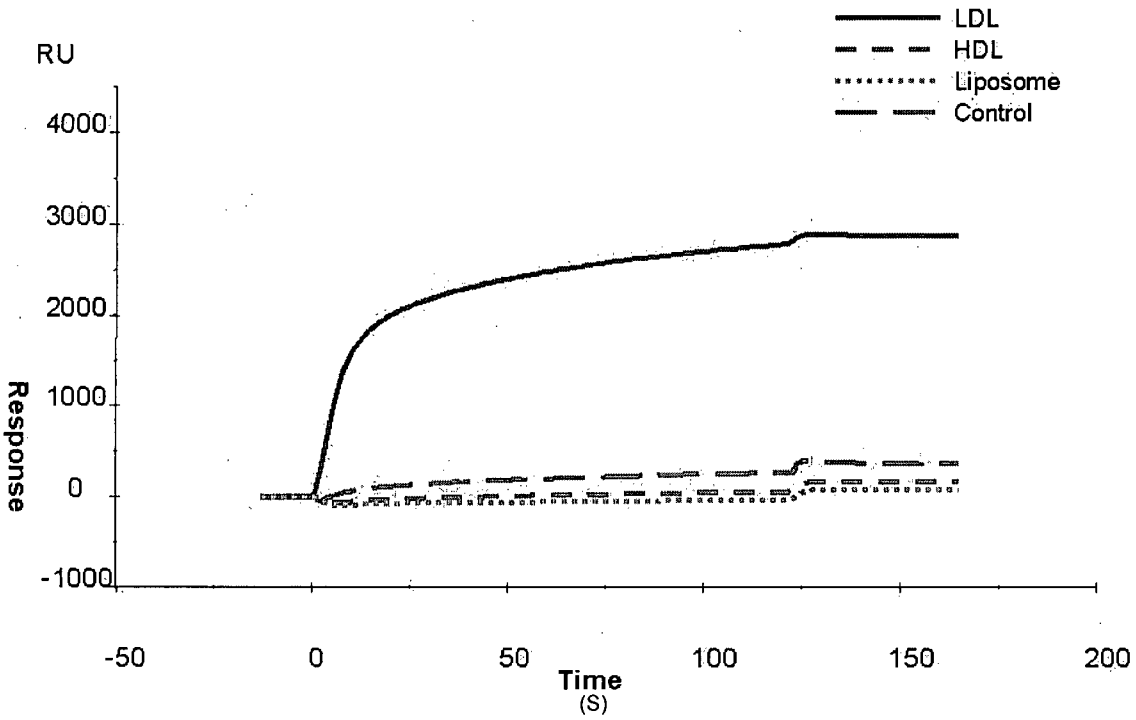


Figure 4-5. Lipopolysaccharide (LPS) control. LPS was used as a positive control to demonstrate binding to lipoproteins. Small amounts of LPS micelle preparation was injected onto the flow cells.

4.4 Discussion

Recent studies have utilised SPR different capacities to study binding characteristic of LCAT to lipoproteins. Due to technical limitations, all these studies have utilised synthetic lipoprotein analogues or chemically modified lipoproteins. Although the findings have added to our understanding of the association of LCAT with lipoproteins, little is known about the interaction of LCAT with unmodified native lipoproteins. The work presented in this chapter makes an attempt to study the binding of rLCAT to plasma lipoproteins.

Biotinylation of lipoproteins by passive diffusion was successful. However, attempts to immobilise these particles to the surface of SA chip resulted in a very low signal that was not sufficient to perform binding studies. The inefficient binding of biotinylated lipoproteins was thought to be due to short distance of biotin label from the surface of the particles. The use of Biotin-LC-DPPE with an extended link between biotin and DPPE resulted in a similar observation suggesting that label availability was not responsible for the lack of binding. Alternatively, the presence of small, unbound particles containing labelled DPPE or loosely associated DPPE on the surface of particles may have contributed to the inefficient binding of lipoprotein to the SA-sensor chip.

Due to difficulties of labeling lipoproteins, a new and experimental chip for hydrophobic interaction was used. This chip utilized a hydrophobic anchoring system (Pioneer L1 chips) that allowed for coating of the gold surface with native lipoproteins and liposomes without any modifications (Figure 4-3). In fact, the original design of the chip, which was to allow formation of membrane bilayers, provided a limited number of hydrophobic anchors and helped to attach similar number of LDL, HDL and liposomes on to the surface of the sensor chip. This was reflected in the immobilization phase,

where the entire maximum signal obtained from LDL was approximately 3 fold larger (Figure 4-3). This phenomenon would allow for a head to head comparison of binding to each particle.

The findings from LCAT binding studies suggest that rLCAT, regardless of source and carbohydrate structure, readily binds to the empty flow channel which was a hydrophobic surface and proteoliposomes (synthetic HDL analogues), while the native lipoprotein fractions (HDL and LDL) show little or no association with the rLCAT. The binding to liposome is in agreement with the previous work by Adimoolam et al which utilised reconstituted HDL and modified LDL particles as well as liposomes to study binding of rLCAT [10]. In contrast to previous findings, there was little or no evidence of dissociation of rLCAT from the liposomes. This was observed independent of source of rLCAT and further complicated the experimental strategy by rendering the chips unusable after 2-3 injection. Attempts to regenerate the sensor chip were unsuccessful and led to defective chips.

Lipopolysaccharide (LPS) complexes were used as positive control for binding to plasma lipoproteins. LPS showed extremely high affinity to the LDL particles whereas there was little association with HDL and liposome particles. This association was not due to non-specific hydrophobic interaction LDL, as there was no evidence of association with empty hydrophobic lane.

This study was limited by a number of factors. Due to irreversible binding of rLCAT and LPS to the immobilised ligands requiring a new sensor chip to be used for every injection – a procedure that required the use of an internal standard to take into account the error inherent in using different chips. In addition, isolated lipoproteins provided reproducible results for only 48 hrs after isolation. Enzyme stability also was a major problem as LCAT activity degraded rapidly at 4-8 ^{o}C which accelerated at 25 ^{o}C.

In conclusion, we have identified a unique method to effectively immobilise unmodified, native plasma lipoproteins to the surface of a sensor ship. This study also suggests that rLCAT does not show an affinity to the native lipoproteins, in contrast to HDL analogues. This may be due to the fact that the isolated HDL particles are the final products rather than the actual substrate as suggested by Kosek et al. In such case, the real substrate particles of LCAT are the nascent HDL and even the early A-I/PL/UC complexes formed as a result of membrane microsolublisation in the interstitial space. Thus, one must exercise caution in hasty extrapolation of the *in vitro* data to physiological conditions where both lipoproteins and LCAT may behave differently than *in vivo*.

Chapter 5

Interaction of S216A and N384Q, the two hyperactive mutants of LCAT

I would like to thank Ms. Sandra McGladdery for performing the pilot experiments, assisting with the kinetic studies, and valuable discussions on data interpretation.

5.1 Introduction

As discussed in Chapter 1, loss of a carbohydrate chain at position 20, 84, or 272 resulted in decreased enzyme activity. The loss of the carbohydrate chain at position 384 by the conversion of Asn to Gln is associated with more than a 2-fold increase in enzyme activity. Complete loss of all carbohydrate chains resulted in an inactive protein. Kinetic analysis of these LCAT mutants showed that the Km of the N384Q and the wild type were similar suggesting that the increase in enzyme activity was due to changes in the Vmax. Therefore, the loss of the carbohydrate chain has been suggested to result in variations in catalytic properties of the enzyme [16].

The Ser-216 residue in LCAT plays an important role in enzyme activity and seems to be closely linked to the active site of the enzyme [13]. It has been reported that Ser-216 has increased activity when it is replaced with an Ala residue but a significant decrease in acyltransferase activity when replaced with Thr while retaining its phospholipase A2 activity [13]. It has been proposed that the Ser-216 is somehow associated with the active site of the enzyme possibly through interactions of Ser-216 and other Ser residues including Ser-181 and Ser-208. It may be that the mutation to Ala-216 reduces the energy required to transfer LCAT from the "closed" to "open" loop and plays an important role in orienting or allowing access to the substrate for effective catalysis [13].

There is little known about the 3-D structure of LCAT, however, we speculate that the two mutations resulting in increased enzyme activity--S216A and N384Q-- function at two separate domains of the enzyme, via two separate pathways. N384Q may alter

the kinetics of the enzyme and at the same time S216A may alter the active site by enhanced access to the substrate. If this is true, then the properties of each individual mutation could equally contribute in an additive fashion producing a protein with activity greater than either individual mutation. Potential clinical benefits of this new construct may extend to treatment of familial LCAT deficiency and modulation of LCAT's role in reverse cholesterol transport and HDL metabolism. A double mutant containing N384Q and a S216T, a mutation resulting in decreased enzyme activity, was also investigated to better understand the interaction of these two putative domains and to demonstrate possible mechanisms by which LCAT functions.

5.1.1 Hypothesis:

Combination of the two hyperactive mutants, N384Q and S216A, will result in an additive effect resulting in further enhancement of LCAT activity.

5.1.2 Objective:

To determine the effects of combining two mutations on the catalytic properties of LCAT.

5.1.3 Specific aims:

- To generate the double LCAT mutants and clone them into pNUT expression vector.
- To express the two double mutants (S216T/N384Q and S216A/N384Q), the three single mutants (S216A, S216T, and N384Q) and the wild type in BHK cells.
- To determine the catalytic properties of the new constructs.

5.2 Materials and Methods

5.2.1 Cloning

Constructs containing LCAT with single and double mutants were generated by PCR based, site directed mutagenesis and standard cloning strategies, as described in previous chapters.

5.2.2 Eukaryotic Cell Culture and Transfection

BHK cells were maintained in conditions described earlier.

5.2.3 LCAT Protein Concentration

The immunoassay of LCAT was carried out by using nitrocellulose membranes as described before (Chapter 2).

5.2.4 Measurement of LCAT Activity

See Chapter 3.

5.3 Results

The quantity of rLCAT secreted from the each of the established cell lines, BHK cells with a different mutant, were comparable to each other (Table 5-1). Newly generated LCAT mutants (S216A/n384Q and S216T/N384Q) did not affect the cellular processing and/or secretion of LCAT into the culture media. This was also true for WT, S216A, S216T, and N384Q species of LCAT, as previously described. This suggests that these individual mutations of LCAT do not (singly or in combination) play a significant role in transcription, translation, intracellular processing and/or secretion of the protein to reduce expression of LCAT.

Table 5-1. Specific activity of rLCAT. Data were obtained from highest BHK producers of rLCAT in the media. Data are the average of duplicate analyses in two distinct experiments.

LCAT	Concentration (μg/ml)	Specific activity (nmol/hr/μg)
Wild type	**9.24**	**3.89**
N384Q	**5.99**	**12.02**
S216A	**8.43**	**8.12**
S216T	**7.61**	**0.94**
S216A/N384Q	**8.91**	**1.50**
S216T/N384Q	**13.62**	**0.65**

Transfected BHK cells at 80% confluence were incubated in serum-free Opti-MEM for 48h. The culture medium was assayed for LCAT activity and protein mass as described in the Materials and Methods. The data represent the analysis of two distinct experiments performed in duplicate with experimental differences less than 10%.

Apo A-I activation curves displayed a simple saturable reaction characteristic. Results from apo A-I activation study of each LCAT mutant helped determine the optimal conditions for our kinetic assessment of WT LCAT and various mutants of the protein.

Although all proteins achieved peak activity at 4 mg per assay, the hyperactive mutants displayed a higher sensitivity to apo A-I. This resulted in peak activation using only 2.0 mg per assay. All the following assays were done with the addition of the appropriate apo A-I content of the reactions (Figure 5-1).

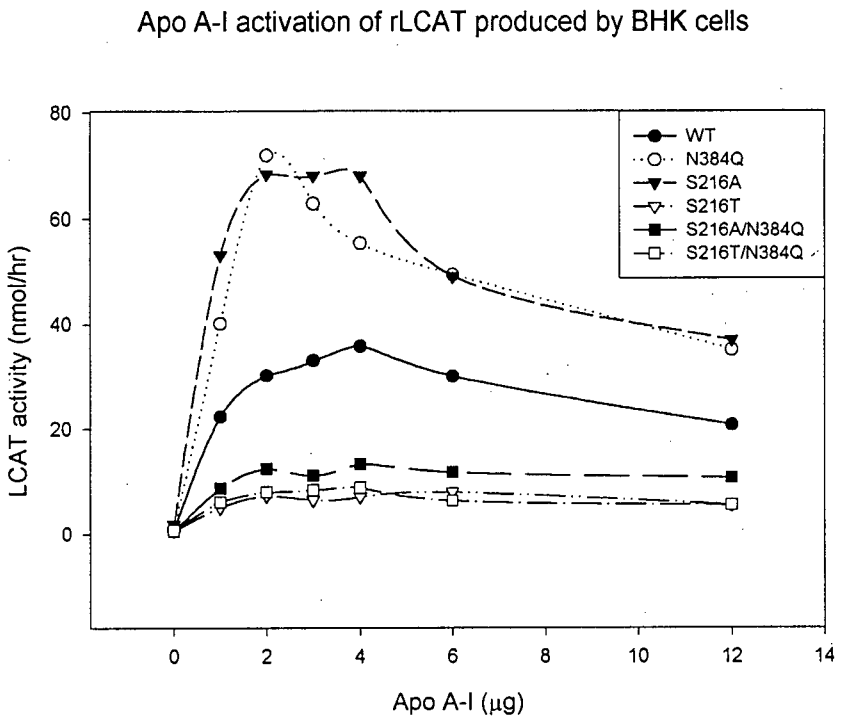


Figure 5-1. Apo A-I activation of LCAT mutants. Increasing amounts of A-I was added to the substrate mixture. LCAT reactions were set-up at 37°C. Each point on the graph represents the mean of duplicate measurements from a single experiment.

Specific activities of each protein revealed the answer to our major question. While WT LCAT activity was set as standard, esterification activity of S216A/N384Q was at 50% of WT. Although unexpected, this mutant will provide us with some new information on the mode of action of LCAT. S216T also had an activity at 50% of WT being closely comparable to S216T/N384Q and S216A/N384Q. This was in contrast to S216A and N384Q which displayed higher rate of esterification at 2.0 and 3.5 fold the WT (Table 5-1).

The effect of substrate concentration was studied by titrating increasing amounts of substrate to the reactions (Figure 5-2). Kinetic parameters of each reaction were determined using the Line Weaver-Burk plot and a summary is presented in Figure 5-3. Both hyperactive LCATs, S216A and N384Q, had the highest corresponding Vmax

values at 4.96 and 4.53, respectively, while the proposed superactive LCAT was nearly inactive.

The Vmax value of N384Q was higher than WT, approximately 2 folds, the Km value of the mutant protein was very similar to the WT control. The increase in Vmax of S216A was accompanied by a marked increase in the Km value. For the remaining mutants, S216T, S216T/N384Q, and S216A/N384Q, both Vmax and Km values were reduced to 50% of WT

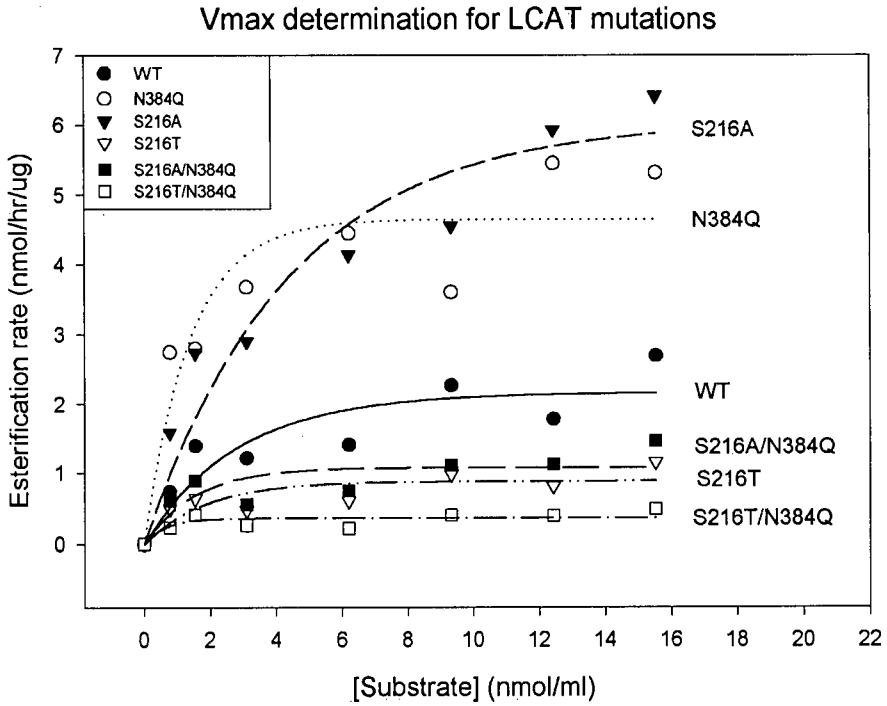


Figure 5-2. Effect of substrate concentration on LCAT activities. Increasing amounts of substrate were added to the reactions and plotted against LCAT activity in nmol of cholesterol est'd/hr/μg of protein. Data obtained from this study will contribute to the kinetic data analyses. Each point on the graph represents the mean of duplicate measurements from a single experiment.

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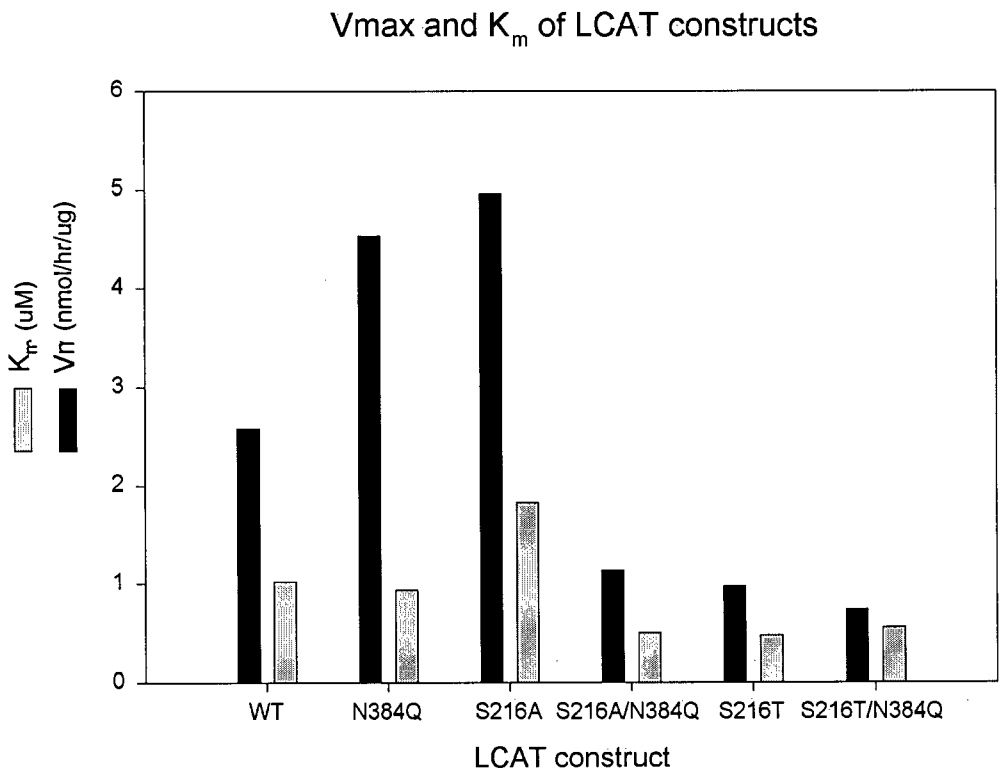

Figure 5-3. Km and Vmax for WT and mutant rLCAT. Km and Vmax values were obtained from the enzyme kinetic experiments using Line Weaver-Burk plot. These parameters are graphed for each mutant including WT. Data represents the mean of duplicate values from a single experiment.

5.4 Discussion

Wild type and mutant proteins secreted by BHK cells did not show any decreased synthesis or secretion by the cells, since the LCAT mass was similar among different transfected lines. Kinetic properties of these proteins were investigated. It was shown that the hyperactive LCAT, N384Q and S216A, displayed the highest Vmax. While N384Q sustains a Km very similar to WT LCAT, the apparent Km of S216A is increased by two fold. This observation indicates that the increased apparent Vmax of N384Q is due to increased efficiency of the enzyme in esterification of UC, as previously proposed [16]. This suggests that the presence of the oligosaccharide moiety on the 384 position has an inhibitory effect on LCAT activity [16]. Since the 3-D coordinates of this carbohydrate moiety is not known, how it exerts its inhibitory affect is not well understood. The 2-fold increase in Vmax of S216A was accompanied by a corresponding increase in Km, which suggests that the increase in apparent Vmax is largely due to the increase in substrate binding ability, unlike N384Q. The results from these single point mutation studies support the notion that a combination of S216A and N384Q might generate a superactive LCAT by combining the individual characteristics of each mutation. The combination of S216A and N384Q resulted in a protein with a 50% reduction in apparent Km and Vmax

Contribution of Ser-216 to the LCAT structure and activity is unclear. Although the proposed 3-D structure of LCAT is now available, spatial co-ordinates of the Ser-216 could not be derived as there was not an appropriate template [8]. The predicted structure suggests that the carbohydrate moiety at the Gln-384 residue lies on the same plane as the catalytic region and can limit access of the substrate to the active site [8].

The originally proposed role of Ser-216 with regards to its involvement in the region of the "lid" domain and its regulatory properties in LCAT surface activation has been challenged by the recent findings [8, 10, 18].

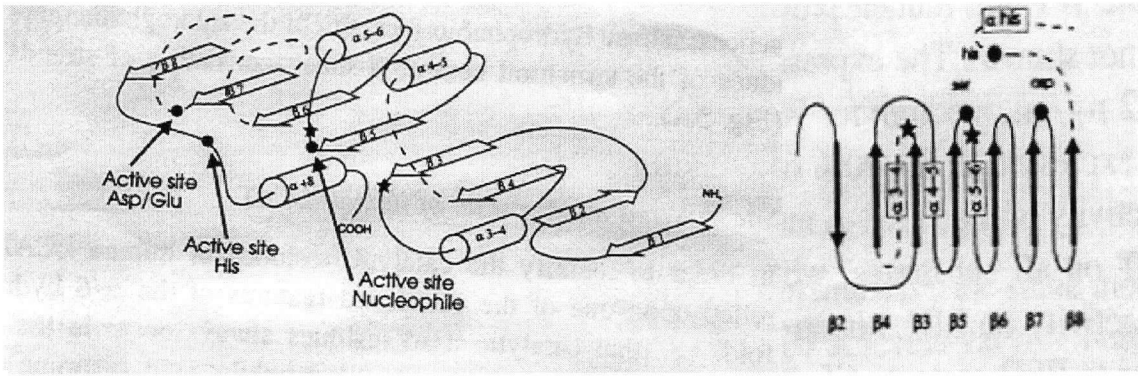

Figure 5-4. Topology diagram of LCAT. *Panel A* represents a typical structure of α/β hydrolase. *B* represents the predicted topology diagram of LCAT. Adapted from Peelman et al, Protein Science (1998), 7:587-599.

If the "lid" domain corresponds to residue 50-74, the potential role of Ser-216 has to be re-evaluated. The current 3-D model of LCAT does not account for a large excursion, residues 208-314 of LCAT that is located between β6 and β7 strands. Secondary structure prediction of this sequence has revealed loops, helices and strands, that is not taken into account in the topology of LCAT (Figure 5-4). This sequence is probably located in the vicinity of the proposed catalytic site. Ser-216 mutations may alter the folding of this region in two ways:

i) This region may still play a role in the interfacial interaction of LCAT with the substrate molecules. Folding alterations due to point mutations of the residue 216 may influence the binding and/or handling of the substrate.

ii) Point mutations of Ser-216 may alter the co-ordinates of Asp 345 of the catalytic triad.

Combination of the N384Q and S216A did not result in an additive effect of their hyperactive properties. The 4th carbohydrate moiety of LCAT may serve a stabilising purpose to some of the small changes in the LCAT sequence, i.e. Ser-216. S216A mutation may rely on this carbohydrate chain for stability and appropriate position of Asp-345.

This observation was also true for S216T which has been previously reported to be less active than WT [13]. These mutations of the Ser-216 appear to result in the inactivation of enhanced capability of the N384Q mutant in generation of CE. This suggests that either:

i. S216A and N384Q mutants do not function exclusive of each other, or
ii. The structure of LCAT cannot accommodate two different mutations, resulting in a structural alteration.

The reduction in Km and Vmax of the double mutants may be due to defective handling of substrate. In light of the current hypothesis -- N384Q does not have the proposed inhibiting carbohydrate chain and S216A provides better access to the active site by maintaining an open conformation of the lid domain -- it is conceivable that a combination of these two mutations may result in a complete lack of restraints for efficient direction of the substrates.

From the thermodynamic point of view, this may not be possible since LCAT may already have been at its peak ability in terms of coupling the phospholipase A2 activity and acyltransferase activity together. The limitation on cholesterol esterification may be due to physical barriers, such as the rate of diffusion of substrates to the enzyme and products away from the enzyme, especially in a reaction environment without a uniform distribution of substrate and limited storage space for the products.

Study of these mutations have provided information on the functional map of the enzyme, in terms of the function of specific amino acid residues and their interactions representative of two different domains of LCAT. In conjunction with previous work, it provides additional information on the global structure and function of the enzyme. In a recent review of the molecular basis of LCAT deficiency syndromes, it was shown that most mutations resulting in FLD are located in the C-terminal half of the protein and the point mutations related to FED are on the N-terminal half of the protein (chapter 1). In addition, a recent work has shown that residue 149 plays an important role in substrate specificity based on PC specificity [247]. Point mutations of the N-terminal half of the protein can affect the substrate binding, specificity, and recognition, whilst the C-terminal mutations may have an effect upon the enzymatic functions responsible for chemical modification mediated by the enzyme.

In the near future, binding studies with synthetic proteoliposomes and native lipoproteins may provide some insight into the mode of action of this protein. Phospholipase A2 activity assessment of our mutants, especially S216T/N384Q, can provide information on the function of Ser-216 and its interaction with Asn- 384.

Chapter 6

Summary and future directions

There is an increasing amount of information on the relationship between functional and structural characteristics of LCAT. Binding of LCAT to plasma lipoproteins has been studied to a lesser extent and only recently has this aspect of LCAT research gained renewed interest. Recently, binding of rLCAT to reconstituted HDL, modified LDL and biotinylated liposomes was quantified using the surface plasmon resonance technique [10, 246]. Adimoolam *et al* proposed that LCAT binds to plasma lipoproteins in a reversible fashion, such that it dissociates after each catalytic cycle. This suggests that LCAT maintains an exchangeable pool that could allow for binding of exogenous rLCAT. Previous work by Pritchard *et al* on the in vivo metabolism rLCAT showed that the majority of exogenous LCAT is cleared rapidly from the circulation [94]. Paradoxically, inability of rLCAT to bind plasma lipoproteins has been suggested to play an important role in the rapid turnover of this enzyme in plasma. In addition, novel FED mutations have been shown to result in a change in substrate (lipoprotein) specificity of LCAT. This thesis was aimed at studying the role of potential structural changes on binding of LCAT to its substrate lipoproteins.

Inability of rLCAT to bind plasma lipoprotein is thought to be due to small differences in protein glycosylation of rLCAT versus plasma LCAT. Recombinant LCAT expressed by BHK cells has been shown to contain slightly different glycosylation pattern than that of plasma LCAT. The first objective of this thesis was to generate/identify a source of recombinant LCAT that would closely mimic plasma LCAT. McArdle 7777 cells were used to produce LCAT from this hepatic derived expression system. The glycosylation pattern of the protein obtained from the liver derived cell line was extensively characterized. The expressed enzyme was uniformly glycosylated with

biantennary oligosaccharide residues, which was significantly different from the glycosylation pattern observed in the plasma and BHK LCATs. Despite the differences in glycosylation pattern, the new rLCAT displays similar kinetic properties to those of plasma and BHK LCATs, suggesting that the differences in glycosylation architecture may not influence activity of the enzyme. These findings also suggest that cells from different species, despite their similar origin may function and utilize alternate pathways in their normal function. Thus, due to the greatest similarity to plasma LCAT, BHK rLCAT was utilized for the subsequent biochemical studies.

In addressing the second objective of this thesis, interaction of LCAT with lipoprotein substrates was studied. This study investigated the binding characteristics of WT and FED plasma LCAT *in vivo*, and rLCAT *in vitro*. Specific experiments were designed to study the lipoprotein binding pattern and activity of plasma LCAT as well as ability of rLCAT to bind plasma lipoproteins *in vitro*. An FPLC system was setup to effectively separate plasma lipoproteins in order to study binding of LCAT to these particles. Using this system efficient separation of individual plasma lipoproteins was achieved. Endogenous LCAT was shown to remain bound to LDL as well as HDL and that beta and alpha LCAT activity co-eluted with LDL and HDL particles, respectively, through two gel filtration columns (Superose 6 & 12) connected in series. This suggests that LCAT is bound to the surface of lipoprotein particles and it does not readily dissociate from those particles. *In vitro* binding studies with whole plasma, total lipoproteins, and individual lipoproteins showed no differential association of rLCAT, either wild type or FED (T123I), with HDL and LDL particles. Although the majority of rLCAT did not bind the lipoprotein particles, the binding pattern of a small fraction of rLCAT (both WT and FED) bound to plasma lipoproteins without any preference for a specific class of lipoproteins. It was also observed that LCAT activity (cholesterol

esterification rate) using the HDL particles did not depend on the concentration of the particle but it was directly correlated to the absolute number of HDL particles. Therefore LCAT activity on HDL (FER_{HDL}) is not concentration dependent. On the other hand, rLCAT activity on LDL (FER_{LDL}) particles indicated a concentration dependent mode of action. Although highly speculative, there is some evidence to support the differential mode of activity hypothesis (Figure 3-10) which states that LCAT bound to HDL particles only utilize the bound particle for generation of CE whereas the LCAT bound to LDL particles are oriented in such a way that the active site of the enzyme is facing away from the particle and can only esterify cholesterol molecules when in contact with the neighboring particle. Although the proposed mechanism is in consistent with the predicted 3-D structure of LCAT, this hypothesis has to be fully explored and confirmed. Surface plasmon studies of rLCAT presented in chapter 4, using native lipoproteins yielded no significant binding to lipoprotein particles. Instead, rLCAT was strongly associated with proteoliposomes. These findings are in conflict with the recent proposed binding kinetics. In fact, LCAT does not appear to dissociate after each catalytic cycle and may not have an exchangeable pool for the following reason:

- The endogenous LCAT co-elutes with plasma lipoproteins.
- Only a small fraction of radiolabelled rLCAT is associated with plasma lipoproteins (physical association).
- Only a small fraction rLCAT is associated with plasma lipoproteins (association detected by activity).
- Although highly speculative, HDL associated LCAT activity is independent of particle concentration.
- Recombinant LCAT does not bind mature, unmodified lipoproteins.

Findings reported by Adimoolam et al was based on a strategy based on artificial

substrate particles which was in agreement with our findings using proteoliposomes.

The final aspect of this thesis was concerned with the relationship of LCAT mutations with its structural and functional characteristics involving study of two very unique mutations, which result in an increase in the LCAT activity. The previous evidence suggested that S216A and N384Q exert their effect based on two independent mechanisms. To further investigate these mutations we hypothesized the combination of two independent mechanisms can lead to an additive effect. The kinetic data obtained from this study showed that the combination of the two mutations does not have an additive effect. Combination of these two mutations resulted in reduction of LCAT activity. Although this effect may be due to lack of independence in mechanism of action or inability of the protein to tolerate sequence alterations, it suggests that the fourth oligosaccharide may influence LCAT function in more ways than just inhibiting substrate accessibility. The predicted 3-D structure of LCAT could not contribute to understanding the role of Ser-216, as this residue was located in a region that shared little homology with any known structure.

In conclusion, this project has made an attempt to investigate the structural characteristics of LCAT that may influence its binding to lipoproteins and activity in plasma. Exogenous LCAT shows no preference in binding to mature LDL and HDL particles, while the endogenous LCAT is bound to the lipoproteins in plasma. This suggests that the mature lipoproteins may not be the optimal binding substrate for LCAT. Carbohydrate moieties of LCAT may influence binding of LCAT to its substrate particles. Furthermore architecture of LCAT oligosaccharide moieties have little influence on the kinetics of LCAT activity. Although hypothetical, the current findings suggest that LCAT is associated with the plasma lipoproteins at the early stages of formation in the interstitium. Specifically, LCAT binds to A-I/PL/UC complexes formed

by membrane micro-solublisation. LCAT is activated by apo A-I and the initial stages of RCT will take place leading to CE accumulation and HDL maturation. These nascent HDL particles perhaps form the best binding substrate for LCAT.

6.2 Future direction

There has been a great deal of research on LCAT function and structure. Studies presented in this thesis have led to formulation of new hypotheses. The future experiments are proposed based on the new findings and hypotheses.

6.2.1 Mode of activity

This thesis provides the preliminary data suggesting that there may be differential mode of activity for LCAT depending on the substrate of choice being HDL or LDL. Possibility of such difference in mode of activity will challenge our current view of LCAT function. Differential mode of activity will enable us to direct and possibly regulate cholesterol esterifcation on specific lipoproteins. In such an event, the current understanding of LCAT activation by apolipoproteins will have to be reassessed. These studies will definitively establish the mode of activity on HDL and LDL particles.

6.2.1.1 Effect of dilution on LCAT activity in LDL and HDL plasma samples

Based on the original hypothesis, if the active site of LCAT is directed onto the particle it is bound to, then, dilution of a constant number of particle will have no impact on the activity. However, if the LCAT activity is altered due to dilution of particles it would suggest that either LCAT does not remain bound to the substrate particles or the lipoprotein bound LCAT is facing outwards directing the active site to the neighbouring particles. Our current data from GF experiments rules out the former. These assays

will take advantage of an anti apo A-I column and PTA precipitation of apo B containing lipoproteins. Serial dilution of ^{3}H-cholesterol labelled apo A-I or apo B depleted plasma would be incubated at 37 ^{o}C to obtain the effect of dilution of FER of LCAT.

6.2.1.2 Use of LCAT inhibitory antibodies

Antibodies directed at the active site of the enzyme can provide important information on whether the orientation of the enzyme on the lipoprotein particles is different on HDL particles compared to LDL. Using epitope specific antibodies to the active site, the LCAT bound to the plasma lipoproteins will be mapped for exposure of its active site. The first strategy would involve inhibition of LCAT activity on the surface of lipoproteins. If the epitope specific Ab cannot inhibit LCAT, that would suggest that the active site is buried in the lipoprotein. However, if the activity is inhibited, the only conclusion may be that the active site is exposed on the surface of the lipoprotein. Also, an HDL or LDL sink ELISA system may be used to determine the binding ability of epitope specific Ab to different classes of lipoproteins.

6.2.2 Surface plasmon resonance using physiological substrates

Experiments presented in this thesis provide the foundation for future experiments to study the binding kinetics of LCAT to the plasma lipoproteins. However, the immediate future steps will involve further characterisation/optimisation of experimental conditions. In long term, SPR is a powerful technique to study interaction of LCAT and its mutants with their substrate particles, activators and inhibitors as well as identification of specific regions responsible for those interactions.

6.2.2.1 Identification of ideal physiological substrate particles

SPR based studies of LCAT have shown strong association of LCAT with synthetic particles, rHDL and liposomes. There has been no successful attempt to show binding of LCAT to its natural substrate, HDL. Findings presented in the current work suggest that LCAT may not bind to mature particles. Instead, LCAT may form a complex with apo A-I or A-I/PL/UC complex to form the nascent HDL. Future studies will have to identify the appropriate particle for binding and the conditions under which LCAT becomes associated with the lipoprotein particles. The initial experiments will concentrate on use of lipoproteins isolated from the lymph. The early A-I/PL/UC complexes may also be generated in tissue culture without endogenous expression of LCAT.

6.2.2.2 Stabilization of purified proteins

Throughout the kinetic and enzymatic experiments, LCAT stability posed a challenge to the experimental design. This issue was a strong factor during the BIAcore (SPR) experiments. Future experiments utilising rLCAT must address this issue in order to obtain reproducible data. It has been suggested that purified LCAT is oxidised resulting in rapid loss of activity in ambient temperature. Strategies such as supplementation of pure material with a reducing cocktail and albumin may be a useful approach.

6.2.2.3 Use of Histidine tagged proteins

Histidine tagged proteins have provided an easy solution to purification of various proteins. Work by Chisholm et al suggests that LCAT with the Histidine tag is active and remains stable under standard conditions for an extended period of time[248]. In addition to the potential of generating large quantities of the purified protein, this rLCAT

may be anchored to the surface sensor chips through its Histidine tag. This would allow for the study LCAT as the stationary phase.

6.2.2.4 Experiments using glycosylation variants of LCAT

Three glycosylation variants of LCAT were described in this thesis. Using the CHO Lec-1 expression system we have generated a glycosylation deficient LCAT that is minimally glycosylated (data not shown). This protein is active and stable for the kinetic studies. Using four glycosylation variants of LCAT, the role of carbohydrate moieties in interaction of LCAT with lipoproteins can be delineated.

6.2.2.5 Experiments using functional and structural mutants of LCAT

As a result of combined efforts from our laboratory many functional and structural mutants of LCAT have been described. Using advanced methods for quantitative determination of binding kinetics of LCAT and its mutants will provide valuable contribution to the current understanding of LCAT function and structure. These findings would provide a better understanding of the natural mutants of LCAT and reclassification of these defects based on their functional deficiencies.

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