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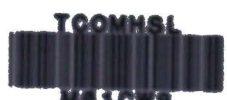
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The relationship between enzymatic activity and conformational properties of the circulating and recombinant forms of human plasma LCAT were examined in the native and denatured states. The two denaturing agents used in this study were guanidine hydrochloride and heat. These studies led to the following conclusions:

(1) Although the alpha helical content of desialylated recombinant LCAT (d-LCAT) is comparable to that of the other two forms of the enzyme (p-LCAT and r-LCAT), the desialylation of LCAT is associated with an increase in the beta sheet and a decrease in beta turn content.

(2) The presence of sialic acids, in addition, seems to influence the local environments of aromatic amino acid residues.

(3) From the denaturation and renaturation studies with guanidine hydrochloride and heat, it appears that the N-glycan structures of p-LCAT and r-LCAT may contribute differentially to the conformational stability of the enzyme.

(4) The alpha helical structure of LCAT may not be involved in maintaining the active conformation of the enzyme.

CONFORMATION PROPERTIES OF CIRCULATING AND

RECOMINANT FORMS OF HUMAN PLASMA LCAT

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CONFORMATIONAL PROPERTIES OF CIRCULATING AND RECOMBINANT FORMS OF HUMAN PLASMA LECITHIN CHOLESTEROL ACYL TRANSFERASE

THESIS

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Master of Science

By

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LIST OF ABBREVIATIONS

p-LCAT: Human plasma Lecithin cholesterol acyltransferase

r-LCAT: Sialylated recombinant human Lecithin cholesterol acyltransferase

d-LCAT: Desialylated recombinant human Lecithin cholesterol acyltransferase

CD spectra: Circular dichroism spectra

CHAPTER I

INTRODUCTION

Physiological Function of LCAT

Lecithin-Cholesterol Acyltransferase (LCAT) (EC 2.3.1.43) is a serine esterase synthesized in liver and secreted into the blood plasma (1). LCAT catalyzes the esterification of cholesterol on the surface of nascent high density lipoprotein (HDL) particles. The cholesterol is converted to cholesteryl ester by the transfer of the acyl group from the sn-2 position of lecithin to the 3-OH group of the cholesterol with the release of lysophosphatidylcholine as shown in figure 1(2, 3). The enzyme exhibits two types of activities namely the acyltransferase activity and a phospholipase A2 activity in the absence of acyl acceptors.

The preferred natural substrate for LCAT is plasma lipoprotein HDL_3 (4). LCAT acts on the surface cholesterol and phospholipid (phosphatidylcholine) of HDL producing core cholesteryl esters. Thus the esterification of cholesterol on HDL promotes the influx of cholesterol from the peripheral tissues and from the other plasma lipoproteins LDL and VLDL (5). LCAT also plays a central role in maintaining the cholesterol homeostasis and is an integral part of reverse cholesterol

transport (6). This process facilitates a cholesterol gradient between peripheral tissues and the liver. Initially the nascent (pre-beta) HDL particles acquire unesterified cholesterol from cell membranes Subsequent to esterification by LCAT, cholesteryl esters from the mature HDL

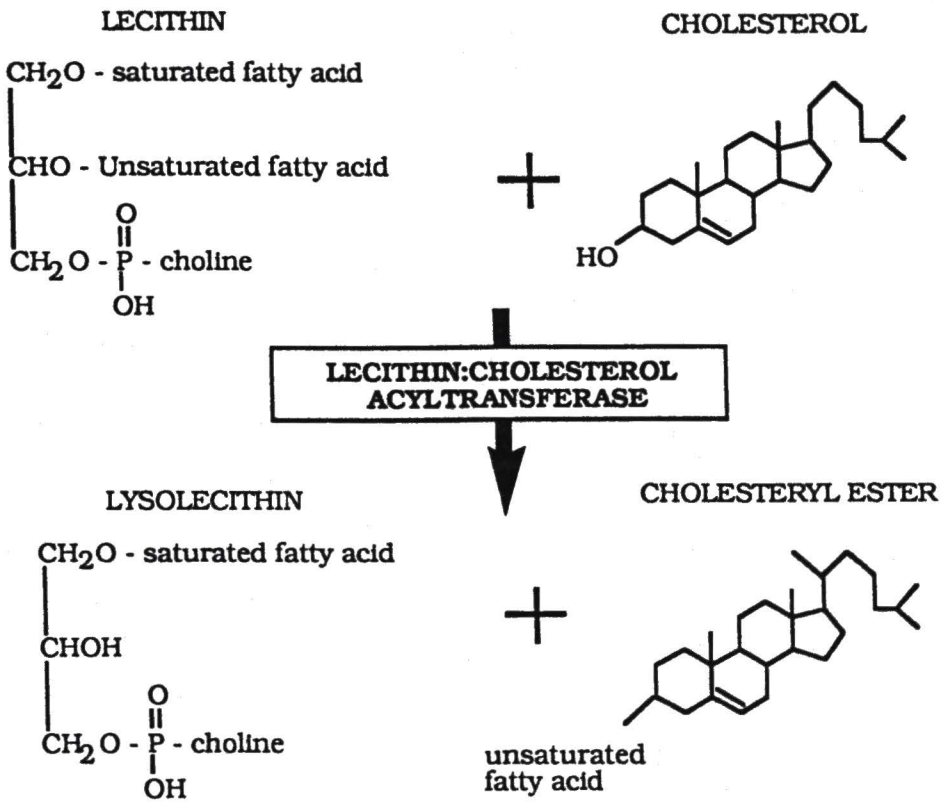


Figure 1

Principal reaction catalyzed by plasma lecithin:cholesterol acyltransferase

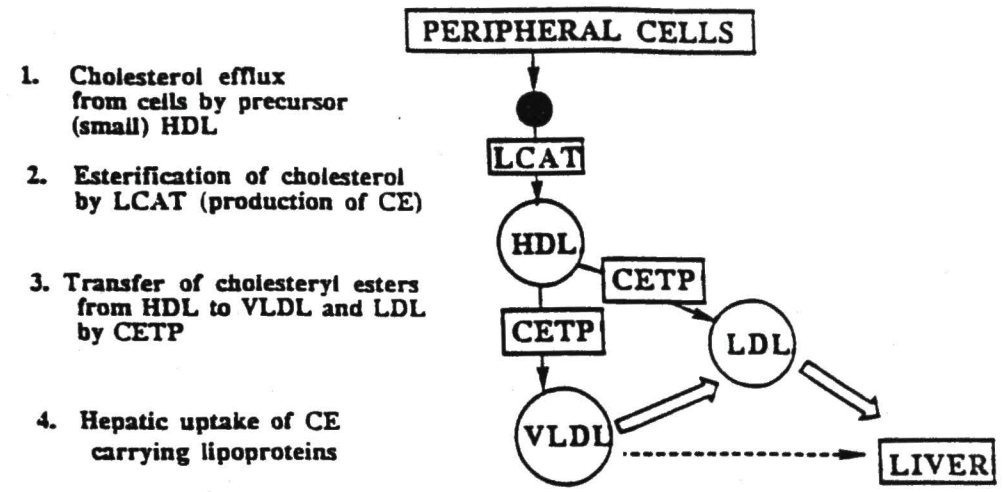
particles are transferred to very low density lipoprotein (VLDL) and low density lipoprotein (LDL) with the aid of cholesteryl ester transfer protein

(CETP) as shown in figure 2 (7, 8). The cholesteryl esters in VLDL and LDL are delivered to the liver by receptor mediated endocytosis where they are further catabolized to bile acids. In endocrine tissues, cholesterol is also utilized in the synthesis of steroid hormones.

LCAT also plays a pivotal role in the interconversion of HDL subclasses and in the maintenance of the lipoprotein structure (9). LCAT in the presence of lipid transfer proteins facilitates the redistribution of the apolipoproteins and the removal of surface lipids, especially cholesterol and lecithin from the remnants of VLDL and Chylomicrons (10) .

LCAT is predominantly activated by apolipoprotein AI (11) , although other apolipoproteins including apo C-I, apo-E and apoA-IV (12-15) may also fulfill this role. The amphipathic alpha helical regions of apolipoproteins that are essential for activation, interact with the phospholipid side chains (lecithin fatty acyl moities). The interaction results in the displacement of the olefinic group from the surface of the substrate complex leading to the reorientation of the sn-2 ester linkage of lecithin. This linkage is subsequently subjected to nucleophilic attack by the oxygen atom of the OH group of the serine 181 residue in theactive site of LCAT (16, 17).

Although LCAT prefers the fatty acids at the sn-2 position of lecithin, the fatty acid present at the sn-1 position may also serve as an acyl group donor for the esterification of cholesterol (3, 18). The acyl donor in



Components of the reverse cholesterol transport pathway

Figure 2

Figure 2 summarizes the components of the reverse cholesterol transport pathway

plasma lipoproteins is most often linoleic acid. The identity of the fatty acid in the sn-1 position affects the rate of hydrolysis of the sn-2 ester bond of lecithin. The enzyme LCAT seems to prefer an unsaturated fatty acid of longer chain length at the sn-2 position of lecithin (3 , 19). The enzyme prefers a sterol acyl-acceptor that has a 3-beta-OH group and a trans configuration of the A/B rings (20).

Congenital LCAT deficiency leads to abnormal lipoprotein metabolism and cholesterol imbalance.

Structure of LCAT

LCAT is a highly hydrophobic glycoprotein composed of a single polypeptide chain with an apparent molecular weight of about 62 Kda. The carbohydrate contributes to about 25% w/w. The total mass of the polypeptide chain (416 amino acid residues) is about 45 Kda. The most abundant amino acids in LCAT are glutamic acid, aspartic acid, glycine and leucine. The C-terminal region of LCAT is rich in proline (21).

LCAT contains six cysteine residues; four are linked in intramolecular disulphide bridges, one between cys 50 and cys 74 and the other between cys 313 and cys 356. The cysteines located at positions 31 and 184 are vicinal in nature and are proximal to the active site of the enzyme. Because the activity of the enzyme is not affected by the substitution of either or both of the cysteine residues by alanine at positions 31 and 184, respectively (22) the cysteines are considered not essential for

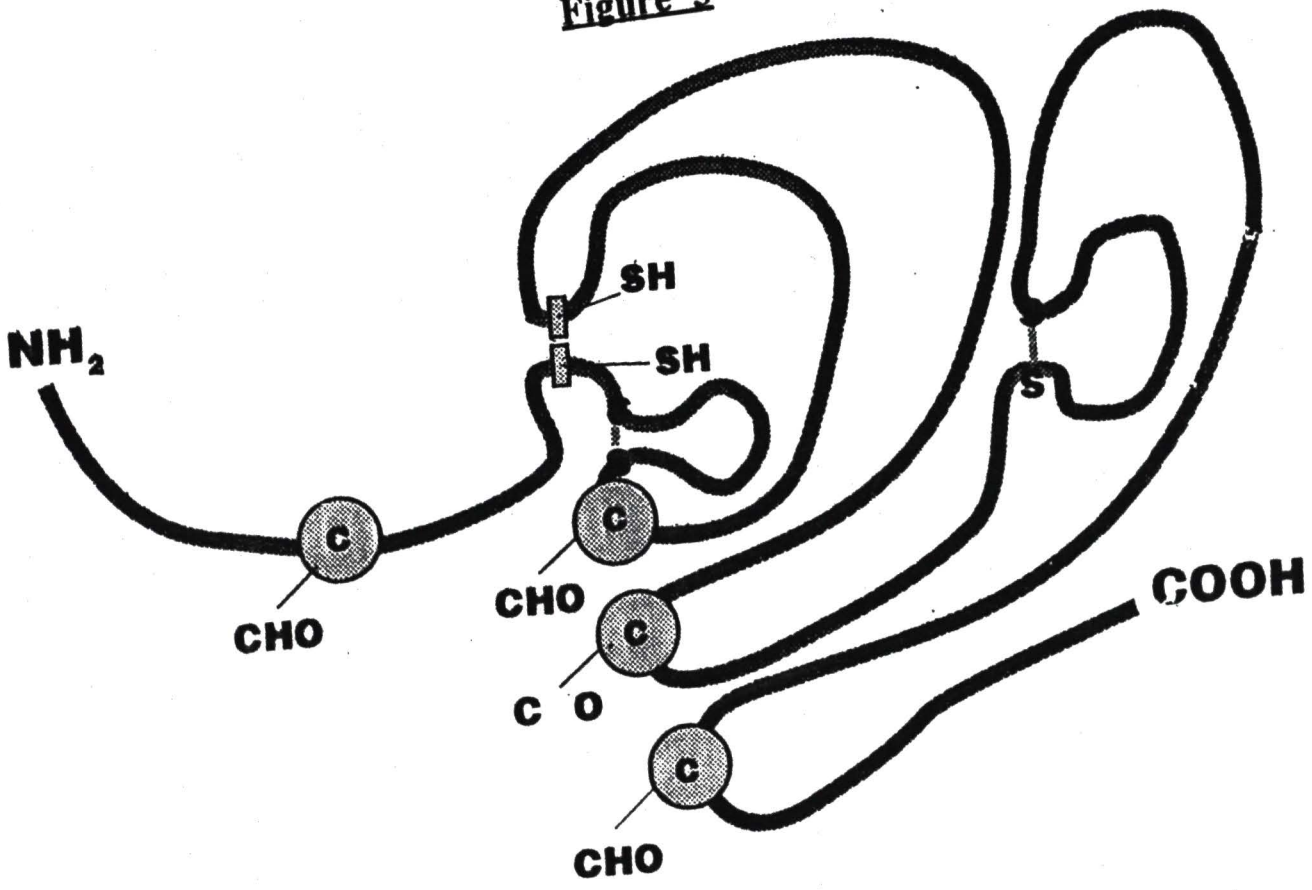
catalysis. However, these residues probably block the access of the substrate to the active site. The disulfide bridges in LCAT are crucial for both activity and secretion of the enzyme (23).

Modification of the enzyme by diethylpyrocarbonate showed that histidine residue participates in both lecithin hydrolysis and esterification of cholesterol (24). When the serine residue is blocked by phenylmethanesulfonyl fluoride (PMSF), both the transacylase and the PLA_2 activities of LCAT are inactivated. It has also been demonstrated from inhibition studies with 1-ethyl-3-(3'-dimethyl aminopropyl)-carbodiimide (EDAC) (25) that potentially three COOH groups are required for the formation of cholesteryl esters . Thus the active site of the enzyme contains a serine (Ser181), histidine(s) and one or more aspartic acid(s) designated as catalytic triad that participates in the proton charge relay system. The structure of LCAT is shown in figure 3.

The carbohydrate on LCAT is composed of 13% hexoses, 6.2% hexoseamines and 5.4% sialic acids (21). Microheterogeneity of LCAT due to variation in the sialic acid content, can be demonstrated by isoelectric focussing (26). Complete desialylation of native human LCAT by treatment with neuraminidase from *Clostridium perfringens* results in the convergence of multiple bands into a single band with a pI of 5.2. Desialylated LCAT exhibits increased enzymatic activity, indicating that the negatively charged sialic acid may interfere with enzyme / substrate binding interactions (26).

Figure 3 is schematic representation of structure of LCAT. LCAT is made of single polypeptide chain represented by dark line. As shown in figure the enzyme has two disulfide bridges and four N-glycosylation sites.

Figure 3



Structure of LCAT

LCAT has four N-linked glycosylation sites located at amino acid residue number 20, 84, 272 and 384 respectively. Each of these sites has the consensus sequence for N-linked glycosylation, Asn-X-Thr/Ser where X is any amino acid other than proline or aspartic acid. By site directed mutagenesis, each of these four sites have been mutated and substituted by glutamine to produce four different individual mutant LCAT enzyme species (27). The mutant enzymes are N-glycosylated at only three of the four potential sites. Mutation of Asn 272->Gln or Thr 274->Ala results in the loss of acyltransferase activity but retention of its phospholipase A_2 activity, thus generating fatty acids but not cholesteryl esters (27). Elimination of N-linked glycosylation at positions 20, 84 and 272, by mutation of the glycosylation consensus sequence by site directed mutagenesis results in a decrease in specific activity of the mutant enzyme as compared to the wild type enzyme. However, elimination of N-linked glycosylation at position 384, results in a two-fold increase in enzyme activity. The mutant enzyme that lacks all the four N-linked glycosylation sites shows markedly decreased secretion of the enzyme (28). It has been deduced by site directed mutagenesis, that Asn 272 (Asn->Thr) is required for intracellular processing while Asn 84 (Asn->Thr) is essential for catalytic activity, but not for the intracellular processing of the enzyme. The N- linked glycosylation at positions 20 and 384 are not required for the intracellular processing, secretion or catalytic activity of the enzyme (29).

The gene for the human LCAT is composed of six exons and five introns that encode for a mRNA of 1550 bases expressed primarily in the liver (30). Mature LCAT is made of 416 amino acids and a hydrophobic leader sequence of 24 amino acids (31).

Several mutations in LCAT result in loss of activity. Deletion of the triplet codon for leucine 300 give rise to a mutant enzyme that leads to Fish eye disease (FED). Although the mutant enzyme has normal catalytic activity compared to the wild type enzyme, deletion of leucine 300 greatly diminishes the secretion of the enzyme (32). Another genetic mutation that causes FED is Thr 123->Ile. This mutation not only affects the amount of LCAT being secreted but also alters the substrate specificity of LCAT from HDL (for wild type enzyme) to LDL (for mutant enzyme) (33). Two different allelic mutations in the gene for LCAT namely Tyr 83 ->stop codon (C->A substitution) and Tyr 156->Asn (T->A transition) also lead to LCAT deficiency. The mutated enzymes may be rapidly catabolized (34).

The extinction coefficient for LCAT as E1% is 21 at 280 nm in 1mM PO4 buffer, pH-7.2. The Stokes radii corresponding to native and desialylated LCATs are 40 A^0 and 36 A^0 and their frictional coefficients are 1.53 and 1.38 respectively (21).

Research Significance

Genetic mutations that lead to LCAT deficiency, are scattered throughout the structure of the enzyme suggesting the presence of several

functionally important structural domains. These domains are likely to be essential in the secretion or the catalytic activity of the enzyme. In order to elucidate the structure-function relationships of the enzyme, it is generally necessary to crystallize and determine its three dimensional structure.

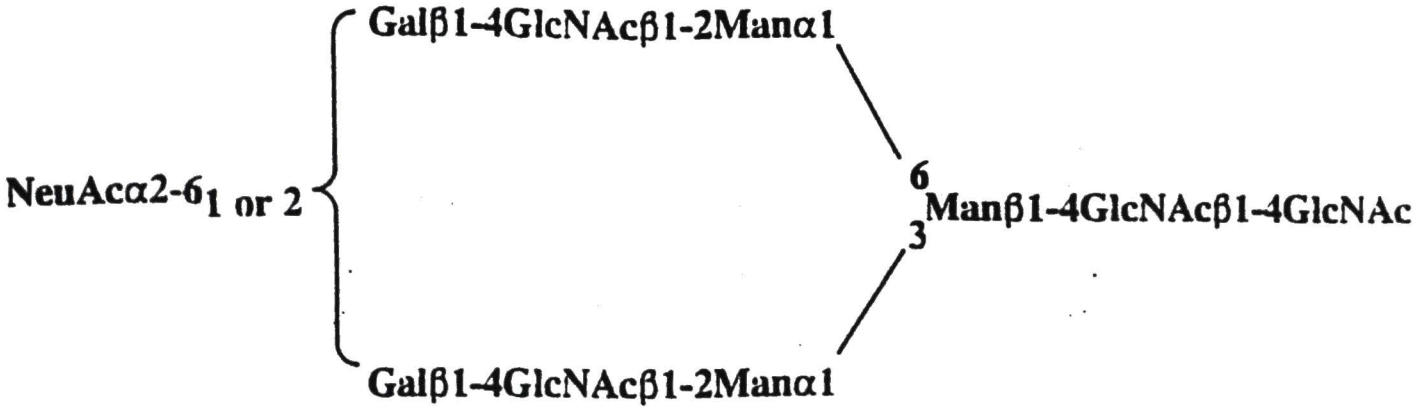
However, our laboratory produces large amounts of the sialylated and desialylated forms of the recombinant human LCAT from baby hamster kidney (BHK) cells. The isolation and purification of the sialylated and desialylated forms of the recombinant LCAT are described under materials and methods. The native human LCAT is purified from cryo-precipitated human plasma by four column chromatography steps as described in materials and method.

Before recombinant LCAT could be used as an appropriate model for elucidating the three dimensional structure of LCAT, it was necessary to establish the fact that the structure and function of the recombinant LCAT were sufficiently similar to native human LCAT. Although it had been confirmed that the recombinant human sialylated LCAT (r-LCAT) resembled the native human LCAT in its basic physical and chemical properties, our laboratory (35) have shown that the sialylated and desialylated recombinant human LCAT and the native human LCAT differed in the structure of the N-Glycan oligosaccharide component as shown in figures 4A and 4B.

By FAB-MS and GC-MS analyses the native human LCAT has a biantennary mono and disialyl N-glycan structure. The desialylated

Figure 4A on page 15 shows the structure of N-glycans isolated from human plasma Lecithin cholesterol acyltransferase (p-LCAT). The p-LCAT has a biantennary mono and disialyl N-glycan structure.

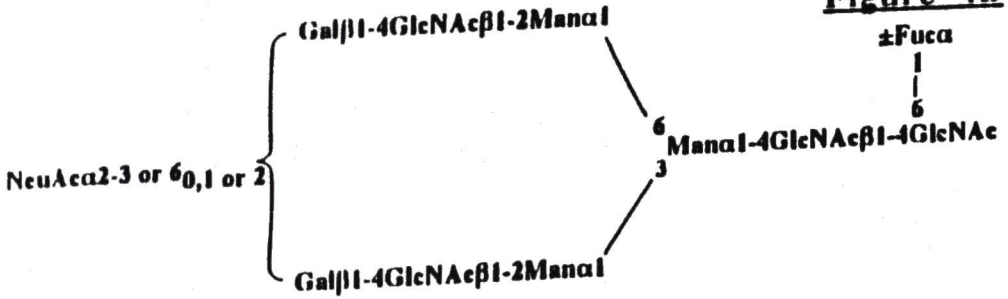
Figure 4A

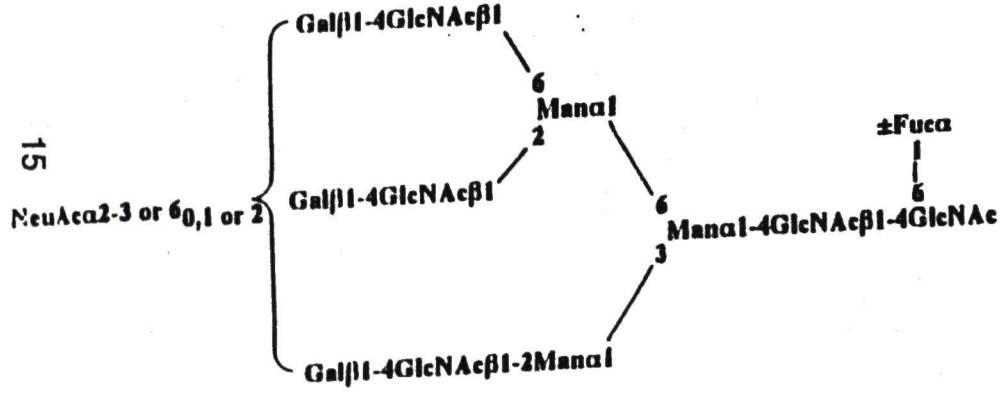


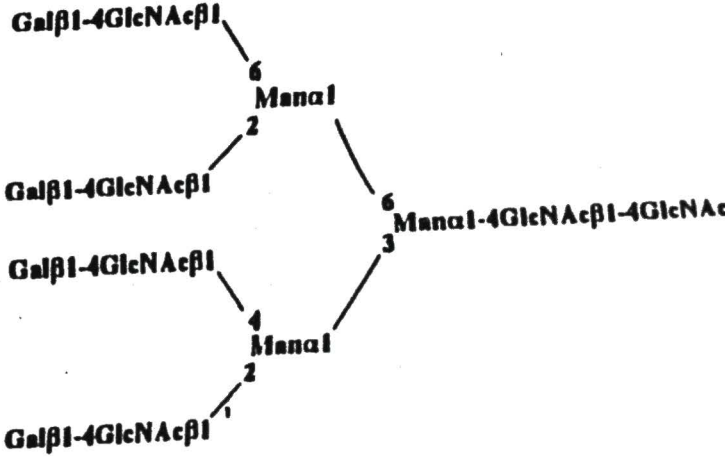
N-Glycans of human plasma LCAT (p-LCAT)

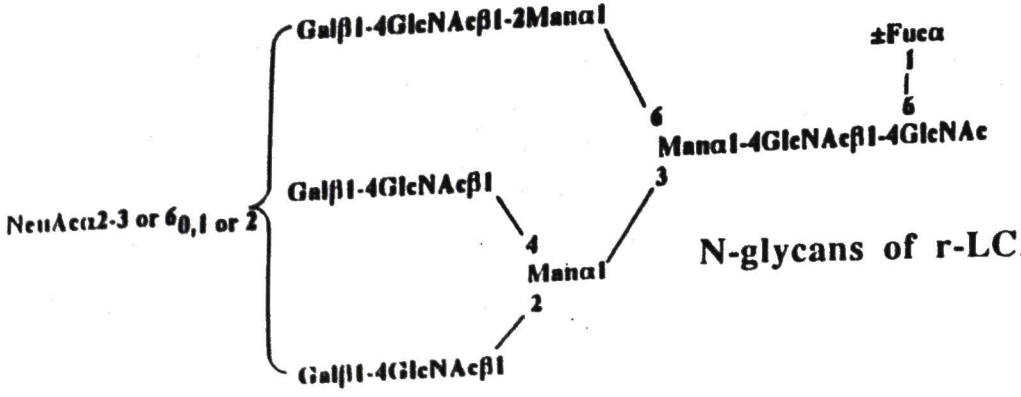
Figure 4B on page 17 shows the structures of N-glycans of sialylated recombinant LCAT (r-LCAT) and that of desialylated recombinant LCAT (d-LCAT). The d-LCAT has a biantennary and triantennary N-glycan structure with and without the core fucose but lacks the terminal sialic acid residues. The N-glycan structure of r-LCAT is very similar to d-LCAT but contains the terminal sialic acid residues in their glycan structure.

Figure 4B









N-glycans of r-LCAT and d-LCAT

recombinant human LCAT (d-LCAT) has a biantennary and triantennary N-glycan structure with and without the core fucose but lacks the terminal sialic acid residues. The N-glycan structure of r-LCAT is very similar to d-LCAT but contains the terminal sialic acid residues in their glycan structure.

The Km and Vmax values for both the native human LCAT and the sialylated human recombinant LCAT are essentially the same. The Km for the d-LCAT is 2-3 times higher than the native human LCAT which is sialylated. Prelimnary comparitive studies of the C-terminal of native p-LCAT, r-LCAT and d-LCAT with carboxypeptidase P did not show any differences in the C-terminal region of these three enzymes, although proline could not be analysed.

Purpose of the Study

The purpose of the present research is to compare the three forms of LCAT in their native and denatured states to determine whether the structural differences in the N-glycan structure of the oligosaccharide component, of the carbohydrate portion of the LCAT and the presence or absence of the terminal sialic acid residues in LCAT would have any influence on the conformational properties of the enzyme. Stability and catalytic activity of the native p-LCAT, r-LCAT and d-LCAT were also monitored. Hence it was also of interest to compare the intrinsic tryptophan emission fluorescence and circular dichroism spectra of the three forms of

the enzyme under native and denaturing experimental conditions. The two denaturing agents used in this study are guanidine hydrochloride and heat.

CHAPTER II

MATERIALS AND METHODS

Materials and Chemicals

Human plasma LCAT (p-LCAT) was isolated and purified from the supenatant of cryoprecipitated human plasma supplied by Carter blood Center, Fort Worth. Recombinant LCAT (r-LCAT) was isolated and purified from the cell culure supernatants of baby hamster kidney cells. Purified r-LCAT was treated with neuraminidase to produce d-LCAT. Neuraminidase and N-Acetyl neuraminic acid were purchased from Boehringer-Mannheim Co, Indianapolis, Indiana. Molecular weight standards for gel electrophoresis was from Pharmacia fine chemicals, Piscataway, New Jersey, while Acrylamide, bisacrylamide, sodium dodecyl sulfate (SDS), ammonium persulfate, beta-mercaptoethanol, glycine, TEMED, Tris base and Coomasie Brilliant Blue G-250 were from Bio-Rad Laboratories, Richmond, California. Bovine serum albumin, and phosphatidyl choline was from Sigma, ultra pure guanidine hydrochloride was from USB, sodium phosphate was from MCB reagents, and [7a-^{3}H] Cholesterol from ICN Biomedicals Inc., Radiochemicals division, Irvine , California.

Methods

Purification of p-LCAT, r-LCAT and d-LCAT

The purification of p-LCAT involved overnight precipitation of one liter of cryoprecipitated plasma with 50 % polyethylene glycol (final concentration of PEG 7%), followed by a combination of phenyl sepharose, Affigel blue and Heparin sepharose column chromatography (36, 37). The subsequent steps involved DEAE-agarose chromatography (38), DEAE-Matrex chromatography (37), a second affigel blue chromatography and hydroxyapatite chromatography (37). The LCAT fractions pooled from hydroxylapatite chromatography gave a single band on SDS-PAGE when stained with Coomasie blue. The sialic acid content of these preparations estimated (39) gave a mean value of about 5.3% .

The BHK cells into which human LCAT c-DNA is stably transfected constitutively secretes r-LCAT (40). The cells were incubated with Cultisphere-G beads in DMEM for 24 hours to permit the attachment of the cells to the beads. After 96 hours, the medium was changed to Opti-MEM for 48 hours. to facilitate the secretion of r-LCAT by the cells.

The purification of r-LCAT was described previously (40). r-LCAT thus isolated and purified by phenyl sepharose column chromatography gave a single band on SDS-PAGE . The mean value of sialic acid content (39) of r-LCAT was 5.30% (triplicates). r-LCAT was desialylated by incubating with neuraminidase *Clostridium. perfringens* for 6 hours at room temperature.

The weight ratio of neuraminidase to r-LCAT was set at 0.1 (26). The desialylated enzyme.(d-LCAT) was purified on a small phenyl sepharose column as described above and was essentially free from sialic acids.

Enzyme assays

The exogenous LCAT substrate (single bilayer non sonicated liposomes) were prepared according to a modification of the method of Batzri and Korn (41). 150 µl of 5 % bovine serum albumin (BSA) and 50 µl of substrate containing [^{3}H] cholesterol were incubated with 10 µl of enzyme aliquots in duplicate for 24 hours at 37^{0}C. The esterification reaction was terminated by addition of 2 ml isopropanol. The lipids were extracted and evaporated under a stream of nitrogen. The dried lipid mixture were redissolved in 60 µl of $CHCl_3$ and spotted on the silica gel coated plastic sheets used for thin layer chromatography (TLC). The solvent used for TLC to separate the free cholesterol and cholesteryl esters was petroleum ether, diethyl ether and acetic acid mixed (90: 10: 1; v/v). The separated lipid bands were visualized by iodine vapors. The zones corresponding to free cholesterol and esterified cholesterol (CE) were cut out and placed in a vial containing scintillation fluid for counting the radioactivity of the sample (42). The radioactivity in cpm corresponding to free cholesterol and esterified cholesterol were separately counted on a scintillation counter. The % of esterified cholesterol (% LCAT activity) was calculated as follows:

$$\% \text{ esterified cholesterol} = \frac{\text{cpm of CE X100}}{\text{cpm of TC (cpm)/10µl of enzyme/24 hr.)}}$$

where TC corresponded to the radioactivity of total cholesterol, TC is the summation of radioactivity of esterified cholesterol (CE) and free cholesterol. The time of incubation was selected to yield linear rates. There was less than 5 % average error between the duplicate assays. The enzyme units were expressed as % cholesterol esterified per 10 µl for 24 hours.

Activity Studies

Four sets, each having 200 µl of r-LCAT (261.8 µg/ml) in 10 mM PO_4 buffer, pH 7.4 were incubated with (sample) and without (control) 3 M and 4 M guanidine hydrochloride for 2 hours. Two sets each containing 125 µl of r-LCAT in 10 mM PO_4 buffer were also incubated with and without 6 M guanidine hydrochloride for 2 hours. The final volume of the incubation mixture was 500 µl. At 0, 15, 30, 60, 90 and 120 minutes after treating with guanidine hydrochloride, 25µl aliquots from the corresponding samples and controls were diluted in 10 mM PO_4 buffer, pH 7.4 to lower the concentration of guanidine hydrochloride from 6M to 0.075M. 5 µl of diluted enzyme samples were incubated with 200 µl of substrate in the presence of 150 µl of 5% BSA for 6 hours.

To determine the concentration of guanidine hydrochloride that had no effect on LCAT activity, five replicate assays were carried out. 50µl

aliquots of r-LCAT (261.8 μg/ml) were mixed with 150 μl of 10 mM PO_4 buffer, pH 7.4 . For the controls, 10μl of enzyme was diluted to 100μl with 10 mM PO_4 buffer, pH 7.4 . For the samples, 10μl of enzyme was diluted with 10 mM PO_4 buffer, pH 7.4 containing 0.6 M guanidine hydrochloride. 10 μl aliquots of diluted enzyme from the control and from the sample were incubated with 50 μl of substrate and 150 μl of 5% BSA to determine the residual enzyme activity. For the sample, the final concentration of guanidine hydrochloride was 0.03M during incubation with substrate. The mean activity (based on 5 replicate assays) in the absence of 0.03M guanidine hydrochloride for r-LCAT was 5.1 ± 0.56 units and 5.4 ± 0.76% units in the presence of 0.03 M guanidine hydrochloride respectively.

The activities of p-LCAT, r-LCAT and d-LCAT were standardized by dilution with 10 mM PO_4 buffer, pH 7.4 so that they each contained the same number of enzyme units. (The activity and protein concentration per 50 μl of p-LCAT, r-LCAT and d-LCAT were 41 %, 45 % and 56 % and 1.7μg, 13.1 μg and 13.7μg respectively).

To monitor the activity changes on denaturation with 6 M guanidine hydrochloride, 50 μl of standardized enzymes (p-LCAT, r-LCAT and d-LCAT) were incubated with 150 μl of 10 mM PO_4 buffer, pH 7.4 that had 8 M guanidine hydrochloride in the sample. The controls had the same amount of enzyme in the same amount of buffer that did not include guanidine hydrochloride. At 0, 5, 10, 15, 30 , 60, 90 and 120 minutes after addition of 6 M guanidine hydrochloride, 10 μl aliquots were withdrawn (in duplicates) and

diluted to 100µl with 10 mM PO_4 buffer, pH 7.4 to lower the guandine hydrochloride concentration from 6 M to 0.6 M. The control and samples were incubated in duplicates of 10 µl of diluted enzyme and 50 µl of substrate plus 150µl of 5% BSA. The final concentration of guanidine hydrochloride in the assay mix was 0.03 M.

To select the temperature at which maximum thermal denaturation occurred and monitor the activity changes during thermal denaturation, 160 µls of the three forms of standardized enzyme samples were heated at 45^0C, 50^0C and 55^0C, respectively for a period of 1 hour (sample). In the controls 160 µl of the corresponding enzyme species were incubated at 37^0C for 1 hour. Aliquots of 10 µl of enzyme were withdrawn at 0, 5, 10, 15, 20, 30 and 60 minutes from the sample and control (duplicates) and incubated with 50 ml of substrate and 150 ml of 5% BSA to test the residual activity.

Circular Dichroism analyses of native and denatured enzyme species in the far u.v. region

The protein concentration for the native and denatured enzyme species were set to 56 µg/ml. The native and thermally denatured enzyme species were dissolved in 1 mM PO_4 buffer, pH 7.4. The chemically denatured enzyme species were in 1 mM PO_4 buffer that had 6 M guanidine hydrochloride. The enzymes samples were heated at 55^0C for 15, 30, 60 and 120 minutes respectively and subjected to CD analyses to monitor the conformational changes. Simultaneously, 10 µl aliquots (in duplicates) of

native and thermally denatured enzyme species were used for LCAT assay to monitor the residual LCAT activity.

For renaturation studies, the enzyme samples were thermally denatured for 2 hours at 55^0C. Subsequently, the denatured enzymes were cooled on ice for about 30 minutes and incubated at 37^0C for 2 hours to allow the enzyme to renature. At the end of 2 hours, 10µl of renatured enzyme were used for LCAT assay and the CD spectra of the renatured enzyme were recorded.

For guanidine hydrochloride denaturation, the enzyme samples were incubated with 1 mM PO_4 buffer containing 6 M guanidine hydrochloride for 15, 30, 60 and 120 minutes respectively. At the specified time points the CD spectra of the denatured enzymes were recorded at 30^0C scanning the wavelength range 250 to 210 nm. For monitoring the activity changes at the specified time points, 10µl of denatured enzymes (in duplicates) originally in 6 M guanidine hydrochloride, were diluted to 100µl with 1 mM PO_4 buffer, pH 7.4 to lower the concentration of guanidine hydrochloride to 0.6 M. From these diluted enzyme samples, 10 µl aliquots of enzyme (duplicates) were assayed to estimate the residual LCAT activity.

For renaturation studies, the enzyme samples that were originally incubated with 6 M guanidine hydrochloride for 2 hours, were dialysed against 2000 ml of 1 mM PO_4 buffer, pH 7.4 for 3 hours, with a change at 1 hour. The renatured enzyme (10µl) was diluted to 100µl with 1 mM PO_4 buffer. The diluted renatured enzyme samples (10µl) were used for LCAT

assay to estimate the activity of the renatured enzyme. The CD spectra corresponding to the renatured enzyme could not be used for evaluating the secondary structure as even the trace amounts of guanidine hydrochloride present interfered with the accuracy of the protein determination; this parameter is crucial for an estimate of the secondary structure of the enzyme by PROSEC.

The CD spectra of the enzymes in their native and denatured states were recorded on an Aviv model 62 HDS spectropolarimeter, supplemented with a RC6 Lauda refrigerated circulating bath to maintain constant compartment temperature. The enzyme samples were placed in 0.1 cm quartz cuvettes, and the CD spectra were scanned and recorded from 250 to 190 nm, at intervals of 1nm and a dwell time of 3 seconds. Each spectrum was the average of 3 repetitions. The corrected spectra were obtained by subtracting the spectrum of the appropriate buffer blank. Ellipticity values recorded in millidegrees by the instrument were converted to molar ellipticity values by the equation:

$$\Theta = [\Theta]_{obs}./[10\{MRC\}l]$$

where $[\Theta]$ is the molar ellipticity in degrees $cm^2/dmol$, $[\Theta]_{obs.}$ is ellipticity recorded by the instrument in millidegrees, MRC is the mean residue concentration of the enzyme, equal to the number of the amino acid residue times the molar concentration of the protein and l is the pathlength in centimeters. The molar ellipticity values were processed by computer to determine protein secondary structural components using PROSEC (supplied

by Aviv). The program determines secondary structural components based on a binary search alogrithm using the reference spectra of Chang et al (43) and estimates alpha helix, beta sheet, beta turn and random coil directly from the instrument data (44). The alpha helical content can be alternatively estimated from $[\Theta_{222}]^{obs}$ using the following equation and poly-L-lysine basis spectra (45).

$$\text{Fraction alpha helix} = \frac{[\Theta_{222}]^{obs} - [\Theta_{222}]^{coil}}{[\Theta_{222}]^{helix} - [\Theta_{222}]^{coil}}$$

where $[\Theta_{222}]^{helix}$ is ellipticity in degrees cm^2/dmol for 0.01% poly-L-lysine, pH 11.1 , in a 1 mm cell and is equal to 35,700 ± 2,800. $[\Theta_{222}]^{coil}$ is assumed to be zero (45).

For the recording of spectra in the near u.v. region, between 250 to 300 nm, the protein concentration was set at 380 µg / ml. Spectra were recorded for the three forms of LCAT in their native and denatured states.

Intrinsic Tryptophan Emission Fluorescence Studies

The fluorescence spectra were recorded on a Schimadzu RF5000U spectrofluorometer that was equipped with water jacketed sample compartment to maintain the temperature of samples in 3 ml quartz cuvettes at 25^0C. The excitation wavelength was set to 280 nm. The wavelength of emission spectra to be recorded was set in the range between 290 and 400 nm. The excitation and emission slit widths were 5nm respectively.

The protein concentration for fluorescence studies was set to 20µgs per ml for all three forms of enzyme. Thermal denaturation was carried out by heating the enzymes at 55^0C for 2 hours. Aliquots of the denatured enzyme samples after heating at 55^0C for 30, 60 and 120 minutes were used to record the emission spectra of thermally denatured enzyme. After recording the spectra, 10 µl of enzyme aliquots from the samples corresponding to the native state and denatured state were used to monitor the changes in LCAT activity.

For monitoring the changes in intrinsic tryptophan fluorescence upon denaturation with 6 M guanidine hydrochloride, the protein concentration was set to 30µgs per ml in 10 mM PO_4 buffer, pH 7.4 for all the three forms of enzyme in their native and denatured states. The fluorescence emission spectra were recorded at time t=0 and t= 1 hour immediately after mixing with 6 M guanidine hydrochloride in 10 mM PO_4 buffer, pH 7.4 in the samples.

The corrected spectra were obtained by subtracting the spectra of the appropriate blanks. The emission maxima and relative fluorescence intensity values were directly obtained from the instrument using the built-in software program provided by Schimadzu.

CHAPTER III

RESULTS

Guanidine Hydrochloride Denaturation:

Comparison of changes in the activities of p-LCAT, r-LCAT and d-LCAT in the native and denatured state.

r-LCAT was incubated in the presence of 3M, 4M & 6M guanidine hydrochloride for 2 hrs at 30^0C. As seen in figure 5 there was only about a 50% reduction in LCAT activity suggesting that the enzyme was relatively resistant to denaturation by guanidine hydrochloride. The estimation of residual enzyme activity involved incubation of the diluted, denatured enzyme with substrate resulting in a guanidine hydrochloride concentration of 0.03 M for 24 hours. Thus the denatured enzyme might have regained its activity during the activity assay. Hence the residual activity of the denatured enzyme might be the net result of denaturation and subsequent renaturation. The enzyme units are defined under materials and method.

In order to compare the effect of guanidine hydrochloride on p-LCAT, r-LCAT and d-LCAT, the activities of the respective preparations were standardized by dilution with 10 mM PO_4 buffer, pH 7.4 so that they each contained the same number of enzyme units. The

Molar Concentration of guanidine hydrochloride versus residual activity of r-LCAT

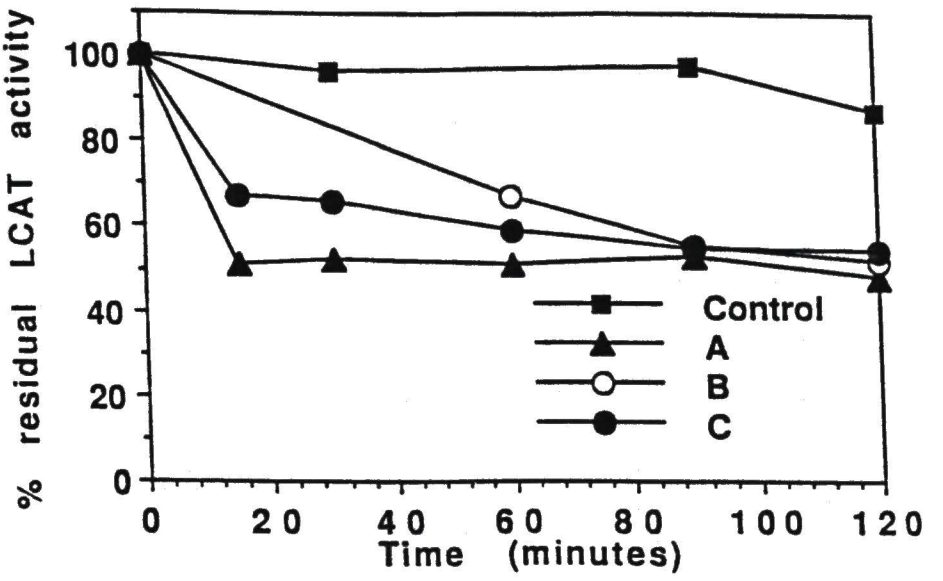


Figure-5

In figure 5 the enzyme r-LCAT in the control is in the native state. A, B and C represent the residual LCAT activities of denatured r-LCAT treated with 3 M, 4 M and 6 M guanidine hydrochloride respectively. The denatured enzyme samples were diluted to give a final concentration of guanidine hydrochloride of about 0.03 M while carrying out the enzyme assays.

activity of these three species of enzymes were determined as described above for r-LCAT (figure 6A & 6B). The residual activities of p-LCAT, r-LCAT and d-LCAT following 2 hour incubation with 6 M guanidine hydrochloride were 70%, 50% and 55% of the controls respectively. The intrinsic tryptophan emission fluorescence spectra and the circular dichroism spectra, used to monitor the structural and conformational changes accompanying the denaturation of these enzymes and the corresponding changes in their enzyme activities are shown in figure 8A, 8B and table-2.

Intrinsic tryptophan fluorescence emission spectra of p-LCAT, r-LCAT and d-LCAT in their native and denatured states

The spectral changes in the intrinsic fluorescence of tryptophan for the three species of enzyme in their native and denatured state were investigated. The wavelength of maximum fluorescence and the corresponding relative fluorescence intensities (RFI) for p-LCAT, r-LCAT and d-LCAT in the native state, were 342.2, 341 and 342 nm and 2.556, 1.464 and 1.891 respectively. The conformational change observed in the presence of 6 M guanidine hydrochloride was accompanied by a red shift in the emission maxima for all the three enzyme forms (p-LCAT, r-LCAT and d-LCAT) to 353.4, 352.9 and 351.4 nm respectively. The shift in emission maxima from 342 nm to 350 nm indicated that all three enzyme species were denatured by 6 M guanidine hydrochloride.

Comparison of activities of the enzyme species in their native state (without guanidine hydrochloride)

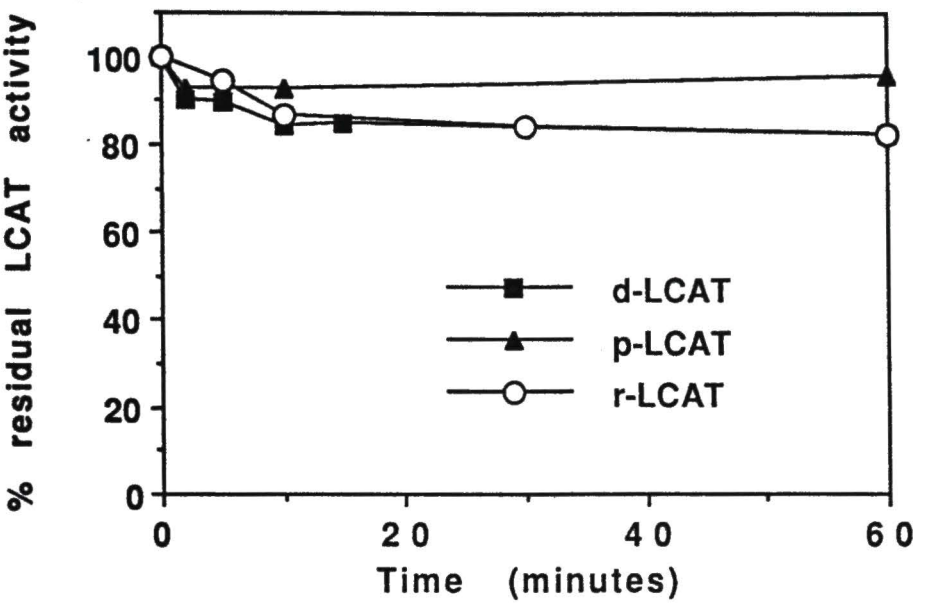


Figure 6A

Figure 6A compares the LCAT activity of r-LCAT, d-LCAT and p-LCAT in their native state. The enzymes were in 10 mM PO_4, pH 7.4 buffer.

Comparison of activities of the enzyme species denatured by 6 M guanidine hydrochloride

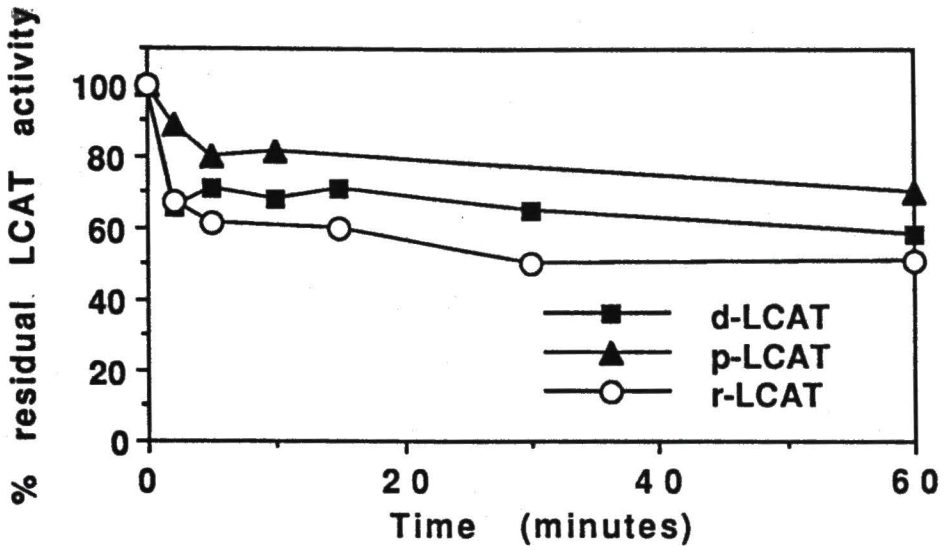


Figure 6B

Figure 6B compares the residual activities of the three forms of enzymes in their denatured states. The scale on X-axis represent the time at which the residual LCAT activity was determined after incubating the respective enzyme species with 10 mM PO_4 buffer, pH 7.4 containing 6 M guanidine hydrochloride.

The red shift in the emission maxima for p-LCAT was accompanied by a decrease in RFI to 94 % of the control, immediately following the addition of 6 M guanidine hydrochloride and to 87 % 1 hour hence. The red shift in emission maximum of the denatured r-LCAT was accompanied by a 27 % decrease in RFI, immediately following the addition of guanidine hydrochloride and in a subsequent increase in RFI to the original value during the 1 hour incubation period. For the d-LCAT, the red shift in emission maximum was accompanied by only a 1% increase in RFI, immediately following the addition of guanidine hydrochloride. No further change was observed during the subsequent 1 hour incubation period.

Circular dichroism (CD) spectroscopy was also used to monitor the conformational changes during denaturation.

Comparison of Circular dichroism spectra of native and denatured species of p-LCAT, r-LCAT and d-LCAT

The protein concentration of sialylated and desialylated r-LCAT and p-LCAT was set at 56 µgs / ml and the circular dichroism spectra for the three species of enzymes were scanned in the far u.v. region between 240 and 190 nm, in their native and denatured states. The % alpha helix calculated from $[\Theta_{222}]^{obs.}$ was used as an index to monitor the conformational changes accompanying the denaturation of the enzyme in the presence of 6 M guanidine hydrochloride.

The secondary structure of native p-LCAT, estimated by a computer program, designated as PROSEC, was 26 % alpha helix, 30 % beta pleated sheet, 10 % beta turns and 34 % of random coil. The alpha helical content calculated from $[\Theta_{222}]^{obs.}$ was 24 % for the p-LCAT in the native state. The CD spectra of the native p-LCAT was compared with that of denatured p-LCAT at 15, 30, 60 and 120 minutes following the addition of 6 M guanidine hydrochloride. The % alpha helix of p-LCAT decreased from 24 % to 5 % upon incubation with 6 M guanidine hydrochloride for two hours. In the native state (no guanidine hydrochloride) the alpha helical content of p-LCAT decreased from 24 % (t=0) to 21 % (t=2hrs.) at 30^0C.

The secondary structural components of r-LCAT in the native state, estimated by PROSEC, were 25 % alpha helix, 39 % beta pleated sheet, 11 % beta turns and 24 % of random coil structure. The alpha helical content, calculated from $[\Theta_{222}]^{obs.}$ was 21 %. The CD spectra of the native r-LCAT was compared with that of denatured r-LCAT recorded at 15, 30, 60 and 120 minutes following the addition of 6 M guanidine hydrochloride. The % alpha helix decreased from 21 % to 7 % upon incubation with 6 M guanidine hydrochloride for two hours. The alpha helix did not change during control incubations with buffer at 30^0C.

The secondary structure of the native d-LCAT estimated by PROSEC, was 24 % alpha helix, 55 % beta pleated sheet, 4 % beta turns and 18 % of random coil structure. The alpha helical content calculated from $[\Theta_{222}]^{obs.}$ was 19 % . The CD spectra of the native d-LCAT was

compared with that of the denatured enzyme at 15, 30, 60 and 120 minutes after the addition of 6 M guanidine hydrochloride. In the presence of guanidine hydrochloride, the % alpha helix decreased from 19 % to 4 % while the alpha helical content of d-LCAT did not change during control incubations in buffer.

Figure 7A and 7B shows the CD spectra of p-LCAT, r-LCAT and d-LCAT in their native and denatured states. The beta pleated sheet and that of beta turn structural component differed between the two (sialylated and desialylated) forms of recombinant LCAT. To verify this observation, the CD analyses were repeated on 8, 6 and 3 replicate samples of desialylated d-LCAT, r-LCAT and p-LCAT respectively (protein concentration was adjusted to 56µgs per ml for all samples). .The statistical analyses (t-test) of the data shown in table-1 validated the originally observed differences in the beta pleated sheet and that of beta turn structures for d-LCAT, r-LCAT and p-LCAT.

The changes in the activity of the respective enzyme forms were correlated with the conformational changes as indicated by CD analysis. Figure 8A shows the changes in alpha helical content for the three enzyme forms on denaturation with 6 M guanidine hydrochloride. Figure 8B shows the changes in residual activities of the denatured enzyme species and their conformational changes by CD. Table-2 shows the conformational and activity changes for p-LCAT, r-LCAT and d-LCAT on denaturation with 6 M guanidine hydrochloride.

Comparison of circular dichroism spectra of enzyme species in their native state

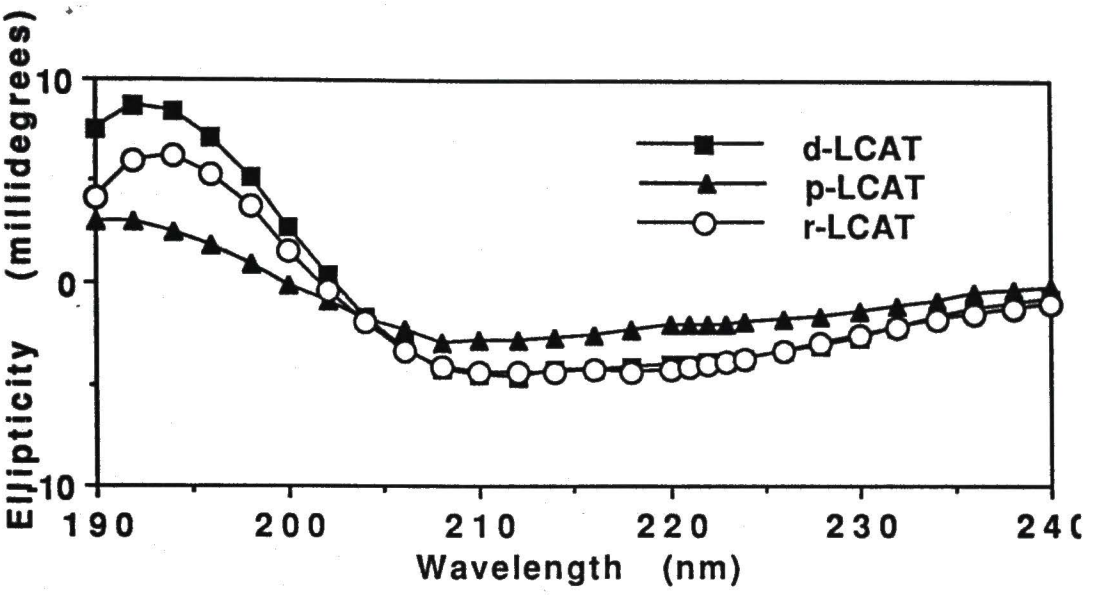


Figure 7A

Figure 7A compares the circular dichroism spectra of p-LCAT, r-LCAT and d-LCAT in their native state recorded at 30^0C in the far u.v. region. The protein concentration was set at 56 micrograms per ml.The enzymes were in 1mM PO_4 buffer pH 7.4.

Comparison of circular dichroism spectra of enzyme species denatured by 6 M guanidine hydrochloride.

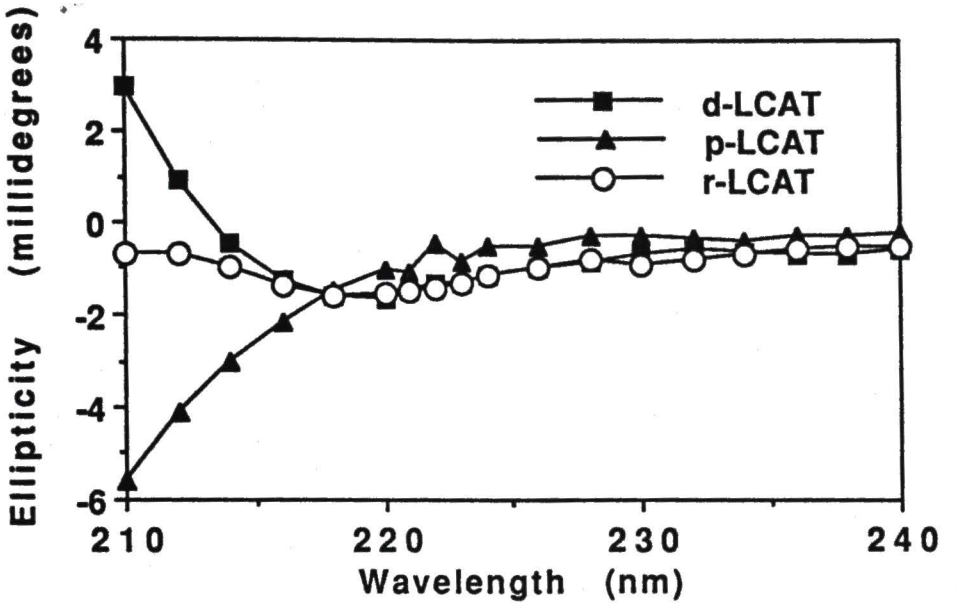


Figure 7B

Figure 7B compares the circular dichroism spectra of p-LCAT, r-LCAT and d-LCAT in their denatured state recorded at time two hours after denaturation with 6M guanidine hydrochloride at 30^0C in the far u.v.region. The protein concentration was set to 56 micrograms per ml. The enzymes were in 1mM PO_4 buffer pH 7.4 that included 6M guanidine hydrochloride.

Comparison of secondary structure of p-LCAT, r-LCAT and d-LCAT

Table-1

Type of enzyme	%alpha helix (O222)	%alpha helix (Prosec)	% beta sheet	%beta turn	%random coil
p-LCAT(3)	24%	27±1.0%	25±3.8%	14±3%	34%
r-LCAT(6)	21%	24±2.9%	39±4.3%*	11±1.6%	26%
d-LCAT(8)	19%	22±1.6%	59±4.4%**	1±1.57%**	18%

* $p \leq 0.005$ **compared to p-LCAT for beta sheet only.**

** $p \leq 0.0005$ **compared to r-LCAT for beta sheet and beta turn**

Table 1 compares the mean values of secondary structural components of p-LCAT, r-LCAT and d-LCAT in their native state. The protein concentration was set at 56 micrograms per ml and the CD spectra was scanned from 240 nm to 190 nm in 1mM PO_4 buffer, pH 7.4. The p values were estimated from the t-test analyses of beta sheet and beta turn secondary structural components of r-LCAT and d-LCAT.

Comparison of alpha helical content of enzyme species denatured with 6M guanidine hydrochloride

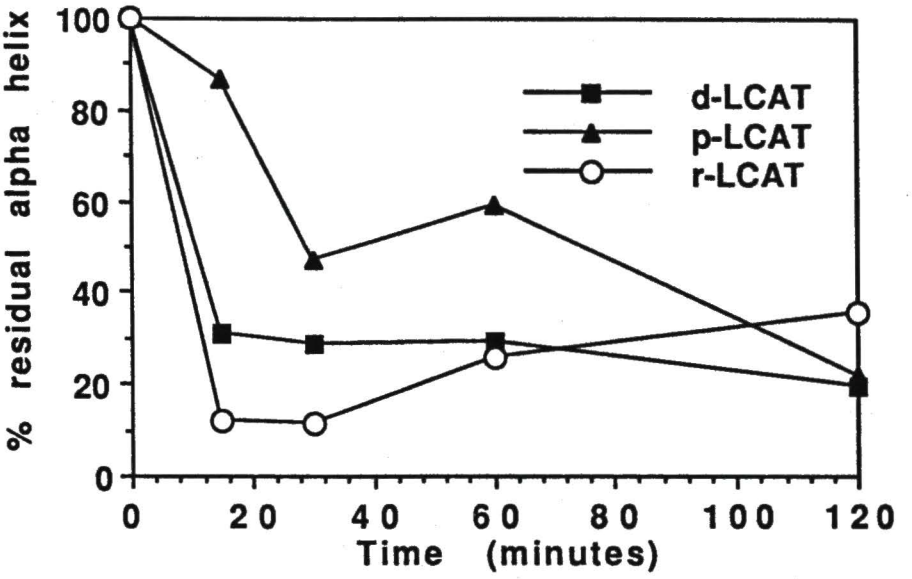


Figure 8A

The changes in the alpha helical content of p-LCAT, r-LCAT and d-LCAT are shown in figure 9A. The circular dichroism spectra of the denatured enzyme species were recorded at 30^0C at 15, 30, 60 and 120 minutes after incubating with 6M guanidine hydrochloride.

Comparison of residual activities of denatured enzyme species

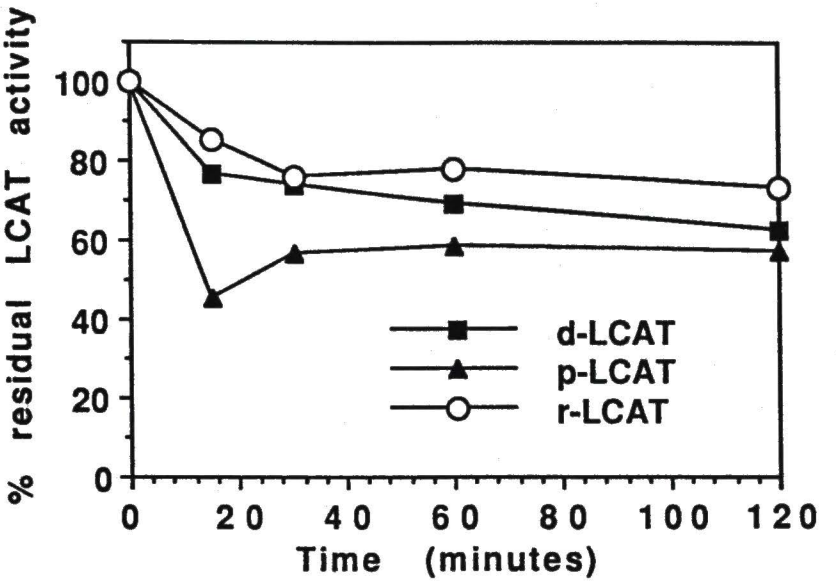


Figure 8B

The changes in the LCAT activity of denatured (p-LCAT, r-LCAT and d-LCAT) as shown in figure 8B were estimated at 15, 30, 60 and 120 minutes after incubating with 6 M guanidine hydrochloride.

Comparison of conformational and activity changes of p-LCAT, r-LCAT and d-LCAT on denaturation with 6 M guanidine hydrochloride

Table 2

The % alpha helix calculated from the observed molar ellipticity corresponding to wavelength 222 nm was used as an index to monitor the conformational changes accompanying denaturation. For guanidine hydrochloride denaturation, the enzyme samples were incubated with 1mM PO_4 buffer containg 6M guanidine hydrochloride for 15, 30, 60 and 120 minutes respectively. At the specified time points the CD spectra of the denatured enzymes were recorded at 30^0C scanning the wavelength range 250 to 210 nm. For monitoring the activity changes at the specified time points, 10µl of denatured enzymes (in duplicates) originally in 6M guanidine hydrochloride were diluted to 100 µl with 1mM PO_4 buffer, pH 7.4 to lower the concentration of guanidine hydrochloride to 0.6 M. From these diluted enzyme samples, 10 µl aliquots of enzyme (duplicates) were assayed to estimate the residual LCAT activity.

Table-2

Time (minutes)	% alpha helix	% residual alpha helix	% residua LCAT activity
		p-LCAT	
0	24 %	100 %	100 %
15	20 %	87 %	45 %
30	11 %	47 %	56 %
60	14 %	59 %	59 %
120	5 %	22 %	57 %
		r-LCAT	
0	21 %	100 %	100 %
15	3 %	12 %	85 %
30	2 %	11 %	76 %
60	5 %	26 %	78 %
120	7 %	35 %	73 %
		d-LCAT	
0	19 %	100 %	100 %
15	6 %	31 %	77 %
30	5 %	29 %	74 %
60	6 %	29 %	69 %
120	4 %	20 %	63 %

When the residual activities of the three enzyme species in their denatured states were compared, the activities of sialylated and desialylated recombinant LCAT were reduced to 73 % and 63 % of their respective original values. The activity of p-LCAT dropped to 57 % of the control on denaturation. The correlation between the % residual alpha helical content and the % residual LCAT activity gave a positive correlation of 0.50 for p-LCAT, 0.81 for r-LCAT and 0.97 for d-LCAT upon denaturation with 6 M guanidine hydrochloride.

The renaturation of the dialysed denatured (6 M guanidine hydrochloride treated) enzyme species could not be monitored by CD as the trace amount of guanidine hydrochloride present in the enzyme dialysate interfered with protein estimation which was crucial to estimate the secondary structure.

The removal of guanidine hydrochloride from the denatured r-LCAT and d-LCAT by dialysis for 4 hours, restored the activities of the apparently renatured enzymes to 91 % and 92 % of their original values. On the other hand, the activity of renatured p-LCAT was only 35 % of the native enzyme.

The CD spectra of sialylated and desialylated recombinant LCAT in the native and denatured states were scanned in the near u.v. region (250 to 300 nm). The CD spectra of the native r-LCAT differed from that of the d-LCAT in the region between 270 to 300 nm. In the native state, the r-LCAT exhibited a number of positive CD bands that are generally

attributed to tryptophan residues in the region between 290 and 300 nm (46). On the other hand, d-LCAT exhibited negative CD bands in the same region that are generally associated with tyrosine residues (46). The CD spectra of denatured r-LCAT differed from that of the native enzyme species. The former exhibited negative CD bands between 290 and 300 nm and positive CD bands in the region between 260 to 270 nm, while the latter exhibited negative CD bands between 260 and 270 nm and positive CD bands between 290 and 300 nm (data not shown). The CD spectra of the denatured enzyme differed from that of native d-LCAT between 260 to 300 nm. The negative CD bands of native d-LCAT were less prominent than those of the denatured d-LCAT in this entire wavelength range. The CD spectra of denatured r-LCAT differed from that of denatured d-LCAT in the entire scanning region. In the native state, the CD spectrum of p-LCAT was quite similar to that of sialylated recombinant LCAT between 275 and 290 nm but it differed in the region between 260 to 270 nm and also between 290 to 300 nm. The spectrum of denatured p-LCAT was similar to the denatured r-LCAT between 260-300 nm while it differed from the spectra of denatured d-LCAT in the entire spectral range. These data suggested that the configuration and the environment of the aromatic amino acid residues may be altered upon desialylation of r-LCAT.

Thermal Denaturation

Enzymatic activities of p-LCAT, r-LCAT and d-LCAT in their native and denatured states.

The changes in LCAT activity were monitored at 5, 10, 15, 20, 30 and 60 minutes for all the three species of enzymes during incubations at 37^0, 45^0, 50^0 and 55^0C. The activity of p-LCAT decreased to 61 % of the initial value at 55^0C, to 65 % at 50^0C and to 67.0 % at 45^0C. The activity of r-LCAT decreased to 30 % of the initial value at 55^0C, to 62 % at 50^0C and to 57 % at 45^0C. The activity of d-LCAT decreased to 40 % of the original value at 55^0C and to 57 % at 50^0C. The activities of p-LCAT, r-LCAT and d-LCAT decreased respectively to 91 %, 88 % and 77 % of the 0 time value during the two hour control incubations at 37^0C. Thus the d-LCAT was somewhat more unstable and perhaps more susceptible to spontaneous denaturation, than the other two enzyme forms (figure 9A &9B). To correlate the changes in enzyme activity with structural and conformational changes during thermal denaturation, all three species of enzymes were subjected to analysis by CD and fluorescence spectroscopy .

Comparison of Intrinsic tryptophan fluorescence emission spectra of p-LCAT, r-LCAT and d-LCAT in their native and denatured states.

The changes in the intrinsic tryptophan emission fluorescence spectra were monitored for the thermally denatured p-LCAT, r-LCAT and d-LCAT, heated to 55^0C for 30, 60 and 120 minutes. There were only very

Comparison of activities of native enzyme species at 37^0C

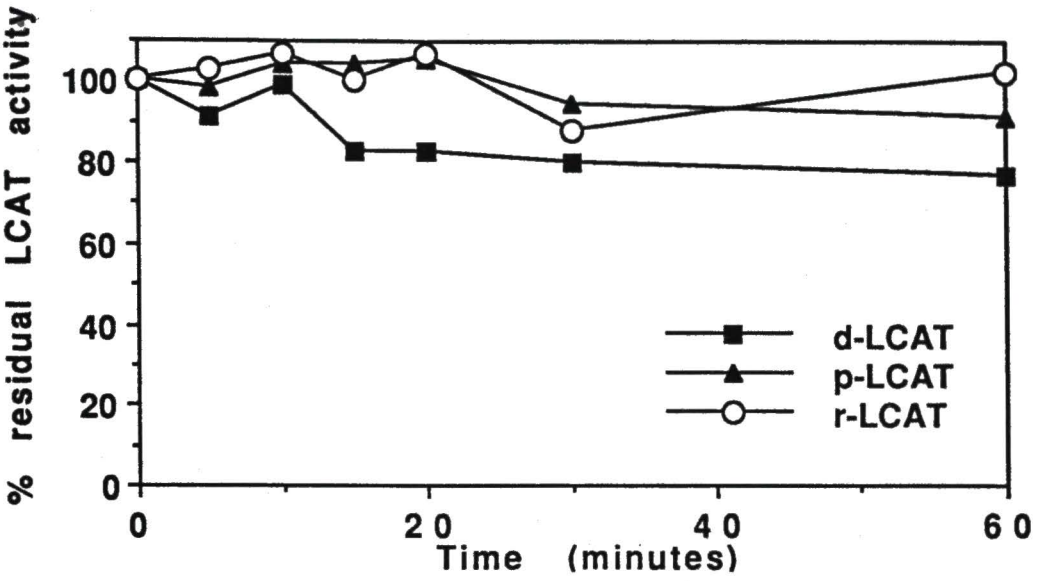


Figure 9A

Figure 9A compares the LCAT activity of p-LCAT, r-LCAT and d-LCAT in their native state at 37^0C. They represent the controls for the thermally denatured enzyme species heated at 55^0C shown in figure 9B. The assay was performed at the time interval of 5, 10, 15, 20, 30 & 60 minutes.

Comparison of activities of thermally denatured enzyme species at 55^0C

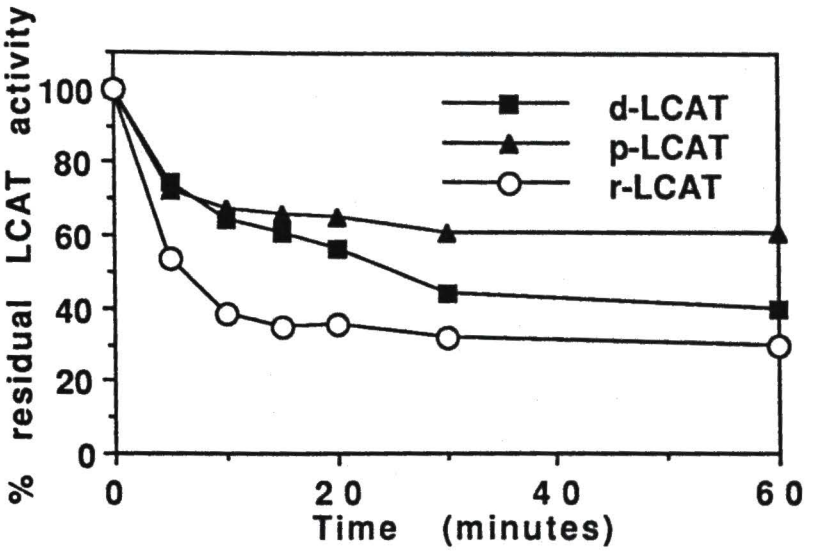


Figure 9B

Figure 9B compares the LCAT activity of thermally denatured p-LCAT, r-LCAT and d-LCAT that were heated to 55^0C. The assay was performed at the time interval of 5, 10, 15, 20, 30 & 60 minutes.

minor shifts in the wavelength of maximum fluorescence between the native and thermally denatured p-LCAT, r-LCAT and d-LCAT. The wavelength of maximum fluorescence was approximately 340 nm for all the three enzyme species in the native state. The RFI were 2.52, 3.22 and 3.35 respectively for desialylated and sialylated recombinant LCAT and p-LCAT in their native states. Upon thermal denaturation the emission maxima increased only slightly (~ 1 nm) for all the three enzyme species. However, an increase in the RFI was observed for all the three enzyme forms upon thermal denaturation. In the denatured state, the RFI increased by 5% for p-LCAT, 12 % for the r-LCAT and 51 % for the d-LCAT. At the same time, enzyme activity was reduced to 37 % of the control for p-LCAT, 33 % for r-LCAT and 26 % for the d-LCAT.

Circular dichroism spectra of p-LCAT, r-LCAT and d-LCAT in their native and denatured states.

The protein concentration of sialylated and desialylated recombinant LCAT and p-LCAT were set to 56µgs / ml and the circular dichroism spectra for the three species of enzymes in their native and denatured states were scanned in the far u.v. region between 240 and 190 nm. The % alpha helix calculated from $[\Theta_{222}]$obs. was used as an index to monitor the structural and conformational changes accompanying the thermal denaturation of the enzyme at 55^0C for 15, 30, 60 and 120 minutes.

The % alpha helix for the native r-LCAT was 21 %. For the

thermally denatured enzyme, it was reduced to 13 % which was equivalent to 62 % of the original alpha helix content of the native enzyme. The % alpha helix in the native d-LCAT was estimated to be 20 % which decreased to 16 % equivalent to 80 % of the original value upon heat denaturation. The % alpha helix calculated for the native p-LCAT was 24 % which decreased to 9 % that was equal to 36 % of the helix content of the native p-LCAT upon heat denaturation. Figure 10A &10B shows the comparison of the CD spectra of the three forms of enzymes in their native and thermally denatured states.

Figure 11A shows the changes in the alpha helical content of the three thermally denatured enzyme species. Figure 11B shows the residual activities of thermally denatured enzyme species. Table-3 shows the conformational and activity changes for all the three enzyme species in their denatured state. The correlation between the % residual alpha helical content and the % residual LCAT activity gave a positive value of 0.85 for p-LCAT, 0.71 for r-LCAT and 0.96 for d-LCAT on thermal denaturation at 55^0C.

The thermally denatured enzyme species were renatured as described in materials and method. The CD spectra of the renatured enzyme samples were recorded following incubation at 37^0C for 2 hours. The mean value of the alpha helix in the renatured enzyme was 19 % (equivalent to 90 % of the alpha helix in the native r-LCAT). This value was restored to 23 %, equivalent to 114 % of the original alpha helix present in d-LCAT. The

Comparison of circular dichroism spectra of native enzyme species at 37^0C

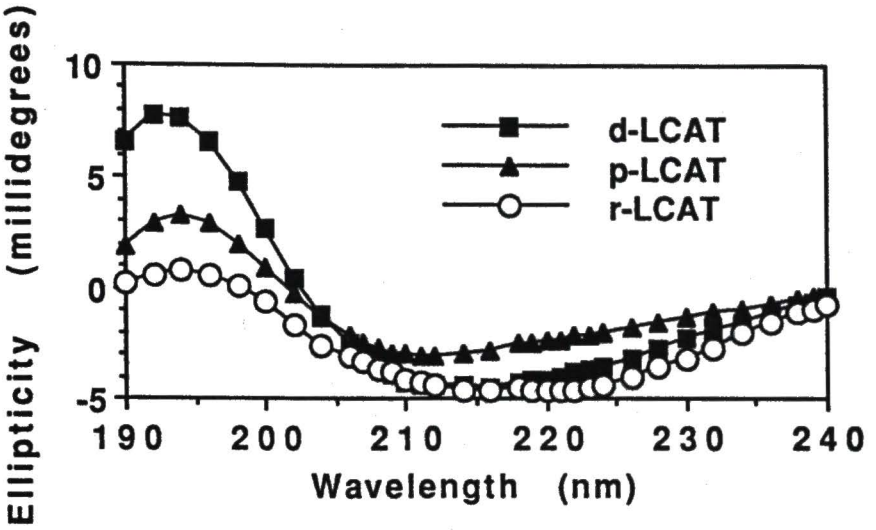


Figure 10A

Figure 10A compares the circular dichroism spectra of p-LCAT, r-LCAT and d-LCAT in their native state recorded at 37^0C in the far u.v.region. The protein concentration was set to 56 micrograms per ml. The enzymes were in 1mM PO_4 buffer pH 7.4.

Comparison of circular dichroism spectra of thermally denatured enzyme species heated at 55^0C for two hours

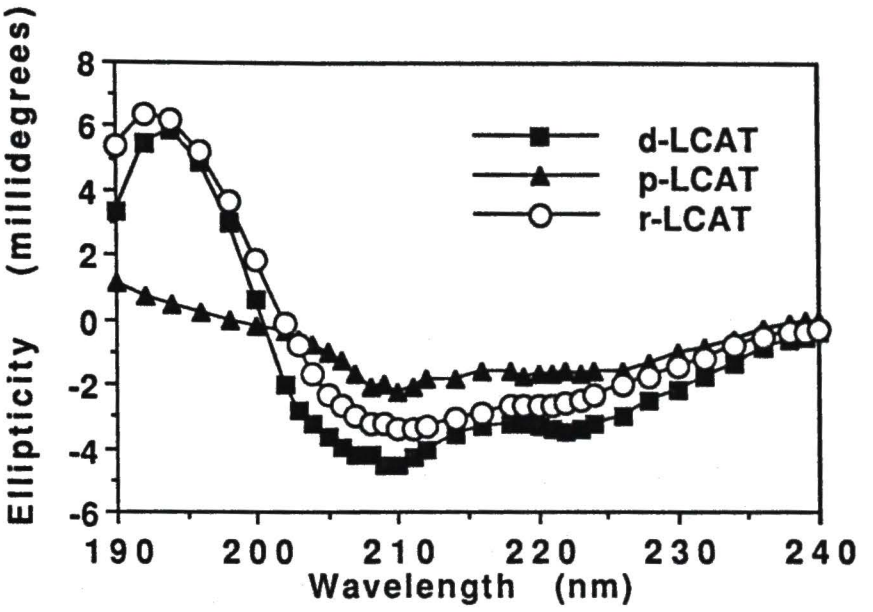


Figure 10B

Figure 10B compares the circular dichroism spectra of p-LCAT, r-LCAT and d-LCAT in their denatured state recorded in the far u.v. region at time two hours after thermal denaturation by heating the enzyme species at 55^0C. The protein concentration was set at 56 micrograms per ml. The enzymes were in 1mM PO_4 buffer pH 7.4

Comparison of changes in alpha helical content of thermally denatured enzyme species

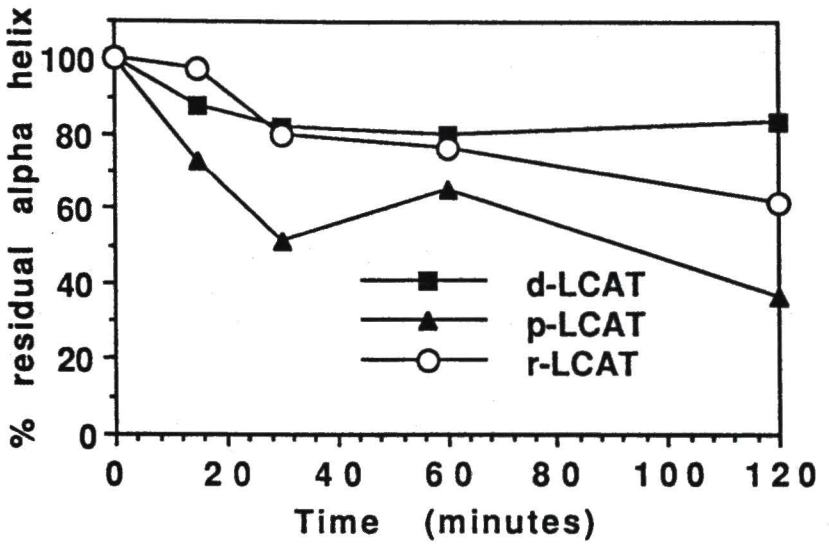


Figure 11A

Figure 11A compares the residual alpha helical content of the thermally denatured enzyme species heated at 55^0C. The circular dichroism spectra of the denatured enzyme species were recorded at15, 30, 60 and 120 minutes after heating at 55^0C

Comparison of residual LCAT activities of thermally denatured enzyme species

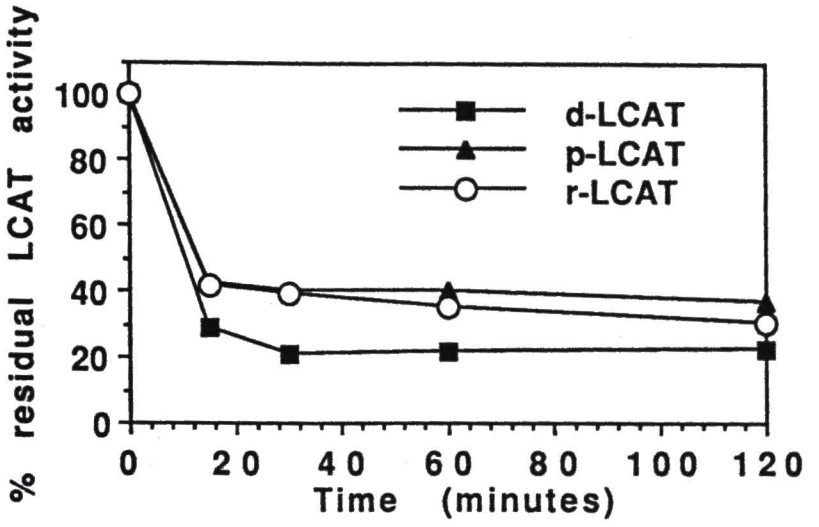


Figure 11B

Figure 11B compares the residual activities of p-LCAT, r-LCAT and d-LCAT that were thermally denatured at 55^0C.The residual activities of denatured enzyme species were estimated at the time interval of 15, 30, 60 and 120 minutes after heating at 55^0C.

Comparison of conformational and activity changes of thermally denatured p-LCAT, r-LCAT and d-LCAT

Table 3

The protein concentration for the native and denatured enzyme species were set to 56 μg/ ml. The native and thermally denatured enzyme species were dissolved in 1mM PO_4 buffer, pH 7.4. The enzyme samples were heated at 55^0C for 15, 30 and 120 minutes respectively and subjected to CD analyses to monitor the conformational changes. The spectra was scanned in the wavelength range 190 to 250 nm. Simultaneously, 10 μl aliquots (in duplicates) of native and thermally denatured enzyme species were used for LCAT assay to monitor the residual LCAT activity.

Table-3

Time (minutes)	% alpha helix	% residual alpha helix	% residual LCAT activity
		p-LCAT	
0	24 %	100 %	100 %
15	17 %	73 %	42 %
30	12 %	51 %	40 %
60	15 %	65 %	40 %
120	9 %	36 %	37%
		r-LCAT	
0	21 %	100 %	100 %
15	21 %	98 %	42 %
30	17 %	80 %	40 %
60	16 %	76 %	35 %
120	13 %	62 %	30 %
		d-LCAT	
0	20 %	100 %	100 %
15	18 %	87 %	29 %
30	16 %	82 %	21 %
60	16 %	80 %	22 %
120	17 %	83 %	22 %

p-LCAT did not regain its activity when renaturation was attempted. The mean value of the alpha helix present in the renatured enzyme was 8 % which accounted for only about 32 % of the helix originally present in the native p-LCAT. However, none of the three renatured enzyme species gained their activities comparable to their respective native states.

CHAPTER IV

DISCUSSION

The amino acid sequence of p-LCAT includes 19 tyrosine residues/mol, 19 phenylalanine residues/mol and 12 tryptophan residues/ mol (30). Earlier data from fluorescence spectroscopy indicated that about 60% of the tryptophan residues were exposed and accessible to the solvent in the native enzyme (21). Spectrophometric titration of the ionizable phenolic side chains of p-LCAT indicated that all the tyrosine residues were inaccessible to solvent at neutral pH but were exposed at higher pH with an apparent pK of 12 following a conformational change. The pH dependent conformational change was preceded by a large change in molar ellipticity at 222 nm at pH 11 (47). The CD spectra of p-LCAT recorded in the far u.v. region indicated that the secondary structure of the enzyme contained 24.0% alpha helix, 27% beta pleated sheet and 49 % remaining structure in1 mM PO_4 buffer, pH 7.2.(47). Raising the ionic strength was reported to have no effect on the overall conformation of the enzyme (47). Doi and Nishida reported that the secondary structure of the enzyme had 18% alpha helix, 53% beta sheet and 29 % disordered structure (26). They

also found that the binding of the enzyme to the lecithin substrate vesicles resulted in no change in secondary structure (26).

During this study, estimated by the computer program PROSEC (Protein secondary structure based on the CD data) , the secondary structure of p-LCAT in its native state had 26.0% alpha helix, 30.0% beta pleated sheet, 10.0% beta turn and 34.0% random coil; thus comparable to earlier published values (26, 47). The secondary structure of r-LCAT in its native state consisted of 25.0% alpha helix, 39.0% beta sheet, 11.00% beta turn and 24.0 % random coil indicating that the overall conformations of the recombinant and circulating enzyme species are quite similar. However, desialylation appears to result in some changes resulting in the secondary structural components of about 24.0 % alpha helix, 55.0 % beta pleated sheet , 4.0 % beta turns and 18.0 % random coil structure. The amount of alpha helix present is similar for all the three species of enzyme in their native states, as estimated by PROSEC and from $[\Theta_{222}]^{obs.}$. These studies indicate that the desialylation of recombinant LCAT results in an increase in the amount of beta pleated sheet structure and a corresponding decrease in the amount of beta turns in the native state as compared to p-LCAT and r-LCAT. Future studies utilizing a more powerful program, BLEND (combining pure secondary structural components to produce a synthetic spectrum using multiple regression analysis and the same standard reference spectra used in PROSEC.) and X-ray crystallography will allow

the verification of the present findings on the secondary structure of LCAT.

Comparison of activity changes following exposure to guanidine hydrochloride and thermal denaturation suggested that functionally p-LCAT was more resistant to thermal and guanidine hydrochloride denaturation than either r-LCAT and d-LCAT.

Because the activities of all three species of enzyme are not completely lost at 55^0C, it was considered that such changes in activity reflected only partial thermal denaturation. Secondly only minor changes in emission maxima and an increase in the relative fluorescence intensity were observed for all the three types of enzyme on thermal denaturation suggesting a heat induced conformational transition between the native and denatured state. These observations confirm that the thermal denaturation was partial.

Structurally, the alpha helix of d-LCAT was most stable to thermal denaturation while p-LCAT was least stable under these conditions. These findings indicated that the desialylation of LCAT could have stabilized the secondary structure of the enzyme. In addition, the tri and tetra antennary structures of the oligosaccharide components of r-LCAT and d-LCAT might be conferring more stability to their respective helices as compared to the biantennary structures of oligosaccharide components of p-LCAT (35). The apparent stabilization of alpha helical structures in d-LCAT was not accompanied by the preservation of enzymatic activity on thermal

denaturation and renaturation suggesting that at least some of the alpha helical structures may not have a major role in the maintenance of the active conformation of the enzyme.

Comparison of the conformational and activity changes for the three species of enzymes during thermal denaturation suggested the following. From figure 11A, 11B and table-3 it is apparent that the thermal denaturation of all the three forms of the enzyme consisted of a fast phase denaturation followed by a slow phase denaturation. The residual activities for all three species of enzymes were comparable on thermal denaturation at 55^0C for two hours. The residual activities of the renatured enzyme species suggested that subtle changes in the structural alignments of active conformation of the enzyme may have been enough to irreversibly inactivate all three forms of the enzyme Structurally, it appears that, the thermal denaturation is reversible for the two forms of recombinant LCAT (r-LCAT and d-LCAT) , while it is irreversible for the p-LCAT. These observations need to be verified by additional, more in depth studies. Functionally, the partial thermal denaturation was irreversible for all the three species of the enzyme. The observed conformational differences between p-LCAT and the two forms of recombinant LCAT to thermal denaturation and renaturation might be due to differences in the configuration and conformation of the oligosaccharide component of p-LCAT and r-LCAT. These components might be contributing

differentially to the conformational stability of the enzyme but not to the appropriate exposure of the active site to the substrate.

Comparison of the intrinsic tryptophan emission fluorescence for all three types of the enzyme in their native states showed the emission maxima approximately 340 nm. According to Burstein et al (1973), this means that some of the tryptophan residues were exposed to the solvent perhaps to facilitate the binding of the lipoprotein substrate (HDL). The relative fluorescence intensities of p-LCAT and r-LCAT in their native state were 3.3 and 3.2 respectively. Hence the amount of the exposed tryptophan residues were likely to be similar for both enzymes. However, the number of exposed tryptophan residues for the d-LCAT in the native state appeared to be less than p-LCAT or r-LCAT as the relative fluorescence intensity in the respective native states appeared to be lower (2.5) than those of the other two enzyme forms. Thus the environments or the configuration of the exposed tryptophan residues of p-LCAT and r-LCAT were likely to be similar while for the d-LCAT these parameters might be somewhat different. The presence or absence of sialic acid thus might play a role in determining the configuration and the local environments of the aromatic amino acid side chains .

During denaturation with 6 M guanidine hydrochloride, all three species of the enzyme were only partially denatured as their residual activities did not approach zero. Alternatively, the renaturation of the denatured enzymes could have occurred during incubation with substrate

24 hours while estimating the residual LCAT activities of denatured enzyme species.

Comparison of the % residual activities and comparison of % residual alpha helices of the three denatured enzyme species (figure 8A and 8B and table-2) led to the following conclusions. The p-LCAT underwent gradual changes in conformation as compared to the abrupt changes in conformation observed for the two forms of recombinant LCAT on denaturation with guanidine hydrochloride. This could be due to the fact that the number of binding sites to 6 M guanidine hydrochloride might be less for p-LCAT as compared to the two forms of recombinant LCAT The residual activities of the two forms of recombinant LCAT seemed to be higher than those of p-LCAT. Perhaps the rates of denaturation and renaturation might be much slower for p-LCAT than for the two forms of recombinant LCAT. The denaturation with guanidine hydrochloride appeared to be functionally reversible for the two forms of recombinant LCAT while it seemed to be irreversible for the p-LCAT. The observed differences between p-LCAT and the two forms of recombinant LCAT to guanidine hydrochloride denaturation and renaturation might be due to differences in the respective configurations and conformation of the oligosaccharide components of p-LCAT and those of r-LCAT.

Examination of the CD spectra recorded in the near u.v region for the r-LCATand d-LCAT in the native and denatured states reflected differences in the environment of the aromatic amino acid residues for the

r-LCAT and d-LCAT. These differences might be attributed to the presence or absence of terminal sialic acid. One might speculate that the observed differences in the configuration of aromatic amino acid side chains between the r-LCAT and d-LCAT might be responsible for the differences in their secondary structure in the native state. The same explanation might hold for the observed differences in the relative fluorescence intensities of intrinsic tryptophan fluorescence of sialylated and desialylated forms of recombinant LCAT in their respective native states.

In conclusion, the presence of sialic acid appears to influence the local environments of aromatic amino acid residues of LCAT that might affect the alignment of beta sheet and beta turn components of LCAT. The structural and functional differences between p-LCAT and the two forms of recombinant LCAT with regard to denaturation and renaturation with 6 M guanidine hydrochloride and by heat might be due to differences in the N-glycan structure of p-LCAT and r-LCAT that contributes differentially to the conformational stability of the respective enzymes but not to the appropriate exposure of the active site to substrate. The alpha helical structures are not likely to have a major role in maintaining the active conformation of the enzyme.

By using the r-LCAT as control, each of the histidine residues in the enzyme may be altered by site directed mutagenesis to produce mutant enzymes. The structure-function relationship of these mutant enzymes

could be compared with that of r-LCAT by CD and fluorescence studies. These studies might help in speculating which and how many histidine residues may be involved in catalysis. r-LCAT can be used as model to study the structure-function relationship of another class of mutant enzymes that causes fish eye disease.

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