STUDIES ON HOG PLASMA LECITHIN:CHOLESTEROL ACYLTRANSFERASE: ISOLATION AND CHARACTERIZATION OF THE ENZYME

DISSEETATION

Presented to the Graduate Council of the North Texas State University in Partial Fulfillment of the Requirements

For the Degree of

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By

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Lecithin:cholesterol acyltransferase (LCAT) was isolated from hog plasma and basic physicochemical properties and functionally important regions were investigated. Approximately one milligram of the enzyme was purified to apparent homogeneity with approximately a 20,000-fold increase in specific activity. In the plasma, hog LCAT was found to associate with high-density lipoproteins (HDL) probably through hydrophobic interactions with apolipoprotein A-I. HDL was the preferred lipoprotein substrate of the enzyme as its macromolecular substrate. The enzyme was found to contain 4 free sulfhydryl groups; at least one of these appeared to be essential for catalytic activity. The enzyme had a tendency to aggregate at high concentrations. More than half of the tryptophan and none of the tyrosine residues of the enzyme were shown to be exposed to the aqueous environment based on fluorescence and absorbance studies, respectively. The enzyme was reacted with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), N-ethylmaleimide (NEM), diisopropylfluorophosphate (DFP) and diethylpyrocarbonate (DEP). Approximately 1 mole of DFP per mole of the enzyme was incorporated into the enzyme. Thirty-four amino acid residues from the amino terminus of the enzyme were sequenced; they were highly hydrophobic in nature and the
first 18 residues were identical to those of human LCAT. The
$[^{3}\text{H}]-\text{DFP}$ labeled enzyme was digested with pepsin and the
labeled peptides were isolated by HPLC. The amino acid
sequences of these peptides were identical to corresponding
segments of human LCAT. At least one of these peptides
appeared to contain the essential serine for the catalytic
activity. Sulphydryl group containing peptides generated by
tryptic digestion were also sequenced from $[^{3}\text{H}]-\text{NEM}$ labeled
peptides. Four radioactive peptides were isolated and their
amino acid sequences were not homologous to any region of human
LCAT. Data obtained from physicochemical characterization,
chemical modification, sequence analysis and immunotitration
suggest that hog LCAT is similar to the human enzyme.
TABLE OF CONTENTS

LIST OF TABLE ........................................................................ v
LIST OF ILLUSTRATIONS ....................................................... vi
LIST OF ABBREVIATIONS .................................................... viii
Chapter
I. INTRODUCTION .............................................................. 1

| Cholesterol and Its Transport |
| Reverse Cholesterol Transport and LCAT |
| Physical and Chemical Properties of LCAT |
| Comparison of Plasma Lipoproteins in Human and Hog |
| Purpose of This Study |

II. MATERIALS AND METHODS ............................................. 16

| Materials |
| Plasma |
| Chemicals |
| Methods |
| Enzyme Purification |
| Characterization of the Enzyme |
| Determination of Substrate |
| Specificity of the Enzyme |
| Tryptophan Determination |
| Thiol Group Titration |
| Reaction of TNB-LCAT with KCN and DTT |
| Concentration Dependence of Inactivation of LCAT by DTNB |
| Phenolic Group Titration |
| Chemical Modification and Radioaffinity Labeling of Enzyme |
| Proteolytic Digestions |
| Isolation of Peptides |
| Amino Acid Sequence Analyses of the Enzyme |
| Reaction of Hog LCAT with Monoclonal Antibody Against Human LCAT |
| Other Methods |

III. RESULTS ......................................................................... 35

| Enzyme Purification |

iii
### Chapter 3

<table>
<thead>
<tr>
<th>Substrate Specificity of the Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physicochemical Properties of the Enzyme</td>
</tr>
<tr>
<td>Molecular Weight of the Enzyme</td>
</tr>
<tr>
<td>Amino Acid and Carbohydrate Compositions of the Enzyme</td>
</tr>
<tr>
<td>Sulfhydryl Groups of the Enzyme</td>
</tr>
<tr>
<td>Tryptophan Residues of the Enzyme</td>
</tr>
<tr>
<td>Phenolic Groups of the Enzyme</td>
</tr>
<tr>
<td>Chemical Modification of the Enzyme</td>
</tr>
<tr>
<td>Radioaffinity Labeling and Quantitation of DFP-Reactive Serine Residue</td>
</tr>
<tr>
<td>Amino Acid Sequence of the Enzyme</td>
</tr>
<tr>
<td>Amino Terminus</td>
</tr>
<tr>
<td>Peptic Peptide: DFP-Reactive Serine Peptide</td>
</tr>
<tr>
<td>Tryptic Peptides: NEM-Reactive Cysteine Peptides</td>
</tr>
<tr>
<td>Immunoreactivity of Hog LCAT with Monoclonal Antibody Against Human LCAT</td>
</tr>
</tbody>
</table>

### IV. DISCUSSION

- Purification of the Enzyme
- Substrate Specificity of the Enzyme
- Physicochemical Properties of the Enzyme
- Primary Structure of the Enzyme
- Comparison of the Hog and Human LCAT.

### Appendix

- Appendix

<table>
<thead>
<tr>
<th>Bibliography</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>I.</td>
</tr>
<tr>
<td>II.</td>
</tr>
<tr>
<td>III.</td>
</tr>
<tr>
<td>IV.</td>
</tr>
<tr>
<td>V.</td>
</tr>
<tr>
<td>VI.</td>
</tr>
<tr>
<td>VII.</td>
</tr>
<tr>
<td>VIII.</td>
</tr>
</tbody>
</table>
# List of Illustrations

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Postulated Mechanism for the Transport of Cholesterol from Peripheral Cells to the Liver in Human and Rat.</td>
<td>8</td>
</tr>
<tr>
<td>2.</td>
<td>Representative Column Chromatographic Profiles Obtained from the Phenyl-Sepharose, DE-52 Cellulose and Hydroxylapatite Columns for Purification of LCAT from Hog Plasma.</td>
<td>37</td>
</tr>
<tr>
<td>3.</td>
<td>Polyacrylamide Gel Electrophoreses of Purified Hog LCAT.</td>
<td>41</td>
</tr>
<tr>
<td>4.</td>
<td>The Rate of Esterification as a Function of Free Cholesterol Concentration in the Substrates.</td>
<td>44</td>
</tr>
<tr>
<td>5.</td>
<td>Distribution of LCAT in Hog Plasma.</td>
<td>47</td>
</tr>
<tr>
<td>6.</td>
<td>Fluorescence Quenching of Hog LCAT Caused by the Incubation with Phosphatidylcholine Micelles, Mixed Micelles and Proteoliposomes.</td>
<td>50</td>
</tr>
<tr>
<td>7.</td>
<td>Time Course of Thiol Group Titration with Purified Hog LCAT.</td>
<td>55</td>
</tr>
<tr>
<td>8.</td>
<td>Concentration Dependence of DTNB Inactivation of Purified Hog LCAT with and without Dialysis against 2 mM DTT.</td>
<td>60</td>
</tr>
<tr>
<td>9.</td>
<td>Fluorometric Determination of Tryptophan Content in Hog LCAT in Native and Denatured Conditions.</td>
<td>62</td>
</tr>
<tr>
<td>10.</td>
<td>Spectrophotometric Titration of Purified Hog LCAT and N-acetyl-L-tyrosinamide at 295 nm.</td>
<td>64</td>
</tr>
<tr>
<td>11.</td>
<td>Chemical Modifications of Cysteine, Serine, and Histidine Residues of Hog LCAT.</td>
<td>67</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>12.</td>
<td>Representative Plots of the Yields per Cycle of PTH-amino Acid Derivatives from the Automated Sequence Analyses</td>
<td>72</td>
</tr>
<tr>
<td>13.</td>
<td>Elution Pattern after Reversed-phase HPLC Separation of Peptides Derived from Peptic Digestion with $[^3H]$-DIP-LCAT</td>
<td>76</td>
</tr>
<tr>
<td>15.</td>
<td>Elution Profile from Reversed-phase HPLC Separation of Peptides Derived from the Reduction of the Peptide #2 of the Tryptic Digest</td>
<td>85</td>
</tr>
<tr>
<td>16.</td>
<td>Inhibition of Hog and Human LCAT Activity by Monoclonal Antibody against Human LCAT</td>
<td>87</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
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</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>DEP</td>
<td>diethyl pyrocarbonate</td>
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</tr>
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<td>DFP</td>
<td>diisopropylfluorophosphate</td>
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</tr>
<tr>
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<td>diisopropylphosphoryl-</td>
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</tr>
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<td>5,5'-dithio-bis-(2-nitro-benzoic-acid)</td>
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</tr>
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</tr>
<tr>
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<td>Endo-β-N-acetylglucosaminidase H</td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoproteins</td>
<td></td>
</tr>
<tr>
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<td>high performance liquid chromatography</td>
<td></td>
</tr>
<tr>
<td>IDL</td>
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<td></td>
</tr>
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<td>lecithin:cholesterol acyltransferase</td>
<td></td>
</tr>
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<td>NEM</td>
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</tr>
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<td>PEG</td>
<td>polyethylene glycol</td>
<td></td>
</tr>
<tr>
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<td>Phenylisothiocyanate</td>
<td></td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonylefluoride</td>
<td></td>
</tr>
<tr>
<td>PTH</td>
<td>phenylthiohydantoin</td>
<td></td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate</td>
<td></td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
<td></td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
<td></td>
</tr>
<tr>
<td>TFMS</td>
<td>trifluoromethanesulfonic acid</td>
<td></td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoproteins</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION

The studies described in this dissertation are concerned with lecithin:cholesterol acyltransferase (LCAT); an enzyme that is involved in cholesterol metabolism. LCAT was purified to homogeneity from hog plasma and characterized including determination of the primary structure of the amino-terminus and diisopropylfluorophosphate (DFP)- and N-ethylmaleimide (NEM)-labeled peptides derived from proteolytic digestions. In this chapter, the current state of knowledge regarding the properties of the enzyme and its reaction are discussed.

Cholesterol and Its Transport

Cholesterol is an ambivalent molecule. It is useful in the building of cell membranes, but it is also potentially dangerous when it accumulates in the artery. Due to its poor water-solubility, cholesterol cannot be readily mobilized and its deposition may eventually lead to the development of atherosclerotic plaques. Cholesterol in the plasma is transported as a component of lipoprotein particles. These are structural aggregates of lipid and protein, consisting of a non-polar core of cholesteryl ester and triglyceride surrounded by a polar shell of free (unesterified) cholesterol, phospholipid and protein (Eisenberg and Levy, 1975; Morrisett et al., 1977). The lipoproteins can be
separated into a number of classes based on their respective bouyant densities (For review, Mahley and Innerarity, 1983; Havel et al., 1980). There are five major classes of lipoproteins in the human system: chylomicrons, very low-density lipoproteins (VLDL, d<1.006 g/ml), intermediate-density lipoproteins (IDL, d=1.006-1.019 g/ml), low-density lipoproteins (LDL, d=1.019-1.063 g/ml) and high-density lipoproteins (HDL, d=1.063-1.21 g/ml).

Chylomicrons (diameter > 100 nm) are synthesized in the small intestine following absorption of dietary lipids to provide a vehicle for transport of triglycerides from the site of absorption to various cells of the body. The major protein component of the chylomicron particle is apolipoprotein (apo) B-48. The triglycerides of chylomicrons are hydrolyzed in the capillaries of endothelial cells by interaction with an enzyme, lipoprotein lipase (Bier and Havel, 1970; LaRosa et al., 1970). The resulting cholesterol-enriched particles are referred to as chylomicron remnants; these are rapidly cleared by the liver (Cooper and Yu, 1978; Sherrill and Dietschy, 1978). The chylomicron remnants provide lipid constituents for the synthesis of some of the higher density lipoproteins in the liver such as VLDL and HDL. VLDL are synthesized in the liver; they transport and redistribute triglycerides and cholesterol from the liver to various tissues. The major protein in the particle is apoB-100. VLDL triglycerides are also hydrolyzed by lipoprotein lipase on the endothelial surface and subsequently by hepatic triglyceride lipase.
generating a series of smaller, cholesterol-enriched particles, IDL and LDL. The IDL are formed in the blood from the partial hydrolysis of VLDL by lipoprotein lipase (Reardon et al., 1978). The LDL represent the end products of VLDL catabolism and the major cholesterol-transporting lipoprotein in the plasma. The mechanism of conversion from IDL to LDL is not yet fully understood, although hepatic lipase has been proposed to play a role in this step (Kuusi et al., 1979).

HDL are synthesized in the liver as discoidal particles called 'nascent' HDL. These precursors are converted to mature HDL in the plasma by accepting lipids and proteins from lower density lipoproteins and by the action of LCAT. The mature HDL have the greatest density among lipoproteins due to a relatively high protein content, with apoA-I being the major protein constituent. More than 10 different proteins have been identified on the surface of the HDL particle. HDL are involved in the transport of cholesterol from the periphery back to the liver, a process that has been termed as reverse cholesterol transport.

At least 10 apolipoproteins (apo-) have so far been identified that have unique functions in regulating the metabolism of lipoproteins (Table I). Three major roles of apoproteins are 1) uptake of lipoproteins by specific tissues; 2) cofactors for enzymes in lipoprotein metabolism; 3) the maintenance of structure of lipoproteins. First, the transport and redistribution of lipids by apoproteins are achieved by the recognition of specific apoproteins by
<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>Molecular Weight</th>
<th>Conc. in Plasma (mg/dl)</th>
<th>Origin</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-I</td>
<td>28,000</td>
<td>90-103</td>
<td>Intestine, liver</td>
<td>LCAT activator</td>
</tr>
<tr>
<td>A-II</td>
<td>17,000</td>
<td>30-50</td>
<td>Intestine, liver</td>
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<td>A-IV</td>
<td>46,000</td>
<td></td>
<td>Liver</td>
<td>Unknown</td>
</tr>
<tr>
<td>B-100</td>
<td>400,000</td>
<td>80-100</td>
<td>Liver</td>
<td>Neutral lipid transport</td>
</tr>
<tr>
<td>B-48</td>
<td>264,000</td>
<td>5</td>
<td>Intestine, liver</td>
<td>Neutral lipid transport</td>
</tr>
<tr>
<td>C-I</td>
<td>6,500</td>
<td>4-7</td>
<td>Liver</td>
<td>LCAT activator</td>
</tr>
<tr>
<td>C-II</td>
<td>8,800</td>
<td>3-8</td>
<td>Liver</td>
<td>LCAT activator</td>
</tr>
<tr>
<td>C-III</td>
<td>8,750</td>
<td>8-15</td>
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<td>Unknown</td>
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<td>D</td>
<td>20,000</td>
<td></td>
<td>Liver</td>
<td>Unknown</td>
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<td>E</td>
<td>34,000</td>
<td>3-7</td>
<td>Liver</td>
<td>Receptor mediated lipoprotein catabolism</td>
</tr>
</tbody>
</table>
lipoprotein receptors on the cell surface. For example, apoB-100 and E have been shown to mediate the interaction of lipoproteins with apoB,E (or LDL)-receptors of the liver and extrahepatic tissues and apoE receptors of the liver (Goldstein and Brown, 1977; Blum et al., 1982; Innerarity et al., 1984). Secondly, apoproteins play critical roles as cofactors for enzymes involved in lipoprotein metabolism. The LCAT-catalyzed reaction is activated by apoA-I (Fielding et al., 1972). It is now apparent that other apoproteins also exhibit cofactor potential, including apoA-II, C-I, C-II, C-III (Jonas et al., 1984) and A-IV (Steinmetz and Utermann, 1983). Catalytic reaction of lipoprotein lipase requires the presence of apoC-II (LaRosa et al., 1970; Havel et al., 1970). Thirdly, various apoproteins, for example apoA-I and B for HDL and VLDL, respectively, have been shown to stabilize the micellar structure of the lipoproteins (Segrest et al., 1974; Sparrow and Gotto, 1982).

Cholesterol enters the plasma mainly as a component of newly synthesized lipoproteins such as chylomicrons (Ashworth and Johnston, 1963), VLDL (Cardell et al., 1967; Hamilton et al., 1967) and HDL (Marsh, 1974; Noel and Rubinstein, 1974). In addition, cholesterol may enter the plasma by another process known as reverse cholesterol transport. This latter mechanism does not involve the secretion of newly formed lipoproteins, but is facilitated by a concentration gradient between plasma membranes of the peripheral tissues and the liver. This pathway will be discussed in more detail below.
Cholesterol is removed from the plasma predominantly as a consequence of tissue uptake of LDL by the LDL-receptor pathway (for a recent review, see Brown and Goldstein, 1986). The cholesterol transported in remnant particles is cleared by specific hepatic receptors as mentioned above (Cooper and Yu, 1978). The cholesterol in HDL is catabolized mainly by the liver (Roheim, et al., 1971) by the reverse cholesterol transport pathway, although adrenals, kidney and spleen also utilize appreciable amounts of this lipoprotein (Ose et al., 1979).

Reverse Cholesterol Transport and LCAT

Nearly all extrahepatic tissues synthesize cholesterol and also acquire cholesterol from extracellular fluids. Excess free cholesterol readily exchanges between lipoproteins and cellular membranes (Murphy, 1962). Cholesterol is removed from the cells in the unesterified form. HDL can serve as an efficient acceptor in this process (Stein et al., 1975) because, subsequent to the uptake of cholesterol by HDL, most of it is converted to the esterified form and thus "trapped" in HDL by the action of LCAT (Glomset, 1968). Newly formed cholesteryl esters are thus incorporated into the core of HDL particles. The continuing esterification of the free cholesterol in HDL therefore provides a concentration gradient of free cholesterol between plasma membranes and lipoproteins. This gradient facilitates a net movement of free cholesterol from cell membranes to the liver. The cholesteryl esters formed by the LCAT reaction in HDL are first transferred to
LDL and VLDL (Goodman, 1964; Nichols and Smith, 1965) by another catalyst called cholesteryl ester transfer protein (Chajek and Fielding, 1978). The VLDL (and eventually LDL) thus become enriched in cholesteryl esters and finally LDL are taken up by receptor-mediated endocytosis (Brown and Goldstein, 1976). Because the liver is the only organ in the body from which significant amounts of cholesterol can be excreted (either as cholesterol or as bile acids), the ultimate catabolism of cholesterol takes place in hepatocytes. It has been suggested that the liver plays an important quantitative role in removing HDL (Van Berkel and Van Tol, 1978), LDL (Pittman et al., 1979) and VLDL (Faergeman and Havel, 1975) as well as chylomicron remnants (Sherrill, 1980). Therefore, the transesterification of cholesterol by LCAT on HDL facilitates the removal of cholesterol from peripheral cells to the liver (Figure 1).

LCAT (EC 2.3.1.43) is synthesized in the liver (Nordby et al., 1976) and circulates in the plasma apparently in association with HDL (Fielding and Fielding, 1971). ApoA-I is a very potent activator of LCAT (Albers et al., 1976). The LCAT reaction occurs in two steps. The first is a phospholipase A$_2$ reaction (Aron et al., 1978) producing lysolecithin and an acyl-enzyme intermediate. The second is a transfer of the acyl group to cholesterol to form a cholesteryl ester (Yokoyama et al., 1980). The activation by apoA-I may be the initial step of the reaction (Yokoyama et al., 1980). LCAT possesses a broad acyl group specificity.
Figure 1.—Postulated mechanism for the transport of cholesterol from peripheral cells to the liver in human and rat.

In human, HDL acquires unesterified cholesterol from the peripheral cell membranes and phospholipids from VLDL and chylomicrons. LCAT reacts with the circulating HDL to form cholesteryl esters from unesterified cholesterol and phosphatidylcholine. The cholesteryl esters formed by the action of LCAT are transferred to the lower density lipoproteins by cholesteryl ester transfer protein and finally catabolized in the liver.

In rat, hog and some other animals, there is only minimal transfer activity of cholesterol esters from HDL to lower density lipoproteins. The cholesteryl esters formed by the LCAT reaction are believed to be transported to the liver by HDL. Here, the rat system is depicted in comparison with the human as the hog and rat systems are essentially identical regarding the components of reverse cholesterol transport.

FC, unesterified cholesterol; CE, cholesteryl esters; PL, phospholipids; CETP, cholesteryl ester transfer protein; LPL, lipoprotein lipase; HTGL, hepatic triglyceride lipase.
generally favoring polyunsaturated fatty acyl groups. Linoleic and arachidonic acids are especially favored (Sgoutas, 1972). The enzyme generally requires a molar ratio of lecithin to cholesterol above 3 to 1 for maximal activity (Sgoutas, 1972). This ratio may be of importance for cholesterol esterification in vivo as HDL is the only lipoprotein with such a ratio and it is also the best lipoprotein substrate for LCAT (Fielding and Fielding, 1971).

**Physical and Chemical Properties of LCAT**

LCAT has been purified by a number of laboratories from human (Chong et al., 1981; Chung et al., 1979; Albers et al., 1976; Doi and Nishida, 1981), hog (Park and Lacko, 1986; Knipping, 1986) and rat (Pownall et al., 1985) plasma. The molecular weight of the enzyme has been estimated to be 60,000–69,000. The enzyme was shown to be a glycoprotein containing a relatively large amount of carbohydrate (approximately 25 percent, w/w). Mannose, galactose, glucosamine and sialic acid were found as components of the enzyme. LCAT may have as many as five isoforms with isoelectric points ranging from 4.5 to 5.5 (Albers et al., 1979; Doi and Nishida, 1981; Chong et al., 1981). Recently, human LCAT has been cloned (McLean et al., 1986) and the entire amino acid sequence of the enzyme has been deduced from the cDNA sequence (see Appendix). The enzyme contains 416 amino acids including 6 cysteine and 9 methionine residues. McLean et al. have shown that the amino acid sequence -Ile-Gly-His-Ser-Leu-Gly- (residues 178–183) in human LCAT was
identical to a segment of porcine pancreatic lipase (residues 149-154); which has been shown to be the interfacial active site of the enzyme (Guidoni et al., 1981). Rat lingual triglyceride lipase has also been shown to contain a similar sequence (-Val-Gly-His-Ser-Gln-Gly-) (Docherty et al., 1985). The involvement of a serine residue in the LCAT reaction has been previously suggested (Chong et al., 1983; Nakagawa et al., 1977). A recent study by Jauhiainen and Dolphin (1986) has shown that serine and histidine residues are involved at the active site of human LCAT and that they are responsible for the phospholipase A\textsubscript{2} activity of the enzyme. Jauhiainen and Dolphin have also postulated that two sulfhydryl groups are involved in the transacylase activity of LCAT. According to this mechanistic scheme, a single serine and histidine residue within the active site catalyzes the hydrolysis of the sn-2 ester bond of lecithin with the formation of a transient fatty acyl-enzyme oxyester. The fatty acyl moiety is then transesterified onto one of the 2 cysteine residues forming a thioester which has the ability to transfer the fatty acyl group to cholesterol, forming cholesteryl ester. This putative mechanism, however, has yet to be confirmed.

Comparison of Plasma Lipoproteins in Human and Hog

The hog (Sus domesticus) has been judged as a suitable animal model for atherosclerosis research on the basis of the following criteria: (a) resemblance of its serum lipoproteins to human lipoproteins, (b) its susceptibility to
atherosclerosis, (c) availability and (d) cost (Babbitt and Levy, 1965; Lee and Alaupovic, 1970; Scanu, 1965). Earlier work has shown that cholesterol feeding in miniature swine resulted in hypercholesterolemia with distinctive hyperlipoproteinemia and the subsequent development of atherosclerosis (Mahley et al., 1975). Hog serum contains three classes of lipoproteins resembling the VLDL, LDL and HDL in human serum. Although the density distribution and the apoprotein components are somewhat different in the lipoproteins from the two species, the lipid content and particle size of the corresponding classes from the two sources are quite similar (Mahley et al., 1974).

The chemical composition of hog LDL and HDL resembles their human counterparts (Fidge, 1973). Jackson et al. (1973) have shown that the major apoprotein from hog and human HDL (apoA-I) have very similar physical, chemical, immunological and physiological properties. It has also been shown that the major species of apoprotein C isolated from hog serum is a polypeptide similar in amino acid composition to apoC-II from human serum and that both polypeptides are capable of activating lipoprotein lipase (Knipping et al., 1975). In addition, the major apoprotein of hog LDL cross-reacted with antiserum raised against human apoB (Goldstein et al., 1977). This apoprotein was also a major component of hog apo-VLDL accounting for about 50 percent of its protein mass. Kalab and Martin (1968) have shown that both hog and human VLDL have pre-beta electrophoretic mobility on agarose gels and that the
amino acid composition of the human and hog apo-VLDL were similar.

An essential distinction between the hog and human lipoprotein system is the heterogeneity of the density classes of hog LDL. Mahley and Weisgraber (1974) found that LDL distributed principally in the density range 1.03-1.08 g/ml and that HDL extended to the lower limiting density of 1.07 g/ml. Janado et al. (1966) observed that hog LDL contained two major components, LDL₁ and LDL₂ (relative ratio: 2:1), which formed discrete boundaries on ultracentrifugation. In contrast, Jackson et al. (1976) reported that there was no difference between LDL₁ and LDL₂ regarding protein composition and immunological properties. However, there was a difference in molecular size and ultracentrifugal properties. In addition, no apoA-II was detected in delipidated hog HDL when it was chromatographed on Sephadex G-150. Another major difference between the metabolism of human and hog lipoproteins is in the transfer of cholesteryl ester from HDL to lower density lipoproteins. There is only minimal transfer of cholesteryl ester in hog plasma (Ha et al., 1981), whereas this is a key event of reverse cholesterol transport in human plasma.

Purpose of This Study

As described above, LCAT catalyzes one of the key reactions in lipoprotein metabolism, especially in facilitating cholesterol catabolism. The enzyme exhibits a number of unique features including a requirement for
macromolecular substrates (Akanuma and Glomset, 1968) and a polypeptide cofactor in vitro (Fielding et al., 1972).

Despite the importance of the enzyme, its characterization has not progressed rapidly mainly due to difficulties in the preparation of sufficient amounts of homogeneous enzyme.

Most of the data on LCAT to date has been obtained from studies of the human enzyme. Although no systematic search for LCAT in different animal species has yet been performed, cholesterol esterifying activity has been found in the plasma of reptiles and amphibians in addition to mammals and birds (Chapman, 1980). The lipid transport system of the hog is largely similar to that of human, although it exhibits a number of differences in the apoprotein spectrum and apparently in the pathway of reverse cholesterol transport. Despite these differences between the two species, the isolation of LCAT from sources other than human plasma should provide an opportunity for comparative studies that will facilitate the elucidation of the structure-function relationships of this enzyme system. In addition, comparison of the properties of the enzyme between the two species should provide much needed information concerning the mechanism of action of the enzyme and the evolutionary aspects of lipoprotein metabolism.

The present studies were designed with the following objectives:

(A) To purify LCAT to homogeneity from hog plasma.

(B) To determine the basic characteristics of the
enzyme. These include substrate specificity, molecular weight, carbohydrate composition and amino acid composition of the enzyme.

(C) To identify and characterize the functionally important amino acid residues of the enzyme. This involves the studies of the reactivity of cysteine, serine, histidine, tryptophan and tyrosine residues of the enzyme.

(D) To attempt to identify potential residues involved in the catalytic activity and determine the amino acid sequence of these regions of the enzyme.
CHAPTER II

MATERIALS AND METHODS

Materials

Plasma

Fresh hog plasma was isolated from blood obtained from a local slaughter house by centrifugation at 1000 x g at 15 °C for 20 min. Fresh and frozen plasma (-20 °C) were used for these studies. The frozen specimens retained their LCAT activities for up to 3 months.

Chemicals

Calf serum fetuin, anthrone, 2-thiobarbituric acid, N-acetyleneuraminic acid, phenylmethylsulfonylfluoride (PMSF), trypsin, chymotrypsin, pepsin, pronase, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), dithiothreitol, N-ethylmaleimide, diisopropyl fluorophosphosphate and cholesterol/triglyceride aqueous standards were purchased from Sigma Chemical Co., St. Louis, Missouri. Acrylamide, bis-acrylamide, riboflavin, ammonium persulfate, N,N,N',N'-tetramethylethylene diamine, 2-mercaptoethanol hydroxylapatite and Coomassie Blue were from Bio-Rad, Richmond, California. Phenyl-Sepharose and protein markers were from Pharmacia Fine Chemicals and cyanogen bromide, anisol and trifluoromethanesulfonic acid (TFMS) were purchased
from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin. Polyethylene glycol-6000 and sodium arsenite were obtained from Matheson, Colman and Bell, East Rutherford, New Jersey and DE-52 was from Whatman. Cholesterol and triglyceride assay kits were purchased from Boehringer-Mannheim Co., Indianapolis, Indiana and [1,2-\(^{3}\)H]-cholesterol and \([^{3}\text{H}]\)-DFP were obtained from Amersham/Searle. \([^{3}\text{H}]\)-NEM was a product of New England Nuclear. D(+)mannose and D(+)galactose were obtained from Pfanstiehl Lab., Waukeagen, Illinois and glucosamine-HCl was purchased from Nutritional Biochemical Corp., Cleveland, Ohio. Most of reagents for the sequence analysis was obtained from Pierce Chemical Co., Rockford, Illinois. Endo-β-N-acetylglucosaminidase-H was obtained from Miles, Elkhart, Indiana. All other chemicals were products of Fisher Scientific Co. and were of reagent grade.

Methods

**Enzyme Purification**

**Step 1. Polyethylene Glycol-6000 (PEG-6000) Precipitation** - One liter of fresh plasma was treated with a 50 percent solution of PEG-6000 to achieve a final concentration of 6 percent (w/v). The suspension was stirred for 3-4 hours at 4 °C and subsequently centrifuged in a Sorvall RC-5B Refrigerated Super Speed Centrifuge at 7000 rpm for 20 min using a Type GSA rotor (Sorvall). The PEG-6000 supernatant solution (PEG-supernatant) was carefully decanted and the precipitate was discarded. The conductivity of the
supernatant was adjusted to 25-28 mMHO with a solution of 5 M NaCl and the pH was adjusted to 7.4, with 5 M NaOH.

**Step 2. Phenyl-Sepharose Chromatography** - The PEG-supernatant was applied to a phenyl-Sepharose column (5 x 25 cm) that had been previously equilibrated with 5 mM sodium phosphate buffer, pH 7.4, containing 0.3 M NaCl. Following the application of the sample, the column was washed with 5 mM sodium phosphate buffer, pH 7.4, containing 50 mM NaCl until the absorbance at 280 nm was less than 0.2. The enzyme was eluted by deionized water (DI-H$_2$O). Absorbance at 280 nm, conductivity and enzyme activity were monitored. The fractions containing the enzyme activity were pooled for further purification. The chromatography was conducted at 4 °C at a flow rate of 320 ml per hour. The phenyl-Sepharose column was regenerated after each cycle by washing with two column-volumes of ethanol, four column-volumes of butanol, two column-volumes of ethanol and four column-volumes of deionized water in succession.

**Step 3. DE-52 Chromatography** - The enzyme fractions from the phenyl-Sepharose column were diluted with 0.5 mM sodium phosphate buffer, pH 7.4, to achieve a conductivity of less than 2 mMHO, the optimal ionic strength of the loading sample on the ion-exchange column. This column (2.5 x 30 cm) had previously been equilibrated with 5 mM sodium phosphate buffer, pH 7.4, and was subsequently eluted with a linear NaCl gradient using one liter of 50 mM sodium phosphate buffer and 500 ml of the same buffer containing 130 mM NaCl, pH 7.4. The
fractions with high enzyme activity were pooled and concentrated by vacuum dialysis to a volume of 1 ml. The concentrated sample was dialyzed at 4 °C overnight against 1 mM sodium phosphate buffer, pH 7.4. The column was regenerated by washing with 0.3 M NaCl. After every three cycles of the operation, the exchanger was unpacked from the column and regenerated by washing with two column-volumes of 0.5 N NaOH, ten column-volumes of DI-H₂O, two column volumes of 0.5 N HCl and ten column volumes of DI-H₂O.

**Step 4. Hydroxyapatite Chromatography** - A hydroxyapatite column (2.5 x 20 cm) was equilibrated with 1 mM sodium phosphate buffer, pH 7.4. The enzyme pool from the DE-52 chromatography was divided in two equal parts and one part was loaded on the column. A linear gradient was applied from 1 mM to 0.1 M sodium phosphate (500 ml, each) pH 7.4, at a flow rate of 20 ml/hr. After three cycles, the column was unpacked and the hydroxyapatite suspended in 1 liter of 0.2 M sodium phosphate buffer, pH 7.4 in a graduate cylinder. The slurry was shaken gently for 10 minutes and allowed to sediment. The fine particles in the buffer layer were removed and the column material was resuspended in DI-H₂O. This procedure was repeated 3 times and then the hydroxyapatite was packed into the column under gravity. All buffers used in hydroxyapatite chromatography were degassed.
Characterization of the enzyme

**Determination of Substrate Specificity**

Lipoprotein substrate specificity of the enzyme was determined by incubation of the purified enzyme with individual lipoproteins (VLDL, LDL and HDL). Hog plasma was prepared as described earlier and treated with 1 mM DFP to inactivate LCAT. Lipoproteins were isolated according to the method by Rudel et al. (1974). The lipoprotein preparations were concentrated to 2 ml using Aquacide (Cal–Biochem.) and dialyzed against 0.01 M Tris, pH 7.4, containing 0.15 M NaCl and 0.005 M EDTA. The free cholesterol concentration of the lipoproteins was determined enzymatically using a reagent kit from Boehringer-Mannheim. Serum albumin-$[^3]H$-cholesterol emulsion was prepared by mixing 60 μl of 0.1 mCi/ml of $[^3]H$-cholesterol with 4 ml of 8 percent fatty acid-free serum albumin (BSA) slowly while stirring under a nitrogen stream (Stokke and Norum, 1971). The rate of cholesterol esterification for the individual lipoprotein preparations was determined as described by Jahani and Lacko (1982). Aliquots of each lipoprotein containing 20 to 200 nmol/ml of free cholesterol were preincubated in duplicate with 20 μl of fatty acid free BSA-$[^3]H$-cholesterol emulsion at 4 °C overnight. The final assay volume was adjusted to 200 μl and the reaction was initiated by the addition of 0.54 μg of purified LCAT. The incubation was carried out at 37 °C for 6 hours and the enzyme activity was calculated as nmol per milliliter per 6
hours. The incubation period and the amount of enzyme in the reaction mixture were selected to obtain a linear rate of cholesterol esterification. The rate of esterification was calculated by multiplying the fractional rate (percent cholesterol esterified per time) by the free cholesterol concentration of each sample.

In order to establish the distribution of the enzyme in the plasma, whole plasma was fractionated on a gel filtration column (Bio-Gel A-5m). A sample of fresh plasma (3 ml) was mixed with \([^3H]\)-cholesterol (0.01 mCi/ml). \([^3H]\)-cholesterol in acetone was dried under a nitrogen stream in a 15-ml vial and the plasma sample was placed in the vial while being stirred under the nitrogen stream. The labeling was conducted at 4 °C for 1 hour. The labeled plasma was applied to a Bio-Gel A-5m column (1.5 x 90 cm) equilibrated with 0.01 M Tris buffer, pH 7.4, containing 0.15 M NaCl and 0.005 M EDTA. The chromatography was carried out with a flow rate of 25 ml/hr and 2-ml fractions were collected. The radioactivity of each fraction was counted and the enzyme activity was assayed according to the method of Chen and Albers (1982).

Affinity of hog LCAT toward an artificial substrate was determined by a fluorometric method. These experiments were designed to determine the difference in the relative fluorescence of hog LCAT alone and in the presence of artificial substrates: phosphatidylcholine (PC) micelle, PC-cholesterol mixed micelle and apoA-I-PC-cholesterol proteoliposome. Each artificial substrate was incubated with
the purified LCAT and the fluorescence quenching was monitored. The PC and mixed micelles were prepared by the method of Batzri and Korn (1973). The PC and cholesterol content of the mixed micelle was 250 to 12.5 (molar ratio). Proteoliposomes were prepared according to the method of Chen and Albers (1982). These artificial substrates were subjected to the Sepharose-4B column chromatography (2.5 x 45 cm) using 10 mM Tris containing 0.14 M NaCl, pH 7.4. The major absorbance peak at 210 nm representing a relatively homogeneous pool of the particles was collected. Following concentration by vacuum dialysis, the liposomes were subjected to fluorometry. The intrinsic fluorescence of the LCAT solution (10 ug) and the liposome solutions was determined, respectively. Small aliquots of concentrated liposomes were added to the LCAT solution and incubated at 37 °C for 4 hours to monitor the fluorescence quenching. The relative fluorescence was corrected for the increase in volume. Data were expressed as a percent LCAT fluorescence quenched by the addition of increasing amounts of liposomes as calculated by an equation:

\[
\frac{(F_a + F_b) - F_c}{F_a} \times 100
\]

Where \( F_a \), \( F_b \) and \( F_c \) represent the relative intensities of fluorescence of LCAT solution alone, liposome alone and LCAT with liposome, respectively. The amount of liposome added was expressed as miligrams of PC in each liposome.
Tryptophan Determination

The tryptophan content of the enzyme was determined by the fluorometric method described by Sasaki et al. (1975). The enzyme (20-30 ug) was lyophilized and redissolved in 100 ul of 0.02 M diethanolamine, 0.02 M triethanolamine, pH 8.0. The vials containing samples were capped and immersed in a shallow boiling water bath for 5 min to denature the protein. The vials were cooled to room temperature and 5 ul of freshly prepared solutions of chymotrypsin and pronase solution (0.05 percent, w/v each) were added (1/100 of LCAT mass). The mixture was incubated overnight at room temperature and diluted to two ml with 6 M urea in 0.02 M diethanolamine, 0.02 M triethanolamine pH 9.2. The samples were placed in fluorometric quartz cuvettes and the relative intensity of emission at 335 nm with excitation at 288 nm was recorded. In order to correct for the possible internal quenching, aliquots of a standard tryptophan solution (1-15 nmol) were added directly to the sample cuvette and the relative intensity of this mixture was extrapolated to zero addition. In order to estimate the exposure tryptophan residues under the non-denaturing conditions, a sample of the undigested active enzyme (20-30 ug) was subjected to the same fluorometric analysis under conditions where enzyme activity was preserved.

Thiol Group Titration

The sulfhydryl groups of the enzyme were titrated with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) essentially as described by Kemp and Forest (1968). The formation of the
thionitrobenzoate (TNB) anion was monitored by its absorbance at 412 nm with a Cary 210 double beam spectrophotometer. The titrations were performed in 0.3 M sodium phosphate buffer, pH 8.0, in the presence and absence of 2 percent (w/v) SDS. The extinction coefficient of the TNB ion at 412 nm (13.6 mM$^{-1}$ cm$^{-1}$) (Ellman, 1959) was used to calculate the number of reactive thiol group per mole of enzyme.

**Reaction of TNB-LCAT with KCN and DTT**

The reversal of the DTNB reaction to regenerate the enzyme activity by KCN was attempted as described by Kiick et al. (1984). Purified hog LCAT (0.45 mg) was allowed to react with 1 mM DTNB for 20 min at room temperature and then KCN was added to final concentration of 10 mM. After 10 min, DTT to final concentration of 10 mM was added; in the control KCN was omitted. Enzyme assays were carried out with aliquots of the reaction mixtures at each step. All reactions were performed in a 300 ul volume at 25 °C in 50 mM sodium phosphate buffer, pH 8.0. As soon as the aliquots were taken, the tubes were placed on ice and the substrate was added. Enzyme assays were carried out in duplicate (incubation time, 4 hours) using proteoliposome substrate (see enzyme assay section). The enzyme activities were compared with those of the control samples that contained the enzyme and buffer. When KCN was added to the reaction mixture, the whole reaction mixture was placed in a 1-ml cuvette and changes in the absorbance at 412 nm were monitored to demonstrate the substitution of cyanide ion for TNB-LCAT.
Concentration Dependence of Inactivation by DTNB

Different concentrations of the purified hog LCAT (50-300 ug/ml) were incubated with DTNB (0.7 mM) for 20 min at 24°C and the enzyme assays were carried out with aliquots of each reaction mixture. Total reaction volume was adjusted to 250 ul with 10 mM sodium phosphate buffer, pH 7.0. The enzyme activity from each reaction mixture was compared with the activity of the control which did not contain DTNB. In order to confirm possible thiol involvement in the protection of enzyme activity from the DTNB inactivation at high enzyme concentration, the purified enzyme (0.42 mg/ml) was dialyzed against 2 mM DTT in 50 mM sodium phosphate buffer, and subsequently against 5 mM sodium phosphate buffer, pH 7.4 with a nitrogen bubbling through the buffer. The enzyme was titrated with DTNB as described earlier with increasing concentrations of DTNB (10-90 uM). An aliquot of each reaction mixture was subjected to enzyme assay. The starting reaction volume was adjusted to 1 ml and aliquots of DTNB in 5 mM sodium phosphate buffer, pH 7.4, were added to give a desired final concentration. Absorbance at 412 nm and LCAT activity were monitored following each addition of DTNB. In the control, 5 mM sodium phosphate buffer was used instead of the enzyme solution.

Tyrosine Titration

For the tyrosine residue titration, 0.5 ml of purified LCAT (0.52 mg/ml in 10 mM sodium phosphate buffer, pH 7.2) was
diluted up to 2 ml with the same buffer. The enzyme solution was placed in a double-beam spectrophotometer in a 1 cm cell. The pH of the solution was gradually increased by the addition of small aliquots (1-14 ul) of KOH solutions (3-12 M). When the absorbance at 295 nm reached a plateau, the enzyme was back-titrated by the addition of aliquots (1-23 ul) of HCl (1-12 M). After titration, the same volume of saturated guanidine hydrochloride was added to the solution and the pH was adjusted at 13.7 with 12 N KOH. The changes in the absorbance at 295 nm were corrected for the increase in sample volume. The concentration of tyrosine residues was calculated from the recorded absorbance using molar extinction coefficient of tyrosine at 295 nm of 2,300 (Beaven and Holiday, 1952). N-acetyl-L-tyrosinamide was used as a standard.

Chemical Modification and Radioaffinity Labeling of the Enzyme

Serine, cysteine and histidine residues have been implicated as the amino acid residues at the active site of human LCAT (Jauhiainen and Dolphin, 1986). The serine residue of hog LCAT was modified by diisopropyl fluorophosphate (DFP) essentially as described by Parkin et al. (1982). LCAT in 1 mM sodium phosphate buffer, pH 7.4 (0.32 mg in 1 ml) was incubated with 0.5 - 10 mM DFP for 30 min at 24 °C. The excess DFP was removed from the reaction mixture by dialysis against 5 mM sodium phosphate, pH 7.4. Activity of the enzyme was measured using proteoliposome substrate (Chen and Albers,
1982). The time course of DFP inactivation was performed by incubation of 30 ul of LCAT (0.32 mg/ml) with 0.5 mM DFP (final concentration) for 0 to 30 min at 24 °C. Quantitation of the modified serine and its relationship to LCAT activity was carried out using \(^{3}\text{H}\)-DFP (0.3 mCi/mg of enzyme) followed by assays under the condition described above. The same amount of radiolabeled DFP was added 3 times with 20 min incubation each addition. The disappearance of the enzyme activity was monitored by enzyme assays at different times from 2 min to 30 min following the addition of radioactive DFP. After incubation, the reaction mixture was dialyzed extensively against 0.1 M acetic acid, pH 3.0. The number of modified serine residues was calculated by counting the radioactivity incorporated into the enzyme.

The cysteine residues of hog LCAT were modified by N-ethymaleimide (NEM) using the same approach as described above for the modification of serine by DFP. The enzyme solution (0.32 mg in 1 ml) was extensively dialyzed against 2 mM DTT with nitrogen bubbling. The concentration range of NEM was 0.5-30 mM and the time course of NEM inactivation was performed identically to that of DFP inactivation. For the quantitation of the modified cysteine residues in LCAT, \(^{3}\text{H}\)-NEM was incubated with intact hog LCAT in the presence of 6 M guanidine hydrochloride or guanidine hydrochloride plus 2 mM DTT. When DTT was used, the nitrogen bubbling was continued during dialysis to prevent reoxidation.

The histidine residues of proteins have been shown to be
modified by diethylpyrocarbonate (DEP) (Miles, 1977). The stock DEP solution was prepared (100 mM) using anhydrous ethanol and control experiments were carried out by incubating the enzyme with ethanol alone. The amount of ethanol used in this experiment (maximum concentration, 5 percent, v/v) did not affect the enzyme activity. For the time course of DEP inactivation, 0.5 mM DEP was incubated with hog LCAT (0.32 mg/ml) for 0 to 30 min. at 24 °C.

**Proteolytic Digestions**

Trypsin and pepsin were used for the digestion of \[^{3}H\]-NEM- and \[^{3}H\]-DFP-labeled hog LCAT, respectively.

For the tryptic digestion, the radiolabeled enzyme was dialyzed against 10 mM ammonium bicarbonate buffer, pH 7.8, and lyophilized in a conical-shaped vial (Reacti-Vial). The lyophilized enzyme was redissolved in 10 mM Tris buffer, pH 7.0 to adjust the final concentration of the enzyme to 1 mg/ml. Solid trypsin was weighed and dissolved in the same buffer (concentration, 1 mg/ml). The trypsin solution was added to the enzyme solution to give an approximate ratio of 1/20 (w/w, trypsin/LCAT). The mixture was vortexed for 1 min followed by the incubation at 37 °C for 10 min. After the incubation period, the same amount of trypsin was added and the incubation continued for another 10 min. During the incubation, the reaction mixture was mixed every 5 min on a vortex. The tryptic digestion was stopped by the addition of glacial acetic acid (final concentration, 15 percent, v/v) and the reaction mixture was placed on ice for 2 hours. Then, the
mixture was transferred to a 1.5 ml conical plastic vials and centrifuged for 15 min. The supernatant solution was collected and the undigested pellet was discarded. The degree of digestion was estimated as follows: aliquots of the sample (10 µl each) were placed on a piece of filter paper (diameter, 3 cm, Whatman #1) before the addition of trypsin, 10 min after trypsin addition and after the reaction was completed. The filters were dried thoroughly and subsequently soaked in hot (80 °C) 10 percent (w/v) trichloroacetic acid (TCA) solution for 2 hours. Then the filters were gently shaken in the TCA solution and washed twice in beakers containing 20 ml of ethanol and 20 ml of diethyl ether. After drying, the filters were placed in a scintillation vial and the radioactivity remaining on the filters was determined. Protein molecules are expected to stay on the filter as a precipitate whereas peptides are washed out. The radioactivity on the filter containing the sample prior to the tryptic digestion thus is presumed to represent the amount of total protein; the sample following the digestion represents the remaining undigested protein due to the incomplete digestion.

For the peptic digestion, 2 ml of [3H]-DFP labeled enzyme (0.32 mg/ml) as described above was incubated with pepsin (1/10 of the enzyme mass in 0.1 M acetic acid solution, pH 2.0) at 37 °C for 30 min (Boyer, 1971). The diisopropylphosphoryl (DIP)-LCAT was extensively dialyzed against the acetic acid solution before the addition of the pepsin. The pepsin solution was added three times
individually (1/30 of enzyme mass per addition) and the mixture was allowed to react for 10 min each addition. The reaction was stopped by the addition of 1 drop of 6 M NaOH. The sample was vortexed every 5 min during the incubation. The extent of digestion was estimated as described for the tryptic digestion.

**Isolation of Peptides**

The peptides from the proteolytic digests were separated by HPLC. The samples were lyophilized and redissolved in 100 ul of 0.1 percent trifluoroacetic acid (TFA). The samples were injected into an HPLC (Bio-Rad Model 1330 and 1305) equipped with a Bio-Rad C_{18} reversed-phase column (4.6 x 250 mm, 5 \text{ um bead size}). The peptide isolation was achieved by a gradient buffer system from 100 percent Buffer A (0.1 percent aqueous TFA) to 40 percent Buffer-A (60 percent Buffer B, which is 0.1 percent TFA in acetonitrile) in 90 min. This was controlled by a computer program from Bio-Rad (Bio-Rad Laboratory Gradient Processor System) interfaced with an Apple IIe computer. The absorbance at 220 nm was recorded to monitor the elution of peptides. The peptides from the HPLC were collected manually. Efforts were made to collect the peptide peak free of contaminants by changing the collection tube frequently and the tubes were individually subjected to the analyses.

**Amino Acid Sequence Analyses of the Enzyme**

Automated Edman degradation (Edman, 1950) was performed
on Applied Biosystems 470A gas phase sequencer with on-line ABI 120 PTH-analyzer. In order to improve the yield and confidence of the heat-labile serine and threonine residues assignment, sequencing was repeated with a program in which temperatures were decreased by 5 °C. During the sequencing of whole enzyme for the N-terminal residues and \(^{3}H\)-NEM labeled tryptic peptides, polybrene was used as a carrier. The \(^{3}H\)-DFP-labeled peptic peptides (less than 100 pmol) were sequenced on TFA-treated glass fiber filter discs in the absence of the carrier polybrene. PTH-amino acids were separated on an ABI Spheri-5 PTH column (C\(_{18}\), 5 µm, 2.1 x 220 mm) using eluent from the same manufacturer. Those are: eluent A; 38 ml of 3 M sodium acetate buffer, pH 3.8, 9 ml of 3 M sodium acetate buffer, pH 4.6 and 1000 ml of 5 percent tetrahydrofuran in water: eluent B; neat acetonitrile. Separation was carried out at 59 °C with a linear gradient from 10 percent B to 50 percent B in 25 min. PTH-amino acids were identified and quantitated by comparison with PTH-amino acid standards.

**Reaction of Hog LCAT with Monoclonal Antibody against Active Site Region of Human LCAT**

Purified hog human LCAT (8-10 µg) were incubated with various amounts of the concentrated tissue culture medium which is from the preparation of the monoclonal antibody against human LCAT (protein concentration, 23.1 mg/ml). The amount of antibody varied from 0.1 mg to 0.5 mg of protein. The incubation was carried out at 37 °C and the residual LCAT
activity was measured as described by Chung et al. (1979).

**Other Methods**

**Preparation of HDL Substrate and LCAT Assay** - The HDL was prepared as described previously (Rudel, et al., 1974). The HDL solution containing approximately 50 mg of apoproteins was mixed with 10 ml of an emulsion of [1,2-\(^3\)H]-cholesterol (5 uCi/ml) in 10 percent (w/v) fatty acid-free BSA (Lacko et al., 1973). To this emulsion, a solution of BSA in a buffer was added to achieve a final volume of 100 ml and a final BSA concentration of 2 percent (w/v). The buffer for the substrate was 0.01 M tris, 0.005 M EDTA, 0.15 M NaCl and 0.005 M 2-mercaptoethanol, pH 7.4 (all final concentrations). Assays were carried out using the method shown previously (Glomset and Wright, 1963; Lacko et al., 1973). Samples of 5-10 ul containing 0.02-0.5 ug of LCAT were incubated with 200 ul of the HDL substrate at 37 °C. The reaction time was adjusted so that initial rates (not exceeding 5 percent esterification) were obtained. Assays were performed yielding an average error of less than 5 percent in duplicate. The assay mixture contained approximately 740,000 dpm/ml of the radioactivity amounting to a cholesterol concentration of 0.036 nmol/ml. At the end of the incubation period, the reaction was stopped by the addition (4 ml) of chloroform : methanol (2:1, v/v). The lipid extract was heated for 15 min at 60 °C and filtered on a 30-ml coarse sintered glass funnel. The residue was washed twice with 2 ml of the chloroform : methanol and then evaporated under a nitrogen stream. The dry
residue was dissolved in a small amount of chloroform : methanol mixture and evaporated again. The lipids were finally dissolved in 30 ul of n-heptane and applied to a silica gel sheet (20 x 10 cm) with a plastic backing for thin layer chromatography (TLC). The TLC was carried out in a solvent mixture petroleum ether : diethyl ether : acetic acid (90:10:1, v/v/v). The lipids were visualized by exposing the air-dried plate to iodine vapors. After evaporation of the excess iodine, the zones containing cholesterol and cholesteryl ester were cut out of the plate and placed in scintillation vials for radioactivity counting. The radioactive zone containing cholesteryl esters were analyzed and the percent esterification was established for each assay. The rate of esterification was computed as a product of the percent esterification per hour and that of the unesterified cholesterol concentration in the sample.

**Protein Determination** - Determination of protein concentration was carried out as described by Bradford (1976) using fetuin for LCAT and BSA for the other proteins as standards.

**Analytical Gel Electrophoreses** - The electrophoreses were carried out in the presence of 0.1 percent (w/v) SDS and 7 M urea using the methods described by Lamml (1973) and Kane (1973), respectively.

The methods for molecular weight determination, deglycosylation, carbohydrate analyses and amino acid composition analysis of hog LCAT are not described in this
dissertation because the detailed methods have been presented in the author's Master's thesis (Park, 1984).
CHAPTER III

RESULTS

Enzyme Purification

The procedure described here is an improved version of an earlier published method (Park, 1984).

Step 1. PEG-6000 precipitation - It has been established that polymers such as heparin, dextran sulfate (charged polymer), dextran and polyethylene glycol (neutral polymer) precipitate lipoproteins selectively (Burstein and Scholnick, 1973; Iverius, 1968; Viikari, 1976). The charged polymers tend to form molecular complexes with lipoproteins, especially in the presence of divalent cations. Iverius and Laurent (1967) reported that VLDL and LDL precipitate from the plasma at a concentration of 4 percent PEG-6000, but HDL does not precipitate until 12-15 percent PEG. This difference allowed the separation of HDL-LCAT complexes from VLDL and LDL at the early stage of the purification procedure. Six percent PEG-6000 (w/v) precipitated VLDL and LDL effectively without altering the enzyme activity. As much as 96 percent of the original LCAT activity was recovered from the PEG-supernatant.

Step 2. Phenyl-Sepharose Chromatography - In this step, the hydrophobic affinity between the HDL-LCAT complex and the phenyl group of the column materials was utilized by varying the ionic strength of the elution buffer. The elution
patterns of the chromatography are shown in Figure 2-A. A large amount of plasma protein was bound to the column in the presence of a high ionic strength medium as the PEG-supernatant was loaded onto the column. As the column was washed with 5 mM sodium phosphate buffer, pH 7.4, containing 50 mM NaCl, most of the contaminating proteins was eluted leaving the enzyme on the column. The LCAT activity was subsequently eluted by applying deionized water to the column. A more than 200-fold purification was achieved by this step. Mainly HDL components and LCAT were in the enzyme preparation. The enzyme may be protected from the possible inactivation in the high ionic strength buffer (Doi and Nishida, 1981) due to its association with HDL at this stage. The phenyl-Sepharose exhibited a large capacity (approximately 250 mg/ml of gel) for the binding of proteins and was highly efficient as no further treatment of the enzyme pool, such as concentration and/or dialysis, was required before proceeding to the next step of the purification.

**Step 3. DE-52 Cellulose Chromatography** - This step was designed to utilize the charge differences between the enzyme and the contaminants present in the sample. Most of the contaminants (mainly HDL constituents) were eluted from the column at low ionic strength. The enzyme activity appeared following the elution of two large absorbance peaks (Figure 2-B). The fractions containing LCAT activity had a relatively small amount of protein (absorbance at 280 nm, less than 0.05). About 15 percent of total enzyme activity was eluted
Figure 2. Representative column chromatographic profiles obtained from the phenyl-Sepharose (A), DE-52 cellulose (B) and hydroxylapatite (C) columns for the purification of LCAT from hog plasma. Approximately 980 ml of PEG-supernatant was loaded on a phenyl-Sepharose column (5 x 25 cm) equilibrated with 5 mM sodium phosphate buffer, pH 7.4, containing 0.3 M NaCl. The column was washed with 5 mM sodium phosphate buffer, pH 7.4, containing 0.05 M NaCl and the enzyme was eluted with deionized water. The enzyme fractions were diluted with 0.5 mM sodium phosphate buffer, pH 7.4 and loaded on a DE-52 column (2.5 x 30 cm) equilibrated with 5 mM sodium phosphate buffer, pH 7.4. A linear NaCl gradient from 50 mM sodium phosphate buffer, pH 7.4 (1000 ml), to the same buffer containing 0.13 M NaCl (500 ml) was then applied. The enzyme pool was subjected to the hydroxylapatite chromatography (column size, 2.5 x 20 cm) after concentration and dialysis against 1 mM sodium phosphate buffer, pH 7.4. A linear phosphate gradient was applied from 1 mM to 0.1 M, pH 7.4 (500 ml each) for the enzyme elution. (See text for the detailed information). (A280), (LCAT activity) and (conductivity).
in the final peak generated by washing with 0.3 M NaCl in 5 mM sodium phosphate buffer, pH 7.4. This protein peak was discarded because the interaction between enzyme and the contaminants was strong rendering the following purification step inefficient. The volume of enzyme pool could be reduced by utilizing a DE-52 cellulose column (2.5 x 5 cm). Approximately 400 ml of DE-52 pool was passed through the short column in 2 hours. The bound enzyme at low ionic strength medium was eluted with 0.15 M NaCl in sodium phosphate buffer, pH 7.4 (final volume, 25 ml). The enzyme activity was well-preserved (recovery, 92 percent) and more than 90 percent of total protein was recovered by this concentration method. The enzyme pool was further concentrated up to 5 ml using vacuum dialysis for the final step of the purification. The enzyme pool contained mainly LCAT, serum albumin and apo-HDLs as judged by SDS-PAGE.

Step 4. Hydroxylapatite Chromatography - The chromatographic profile of this step is shown in Figure 2-C. The dialyzed enzyme pool from the DE-52 cellulose chromatography was subjected to this step. An elution pattern essentially identical to the previously reported one was observed (Park and Lacko, 1986; Park, 1984). In order to eliminate the most persistent contaminant (apoA-I), the enzyme pool from the previous step was divided into two parts to reduce the protein loading on the hydroxylapatite column. A single sharp absorbance peak at 280 nm appeared immediately after the sample application at the early stage of sodium
phosphate gradient (1 mM to 100 mM). This peak contained more than 90 percent of the total enzyme activity and the contaminants were found in the later peak. The enzyme pool routinely exhibited a single band on the SDS-PAGE.

**Evaluation of the Purification Procedure** - The enzyme prepared by the procedure described was consistently homogeneous as judged by polyacrylamide gel electrophoreses in the presence of 0.1 percent of SDS and 7 M urea (Figure 3). In addition, this preparation was found to be free of serum albumin and apoA-I as demonstrated by immunodiffusion experiments using antisera raised against porcine serum albumin and apoA-I. Table II shows the yield and degree of purification produced by the procedure described. Generally up to 1.2 mg of enzyme could be obtained from 1 liter of hog plasma with an approximately 22,000-fold increase in the specific activity. Each step of the purification was highly reproducible over a 2 year period and the whole procedure could be completed in less than ten days. The purified enzyme was stable in the frozen state at -20 °C for at least two weeks or as an ammonium sulfate precipitate (Jahani and Lacko, 1982) for at least 2 months.

**Substrate Specificity of the Enzyme**

The rate of cholesterol esterification was compared using the major hog lipoproteins as substrates for purified LCAT. Figure 4 shows the substrate potential of hog lipoproteins for the LCAT reaction. The lipoprotein substrates (HDL, LDL and VLDL) were used at free cholesterol concentrations ranging...
Figure 3. Polyacrylamide gel electrophoreses of purified LCAT. Approximately 20 μg of protein was applied to the gel and electrophoresis was carried out in the presence of 0.1 percent (w/v) SDS (left) or 7 M urea (right) according to the methods described by Laemmli (1973) and Kane (1973), respectively.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>1,000</td>
<td>142,000</td>
<td>181,000</td>
<td>1.27</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Polyethylene glycol Supt.</td>
<td>975</td>
<td>98,200</td>
<td>174,000</td>
<td>1.77</td>
<td>96</td>
<td>1.4</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>425</td>
<td>450</td>
<td>123,000</td>
<td>272</td>
<td>68</td>
<td>220</td>
</tr>
<tr>
<td>DE-52 Cellulose</td>
<td>386</td>
<td>100</td>
<td>79,000</td>
<td>782</td>
<td>44</td>
<td>620</td>
</tr>
<tr>
<td>Hydroxylapatite**</td>
<td>64</td>
<td>1.15</td>
<td>31,800</td>
<td>27,700</td>
<td>18</td>
<td>22,000</td>
</tr>
</tbody>
</table>

* One unit of enzyme catalyzes the esterification reaction of 1 nmol of unesterified cholesterol per hour at 37 °C with HDL substrate as described in "Methods".

** The DE-52 Cellulose pool was divided by two equal parts and two batches of hydroxylapatite chromatographies were operated for each preparation. The calculations were based on the combined batch.
Figure 4. The rate of esterification as a function of free cholesterol concentration in the substrates. Aliquots of HDL (●), LDL (○) and VLDL (▲) preparations containing 20-200 nmol/ml of free cholesterol were preincubated with 20 ul of fatty acid free BSA-[3H]-cholesterol emulsion. The final assay volume was adjusted to 200 ul and the reaction was initiated by the addition of 0.54 ug of purified hog LCAT. The incubation time was adjusted to obtain the initial rate of cholesterol esterification.
from 20 to 200 nmol/ml. The maximal enzyme activity obtained with HDL as a substrate was about twice that obtained with LDL, whereas the activity with VLDL was less than 20 percent of that found with HDL. The relatively high enzyme activity observed upon incubation with LDL was somewhat surprising as there is generally no apoA-I or other suitable polypeptide cofactor present in this lipoprotein fraction. The observed LCAT activity with LDL as substrate appears to be due to a minor contamination of LDL with apoA-I containing-particles. When the same LDL preparation was first passed through an immunoaffinity column containing immobilized antibody against apoA-I, LDL could no longer serve as a substrate for LCAT.

The distribution of LCAT activity in whole plasma was also investigated. Hog plasma was labeled with \(^{3}\text{H}\)-cholesterol and subsequently fractionated on a molecular sieve column (Bio-Gel A-5m). Three distinct peaks of radioactivity appeared corresponding to the elution volume of lipoprotein standards (HDL, LDL and VLDL). Most of the LCAT activity was associated with the higher molecular weight portion of HDL (Figure 5). These data suggest that hog LCAT is associated with HDL in the plasma and therefore prefers HDL as a substrate.

In order to study the interaction between LCAT and HDL components, fluorometric studies were carried out using artificial substrates. As apoA-I is the predominant protein constituent of HDL, the fluorescence quenching of the tryptophan residues of LCAT were measured upon incubation with
Figure 5. Distribution of LCAT in hog plasma. Three milliliters of hog plasma was labeled with $[^3H]$-cholesterol (0.01 mCi/ml) and loaded onto a Bio-Gel A-5m column (1.5 x 90 cm). The column chromatography was carried out at a flow rate of 25 ml/hr and the radioactivity (●) and LCAT activity (○) were monitored.
macromolecular complexes of lipids and proteoliposomes consisting of phosphatidylcholine, cholesterol and apoA-I. Figure 6 shows the degree of fluorescence quenching of LCAT produced by the addition of the artificial substrates. The emission spectra at 335 nm was monitored followed by the excitation of the molecules in the reaction mixtures at 288 nm (see 'Methods'). The intrinsic fluorescence of LCAT was compared to that of the reaction mixtures where LCAT was incubated with model substrates. Data were expressed as a percent quenching as function of the amount of artificial substrates present. Up to 12 percent of the LCAT fluorescence was quenched by incubating with proteoliposome substrate. No quenching was observed with phosphatidylcholine micelles alone or with phosphatidylcholine : cholesterol mixed micelles. Apparently, the enzyme binds more efficiently to the apoA-I containing particle than to the lipid micelles alone.

Physicochemical Properties of the Enzyme

A large proportion of the results including molecular weight determinations, amino acid composition analysis and carbohydrate analysis of the enzyme has been described in the author's master's thesis (Park, 1984). Here, these findings are summarized to describe the properties of hog LCAT.

Molecular Weight of the Enzyme

The apparent molecular weight of purified hog LCAT was 66,000 ± 3,000. This was in good agreement with the molecular weight determined by HPLC. The molecular weight of the
Figure 6. Fluorescence quenching of hog LCAT caused by the incubation with phosphatidylcholine (PC) micelles (1), mixed micelles (2) and proteoliposomes (3). Intrinsic fluorescences of purified hog LCAT and the liposome vesicles were determined individually. Then, the fluorescence of the mixed solutions were measured after incubation of LCAT with each vesicle at 37 °C for 4 hours. The degree of quenching was expressed as a percent of the difference in the emission fluorescence between before and after the incubation compared to the intrinsic fluorescence of the enzyme. Emission spectra at 335 nm with an excitation at 288 nm were used. The composition of liposome vesicles are: PC to cholesterol was 250 to 12.5 (molar ratio) for the mixed micelles; PC to cholesterol to apo-AI was 250 to 12.5 to 1 (molar ratio) for the proteoliposomes and 100 percent PC for the PC micelles. Homogeneous size of the liposome vesicles were used after the vesicles were subjected to the Sepharose-4B column chromatography. Amounts of the liposome vesicles added are expressed as the amounts of PC added.
polypeptide moiety of the enzyme was determined to be approximately 52,000, which is 78.8 percent (w/w) of total mass of the intact enzyme. The carbohydrate content determined by chemical and enzymatic deglycosylation studies showed a good agreement with the results of carbohydrate analysis (21.4 percent, w/w).

**Amino Acid and Carbohydrate Composition of the Enzyme**

The amino acid compositions of the purified hog and human LCAT are shown in Table III. The molecular weight of the polypeptide moiety of hog LCAT (52,000) was obtained by subtracting the weight of carbohydrate moiety (21 percent, w/w: Table III) from 66,000 and by the chemical and enzymatic deglycosylation studies determined by SDS-PAGE. The enzyme showed a relatively high content of glycine and leucine.

Table III compares the carbohydrate composition of hog and human LCAT. Hog and human LCAT have a relatively large carbohydrate content. Total content of carbohydrate (21.4 percent, w/w) in hog LCAT was in good agreement with the results from the deglycosylation studies, which showed 14,000 molecular weight difference (21.2 percent, w/w) between before and after deglycosylation.

**Sulfhydryl Groups of the Enzyme**

Figure 7 shows a time course of the DTNB titration of hog LCAT at pH 8.0 in the presence and absence of SDS. There were two titrable sulfhydryl groups per mol of LCAT under non-denaturing conditions and 4 residues per mole under
<table>
<thead>
<tr>
<th>Amino Acid Composition</th>
<th>Hog (Residues/100,000 g of Protein)</th>
<th>Hog (Residues/52,000 g of Protein)</th>
<th>Human (Residues/100,000 g of Protein)</th>
<th>Human (Residues/45,000 g of Protein)</th>
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<tbody>
<tr>
<td>Lysine</td>
<td>42.5 (0.47)</td>
<td>22.1 (0.24)</td>
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<td>Histidine</td>
<td>23.4 (0.47)</td>
<td>12.3 (0.44)</td>
<td>28</td>
<td>12</td>
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<tr>
<td>Arginine</td>
<td>41.0 (1.35)</td>
<td>21.3 (0.61)</td>
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<tr>
<td>Aspartic Acid</td>
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<td>47.8 (2.81)</td>
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<td>41</td>
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<tr>
<td>Threonine</td>
<td>59.2 (2.49)</td>
<td>30.8 (1.12)</td>
<td>54</td>
<td>24</td>
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<tr>
<td>Serine</td>
<td>44.5 (3.82)</td>
<td>23.1 (1.72)</td>
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<td>24</td>
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<tr>
<td>Glutamic Acid</td>
<td>101 (7.76)</td>
<td>52.7 (3.49)</td>
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<td>38</td>
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<tr>
<td>Proline</td>
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<td>33.2 (1.44)</td>
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<td>glycine</td>
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<td>43.5 (7.13)</td>
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<td>31.5 (2.74)</td>
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<tr>
<td>Valine</td>
<td>61.3 (2.42)</td>
<td>31.9 (1.09)</td>
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<td>25</td>
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<td>Methionine</td>
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<tr>
<td>Isoleucine</td>
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<td>16.4 (0.32)</td>
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<td>15</td>
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<tr>
<td>Leucine</td>
<td>76.0 (2.18)</td>
<td>39.5 (1.05)</td>
<td>114</td>
<td>50</td>
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<tr>
<td>Tyrosine</td>
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<td>21.2 (0.96)</td>
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<td>19</td>
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<tr>
<td>Phenylalanine</td>
<td>39.1 (1.73)</td>
<td>20.4 (0.78)</td>
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<td>Half-cystine</td>
<td>8.5 (2.85)</td>
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<td>4</td>
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<tr>
<td>Tryptophan</td>
<td>17.3 (3.46)</td>
<td>8.9 (1.80)</td>
<td>23</td>
<td>10</td>
</tr>
</tbody>
</table>

Carbohydrate Composition (% w/w)

- Polypeptide: 78.6
- Carbohydrate: 21.4
- Hexoses: 11.3
- Hexosamines: 1.9
- Sialic Acids: 8.2

-Continued-
<table>
<thead>
<tr>
<th>TABLE III - Continued</th>
</tr>
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</table>

The values are the average of five independent determinations conducted on 24-, 48- and 72 hours hydrolysates. Standard deviations are in the parentheses.

- **a** Adopted from Chong et al., 1983b.
- **b** Aspartic acid is the sum of aspartic acid and asparagine.
- **c** Obtained by the zero-time extrapolation
- **d** Glutamic acid is the sum of glutamic acid and glutamine.
- **e** Determined with Beckman amino acid analyzer (Spackman and Moore, 1958).
- **f** Determined by performic acid oxidation (Hirs, 1967).
- **g** Determined by fluorometric method (Sasaki et al., 1975). Average of two independent determinations.
- **h** Determined using anthrone reagent (Roe, 1955; Spiro, 1966).
- **i** Determined using the colorimetric method after mild acid hydrolysis (Johnson, 1977).
- **j** Determined using thiobarbituric acid method by Skoza and Mohos, 1976.
Figure 7. Time course of thiol group titration with purified hog LCAT. The enzyme solution (0.263 mg/ml of 0.3 M sodium phosphate buffer, pH 8.0) was titrated with 0.4 mM DTNB in the presence (●) and absence (○) of 2 percent of SDS.
denaturing conditions. Performic acid oxidation data for amino acid composition analysis revealed 4 half cystines per mole of the enzyme (Table III).

The substitution of thionitrobenzoic LCAT (TNB-LCAT) with a smaller molecule, cyanide ion, was attempted to show the essential nature of thiol group(s) for LCAT activity. The enzyme was first inactivated by DTNB and it did not regain its activity when the TNB molecule was replaced by the cyanide ion (Table IV). This observation suggests that DTNB reacted with the thiol group(s) essential for the catalytic activity of LCAT. The substitution of cyanide ion for the TNB-LCAT was demonstrated spectrophotometrically by monitoring the release of TNB ion (absorbance at 412 nm) after the KCN solution was added. The reaction mixture was thoroughly dialyzed before the addition of KCN and the absorbance background at 412 nm was thus minimized. An increment in the absorbance was clearly detectable when KCN was added, although it was too small to quantitate.

DTNB could not inactivate the enzyme at enzyme concentrations greater than 0.2 mg/ml. The inactivation of LCAT by DTNB at different enzyme concentrations was thus investigated. The enzyme remained active at the concentrations greater than 0.2 mg/ml even in the presence of 0.7 mM DTNB (data not shown). However, at enzyme concentrations less than 0.1 mg/ml, the enzyme activity was rapidly and nearly completely abolished by the same concentration of DTNB. The concentration of the enzyme that
TABLE IV
REACTION OF TNB-LCAT WITH DTT AND KCN

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Percent Initial Activity</th>
<th>Percent Initial Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DTNB</td>
<td>DTNB</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>1.0</td>
<td>2.1</td>
</tr>
<tr>
<td>25</td>
<td>33.2</td>
<td>0.5</td>
</tr>
<tr>
<td>30</td>
<td>48.5</td>
<td>1.2</td>
</tr>
<tr>
<td>35</td>
<td>43.2</td>
<td>20.8</td>
</tr>
<tr>
<td>40</td>
<td>48.2</td>
<td>37.5</td>
</tr>
</tbody>
</table>

Hog LCAT (0.45 mg) was allowed to react with 1 mM DTNB for 20 min. at room temperature. After extensive dialysis against 50 mM sodium phosphate buffer, pH 7.0, 10 mM DTT or KCN was added to the reaction mixture. Enzyme assays were carried out with aliquots of the reaction mixtures as a function of the reaction time. All reactions were performed in a 300 ul volume at 25 °C in 50 mM sodium phosphate buffer, pH 7.0. The enzyme activities were compared with those of the control samples that contained the enzyme and buffer. The substitution of cyanide ion for the TNB-LCAT was demonstrated spectrophotometrically by monitoring the release of TNB ion (absorbance at 412 nm).
yielded one-half of the maximal activity was found to be approximately 0.16 mg/ml. On the other hand, when the enzyme (0.42 mg/ml) was extensively dialyzed against DTT (2 mM) with nitrogen bubbling, as little as 30 μM DTNB eliminated approximately 50 percent of the enzyme activity even at the high enzyme concentration (Figure 8).

**Tryptophan Residues of the Enzyme**

As LCAT has been proposed to exist in a complex with HDL in the plasma, the enzyme may have hydrophobic amino acid residues exposed to the aqueous environment. The availability of tryptophan residues to the aqueous solution was estimated using fluorometry. Figure 9 shows the tryptophan content (relative intensity of fluorescence) of the native enzyme and protease-digested enzyme. For the protease digestion, the enzyme solution was heated in boiling water for 5 min and subsequently digested with pronase and chymotrypsin. Hog LCAT was found to contain 9 residues of tryptophan per mole of enzyme. Five out of these 9 residues were apparently exposed to the aqueous environment under non-denaturing conditions.

**Phenolic Groups of the Enzyme**

LCAT was subjected to spectrophotometric titration. The pH of the enzyme solution was gradually increased to 13 and the changes in the state of ionization of phenolic groups were monitored spectrophotometrically (295 nm) as described by Chong et al., (1983a). As shown in Figure 10, the tyrosine residues of the enzyme remained unionized up to about pH 11.
Figure 8. Concentration-dependence of DTNB inactivation of purified hog LCAT with (open symbols) and without (closed symbols) dialysis against 2 mM DTT. LCAT activity (triangles) and number of thiol titrated (OD 412 nm) (circles) were monitored.
Figure 9. Fluorometric determination of tryptophan content in hog LCAT in native (B) and denatured (A) conditions. The fluorescence emission was measured at 335 nm with excitation at 288 nm. Aliquots of standard tryptophan solution (1-15 nmol) were added to the enzyme solution and the relative intensity of the fluorescence emission was extrapolated to zero to determine the quantities of tryptophan present in the enzyme. In order to denature the enzyme, solution was boiled and digested with pronase and chymotrypsin as described in 'Methods'. The tryptophan solution was used as a standard (C).
NMOLES OF TRYPTOPHAN ADDED

RELATIVE INTENSITY OF FLUORESCENCE

NMOLES OF TRYPTOPHAN ADDED

2.4 3 4.35 6 9 12 15

A  B  C
Figure 10. Spectrophotometric titration of purified hog LCAT (●) and N-acetyl-L-tyrosinamide (△) at 295 nm. LCAT solution (0.52 mg in 2 ml of 10 mM sodium phosphate buffer, pH 7.2) was titrated by the addition of small increments (1-24 ul) of KOH (3-12 M) (A) and HCl (1-23 ul of 1-12 M) (B) solution.
A characteristic pK value of about 11 was obtained, which is distinct from the apparent pK value of the control N-acetyl-L-tyrosinamide. This phenomenon could be reversed by back-titration of the enzyme, although enzyme activity could not be recovered. The absorbance data allowed the calculation of the number of exposed tyrosine residues per mole of enzyme assuming a molar extinction coefficient of the phenolic group at 295 nm of 2,300 (Chong et al., 1983a). The maximum number of phenolic groups titrated was 13 residues per mole of enzyme. This finding does not agree with the data obtained from amino acid composition analysis that yielded 21 residues per mole. The discrepancy between the two studies was found to be due to the incomplete unfolding of the enzyme by pH changes alone. Consequently, when the enzyme was further denatured by 6 M guanidine hydrochloride, approximately 19 phenolic groups were titrated at pH 13.

Chemical Modifications of the Enzyme

The activity of hog LCAT was modified by utilizing specific reagents reacting with amino acid side chains of the enzyme. As shown earlier, cysteine residue of hog LCAT appeared to be essential for activity. Figure 11 shows the inactivation of hog LCAT by the modification of cysteine (A), serine (B) and histidine (C) residues. The sulfhydryl modifications were accomplished utilizing DTNB and NEM (Figure 11-A, final concentration, 0.5 mM). Hog LCAT was rapidly inactivated by DFP. Eighty percent of total the enzyme activity was abolished in the presence of 0.5 mM DFP within
Figure 11. Chemical modifications of cysteine (A), serine (B) and histidine (C) residues of hog LCAT. N-ethylmaleimide (●) and DTNB (○) for the sulfhydryl, diethylpyrocarbonate for the histidine and diisopropyl fluorophosphate for the serine residues were used. All the concentrations of chemicals were 0.5 mM and the samples were extensively dialyzed against 5 mM sodium phosphate buffer, pH 7.4, after reaction. The enzyme activity remained after the reaction was compared with that of control experiment that does not contain the chemicals.
seconds. Figure 11-B shows the time course of the DFP inactivation (final concentration, 0.5 mM) of the enzyme.

Diethylpyrocarbonate (DEP) selectively modifies histidine residues at low concentrations and at neutral pH (Miles, 1977). The time course of DEP inactivation of hog LCAT is shown in Figure 11-C (final concentration of DEP, 0.5 mM). Ninety five percent of the enzyme activity was abolished in 5 min of incubation. During the reaction, there was increase in absorbance at 245 nm, indicating formation of N-carbethoxyhistidyl derivatives of LCAT (Muhrad et al., 1969). There was no concomitant decrease in the absorbance at 278 nm, indicating that O-carbethoxylation of tyrosine residues had not occurred (Tudball et. al., 1972). These data suggest that at least one histidyl residue of the enzyme is involved in the catalytic activity.

Attempts to protect the enzyme against chemical inactivation were made using HDL and proteoliposome substrates. None of these substrates could protect the enzyme against inactivation via chemical modifications.

Radioaffinity Labeling and Quantitation of DFP-Reactive Serine Residues

The stoichiometry of the inactivation of hog LCAT was determined in order to estimate the number of amino acid residues involved in the chemical modification. [³H]-DFP was employed to quantitate the modified serine residue. When hog LCAT (0.64 mg in 2 ml of 1 mM sodium phosphate buffer, pH 7.4) was incubated with [³H]-DFP (0.3 mCi/mg of enzyme), the enzyme
activity was completely abolished in 5 min. After extensive
dialysis against 0.1 M acetic acid, the radioactivity
corresponding to 0.89 mole of $[^3\text{H}]-\text{DFP}$ per mole was
incorporated into the enzyme.

**Amino Acid Sequence Analyses of the Enzyme**

**Amino Terminus** — The purified enzyme was subjected to
automated Edman degradation as described in "Methods". The
enzyme was extensively dialyzed against 1 mM sodium phosphate
buffer, pH 7.4, before being subjected to sequence analysis.
Samples were spotted on glass fiber filters treated with
polybrene. The polybrene was used as a carrier matrix to avoid
excessive peptide loss due to washout. Thus, 34 amino acid
residues were tentatively identified in the amino terminal
region in a single analysis. The identity of the assigned
amino acids was confirmed by repeated sequencing. Table V
shows the amino terminal sequence of hog and human LCAT. The
sequence of the human enzyme is from the amino acid sequence
deduced from the cDNA sequence by McLean et. al. (1986). The
entire sequence of the human enzyme is shown in the Appendix
(page 117).

The amino terminal residues 20, 30 and 31 could not be
identified. Presumably, one of these positions may be
occupied by cysteine. Free cysteine is not stable under the
reaction condition of sequence analysis and its derivative
cannot be identified at 269 nm, the wave length for the
detection of the PTH-amino acid derivatives. Figure 12-A
shows a representative plot of the yields per cycle from the
### TABLE V

AMINO TERMINAL SEQUENCE OF HOG AND HUMAN LCAT

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</thead>
<tbody>
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</table>

*The sequence of the human enzyme was taken from the deduced sequence from cDNA performed by McLean et al. (1986).

**The amino acid residues indicated by 'X' are those of ambiguous ones in the identification.
Figure 12. Representative plots of the repetitive yields per cycle of PTH-amino acid derivatives from the automated sequence analyses. The yield of the first cycle was taken as 100 percent. A, Amino terminus; B, Peptic peptide #4 (Figure 13); C, Peptic peptide #5 (Figure 13); D, Tryptic peptide #6 (Figure 14); E, Tryptic peptide #5 (Figure 14).
amino terminal amino acid sequencing as well as other sequencing efforts. The yield of the first cycle was taken as 100 percent. The first cycle usually shows a high yield because this residue is subjected to two cycles of phenylisothiocyanate (PITC) coupling. The yields of serine were generally low (Figure 12-A, cycle #19). The low yield of serine was shown to be due to the formation of dehydroalanine and the DTT aduct of PTH-serine (Ingram, 1953). The peak from the DTT-adduct was not used for the calculation of yield, but was very useful in confirming the identity of the serine residue. In contrast, dehydroalanine can not be monitored at 269 nm. The low yield of histidine (Figure 13-A, cycle #11) is an inherent problem of the system and is attributed to the extraction difficulties of ATZ-histidine from the polystyrene matrix (ABI user bulletin #21).

Peptic Peptides: DFP-Reacting Peptides -- The strategy for the sequence analysis of the DFP-reacting peptide was to:
1) label the serine with [³H]-DFP
2) digest the enzyme with pepsin
3) isolate the radioactive peptide by HPLC and
4) sequence it.

As shown previously, the reaction of hog LCAT with DFP results in essentially total loss of the enzymatic activity. The quantitation of [³H]-DFP incorporated into the enzyme indicated approximately one residue of serine per mole reacting with this reagent. The labeling of [³H]-DFP was carried out by the same methods as described earlier for the
chemical modification of the serine. When the purified enzyme
solution (0.64 mg in 2 ml of 1 mM sodium phosphate buffer, pH
7.4) was incubated with 0.1 mCi of $[^3H]$-DFP, the enzyme
activity was totally abolished within 5 min. In order to
force the reaction toward completion, the same amount of
$[^3H]$-DFP was allowed to react with the enzyme twice for 20 min
each. This was followed by the addition of a large excess (30
mM) of unlabeled DFP for 20 min at 24 °C. After an extensive
dialysis against 0.1 M acetic acid, pH 2.0, the radioactivity
incorporated into the enzyme was determined to be 0.89 mole of
DFP per mole of the enzyme. The dialyzed $[^3H]$-DIP-LCAT (final
concentration, 0.43 mg/ml) was subjected to peptic digestion
carried out as described in "Methods". In this manner, more
than 50 percent of the enzyme was eventually digested by
pepsin (1/30 of enzyme mass) within 10 min of incubation. The
reaction was stopped by freezing the reaction mixture by
dry-ice in acetone. The digest was lyophilized and finally
redissolved in 300 ul of 0.1 percent TFA. The peptides were
separated by HPLC as described in "Methods". The
representative chromatographic pattern resolved from the HPLC
is shown on Figure 13. Peptides indicated by numbers in
Figure 13 contained the most of radioactive label. Peaks #4
and 5 were selected for sequencing as the other fractions
contained either insufficient amounts of material for
sequencing or were contaminated as judged by the shape of the
peaks. Peptide #5 was rechromatographed to confirm its
purity. About 200 pmol of the peak #4 and 5 were used for
Figure 13. Elution pattern after reversed-phase HPLC separation of peptides derived from peptic digestion with [3H]-DIP-LCAT. The enzyme was first labeled with [3H]-DFP and dialyzed against 0.1 M acetic acid and then subjected to the peptic digestion. The resulting digest, after lyophilization, was fractionated on a C18 reversed-phase HPLC (4.6 x 250 mm, 5 um bead size, Bio-Rad) equilibrated with 0.1 percent (v/v) aqueous TFA. The flow rate was 1 ml/min. at room temperature. The peptides were eluted using a linear gradient program consisting of 100 percent Buffer A (0.1 percent aqueous TFA) to 40 percent Buffer A (60 percent Buffer B, which is 0.1 percent TFA in acetonitrile) in 90 min. The effluent was monitored at absorbance UV 220 nm and generally 1 ml fractions were collected manually. When the absorbance peaks appeared on the recorder, the fraction size was reduced to 0.3-0.5 ml to avoid the possible contamination by a adjacent peptide. From these fractions, an aliquot of 100 ul was placed in a scintillation vial and dried under the air-stream and used to determine the radioactivity. More than 1,500 cpm/0.1 ml of radioactivity was observed in the absorbance peaks indicated compared to less than 50 cpm/0.1 ml in the other absorbance peaks. The peaks #4 and 5 were sequenced after the peak #5 was repurified using the identical buffer system described above.
sequence analysis by automated Edman degradation. Based on these findings, the amino acid sequences of the peptides containing DFP-reactive serine residue were:

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<th>7</th>
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<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
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<tr>
<td>Pept. #4—Ile Ser Leu Gly Ala Pro Trp Gly Gly Ser</td>
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<tr>
<td>Pept. #5—Tyr Ile X Asp X Gly Phe Pro Tyr X Asp Pro Val</td>
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Repetition of sequence analysis from another preparation gave results consistent with these findings. The repetitive yield in each cycle of Edman degradation is shown in Figure 12-B and 12-C. No radioactive DIP-serine derivative could be detected following the automated Edman degradation. This appears to be due to the reversibility of the DFP reaction under basic conditions.

The amino acid sequences shown above are identical to segments of human sequences deduced from the cDNA of human LCAT (McLean et al., 1986). In the human sequence, residues 207-216 and residues 325-337 are nearly identical to the sequences of peptide #4 and 5, respectively (see Appendix).

Cysteine-Containing Peptides — As shown earlier, hog LCAT was inactivated by sulfhydryl reagents and at least one of these sulfhydryl group(s) appeared to be essential for catalytic activity. Consequently, attempts were made to determine the amino acid sequence of the regions surrounding cysteine residues. The strategy for the sequencing was essentially the same as that described for the DFP-reactive
serine containing peptides. As there is no affinity labeling reagent specific for the catalytic sulfhydryl group, we have decided to use a general sulfhydryl reagent (NEM). Purified enzyme (0.64 mg in 2 ml of 1 mM sodium phosphate buffer, pH 7.4) was extensively dialyzed against 2 mM DTT and subsequently 10 mM NaHCO₃, pH 7.4 in the presence of nitrogen bubbling through the solution. The enzyme was incubated with [³H]-NEM (0.2 mCi) at 24 °C for 20 min. The same amount of [³H]-NEM was added to the solution twice with 20 min incubation following each addition. Then, a large excess of cold NEM (30 mM in 5 percent ethanol) was added to complete the reaction. The enzyme was inactivated within 5 min of the first addition as demonstrated by enzyme assays. The reaction mixture was extensively dialyzed against 10 mM (NH₄)HCO₃, pH 7.8. Trypsin solution (1 mg/ml) was added 3 times in succession (1/30 of the enzyme mass each) and the mixture was incubated for 10 min at 37 °C following each addition of trypsin. Approximately 65 percent of the enzyme was digested in 30 min. The digest was lyophilized and redissolved in 300 µl of 0.1 percent TFA for the HPLC. One third of the sample was subjected to the HPLC as described in the "Methods". Figure 14 depicts a representative chromatographic profile resolved on HPLC. An aliquot of 100 µl from each of these peptide samples was subjected to liquid scintillation counting. The peptides indicated by numbers in Figure 14 contained more than 600 cpm/0.1 ml of radioactive labels compared to less than 50 cpm/0.1 ml in the other absorbance
Figure 14. Elution pattern after reversed-phase HPLC separation of peptides derived from tryptic digestion with $[^3H]$-NEM-LCAT. The enzyme was first labeled with $[^3H]$-NEM and dialyzed against 10 mM ammonium bicarbonate, pH 7.8, and then subjected to the tryptic digestion. The resulting digest was lyophilized and redissolved in 300 ul of 0.1 percent TFA. One third of the sample was used for the HPLC using the identical conditions to those for the peptic peptides isolation. The absorbance peaks indicated by numbers showed more than 600 cpm of radioactivity compared to less than 50 cpm in the other absorbance peaks when an aliquot of 100 ul was subjected to the liquid scintillation counting. The absorbance peaks #3 and 5 were contaminated by the other peptides as judged by inspections of the shape of the peaks. The insets in this figure are the results of rechromatography using the identical conditions.
peaks. As judged by the shapes of the peaks, peptide \#3 and \#5 were contaminated. These two peptide samples were lyophilized and rechromatographed. Insets in Figure 14 show the rechromatography patterns. The highest peaks contained the radioactivity and approximately 200 pmol of the peptides were sequenced as described in 'Methods'. Table VI shows the results of the sequence analyses with the peptides \#2, 4, 5, and 6. After determination of amino acid sequences, the peptide \#1 and 3 were found to be derived from trypsin when the sequences were compared with the known sequence of trypsin.

The amino acid residues indicated by 'X' could not be identified. Residues with an ambiguous identity were also designated as 'X'. These unidentified residues may be cysteine because the PTH-derivative of cysteine is not stable under the sequencing conditions. It was not possible to identify the radioactive residues for the \[^{3}H\]-NEM-cysteine derivative from the sequencing effluent for the same reasons. Peptide \#2 appeared to be homogeneous, but two PTH-amino acids with almost the same signal strength were observed for every sequencing cycle. Accordingly, these observations were considered to represent two peptides that may be linked by a disulfide bond. At cycle \#3, no amino acid could be identified for either one of the peptides; these could be cysteine residues. In order to resolve the identity of this peptide further, it was dialyzed extensively against 2 mM DTT with nitrogen bubbling through the buffer. The sample was
<table>
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<tr>
<th>Peptide Number in Figure 14</th>
<th>Amino Acid Sequence</th>
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<tr>
<td>2 (a)</td>
<td>Met-Leu--X--Gly-Leu-Ala--X--</td>
</tr>
<tr>
<td>(b)</td>
<td>Ile-Val--X--Gln-Tyr-Thr--X-- Leu-Ala</td>
</tr>
<tr>
<td>4</td>
<td>X--Leu-Glu-Phe-Leu--X--Gln-Ser--X</td>
</tr>
<tr>
<td>5</td>
<td>Ala-Pro-Tyr-Pro-Gly-Gln-Ile-Thr--X--Asn-Met-Phe-Ala-Gly-Leu-Gln</td>
</tr>
<tr>
<td>6</td>
<td>Tyr-Phe-Trp-Ile-Asn--X--Leu-Ile</td>
</tr>
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</table>
then lyophilized and redissolved in 1 mM sodium phosphate buffer, pH 7.0 (50 ul) and subsequently subjected to HPLC using the same solvent system as that of the previous separation. Figure 15 shows the HPLC profile of this sample. Two absorbance peaks were revealed at 220 nm with a retention time corresponding approximately 30 percent solvent B, that is about 7 min earlier than the retention time of the original peptide #2 in Figure 14. This indicates that disulfide bond in peptide #2 in Figure 14 may have been reduced by DTT and the resulting two peptides were thus eluted from the HPLC column separately. The shorter retention time of the peaks indicates that the size of the peptides has been reduced. Peptides #1 and #2 in Figure 15 could not be sequenced because of the limited amount of the material available.

When the cysteine containing peptides were compared with the amino acid sequence of human LCAT, generally no sequence homology could be found between the two species.

**Immunoreactivity of Hog LCAT with Monoclonal Antibody against Human LCAT**

In order to compare the nature of the active site region of hog and human LCAT, the respective enzymes were titrated with a monoclonal antibody developed against the active site region of human LCAT. As shown in Figure 16, a concentration dependent inhibition of esterifying activity of both human and hog enzyme was observed in the presence of the monoclonal antibody. The extent of inhibition was almost identical suggesting the similarity in the active site of LCAT from both
Figure 15. Elution profile from reversed-phase HPLC separation of peptides derived from the reduction of the peptide 2 of the tryptic digest. The sample from peak 2 in Figure 15 was dialyzed extensively against 2 mM DTT in the presence of a nitrogen bubbling through the buffer and lyophilized. The peptides were redissolved in 50 ul of 1 mM sodium phosphate buffer, pH 7.0, and subjected to the separation by HPLC using the identical conditions described in the peptic peptides isolation (Figure 14). The composition of the solvent for the elution of the peak 1 and 2 was about 30 percent of acetonitrile, which is about 7 min earlier than the peak 2 in Figure 15.
Figure 16. Inhibition of hog (o) and human (●) LCAT activity by monoclonal antibody against human LCAT. Approximately 8-10 ug of enzymes was incubated with various concentrations of the monoclonal antibody (culture medium). The incubation was carried out at 37 °C using the assay mixture described by Chung et al., (1979). Residual LCAT activity was measured as described by Chung et al., (1979).
Protein in the condition media.
species. This result also emphasizes the significance of the sequence homology between two species in the $[^3\text{H}]-\text{DIP-serine}$ containing peptides shown earlier.
CHAPTER IV

DISCUSSION

Purification of the Enzyme

This study describes a highly efficient purification procedure that yields milligram quantities of homogeneous LCAT from hog plasma. Generation of a large quantity of the enzyme has been a difficult task that originally resulted in the relatively slow progress of research in this area. The purification of the enzyme from a source other than human plasma has also made comparative studies possible.

The purification described here has a number of advantages over the published procedures for the purification of human (Albers et al., 1976, Chung et al., 1979 and Chong et al., 1981) and hog LCAT (Knipping, 1986). Most of the previous purification schemes involve one or more ultracentrifugation steps, which are expensive, time-consuming and limit the amount of the starting material. The approach utilizing column chromatography as described in this study is simple, efficient and easy to scale-up. Based on the specific activity expressed in nmol cholesterol esterified per hour per mg of protein, the final purification of the enzyme was approximately 20,000-fold over the starting plasma with a 17 percent yield. The procedure here is an improved version of the one published previously (Park and Lacko, 1986). The
dodecylamine-agarose and DEAE-agarose column chromatographies were adequately substituted for the phenyl-Sepharose and DE-52 cellulose steps, respectively. Although the theoretical backgrounds of the respective chromatographic steps are quite similar, the operation of the columns is much simpler in the new procedure. For instance, the capacity of the dodecylamine-agarose was much less than phenyl-Sepharose, thus the size of the column and the time of the operation can now be decreased significantly. The delipidation step by Triton X-100 prior to the DEAE-agarose column used in the old procedure is now eliminated. The delipidation method was an improvement compared to the procedure using organic solvent, but the long period of washing the column with various buffers to remove the detergent was tedious and time-consuming. The free enzyme molecules can now be successfully separated from the lipid-bound enzyme by the DE-52 cellulose chromatography without delipidation. As shown in Figure 2-B, a late eluting protein peak with the enzyme activity appears to be the lipid-bound enzyme as shown in the next step of the purification. When the late eluting protein peak from the DE-52 cellulose step was subjected to hydroxylapatite chromatography, the enzyme was associated with the major contaminant (apoA-I) as demonstrated on SDS-PAGE. The association of the enzyme with the contaminant was assumed to be mediated by lipids. The most significant advantage of the new procedure described here over the published method is the elimination of one of the chromatographic steps.
(antibody-agarose column chromatography). When the highly purified enzyme preparation (hydroxylapatite pool) was concentrated for immunoaffinity chromatography, large losses of enzyme occurred. By controlling the amount of protein, a homogeneous enzyme could be obtained by hydroxylapatite chromatography without use of the immunoaffinity column chromatography. About the same quantity of enzyme could be obtained from one liter of plasma by the new procedure as that from two liters of plasma in the published procedure. This procedure has now been successfully used for the purification of human LCAT also and may thus be useful for the purification of the enzyme from other sources.

Substrate Specificity of the Enzyme

HDL has been considered as a superior substrate for LCAT because it contains the activating peptide, apoA-I. However, recently other apoprotein components present in lipoproteins have also been shown to serve as LCAT activators. This development has broadened the possibilities for other lipoproteins to serve as substrates for LCAT. Most of the polypeptides of HDL are able to serve as activators (or cofactors) of LCAT. These include apoA-I (Fielding et al., 1972), apoC-I (Scoutar et al., 1975 and Jackson et al., 1974) and apoA-IV (Steinmetz and Utermann, 1983). However, the action of the enzyme does not seem to be restricted only to HDL, as these apoproteins are also found in VLDL and sometimes in LDL (Abbey et al., 1984 and Zechner et al., 1984). In order to determine the lipoprotein substrate specificity of
hog LCAT, the reaction of purified hog LCAT with lipoproteins isolated from hog plasma was investigated. The findings clearly indicate that HDL is the preferred lipoprotein substrate over LDL and VLDL for hog LCAT. Fielding et al. (1972) reported that HDL was the superior substrate for human LCAT. Recent observations indicate that rat LCAT also prefers HDL over VLDL as a substrate (Lee et al., 1986). In addition, hog LCAT was found to be associated with HDL when whole plasma was fractionated by gel filtration (Figure 5). Furthermore, fluorometric study of hog LCAT indicates that the enzyme interacts with liposomes containing apoA-I, but not with those lacking apoA-I (Figure 6). This interaction may be mediated by hydrophobic forces between hydrophobic amino acid residues of LCAT and apoA-I, possibly through the interaction between tryptophan and other hydrophobic amino acid residues. These observations suggest that hog LCAT catalyzes the transesterification reaction on the HDL particles in vivo as established in the human system (Jahani and Lacko, 1982; Fielding and Fielding, 1971). As only minimal cholesteryl ester transfer activity has been shown in hog plasma (Ha et al., 1981), HDL appears to serve as a vehicle for the transport of cholesteryl esters to the liver for catabolism, whereas lower density lipoproteins play a similar role in human plasma (Glomset, 1979).

Physicochemical Properties of the Enzyme

Basic physicochemical properties of hog LCAT were studied including the nature of sulfhydryl, tyrosyl and tryptophanyl
residues of the enzyme. The molecular weight of intact and deglycosylated enzyme and carbohydrate and amino acid compositions have been reported in the author's master's thesis (Park, 1984). Those are briefly summarized in this dissertation to reflect the overview of hog LCAT.

Purified hog LCAT consistently migrated slightly faster than bovine serum albumin (Mr=67,000) did on SDS-PAGE. The molecular weight determined by HPLC also showed 66,000. The glycoprotein nature of LCAT has previously led to discrepancies in the determination of accurate molecular weight of polypeptide moiety of the human enzyme (Chong et al., 1983). Hog LCAT was shown to contain a considerably high content of carbohydrates (21.4 percent, w/w) as shown in Table III. The oligosaccharide chain of the enzyme may play a significant role in the maintenance of solubility and catabolism of the enzyme, thus required further detailed investigation. The behavior of hog LCAT on SDS-PAGE is puzzling, because even though it contains over 20 percent carbohydrate, its mobility reflects the molecular weight of 66,000. Thus, while human LCAT has shown to have a molecular weight of 60,000 (Chong et al., 1983a), it has a mobility on SDS-PAGE essentially identical to the hog enzyme. It is difficult to estimate a molecular weight of a glycoprotein on SDS-PAGE accurately because it shows a broad diffuse band on the gel.

The spectrophotometric titration data show two and four titrable sulfhyryl groups under native and denaturing
conditions, respectively. Four half-cystines were indicated by performic acid oxidation. A total of four sulphydryl groups were detected by the $[^3\text{H}]$-NEM labeling study when the enzyme was initially dialyzed against 2 mM DTT and denatured in the presence of 6 M guanidine hydrochloride. According to these data, it is unlikely that there is a disulfide bond in hog LCAT. Similar studies performed by Chong et al. (1983b) have suggested that human LCAT may contain a disulfide bond.

Although it has been known for two decades (Norum, 1965) that sulfhydryl reagents inactivate LCAT, it has not yet been shown clearly whether the sulfhydryl group(s) are directly involved in the catalytic activity. A large molecule such as DTNB situated near the active site may cause a steric hindrance and thus retard the approach of the substrates to the active site of the enzyme. In order to resolve this point, thionitrobenzoic-LCAT (TNB-LCAT) was substituted with a smaller molecule, cyanide ion. The disulfide bond formed between TNB and LCAT can be easily cleaved by cyanide ion (Chung et al., 1971) forming a thiocyanate derivative with the enzyme. When the sulfhydryl group is near the active-site, the substitution is expected to result in at least a partial recovery of enzyme activity. As shown in Table IV, the enzyme could not regain its activity by the substitution suggesting that thiol group(s) is(are) essential for the LCAT activity. The recovery of enzyme activity observed after the thiocyanate derivative was treated with DTT is due to the elimination of free thiocyanate by DTT (Jocelyn, 1972). The essential thiol
was regenerated by the reaction of
\[ R_1SCN + R_2SH \rightarrow R_1SH + R_2SCN. \]
This reaction has been demonstrated earlier using cysteine containing peptides obtained from wool (Cuthbertson and Philips, 1945).

DTNB could not inactivate purified hog LCAT at high enzyme concentrations (>0.2 mg/ml). Whereas, it nearly completely inactivated the enzyme at low enzyme concentrations (<0.1 mg/ml). These results suggest that the sulfhydryl group(s) essential for the enzyme activity become unavailable to the DTNB reaction at high enzyme concentrations. This may be due to self-association of the enzyme molecules at high enzyme concentrations. During the self-association process, the essential sulfhydryl group(s) appear(s) to be buried in the interior of the enzyme and thus remain protected from the inactivation by DTNB. In order to test the possibility that the enzyme molecules undergo an intermolecular disulfide bond formation during the self-association process, the enzyme solution (0.42 mg/ml) was dialyzed extensively against DTT (2 mM) and then subjected to DTNB-treatment. As shown in Figure 8, approximately 50 percent of the enzyme activity was abolished by 30 μM DTNB, while the DTT-untreated enzyme remained fully active. These results indicate that changes in the environment of the essential thiol group(s) may occur due to the reaction with DTT. On the other hand, two thiol groups were titrated by DTNB both with and without prior dialysis against a DTT-containing buffer. These data suggest that
there was no apparent reduction of a disulfide bond during the DTT-treatment. These phenomena can not be explained fully at the present time. It is possible that the reduced disulfide bond might have been reoxidized during the dialysis against 5 mM sodium phosphate buffer while removing the unreacted DTT. During this transformation, the essential sulfhydryl group may be partially exposed, thus rendering it available to the DTNB-inactivation. Preliminary data from amino acid sequence analyses indicate that there may be two peptides connected by a disulfide bridge among the tryptic peptides. Although the studies with human LCAT indicated one possible disulfide bond (Chong et al., 1983b) and the amino acid sequence of human LCAT deduced from cDNA showed 6 cysteines in the human enzyme (McLean et al., 1986), the data in this study do not support the existence of a disulfide bond in hog LCAT.

These observations taken together may lead to the following conclusion: Hog LCAT contains 4 sulfhydryl groups. One of these is involved in catalytic activity and all four may be involved in the self-association process that protects the enzyme against DTNB-inactivation at a high enzyme concentrations in vitro. During the self-association process, the sulfhydryl groups may contribute to intermolecular disulfide bond formation. This reaction is likely to be more favorable for those sulfhydryl groups that are not involved in catalytic activity. As a result, the self-association may protect the active sulfhydryl groups from reacting with DTNB. The apparent disulfide linkage observed during the sequence
analysis of the tryptic peptides may also have been artificially generated by the self-association process after purification.

In the fluorescence study, more than half of the tryptophan residues were shown to be exposed to the aqueous medium (Figure 9). This somewhat unusual feature of the enzyme suggests that a large proportion of the tryptophan residues are located on the outer surface of the molecule and thus could be involved in substrate binding. As discussed earlier, the enzyme appears to bind to the macromolecular substrate (HDL) in the plasma, possibly through hydrophobic interactions. The fluorescence quenching studies with artificial substrates suggest that the tryptophan residues of the enzyme and apoA-I may be the main contributors to this interaction (Figure 5). In addition, the amino acid sequence analysis showed that only two out of the first 18 amino acid residues from the amino terminus contained a charged group and this sequence is 100 percent homologous to the same region of the human enzyme. These observations taken together suggest that the amino terminal residues and the tryptophan residues of the enzyme may be involved in substrate binding forming a hydrophobic pocket to accommodate the macromolecular substrate (HDL).

The spectrophotometric titration of phenolic groups in the enzyme showed a large increase in absorbance at 295 nm with an apparent pK of about 11 (Figure 10). In a control experiment using N-acetyltirosinamide, the apparent pK for the
increase in absorbance was about 10. These data suggest that the ionization of the phenolic side chains in the enzyme was delayed, perhaps due to a conformational transition that had taken place prior to the exposure of the tyrosine residues to the aqueous environment. At pH 13.0, approximately 13 of 21 tyrosine residues of the enzyme were exposed. However, nearly all the tyrosines were exposed following treatment of the enzyme with 6 M guanidine hydrochloride. A drastic conformational change including a complete destruction of the secondary structure may thus be required for the complete exposure of the tyrosine residues in hog LCAT. Under physiological conditions, most of the tyrosine residues would thus be shielded from the solvent, suggesting that these residues are not exposed to the surface and are not likely to participate in the interaction between substrate and the enzyme.

Chemical Modification of the Enzyme

Serine, cysteine and histidine residues have been suggested as part of the active site of human LCAT (Jauhiainen and Dolphin, 1986). These amino acid residues of the hog enzyme were therefore subjected to chemical modification. The reagents used in this study for the modification of sulfhydryl groups were DTNB and NEM. DTNB reacts with free sulfhydryl groups of proteins, forming thionitrobenzoic protein and liberating 1 mole of thionitrobenzoate anion for each sulfhydryl group. This anion shows a strong absorbance at 412 nm (extinction coefficient, 13.6 mM\(^{-1}\) cm\(^{-1}\) at pH 8, Ellman,
1959) and it is widely used for quantitation of sulfhydryl groups in proteins as well as in various biological fluids and tissues (Ellman, 1959 and Butterworth et al., 1967). NEM is another frequently used sulfhydryl-reacting reagent. The reaction of maleimides with sulfhydryl groups involves the addition of mercaptide ion to the olefinic double bond of the maleimide ring. The reagent also reacts more slowly with amino groups, via the same general mechanism. Products of both reactions have been isolated and identified (Marrian, 1949 and Smyth et al., 1960). Human LCAT has been shown to be inactivated by sulfhydryl reagents (Norum, 1965; Chong et al., 1983b; Jauhiainen and Dolphin, 1986). In this study, hog LCAT was also shown to be inactivated by sulfhydryl reagents and the sulfhydryl group(s) of the enzyme was found to be essential for catalytic activity. As shown in Figure 11-A, the enzyme was not completely inactivated by DTNB or NEM. The reason for the incomplete reaction may be the steric hindrance due to the bulky structure of DTNB, which apparently fails to react with all the sulfhydryl group(s) at the active site. Similar data were obtained with NEM. NEM, a less bulky reagent than DTNB, inactivated the enzyme up to 80 percent within 5 min of incubation. The possibility of an incomplete inactivation due to self-association was discussed earlier and does not apply here as low concentrations of the enzyme (0.12 mg/ml) were used. These data, together with other findings discussed previously, indicate that sulfhydryl group(s) are likely to be essential for the catalytic activity of the
enzyme. The number of NEM-reacting sulfhydryl groups was estimated using $[^3H]$-NEM labeling. Native, denatured and both denatured and reduced enzymes were subjected to the radiolabeling. In the native state of the enzyme, two sulfhydryl groups were labeled by $[^3H]$-NEM and when the enzyme was denatured by 6 M guanidine hydrochloride, 4 sulfhydryl groups were labeled. These data are in good agreement with the results obtained from the DTNB titration. On the other hand, when the denatured enzyme was reduced by DTT, no further increase in radioactivity was observed upon $[^3H]$-NEM labeling indicating that no disulfide bond was originally present in the enzyme.

The quantitation of essential cysteine residue(s) was unsuccessful due to the self-association problem mentioned above. The only way to assess the modification of essential cysteines was a spectrophotometric method using Ellman's reagent, which allows the simultaneous monitoring of both the thiol groups titrated and enzyme activity. As the enzyme undergoes a self-association process above the enzyme concentration 0.2 mg/ml, it was not possible to monitor the TNB-LCAT formation below this concentration using absorbance measurements at 412 nm.

Specific serine residues of hog LCAT were reacted with DFP. Serine residues, when not involved in catalysis, do not generally react with DFP under these conditions. The special reactivity of these residues is a function of the secondary and tertiary structure of the enzyme and is abolished upon
denaturation. The chemistry involved in this reaction is a nucleophilic attack of the active-site serine hydroxyl group on the electrophilic phosphorus atom of DFP, eliminating fluoride ion and forming a stable diisopropylphosphoryl enzyme derivative. This reaction is similar to the formation of the acyl-enzyme intermediate in the normal hydrolytic reaction of a number of enzymes that also possess a serine at the active site. In this regard, DFP can be viewed as a pseudosubstrate. Although the chemical basis of this reaction is not well understood, DFP has been widely used for the identification of active site serine residue in many enzymes such as chymotrypsin (Jansen et al., 1949), cholinesterase (Kraut, 1977) and lipoprotein lipase (Reddy et al., 1986). As shown in Figure 11-B, the activity of hog LCAT was rapidly abolished by the addition of DFP. When \[^{3}\text{H}]\text{-DFP}\) was added to the enzyme, approximately 1 mole of DFP per mole of enzyme was incorporated indicating that there is 1 residue of serine per mole of enzyme that is reactive with DFP.

The histidine residues of hog LCAT were modified by diethylpyrocarbonate (DEP). This reagent, ethoxyformic anhydride, reacts with the imidazole groups of proteins at neutral pH. This reaction can be monitored by an increase in absorbance at 245 nm, which can be used as a quantitative tool of the extent of the reaction using a molar extinction coefficient of 3,200 (Muhlrad et al., 1969). At acidic pH, DEP also reacts with \(\alpha\)-amino and phenolic groups of protein (Melchior and Fahrney, 1970). The formation of O-carbethoxy
protein derivative with tyrosine residue can be monitored by a concomitant decrease in the absorbance at 278 nm (Tudball et al., 1972). As shown in Figure 11-C, 0.5 mM DEP inactivated hog LCAT sensitively up to 95 percent in 5 min.

Attempts were made to protect the enzyme from the chemical inactivations by preincubation with substrates. Proteoliposome and HDL substrates were used for the protection. However, the protection of the enzyme against the chemical inactivation was unsuccessful. Since the protection experiment can be a critical piece of evidence for the identification of amino acid residues involved in the catalytic activity, more efforts are required to support these preliminary findings. Data from present studies thus can not rule out the possibility that the chemicals utilized reacted with highly reactive amino acid residues that are not directly involved in the catalytic activity; this could result in a conformational transition and subsequently the inactivation of the enzyme. However, the data from the present study are consistent with the view that the amino acid residues identified by their chemical reactivity are involved in the catalytic mechanism. In contrast, human LCAT has been shown to be protected by substrates (Jauhiainen and Dolphin, 1986). This discrepancy between the published findings and the present study can not be reconciled at this time. Similar studies performed in the author's laboratory after this study indicate that the enzyme may be protected from chemical inactivations by substrates under certain conditions (Jung and
Lacko, unpublished observations, 1987).

Primary Structure of the Enzyme

Partial amino acid sequence of hog LCAT was determined using the intact enzyme, peptic peptides for the $[^3\text{H}]$-DIP labeled peptides and tryptic peptides for the $[^3\text{H}]$-NEM labeled peptides. Approximately 20 percent of the entire primary structure was sequenced in this manner. The sequence analyses were carried out by automated Edman degradation (Edman, 1950) in a gas-phase sequencer. Using this highly efficient system, about 100 pmol of protein or peptide could be sequenced requiring one hour per residue in a totally automated mode.

As shown in Table V, up to 34 amino acid residues from the amino terminus of hog LCAT were sequenced. Beyond residue 34, the sequencing began to yield ambiguous results and at that point the analysis was discontinued. It is likely that the residue 31 in the hog enzyme is cysteine as the homology of the amino terminal sequence of hog and human enzymes is very high. The human enzyme has a cysteine residue in position 31. One of the residues 20-22 is proposed to be the site of carbohydrate attachment (McLean et al., 1986). Consequently, the assignment of residues 20 and 21 might be difficult as this portion is not identical in hog LCAT. Actually, small amounts of asparagine were detected in cycle #20. This indicates that some of the asparagine at residue 20 may have lost their carbohydrate moieties during the preceding sequencing steps and thus appeared as asparagine during cycle #20. As shown in Table V, this region of the
enzyme was highly conserved compared to the sequence of human LCAT (McLean et al., 1986). The first 18 amino acid residues from the amino terminus of hog and human enzymes are identical. Only 4 out of 34 residues from the amino terminus were found to be different between the amino terminal regions of LCAT from these two species. The evolutionary conservation of this region suggests a functional role of the amino terminal segment of the enzyme. This region of the enzyme is highly hydrophobic as seen in Table V. Therefore, one may speculate that the amino terminal portion of LCAT is part of a binding domain for hydrophobic lipid substrate as was originally suggested by Doi and Nishida (1981b). This region was shown to be the most hydrophobic region in the entire enzyme as demonstrated by a computer program (McGENE, Applied Genetic Technology) with human LCAT sequence. Using this program, the secondary structure from the residue 9 to 26 was predicted. According to the prediction, residue 9 (Pro) and residues 22-26 (Thr-Ile) are located inside and the residues 19-21 (Pro-Asn) are at the outside of three dimensional structure of the enzyme. Residue 9, 13, 14, 16, 17, 23, 24 are \( \beta \)-turn, residues 10-12, 15, 18-21 are random coil and residues 25, 26 are \( \beta \)-pleated sheet. In these analyses, residue 20 was assumed to be Tyr because it was the most probable designation by the inspection of the chromatogram from the sequencer. When human LCAT was examined in this manner, identical results were obtained even though there are 4 amino acid replacements compared to the hog enzyme (residues
19-21 and 23). The first and last 8 residues could not be analyzed due to limitations of the program.

Another region of the enzyme investigated in this study was peptide fragments presumably bearing the active serine residue that rapidly reacts with DFP. Upon reacting the hog enzyme with this reagent, two labeled peptic peptides were isolated and sequenced. The sequence data (peptide #4 and 5 in Figure 14) were obtained from triplicate determinations of peptide #4 and from a single run of peptide #5 as both peptides were highly homologous with corresponding sequences of human LCAT (see Appendix). As shown earlier, these two peptides had identical sequences to known segment of the human enzyme sequence. It was not possible to identify the radioactive DIP-serine from the sequencing effluent because the DFP-reaction was reversible at alkaline pH. However, the peptide #4 appears to contain the essential serine for the catalytic activity of the enzyme. This peptide is 100 percent homologous with the residue 207-216 in human LCAT and also shows a great deal of homology with the active-site peptide of bovine milk lipoprotein lipase (Verger, 1984) and porcine pancreatic lipase (Verger, 1984; Guidon et al., 1981). Table VII shows the summary of the sequence homology of those enzymes. These data support the proposal of Maraganore and Heinrikson (1986) who suggested that the segment containing residues 209-223 includes the catalytic serine of human LCAT. Maraganore and Heinrikson further proposed that so called "interfacial hexapeptide" (Ile-Gly-His-Ser-Leu-Gly) which is
# Table VII

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<td>Human LCAT&lt;sup&gt;a&lt;/sup&gt; (Residue 207-223)</td>
<td>Ile-Ser-Leu-Gly-Ala-Pro-Trp- -Gly-Gly-Ser-Ile-Lys-Pro-Met-Leu-Val-Leu</td>
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<td>Lipoprotein Lipase&lt;sup&gt;b&lt;/sup&gt; Active site Peptide</td>
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<td><strong>Peptide #5 from Peptic Peptides of Hog LCAT</strong></td>
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<td>Human LCAT (Residue 325-337)</td>
<td>Tyr-Ile-Tyr-Asp-His-Gly-Phe-Pro-Tyr-Thr-Asp-Pro-Val</td>
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<sup>a</sup> From McLean et al., 1986.

<sup>b,c</sup> From Verger et al., 1984.
completely conserved in the pancreatic lipase, lipoprotein lipase and LCAT, may serve as a binding site for macromolecular substrate.

Although the precise roles of these respective serine residues in the LCAT mechanism remain to be resolved, these data suggest that the catalytic serine is indeed at the location proposed by Maraganore and Heinrikson (1986). The other DFP-reacting peptide (peptide #5 in Figure 13) contains three tyrosine and one threonine residues. In addition, there are two unidentified residues that could serve as potential reactive sites for DFP. This peptide is likely to represent a non-specific but highly exposed DFP reacting amino acid side chain in LCAT and the assignment of its role in the LCAT mechanism (if any) must await further investigation.

As discussed earlier, the sulfhydryl groups of the enzyme appear to be important for the catalytic activity of LCAT. Accordingly, attempts have been made to isolate and sequence of the cysteine containing peptides. The enzyme was labeled with $[^3H]$-NEM and fragmented by trypsin. The tryptic digestion with the radiolabeled enzyme followed by HPLC yielded six unique radioactive peptides (Figure 14). The results of the sequence analyses of those peptides are shown in Table VI. Since a large amount of trypsin (1/10 of LCAT, w/w) was used for the digestion of the enzyme, it was possible that some of peptides isolated by HPLC were derived from trypsin. When the obtained sequences were compared with the amino acid sequence of trypsin, it was found that the peptide
#1 and 3 of Figure 15 were in fact from trypsin. The radioactivity observed in those peptides appears to be from contamination by adjacent peptides which are highly radioactive. Peptide #2 appears to be a disulfide containing peptide. Although there is no supporting evidence available for the presence of the disulfide bond in native hog LCAT, the reduction of the peptide #2 gave two isolated peaks upon rechromatography by HPLC with a much shorter retention time than the original peptide before the reduction (Figure 15). This indicates that the peptide #2 has been reduced. The presence of a disulfide bond has been suggested for the human enzyme (Chong et al., 1983b). Thorough chemical analyses on the sulfhydryl groups and disulfide bond performed in this study could not identify a disulfide linkage in hog LCAT. The putative disulfide bond observed in this study could be generated artificially due to the self-association property of the enzyme as discussed earlier sections. None of the tryptic peptides sequenced has shown any homology with the published sequences of human LCAT that surround cysteine residues. The lack of the sequence homology is puzzling especially because Jauhiainen and Dolphin (1986) proposed central roles of specific cysteine residues in the LCAT mechanism. Similar studies are in progress in the author's laboratory in order to confirm these data.

The amino terminal and the DFP labeled serine-containing peptides may be a functionally very important part of the enzyme. It has been well-established that serine and
histidine residues are involved in the catalytic processes of serine proteases and esterases (cholinesterases). LCAT appears to resemble this type of mechanism at least in the phase of phospholipase A<sub>2</sub> type half-reaction. The mechanistic information concerning the description of acylation and deacylation steps of the serine proteases includes (1) The imidazole group (pK<sub>a</sub>=7) of the histidine residue serves as a good general base catalyst to increase the nucleophilicity of serine (pK<sub>a</sub>=13.5) (Bender and Kezdy, 1965), (2) An acyl-enzyme intermediate is formed during the hydrolysis, (3) A base (imidazole group) initiates the deacylation reaction, (4) The deacylation is carried out by a nucleophilic attack of a water molecule. The involvement of sulfhydryl group(s) in the catalytic activity suggests that there may be a sequential transfer of acyl groups during the transacylation process from thioesters to a serine residue forming oxyesters or vice versa as proposed by Stoops and Wakil (1980 and 1981) for the fatty acid synthase. Cholesterol may be in a 'hydrophobic pocket' which is located at or near the active site of the enzyme and the esterification reaction takes place between the acyl-enzyme intermediate and the hydroxyl group of cholesterol. The water molecule may compete as the acyl acceptor resulting in the hydrolysis of the acyl-enzyme intermediate.
Comparison of the Hog and Human LCAT

Table VIII compares the characteristics of hog LCAT that resulted from this study and those of human enzyme shown in the literature. The similarity of the enzyme from two species was first evidenced by the fact that both enzymes can be purified by nearly identical procedures.

The molecular weights of the enzymes from the two species are similar. The molecular weight of human enzyme has been shown to be between 59,000 and 69,000 by different investigators. These differences could be the result of the methods used, the purity of the enzyme or experimental error.

The carbohydrate composition of the hog enzyme is also quite similar to that of the human enzyme. The glycoprotein nature of the enzyme may be important in maintaining the solubility of the enzyme in the circulation. It may also play a significant role in the catabolism of the enzyme (Sharon, 1980; Gibson et al., 1980; Sharon and Lis, 1981). The liver contains receptors which specifically recognize the galactose residue of the complex type of glycoproteins. The galactose residue is usually located at the next to sialic acid that commonly represents the terminal carbohydrate of a glycoprotein. Asialoglycoprotein is produced by the action of neuraminidase and the glycoprotein is then taken up by the liver via the galactose specific receptor. Since LCAT is believed to be one of the components of HDL and HDL is mainly catabolized in the liver (Pittman et al., 1979 and 1982; Carew et al., 1982), the enzyme may be catabolized through such a
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*Sources of data for human LCAT:

1. Chong et al. (1983a)
2. Chong et al. (1983b)
3. Fielding (1972)
5. McLean et al. (1986)
The state of cysteine and cystine residues were discussed extensively in the previous section. Four sulfhydryl groups were identified in this study, while human LCAT was shown to contain one additional disulfide bond.

The amino acid composition of the hog enzyme was generally similar to that of the human enzyme except for the number of lysine, glutamic acid and leucine residues. This may be due to the difference in the molecular weight of respective polypeptide moieties of the two enzymes. In order to assess the similarities of the enzyme from human, hog, and rat plasma, the following formula recommended by Pownall et al. (1985) was used to calculate a correlation coefficient:

\[
R_{kl} = \frac{\sum X_{ik} X_{il}}{\left(\sum X_{ik} \sum X_{il}\right)^{1/2}}
\]

where the mole fraction of amino acid residue, \(i\) of one type of LCAT, \(X_{ik}\), was compared to the mole fraction of amino acid residue \(i\) of another species of LCAT, \(X_{il}\). Using this type of analysis, the following correlations were found.

- Human vs. hog; \(R_{kl} = 0.983\)
- Hog vs. rat; \(R_{kl} = 0.993\)
- Human vs. rat; \(R_{kl} = 0.997\)

In addition, amino acid sequences of amino termini and the segments containing DFP-reactive serine showed a high sequence homology between hog and human LCAT. These data indicate a high degree of similarity between the enzymes from different receptor system.
sources.

Both human and hog LCAT prefer HDL as a substrate. In human, there are different subclasses of HDL (HDL1, HDL2 and HDL3). Human LCAT has been shown to prefer the HDL3 which is a denser particle than the other HDL species (Jahani and Lacko, 1982). However, there is no such apparent heterogeneity in hog HDL. This suggests that the products of the LCAT reaction are stored in the same population of HDL particles. This is necessary considering that there is no cholesteryl ester transfer between lipoproteins in the hog system. Therefore, the cholesteryl ester transfer complex proposed by Fielding and Fielding (1980) does not exist in hog plasma. This system, in human plasma, was suggested to be composed of apoproteins, LCAT, cholesteryl ester transfer protein and complimentary lipid components (Chajek and Fielding, 1978). Consequently, hog LCAT catalyzes the transesterification reaction on the HDL and the HDL serves as a cholesterol carrier from peripheral tissues to the liver in the hog system.

The results of the chemical modification and the amino acid sequence studies strongly indicate that the functionally important regions of the enzyme are highly-conserved during the evolution. The immunoreactivity of both human and hog LCAT against the monoclonal antibody raised against the active site region of human LCAT shows an identical extent of inhibition. This also suggests that the two enzymes have very similar, if not identical, active sites. Although there are
essential distinctions in the lipoprotein metabolism between human and hog, this study shows that hog is a good model for the comparative study of LCAT.
### Amino Acid Sequence of Human LCAT

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The data were adopted from McLean et al., 1986.

The regions of the sequence mentioned in the text are underlined.
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