Purification and Characterization of Human Plasma Lecithin:Cholesterol Acyltransferase†

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ABSTRACT: A highly purified (approximately 12 000-fold) homogeneous preparation of human plasma lecithin:cholesterol acyltransferase (LCAT) with 16% yield was obtained by a combination of density ultracentrifugation, high density lipoprotein affinity column chromatography, hydroxylapatite chromatography, and finally chromatography on anti-apolipoprotein D immunoglobulin-Sepharose columns to remove apolipoprotein D. This enzyme preparation was homogeneous by the following criteria: a single band by polyacrylamide gel electrophoresis in 8 M urea; a single band on sodium dodecyl sulfate gel electrophoresis with an apparent molecular weight of 68 000 ± 1600; a single protein peak with a molecular weight of 70 000 on a calibrated Sephadex G-100 column. Its amino acid composition was different from human serum albumin and all other apoproteins isolated from lipoprotein fractions.

Human plasma contains an enzyme that can catalyze esterification of lipoprotein cholesterol by transferring fatty acids from the 2 position of lecithin to the hydroxyl group of unesterified cholesterol (Glomset, 1968, 1972). There is good evidence that this lecithin:cholesterol acyltransferase enzyme (EC 2.3.1.43) reacts preferentially with plasma high density lipoproteins and that it forms most of the cholesterol esters of human plasma lipoproteins (Glomset, 1972, 1973). However, the physiological role of the enzyme remains to be clarified. Furthermore, because of the difficulties involved in isolation of purified lecithin:cholesterol acyltransferase, little is known about its physical and chemical properties. Fielding and Fielding (1971) and Fielding (1974) purified the enzyme 2500- to 6000-fold in 10–20% yield by a combination of salt precipitation, ultracentrifugation, and adsorption chromatography. Glomset (1972), using similar methods along with ion exchange and affinity chromatography (Akanuma and Glomset, 1968), reported a 1200-fold purification. Utilizing similar procedures, Lacko et al. (1974) recently reported a 4000-fold purification with a 5% yield. Kostner (1974) reported a 15 000-fold purified enzyme, although he did not characterize the preparation. The objective of this study was to prepare highly purified homogeneous preparations of active lecithin:cholesterol acyltransferase and to present some physical and chemical properties of this enzyme.

Methods and Materials

For preparation of substrate, high density lipoprotein (HDL)[1] of density (d) 1.125–1.21, or HDL_3, was isolated from individual donors by sequential ultracentrifugation. Specifically, the nonprotein solvent density of plasma from a normolipidemic fasting adult was adjusted to 1.125 g/ml with solid KBr. Ultracentrifugation was then performed in a 60 Ti rotor at 50 000 rpm at 10 °C for 24 h. The top 9 ml of each tube was aspirated after tube slicing and the bottom fraction adjusted to 1.21 g/ml with solid KBr, centrifuged at 50 000 rpm for 28 h, and the top HDL_3 fraction aspirated. This HDL_3 fraction was recentrifuged twice at 50 000 rpm for 40 h, then was dialyzed against 0.01 M Tris-HCl and 1 mM EDTA (pH 7.4) (Tris buffer), diluted 1:1 with 6% fatty acid free human serum albumin, and heat inactivated for 25 min at 56 °C. [4-^{14}C]Cholesterol (sp act. 57 mCi/mmol) obtained from New England Nuclear was purified by thin-layer chromatography on silica gel 60 F254 (EM Laboratories, Inc., Elmsford, N.Y.) plates, using the solvent system of petroleum ether–ether–acetic acid (135:15:1.5, v/v/v). A human serum albumin stabilized emulsion of labeled cholesterol was then prepared (Stokke and Norum, 1971). The human serum albumin–cholesterol emulsion was added to the HDL_3 preparation and shaken for 4 h at 37 °C in sealed containers flushed with N_2. The labeled HDL_3 substrate containing 1.0 mg of HDL_3 protein/ml and 0.5 μCi of [^{14}C]cholesterol/ml was stored at −20 °C in 0.2-ml aliquots in sealed Wheaton vials.

After each step of the enzyme isolation procedure, aliquots of the enzyme preparation were quick frozen in dry ice and acetone and then the samples from each step thawed and assayed together. Lecithin:cholesterol acyltransferase activity was assayed as the synthesis of labeled cholesterol esters by incubation of test samples with the ^{14}C-labeled HDL. The incubation mixture contained 0.2 ml of HDL_3 substrate, 0.2 ml of enzyme source or buffer control, and 4 μl of 1 M β-mercaptoethanol. The enzyme reaction was stopped by adding 20 vol of chloroform–methanol (1:1, v/v). The solvent phase was recovered by centrifugation, dried with anhydrous sodium sulfate, and evaporated under a stream of N_2. Lipids were redissolved with 0.4 ml of hexane, and a 40-μl fraction applied to silica gel plate which was developed in petroleum ether–ether–acetic acid (135:15:1.5, v/v/v). The cholesterol ester bands were visualized with iodine vapor and recovered by scraping and the radioactivity determined in glass vials containing 10 ml of scintillation fluid (4 g of 2,5-diphenyloxazole, 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene/l. of toluene) in a Model 6868 Nuclear-Chicago liquid scintillation spectrometer. Test samples were diluted with Tris buffer to give an enzyme concentration sufficient to convert approximately

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[1] Abbreviations used are: HDL, high density lipoprotein; VHDL, very high density lipoprotein.

Table I: Purification of Human Plasma Lecithin:Cholesterol Acyltransferase.

Fraction	Total Protein (mg)[a]	Total Act. (Units)[b]	Sp Act. (Units per mg of Protein)	Yield (%)	Purification (Fold)
Plasma (1 l.)	71 000	6089	0.086		
Density 1.21–1.25 g/ml ultracentrifuge fraction	2 749	6074	2.21	100	26
HDL affinity column eluate	90	3075	34.2	50	398
Hydroxylapatite eluate	8	1192	[illegible].9	20	1 732
Anti-D affinity column eluate	0.9	956	1062	16	12 349

[a] By the method of Lowry et al. (1951). [b] One unit of enzyme catalyzed the esterification of 1 nmol of unesterified cholesterol/h from high density lipoprotein (d = 1.125–1.21 g/ml) substrate at 37 °C and pH 7.4.

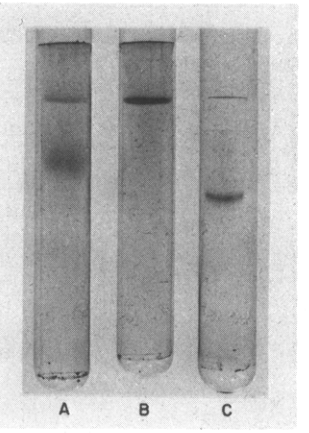

FIGURE 1: Polyacrylamide gel electrophoresis of lecithin:cholesterol acyltransferase preparations. (A) Hydroxylapatite eluate on sodium dodecyl sulfate acrylamide gel (Weber et al., 1972). Twenty micrograms of protein was preincubated with 1% sodium dodecyl sulfate and 1% β-mercaptoethanol for 2 min at 100 °C. (B) Anti-apolipoprotein D affinity column eluate on sodium dodecyl sulfate acrylamide gel. Thirty micrograms of protein was preincubated with 1% sodium dodecyl sulfate and 1% β-mercaptoethanol for 2 min at 100 °C. (C) Anti-apolipoprotein D affinity column eluate on 7.5% acrylamide gel (Davis, 1964) in 8 M urea. Thirty micrograms of protein in 8 M urea was preincubated with 1% β-mercaptoethanol for 30 min at 23 °C.

5% of the labeled cholesterol to cholesterol ester in 4 h. Under this assay condition, enzyme activity was linear with time for at least 4 h. Furthermore, the calculated cholesterol esterifying capacity of the test sample was independent of the dilution at which it was assayed provided the conversion of unesterified cholesterol to cholesterol ester was less than 6%.

For isolation of lecithin:cholesterol acyltransferase, the nonprotein solvent density of plasma was raised to a density (d) of 1.25 g/ml with KBr and centrifuged 28–30 h at 50 000 rpm ($8.4 \times 10^5 g$ per min). The top $d < 1.25$ g/ml fraction was aspirated after tube slicing and centrifuged at d = 1.21 g/ml at 50 000 rpm for 44 h. The bottom 27 ml, constituting the d = 1.21–1.25 plasma fraction, was quick frozen at −70 °C and retained for lecithin:cholesterol acyltransferase isolation. The top 9 ml of each tube or the $d <$ 1.21 g/ml plasma fraction was aspirated and then recentrifuged at d = 1.125 g/ml at 50 000 rpm for 28 h for HDL_3 isolation. The bottom d = 1.125–1.21 HDL_3 fraction was dialyzed against 0.1 M sodium carbonate buffer (pH 9.0) and coupled to Sepharose 4B (Porath et al., 1967). Approximately 1.2 mg of HDL_3 was coupled per ml of packed Sepharose. After coupling, the nonreacted groups of the activated Sepharose were blocked with 0.5 M glycine (pH 9.0). Following the conditions of Akanuma and Glomset (1968) the d = 1.21–1.25 plasma fraction, or very high density lipoprotein (VHDL) fraction, was passed through the Sepharose-HDL column. The bound lecithin:cholesterol acyltransferase was eluted with 0.5 mM sodium taurocholate as suggested (Akanuma and Glomset, 1968). After dialysis against 1 mM phosphate buffer (pH 7.4) to remove the sodium taurocholate, the lecithin:cholesterol acyltransferase preparation was passed through a hydroxylapatite column (Glomset and Wright, 1964) equilibrated with 1 mM phosphate buffer. The protein eluted from the column was subsequently passed through an imunoglobulin-Sepharose 4B column prepared in the same manner as the HDL-Sepharose column and equilibrated with 0.01 M Tris-HCl–0.14 M NaCl–1 mM EDTA (pH 7.4). The apolipoprotein D bound to the column was eluted with 0.2 M glycine-HCl (pH 2.2). Immunoglobulin was isolated from specific anti-apolipoprotein D sera by precipitation with 18% and then 12% sodium sulfate and DEAE-cellulose chromatography (Levy and Sober, 1960) equilibrated with 0.02 M phosphate buffer (pH 6.8).

Apolipoprotein D was isolated from delipidized HDL_3 by hydroxylapatite chromatography (McConathy and Alaupovic, 1973). Rabbits were immunized with 500 μg of apoprotein D as described (Albers and Dray, 1968). Antisera to apolipoproteins A-I, A-II, and B and to Lp(a) lipoprotein were the same as used previously (Albers et al., 1975, 1976; Albers and Aladjem, 1971; Albers and Hazzard, 1974).

Protein was determined by the method of Lowry et al. (1951) and cholesterol was measured by an AutoAnalyzer method (Lipid Research Clinics Program, 1974). Molecular weight determinations were made using polyacrylamide gel electrophoresis with sodium dodecyl sulfate (Weber et al., 1972). Phosphorylase A, human serum albumin, catalase, ovalbumin, chymotrypsin, and RNase were used as standards. Polyacrylamide gel electrophoresis was performed (Davis, 1964) in the presence of 8 M urea.

Amino acid composition was determined with a JLC-5AH automatic analyzer using a single column packed with Durrum DC-1A resin. Protein samples were hydrolyzed in 6 M HCl for 24 h at 110 °C under N_2. No corrections were made for losses during hydrolysis. Half-cystine was determined independently as cysteic acid in performic acid oxidized samples (Moore, 1963).

Results

The lecithin:cholesterol acyltransferase was purified from 1 l. of pooled human plasma. All procedures were carried out at 4 °C. Composite results from three experiments are shown in Table I. The d = 1.21–1.25 g/ml plasma fraction (very high density lipoprotein or VHDL) isolated by ultracentrifugation contained the bulk of the plasma enzyme activity but less than 4% of the total protein. The HDL-Sepharose column bound less than 4% of the total protein of the VHDL plasma fraction but bound >99% of the enzyme activity. Approximately 50% of the VHDL lecithin:cholesterol acyltransferase activity was eluted with 0.5 mM sodium taurocholate. Further purification was carried out by chromatography on hydroxylapatite in 1 mM potassium phos-

Table II: Amino Acid Composition of Human Plasma Lecithin:Cholesterol Acyltransferase.[a]

Amino Acid	Mean (Range)
Lys	27 (25–29)
His	20 (19–21)
Arg	34 (30–37)
Asp	78 (74–80)
Thr	47 (45–50)
Ser	48 (46–49)
Glu	87 (83–89)
Pro	59 (57–62)
Gly	74 (65–83)
Ala	59 (55–63)
Half-Cys[b]	15 (13–17)
Val	61 (48–75)
Met	17 (16–17)
Ile	34 (31–38)
Leu	83 (77–89)
Tyr	17 (16–18)
Phe	26 (24–28)

[a] Analysis on two preparations, two analyses per preparation. Values are expressed as moles of amino acid per 100 000 g of protein. [b] Determined by performic acid oxidation.

phate buffer (pH 7.4). Approximately one-third of the enzyme activity passed directly through the column. This preparation was tested in gel diffusion at a protein concentration of 0.4–0.6 mg/ml against anti-human serum albumin, anti-human immunoglobulins, anti-apolipoprotein B, anti-Lp(a) lipoprotein, anti-apolipoprotein A-I, anti-apolipoprotein A-II, and anti-apolipoprotein D made in rabbits. The lower limit of detection of immunodiffusion analysis is approximately 2 μg of protein/ml. Only apolipoprotein D could be detected in this lecithin:cholesterol acyltransferase preparation. Therefore, the preparation was passed through the anti-apolipoprotein D affinity column. This final procedure yielded an enzyme preparation with about 12 000-fold purification and 16% yield. This highly purified enzyme preparation was homogeneous in charge and size (Figure 1) with the mobility on sodium dodecyl sulfate and urea polyacrylamide gels nearly identical with human serum albumin. The apparent molecular weight was 68 000 ± 1600 (n = 7) as determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. On a calibrated Sephadex G-100 column equilibrated with 0.1 M Tris-HCl–0.15 M NaCl–1 mM EDTA (pH 8.0) the preparation eluted with an apparent molecular weight of 70 000 (assuming a globular protein with a partial specific volume of 0.70–0.75 ml/g and hydrated with approximately 0.3 g of water/g of protein). The amino acid analysis of the purified lecithin:cholesterol acyltransferase preparation is shown in Table II. The amino acid composition differed from all previously reported fractions obtained from VHDL or HDL and also differed from human serum albumin.

Discussion

This study represents the first report on a highly purified homogeneous preparation of human plasma lecithin:cholesterol acyltransferase. This lecithin:cholesterol acyltransferase preparation was found homogeneous by three different criteria: (1) a single band on polyacrylamide gel electrophoresis in the presence of 8 M urea; (2) a single band on sodium dodecyl sulfate gel electrophoresis; and (3) a single protein peak on Sephadex G-100 chromatography. It also had approximately the same apparent molecular weight on both sodium dodecyl sulfate gel electrophoresis and Sephadex chromatography suggesting that the lecithin:cholesterol acyltransferase enzyme is a single polypeptide chain of mol wt 68 000–70 000. Lacko et al. (1974) reported the isolation of a protein preparation with lecithin:cholesterol acyltransferase activity which had an apparent mol wt of 95 000 on calibrated G-100 columns and approximately 50 000 on sodium dodecyl sulfate gel electrophoresis. Thus, Lacko's preparation appears to have somewhat different properties than ours. Furthermore, his preparation was not homogeneous by standard polyacrylamide gel electrophoresis.

The major protein contaminate in the hydroxylapatite eluate was apolipoprotein D. This glycoprotein is found not only in the very high density lipoprotein fraction but is also a consistent constituent of HDL_3 (McConathy and Alaupovic, 1973). Both HDL_3 and VHDL are good substrates for lecithin:cholesterol acyltransferase (Fielding and Fielding, 1971). What role apolipoprotein D plays in the lecithin:cholesterol acyltransferase reaction is not clear but it has been suggested as a specific carrier of lysolecithin, a product of the lecithin:cholesterol acyltransferase reaction (Olofsson and Gustafson, 1974), whereas Kostner (1974) has suggested that it is an activator of the lecithin:cholesterol acyltransferase reaction. Now that a method of obtaining highly purified lecithin:cholesterol acyltransferase in reasonable yield is available, studies can be initiated to elucidate the interaction of lecithin:cholesterol acyltransferase with the various purified apolipoproteins with well-defined substrates such as used in the studies by Soutar et al. (1975).

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Inactivation of Horse Liver Alcohol Dehydrogenase by Modification of Cysteine Residue 174 with Diazonium-1*H*-tetrazole†

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ABSTRACT: Diazonium-1*H*-tetrazole was tested as a potential active-site-directed reagent for amino acid residues involved in catalysis by alcohol dehydrogenase. In a novel reaction with a protein, diazonium-1*H*-tetrazole inactivated the enzyme selectively, and almost stoichiometrically, by reacting with the sulfur of a cysteine residue, Cys-174. As a model compound, the tetrazole adduct of free cysteine was prepared. Elementary and spectral analyses of the adduct were consistent with the structure 5-tetrazoleazo-*S*-cysteine. The adduct absorbs light with a maximum at 316 nm, and is destroyed by irradiation at this wavelength. The inactivated enzyme still bound NADH as determined by difference spectroscopy, but did not enhance the fluorescence of the bound NADH as did native enzyme. X-ray crystallographic studies of free enzyme have shown that Cys-174 coordinates the zinc at the active site (Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T., and Brändén, C.-I. (1974), *FEBS Lett. 44,* 200–204). The modified enzyme is probably inactive because the large, negatively charged tetrazole ring interferes sterically or electrostatically with the binding of substrates or with hydride transfer.

Several amino acid residues located at the active sites of horse liver alcohol dehydrogenase (EC 1.1.1.1) have been identified by chemical modification studies and by x-ray crystallography, but only the zinc ions at the active sites are thought to be involved in the transfer of hydride ion from ethanol to NAD^+ in the ternary complex (Eklund et al., 1974). Some proposals for the mechanism of alcohol dehydrogenases involve removal of the proton from the hydroxyl group of the alcohol by a basic group, possibly with formation of a zinc alkoxide (Eklund et al., 1974; Wang, 1968; McFarland and Chu, 1975; Mildvan, 1970; Klinman, 1975). Since various pH dependency studies have shown that one or more groups on the liver enzyme with pK values from 6.4 to 7.6 must be unprotonated for maximum activity, the base may be an imidazole (Brooks et al., 1972; Plapp et al., 1973; Shore et al., 1974). To modify such a base, we chose to use diazonium-1*H*-tetrazole, which reacts with histidine and tyrosine residues to form readily identifiable products (Horinishi et al., 1964; Sokolovsky and Vallee, 1966; Takenaka et al., 1969). Furthermore, DHT[1] is similar to tetrazole, which is a competitive inhibitor against ethanol with an inhibition constant of 3.2 mM (Theorell et al., 1969). Thus, DHT might be an active-site-directed inactivator, binding to the enzyme–NAD^+ complex as does pyrazole (Theorell and Yonetani, 1963). Nitrogenous bases, such as imidazole and pyrazole, also bind (weakly) at the zinc site on the enzyme in the absence of NAD^+ (Sigman, 1967); thus, DHT might also bind to and react with free enzyme.

Experimental Procedure

Materials. Chemicals were obtained from the following sources: NAD^+ and NADH for kinetics, Boehringer Mannheim; NAD^+ and NADH, Sigma; cysteine (free base), Cyclo Chemicals; 5-amino-1*H*-tetrazole, Eastman or Aldrich; acetaldehyde, 2-mercaptoethanol, and pyrazole, Eastman; and ethylenimine and all sequencing reagents, Pierce. Horse liver alcohol dehydrogenase was purified by a modification of the procedure of Theorell et al. (1966).

Preparation of Tetrazole Azoenzyme. DHT was freshly prepared and adjusted to pH 2 to 3 with 6 N NaOH (Horinishi et al., 1964). The general modification procedure was to add DHT (2.4 to 250 μM in water) to an equal volume of alcohol dehydrogenase (at a concentration of 1 to 10 mg per ml in 1.0 M potassium phosphate buffer, pH 6.8), while the

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[1] The abbreviation used is: DHT, diazonium-1*H*-tetrazole.