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A cholesteryl ester transfer complex in human plasma

(lecithin:cholesterol acyltransferase/cholesteryl ester transfer protein/low density lipoprotein/high density lipoprotein)

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ABSTRACT **Immunoadsorption affinity chromatography has been used to define the structure of lipoproteins in human plasma containing lecithin:cholesterol acyltransferase (EC 2.3.1.43) (LCAT) and transfer protein (apo D). The whole of LCAT was adsorbed by antibodies specific for apo D and for apo A-1, indicating that the enzyme is present in plasma exclusively as a complex with its cofactor (apo A-1) and product transfer protein (apo D). About 80% of apo D (but no LCAT) was removed by antibody to apo A-2, indicating the presence of most of apo D in the form of an enzyme-free complex with apo A-1 and apo A-2. After removal of LCAT with antibody to apo D, plasma was unreactive as a substrate with isolated LCAT, but substrate activity was generated by ultracentrifugal flotation with either intact or adsorbed plasma. The apparent stoichiometry of the complex with LCAT (LCAT:apo A-1:apo D) was 1.0:0.9:1.8; that of the complex containing apo A-1, apo A-2, and apo D was 3.9:2.2:1.0.**

Lecithin:cholesterol acyltransferase (EC 2.3.1.43) (LCAT) in human plasma catalyzes the synthesis of almost the whole of plasma cholesteryl ester content. Its activity is mediated via an apoprotein cofactor (apo A-1) (1), which is a component of lipoproteins isolated within the high density (HDL) range (2). Most plasma cholesteryl esters are associated with low density lipoprotein (LDL), which contains little or no apo A-1 and is not a substrate for LCAT (3). These esters are delivered to LDL through the activity of a cholesteryl ester transfer protein (apo D) recently identified and isolated (4). The rates of cholesteryl ester synthesis and transfer in plasma are essentially equal (4), suggesting synergism between these reactions and possible structural association of the transferase and transfer proteins. However, after centrifugal flotation, LCAT was recovered in the $\rho > 1.21$ g/cm^3 density fraction whereas the greater part of transfer activity was present in the $1.063 < \rho < 1.21$ g/cm^3 density range (4, 5). A complex containing LCAT and apo D, if present in whole plasma, would play a key role in the regulation of plasma cholesterol metabolism, yet centrifugation is effective in dissociating plasma lipoprotein complexes (6). In this research, immunoadsorption chromatography has been used to determine the properties and apoprotein stoichiometry of complexes containing LCAT and apo D. Although absence of binding of one protein to immobilized antibody of another may be only ambiguous evidence for lack of association (because the complex might have dissociated during chromatography), binding of one protein to antibody of another is strong evidence that a complex exists in that form in the medium. Such evidence is here obtained for the association of LCAT and apo D.

MATERIALS AND METHODS

Preparation and Assay of Lipoprotein Apoproteins. Apo D was isolated as described (4) from human plasma HDL. It was a single species by polyacrylamide gel electrophoresis and was immunologically and chemically identical to the active cholesteryl ester transfer protein purified from plasma by affinity chromatography (4). Apo A-1 and apo A-2, the major apoproteins of HDL, were isolated from delipidated HDL by molecular sieve chromatography in 10 mM Tris·HCl, pH 8.2/6 M urea, followed by DEAE-cellulose chromatography in the same buffer with a gradient of 0.01–0.1 M NaCl (2). LDL was isolated within the density limits 1.02–1.04 g/cm^3, and its component apo B protein was precipitated with 50% (vol/vol) tetramethylurea (7). Antisera to these apoproteins were raised by injecting 100–500 μg of antigen in 50% Freund's complete adjuvant into New Zealand rabbits; titers were boosted once or twice under the same conditions at two weekly intervals. Antisera obtained by venous bleeding were used for radial immunodiffusion assay of apoprotein levels in human plasma and plasma fractions. Assays for apo A-1, apo A-2, and apo B were identical to those described (8, 9). For assay of apo D, 2-mm layers of 0.6% agarose (Bio-Rad) were prepared on glass plates in 50 mM barbital buffer (pH 8.6) containing 4% (vol/vol) antiserum. Antigen was added in 1% Triton WR-1339 to the 2-mm sample wells. Precipitin rings reached maximal diameter within 24 hr at room temperature; (diameter)^2 was linear with the mass of added antigen up to 10^3 ng. The level of apo D expressed was not increased by addition of 1 mM sodium decyl sulfate or 50% (vol/vol) tetramethylurea.

To prepare immobilized antibodies, we fractionated antiserum with sodium sulfate (12–18% saturation), dissolved the precipitate in 175 mM phosphate buffer (pH 6.3), dialyzed the solution against the same buffer, and passed it through a column of DEAE-cellulose (10). The gamma globulin fraction in the void volume, dialyzed into 0.1 M $NaHCO_3$/0.5 M NaCl, pH 7.8, was mixed with activated CNBr-Sepharose 4B (Pharmacia) at a ratio of 1 g of dry gel per 10 mg of protein (11). Columns were equilibrated with 10 mM Tris·HCl/0.15 M NaCl/1 mM disodium EDTA, pH 7.4. To absorb out lipoproteins containing individual antigens, we added plasma (usually 0.5–2 ml) to 10–20 × 1.2 cm columns of immobilized antibody at 4°C; the flow rate was 3–4 ml/hr. Fractions containing >98% of added proteins were pooled and assayed for LCAT, transfer protein activity, and lipoprotein antigens. Under the conditions described, the whole of detectable antigen was removed by pas-

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Abbreviations: LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; apo A-1, coprotein of LCAT; apo D, cholesteryl ester transfer protein.

sage through the corresponding immobilized antibody. Because there was no detectable crossreaction between the antisera used in these experiments, the nature of multi-apoprotein complexes in plasma could be determined in terms of the simultaneous removal of LCAT activity or other apoprotein antigens.

Isolation and Assay of LCAT. Enzyme activity was isolated from human plasma as detailed elsewhere (5). The product was homogeneous by polyacrylamide gel electrophoresis, and transferase activity comigrated with the only protein band. The activity of purified transferase was wholly (>99.8%) dependent upon the presence of added apo A-1 (5). It contained no detectable apo A-1, apo A-2, apo B, or apo D. LCAT activity was assayed by two procedures, one designed to determine transferase against a standard liposome substrate under conditions independent of plasma lipoprotein or lipid concentration, the other to determine the rate of endogenous esterification of lipoprotein-free cholesterol in plasma. In the first, liposomes containing egg lecithin (Sigma) and cholesterol (molar ratio 6/1) were prepared with the French pressure cell (5). The cholesterol moiety was labeled with [1,2-^{3}H]cholesterol (New England Nuclear) (1.5×10^5 dpm/μg). The liposomes were activated by incubation (1 hr, 37°C) with apo A-1 (1.0 μg/12 μg of lecithin). Assay was in the presence of 50 mM Tris·HCl (pH 7.4) and 5% (wt/vol) recrystallized human albumin (12). Assays of plasma (1–10 μl) with this substrate (0.2 ml) were linear for 5 min. The reaction was stopped by addition of chloroform and methanol (13). Production of labeled cholesteryl ester was determined by thin-layer chromatography of portions of the chloroform phase on silica gel layers on glass plates developed in hexane/diethyl ether/acetic acid, 83:16:1 (vol/vol). Cholesteryl ester radioactivity was determined by liquid scintillation spectrometry (5). A reaction plot of Michaelis–Menten form was obtained for production of labeled cholesteryl ester for each of several substrate liposome concentrations, and the apparent V_{max}/K_m ratios were plotted as a function of liposome cholesterol to obtain the calculated maximal reaction velocity for the rate of LCAT with pure liposome substrate (apparent K_m 15.2 $\pm$ 1.1 μg of cholesterol ml^{-1}). The assay gave equivalent rates for endogenous and added purified transferase. When plasma (activity 6.05 nmol ml^{-1} min^{-1} cholesterol esterified in the liposome assay) was mixed with isolated LCAT to give an expected total activity of 13.33 nmol ml^{-1} min^{-1}, activity recovered from the mixture was 13.50 nmol ml^{-1} min^{-1}. When both plasma and plasma mixed with pure LCAT were incubated at 37°C, the proportion of recovered activity after 3 hr was the same (0.87 and 0.88, respectively, of that originally present). Finally neither added purified HDL nor LDL (5 μg of lipoprotein protein per μl of plasma) had significant effect on the level of LCAT assayed in plasma with the liposome assay.

In the second assay procedure, LCAT activity was assayed in terms of loss of free cholesterol from plasma, measured chemically with cholesterol oxidase (14). Samples of plasma (50–100 μl), before and after incubation at 37°C, were extracted with chloroform and methanol (13), and portions of the chloroform phase (containing 1–10 μg of cholesterol) were assayed in triplicate (reproducibility $\pm$ 1.1% over this range). Rates of loss of cholesterol were linear over the 10-min incubation period.

Measurement of Cholesteryl Ester Transfer Activity. The level of transfer protein was assayed not only immunologically as apo D but also biologically in terms of the rate of transfer of cholesteryl ester to very low density lipoprotein (VLDL) and LDL at 37°C. Plasma was brought to 20 mM with Tris·HCl buffer (pH 7.4); at intervals samples (0.5 ml) were mixed with 0.1 vol of heparin/$MnCl_2$ (2250 International Units of heparin per ml/1.0 M $MnCl_2$) to precipitate LDL and VLDL (15). Samples of supernatant (which were unreactive by immunoassay with anti-apo B antiserum) were assayed for their cholesteryl ester content with cholesterol esterase and oxidase (14). LCAT was either inhibited with 2 mM dithiobisnitrobenzoic acid (16) and transfer activity was measured as the decrease in soluble cholesteryl ester with time, or else, in the presence of LCAT activity, transfer was expressed as the difference between total and heparin/$MnCl_2$-soluble cholesteryl ester, again as a function of time.

RESULTS

When plasma was treated with heparin/$MnCl_2$ to precipitate lipoproteins containing apo B (VLDL and LDL), the level of LCAT remaining in the supernatant solution did not decrease from that of plasma (96–100% of original values, three experiments), indicating that the major acceptor of cholesteryl ester, and the esterifying system, were not structurally associated. There was also no loss of apo D from the plasma when VLDL and LDL were precipitated. Similar experiments were carried out in which apo B-containing lipoproteins were removed by immunoadsorption with antibody to apo B; under conditions in which all detectable apo B-containing lipoprotein was removed from plasma, there was no loss of LCAT activity and no loss of cholesteryl ester transfer protein assayed either by its transfer activity, as described above, or immunologically as apo D.

When plasma was passed through a column of immobilized antibody to apo D under conditions where all detectable antigen was removed, the plasma retained no detectable LCAT activity even though, when isolated LCAT was passed through, the enzyme activity was fully recovered in the column effluent. The plasma retained no cholesteryl ester transfer activity, assayed either in terms of loss of heparin/$MnCl_2$-soluble cholesteryl ester, assayed chemically after the inhibition of LCAT, or with the transfer of [^{3}H]cholesteryl ester label from HDL to LDL, measured as described (4). However, essentially the whole of plasma-free and ester cholesterol was recovered (Table 1), and when isolated LCAT was passed through the column under the same conditions, it was quantitatively recovered in the column effluent. These experiments indicate that the whole of plasma LCAT was associated with apo D. As shown in Fig. 1, when plasma was passed through the anti-apo D affinity column under conditions in which only a fraction of apo D was removed, there was also a partial and proportionate removal of both cholesteryl ester transfer activity and LCAT. When purified LCAT was added either to whole plasma or to plasma from which part or all of detectable apo D antigen had been

Table 1. Effects of immunoabsorption on plasma concentrations of LCAT, transfer protein, and cholesterol

	Original plasma	Eluate	%
Protein, mg	67.4	67.98	100.9
Apo D, μg	65.7	<2.0	<2.5
Unesterified cholesterol, μg	365	348	95.9
Ester cholesterol, μg	1490	1459	98.5
LCAT, nmol min^{-1}	6.40	<0.04	<0.6

Values are the means of three determinations; 1.25 ml of plasma containing 1/20 vol of 0.2 M sodium citrate was applied to a column of immobilized antibody to apo D as described in the legend to Fig. 1. The total of eluate fractions containing detectable protein or cholesterol was pooled and assayed for cholesterol, protein, or transferase content relative to that of the applied plasma. Apo D was determined by radial immunodiffusion assay, protein by the Lowry procedure, and LCAT by using lecithin–cholesterol liposomes.

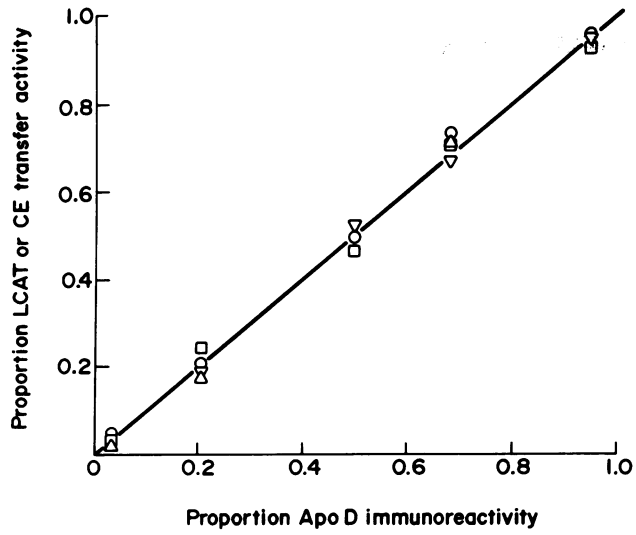

FIG. 1. LCAT and cholesteryl ester transfer rates in plasma partially depleted of apo D. Plasma (1.5–12 ml) was passed through an anti-apo D immunoabsorbent affinity column (1.2 × 40 cm) of anti-apo D IgG covalently complexed with CNBr-Sepharose 4B. The column was equilibrated in 0.15 M NaCl/10 mM Tris·HCl/1 mM disodium EDTA, pH 7.4. The flow rate was 10 ml/hr. Under these conditions the column removed 120–150 μg of apo D antigen, recovered if required with 3 M NaCNS (5). The pool eluate passing through the column was assayed for LCAT activity in terms of either cholesteryl ester formation with [^{3}H]cholesterol liposomes (□) or decrease in total plasma-free cholesterol (○); cholesteryl ester transfer was measured either in terms of the rate of transfer of labeled cholesteryl ester from [^{3}H]cholesterol-labeled HDL to unlabeled LDL (5) (△) or in terms of the decrease in heparin/$MnCl_2$-soluble cholesteryl ester assayed chemically (▽). CE, esterified cholesterol.

removed, there was no increase in the rate of esterification of cholesterol (Fig. 2) even though the added activity, assayed with liposome substrate at the end of the incubation period, was recovered from the plasma medium. In control experiments,

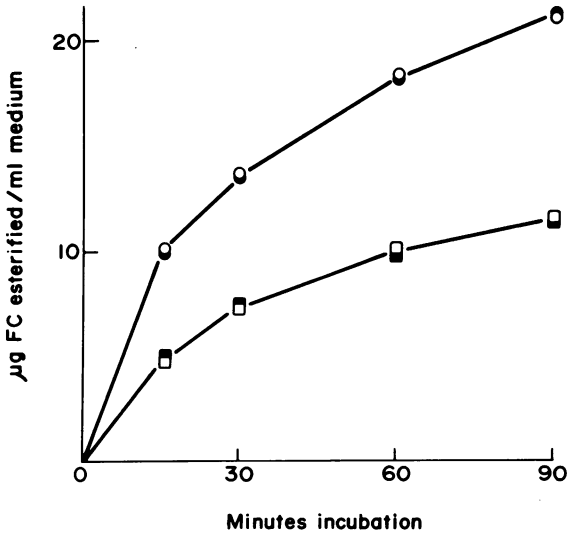

FIG. 2. Effect of added pure LCAT on the rate of esterification of free cholesterol (FC) in whole plasma (○, ●) and plasma depleted (48%) of apo D (□, ■). Purified LCAT was added to plasma or plasma partially depleted (48%) of apo D in an amount equivalent to 6.9 nmol of cholesterol esterified per ml of plasma per min (units) by liposome assay; the original plasma contained 6.1 units/ml and the partially depleted plasma 3.0 units/ml of endogenous LCAT activity with the same assay. Values represent free cholesterol loss on incubation determined with cholesterol oxidase in the absence (○, □) or presence (●, ■) of added LCAT.

when the reactive groups of Sepharose-CNBr were blocked with ethanolamine (11), albumin, or fibrinogen, there was no removal of LCAT, apo D, or transfer activity from plasma during column chromatography. These results suggest the absence of substrate for LCAT in plasma beyond that already complexed with the enzyme and the simultaneous removal of LCAT and substrate by antibody to apo D. Because HDL prepared by ultracentrifugal flotation is a substrate for the enzyme (3), the substrate properties of HDL prepared by centrifugation from intact and apo D-free plasma were compared. Although plasma depleted of apo D was not a substrate for LCAT, its HDL fraction ($1.063 < \rho < 1.21$ g/cm^3) after dialysis into 0.15 M NaCl/1 mM disodium EDTA, pH 7.4, assayed in the presence of 5% albumin, was as effective a substrate for isolated LCAT as HDL from the same plasma not subjected to column chromatography; at an HDL-free cholesterol concentration of 25 μg ml^{-1}, the ratio of esterification rates was 0.96 ± 0.11 (six determinations). This result indicates that the substrate properties of HDL in reaction with pure LCAT are generated during its isolation; this change may be effected by the high concentration of salt used during centrifugation or by centrifugal shearing forces *per se*.

Experiments described above suggested no association of LCAT and apo D with lipoproteins containing apo B. The relationships between these and apo A-1 and apo A-2, the major HDL apoproteins, are shown in Table 2. All (> 99%) of both LCAT activity and apo D were removed by immobilized anti-apo A-1 under conditions where the whole of detectable apo A-1 antigen was removed from plasma, indicating that both LCAT and transfer protein were present in whole plasma exclusively in association with apo A-1. On the contrary, no LCAT was removed by anti-apo A-2 even when the whole of apo A-2 was removed, whereas under the same conditions 80 ± 2% of total plasma apo D was adsorbed. These results indicate the presence of two species of complex containing apo D: one containing apo D, apo A-1, and the whole of plasma LCAT; the other containing apo D, apo A-1, and apo A-2. The stoichiometry of apoproteins in these complexes was determined by differential immunoadsorption column chromatography (Table 2). The apo D content of the first complex (20% of plasma apo D) was 0.35 ± 0.02 nmol ml^{-1} (three experiments); apo A-1 associated with this complex was determined as the level of apo A-1 removed by anti-apo D after prior removal by chromatography of all species containing A-2. This represented 0.19 ± 0.02 nmol ml^{-1} of apo A-1. The level of LCAT in the complex, calculated from the specific activity of the isolated enzyme and its plasma level (both with the liposome assay), was 0.17 ±

Table 2. Proportion of apoproteins and LCAT remaining after complete removal of homologous antigen by antibodies to apo A-1, apo A-2, and apo D

Antibody	Proportion of apoprotein or activity remaining			
	Apo A-1	Apo A-2	Apo D	LCAT
Anti-apo A-1	—	0.01	0.01	<0.01
Anti-apo A-2	0.09	—	0.20	1.00
Anti-apo D	0.91	0.80	—	<0.01

Values are means of determinations on three plasma samples (SD was <0.01 in each case). Whole plasma apoprotein concentrations were: apo A-1, 1371 μg ml^{-1}; apo A-2, 304 μg ml^{-1}; and apo D, 58 μg ml^{-1}. LCAT activity (liposome assay) was 4.74 nmol ml^{-1} min^{-1} cholesterol esterified at 37°C. The specific activity of isolated LCAT in these experiments was 507 nmol min^{-1} mg^{-1} protein in the same assay. Molecular weight values used in calculation are: apo A-1, 28,000; apo A-2, 18,000 (17); apo D, 32,000; LCAT, 65,000 (these experiments). After removal of apo A-2 with homologous antibody, further apo A-1 removed by chromatography on anti-apo D was 5.5 ± 1.0 μg ml^{-1}.

0.02 nmol^{-1}. The calculated stoichiometry (apo A-1:LCAT:apo D) was therefore 1.0:0.9:1.9. The composition of the complex containing apo D, apo A-1, and apo A-2 was determined similarly. Its content of apo D was 1.5 ± 0.1 nmol ml^{-1}; its apo A-1 content (that apo A-1 removed by anti-apo D minus the apo A-1 content of the complex containing LCAT) was 5.5 ± 0.2 nmol ml^{-1}; and its apo A-2 content (that apo A-2 removed by anti-apo D) was 3.3 ± 0.2 nmol ml^{-1}. The resulting stoichiometry (apo D:apo A-1:apo A-2) was therefore 1.0:3.8:2.2.

DISCUSSION

In earlier research it had been concluded that LCAT, reacting with bulk HDL, generated cholesteryl esters which were then transferred nonenzymatically to VLDL and LDL (18). The second step of this reaction is now known to be mediated via the transfer protein (4). There is, however, considerable kinetic evidence from studies *in vivo* that only a very small pool of lipoprotein cholesterol is directly involved in ester synthesis and transfer (19, 20). Furthermore, in Tangier disease, a condition where HDL is congenitally essentially absent (21), the rate of formation and transfer of cholesteryl ester is almost normal. The present study provides evidence, first, that the substrate activity of HDL with LCAT is generated during centrifugal isolation (possibly through the formation of new associations of apoproteins and lipids); and second, that in whole plasma LCAT and apo D form a complex of defined stoichiometry that both synthesizes and distributes cholesteryl ester to the acceptor lipoproteins, including bulk HDL. This association is here called the "cholesteryl ester transfer complex." Its apparent molecular weight by gel filtration is approximately 220,000 (unpublished experiments). The apoprotein stoichiometry described above would contribute a minimal protein molecular weight of approximately 160,000; the balance may be contributed by lipids. This is also suggested by the removal of small amounts of cholesterol and cholesteryl ester by anti-apo D chromatography (Table 1) and by the recovery of plasma LCAT in ^2H_2O/CsCl as a complex of ρ = 1.23–1.25 g/cm^3 (5). However, in view of the high metabolic activity of the complex, such lipid would be present as a highly labile component in contrast to the defined stoichiometry of apoproteins. By techniques similar to those described here, it should now be possible to determine the concentration and activity of transfer complex in abnormalities of plasma cholesterol metabolism, such as those involving high levels of cholesteryl ester.

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