PURIFICATION AND SUBSTRATE SPECIFICITY OF LECITHIN–CHOLESTEROL ACYL TRANSFERASE FROM HUMAN PLASMA

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1. Introduction

Lecithin–cholesterol acyl transferase is active in the production of cholesterol esters and lysolecithin from the free cholesterol and lecithin moieties of the mammalian plasma lipoproteins, and is the major source of human plasma cholesterol esters [1, 2]. In the present research, this enzyme has been purified 2500-fold from human plasma. The purified enzyme is active only against the HDL_3 and VHDL classes of plasma lipoproteins. It has a half life of 3 hr at 4°. The protein moiety of these classes is required for the significant esterification of cholesterol in sonically dispersed lipid emulsions.

2. Materials and methods

LCAT activity was assayed as the synthesis of labeled cholesterol esters in whole plasma or isolated lipoproteins labeled by incubation with albumin-bound ^{3}H-cholesterol [3]. The cholesterol was purified in the laboratory by thin-layer chromatography to remove radioactivity in the cholesterol ester band. Either native plasma or lipoproteins, or samples heated at 58° for 30 min to inactivate endogenous LCAT activity [3], was used in the assay medium.

Abbreviations:
VLDL : very low density lipoprotein $d < 1.006$
LDL : low density lipoprotein $1.019 < d < 1.063$
HDL_2 : high density lipoprotein $1.063 < d < 1.12$
HDL_3 : high density lipoprotein $1.12 < d < 1.21$
VHDL : very high density lipoprotein $1.21 < d < 1.25$
LCAT : lecithin–cholesterol acyl transferase

Each was labeled to a specific activity in unesterified cholesterol of 0.01 μCi/μg. Following incubation with the enzyme samples (0.20 ml assay), total lipid was extracted with 5 ml chloroform–methanol (1:1 v/v). Aliquots were developed on silica gel G plates in hexane–diethyl ether–acetic acid (83:16:1 v/v). The cholesterol ester bands visualized with iodine vapour were scraped off and radioactivity determined in glass vials containing 10 ml of scintillation fluid (100 g naphthalene, 10 g POP, 0.5 g POPOP/liter toluene) in a Series 3000 Packard liquid scintillation spectrometer with automatic standard. Lipoprotein fractions (VLDL, LDL, HDL_2, HDL_3, VHDL) were isolated by ultracentrifugal flotation in KBr–NaCl solutions [4]. Delipidation of HDL_2, HDL_3 and VHDL was carried out with ethanol–ether (2:1 v/v) and sonicated dispersions of the extracted lipoprotein lipids were prepared in 0.145 M NaCl [5]. Protein was measured by the ninhydrin method [6] after hydrolysis in 6 N HCl at 110° for 21 hr, and also according to Lowry [7]. Phospholipids and cholesterol in lipoprotein lipids were determined according to the regular laboratory procedures [8, 9]. Recrystallized bovine serum albumin was obtained from Armour, Chicago. Deuterium oxide (99.7% D_2O) and hydroxyl apatite (Biogel HT) were purchased from Biorad, Richmond, California, and caesium chloride (99+% CsCl) from Matheson, Ohio.

3. Results

LCAT activity was purified from the fresh plasma of healthy non-fasting male donors. (Fasting plasma

preincubated with 1/40 vol. intralipid 10% triglyceride emulsion (Vitrum, Sweden) was also successfully used in preliminary experiments). All manipulations with enzyme fractions were carried out at 4–5° except where stated. 100 ml portions of plasma were obtained from blood collected into 1/20 vol. 0.2 M sodium citrate after centrifugation for 20 min at 2,000 *g*. These were brought to 66% saturation of $(NH_4)_2SO_4$ by addition of the solid salt. After standing in ice for 60 min, the precipitate was centrifuged down at 20,000 rpm for 20 min and then dispersed in 25 ml of D_2O–0.005 M EDTA, pH 7.2 ('D_2O–EDTA'). After stirring for 60 min, the undissolved residue was removed by centrifugation as before. The supernatant contained the bulk of LCAT activity (table 1). Solid CsCl was added to 14.5% w/v and 3 ml samples were layered under the same volume of D_2O–EDTA–14.5% CsCl (d = 1.21) in the cellulose nitrate tubes of a Spinco 40.3 rotor. Centrifugation was for 36 hr at 40,000 rpm at 5–6° in a Type L2-65 ultracentrifuge. After the run, the tubes were sliced (Beckman tube slicer) to isolate the clear intermediate zone between the supernatant orange band containing the bulk of the lipoprotein, and the yellow infranatant of plasma albumin. The active fraction was then mixed with 0.1 g CsCl/ml and layered as before under an equal vol. of D_2O–EDTA–18% CsCl w/v (d = 1.25). Centrifugation was for 36 hr at 35,000 rpm. The clear intermediate zone was again removed by slicing and contained the bulk of LCAT activity purified about 350-fold from the plasma in 30–35% yield (table 1). Addition of 2-mercaptoethanol (1 mM final concentration) during the centrifugation procedure did not increase purification or recovery. Further purification was carried out by chromatography on hydroxyl apatite in 0.02 M potassium phosphate buffer containing 27% w/v NaBr, pH 7.2. After equilibration of 0.9 X 5.0 cm columns with this buffer, in which medium dissociation of enzyme substrate complex in the LCAT system is maximal [10], 1 ml of the active fraction from the preceding step was made 0.02 M in

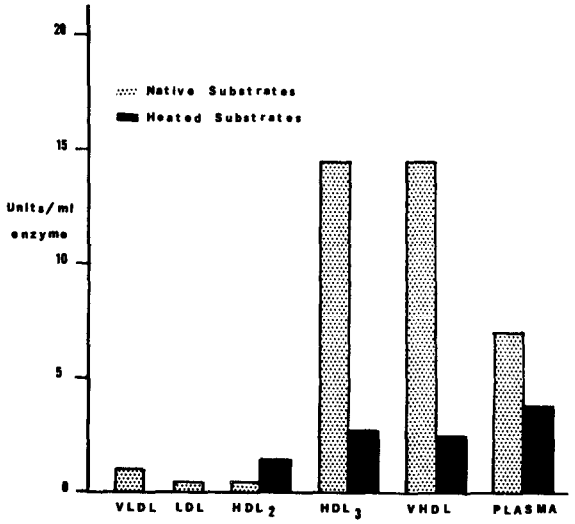

Fig. 1. LCAT activity against human plasma and lipoprotein fractions. Assays contained 0.02 ml lipoprotein solution (100 μg free cholesterol/ml, 0.01 μCi/μg) + 0.02 ml 10% bovine serum albumin in 0.145 M NaCl, pH 7.4 + 0.155 ml 0.145 M NaCl + 0.005 ml purified LCAT solution or potassium phosphate–NaBr buffer. Control tubes without added LCAT were assayed with each lipoprotein fraction. Incubation was for 3 hr at 37°, after preincubation (without added LCAT) for 30 min at 37°.

Table 1
Purification of lecithin–cholesterol acyl transferase.

Fraction	Vol. (ml)	Total activity (units*)	Total protein (mg**)	Activity/mg protein	Yield	Purification (fold)
Original plasma	100	6600	6230	1.06	1.00	1.00
Soluble fraction from $(NH_4)_2SO_4$	59	5185	3735	1.38	0.80	1.3
Ultracentrifuge fraction	12.5	2113	5.5	384.2	0.32	362
Hydroxyl apatite eluate	16.0	1183	0.40	2956	0.18	2788

* 1 unit of enzyme catalyzed the esterification of 1 nmole unesterified cholesterol/hr from whole native plasma at 37° and pH 7.4.
** By the ninhydrin method [6].

phosphate buffer and 27% w/v in NaBr and passed into the column. The flow rate was 1 ml/hr, and 1 ml fractions were collected. The column was washed with 5 vols. of potassium phosphate–NaBr buffer, then with 5 vols. of the same buffer made 0.05 M in sodium citrate. 25–50% of added enzyme activity passed directly through the column. This was inactive against extracted lipoprotein lipids in the absence of the lipoprotein protein moiety, as described below. The remainder of applied activity was eluted in the citrate fraction. This latter fraction did not show a requirement for lipoprotein protein, as shown by activity against lipoprotein lipids alone. A final purification of about 2500-fold in 10–15% yield was obtained (table 1) in terms of protein as measured by the ninhydrin reaction after acid hydrolysis [6]. Protein determination by the method of Lowry [7] gave calculated purifications of up to 10,000-fold presumably due to a low content of tyrosine in the purified LCAT fraction. The purified enzyme had a half-life of activity of 3 hr at 4°. The activity of the purified LCAT fraction against the lipoproteins of the plasma was determined. As shown in fig. 1, only HDL_3 and VHDL were effective substrates for this enzyme. Small amounts of cholesterol esterification (0–5% the rate of HDL_3, 4 experiments) were usually seen in VLDL, LDL and HDL_2. In view of the routine use of whole heated human plasma suggested for the assay of LCAT [3], it was of interest to determine whether heating affected the rates of esterification obtained. A significantly reduced rate of esterification was found in whole plasma, HDL_3 and VHDL (fig. 1).

The delipidated proteins of HDL_3 and VHDL were cofactors for the effective esterification of the cholesterol fraction of sonicated emulsions of HDL_2, HDL_3 or VHDL lipids (fig. 2). Under the given assay conditions, maximal activity was obtained at 50 μg lipoprotein protein/ml assay medium, representing a protein:phospholipid ratio of 1.0:0.5. Under the same conditions, delipidated HDL_2, in assays containing the lipids of HDL_2, HDL_3 or VHDL, permitted a lower, but significant rate of esterification of cholesterol (20–35% HDL_3, 4 experiments). Significant cofactor activity was always present in the $d > 1.25$ infranate, even after extensive centrifugation to remove VHDL (for 48 hr at 40,000 rpm in the 40.3 rotor of the ultracentrifuge). These cofactor activities were obtained whether the ^{3}H-cholesterol label was

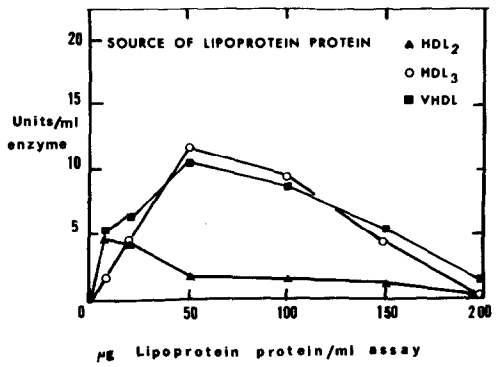

Fig. 2. LCAT activity against HDL_3 lipids in the presence of delipidated lipoprotein protein. Assays contained 0.02 ml sonicated HDL_3 lipids (100 μg free cholesterol/ml, 0.01 μCi/μg) + 0.02 ml 10% bovine serum albumin in 0.145 M NaCl, pH 7.4 + 0.05 ml of delipidated lipoprotein protein solution (0.145 M NaCl, 0.005 M NH_4OH–NH_4Cl, pH 8.1) + 0.105 ml 0.145 M NaCl + 0.005 ml purified LCAT or buffer solution. Assay conditions were as in fig. 1.

added by equilibration with cholesterol-bound albumin, or incorporated directly into the extracted lipoprotein lipid before its dispersion sonication.

4. Discussion

The foregoing experiments demonstrate a cofactor protein requirement for the esterification of cholesterol by purified lecithin–cholesterol acyl transferase, and also reveal an unexpected specificity in its interaction with the plasma lipoprotein classes. The preparation of enzyme by flotation with endogeneous lipid allows a purification similar to that recently obtained by use if added lecithin micelles [11]. However, in the present research, no protection of activity by 2-mercaptoethanol has been obtained. Possibly the effect described represents a stabilization of the added lipid against oxidation. The fraction isolated by either method has no protein cofactor requirement for the esterification of cholesterol. The further purification obtained in the present research by hydroxyl apatite chromatography of the active fraction, has made use of the observations of Akanuma and Glomset [10] that LCAT is passed through such columns under conditions of low phosphate concentration when much other plasma protein is retained.

The final product shows an absolute requirement for lipoprotein protein cofactor (fig. 2), similar to that required for the triglyceride hydrolase activity of purified lipoprotein lipase [12]. No significant differences were found in the activities of HDL_3 and VHDL as substrates and cofactors of LCAT activity (figs. 1 and 2). Native HDL_2 was almost inactive (fig. 1) although the delipidated protein moiety, in the presence of its own or HDL_3 lipids, showed a significant activity (fig. 2). It appears likely that the lack of esterification of HDL_2 cholesterol by LCAT represents not substantial lack of a specific protein cofactor, but is rather, at least in part, a feature of the native lipoprotein structure. The quite different activity of native HDL_2 and HDL_3 in cholesterol esterification, suggests that these may represent separate intermediates in the metabolism of plasma lipoprotein lipids.

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