Cholesteryl ester transfer protein

Size of the functional unit determined by radiation inactivation

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Radiation inactivation was used to determine the functional M_r of cholesteryl ester transfer protein (CETP) in rabbit plasma from control and irradiated animals. This technique reveals the size of the functional unit required to carry out the transfer function. The functional M_r was calculated to be 70000 ± 3000 (mean $\pm$SD) for both control and irradiated rabbits. This result is in accordance with the M_r obtained by a completely different method, namely SDS-polyacrylamide gel electrophoresis of a partially purified (110-fold) rabbit CETP. The p*I* of this CETP was found by isoelectric focusing to be equal to 5.95. The results suggest that the functional unit of this enzyme is the monomer.

Cholesteryl ester transfer protein *Radiation inactivation* *Target theory* M, *Isoelectric point*

1. INTRODUCTION

Over the past few years various studies have been carried out concerning the transfer of different lipid constituents between plasma lipoproteins as catalyzed by specific proteins [1-3]. Cholesteryl ester, phospholipid and triglyceride transfers were studied either separately or in conjunction with one another. Sometimes, the exchanges of apolipoproteins and free cholesterol [4] were also involved in these studies.

As yet it is not perfectly clear whether all the lipid transfer activities are located on the same protein, or if different proteins are specific for different lipid transports. On the other hand, cholesteryl ester transfer protein is associated with other plasma components as a lipid-protein complex. Therefore divergent M_r values of 150000 [3] and 66000 [5] have been proposed for the transfer protein(s). This report aims to characterize particularly the protein responsible for the transfer of cholesteryl ester by exploring with 2 entirely different methods for M_r determination.

The radiation inactivation method allows one to determine in situ the M_r of enzymes without prior purification as reported [6]. The shape of the protein does not influence the results. This method is based on the effect of primary ionizations of high-energy radiation (10^4 rad/min) [7] which inactivates an enzyme by a direct hit. Analysis by 'target theorey' of the enzyme inactivation after exposure to different doses of γ-rays gives the size of the functional unit necessary for catalytic activity [8]. This technique has been used to determine the functional M_r of lipoprotein lipase in rat heart and adipose tissue [9].

2. MATERIALS AND METHODS

Male New Zealand white rabbits were used either as control or as irradiated animals. the latter

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Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; DTNB, dithiobisnitrobenzoic acid; LPL, lipoprotein lipase

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received a single whole-body dose of 800 rad from a linear electron accelerator (LD_{50} in 30 days). Blood was collected from either control or irradiated rabbits (16 h after irradiation) by intracardiac puncture into EDTA (final concentration 1 mg/ml).

The different classes of plasma lipoproteins were separated by sequential ultracentrifugation at the following density intervals: <1.006 = chylomicrons, <1.006 = VLDL, 1.006–1.063 = LDL, 1.063–1.21 = HDL.

Labelling of lipoproteins was as described by Marcel et al. [10]. A stabilized [^{3}H]cholesterol (Amersham) emulsion in 5 mM Tris-EDTA, containing 0.05% NAN_3 and 2% fatty-free bovine serum albumin buffer was incubated with total plasma for 1 h at 37°C in the presence of DTNB (final concentration 0.5 mM). β-Mercaptoethanol (final concentration 15 mM) was added to stimulate LCAT activity. After 20 h of incubation, plasma labelled lipoproteins were separated by ultracentrifugation and dialysed against a buffer containing 5 mM Tris-HCl, 0.5 mM EDTA, 150 mM NaCl, pH 7.4.

CETP was purified by hydrophobic chromatography on phenyl-Sepharose CL4B [5], cation-exchange chromatography on Cm52 carboxymethylcellulose [11] and by gel filtration on Sephacryl S300. Equilibration and elution of the last gel were realized with a solution of 0.1 M Tris-HCl, 0.5 M NaCl, 0.2% NaN_3, pH 8.0. The active fractions after each step were pooled and dialysed against Tris-HCl/EDTA/NaCl buffer as above. SDS-polyacrylamide gel electrophoresis was performed at the different purification steps as well as analytical isoelectric focusing.

CETP activity was measured as the ability of protein fractions to promote the transfer of radiolabelled cholesteryl ester from HDL to VLDL at 37°C. Labelled HDL (33 μg cholesteryl ester) were incubated with unlabelled VLDL (33 μg cholesteryl ester) in the presence of CETP (3.5 mg protein) in a final volume of 1 ml saline solution containing 0.01% NaN_3 for 3 h at 37°C. 0.5 mM DTNB was added to inhibit LCAT activity [9]. Incubation was stopped by transfer to crushed ice and by immediately diluting with 0.9% NaCl solution. VLDL were reisolated by centrifugation at 105 000 $\times$ g for 18 h at the density of 1.006. Lipids were analysed after extraction by thin-layer chromatography for their radioactive esterified and free cholesterol content.

For radiation inactivation, aliquots (0.3 ml) of the plasma fraction of $d>1.21$ g/ml either from control or irradiated rabbits were lyophilized in 1.5-ml Eppendorf microfuge tubes. The tubes were irradiated at room temperature (26 $\pm$ 2°C) in a ^{60}Co irradiator (Gammacell model 220, Atomic Energy of Canada, Ottawa) delivering about 2.5 Mrad per h [6].

The irradiator was calibrated with enzymes of known radiation sensitivities according to Beauregard and Potier [7]. Three tubes were exposed to each radiation dose. Non-irradiated tubes served as controls. The logarithm of remaining enzyme activity was plotted vs radiation dose and a regression line was drawn through the points.

The empirical equation of Kepner and Macey [6] was used to relate the radiation dose (in Mrad) necessary to inactivate CETP to 37% of its initial activity (D_{37}) to the M_r:

$$M_r = 6.4 \times 10^5 \times 1/D_{37}$$

3. RESULTS AND DISCUSSION

The radiation inactivation of rabbit CETP from the plasma fraction of $d>1.21$ g/ml is shown in fig.1. The linear regression curves gave a correlation parameter of 0.9779 and 0.8993 for control and irradiated rabbits, respectively. From these curves, the radiation dose leading to reduction to 37% of initial activity (D_{37}) was obtained at 9.0 Mrad for control rabbits. This allowed an estimate of 70 000 $\pm$ 3000 for the functional M_r of rabbit CETP to be made. Similar values (8.9 Mrad) were obtained for rabbits after a total-body irradiation.

Partial purification of rabbit CETP by chromatography using phenyl-Sepharose, CM-cellulose and Sephacryl S300 as supports allowed a 111-fold enrichment with 40% recovery from the plasma fraction of $d>1.21$ g/ml of control rabbits. Comparable yields were obtained with irradiated rabbits (106-fold purification, 22% recovery). Purified fractions were run on SDS-polyacrylamide gels and the electrophoresis produced a band at about M_r 70 000 in both control and irradiated rabbits which possessed the CE transfer activity (fig.2).

Polyacrylamide gel isoelectrofocusing patterns of both CETP from control and irradiated rabbits

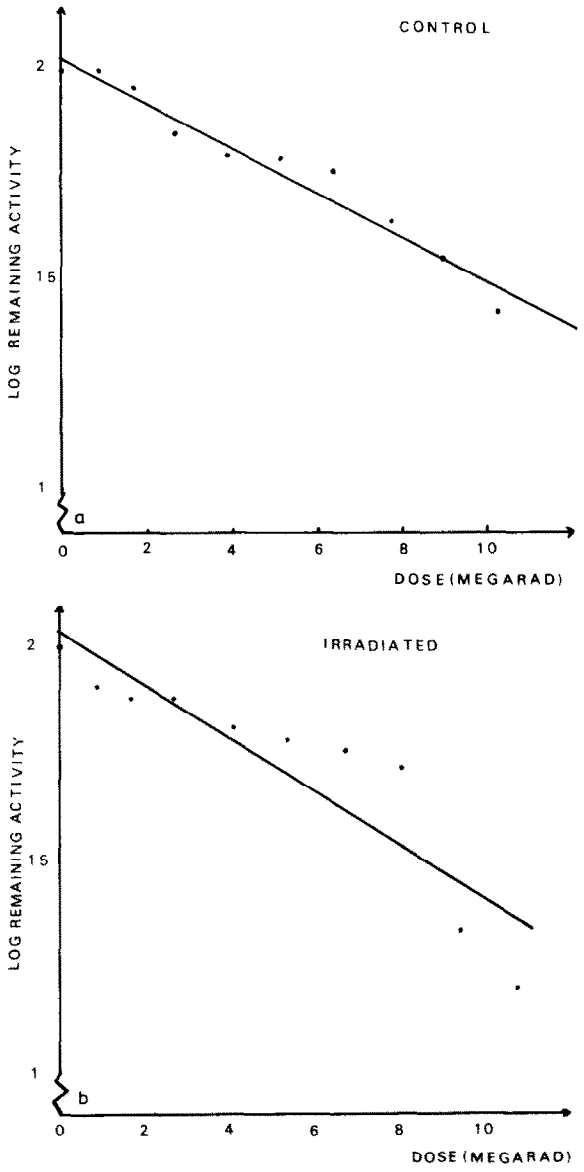

Fig.1. Radiation inactivation of CETP from control (a) and irradiated (b) rabbits. The regression lines were fitted by the least-squares method.

show that active fractions produced a prominent band at pH 5.95 (fig.3).

The rabbit CETP studied here has an apparent M_r of 70000 and a pI of 5.95 and promotes the transfer of labelled esterified cholesterol from HDL to VLDL. CETP was partially purified (110-fold) but this modest purification procedure (3 steps) should be compared to that obtained by Ab-

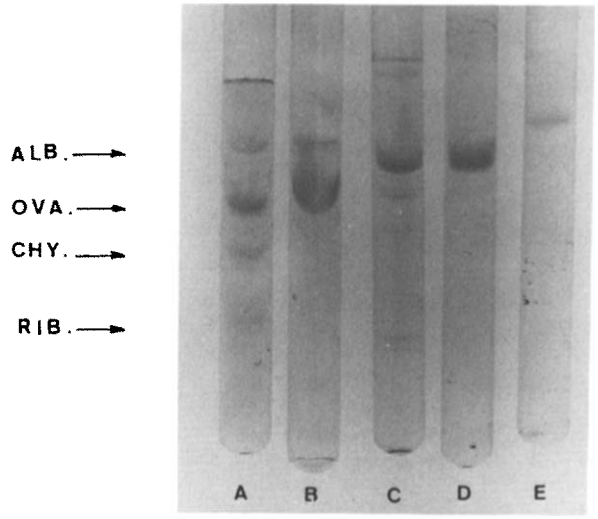

Fig.2. SDS-polyacrylamide gel electrophoresis of rabbit CETP. The dialyzed fractions from the differnt purification steps were lyophilized and run on 10% polyacrylamide gels at pH 8.8 in the presence of 0.01% SDS. (A) Protein standards, (B) $d > 1.21$ g/ml protein fraction, (C) phenyl-Sepharose pool, (D) CM-cellulose pool, (E) Sephacryl S300 pool.

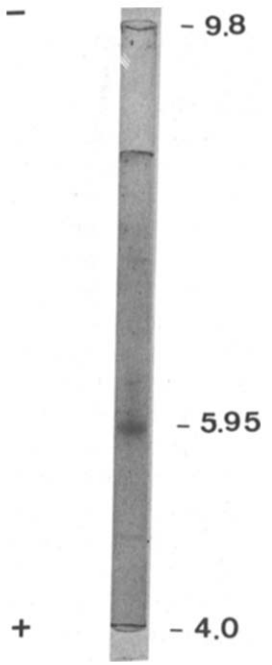

Fig.3. Analytical isoelectric focusing of rabbit CETP. Samples were run on 12 cm, 7.5% polyacrylamide gels containing 6.8 M urea and 2% ampholines, pH 3.5–10.

bey et al. [11] (600-fold with 5 steps). We agree with Abbey et al. [11] that the purification level of CETP from rabbit plasma is smaller than that from human plasma, because of the presence in the latter of an inhibitor of CETP [12]. This inhibitor might lead to an underestimate of the original CETP activity in total plasma, resulting in an overestimation at the final purification step.

Our studies on M_r of CETP by 2 methods entirely distinct in principle, namely radiation inactivation and SDS-polyacrylamide gel electrophoresis, which gave concurrent results are in favour of an M_r around 70 000. Very similar data were obtained by Abbey et al. [11]who found an M_r of 68 000 for rabbit CETP. Calvert and Abbey [13] proposed an M_r of 69 000 for human CETP whereas Morton and Zilversmit [5] showed 2-bands: one of M_r 58 300 and another of M_r 66 400. These 2 proteins would be different molecular forms of the same transfer protein. Furthermore, other groups found similar values, such as Tall et al. [14] and Ihm et al. [15] who put forward the values of M_r 63 000 and 61 000, respectively for CETP from human plasma. The combination of data obtained by the radiation inactivation method and SDS-PAGE suggests that the functional unit of this enzyme is a monomer.

Concerning the isoelectric point, Rajaram et al. [16] have measured a p*I* of 9. However, Morton and Zilversmit [5] proposed a p*I* of 9.1 for CETP measured in the absence of urea, while in the presence of urea the value was 4.8. Human CETP was given a p*I* around 5 by Pattnaik et al. [17]. According to Wetterau and Zilversmit [18], bovine CETP gave measurements of p*I* between 5.2 and 5.6.

Previous results demonstrated that whole-body irradiation caused several lipid disorders in rabbit. There was accumulation of VLDL, appearance of threonine-poor apolipoproteins and loss in LPL activity [19,20]. Furthermore, the transfer of CE from HDL to VLDL seems to be enhanced (unpublished). However, our results show that the CETP in irradiated rabbits seem to suffer no alteration since the global activity remains intact and the M_r and p*I* are not modified compared to control rabbits.

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