Physical and kinetic characterization of recombinant human cholesteryl ester transfer protein

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Cholesteryl ester transfer protein (CETP) mediates the exchange of triglycerides (TGs), cholesteryl esters (CEs) and phospholipids (PLs) between lipoproteins in the plasma. In order to better understand the lipid transfer process, we have used recombinant human CETP expressed in cultured mammalian cells, purified to homogeneity by immunoaffinity chromatography. Purified recombinant CETP had a weight-average relative molecular mass (M_w) of 69 561, determined by sedimentation equilibrium, and a specific absorption coefficient of $0.83 \text{ litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$. The corresponding hydrodynamic diameter (D_h) of the protein, determined by dynamic light scattering, was 14 nm, which is nearly twice the expected value for a spheroidal protein of this molecular mass. These data suggest that CETP has a non-spheroidal shape in solution. The secondary structure of CETP was estimated by CD to contain 32% α -helix, 35% β -sheet, 17% turn and 16% random coil. Like the natural protein from plasma, the recombinant protein consisted of several glycoforms that could be

only partially deglycosylated using N-glycosidase F. Organic extraction of CETP followed by TLC showed that CE, unesterified cholesterol (UC), PL, TG and fatty acids (FA) were associated with the pure protein. Quantitative analyses verified that each mol of CETP contained 1.0 mol of cholesterol, 0.5 mol of TG and 1.3 mol of PL. CETP mediated the transfer of CE, TG, PL, and UC between lipoproteins, or between protein-free liposomes. In dual-label transfer experiments, the transfer rates for CE or TG from HDL to LDL were found to be proportional to the initial concentrations of the respective ligands in the donor HDL particles. Kinetic analysis of CE transfer was consistent with a carrier mechanism, having a K_m of 700 nM for LDL particles and of 2000 nM for HDL particles, and a k_{cat} of 2 s^{-1} . The K_m values were thus in the low range of the normal physiological concentration for each substrate. The carrier mechanism was verified independently for CE, TG, PL and UC in 'half-reaction' experiments.

INTRODUCTION

The movement of neutral lipids and phospholipids (PLs) between lipoprotein particles is mediated by cholesteryl ester transfer protein (CETP), a glycoprotein found in plasma (for reviews, see [1–3]). CETP-mediated exchange of cholesteryl esters (CEs) and triglyceride (TG) is thought to be important in determining the plasma levels and composition of low-density lipoproteins (LDL), very-low-density lipoproteins and high-density lipoproteins (HDL). An understanding of the molecular process whereby CETP moves lipids between lipoproteins could provide fundamental insights into the regulation of normal lipid metabolism and the development of atherosclerosis.

In many early studies characterizing CETP, protein that was partially purified from human plasma was used [4,5]. More recently, multi-step purification methods have been reported for CETP from human or rabbit plasma [6–11], and a few studies have reported the use of recombinant human protein [12–14] or cynomolgus monkey protein [15] produced in cultured mammalian cells transfected with a cDNA for CETP. As deduced from the cDNA sequence [16], CETP contains 438 amino acids, with four potential glycosylation sites, and seven cysteines. Differential glycosylation contributes to heterogeneity [17]. A detailed mechanism for lipid transfer has not been presented but much of the available evidence indicates that CETP probably acts by a carrier or Ping Pong mechanism as opposed to sequential models involving CETP-mediated ternary complex formation or lipoprotein fusion. Conflicting kinetic data based on partially purified CETP have been used in support of either the carrier

model [18] or the ternary complex model [5]. A recent analysis of recombinant cynomolgus monkey CETP according to a symmetrical lipid exchange model supports the carrier mechanism [19]. The carrier mechanism is further supported by lipid-binding studies [20], the stoichiometry of CE and TG exchange [21], the specificity for certain lipids [22–24], preferential inhibition of either CE or TG by monoclonal antibody or other inhibitors [4,19,24,25], and physical studies with lipid interfaces [13].

The present studies are intended to characterize the biochemistry *in vitro* and the physical properties of highly purified recombinant human CETP. The native molecular mass of CETP is of particular interest, since conflicting reports in the literature have provided evidence that CETP exists as a monomer [11,14] or as a dimer [26]. The data presented in this paper demonstrate a monomeric structure for CETP, but suggest that it has a non-spheroidal shape. In addition, a detailed kinetic analysis of CE transfer was performed in order to obtain accurate kinetic constants for the lipid-transfer process. The kinetic data, in addition to half-reaction experiments, indicate that CETP-mediated lipid transfer occurs by a carrier mechanism.

EXPERIMENTAL

Production of CETP

A CHO cell line that was stably transfected with human CETP cDNA [12] was obtained from Dr. Alan Tall (Columbia University, New York, NY, U.S.A.). The cDNA was used also to transfect BHK cells using methods described previously [27].

Abbreviations used: CETP, cholesteryl ester transfer protein; CE, cholesteryl ester; TG, triglyceride; UC, unesterified cholesterol; PL, phospholipid; FA, fatty acid; LDL, low-density lipoproteins; LDLc, low-density lipoprotein cholesterol; HDL, high-density lipoproteins; HDLc, high-density lipoprotein cholesterol.

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Both cell lines expressed about 2–4 $\mu\text{g/ml}$ CETP, measured with ELISA [28], when grown in serum-free medium containing transferrin and BSA complexed with linoleic, oleic, palmitic and arachidonic acids. Cells were grown on microcarrier beads in 12 l suspension culture reactors with O_2 and pH control. Cell-conditioned medium was collected at 4 °C in 100–200 litre batches, concentrated 20–40-fold on spirally-wound ultra-filtrative membranes of 30000 nominal molecular-mass cut-off (Amicon/W. R. Grace) and stored at –20 °C.

Generation of anti-CETP monoclonal antibody and affinity column preparation

A hybridoma producing anti-CETP monoclonal antibody M468 was obtained after immunization of mice with purified CETP. M468 was identified as a mouse IgG that reacted specifically with CETP in SDS/PAGE immunoblots (Western blots) of partially purified CETP, but that had no effect on CE transfer activity. To obtain large quantities of M468, the hybridoma cells were injected into the peritoneal cavity of mice to generate ascites fluid or were grown in large-scale serum-free perfusion cell cultures. IgG was purified by precipitation with caprylic acid followed by $(\text{NH}_4)_2\text{SO}_4$ precipitation [29].

Immunoaffinity purification of CETP

Purified M468 IgG was coupled to CNBr-activated Sepharose 4B (Sigma) according to the manufacturer's directions. Concentrated conditioned medium containing CETP was applied to the column, washed with 0.01 M Na_2HPO_4 , pH 7.4, containing 1.1 M NaCl until the A_{280} reached baseline, and then was eluted with 25 mM glycine, pH 3.0. Fractions collected were immediately neutralized by the addition of 1 M Tris/HCl, pH 8.0. The lipid-transfer activity of the collected fractions was measured and those with activity were pooled. The active CETP pool was then dialysed against either distilled water or 10 mM NH_4HCO_3 , pH 7.8.

Sedimentation equilibrium analysis of molecular mass

Sedimentation equilibrium experiments were performed using CETP solutions (0.7 mg/ml) in 10 mM NH_4HCO_3 , pH 7.4. Because the protein concentration was less than 5 mg/ml, Dextran T-40 (Pharmacia) was added to a final concentration of 5 mg/ml in order to stabilize the protein density gradient and prevent convective mixing during rotor deceleration, as described previously [30,31]. The CETP/Dextran T-40 solution (125 μl) was centrifuged at 17750 g for 24 h using an Airfuge (Beckman Instruments) in a thermostatically controlled chromatography cabinet equilibrated at 5 °C. The measured temperature of the mixtures after centrifugation was 7.5 °C.

After centrifugation, a computer-controlled micromanipulator was used to withdraw 10 μl fractions from the top of the solution column using a 100 μl gas-tight syringe connected by Teflon capillary tubing to a 24 gauge needle. The protein concentration in each fraction was assayed using a Bradford protein reagent kit (BioRad). The weight-average molecular mass (M_w) of CETP was determined from a plot of log absorbance at 596 nm versus the square of the radial distance of each fraction relative to the solution column meniscus (r_m^2). The slope of this plot, σ , was used to calculate M_r :

$$M_r = 2\sigma RT / (1 - \nu\rho)\tilde{\omega}^2$$

where R is the molar gas constant, T is the absolute temperature, ν is the partial specific volume, ρ is the solution density and $\tilde{\omega}$ is the angular speed. A constant ρ of 1.013 g/ml was estimated from

density measurements of 5 mg/ml Dextran T-40 solution as a function of temperature. A ν value of 0.69 ml/g for CETP at 7.5 °C was obtained by temperature correction [32] of a previous determination of ν for CETP at 20 °C [11]. The M_r for the spheroidal protein BSA was determined under identical conditions used for CETP, and yielded a value of $M_r = 74766$. This value is 11.6% higher than the theoretical value of 67000 and, as expected, reflects contributions from minor amounts of protein aggregates.

Dynamic light-scattering analysis of hydrodynamic diameter

The hydrodynamic diameter for 0.7 mg/ml CETP in 10 mM NH_4HCO_3 , pH 7.4, was determined by dynamic light scattering [33]. The dynamic light-scattering system consisted of a 128-channel Model BI2030AT digital correlator (Brookhaven Instruments), coupled to a goniometer and fibre-optic light collection system. Scattered light was collected at a fixed scattering angle of 90°. All measurements were made at 20 ± 1 °C. Prior to analysis, solutions were centrifuged in a Model 5414 microcentrifuge (Eppendorf) to sediment dust particles.

Measured and calculated baselines of the intensity autocorrelation function agreed to within 0.1%. The analysis of autocorrelation functions was performed with standard dynamic light scattering software (Brookhaven Instruments) which employed single-exponential fitting, cumulants analysis and non-negatively constrained least-squares particle size distribution analysis routines [34].

Determination of specific absorption coefficient

CETP solutions at approx. concentrations of 0.7–0.9 mg/ml, in 10 mM NH_4HCO_3 , pH 7.6, were centrifuged using a microfuge (Fisher Scientific, Model 235C) for 5 min to remove micro-particulates. Portions of each sample were then removed, pipetted directly into pre-weighed hydrolysis tubes and subjected to quantitative amino acid analysis. CETP concentration was calculated from the sum of the pmoles of amino acids using a theoretical M_r of 53107.75 for CETP [16]. The A_{280} for each sample was also determined using a diode array spectrophotometer (Hewlett-Packard 8452A). In order to correct for light scattering, absorbance measurements were also taken at 340, 360, 380, 400 and 420 nm. A plot of absorbance versus $1/\lambda^4$ was generated, and the amount of light scatter for each sample was determined by linear extrapolation to 280 nm. The light-scattering values were subtracted from the original A_{280} values to give the absorbance due to the protein.

Secondary structure analysis

CD in the far UV (190–250 nm) was used to estimate the secondary structure of CETP. Samples of CETP were dialysed in 50 mM NaHCO_3 . A_{280} was used to determine CETP concentrations, as described above. CD spectra were collected at 20 °C in a Jasco J-500C spectropolarimeter using 0.5 mm cylindrical cells. The spectropolarimeter was routinely calibrated with D-10-(+)-camphor sulphonic acid at 290 nm. The CD spectra (average of 4 scans) were baseline corrected and converted into mean residue molar ellipticity using a mean residue M_r of 111.6. A least-squares-fitting procedure incorporating the constraint that the sum of the proportions of secondary structural forms is 100% was used to estimate secondary structure composition [35]. The helix content was also estimated from the ellipticity at 222 nm [36]. A sample of horse myoglobin was analysed in parallel with the CETP samples and was found to agree with literature values (75–80% α -helix) [35].

No corrections were made to the CD spectra for contributions due to carbohydrate. The effect of carbohydrate on protein CD has often been shown to be relatively minor [37–39]. Nevertheless, a simple calculation for CETP is useful to justify this assumption. Given the theoretical M_r of 53 107 and the observed M_r of about 65 000 for the largest glycoform in SDS/PAGE, the maximum contribution to the M_r from carbohydrate could be about 12 000. If the entire carbohydrate portion were composed of 2-acetamido-2-deoxy- α -D-glucopyranose units ($M_r = 236$), then there could be, at most, 50 mol per mol of CETP. Given our knowledge of N-linked carbohydrate structures, this is likely to be an overestimate by at least one order of magnitude. CETP has 476 amino acid residues, or 474 peptide bonds. Each acetamido residue could contribute a $\Delta\epsilon$ of 1–2 (maximum about 210 nm) [40], while each peptide residue would contribute a $\Delta\epsilon$ of 3–7 [41]. Thus in the worst case, it can be estimated that the peptide amide would contribute a $\Delta\epsilon = 474 \times 3 = 1422$, and the carbohydrate amide could contribute $\Delta\epsilon = 50 \times 2 = 100$, or about 7% to the CD amplitude. At other wavelengths the contribution of carbohydrate would be less. Therefore, given the likely carbohydrate composition, the CD contribution from carbohydrate amides would probably be less than 1% of the observed CD amplitude.

CE transfer assay

Activity was monitored by determining the transfer of [^3H]CE from donor HDL₃ to acceptor LDL as described previously [28]. Briefly, assays were performed in 200 μl volumes in 96-well filter plates (0.65 μm ; Multiscreen DPB, Millipore) containing LDL (50 $\mu\text{g}/\text{ml}$ total cholesterol), [^3H]CE-labelled HDL (6 $\mu\text{g}/\text{ml}$ total cholesterol) and 0.1–1 $\mu\text{g}/\text{ml}$ CETP. After 2 h at 37 °C, LDL was specifically precipitated by adding 50 μl of dextran sulphate precipitating reagent (Sigma Chemical Co., St. Louis, MO, U.S.A.). After filtration, the amount of [^3H]CE present in LDL was determined by liquid-scintillation counting (Topcount, Packard Instruments). Corrections for non-specific precipitation were made by omitting CETP from identical sample wells. To study CETP kinetics, the assay described above was modified by varying the [^3H]CE–HDL concentration while holding the LDL concentration constant.

Preparation of dual-labelled HDL

HDL and plasma proteins were obtained from fresh plasma as described previously [28]. Lipid dispersions containing [1,2,6,7- ^3H]cholesterylolate (60–100 Ci/mmol, New England Nuclear) and carboxyl-[^{14}C]triolein (80–120 Ci/mmol, New England Nuclear) were made using methods described previously [21] and added to the plasma fraction with d 1.11. After flushing with N_2 , the mixture was incubated overnight at 37 °C to allow for transfer of radiolabelled lipids into the HDL particles. To obtain the desired HDL₃ fraction, the density was adjusted to 1.21 g/ml with solid NaBr and subjected to the same centrifugation conditions outlined above. The top one-third of the sample in each tube was pooled and dialysed as described above. Total cholesterol and TG concentrations were determined by enzymic methods (Sigma). Typically, about 60% of the [^3H]CE and 50% of the [^{14}C]TG were incorporated into the HDL₃ fraction.

Dual-label CE and TG transfer assay

The dual-labelled HDL₃ donor particles described above were used to measure the simultaneous transfer of CE and TG to LDL acceptor particles. LDL (57 $\mu\text{g}/\text{ml}$ total cholesterol), [^3H]CE and [^{14}C]TG–HDL (7.1 $\mu\text{g}/\text{ml}$ total cholesterol), and CETP (0.1–1 $\mu\text{g}/\text{ml}$) were incubated at 37 °C in 200 μl of assay buffer (10 mM Tris, pH 7.4, containing 1 mM EDTA, 0.14 M NaCl and 10 mg/ml BSA). LDL was selectively precipitated as described above for the single-label assay. The plate was mixed by vortex, incubated for 10 min at room temperature, and then subjected to centrifugation at 700 g and 4 °C (Beckman J-6B) for 30 min. The amount of radioactivity that remained associated with HDL was determined by removing a 150 μl aliquot of the supernatant from each well for liquid-scintillation counting. The amount of [^3H]CE or [^{14}C]TG transferred from the donor HDL particles to the acceptor LDL particles was calculated relative to the blank samples that did not contain CETP.

Kinetics of CETP-mediated CE and TG transfer

The kinetic model used to analyse the transfer of CE and TG from HDL to LDL is analogous to a dual-substrate, group-transfer enzyme reaction:

According to this model, the donor substrate is HDL containing [^3H]CE. The acceptor substrate is LDL. Initial rate conditions for the transfer of radiolabel from HDL to LDL were established with respect to time and CETP concentration. The steady-state rate equation describing a Ping Pong Bi Bi, unidirectional, heterotypic transfer of lipid from HDL to LDL is given by:

$$\frac{v}{V_{\max}} = \frac{[\text{HDL}][\text{LDL}]}{K_{m, \text{LDL}}[\text{HDL}] + K_{m, \text{HDL}}[\text{LDL}] + [\text{HDL}][\text{LDL}]} \quad (1)$$

where [HDL] and [LDL] are the respective concentrations of the donor and acceptor particles, v is the initial rate of transfer, $V_{\max}$ is the maximal rate of transfer, and $K_{m, \text{LDL}}$ and $K_{m, \text{HDL}}$ are the respective Michaelis constants for heterotypic transfer of the radiolabelled lipid ([42], pp. 608–612). Lineweaver–Burk-type linear transformations of this equation can be generated for the cases where LDL is varied at different constant HDL concentrations, or where HDL is varied at different constant LDL concentrations. Either approach would produce sets of parallel lines according to this model ([42], p. 610). The alternative random Bi Bi model, in which ternary complex formation is part of the transfer mechanism, would produce intersecting sets of lines. Thus the two models can be distinguished based on the type of double-reciprocal plots obtained. The apparent K_m and $V_{\max}$ values obtained at each fixed substrate concentration can be used to generate secondary plots to obtain the actual K_m and $V_{\max}$ values ([42], p. 610).

However, eqn. (1) does not take into account the effects of homotypic transfer between HDL particles or between LDL particles described previously [43]. For the present CETP assay methods, homotypic transfer would be analogous to competitive substrate inhibition ([42] p. 830). Thus eqn. (1) can be corrected for homotypic transfer by the introduction of K_i terms according to the following equation:

$$\frac{v}{V_{\max}} = \frac{[\text{HDL}][\text{LDL}]}{K_{m, \text{LDL}}[\text{HDL}] \left(1 + \frac{[\text{HDL}]}{K_{i, \text{HDL}}} \right) + K_{m, \text{HDL}}[\text{LDL}] \left(1 + \frac{[\text{LDL}]}{K_{i, \text{LDL}}} \right) + [\text{HDL}][\text{LDL}]} \quad (2)$$

In eqn. (2), the K_i terms are equivalent to the respective Michaelis constants for homotypic transfers. When $[\text{LDL}] < K_{i,\text{LDL}}$ and $[\text{HDL}] < K_{i,\text{HDL}}$, then eqn. (2) reduces to eqn. (1). Thus eqn. (1) will describe approximately the transfer process at low substrate concentrations, and the double reciprocal plots will be parallel. However, if either or both substrates are increased substantially above the respective K_i values, then the denominator terms will become increasingly large, and the apparent rate of transfer from HDL to LDL will diminish. Homotypic transfer between donor HDL particles will cause the double-reciprocal plots to become non-linear and curve upward, whereas homotypic transfer between acceptor LDL particles will produce intersecting, non-parallel lines.

The molar concentrations of LDL and HDL particles used in the assays were calculated from the total cholesterol concentrations present in each preparation. It was assumed that the M_r of HDL₃ was 1.75×10^5 , and that the total cholesterol concentration was 15% by weight. Likewise, LDL was assumed to have an M_r of 2.6×10^6 , and to contain 47% cholesterol by weight.

Chromatographic CETP lipid-binding assay

A modification of a chromatographic method described previously [20] was used to determine the transfer of lipid monomers from donor liposomes to CETP, and from CETP to acceptor liposomes. Lipid donor particles containing a ^3H -labelled lipid were incubated with CETP in the presence of 0.1% (w/v) BSA in 10 mM Tris, pH 7.4, containing 1 mM EDTA, 0.14 M NaCl and 0.1% NaN_3 (TESA) buffer at 37 °C. CETP was then separated from the liposomes by size-exclusion chromatography using an HR 10/30 Superose 12 column with a FPLC system (Pharmacia). Flow rates were 1 ml/min, and 2 ml fractions were collected.

Liposomes (small unilamellar vesicles) containing 72 mol % phosphatidylcholine, 25 mol % cholesterol and 3 mol % of cholesteryl oleate were prepared as follows. The appropriate amounts of lipid were transferred from stock solutions into a glass tube and dried under N_2 . For preparation of radiolabelled liposomes, 15 μCi of [^3H]cholesteryl oleate, [^3H]cholesterol, [^3H]triolein or [^3H]phosphatidylcholine was included. The dried lipids were then dissolved in ethyl acetate, dried again under N_2 (3 times to ensure complete removal of the solvent) and lyophilized overnight. The resulting lipid film was dissolved in absolute ethanol with vortex mixing and sonication, and then rapidly injected into TESA buffer while vortex mixing so that the final concentration of ethanol was 7.5% (v/v). Ethanol was removed by dialysis against TESA buffer. The dialysed liposomes were analysed for recovery of lipids by liquid scintillation counting and lipid phosphorus analysis. Liposomes were stored for up to 6 days at 4 °C.

RESULTS AND DISCUSSION

Purification of CETP by immunoaffinity chromatography

BHK or CHO cells that were stably transfected with the CETP cDNA gene secreted about 1–4 $\mu\text{g}/\text{ml}$ CETP into serum-free medium, as determined by ELISA. After concentration by ultrafiltration (20–40 fold), a single-step immunoaffinity chromatography procedure with the monoclonal antibody M468 was used to purify CETP to homogeneity. Typical recoveries were about 40% with 150-fold purification (results not shown). The lipid transfer activities, appearance in SDS/PAGE, amino acid

Figure 1 SDS/PAGE of purified CETP and CETP treated with N-glycosidase F

CETP samples were reduced with β -mercaptoethanol and analysed by SDS/PAGE using a 10% gel followed by staining with Coomassie Blue G250. Lane A contains molecular-mass standards (M_r): phosphorylase b (95 000), BSA (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and α -lactalbumin (14 400). Lane B contains 4 μg of CETP, purified by mAb immunoaffinity chromatography. Lane C contains 4 μg of CETP after deglycosylation using denaturing conditions [0.1% (w/v) SDS at 37 °C, followed by 0.5% (v/v) Nonidet P40 and 2 units of N-glycosidase F and incubated overnight at 37 °C]. Lane D contains 4 μg of CETP that was deglycosylated using non-denaturing conditions (2 units of N-glycosidase F at 37 °C, overnight).

compositions and lipid compositions of the CETP from BHK or CHO cells were indistinguishable, so proteins from both sources were used for the following studies.

Deglycosylation of CETP

SDS/PAGE analysis of CETP produced by immunoaffinity chromatography indicated that the preparation was free of contaminating proteins. Two major isoforms of M_r , 65 000 and 62 000, and a minor isoform of M_r , 59 000 were present (Figure 1, lane B). Treatment with the enzyme N-glycosidase F under non-denaturing conditions (Figure 1, lane D) or under denaturing conditions (Figure 1, lane C) produced bands of M_r , 56 000, M_r , 53 000 and M_r , 51 000. The extent of deglycosylation was enhanced by denaturation, shown by the increased amount of the M_r , 51 000 band and corresponding decreases in the bands of higher molecular mass when deglycosylation was performed under denaturing conditions. The heterogeneity with regard to glycosylation and the resistance to complete deglycosylation are consistent with previous reports [17].

CETP molecular-mass and hydrodynamic diameter analysis

The weight-average relative molecular mass (M_w) of CETP was obtained from the slopes of sedimentation equilibrium plots shown in Figure 2. Duplicate analyses produced M_r values of 67 100 and 72 000, giving an average value of 69 550, indicating that recombinant CETP exists as a monomer under these solution conditions. The native M_r obtained by sedimentation equilibrium agrees closely with the molecular mass obtained for denatured CETP using SDS/PAGE. These data are also in excellent agreement with sedimentation equilibrium experiments using CETP from human plasma [11] and with cross-linking and fluorescence energy transfer experiments using recombinant CETP [14].

Figure 2 Determination of CETP molecular mass by sedimentation equilibrium

Duplicate analyses were performed using 0.7 mg/ml of CETP in 10 mM NH_4HCO_3 , pH 7.4. Linear regression analysis of the data from two experiments yielded M_r of 67144 (◆) and 71978 (■).

Figure 3 Determination of CETP hydrodynamic diameter by dynamic light scattering

The intensity-weighted differential hydrodynamic diameter distribution of 0.7 mg/ml CETP in 10 mM NH_4HCO_3 , pH 7.4, was calculated using the method of non-negatively constrained least-squares. Peak maxima for the two modes were 14 nm and 60 nm.

The corresponding hydrodynamic diameter (D_h) of CETP was determined by dynamic light scattering. Calculation of intensity-weighted hydrodynamic diameter distributions via non-negatively constrained least-squares analysis produced the distribution shown in Figure 3. A bimodal distribution of hydrodynamic diameters was found with average D_h of 14 nm and 60 nm. Calculation of weight percentage protein corresponding to the 14 nm and 60 nm modes was 96 % and 4 % respectively. The corresponding hydrodynamic diameter distribution of a BSA standard yielded modes at 7 nm and 41 nm, with weight percentage protein content of 98 % and 2 % respectively. The chemical identity of the minor components in either protein preparation was not determined, but small amounts of aggregated protein and/or lipid micelles are the most likely possibilities. The D_h equal to 7 nm for BSA is identical with previous measurements [44,45]. Since BSA and CETP are comparable in molecular mass (M_r , 67000 and M_r , 69561 respectively), the light scattering results represent an anomaly. Takahashi et al. [26] reported a D_h of 9.88 nm for CETP, and suggested that dimerization of CETP was responsible for the anomalously high result. However, this seems unlikely given the other physical evidence supporting a monomeric structure for CETP. An alternative explanation is that the shape of CETP in solution is highly dys-symmetrical or rod-like, resulting in a larger apparent hydrodynamic diameter than spherical proteins of similar molecular mass. Further structural studies will be needed to resolve this issue.

Specific absorption coefficient of CETP

CETP concentrations were determined by quantitative amino analysis. The absorbance at 280 nm of the same solutions was measured. After correction for light scattering, the data were used to calculate a specific absorption coefficient of $0.83 \pm 0.02 \text{ litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ (means $\pm$ S.D. of 9 independent determinations).

Secondary structure analysis of CETP

The results of two different methods used to calculate secondary structure from the CD spectrum (not shown) are summarized in Table 1. The published data for CETP purified from rabbit and human serum are included for comparison. The α -helix content for recombinant CETP was estimated to be 23 % from the ellipticity at 222 nm. This is similar to our estimate of 25 % for rabbit CETP and 26 % for human CETP, calculated from published CD spectra [8,11]. The recombinant human CETP CD spectrum was also analysed by a least-square-fit procedure that

Table 1 Secondary structure of CETP

*The α -helix content of recombinant human CETP was estimated from the CD spectrum using the θ_{222} method [36]. The estimates for human serum and rabbit serum CETP were derived using the spectra published by Ko et al. [8] and Ohnishi et al. [11]. †Secondary structure analysis of recombinant human CETP was performed by a least-squares-fitting method [35]. The fraction of each type of secondary structure is compared with the values published for human serum CETP [11] and rabbit serum CETP [8].

	θ_{222} Method		†Secondary structure analysis		
	% α -helix	% α -helix	% β -sheet	% Turn	% Random coil
Recombinant human CETP	23	32	35	17	16
Human serum CETP*	26	29	38	10	23
Rabbit serum CETP*	25	30	39	12	19

Figure 4 Time course and CETP-concentration dependence for CE and TG transfer from HDL to LDL

A dual-label transfer assay was used to follow the time course and CETP-concentration dependence of [^3H]CE (■) and [^{14}C]TG (●) transfer from HDL to LDL. In (A) and (B), the mass amount (nmol) of each lipid transferred from HDL to LDL was calculated based on the respective specific radioactivity of the labelled lipids. In (C) and (D), the same data are plotted as a percentage of the total amounts of each lipid present in the donor HDL particles. An incubation period of 60 min at 37 °C was used in the CETP concentration experiment (A and C). The concentration of CETP used in the time course experiment (B and D) was 220 ng/ml. Other conditions were as described in the Experimental section.

uses reference spectra for known protein structures for determination of α -helix, β -pleated sheet, turn and random coil. This analysis yielded an estimate of 32 % α -helix, 35 % β -pleated sheet, 17 % turn and 16 % random coil. There is good agreement in the secondary structure estimates for the recombinant CETP used in the present study and the estimates reported previously for the two plasma-derived preparations.

Kinetic analysis of CETP-mediated CE and TG transfer

A dual-label assay that monitors CETP-mediated transfer of both [^3H]CE and [^{14}C]TG from HDL to LDL was used to determine the CETP concentration dependence (Figure 4A) and the time course (Figure 4B) of transfer by CETP. In the results of the experiment shown in Figure 4(A), the concentration of CETP was varied between 30 and 840 ng/ml in a 60 min assay. CE and TG transfer increased linearly with respect to CETP concentration, up to about 200 ng/ml. Above 220 ng/ml CETP, the plots became non-linear even though CE and TG transfer continued to increase. The time-course of transfer using 220 ng/ml CETP shows that the rates of transfer for both CE and TG were linear with respect to incubation time for at least 60 min, and then continued to increase non-linearly for up to 240 min (Figure 4B).

The ratio of transferred CE to transferred TG was 3–4:1 at all CETP concentrations and all time points (Figures 4A and

Figure 5 Kinetic analyses of CETP-mediated CE Transfer

(A) Direct plot: the initial rates of [^3H]CE transfer from HDL to LDL were determined as a function of HDLc concentration. The LDLc concentrations were: 320 nM (■); 160 nM (●); 80 nM (▲); 40 nM (◆). (B) Lineweaver–Burk plot: the data from the direct plot in (A) were replotted in double-reciprocal form. (C) Secondary double-reciprocal plot: $1/K_{m,app,HDL}$ versus $1/[LDL]$. The apparent K_m values for HDL (x-axis intercepts) from the Lineweaver–Burk plot (B) were plotted against the reciprocal of the LDLc concentrations. The three points (■) represent values for 40, 80 and 160 nM LDLc. The K_m for HDLc is obtained from the y-axis intercept and the K_m for LDLc is obtained from the x-axis intercept of this plot. (D) Secondary double-reciprocal plot: $1/[LDL]$ versus $1/V_{max,app}$. The apparent V_{max} values (y-axis intercepts) from the Lineweaver Burk plot (B) are plotted against the reciprocal of the LDLc concentrations. The three points (■) represent values for 40, 80 and 160 nM LDLc. V_{max} is obtained from the y-axis intercept of this plot.

4B). This ratio corresponded closely to the 3:1 concentration ratio of CE to TG found in the HDL donor particles. Thus the relative rate of transfer of the two lipids appeared proportional to the relative concentrations of the two lipids in the donor HDL particles. This point is further emphasized in Figures 4(C) and 4(D) in which the data from Figures 4(A) and 4(B) are re-plotted to show the amounts of each lipid transferred as a percentage of their respective starting concentrations in the donor HDL particles. The CE and TG curves are superimposable, verifying that the relative efficiency of transfer for CE and TG was strictly proportional to the relative concentrations of the two lipids in the HDL particles.

In order to determine the kinetic constants associated with heterotypic transfer of [^3H]CE from HDL to LDL, transfer was measured as a function of varying HDL-cholesterol (HDLc) concentrations at four different constant LDL-cholesterol (LDLc) concentrations (Figure 5A). The curves appeared hyperbolic and the rates saturable as the HDLc concentrations were increased. When the HDL concentrations were increased several-fold above those shown in Figure 5(A), the rates of CE and TG transfer from HDL to LDL decreased (results not shown). This is consistent with eqn. (2), which predicts that homotypic transfer between HDL particles should compete with heterotypic transfer from HDL to LDL. Similar results were obtained when LDL

was used as the varied substrate and HDL held constant (results not shown).

A Lineweaver–Burk plot of data from Figure 5(A) is shown in Figure 5(B). At the three lower LDL concentrations (40, 80, and 160 nM) the lines appear parallel, consistent with the carrier (Ping Pong Bi Bi) mechanism of eqn. (1). The slope of the line for 320 nM LDLc appears to deviate from those of the other three lines and intersects the y axis near the 160 nM line, consistent with competitive substrate inhibition (homotypic transfer) by LDL (eqn. 2). A statistical test using covariance analysis was performed to determine if the slopes of the lines were significantly different, or by default, parallel [46]. First, when all four lines were analysed to test for the heterogeneity of slopes, they were straight lines ($r^2 = 0.996$), but were not parallel ($P = 0.0017$) due to inclusion of the 320 nM LDLc group. If the high LDLc group is omitted, the analysis indicates that the slopes of the other three lines are not different ($r^2 = 0.995$, $P = 0.404$). The lines are, therefore, parallel. Thus the kinetics are consistent with the carrier model of eqn. (1) and eqn. (2) and are not consistent with the alternative sequential model [47]. The latter would have produced intersecting lines at all LDLc concentrations in the double-reciprocal plots.

Since the apparent K_m and V_{max} values obtained from the Lineweaver–Burk plots (Figure 5B) were dependent upon the concentration of the fixed substrate used, the actual V_{max} and K_m values were obtained from secondary double-reciprocal replots. In Figure 5(C), $K_{m,app}$ obtained from the x-axis intercepts of Figure 5(B) for the 40, 80 and 160 nM LDLc data are replotted against $1/[LDL]$. From the y-intercept of the resulting straight line in Figure 5(C), it is estimated that the K_m for HDL is 2000 nM and from the x-intercept, the K_m for LDL is estimated to be 700 nM (calculated as HDL and LDL particles). When these values are converted into mg/dl cholesterol, the K_m s are 80 mg/dl for LDLc and 5 mg/dl for HDLc. Thus the K_m values estimated for both substrates are within the normal physiological ranges found in plasma.

A replot of $1/V_{max,app}$ versus $1/[LDL]$ also produced a straight line (Figure 5D). From the y-intercept of this plot, it is estimated that the V_{max} is $76 \text{ pmol} \cdot \text{min}^{-1}$. Since the assays contained 0.6 pmol of CETP, the rate constant for CE transfer is estimated to be $k_{cat} = 2 \text{ s}^{-1}$, a rate that is well within the range for true enzymic reactions. The linearity of the Lineweaver Burk plots and the secondary replots provide further support for the Ping Pong Bi Bi mechanism. The estimates of V_{max} and k_{cat} are undoubtedly minimal values for total lipid-transfer activity for several reasons. First, only radiolabelled CE was measured, but TG, PL and unesterified cholesterol (UC) are also transferred simultaneously. Also, the rates of homotypic transfer of lipid between HDL particles and between LDL particles could be appreciable. Finally, CETP also transfers lipid in the reverse direction. Thus the rate of total lipid transfer by CETP could be as much as an order of magnitude faster than indicated by the k_{cat} estimated for CE transfer from HDL to LDL.

Similar results to those presented here, including homotypic substrate inhibition, were obtained for microsomal transfer protein which shuttles TG between membranes [48]. Two other studies have used a kinetic approach to analyse the CETP transfer mechanism, although neither study utilized purified CETP. The conclusions of Barter and Jones [18] were similar to ours, even though the methods of analysis were different. In contrast, Ihm et al. [47] concluded that the CETP transfer mechanism is sequential since intersecting lines were obtained in a double-reciprocal plot [47]. However, only two fixed substrate concentrations were used in that study. The results presented in this paper, as well as those obtained with microsomal transfer

Figure 6 Binding of [^3H]-CE, -TG, -UCL or -PL to CETP

Liposomes containing ^3H -labelled lipids were incubated alone (■) or with $60 \mu\text{g}$ CETP (●) for 15 h at 37°C . The samples were applied to a Superose 12 size-exclusion column and the radioactivity in fractions eluting from the column was measured by liquid scintillation spectrometry. The radiolabelled lipids in the liposomes were: (A) [^3H]cholesteryl oleate; (B) [^3H]triolein; (C) [^3H]cholesterol; (D) [^3H]phosphatidylcholine. The arrowheads indicate the elution positions of control liposomes, detected by light scattering, or CETP, detected by ELISA.

protein, demonstrate that it is necessary to use a wide range of substrate concentrations in order to delineate the kinetic mechanism by this method, since homotypic transfer processes can cause non-parallel or non-linear plots at some substrate concentrations. Recently, Epps et al. [19] have presented a detailed kinetic analysis of cynomolgus monkey CETP and their data were consistent also with the carrier model. The K_m for LDL acceptor particles (700 nM) was approx. 3-fold lower than the K_m for HDL donor particles (2000 nM), implying that the LDL particle is a slightly more effective substrate than the HDL particle. Interestingly, when the K_m values are converted from molar concentrations of lipoprotein particles to mg/dl cholesterol, they fall within the low end of the normal physiological range for the plasma concentrations of each substrate; 5 mg/dl HDLc and 80 mg/dl LDLc. In contrast with the present approach, other investigators have used cholesterol pools instead of particle concentration to describe kinetic data [12,18,47]. That

approach can lead to misleading interpretation of kinetic data, since the molecular masses and compositions of HDL and LDL differ substantially. For example, Barter and Jones [18] concluded that the probability of transfer from the HDLc pool was about 30-fold higher than from the LDLc pool, implying that HDL was the favoured substrate. When the present K_m values are converted from particle concentration to cholesterol concentration, the resulting values for K_m become 5 mg/dl HDLc and 80 mg/dl LDLc. The 40-fold ratio of these values is approx. the same as the 30-fold probability ratio reported by Barter and Jones [18].

Transfer of lipid from liposomes to CETP and from CETP back to liposomes

CETP purified from serum-free cell culture medium was extracted and analysed by two-dimensional TLC. All of the major lipid classes normally associated with lipoproteins were present, including CE, TG, PL and UC (results not shown). Quantitative determination of the lipids associated with CETP produced the following results: TG, 0.5 mol/mol CETP (measured as glycerol); PL, 0.9 mol/mol CETP (measured as choline) or 1.3 mol/mol CETP (measured as lipid phosphate); UC 1.0 mol/mol CETP (measured as cholesterol). CE could not be measured, because the detergent used to dissolve the dried lipid extract inactivated the esterase used in the two-step analysis of CE.

Figure 6 shows a series of experiments in which CETP was incubated with donor liposomes containing either [3 H]CE, [3 H]TG, [3 H]UC or [3 H]phosphatidylcholine. The CETP was then separated from the liposome mixtures by size-exclusion chromatography. In individual experiments, CETP extracted and bound all four of the radiolabelled lipids that were loaded into the liposomes, shown by the co-elution of radiolabelled lipid with CETP. The labelled lipid that co-eluted with CETP was shown, after Bligh-Dyer extraction and TLC, to be radiochemically intact. In the absence of CETP, all of the radioactivity eluted exclusively with the liposomes.

The kinetic data for CE and TG transfer presented above are consistent with the carrier mechanism. According to this model, the transfer process consists of two half-reactions. In the first half-reaction, CETP associates with a donor lipoprotein particle, extracts a lipid particle, and then dissociates from the lipoprotein with a bound lipid molecule. Thus the binding data shown in Figure 6 can be regarded as the first half of the transfer process for CE, TG, PL or UC. In order to determine if CETP can then transfer the lipid molecules to acceptor lipoprotein particles, isolated radiolabelled CETP-lipid intermediates (corresponding to fractions 20–25, shown in Figure 6) were incubated with fresh acceptor liposomes. The entire sequence for CE is shown in Figure 7. In Figure 7(A), [3 H]CE donor liposomes alone are shown to elute with the column void volume (fractions 8–12). CETP elutes in fractions 20–28, determined by immunoassay (Figure 7B). When radiolabelled liposomes were incubated with CETP, a peak representing a [3 H]CE–CETP complex was eluted in fractions 20–28 (Figure 7C). In order to determine if the [3 H]CE–CETP complex was stable, these fractions were pooled and re-injected, and again eluted in the same fractions (Figure 7D), indicating that the [3 H]CE did not readily dissociate from CETP. When the isolated [3 H]CE–CETP complex was incubated with fresh, unlabelled acceptor liposomes, radiolabelled lipid was transferred from the CETP to the liposomes, indicated by the presence of radioactivity in the liposome fractions (Figure 7E). Similar data were obtained for TG, phosphatidylcholine and UC (results not shown). Thus CETP extracted from liposomes all four of the lipid types tested, and the bound lipid was transferred to fresh acceptor liposomes.

Figure 7 Half-reactions: transfer of [3 H]cholesteryl oleate from liposomes to CETP and from CETP to liposomes

Mixtures of CETP and donor liposomes containing [3 H]cholesteryl oleate were incubated for 2 h at 37 °C and then fractionated by Superose 12 size-exclusion chromatography. The fractions were assayed for [3 H]cholesteryl oleate by liquid scintillation spectrometry and for CETP mass by ELISA. (A) Control showing elution of [3 H]cholesteryl oleate donor liposomes alone (25 nmol of total lipid); (B) control showing elution of CETP alone (60 μ g); (C) transfer of [3 H]cholesteryl oleate from donor liposomes to CETP. CETP and [3 H]cholesteryl oleate donor liposomes were separated after a 2 h incubation at 37 °C. The presence of the radiolabel in the CETP fractions indicates the association of [3 H]cholesteryl oleate with CETP. This procedure was repeated 3 times in order to obtain enough CETP–[3 H]cholesteryl oleate complex for re-chromatography. (D) Control showing that [3 H]cholesteryl oleate remains bound to CETP. Fractions 20–28 from the experiment in (C) containing both CETP and [3 H]cholesteryl oleate were pooled and re-chromatographed. (E) Transfer of [3 H]cholesteryl oleate from CETP to acceptor liposomes. The CETP–[3 H]cholesteryl oleate fractions from (C) were incubated for 2 h at 37 °C with unlabelled acceptor liposomes before chromatography. CO, cholesteryl oleate.

These experiments demonstrated that CETP could bind and transfer all four of the lipid classes, including UC, and verified the previous report showing that partially purified CETP from serum could bind and transfer CE and TG [25]. The binding and

transfer of UC is of particular interest, because it has not been considered previously to be a substrate for CETP [49]. The transfer of CE and TG by CETP have been shown to be dependent upon the concentration of UC in donor and acceptor lipoproteins, although the mechanism for this effect was not clear [50,51]. The present results, showing direct binding of UC by CETP, suggest that CE and TG transfer could be regulated by either competition for binding sites by cholesterol or by an allosteric mechanism.

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