Eur Biophys J (1996) 25: 31–36

ARTICLE

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Penetration of an emulsion surface by cholesteryl ester transfer protein

Received: 29 March 1996 / Accepted: 30 May 1996

Abstract Quenching of the intrinsic fluorescence of cholesteryl ester transfer protein (CETP) by spin labelled fatty acids (5-NS and 16-NS) was investigated to determine the degree to which the protein penetrated the phospholipid monolayer surface of a lipid emulsion. When bound to the phospholipid surface approximately 50% of the fluorophores of the transfer protein were accessible to quenching by 5-NS whose nitroxy group locates near the monolayer surface. On the other hand, only 22% of the fluorophores of CETP were accessible to quenching by 16-NS whose nitroxy group locates deeper in the surface monolayer. Quenching of the CETP fluorescence by an aqueous phase quencher (acrylamide) shows that the protein undergoes a conformational change on binding which increases the proportion of the tryptophan residues exposed to the aqueous phase. The results indicate that CETP does not penetrate the lipid surface to a significant degree.

Key words Cholesteryl ester transfer protein · Lipid emulsion · Fluorescence quenching · Lipoproteins

Abbreviations CETP, cholesteryl ester transfer protein · egg PC, egg yolk phosphatidylcholine · PC, phosphatidylcholine · PL, phospholipid · CE, cholesteryl ester · CO, cholesteryl oleate · 5-NS, 5-nitroxystearic acid · 16-NS, 16-nitroxystearic acid · TO, triolein · VLDL, very low density lipoproteins · LDL, low density lipoproteins · HDL, high density lipoproteins · HDL_3, high density lipoprotein subfraction 3 · Apo AI, apolipoprotein AI

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Introduction

The prime function of human plasma cholesteryl ester transfer protein (CETP) is to transfer newly esterified cholesterol from high density lipoprotein to triglyceride-rich lipoproteins (LDL, VLDL) with a corresponding transfer of triglycerides in the reverse direction (Barter et al. 1990 b; Barter and Rye 1994). It plays a central role in reverse cholesterol transport which moves cholesterol from peripheral tissues to the liver (Barter and Rye 1994; Tall 1993). CETP cDNA codes for a polypeptide of Mr 53,000 (476 residues) (Drayna et al. 1987). However, the apparent molecular weight determined by SDS-electrophoresis is 74,000 (Hesler et al. 1987), the higher value reflecting glycosylation at 4 potential asparagine glycosylation sites (Drayna et al. 1987; Stevenson et al. 1993). Biological activity is associated with the C-terminal domain since deletion of amino acid residues between Phe-463 and Leu-475 results in mutant proteins with defective transfer activity (Wang et al. 1992). Deletion of up to 66 residues from the C-terminus results in mutants with defective activity and lipid binding (Au-Young and Fielding 1992). It remains to be determined if there are separate domains or subdomains responsible for lipid binding and transfer activity. However, it would appear that the protein can bind to all lipoprotein classes with similar affinity ($K_d = 20-25$ nM) but with substantially different binding capacities (Morton 1985).

Two mechanisms have been proposed for CETP-mediated lipid transfer. CETP may bind to the surface of a donor lipid particle, bind cholesteryl ester from the lipid phase, dissociate from the surface, and diffuse through the aqueous phase to bind to an acceptor particle where it releases its substrate (Barter and Jones 1980). Alternatively, the transfer may occur via the formation of a transient ternary complex (donor-CETP-acceptor) (Ihm et al. 1982). Regardless of the mechanism, CETP activity can be modulated by changing physicochemical characteristics of the lipid surface of donor and acceptor particles. Factors such as surface charge (Nishida et al. 1993; Rajaram et al.

1994a), phospholipid head group (Rajaram et al. 1994a), the presence of subsidiary lipids such as unesterified cholesterol (Morton 1988; Rajaram et al. 1994b), and fatty acids (Nishida et al. 1993; Rajaram et al. 1994a; Barter et al. 1990a), as well as apolipoprotein composition (Miller et al.1991; Ohnishi and Yokoyama 1993) have been identified using either plasma lipoproteins or lipid emulsions as model donors and acceptors.

How does CETP access a substrate (cholesteryl ester) which is located principally in the core compartment of lipoproteins or their emulsion analogues? Cholesteryl esters have only limited solubility in the surface phospholipid monolayer of these structures (3–4 mol%) (Miller and Small 1983; Hamilton et al. 1983). Two situations are envisaged. Either the protein remains on the lipid surface and removes small amounts of cholesteryl ester present in the monolayer with subsequent redistribution of cholesteryl ester from the core to the monolayer compartment, or it penetrates the surface monolayer to gain direct access to substrates in the core compartment. The tryptophan fluorescence of CETP provides us with an intrinsic probe to examine these alternatives. Genomic sequencing of CETP shows that it possesses tryptophan residues at positions 105, 106, 162, 264 and 299 (Drayna et al. 1987). Microemulsions which consist of a surface monolayer of phospholipid enclosing a core of neutral lipid (cholesteryl ester and/or triacylglycerol) are useful models for the study of apolipoprotein-lipid interactions (Tajima et al. 1988; Li and Sawyer 1992). In the present study we examine the binding of CETP to microemulsions which mimic the lipid phase of lipoproteins but lack their extensive lipid heterogeneity. Fluorescence techniques are used to measure the accessibility of tryptophan residues in CETP bound to the emulsion surface to quenchers in the lipid or aqueous phases. The experiments indicate that CETP does not penetrate the surface monolayer to a significant degree.

Materials and methods

Chemicals. Egg phosphatidylcholine (egg PC) was from Lipid Products, South Nutfield, UK, and 5-nitroxy stearic acid (5-NS), 16-nitroxy stearic acid (16-NS) and N-acetyltryptophanamide from Aldrich Chemicals, Milwaukee, MI. Cholesteryl oleate (CO) was from Sigma, St Louis, MO. Triolein was from Nu-Chek-Prep Inc., Elysian, MN.

Lipid emulsions. Lipid emulsions consisting of a surface monolayer of egg PC enclosing a core compartment of neutral lipid (CO and TO) were prepared by a pressure extrusion technique developed in our laboratory (Drew et al. 1990). Briefly, lipid mixtures (egg PC, CO and TO in a molar ratio of 50:38:1) in $CHCl_3$/MeOH (1:1 v/v) were placed in glass vials and the solvent was removed by evaporation under a stream of nitrogen. The material was vacuum desiccated at room temperature for 24 h. The lipid mixtures were dispersed in Buffer A (10 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, 1.0 mM EDTA and 3.0 mM NaN_3) to a total lipid concentration of 0.4% (w/v) and sonicated at maximum power in a MSE Soniprep for 15 min under a nitrogen atmosphere. A thermostatted water bath was used to maintain the sonication vial at 51–60 °C, this being about 5 °C above the transition temperature of the highest melting species present, namely, CO (46 °C), (Ginsburg et al. 1982). The sonicated lipid suspension was centrifuged for 10 min in a bench top centrifuge (3000 rpm) and subjected to pressure extrusion through a 100 nm pore size polycarbonate filter using a hand-held Avestin LiposoFast Basic Extrusion Apparatus (Avestin Inc., Ottawa, ON, Canada). Extrusion was carried out in a temperature controlled cabinet maintained at 50 °C. After 11 passages through the filter, the emulsions were separated from excess phospholipid using a discontinuous sucrose gradient (Bengtsson and Olivecrona 1980). The density of the extruded material was raised to 10% (w/v) with respect to sucrose, gently layered under Buffer A in 13.5 ml centrifuge tubes, and subjected to ultracentrifugation using a Beckman 70.1 Ti rotor (40,000 rpm, 20 °C, 30 min). The emulsion fraction was recovered as the top 1 ml of the solution by tube slicing. A phospholipid-rich fraction is present as a discrete band just above the sucrose-buffer interface. The emulsion recovered from the above step was applied beneath a preformed 0–8% linear gradient of sucrose and subjected to ultracentrifugation (6000 rpm, 20 °C, 120 min) using a Beckman SW-40 rotor. The resulting gradient distribution was recovered from the bottom of the tube by collection of 1 ml fractions. The fractions were assayed for phospholipid, cholesteryl ester and triacylglycerol. The first 3 fractions were pooled and dialysed against Buffer A. The average molar ratio of core to surface lipids [(TO+CO):PC] for these three fractions was 3. The particle diameter calculated from the molar volumes of the components (Mims et al. 1986) was 41.2 nm. Emulsions were used within 4 days of preparation.

Fluorescence quenching. Fluorescence measurements were made at 25 °C using a Perkin Elmer LS-5 spectrofluorometer equipped with a thermostatted cuvette holder (excitation 280 nm; emission 334 nm). Quenching of the intrinsic fluorescence of CETP in the presence and absence of the emulsion was measured by progressive addition of small aliquots of quencher. Paramagnetic fatty acid quenchers were added from a stock solution in methanol. The final concentration of methanol in the cuvette was not more than 1.7% (v/v). Recrystallised acrylamide was added from a stock solution in Buffer A. Fluorescence intensities were corrected for light scatter and dilution. For dynamic quenching, the decrease in fluorescence intensity is described by the Stern-Volmer equation: $[(I_0/I)-1] = K_{sv}[Q]$, where I_0 and I are the intensities in the absence and presence of the quencher, respectively, K_{sv} is the Stern-Volmer quenching constant, and [Q] is the concentration of the quencher. If there are two populations of fluorophores, one of which is inaccessible to quencher, the Stern-Volmer equation is modified to the form (Lehrer 1971):

$$I_0/\Delta I = 1/(f_a K_{sv}[Q]) + 1/f_a \quad (1)$$

where $\Delta I = I_0 - I$, and f_a is the fraction of accessible fluorophores. A linear plot of the above equation yields the value of f_a from the ordinate intercept. A different measure of the accessibility of tryptophan residues of CETP to solvent was obtained by comparing the Stern-Volmer quenching constant with that for N-acetyltryptophanamide (Weinberg 1988), recognising that K_{sv} is the product of the fluorescent lifetime and the bimolecular quenching constant.

Binding of CETP to emulsions. The ratio of PL to CETP at which all the protein is bound to the lipid surface was determined by measuring the quenching of the intrinsic fluorescence of CETP on binding to an emulsion surface containing the paramagnetic quencher 5-NS. Measurements were made at 25 °C (excitation 280 nm; emission 334 nm), and intensities were corrected for dilution and light scatter. The PL : CETP ratio at which no further quenching occurs indicates the point at which all the CETP is bound to the emulsion surface.

CETP. CETP was purified from human plasma as described by Barter et al. (1990 c). On SDS-PAGE, the protein migrated as a doublet accounting for 85% of the material. The migration of purified CETP as a doublet in electrophoresis gels has been reported previously by Ohnishi et al. (1990). Both species are recognised by an anti-CETP monoclonal antibody (Ko et al. 1993), and both possess lipid transfer activity (Ohnishi et al. 1990). Our preparation had no detectable lecithin : cholesterol acyltransferase activity and was devoid of apo AI as measured by immunoassay.

Analytical methods. Concentrations of phosphatidylcholine, triacylglycerol and cholesterol were measured with enzymatic kits (Boehringer Mannheim GmbH, FRG) used in conjunction with a Cobas-Bio centrifugal analyser (Roche Diagnostics, Zurich, Switzerland). Protein concentrations were determined by the method of Lowry et al. (1951). CE transfer activity of fractions during the purification of CETP was measured by monitoring the transfer of ^{3}H-CE from donor HDL_3 to acceptor LDL according to published methods (Albers et al. 1984).

Results

Binding of CETP to lipid emulsions

The intrinsic fluorescence of CETP is quenched by 12% on binding to an emulsion surface with no significant change in the emission maximum (results not shown). However, CETP fluorescence was quenched by about 50% when the protein is bound to an emulsion containing 5-NS in its surface monolayer. Advantage was taken of this more substantial quenching to determine conditions under which all the CETP was bound to the lipid surface. This information was required for the design of experiments aimed at measuring the accessibility of tryptophan residues in bound CETP and ensured that free or unbound CETP did not contribute to the fluorescence signal. The binding of lipid emulsion to the transfer protein is shown in Fig. 1. Double reciprocal analysis of this data shows that at the molar ratios of phospholipid to CETP used in the quenching studies (232) more than 95% of the CETP is bound to the emulsion. In all quenching experiments described below the ratio was kept at this level.

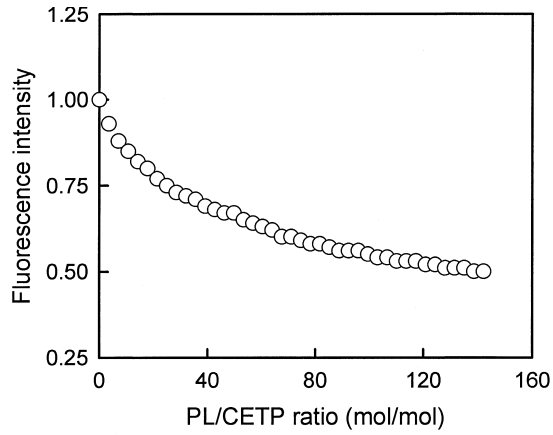

Fig. 1 Titration of CETP with a lipid emulsion containing 5-NS. Aliquots of lipid emulsion (0 to 34 μM PL) were added to a sample of buffer or CETP (0.25 μM). The quenching of fluorescence intensity at each concentration of the lipid emulsion, normalised with respect to the unquenched sample, is plotted against PL/CETP ratio. Results are representative of three separate titrations

Quenching by doxyl stearates

The n-doxyl stearates have been used extensively as depth sensitive probes of lipid bilayers and of the monolayer surface of lipid emulsions (Li et al. 1990; Li and Sawyer 1992). The paramagnetic doxyl stearates are effective quenchers of intrinsic protein fluorescence, and we use them here to indicate the relative average depth of penetration of the monolayer surface of the emulsion by aromatic residues of CETP. The quenching data for 5-NS and 16-NS are presented in Fig. 2 in the form of the modified Stern-Volmer plot (Eq. [1]). The percentage accessibility of the fluorophores to the quencher as determined from the ordinate intercepts of these plots was 49% for 5-NS and 22% for 16-NS. Thus, the tryptophan residues in CETP are more than twice as accessible to quenching by 5-NS than 16-NS. The results suggest that on binding to the lipid emulsion, the tryptophan residues of CETP are, on average, closer to the lipid-water interface than to a region deeper in the surface monolayer.

Quenching by acrylamide

The possibility that tryptophan residues in CETP become buried at the protein-lipid interface was examined by meas-

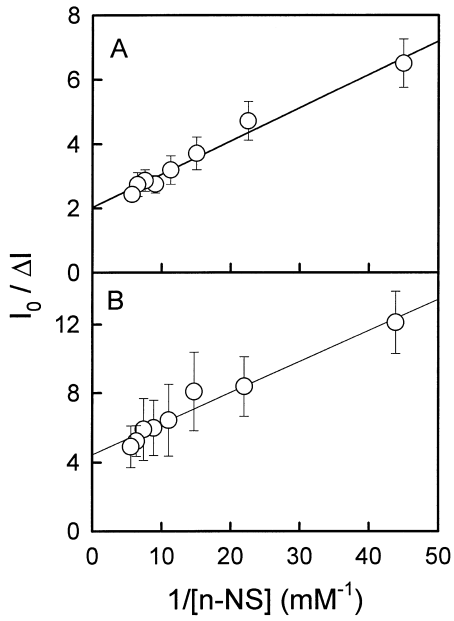

Fig. 2 A, B Fluorescence quenching of CETP bound to an emulsion surface by lipid phase quenchers (**A** 5-NS; **B** 16-NS). To a mixture of CETP and emulsion (molar ratio of phospholipid to protein 232) was added increasing amounts of quencher (0 to 0.18 mM). Fluorescence intensities at each concentration of the quencher were corrected for dilution and scatter and analysed according to modified Stern-Volmer equation. Data shown are average (±SD) of four experiments

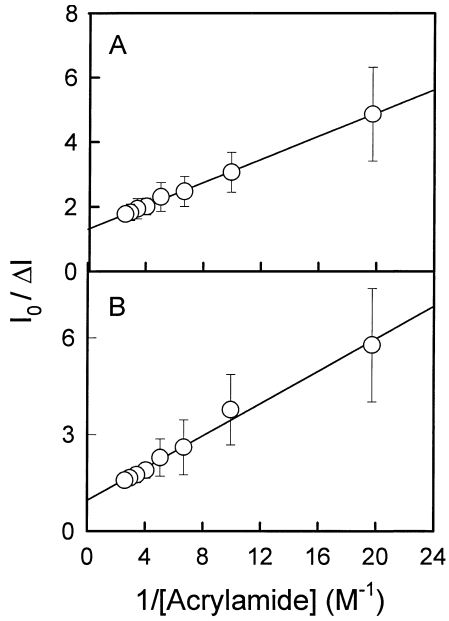

Fig. 3 A, B Fluorescence quenching by acrylamide. To a sample of CETP either in buffer (**A**) or bound to an emulsion surface (**B**) was added increasing amounts of acrylamide (0 to 0.4 M). Other details are as in the caption to Fig. 2. Results are the average of eight experiments (±SD)

uring the accessibility of the fluorophores to a quencher in the aqueous phase in the presence and absence of the emulsion. Figure 3 presents the results in the form of modified Stern-Volmer plots when acrylamide is used as the aqueous phase quencher. In aqueous solution, 81% of the tryptophan fluorophores in CETP are accessible to the quencher. When CETP is associated with the emulsion, 100% of the fluorophores are accessible to quenching by acrylamide. For acrylamide quenching, the ratios K_{sv}(CETP) : K_{sv} (N-acetyl-tryptophanamide) were 0.37 and 0.17 for the transfer protein in solution and bound to the emulsion, respectively. This ratio measures the quenching efficiency of the accessible fluorophores in the protein relative to N-acetyl tryptophanamide in free solution.

Discussion

Frequently, a protein which interacts with a lipid surface undergoes a conformational change which increases its α-helical content, and causes a blue shift and an increase in the quantum yield of its intrinsic fluorescence. Apolipoprotein A I and lipoprotein lipase are examples of this behaviour (Gwynne et al. 1975; McLean and Jackson 1985). CETP appears to be an exception in this regard. Ohnishi's group have reported that the binding of human or rabbit CETP to a microemulsion causes little change in α-helix (Ohnishi et al. 1994; Ko et al. 1993). Our observation of a lack of a blue shift in fluorescence emission and a quenching rather than an enhancement of intrinsic fluorescence on binding to a microemulsion supports the view that the tryptophan residues in CETP do not experience a more hydrophobic environment within the lipid-protein complex.

The penetration of CETP into the surface of a lipid emulsion is represented by the two extreme models depicted in Fig. 4. The following evidence supports the view that CETP does not penetrate the surface monolayer of emulsions to any significant degree (Model A, Fig. 4).

1. The binding of CETP to the emulsion does not result in a blue shift in the tryptophan fluorescence that normally accompanies the transfer of fluorophores to a lipid environment of low dielectric constant; nor is there any enhancement of tryptophan fluorescence that is usually associated with such a change. However, these effects may be partially masked by the fact that the CETP fluorescence is already considerably blue shifted in the unbound protein. Nevertheless, the 12% quenching observed on binding CETP to the emulsion surface suggests that the tryptophan residues in the bound protein are in a more hydrophilic environment brought about by a conformational change within the protein.

2. When CETP is bound to the emulsion surface, a higher proportion of the tryptophan residues is accessible to a quencher located near the lipid surface (49% for 5-NS) than a to a quencher located deeper in the surface monolayer (22% for 16-NS).

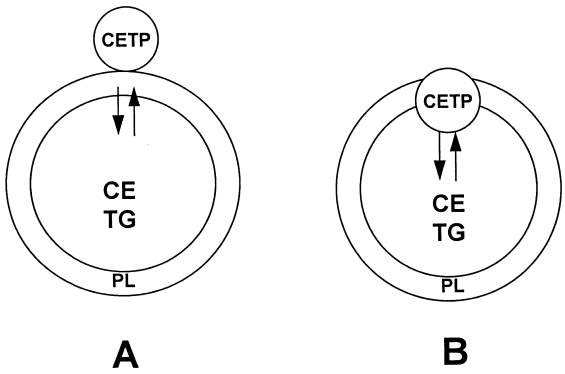

Fig. 4 A, B Two extreme models for the binding of CETP to the emulsion surface. In Model **A**, CETP does not penetrate the surface monolayer but removes small amounts of cholesteryl esters soluble in that compartment, thereby inducing repartition of cholesteryl esters from the core compartment. In Model **B**, the CETP penetrates the surface monolayer for direct access of cholesteryl esters in the core compartment

3. Tryptophan residues in CETP are not protected from an aqueous phase quencher when the protein is bound to the emulsion. On the contrary, a higher proportion of tryptophan residues are accessible to quenching from the aqueous phase when the protein is bound to the lipid surface. Nevertheless, the efficiency of quenching the accessible residues (relative to the quenching of N-acetyl-tryptophanamide in solution) is decreased on binding. These results support the conclusion in (1) above that CETP undergoes a conformational change on association with the emulsion.

The Lehrer treatment of Stern-Volmer quenching assumes that the fluorphores are equally absorbing and have similar lifetime characteristics (Lehrer 1971). However, the multiexponential nature of tryptophan emission is well known and it is unlikely that the lifetime characteristics of the 5 tryptophan residues in CETP are identical. Moreover, the quenching mechanism may be a mixture of both static and dynamic processes. The interpretation described above therefore requires confirmation by other techniques. However, the conclusion that CETP does not penetrate the lipid surface to a significant degree is supported by the results of recent surface balance measurements which show that the exclusion pressure for CETP is lower than for other apolipoproteins and that CETP is much less effective in increasing the surface pressure of monolayers than apolipoproteins such as apoAI (Weinberg et al. 1994). It has been argued that the surface pressure of lipoproteins may be quite high (≥29 mN/m). Thus, the activation energy for the desorption of CETP from the lipid surface may be sufficiently low to enable the protein to associate and dissociate with the lipid surface without penetration of that surface to any significant degree.

Acknowledgements This work was supported by the National Health and Medical Research Council of Australia.

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