Effects of charged lipids on the interaction of cholesteryl ester transfer protein with lipid microemulsions

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This study reports the effects of charged lipids on the transfer of cholesteryl-1-pyrene decanoate (Py-CE) between apolipoprotein-free microemulsion particles mediated by cholesteryl ester transfer protein (CETP). The surface charge characteristics of microemulsion particles composed of cholesteryl oleate and egg yolk phosphatidylcholine were altered by incorporating phosphatidylserine, oleate or stearylamine into the phosphatidylcholine that forms the surface monolayer of the particle. The transfer of Py-CE was measured continuously by following the decrease in excimer fluorescence that accompanies the transfer of the probe from donor to acceptor particles [Rajaram, Chan and Sawyer (1994) *Biochem. J.* **304**, 423–430]. The inclusion of 20 mol % phosphatidylserine relative to the phospholipid in the surface monolayer of the emulsion caused a 64 % decrease in the first-order rate constant describing the transfer. An increase in ionic

strength caused a partial reversal of this effect, indicating that electrostatic factors are only partially responsible for the interaction with lipid. Complete inhibition of transfer was observed when 10 mol % sodium oleate was incorporated into the surface monolayer. The incorporation of stearylamine into the emulsion caused a 32 % increase in the transfer rate. The binding of CETP to the different emulsion surfaces was also examined using a surface plasmon resonance biosensor. The presence of negatively charged lipid (phosphatidylserine or oleic acid) decreased the rate of association of CETP with the emulsion without a significant change in the dissociation rate constant. The presence of the positively charged lipid stearylamine increased the rate of association of CETP with the lipid surface. It is concluded that a negative surface charge on the monolayer decreases the rate of transfer by decreasing the affinity of CETP for these particles.

INTRODUCTION

Human plasma cholesteryl ester transfer protein (CETP) promotes the transfer and exchange of neutral lipids among plasma lipoproteins [1]. Importantly, it mediates the transfer of cholesteryl esters synthesized by the action of lecithin:cholesterol acyltransferase from high-density lipoproteins (HDLs) to triacylglycerol-rich lipoproteins. In doing so, it influences the distribution of cholesterol between lipoprotein classes and plays an important role in the reverse transport of cholesterol from peripheral tissues to the liver, although the importance of this reaction *in vivo* has yet to be explored. The molecular mechanism of the transfer is unknown. Both shuttle and ternary complex (donor–CETP–acceptor) mechanisms have been proposed [2,3]. The former involves the dissociation of CETP from the surface of a donor particle, its diffusional transfer through the aqueous phase and then its association with an acceptor particle. In the alternative hypothesis, transfer occurs during the transient formation of a ternary complex between CETP and the donor and acceptor particles. In both models, the primary event is the binding of CETP to the donor surface. However, it is not known whether the extent of binding is rate-limiting in the transport kinetics. Using synthetic microemulsions, Ohnishi and colleagues [4,5] showed that saturation of the surface of the emulsion with apolipoprotein AI (apo AI) or apo AII increased the rate of exchange by a factor of 7.5. The presence of apo AI did not change significantly the binding capacity or the binding constant for CETP. In the case of apo AII, an increase in the rate of CETP-mediated transfer was correlated with increased binding to the emulsion surface [6,7]. Nishida and colleagues [8] also studied the relationship between the transfer activity of CETP and its affinity for chemically modified lipoproteins. Increasing

the negative charge on low-density lipoprotein (LDL) by succinylation or by treatment with fatty acids [8] increased the transfer rate as well as the affinity of the transfer protein for this lipoprotein fraction. On the other hand, similar modification of HDL had no effect on either the rate of transfer of cholesteryl ester (CE) or its affinity for CETP. Similarly, sodium oleate enhances the capacity of CETP to transfer CE from HDL to LDL or very-low-density lipoprotein (VLDL) [9], but not of the core lipids in the reverse direction [10]. Thus apolipoproteins may affect the transfer rate in unique ways which may or may not relate to their effects on the binding of CETP to the lipid surface.

Recently we reported the use of a surface plasmon resonance (SPR) biosensor to measure the interaction of CETP with lipid emulsions [11]. In the present study we apply SPR to measure the association/dissociation kinetics of the CETP–lipid interaction, and direct attention to the question of whether the presence of charged lipids in the emulsion affects the binding of CETP and hence influences transfer activity. To separate factors relating to surface charge from those due to apolipoproteins, we measure the transfer of CE between apolipoprotein-free donor and acceptor microemulsions whose surface is modified by the presence of sodium oleate, stearylamine or phosphatidylserine (PS). We present evidence that a negatively charged lipid on the surface monolayer of these particles decreases the transfer rate by decreasing the binding of CETP to the emulsion surface.

EXPERIMENTAL

Materials

Cholesteryl-1-pyrene decanoate (Py-CE) was purchased from Molecular Probes. Egg-yolk phosphatidylcholine (PC) and bovine PS were from Lipid Products, South Nutfield, U.K.

Abbreviations used: CE, cholesteryl ester; CETP, CE transfer protein; PC, egg phosphatidylcholine; PS, phosphatidylserine; Py-CE, cholesteryl-1-pyrene decanoate; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; apo AI, apolipoprotein AI; SPR, surface plasmon resonance.

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Stearylamine, cholesteryl oleate, sodium oleate and bovine IgG (Cohn fraction III; 99%) were from Sigma. The BIAcore CM5 sensor chip and the amine coupling kit were purchased from Pharmacia Biosensor (Uppsala, Sweden).

Microemulsions

Microemulsions comparable in size to LDL particles were prepared essentially according to the method of Ginsburg et al. [12]. Lipid mixtures (equimolar amounts of PC and cholesteryl oleate in chloroform/methanol, 1:1, v/v) were placed in glass vials, dried under a stream of nitrogen and vacuum-desiccated at room temperature for 24 h. For emulsions containing labelled CE, the initial lipid mixture also contained Py-CE (15% by weight of total CE). In the case of lipid emulsions containing PS, the initial lipid mixture contained 5, 10 or 20 mol% PS relative to total phospholipid. The lipid mixtures were dispersed in TBS (10 mM Tris/HCl buffer, pH 7.4, containing 0.15 M NaCl, 1.0 mM EDTA and 3.0 mM NaN₃) to a total lipid concentration of 0.1% (w/v) and sonicated for 120 min under a nitrogen atmosphere. A thermostatted water bath was used to maintain the sonication vial at 51–60 °C, i.e. at temperatures not less than 5 °C above the transition temperature of the highest-melting-point species present, namely cholesteryl oleate (46 °C [12]). The sonicated suspension was purified by ultracentrifugation and size-exclusion chromatography [13] to obtain a preparation of emulsion particles with a mean particle diameter of 14.1 ± 1.1 nm, as calculated from the molar volumes of the components [14]. All preparations were dialysed against TBS and used within 4 days of preparation to avoid the aggregation of particles that is known to occur during longer periods of storage [15].

Oleate or stearylamine was incorporated into the emulsion as follows. A weighed amount of sodium oleate was dispersed in TBS and briefly heated to 70 °C under N₂ and then cooled to room temperature before use. Aliquots of this solution were added to the incubation mixture. Stearylamine was added as an ethanolic solution. The final concentration of ethanol in the incubation was less than 2% (v/v), and this had no effect on the transfer of Py-CE in control experiments.

Continuous fluorescence assay for CE transfer

At high local concentrations, and because of its long lifetime, the first singlet state of pyrene forms excimers with the ground-state species. The excimers have a characteristic fluorescence emission at 470 nm which can be used to monitor changes in local concentrations of the probe. Pyrene–lipid conjugates have been used previously to measure the activity of phospholipid transfer proteins, and more recently to follow the CETP-mediated transfer of Py-CE [4,13]. In the present case, the Py-CE microemulsion (donor) and unlabelled emulsion (acceptor) were mixed with CETP in a microcuvette and incubated at 37 °C. The ratio of donor to acceptor was 1:8 (based on the concentration of CE), to avoid both the contribution of back-transfer from acceptor to donor and the generation of excimers in the acceptor particles. The transfer of Py-CE from donor to acceptor was measured as the decrease in the excimer fluorescence at 470 nm [13]. Excitation was at 340 nm. The decrease in excimer fluorescence in the absence of CETP was always < 2%. The control data set obtained in the absence of CETP was subtracted from each data set obtained in the presence of CETP. The resulting curves were fitted to a single-exponential function using a non-linear least-squares fitting procedure:

$$F(t) = A \exp(-k_t t) + c \quad (1)$$

$F(t)$ is the excimer fluorescence at time t , k_t is the apparent first-

order rate constant, A is a pre-exponential factor representing the amplitude of the decay and c is determined by the final equilibrium level of Py-CE. Fluorescence measurements were made at 37 °C with a Perkin-Elmer LS-5 spectrofluorimeter equipped with a thermostatted cell holder.

Biosensor analysis

Biosensor experiments were carried out with a BIAcore instrument (Pharmacia Biosensor AB) using the standard CM5 sensor chip coated with CM-dextran on the gold surface. Immobilization of protein was carried out using the BIAcore amine coupling protocol provided with the Pharmacia coupling kit. The buffers used with the BIAcore machine were the following: running buffer [10 mM Hepes, pH 7.4, 150 mM NaCl, 3.4 mM EDTA (HBS)], regeneration buffer (HBS containing 0.005% surfactant P-20), coupling buffer (10 mM sodium acetate, pH 4.5) and deactivation solution (1 M ethanolamine). All wash solutions were degassed and filtered through a 0.22 μ m-pore-size filter. Surfactant-free HBS was used as the running buffer for all experiments. The flow rate was 5 μ l/min. Surface regeneration was carried out by injecting 40 μ l of the regeneration buffer. Non-specific binding was assessed separately by injecting the emulsion over a surface either with immobilized IgG or with no ligand attached. Maintenance procedures for unclogging the microfluidic cartridge, desorption and system sanitization were carried out as recommended by the manufacturer.

Analysis of biosensor kinetic data

When monochromatic plane-polarized light is directed at a glass surface coated with a gold film, SPR occurs at a precise angle of incidence and causes interaction between the evanescent wave and free oscillating electrons (plasmons) in the metal film. The angle is dependent on the refractive index of the medium within 300 nm of the metal surface, and thus changes in the refractive index of the solution bathing the surface of the sensor will affect the resonance angle. Binding of the emulsion to the sensor surface results in a change in the resonance angle, measured in terms of resonance units. The ascending and descending sides of the sensorgram correspond to the association and dissociation phases of the reaction respectively, and were analysed using the integrated forms of the rate equations. The dissociation rate (k_d) is given by:

$$R_t = R_0 e^{-k_d t} + R_\infty \quad (2)$$

R_0 , R_t and R_∞ are the biosensor responses at time zero, time t and time ∞ respectively. The association kinetics are given by:

$$R_t = R_{eq}(1 - e^{-k_{obs} t}) + R_\infty \quad (3)$$

where R_{eq} is the plateau level of association and $k_{obs} = (k_a C_A + k_d)$, k_a being the association rate constant and C_A the molar concentration of analyte. Data were fitted using the non-linear least-squares procedure available within the SigmaPlot program.

Eqns. (2) and (3) assume that the analyte is univalent in terms of binding to the immobilized component. When the analyte is multivalent, the equations of Ward et al. [16] can be used to determine k_a . In the present case, the lipid particle has the potential to bind more than one CETP molecule, although steric considerations at the surface make it unlikely that all sites are occupied. The concentration of analyte (C_A), in terms of the concentration of binding sites, is unknown, and it is not possible to determine k_a without some assumptions as to valency. We therefore prefer to describe the association kinetics in terms of the product $k_a C_A$, recognizing that a change in this value can be caused by a change in C_A and/or a change in k_a . The data for

kinetic analysis were not used within 30 s of sample reaching the sensor surface or within 60 s of applying the buffer wash, so as to avoid errors due to the substantial refractive index differences between sample and buffer.

Other methods

The concentration of cholesterol was measured with an enzymic kit (Boehringer Mannheim) used in conjunction with a Cobas-Bio centrifugal analyser (Roche Diagnostics, Zurich, Switzerland). The concentration of phospholipid phosphorus was determined according to the method of Bartlett [17]. CETP was purified from human plasma as described by Barter et al. [9]. On SDS/PAGE, the protein migrated as a doublet which accounted for more than 85% of the material. The final preparation had no detectable lecithin:cholesterol acyltransferase activity and was devoid of apo AI, as measured by competitive ELISA. The CE transfer activity of fractions during the purification of CETP was measured by monitoring the transfer of [^3H]CE from donor HDL subfraction 3 to acceptor LDL according to published methods [18]. The transfer activity of CETP varied between preparations. However, assays using the same preparation were reproducible over a period of several weeks when the protein was stored at -80°C . Protein concentrations were determined by the method of Lowry et al. [19].

RESULTS

Effect of PS on the transfer of Py-CE between emulsion particles

PS is negatively charged at physiological pH [20] and is frequently used to confer charge on synthetic phospholipid bilayers [21]. To determine if negative charge on the surface monolayer of an emulsion affects CETP activity, the transfer of Py-CE was examined using cholesteryl oleate/PC emulsions containing various amounts of PS relative to total phospholipid, but having the same PS content in donor and acceptor. In the present experiments the PS content of the emulsion preparation was

Figure 1 Transfer of fluorescent CE between cholesteryl oleate/PS/PC emulsions

Aliquots of Py-CE-labelled donor emulsion (9 nmol of CE) and unlabelled acceptor emulsion (75 nmol of CE), both containing the same amount of PS relative to total phospholipid, were mixed with CETP (27 $\mu\text{g}/\text{ml}$; final volume 600 μl). Changes in the relative excimer fluorescence (rel. excimer fluor.) were monitored continuously for 30 min. Curve 1, control (no CETP; 0 mol% PS); curve 2, 20 mol% PS; curve 3, 10 mol% PS; curve 4, 0 mol% PS. The solid lines are single-exponential fits to eqn. (1). Results are representative of four different emulsion preparations.

Table 1 Effects of surface modifiers on the rate of transfer of Py-CE between emulsion particles

The transfer of Py-CE from donor to acceptor emulsion (both containing the same level of the surface modifier) was monitored at 37°C for 60 min. Apparent first-order rate constants for Py-CE transfer were obtained by fitting the excimer intensity data to eqn. (1), as described in the Experimental section; S.D. values were within ± 0.005 . The excimer intensity for the final equilibrium level (c in eqn. 1) is expressed as a fraction of initial fluorescence intensity; S.D. values were within ± 0.06 . The concentration of PS (mol%) is relative to the total phospholipid used to prepare the microemulsion. CETP used in the incubations with oleic acid was from a different protein preparation. Other details of experimental conditions are given in the legend to Figure 1.

Surface modifier	Concn. (mol%)	Rate constant (min^{-1})	c
PS	0	0.044	0.65
	5	0.028	0.66
	10	0.026	0.67
	20	0.016	0.68
Oleic acid	0	0.012	0.75
	5	0.008	0.80
Stearic acid	0	0.037	0.64
	14	0.049	0.62

based on the molar ratios of phospholipids originally included in the sonication mixtures (see the Experimental section). In the absence of CETP, the excimer fluorescence intensity of Py-CE in the donor remained constant regardless of the PS content of the emulsion particles. Addition of CETP initiated a decrease in excimer fluorescence (Figure 1). A single-exponential function (eqn. 1) is sufficient to adequately fit the experimental data. As the PS content of the emulsion increased there was an inhibition of CETP-mediated Py-CE transfer. At 20 mol% PS, the Py-CE transfer at 60 min was reduced by 42%. The apparent first-order rate constant decreased from 0.044 min^{-1} in the absence of PS to 0.016 min^{-1} at 20 mol% PS. The rate constants are summarized in Table 1. The presence of PS did not change the equilibrium excimer intensity (c) at infinite time (eqn. 1; Table 1).

In separate experiments (Figure 2), we determined whether the donor and acceptor particles contributed equally to the charge effect by measuring Py-CE transfer between heterologous donor and acceptor particles. In agreement with the results in Figure 1, the inclusion of 20 mol% PS in both donor and acceptor particles decreased the transfer relative to that between neutral emulsions by 55% at 60 min. When only the acceptor contained 20 mol% PS, the reduction in transfer was 48% at 60 min, and when only the donor contained 20 mol% PS the reduction in transfer was only 4% at 60 min. If both donor and acceptor contribute equally to the charge effect, the results should reflect the 8-fold excess of acceptor over donor. Thus when the acceptor is charged, the reduction in transfer is about eight-ninths that when both donor and acceptor are neutral ($55\% \times 8/9 = 49\%$). When the donor is charged, the transfer should be about one-ninth that of the neutral donor/neutral acceptor control ($55\% \times 1/9 = 6\%$). These values are in broad agreement with those found experimentally. Thus the donor and the acceptor contribute equally to the charge effect. However, the effect is greatest when the acceptor is charged, since it is present in an 8-fold excess over the donor.

Effect of ionic strength on the transfer of Py-CE between charged microemulsions

Univalent cations such as K^+ bind to the head group of PS, rendering the microenvironment of the negatively charged mem-

Figure 2 Py-CE transfer between charged and uncharged lipid particles

Transfer of fluorescent CE from donor to acceptor emulsions was measured by monitoring changes in relative excimer fluorescence (rel. excimer fluor.) as described in the legend to Figure 1. The PS content of the negatively charged emulsion was 20 mol% relative to total phospholipid. Curve 1, donor and acceptor neutral; curve 2, donor negatively charged and acceptor neutral; curve 3, donor neutral and acceptor negatively charged; curve 4, donor and acceptor negatively charged; curve 5, control (no CETP). The solid lines are the fits to eqn. (1); experimental points have been omitted for clarity.

brane surface less polar [22] without affecting the packing of the acyl chains [23]. To confirm that it was the negative surface charge that decreased the transfer of CE between emulsion particles, the monolayer surface potential was reduced by increasing the ionic strength of the aqueous phase. Py-CE-labelled donor and unlabelled acceptor emulsions, each containing 20 mol% PS relative to total phospholipid, were incubated with CETP. The ionic strength of the incubation medium was varied by the addition of 0.1–0.4 M KCl. In the absence of CETP, the excimer fluorescence of Py-CE in the donor remained steady at

Figure 3 Effect of ionic strength on Py-CE transfer between cholesteryl oleate/PS/PC emulsions

Py-CE-labelled microemulsions composed of cholesteryl oleate/PC and PS (20 mol% relative to total phospholipid) were dialysed against 10 mM Tris/HCl buffer, pH 7.4, containing 0.1 M KCl, 1.0 mM EDTA and 3.0 mM Na₂S₂O₃, and the transfer reaction was initiated by the addition of CETP (curve 4). The ionic strength of the incubation mixture was increased by adding a concentrated solution of KCl to final KCl concentrations of 0.2 M (curve 3), 0.3 M (curve 2) and 0.4 M (curve 1). Other details are as described in the legend to Figure 1. The solid lines are the fits to eqn. (1); experimental points have been omitted for clarity.

Figure 4 Biosensor analysis of the interaction of immobilized CETP with a cholesteryl oleate/PC microemulsion

(A) Sensorgram showing the binding of emulsion to CETP immobilized on the biosensor surface. Association is initiated by the injection of the emulsion sample at point a. Dissociation is induced by the flow of buffer at point b. Point d marks the beginning of regeneration of the surface. The contribution of the refractive index of the emulsion suspension to the biosensor response is indicated by the substantial drop in resonance that occurs between points b and c. Data for the association (B) and dissociation (C) reactions were analysed according to eqns. (3) and (2) respectively. The solid line is the non-linear least-squares fit to the data points (○).

all concentrations of KCl used in the incubation. Increasing the salt concentration from 0.1 to 0.4 M KCl increased the CETP-mediated transfer of Py-CE (Figure 3). At 0.3 M KCl, the inhibition of Py-CE transfer due to PS was partially reversed. Higher concentrations of potassium did not relieve the inhibition of Py-CE transfer further (Figure 3).

Effects of other modifiers of surface charge on Py-CE transfer

The influence of membrane surface charge was explored further by monitoring the transfer of Py-CE between microemulsions possessing different modifiers on their surface. Oleic acid can also confer a negative charge on the emulsion surface. However, since fatty acids modify the microviscosity of the membrane differently, the effect of oleic acid on Py-CE transfer was determined. Sodium oleate at levels of 0, 5, 10 and 15 mol% relative to total phospholipid was added to mixtures of a Py-CE-labelled donor emulsion and unlabelled acceptor, and the CETP-mediated transfer of Py-CE was followed by measurement of excimer

Table 2 Effect of emulsion surface charge on the rates of association and dissociation for binding to immobilized CETP

Kinetic parameters for the interaction between emulsion and CETP were determined using the BIAcore biosensor. The volume (15 μ l) and the phospholipid concentration (2.86 mM) of the emulsion injected was constant for all runs. The concentration of PS (mol%) is relative to the total phospholipid used to prepare the microemulsion, whereas the concentrations of oleic acid and stearylamine (mol%) are relative to phospholipid in the final microemulsion. The sensorgram data were analysed as described in the Experimental section. The results are means $\pm$ S. D. of at least four determinations. Significance of difference compared with the control: * $P < 0.05$; ** $P < 0.001$.

Emulsion	$10^3 \times k_d$ (s^{-1})	$10^3 \times k_a C_A$ (s^{-1})
Control	6.00 ± 0.78	8.06 ± 0.91
PS (20 mol%)	4.78 ± 0.50	$6.77 \pm 0.88^*$
Oleic acid (5 mol%)	5.38 ± 0.61	$2.74 \pm 0.80^{**}$
Stearylamine (14 mol%)	4.08 ± 1.02	$11.29 \pm 0.91^{**}$

fluorescence. The presence of 5 mol% oleate in the surface monolayer caused a 33% decrease in the transfer rate (Table 1). At 10 and 15 mol% oleate, the transfer of Py-CE was completely inhibited. Stearylamine also partitions in the surface monolayer, but provides a positive charge to the emulsion surface [24]. Stearylamine was added to Py-CE-labelled donor emulsions and unlabelled acceptor emulsions at levels of 0, 3.5, 7 and 14 mol% relative to total phospholipid. The CETP-mediated transfer of CE at 60 min increased with increasing levels of this surface modifier. The rate constant for the transfer of Py-CE increased by 32% on the addition of 14 mol% stearylamine (Table 1). While the presence of these charged species in the surface monolayer significantly affected the rate constants, it is important to note that in each case the excimer intensity for the final equilibrium reached a level comparable with that of the respective control (Table 1).

Interaction of emulsion with immobilized CETP

For proteins which interact transiently with lipid surfaces, it is possible to use SPR to determine whether a change in the composition of the lipid particle leads to changes in the association and dissociation kinetics. Interaction with immobilized CETP was studied over a concentration range of the emulsion phospholipid of 650–3000 μ M; values of k_d and $k_a C_A$ were independent of emulsion concentration within this range (results not shown). In the present experiments the volume (15 μ l) and the concentration of phospholipid in the sample injected (2.86 mM) were identical in all runs. Figure 4(A) shows a typical sensorgram of this system. The rapid and substantial jump in the biosensor response which precedes the association and dissociation kinetics has been noted previously [11], and is due to the refractive index of the emulsion suspension. Values of k_d and $k_a C_A$ were derived by fitting the data points to eqns. (2) and (3) respectively, and are summarized in Table 2. Typical fits of the association and dissociation sections of a sensorgram are shown in Figures 4(B) and 4(C) respectively. The inclusion of 20 mol% PS or 5 mol% oleic acid in the emulsion decreased the value of $k_a C_A$ by 16% and 66% respectively (Table 2). In contrast, inclusion of 14 mol% stearylamine in the emulsion monolayer increased the value of $k_a C_A$ by 40%. There were no significant effects of these additions on the dissociation rate constant (Table 2).

DISCUSSION

The interaction of a protein with a lipid surface can be mediated by electrostatic and/or hydrophobic interactions. In the case of apolipoproteins, amphipathic α -helices are believed to play an important role by presenting a hydrophobic face to the lipid phase and a hydrophilic surface to the solvent [25]. CETP is unique in that it is one of the most hydrophobic proteins known [26], yet it remains soluble in aqueous solvents and possesses only two putative amphipathic helices (residues 264–276 and 465–476). Part of this solubility can be attributed to glycosylation, there being four potential asparagine-linked glycosylation sites in the sequence (residues 88, 240, 341 and 396) [26]. It is therefore probable that hydrophobic interactions are a major contributor to the binding of CETP to lipid surfaces. The present studies were aimed at determining whether electrostatic interactions also contribute to the interaction energy.

The results demonstrate that the charge on the lipid surface can affect the ability of CETP to transfer Py-CE between synthetic microemulsions; transfer activity is inhibited by negative charge and favoured by positive charge. The effect of charge agrees with expectations based on the isoelectric point of CETP, which is in the range 5.0–5.2 [27,28]. The observation that an increase in ionic strength partially reverses the effect of PS establishes an electrostatic component of the interaction. However, the failure of salt to completely reverse the effect of PS points to the involvement of additional factors. The finding that the donor and acceptor particles contribute about equally to the charge effect, at least when PS provides the negative charge, indicates that the transfer is controlled by the equilibrium binding of CETP to both donor and acceptor particles. This model also predicts that the acceptor competes with the donor for the binding of CETP such that the transfer rate decreases with increasing acceptor concentration, an observation that we have also made experimentally (results not shown). The charge modifiers had no effect on the final equilibrium levels of excimer fluorescence in the transfer assays (c ; Table 1). The failure to observe excimer levels lower than 62–75% of the original values suggests the existence of an unexchangeable pool of Py-CE, an observation that has also been made in the case of the spontaneous exchange of unesterified cholesterol between bilayer vesicles [29].

In the case of negatively charged phospholipid bilayers, univalent cations such as potassium act as counter-ions which effectively reduce the area per phospholipid molecule and the surface charge density, without affecting the physical state of the phospholipid acyl chains [30–32]. It is not known if similar effects occur at the surface of phospholipid/CE lipid emulsions. However, we note that oleic acid is much more effective than PS in decreasing the transfer rate. The interfacial pK of oleic acid is higher than that of PS, and thus simple electrostatics cannot explain the greater effect of oleic acid. However, the microviscosity of the membrane will be modified differently by the addition of each negatively charged species, oleic acid being an effective fluidizer of PC bilayers [33]. The concentration of oleic acid in the aqueous phase will be finite but extremely small due to the large partition coefficient of this fatty acid into lipid bilayers (400 000–790 000 [34]). Nevertheless, given the large average hydrophobicity of CETP, the question arises as to whether the protein itself can bind fatty acids. We recently reported a new and sensitive method for measuring the binding of fatty acids to proteins [35]. The method is based on the ability of a fatty acid binding protein to increase the apparent critical micelle concentration of the fatty acid. No binding of oleic acid to CETP could be detected by this method (results not shown).

Thus the effect of oleic acid on the transfer activity of CETP cannot be explained in terms of specific binding of the fatty acid to the protein.

We have not observed any increase in light scattering when CETP is added to emulsion mixtures (results not shown). It is therefore unlikely that CE transfer takes place within a transient ternary complex. In the alternative shuttle model of CETP action, the surface charges on the donor and acceptor will not affect the rate of diffusion of CETP through the aqueous phase, but will affect the on- and off-rate constants that control the binding of CETP to the lipid surfaces. In an ideal system, the surface charge would affect both association and dissociation kinetics, whereas we find that a negative surface charge decreases the on-rate constant without affecting the off-rate constant. Two factors are relevant to this observation. Firstly, when the analyte (emulsion) possesses multiple binding sites, the on-rate is determined by the product of the on-rate constant and the concentration of sites on the emulsion. Thus the effect of a negative surface charge may be to decrease the binding capacity and/or to decrease the on-rate constant. Secondly, the protein species which binds to the emulsion surface may not be that which dissociates from it. The quenching of CETP fluorescence by an aqueous phase quencher (acrylamide) shows that the protein undergoes a conformational change on binding which increases the proportion of tryptophan residues exposed to the aqueous phase [36]. Proteins which interact with lipid surfaces frequently undergo conformational changes on binding. For example, apo AI and lipoprotein lipase increase their content of α -helix [37,38]. Thus the differential effects of surface charge on the association and dissociation rate constants may be a consequence of the conformational change in the protein.

Comparison of the transfer rate with binding kinetics determined with the SPR biosensor must be made with caution. The kinetic constants determined by the SPR biosensor may not relate to those operating in free solution. The biosensor experiment is subject to uncertainties arising from possible heterogeneity of CETP immobilization, since not all CETP molecules may be conjugated to the CM-dextran surface through the same amino group, thereby causing heterogeneity in binding affinity. Moreover, the degree to which the emulsion particle might penetrate or distort the dextran matrix of the biosensor is not known. Diffusion-limited kinetics may also be a problem because of the large size of the emulsion particle. Collectively, these problems are thought to contribute to differences between solution-phase and solid-phase methods and to deviations from pseudo-first-order behaviour in the analysis of the ascending and descending sides of the sensorgram [39]. Nevertheless, if experimental conditions (e.g. flow rate, temperature) are kept constant, the derived rate constants are useful comparative indicators of the effects of surface charge.

Apart from affecting the binding of CETP to the lipid surface, the surface charge may also influence CETP action by imposing conformational constraints on that domain of the protein which confers transfer activity. There is strong evidence that the C-terminal domain of CETP, which contains one of the two putative amphipathic helices, is involved in both lipid binding and lipid transfer. Deletion and site-directed mutagenesis of the C-terminal amphipathic helix shows that it is necessary for both lipid binding and transfer activity [40,41]. A monoclonal antibody (TP2) directed against the 26-residue C-terminal domain neutralizes both transfer activity and the binding of neutral lipid [42,43]. However, two other monoclonal antibodies (TP12 and TP18), whose epitopes are distant from the C-terminus, also partially inhibit CE transfer [44]. It is suggested that these domains, which are probably involved in lipoprotein binding,

may be in close proximity to the C-terminus in the native structure [44]. Wang et al. [41] have suggested that monoclonal antibody TP2 may bind to and constrain the polar face of the amphipathic helix, thus interfering with lipid transfer. We suggest that a negatively charged lipid monolayer may act similarly by constraining the negatively charged face of the C-terminal helix, which is rich in hydrophilic residues (Glu⁴⁶⁵, His⁴⁶⁶, Asp⁴⁷⁰, Ser⁴⁷⁶), and by inducing the hydrophobic face of this domain to adopt a conformation such that the lipid substrate cannot be easily accessed. Similarly, a positive surface charge would favour binding of this domain or allow the correct orientation of the amphipathic helix on the lipid surface. CETP has an extremely high hydrophobic index, and its interaction with lipid surfaces is likely to be mediated primarily by hydrophobic interactions. Nevertheless, the present study suggests that ionic interactions play a moderating role in the model emulsion system. Whether surface charge has any role in determining CETP activity *in vivo* is not known. PS and phosphatidic acid represent only 3% of HDL phospholipids [45] and there are only small amounts of sialic acid present (5.5 μ g/mg of protein) [46]. Thus these components are unlikely to determine the binding and hence activity of CETP in lipoprotein systems. On the other hand, the unesterified fatty acid content of HDL represents 15% of the total plasma unesterified fatty acid, and can increase the electrophoretic mobility of this lipoprotein [47]. Recent evidence indicates that non-esterified fatty acids can modulate the CETP-mediated transfer of CE from HDL to LDL/VLDL, but not in the reverse direction [48]. Thus the presence of fatty acids in HDL, LDL and VLDL is likely to be of greater physiological relevance to the action of CETP than is the presence of negatively charged phospholipids. It remains to be determined whether apo AI and apo AII, both of which enhance CETP-mediated lipid transfer in model emulsion systems [4,5], exert a similar effect in lipoprotein systems.

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