Evidence That Cynomolgus Monkey Cholesteryl Ester Transfer Protein Has Two Neutral Lipid Binding Sites*

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Two inhibitors of cynomolgus monkey cholesteryl ester transfer protein were evaluated. One, a monoclonal antibody made against purified cynomolgus monkey cholesteryl ester transfer protein, was capable of severely inhibiting triglyceride transfer, but had a variable effect on cholesteryl ester transfer. At low antibody to antigen ratios, there was what appeared to be a stoichiometric inhibition of cholesteryl ester transfer, but at high antibody to antigen ratios the inhibition of cholesteryl ester transfer was completely relieved, even though triglyceride transfer remained blocked. Fab fragments of the antibody had no effect whatsoever on cholesteryl ester transfer, but were capable of completely blocking triglyceride transfer. The other inhibitor, 6-chloromercurocholate, severely inhibited cholesteryl ester transfer with minimal inhibition of triglyceride transfer. When both inhibitors were added to the assay, both cholesteryl ester and triglyceride transfer were inhibited; an indication that the inhibitors did not compete for the same binding site on cholesteryl ester transfer protein.

When the antibody was given subcutaneously to cynomolgus monkeys at a dose which inhibited triglyceride transfer in the plasma by more than 90%, there was no detectable effect on the high density lipoprotein (HDL) cholesterol level, but the HDL triglyceride levels decreased from 13 ± 2 to 1 ± 0 mol/mol of HDL (mean ± S.D.); an indication that the antibody uncoupled cholesteryl ester and triglyceride transfer in vivo. The 6-chloromercurocholate could not be evaluated in Vivo because it is a potent leukincholesterol acyltransferase inhibitor. The fact that cholesteryl ester transfer can be inhibited without effect on triglyceride transfer and, conversely, that triglyceride transfer can be inhibited without effect on cholesteryl ester transfer indicates that these two lipids are not transferred by a single, non-discriminatory process.

Cholesteryl ester transfer protein is a plasma protein that catalyzes the redistribution of neutral lipids, triglycerides, cholesteryl esters, retinyl esters, and possibly other lipophilic compounds, among lipoproteins (1). In its absence, neutral-lipid transfer between lipoproteins is essentially nonexistent. The mechanism by which CETP1 catalyzes the redistribution of neutral lipids is not entirely understood. Two models have been proposed: the shuttle model (2) in which CETP acts as a carrier, transporting neutral lipids back and forth between donor and acceptor; and the ternary complex model (3) in which donor, acceptor, and transfer protein come together in a single complex for neutral-lipid exchange. The shuttle model has the transfer process divided into 7 steps: association (or docking) of CETP with the donor lipoprotein; transfer of the neutral lipid from the donor to CETP; dissociation of CETP-lipid complex from the donor; diffusion of the complex through the surrounding medium; docking of the CETP-lipid complex with the acceptor; transfer of the CETP-associated lipid to the acceptor; and finally, desorption of CETP from the acceptor. If the donor and acceptor are identical, then there are only 4 elementary steps: association, exchange, dissociation, and diffusion. With the exceptions of the dissociation and diffusion components, the same processes are required in the ternary model, although not necessarily in the same order. Finally, it is not known whether CETP dissociates from donors or acceptors without neutral lipid; the inability to do so would categorize it as an "exchange" protein rather than a "transfer" protein.

CETP activity has been detected in the plasma of several species (4-10). Of those, the cynomolgus monkey protein is the most similar in primary structure to the human protein (9). There are just 20 amino acids out of 476 which are different between the two proteins and all but two of these substitutions are conservative. Most noteworthy, however, is the observation that the carboxyl-terminal 38 amino acids of the two proteins are identical, for that is the region of CETP thought to be essential for lipid transfer (11-15). This high degree of homology between the human and cynomolgus monkey proteins implies that observations made using the monkey protein are germane to our understanding of human CETP structure and function.

An unanswered question regarding human CETP is whether it has more than one neutral lipid binding site. Early studies with both crude and purified CETP preparations indicated that some mercury-containing compounds were capable of inhibiting triglyceride transfer without inhibiting cholesteryl ester transfer (16, 17); an observation compatible with the premise that two neutral lipid binding sites were present. The present paper addresses that question using cynomolgus monkey CETP as the transfer protein, and shows that, in vitro, not only can triglyceride transfer be inhibited without effect on cholesteryl ester transfer, but also that cholesteryl ester transfer can be inhibited without effect on triglyceride transfer. In addition, we present evidence that cholesteryl ester transfer can be uncoupled from triglyceride transfer in vivo.

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1 The abbreviations used are: CETP, cholesteryl ester transfer protein; LPDP, lipoprotein deficient plasma; LDL, low density lipoproteins; HDL, high density lipoproteins.
MATERIALS AND METHODS

Reagents—HDL was isolated from cynomolgus monkey plasma by ultracentrifugation (at 1 063–1.225 g/ml; (9)) and then passed over a 2.6 × 30-cm column of Sephadex G-25 (Pharmacia, Uppsala, Sweden) to remove residual LDL and LP(a). Lipoprotein-deficient plasma (LPDP) was prepared by adjusting the density of fresh plasma from cynomolgus monkeys to 1.225 g/ml with solid KBr and centrifuging at 285,000 × g for 48 h. The top one-third of the tubes' contents was removed by spinning. The bottom fraction was dialyzed extensively against phosphate-buffered saline and stored at 4°C. Human LDL containing [4-14C]cholesteryl oleate and [9,10-3H]triolein was used as the donor and cynomolgus monkey HDL was used as the acceptor.

Each incubation tube contained LDL, HDL, [3H]triolein was used as the donor; and cynomolgus monkey HDL was used as the acceptor. The reaction was started by the addition of CETP and the redistribution of the radioactivity was followed by taking samples at various times, remov-

In Vitro Studies—The CETP assay used here has been described in detail previously (9) and is a modification of that originally described by Pattnaik et al. (21) and modified by Morton and Zilversmit (17). Briefly, radiolabeled lipids are incorporated into a “donor” lipoprotein and that donor is then incubated with an “acceptor” lipoprotein in the presence of a source of CETP. In the present study, cynomolgus monkey LPDP or purified recombinant cynomolgus monkey CETP was used as CETP source. Human LDL labeled with [4-14C]cholesteryl oleate and [9,10-3H]triolein was used as the donor, and cynomolgus monkey HDL was used as the acceptor. Each incubation tube contained LDL, HDL, a source of CETP and incubation buffer (Tris-HCl (50 mm, pH 7.4) containing bovine albumin (1%), NaCl (150 mm), and NaN3 (3 mm)). The amount of LDL- or HDL-associated lipid in the assay tube was 700 μl. The reaction was started by the addition of CETP and the redistribution of radioactivity was followed by taking samples at various times, removing the LDL by precipitation, and quantifying the radioactivity in the supernatant.

In the absence of CETP, redistribution of radioactivity was essentially zero. In the presence of CETP, the distribution of the radioactive lipids between the LDL and HDL approached equal specific activity. This occurred regardless of whether the radioactive lipid was initially in LDL or HDL. The redistribution kinetics were those of a two-pool, closed system, in which there was no appreciable net mass transfer.

Table I Lipid composition of LDL and HDL used for in vitro cholesteryl ester and triglyceride transfer measurements

| Lipoprotein | Free cholesterol (nmol) | Cholesterol ester (nmol) | Triglyceride (nmol) | Phospholipid (nmol) |
|-------------|-------------------------|--------------------------|--------------------|---------------------|
| LDL         | 7.94                    | 19.68                    | 4.92               | 16.64               |
| HDL         | 8.08                    | 18.30                    | 3.30               | 32.11               |

Table I Values are nanomoles of LDL- or HDL-associated lipid in the assay tube.

In Vivo Studies—Adult, male cynomolgus monkeys obtained from the Upjohn Colony were used for these studies. In study 1, four monkeys received CMTP-2 and 4 others received CMTP-1. In study 2, three monkeys received CMTP-2. In each instance the antibody was dissolved in sterile saline and passed through a 0.45-μm Millipore filter prior to injection. Thirty mg of protein in a final volume of 12 ml was injected into each monkey subcutaneously, between the shoulder blades. In study 1, 7-ml blood samples were taken just prior to administration of the antibody (t = 0) and at 0.25, 1, 2, 3, 4, 7, 9, and 11 days after antibody administration. In study 2, a 24-ml blood sample was taken from each monkey 7 days prior to antibody administration and a second 24-ml blood sample was taken 48 h after antibody administration. All blood samples contained EDTA at 1 mg/ml and azide at 0.02% and were kept at 4°C until analyzed.

Statistics—The effects of subcutaneous administration of CMTP-2 on the plasma lipids of the cynomolgus monkey (Table II) were evaluated using a one-way, repeated measures analysis of variance and Dunnett's test for multiple comparisons (23). The effects of CMTP-2 treatment on the size and composition of HDL were evaluated using the paired t test (23).
RESULTS

Fig. 2A shows the effect of increasing CMTP-2 concentration on the redistribution of radioactivity between LDL and HDL, as measured with cynomolgus monkey LPDP as the source of CETP. Triglyceride transfer was progressively inhibited with increasing amounts of the antibody, whereas cholesteryl ester transfer was progressively inhibited only at antibody concentrations \(<16 \text{ nM}\). Beyond this point, the inhibition decreased with increasing antibody concentration until at concentrations near 100 \text{ nM}, cholesteryl ester transfer was almost normal. Subsequent studies (not shown) demonstrated that at CMTP-2 concentrations above 1 \text{ \mu M}, cholesteryl ester transfer was not inhibited whatsoever.

The icons in the lower portion of the graphs are intended to show the theoretical relationship between antigen (depicted as the gray globular structure) and antibody (depicted as the black y-shaped structure) at various antibody concentrations. If the affinity of the antibody for CETP is high, then at antibody concentrations below stoichiometry the antibody should be saturated with CETP, i.e. both antigen binding sites should be occupied. This form is depicted by the icon (a) in the lower left of Fig. 2A. As the antibody concentration increases, the degree of saturation decreases, such that at antibody concentrations well above stoichiometry only one of the antigen binding sites should be occupied. That form of the antibody-CETP complex is depicted by the icon (b) in the lower right portion of Fig. 2A.

We hypothesized that only the saturated form of the antibody (Fig. 2A, icon a) is capable of inhibiting cholesteryl ester transfer, and the loss of inhibition evident at high antibody concentrations (\(<16 \text{ nM}\)) coincides with the accumulation of the hemisaturated form of the antibody (Fig. 2A, icon b). To test this premise, we produced antibody binding (Fab) fragments of CMTP-2 and evaluated the effects of those Fab fragments on cholesteryl ester and triglyceride transfer. The results of those experiments are shown in Fig. 2B. The CMTP-2 Fab fragments, like the intact antibody, were capable of completely inhibiting triglyceride transfer, but no inhibition of cholesteryl ester transfer was detectable, even at Fab concentrations near 1 \text{ \mu M}.

These experiments not only support the hypothesis that both antigen binding sites on CMTP-2 must be occupied for cholesteryl ester transfer to be inhibited, but also demonstrate that CETP-mediated cholesteryl ester transfer can be uncoupled from triglyceride transfer.

To eliminate the possibility that a distinct protein was responsible for each type of neutral lipid transfer, the Fab studies were repeated using recombinant cynomolgus monkey CETP that had been purified from the media of Chinese hamster ovary cells expressing the protein. Fig. 3 shows the results of those experiments. Note that, as with LPDP, there was a selective inhibition of triglyceride transfer by the Fab fragments and no inhibition of cholesteryl ester transfer whatsoever. Thus, triglyceride transfer could be selectively inhibited regardless of whether LPDP, or purified, recombinant CETP was used as the source of transfer activity.

The question then arose as to whether the converse condition could be created, i.e. whether cholesteryl ester transfer could be

Table II

| Plasma lipids | Time after antibody injection (days) |
|--------------|-------------------------------------|
|              | 0         | 0.25      | 1         | 2         | 3         | 4         | 7         | 9         | 11        |
| Triglyceride | 43 ± 17   | 34 ± 13a  | 22 ± 11a  | 29 ± 14a  | 36 ± 24a  | 24 ± 8a   | 21 ± 5a   | 51 ± 36   | 38 ± 19   |
| Cholesterol  | 117 ± 27  | 115 ± 24  | 103 ± 23  | 112 ± 23  | 116 ± 24  | 117 ± 29  | 123 ± 24  | 114 ± 21  | 112 ± 17  |
| HDL          | 19 ± 3    | 12 ± 3a   | 4 ± 1a    | 1 ± 1a    | 4 ± 1a    | 3 ± 1a    | 4 ± 4a    | 10 ± 4    | 14 ± 4    |
| Cholesterol  | 58 ± 11   | 60 ± 14   | 57 ± 11   | 66 ± 13   | 65 ± 12   | 64 ± 14   | 57 ± 8    | 52 ± 8    | 51 ± 4    |

*Indicates that the value was significantly (p < 0.05) less than the t = 0 value.

Fig. 2: Effect of CMTP-2 and CMTP-2 Fab on CETP-mediated neutral lipid transfer. The indicated amount of antibody (A) or Fab (B) was added to the standard CETP assay ingredients (see "Materials and Methods") and those mixtures incubated at 37 °C for 3 h, after which the reaction was stopped and the amount of radioactivity present in the HDL quantified. Cynomolgus monkey LPDP (100 \mu l) was the source of CETP. Control tubes (containing no antibody or Fab) were run in parallel and the HDL radioactivity in a given assay tube expressed as a percentage of the HDL radioactivity in the control tube. The latter values were: [4-14C]cholesteryl ester (filled squares), 9,917 dpm; [9,10-3H]triglyceride (open squares), 18,921 dpm. The icons (a-d) in the lower portion of the graphs represent: a, antibody with both antigen binding sites occupied with CETP; b, antibody with only one of the antigen binding sites occupied with CETP; c and d, Fab fragments occupied with CETP.
inhibited without affecting triglyceride transfer. Fig. 4 shows the effects of the compound 6-chloromecuric cholesterol (U-617) on neutral lipid transfer mediated by cynomolgus monkey LPDP. Those data show that selective inhibition of cholesteryl ester transfer is indeed possible. Separate studies of U-617 inhibition of cholesteryl ester transfer using purified, recombinant cynomolgus monkey CETP and synthetic donors and acceptors (19), indicated that U-617 was a competitive inhibitor of cholesteryl ester transfer that had no detectable effect on triglyceride transfer.

To determine whether the CMTP-2 Fab binding site on cynomolgus monkey CETP overlapped the U-617 binding site, monkey LPDP was preincubated with high concentrations of the Fab fragments after which the effects of U-617 on lipid transfer were measured. As expected, triglyceride transfer was inhibited greater than 95%; the effects on cholesteryl ester transfer are shown in Fig. 5. Note that the Fab fragments did not block the ability of the compound to inhibit cholesteryl ester transfer and, in fact, appeared to increase the potency of U-617 somewhat. That indicates that the site on CETP at which U-617 binds is different from that which binds the Fab.

Given the observations that the transfer of cholesteryl ester and triglyceride could be selectively inhibited in vitro, the question arose whether the same would occur in vivo. Previous studies (25–27) indicated that inhibition of cholesteryl ester transfer resulted in an increase in HDL-cholesterol levels, but it has not been firmly established what effect selective inhibition of triglyceride transfer would have on plasma lipoprotein levels. To see whether triglyceride transfer could be inhibited in vivo to a significantly greater extent than cholesteryl ester transfer, 30 mg of purified CMTP-2 were administered to each of four cynomolgus monkeys subcutaneously. Analysis of plasma samples taken from those monkeys at various times after antibody administration indicated that triglyceride transfer was inhibited approximately 90% from 24 to 96 h and that it was inhibited to a much larger extent than cholesteryl ester transfer (Fig. 6).

Table II shows the effects of antibody administration on the plasma cholesterol and triglyceride concentrations in those monkeys. Note that there was not a significant effect of the antibody on either total or HDL cholesterol levels; however, there was a clear decrease in total plasma triglyceride concentrations as a result of antibody treatment, and that decrease appeared to be due largely to the decrease in the HDL fraction. In separate studies run in parallel with these, four other monkeys were injected each with 30 mg of CMTP-1, a monoclonal antibody to cynomolgus monkey CETP which had no detectable

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effect on either triglyceride or cholesteryl ester transfer in vitro, and in these studies no effect on total or HDL triglyceride or cholesteryl concentrations was detected. That indicates that the change in HDL triglycerides evident in Table II was due to inhibition of CETP-mediated triglyceride transfer by CMTP-2.

To evaluate more carefully the effects of CMTP-2 administration on the structure of the HDL, three different monkeys were given 30 mg each of the antibody subcutaneously, and a blood sample taken 48 h later for HDL composition analysis. The results of those analyses are contained in Table III and show that treatment with the antibody for 48 h resulted in an HDL that was virtually devoid of triglyceride, but was otherwise unaffected. That was taken as evidence that triglyceride transfer was inhibited well out of proportion to cholesteryl ester transfer, and therefore, that the antibody also uncoupled cholesteryl ester and triglyceride transfer in vivo. It also indicated that essentially all of the triglyceride in the HDL of the cynomolgus monkey was transferred there from other triglyceride-rich lipoproteins (e.g. very low density lipoprotein and chylomicrons) by CETP.

**DISCUSSION**

The studies reported here demonstrate that the CETP-mediated transfer of cholesteryl ester can be inhibited without effect on triglyceride transfer and, conversely, triglyceride transfer can be inhibited without effect on cholesteryl ester transfer. From this we conclude that the two lipids are not transferred by a single, non-discriminatory process. Given that the same protein is responsible for the transfer of both types of neutral lipid, the question becomes, how is that discrimination possible? We would propose, based on currently accepted models of the transfer process (2, 3), that two specific, nonoverlapping binding sites are involved in the transfer of the two classes of lipid.

An underlying assumption here is that both of the inhibitors presented in this paper block transfer by interacting directly with CETP, and not by altering the donor or acceptor. In the case of U-617, we base that deduction on previous studies which showed that U-617 is a competitive inhibitor which probably reacts with an unpaired cysteine in CETP. In the case of CMTP-2, we know that the antibody recognizes an epitope on CETP, since it was obtained by screening hybridomas using a direct binding assay. Finally, if either or both of the current models of the transfer process (2, 3) are correct, then the inhibitors must be interacting directly with the protein, for it would not be possible to selectively inhibit transfer of one or the other lipid by blocking the docking, dissociation, or diffusion steps.

To confirm that the selective inhibition detected using the in vitro assay was, in fact, real, and not an artifact of the assay, we evaluated the effect of the inhibitor of triglyceride transfer in vivo. The reasoning was that if selective inhibition is occurring, it should manifest itself as a selective change in the lipoprotein composition. For example, previous studies (25–30) have shown that inhibition of cholesteryl ester transfer causes an increase in the plasma HDL-cholesterol levels. If, as indicated by the in vitro assay, CMTP-2 at high concentrations inhibits triglyceride transfer but not cholesteryl ester transfer, in vivo administration of the antibody should change the HDL triglyceride content with no effect on the HDL cholesterol levels. That is precisely what occurred: the triglyceride content of HDL was reduced more than 10-fold by antibody treatment while the cholesterol content of HDL remained essentially unchanged. Thus, CETP-mediated cholesteryl ester and triglyceride transfer can be uncoupled in vivo, in full agreement with the two-site hypothesis.

Others (16, 17, 31) have reported that CETP-mediated triglyceride transfer could be selectively inhibited, but it has

**Table III**

Composition of HDL before and after administration of monoclonal antibody CMTP-2 to cynomolgus monkeys.

CMTP-2 (30 mg) was administered subcutaneously to 3 male cynomolgus monkeys and blood samples for HDL isolation collected 48 h later (pretreatment, blood samples were taken 1 week before antibody injection). The mean (±S.D.) Stokes’ radius of the HDL was determined as described previously (22) and was 49.5 ± 0.1 Å. No effect of CMTP-2 treatment on Stokes’ radius was detected. Neither was there any effect of antibody treatment on the mean HDL density (1.123 ± 0.006 g/ml; calculated as described by Schumaker (35) or the mean HDL molecular mass (342 ± 18 kDa; calculated as described by Shen et al. (36)).

| Protein    | Total       | ApoA-I      | Other       | Phospholipid | Cholesterol | Cholesteryl ester | Triglyceride |
|------------|-------------|-------------|-------------|--------------|-------------|------------------|-------------|
| **Pretreatment** |             |             |             |   |    |  | |
| WT %       | 40.4 ± 2.5  | 29.5 ± 3.0  | 10.9 ± 4.3  | 33.6 ± 1.8   | 4.8 ± 0.4   | 18.0 ± 1.5       | 3.2 ± 0.5   |
| Mol/mol HDL| 8.0 ± 1.5   | 3.5 ± 0.4   | 4.5 ± 1.8   | 153 ± 8      | 42 ± 4      | 95 ± 8           | 13.2 ± 2    |
| **Posttreatment** |           |             |             |   |    |  | |
| WT %       | 39.0 ± 0.4  | 30.7 ± 2.2  | 8.3 ± 1.8   | 35.9 ± 1.9   | 4.0 ± 0.1   | 20.8 ± 1.6       | 0.3 ± 0.0   |
| Mol/mol HDL| 7.1 ± 0.5   | 3.7 ± 0.3   | 3.4 ± 0.8   | 164 ± 9      | 35 ± 1      | 109 ± 8          | 1 ± 0       |
| Prob > t (mol/mol HDL) | 0.415 | 0.361 | 0.406 | 0.051 | 0.075 | 0.103 | 0.007 |
Table IV

Comparison of triglyceride and cholesteryl ester transfer activities of LPDP from three species

| Cholesterol ester | Triglyceride | CET/G ratio |
|-------------------|--------------|-------------|
| Hamster           | 0.167        | 0.221       | 0.76       |
| Cynomolgus monkey | 1.180        | 0.237       | 4.98       |
| Human             | 0.593        | 0.079       | 7.51       |

been argued (32) that, because triglycerides have a larger molecular volume (1600 Å³) than cholesteryl esters (1090 Å³), a given inhibitor could partially obstruct access to a single binding site such that the cholesteryl esters could exchange but not the triglycerides. However, in these and previous studies,2 U-617 was found to inhibit transfer of the smaller molecule but not that of the larger. Therefore, that explanation is not adequate in this instance. On the other hand, by postulating the existence of two neutral lipid binding sites, one can invoke the same principle to explain the selectivity of U-617 for cholesteryl esters; i.e. that a second, but smaller, neutral lipid binding site exists on CETP which is accessible to cholesteryl esters, but not triglycerides, and with which U-617 interacts specifically.

If discrete binding sites exist, and each of the inhibitors used here is, in fact, selective for a distinct site, then the binding of one inhibitor should not reduce the effectiveness of the other. Incubation of CETP with CMTP-2 Fab fragments completely blocks triglyceride transfer, but does not interfere with the ability of U-617 to inhibit cholesteryl ester transfer. Thus, the binding sites of the two inhibitors do not overlap; a conclusion in full agreement with the two binding site hypothesis.

Finally, the two-binding site hypothesis is entirely compatible with the observation that CMTP-2 inhibition of cholesteryl ester transfer was reversible beyond a certain antibody concentration, but triglyceride transfer was not. We would propose that the site principally responsible for triglyceride transfer is the epitope recognized by the antibody (i.e. blocked by attachment of the antigen binding region of the antibody) and that the cholesteryl ester binding site is a separate site which does not react with the antibody, but is located such that access to it is hindered by a second CETP molecule bound to the same antibody (Fig. 2A, icon a). Thus, at low antibody concentration (below 16 nM; Fig. 2A), both antigen binding sites are occupied and cholesteryl ester transfer is inhibited; however, as the antibody concentration increases beyond 16 nM and the unoccupied form of the antibody (Fig. 2A, icon b) becomes the predominant species, access to the cholesteryl ester transfer site is restored and inhibition of cholesteryl ester transfer is relieved. The fact that CMTP-2 Fab fragments had no observable effect on cholesteryl ester transfer is taken as support for this explanation of the reversible transfer, and therefore, for the premise that topologically distinct, neutral lipid binding sites exist on cynomolgus monkey CETP.

If CETP does, in fact, contain a distinct binding site for each of the two substrates, then there would be the possibility that genetic drift could alter the protein such that the ratio of the specificities toward the two substrates would be different for different species. Such species to species differences should be less evident in the case of a single, non-discriminatory binding site. Comparing CETP activity from three species and using identical assay conditions for measuring those activities, we do, indeed, find substantial species-dependent differences in the substrate specificities. As shown in Table IV, the ratio of transfer rates, \( V_{\text{CE}} / V_{\text{TG}} \), ranges from 0.76 for hamster CETP to 7.5 for the human CETP. Thus, substrate specificity is an intrinsic property of the protein and that specificity can vary consider-ably among species, suggesting divergent adaptation of the two sites.

There is abundant precedent for the existence of multiple lipid binding sites on a single protein. Probably the best example of this is plasma albumin, which has two major binding domains (designated subdomains IIA and IIA (33)), and possibly multiple lipid binding sites within a subdomain (34). The experiments described here suggest that two or more neutral lipid binding sites may also exist on cynomolgus monkey CETP, but offer no information as to whether these represent distinct structural domains, or separate binding sites within a single domain. They do, however, suggest that the binding sites are dissimilar, since one appears to have a higher affinity for triglycerides and the other a higher affinity for cholesteryl esters.

Morton and Zilversmit (16) have argued against the two-site hypothesis, based largely on the observation that increasing the cholesteryl ester concentration in the donor particle decreased the rate of transfer of triglycerides (Ref. 16, Table II) which they interpreted as indication that the two lipids were competing for a single site. However, since increasing the fraction of cholesteryl esters in the lipid core automatically decreased that of the triglycerides, these observations may simply reflect the fact that the transfer rate of each substrate depends on the concentration of that substrate in the donor, i.e. the triglyceride transfer rate may have decreased, not because of competition for the binding site, but simply because the triglyceride concentration in the donor was reduced. Thus, these data do not prove that these two lipids compete with each other for a single binding site. Nonetheless, we would agree with Morton and Zilversmit (16) that the two lipids are probably mutually exclusive (binding at one site precludes binding at the other), for otherwise it is difficult to explain the “net mass transfer” phenomenon. Mutual exclusivity could occur as a result of conformational changes induced in one site when the other is occupied; or, because of their spatial relationship, CETP may simply be unable to present both sites to the lipid donor simultaneously. In the latter case, one would have to hypothesize that, once either site becomes occupied, CETP dissociates, so that only 1 mol of a given lipid would be transferred at a time, otherwise net mass transfer could not occur.

The single most compelling evidence that two sites exist is the observation that exchange of either lipid can continue, even though transfer of the other is blocked. Several studies (16, 17, 24, 31, 37) have demonstrated that this can occur. Ko et al. (24), for example, produced a monoclonal antibody against rabbit CETP that had an inhibitory pattern which was very similar to that observed with CMTP-2, i.e. at high antibody concentrations triglyceride transfer was completely blocked, but cholesteryl esters continued to exchange. Despite these results, those authors proposed that there was but one lipid binding site, and invoked the steric hindrance hypothesis (discussed above) to explain their observations. However, this seems unlikely given the observations that compounds such as U-617 and PD 140195 (37) can selectively inhibit transfer of the smaller of the two lipids. Ko et al. (24) also observed that, even though cholesteryl ester exchange continued after triglyceride exchange was blocked, net mass transfer of cholesteryl ester appeared to have been blocked. Thus, by blocking one site, one apparently renders CETP incapable of net mass transfer.

Although no direct evidence exists for the one or two lipid binding sites on CETP, many of the observations reported to date are difficult to reconcile with a single binding site, whereas all observations are consistent with the two-site hypothesis. The question of one or two sites is more than academic and leads to the physiologically important question of
whether cholesteryl ester and triglyceride binding and transfer are mutually exclusive, or can a ternary CETP-CE-triglyceride complex be formed? We feel that the mutual exclusion of the two lipids from CETP is probably real but is due to the fact that CETP is unable to present both binding sites simultaneously to the lipid donor.

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