Kinetics and Inhibition of Lipid Exchange Catalyzed by Plasma Cholesteryl Ester Transfer Protein (Lipid Transfer Protein)

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ABSTRACT: The cholesteryl ester transfer protein-catalyzed cholesteryl ester transfer is inhibited by two compounds identified by a large-scale screening of cholesterol backbone-containing molecules. Kinetic analysis shows that U-95,594, an amino steroid, inhibits competitively the cholesteryl ester transfer protein-catalyzed transfer of both cholesteryl esters and triglycerides, as well from high-density lipoproteins as from synthetic microemulsions. In contrast, U-617, an organomercurial derivative of cholesterol, inhibits competitively the transfer of cholesteryl ester from either donor but is without any effect on triglyceride transfer. In addition to the rapid, competitive inhibition of cholesteryl ester transfer, U-617 also slowly and reversibly reacts with cholesteryl ester transfer protein to produce an additional 10-fold decrease in cholesteryl ester transfer activity but, again, without effect on triglyceride transfer.

Cholesteryl ester transfer protein (CETP),[1] or lipid transfer protein (LTP), catalyzes the exchange of cholesteryl esters (CE) and triglycerides (TG) among lipoproteins (Zilversmit *et al.*, 1975; Rye & Barter, 1992). The primary result of CETP action is the exchange of the CE produced by lecithin: cholesterol acyltransferase (LCAT) in high-density lipoproteins (HDL) for TG from TG-rich lipoproteins. Thus, CETP plays a major role in the regulation of HDL-CE's, and this has been documented both in humans (Whitlock *et al.*, 1989) and in rabbits (Koizumi *et al.*, 1985). Because of the probable contribution of CETP to atherogenesis (Pape *et al.*, 1991; Marotti *et al.*, 1992), inhibitors of the cholesteryl ester exchange process are potential therapeutic agents. Ideally, drug candidates with CETP-inhibitory activity should be specific for the protein and competitive with respect to the neutral lipid substrates. In spite of extensive search, very few inhibitors of CETP have been detected to date; antibodies (Hesler *et al.*, 1988), certain cationic detergents (Nishida *et al.*, 1993), and one hydrophobic thiol (Bisgaier *et al.*, 1994) have been shown to inhibit the CETP-mediated transfer process under given experimental conditions. Mere observation of a decrease in the transfer rate, however, does not demonstrate the competitive nature of the inhibition. In fact, inhibition of the CETP-mediated transfer and, in general, that of any transfer protein, poses a challenging kinetic problem. Due to the complexity of the exchange processes, competitive binding at the active site of the protein is only one of the many possible inhibitory pathways; inhibition may also occur at any of the several steps along the exchange pathway. It is then not trivial to analyze the kinetics of inhibition and to delineate from there the inhibitory pathway. The rate equation for symmetrical lipid exchange between identical lipid particles (van den Besselaar *et al.*, 1975)—already invoking several simplifying assumptions—illustrates the complex nature of the process:

$$V_o = \frac{k_1[L_1]k_2[L_2]P_{tot}}{(k_1[L_1] + k_2[L_2])\left(1 + \frac{k_1}{k_{-1}}[L_1] + \frac{k_2}{k_{-2}}[L_2]\right)} \quad (1)$$

In this equation, V_o is the initial rate of transfer of the exchangeable lipid from the donor to the acceptor particles, P_{tot} is the total amount of transfer protein, $[L_1]$ is the concentration of donor lipid particles, $[L_2]$ is the concentration of the acceptor lipid particles, and k_1, k_{-1}, k_2, and k_{-2} are rate constants for the association and dissociation from the donor and acceptor particles. According to this equation, inhibition of the exchange reaction can be achieved either by modification of the surface of the donor and/or acceptor particles or by competition with the binding of CETP to the donor and/or acceptor particles. Modification of the particle surface, by the restructuring of the surface lipids, may either prevent the binding of CETP or increase the binding of CETP to the extent that it will not dissociate from the surface. In assays where the donor and acceptor particles are separated by precipitation, surface modification may also create confusing artifacts by changing the solubility properties of the donors and acceptors. Inhibitors of CETP that would act through modification of the lipoprotein surfaces are undesirable from a therapeutic standpoint since their action is hardly going to be limited to CETP activity alone. Besides the mechanistic problems associated with the search for CETP inhibitors, one also faces the problem of the possible existence of multiple binding sites, namely, those severally specific for HDL, LDL, CE, and TG. Also, the CETP-catalyzed lipid exchange is asymmetric, and the equations

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[1] Abbreviations: CETP, cholesteryl ester transfer protein; LTP, lipid transfer protein; CE, cholesteryl ester; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LCAT, lecithin: cholesterol acyltransferase; LPDP, lipoprotein-deficient plasma.

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governing such a process are rather complex (see the Appendix). Last, but not least, inhibitors of the CETP-mediated lipid transfer are likely to be highly hydrophobic, and thus they should partition between the aqueous phase and the lipid particles. One thus also has to determine whether the active species of the inhibitor is the one in the lipid phase or the one in the aqueous phase.

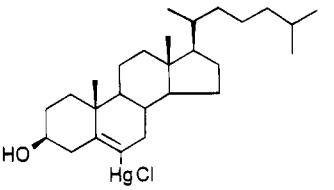
In the present work, we describe the results of our efforts to identify competitive, small-molecule inhibitors of CETP activity. Candidates for competitive CETP inhibitors were identified from the collection of compounds of The Upjohn Co. using a large-scale radioactive assay screening process. Two of the best candidates, synthesized by Upjohn chemists, U-95,594 and U-617, were then studied from the point of view of the mechanism of their inhibitory action. We have utilized assays for measuring both inhibition of the transfer reaction and for identifying agents that are other than competitive. A continuous recording fluorescence assay (Epps *et al.*, 1995) was used to monitor lipid transfer mediated by CETP from cynomolgus monkeys (Sarcich *et al.*, 1995). Kinetic equations were derived for analyzing the donor and acceptor dependencies of lipid transfer. Simultaneously, LDL precipitation and LCAT activity were also measured in order to identify nonspecific lipid surface modifiers. By the use of these assays, we were able to ascertain the competitive nature of the two inhibitors of CETP, one of which is active at nanomolar concentrations. The procedures which we describe are general and conducive to the identification of competitive inhibitors of CETP and other transfer proteins.

MATERIALS AND METHODS

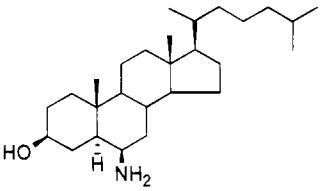
Powdered hen egg L-α-phosphatidylcholine was purchased from Avanti Polar Lipids. Triolein, 1,3-diolein, and oleic acid were from Sigma Chemical Co. Hexabromotriolein and Bodipy-TG were synthesized as described previously (Epps *et al.*, 1995). [*cholesteryl-4-*^{14}*C*]Cholesteryl oleate, 45−60 mCi/mmol, and triolein, [9,10-^{3}H(N)]-[$CH_3(CH_2)_7CH{=}CH(CH_2)_7CO]_3O_3C_3H_5$, 10−30 Ci/mmol, were purchased from New England Nuclear. Cholesteryl 4,4-difluoro-4-bora-3a,-4a-diaza-*s*-indacenyl-3-dodecanoate (Bodipy-cholesteryl ester) and 5-butyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacenyl-3-nonanoic acid (C_4-Bodipy), were obtained from Molecular Probes as solids and dissolved in chloroform to make stock solutions. The inhibitors U-617 and U-95,594, whose structures are shown in Figure 1, were synthesized and purified by chemists at The Upjohn Co. and obtained as solids from the Biological Screening Office. Water was purified with a Corning Mega-Pure all-glass distillation apparatus, and all solvents used were HPLC grade from Burdick and Jackson. The buffer used for all experiments was 10 mM phosphate, 150 mM NaCl, 0.1 mM EDTA, and 0.05% sodium azide, pH 7.4.

Recombinant cynomolgus monkey CETP (Sarcich *et al.*, 1995) was provided by Jean L. Sarcich and Alfredo Tomasselli. CETP from human and monkey plasma was obtained as described (Pape *et al.*, 1991).

CETP activity was measured both by a fluorescence assay (Epps *et al.*, 1995) and by a radioactivity assay (Pape *et al.*, 1991). Inhibitors were added to the reaction mixtures from stock concentrated DMSO solutions. The maximum final DMSO concentration in the reaction mixture was 0.2% (v/v); it had no measurable effect on the rate of CETP-catalyzed



U-617



U-95594

FIGURE 1: Chemical structures of CETP inhibitors.

lipid transfer. The inhibitors used in this study are strongly amphiphilic, containing strong hydrophobic and hydrophilic groups. It is likely that they locate preferentially in the surface monolayer of the emulsion particles. For this reason, the concentration of the inhibitor is expressed in terms of mol %, with respect to the total phospholipid of both donor and acceptor.

For the analysis of inhibition using the radioactivity assay data, we expressed the residual activity in the presence of inhibitor as the fraction, F, of substrate transferred in the presence of inhibitor, P_i, with respect to that in the absence of inhibitor, P_u. This ratio was routinely measured under conditions where the redistribution of radioactivity of the uninhibited reaction was *ca.* 75% of the equilibrium value. Since the reaction can be approximated as a first-order process, the quantity of label transferred, P, is given by:

$$P = P_\infty(1 - e^{-kE_o t}) \tag{2}$$

where P_∞ is the quantity transferred at equilibrium, k is the second-order rate constant of the CETP-catalyzed transfer, and E_o is the concentration of active, uninhibited CETP. Then, if the inhibition is competitive,

$$E_o = \frac{E_{tot}}{1 + \frac{I}{K_i}} \tag{3}$$

where E_{tot} is the analytical CETP concentration, I is the inhibitor concentration, and K_i is the dissociation constant of the CETP−inhibitor complex. Substitution into eq 2 gives

$$P_i = P_\infty\left(1 - e^{\frac{-kE_{tot}}{1+\frac{I}{K_i}}t}\right) \tag{4}$$

When a single time point is measured, then t = constant and, with P_u maintained constant, the ratio, F, becomes

$$F = \frac{P_\infty}{P_u}\left(1 - e^{-\frac{\text{constant}}{1+\frac{I}{K_i}}}\right) \tag{5}$$

The results of the inhibition experiments were then analyzed in terms of this equation, using a nonlinear least squares method.

The radiolabeled HDL for the LCAT assay was prepared by adding 50 μCi of [4-^{14}C]cholesterol solubilized in lecithin−taurocholate micelles to 30 mL of plasma from which lipoproteins other than HDL had been removed by centrifugation. Before the addition of the radiolabeled cholesterol, the LDL-free plasma was first dialyzed extensively against PBS which also contained 5,5′-dithiobis(2-nitrobenzoic acid), DTNB (0.4 g/L). The dialysis removed the salt used for ultracentrifugation, and DTNB destroyed any endogenous LCAT activity. After addition of the lecithin−taurocholate−[4-^{14}C]cholesterol micelles, the plasma was dialyzed against 1 L of DTNB-containing PBS kept at 37 °C for 18 h. Thereafter, the mixture was dialyzed at 4 °C for 48 h against 3 × 1 L of buffer each. The HDL was then isolated by ultracentrifugation, dialyzed against PBS, and stored at 4 °C. The final solution contained *ca.* 8000 dpm of [4-^{14}C]cholesterol/μL; more than 97% of this migrated with free cholesterol on TLC.

The LCAT assay mixture consisted of 50 mM Tris-HCl buffer, pH 7.40, 0.15 M NaCl, 1% (w/v) BSA, 0.02% (w/v) NaN_3, 20 μL of [4-^{14}C]cholesterol-labeled HDL, 100 μL of fresh cynomolgus monkey plasma, and 7 μL of the CETP inhibitors dissolved in DMSO, to yield a final volume of 700 μL. The assay mixture was incubated at 37 °C for 90 min, and then the reaction was stopped by injecting the entire mixture into 2 mL of ethanol. Four milliliters of hexane was added and the mixture vigorously shaken for 1 min. The mixture was then centrifuged at 1500 rpm (300*g*) for 15 min. Three milliliters of the hexane phase was blown to dryness under N_2.

The lipids were solubilized in 250 μL of chloroform, applied to a TLC plate, and developed by petroleum ether: ethyl acetate (80:20, v/v). The radioactivity was visualized and quantitated using the Molecular Dynamics PhosphorImager instrument. Only two radioactive bands were evident, one comigrating with free cholesterol and the other with cholesteryl esters. The radioactivity in the ester band was expressed as a fraction of the total radioactivity on the plate. Because fresh plasma was used daily, often taken from different monkeys with varying levels of LCAT in their plasma, it was necessary to normalize the data for day-to-day or monkey-to-monkey comparisons. Control assays, with no inhibitor present, were run in duplicate with each set of assays, and the degree of inhibition produced by a given compound was measured as a fraction of the control velocity.

RESULTS

Kinetics of CETP-Catalyzed Bodipy Lipid Transfer. The time-dependent transfer of Bodipy-TG or Bodipy-CE from donor to acceptor microemulsions was measured by monitoring the accompanying increase in Bodipy fluorescence. As shown in Figure 3, addition of CETP to the donor/acceptor mixture produced a rapid increase in fluorescence which reached a constant value after a period of time. The data were analyzed in terms of a first-order reaction using a nonlinear least squares-fitting program (Yamaoka *et al.*, 1981). The agreement of the experimental data points with the theoretical curves and the randomness of the residuals throughout the time course of the reaction indicate that the

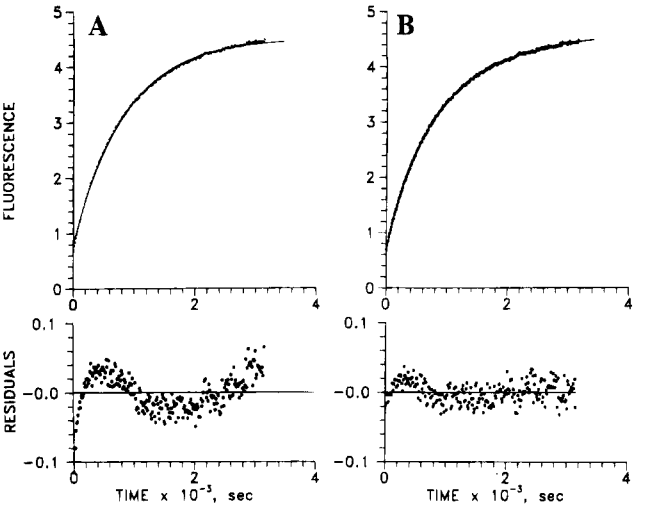


FIGURE 2: Kinetics of CETP-catalyzed transfer of labeled TG. (A) The time dependence of TG transfer as analyzed according to a first-order rate model. (B) Analysis of the same data according to eq 19 in the Appendix. The solid lines represent the theoretical fits to the experimental data points. The reaction mixture contained 4.5 μg of recombinant cynomolgus monkey CETP, TG donor particles containing 5 μg of phospholipid, and TG acceptor particles containing 30 μg of phospholipid, in a final volume of 2 mL of assay buffer. All procedures were as described in Materials and Methods.

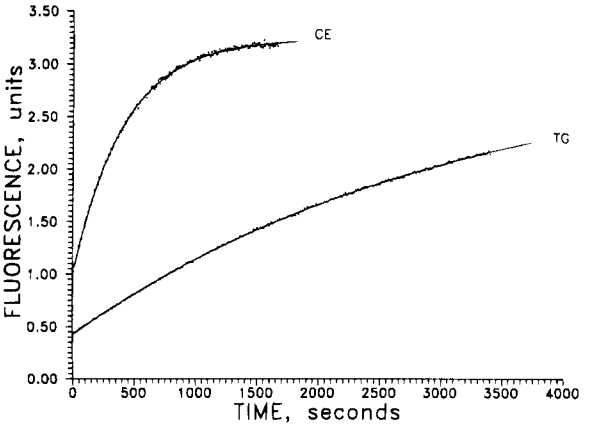


FIGURE 3: Kinetics of CETP-mediated transfer of CE and TG in the fluorescence assay. Lipid transfer (CE and TG) mediated by CETP was monitored by observing the time-dependent increase in Bodipy fluorescence after transfer from donor, 5 μg of phospholipid, to acceptor, 30 μg of phospholipid, particles in a final volume of 2 mL of assay buffer. The transfer reaction was initiated by the addition of 0.75 μg (5 nM) of recombinant monkey CETP, and the fluorescence was measured with excitation at 490 nm and emission at 530 nm as described in Materials and Methods. The solid lines represent the theoretical fits to the experimental data points using a first-order rate model.

transfer can be approximated by a first-order reaction model. Under conditions of identical donor, acceptor, and CETP concentrations, the pseudo-first-order rate constant for transfer of Bodipy-CE was 2.45 ± 0.01 × 10^{-3} s^{-1}, significantly larger than that for transfer of Bodipy-TG, 0.32 ± 0.02 × 10^{-3} s^{-1}. As reported previously (Epps *et al.*, 1995), with either of the Bodipy donors, the initial rates of transfer and also the experimental pseudo-first-order rate constants were linear with CETP concentration over at least the range of 0.125−1 μg/mL (data not shown).

In the analysis of the kinetics of triglyceride transfer according to a first-order reaction scheme, a slight but

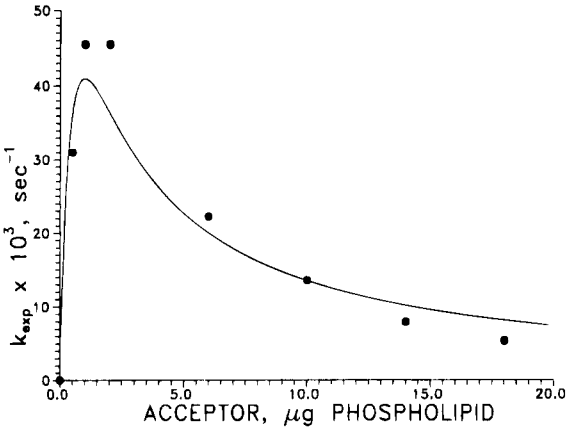


FIGURE 4: Kinetics of CE transfer as a function of acceptor concentration. The kinetics of fluorescent CE transfer were measured as a function of acceptor particle concentration at constant donor concentration (0.5 μg of phospholipid). The experimental rate constants were calculated using a pure first-order rate model and then analyzed using eq 20 in the Appendix. The solid line represents the theoretical fit to the experimental data. The reaction was initiated by the addition of 0.75 μg of recombinant cynomolgus monkey CETP.

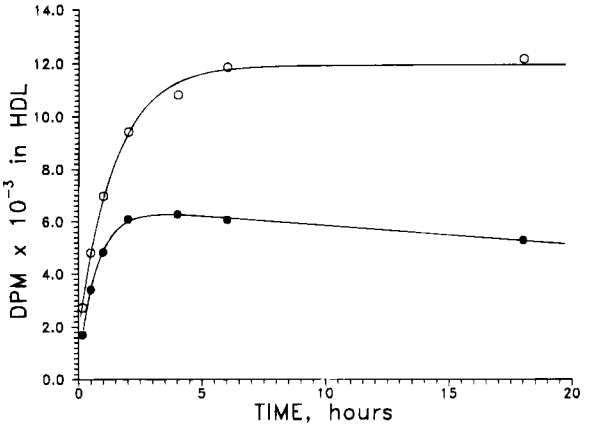


FIGURE 5: Kinetics of lipid transfer in the radioactivity assay. The CETP-mediated transfer of radioactive CE and TG was monitored as described in Materials and Methods using cynomologous monkey lipoprotein-deficient plasma as a source of CETP. The solid lines represent the theoretical fits to a first-order rate model: (●) CE and (○), TG.

systematic deviation was always present. For this reason, the kinetics of exchange were also analyzed in terms of the reaction scheme proposed in eq 9 of the Appendix. This scheme leads to eq 19 which relates the fluorescence of the reaction mixture to time. The experimental data were analyzed using this equation and a nonlinear least squares-fitting program. Provided that the experimental noise is truly random, the inversion of the dependent and independent variables should not lead to significant changes in the best-fit parameters. The experimental data were invariably fully consistent with eq 19. We illustrate these data analyses in Figure 2 for the case of exchanging labeled for unlabeled TG. Analyzing the same data sets in terms of a simple, first-order equation yielded a fit which was statistically inferior to that obtained by eq 19. This analysis and residuals are shown in panel B of Figure 2. Nevertheless, the first-order rate constants calculated from the two schemes were virtually identical, provided that the first 10% of the reaction was excluded from the simple, first-order analysis (data not shown). Consequently, unless indicated otherwise, the reactions presented in this paper were analyzed by the simple, first-order reaction model. In our experience, the deviations from first-order kinetics were always more apparent for TG transfer, presumably because the unlabeled TG transferred into the donor competes better against labeled TG than against labeled CE.

The dependence of k_{exp} on the acceptor concentration was measured under conditions where the donor and the CETP concentrations remained constant. As shown in Figure 4, the experimental results did show a maximum, as expected from eq 20 of the Appendix. When analyzed by nonlinear least squares fit, the data were found to be consistent with this equation, although the experimental scatter was rather large. The results show, nevertheless, that, because of the strong binding of CETP to the acceptor particles, the descending limb of this curve is the only portion accessible to our experimental method. Similarly, the donor concentration dependency displays a clear descending limb, while the ascending portion is not readily accessible to our experimental method (data not shown). One may nevertheless conclude that, at least within our experimental range, the kinetics are consistent with the scheme proposed in the Appendix.

Kinetics of Radioactive Lipid Transfer. We also evaluated the kinetics of radioactive lipid transfer from serum LDL to HDL, catalyzed by CETP from lipoprotein-deficient plasma of cynomolgus monkey. The time-dependent increase of radioactivity in HDL produced by the addition of CETP was measured as described in Materials and Methods. The results of one such experiment are shown in Figure 5; they were analyzed by nonlinear least squares using a first-order reaction model and a slight linear drift. The data were consistent with that model, as evidenced by the agreement of the theoretical curves with the experimental data points. We calculate pseudo-first-order rate constants of 1.32 ± 0.06 and 0.69 ± 0.06 h^{-1} for CE and TG transfer, respectively. The ratio of these rate constants is much closer to 1 than that of the rate constants measured with synthetic donor and acceptor particles, reflecting perhaps the chemical potential differences between lipid particles of different origin and composition. The slight linear drift observed after completion of the transfer is probably due to a slow remodeling of the acceptor particles which changes their precipitation properties.

Kinetics of Inhibition by U-95,594. A competitive inhibitor that blocks the active site of CETP decreases the concentration of CETP available for transfer activity. Thus, transfer in the presence of a competitive inhibitor should still obey first-order kinetics, but the rate constant should be smaller than that of the uninhibited reaction. We have measured the action of the inhibitors under acceptor concentrations where most of the CETP is bound to the donor/acceptor particles, *i.e.*, in the descending limb of the acceptor dependency curve. Under these conditions, the binding of a *competitive* inhibitor to CETP is mitigated by the binding of CETP to the donor and acceptor surfaces. Consequently, the rate constant decreases with increasing inhibitor concentration, but the apparent K_i calculated from the data is higher than the true K_i. On the other hand, inhibition that occurs by blocking the donor and/or acceptor surfaces would reveal itself as an increase in the rate constant, since a decrease in the available surface is formally equivalent to a decrease in the acceptor concentration.

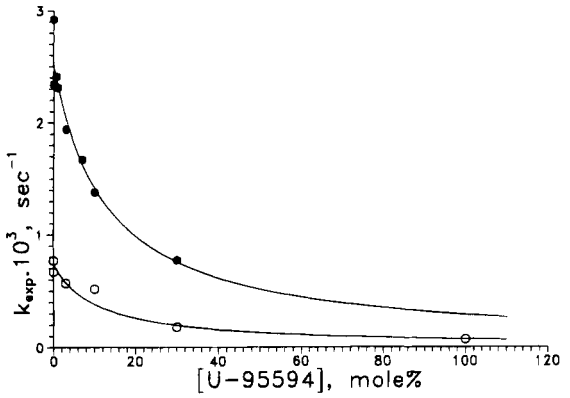


FIGURE 6: Inhibition of lipid transfer by U-95,594. Inhibition of CE and TG transfers was evaluated as described in Materials and Methods. The reaction mixtures contained Bodipy-CE or -TG donors (5 μg of phospholipid), acceptors (30 μg of phospholipid), and a fixed amount of inhibitor added in 4.6 μL of DMSO. Lipid transfer was initiated by the addition of 0.32 μg of recombinant cynomologous monkey CETP. The first-order rate constants for CE transfer (●) were calculated as described in Materials and Methods, while those of TG transfer (○) were calculated using eq 19 of the Appendix. The inhibition of either transfer was analyzed in terms of a simple, competitive, hyperbolic model. The solid lines represent the theoretical fits to the experimental data points.

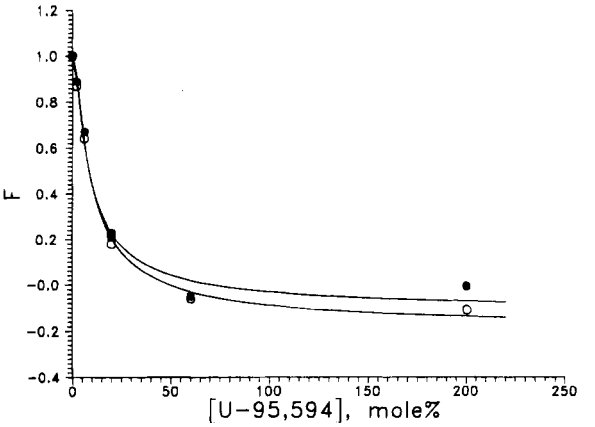


FIGURE 7: Inhibition of lipid transfer by U-95,594 in the radioactivity assay. The inhibition of radioactive lipid transfer by U-95,594 was assayed as described in Materials and Methods. The ratio of transfers with and without inhibitor, F, was analyzed as a function of inhibitor concentration using eq 5: (●) CE and (○) TG.

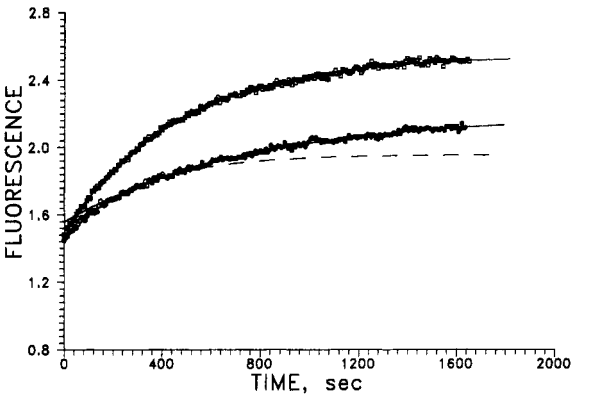


FIGURE 8: Kinetics of inhibition of CE transfer by U-617. The full time courses of the uninhibited and inhibited (0.3 mol % U-617 with respect to phospholipid added in 4.6 μL of DMSO) fluorescence CE transfer reactions were measured using CE donors (5 μg of phospholipid) and acceptors (30 μg of phospholipid) and 0.32 μg of recombinant cynomolgus monkey CETP in a final volume of 2 mL of assay buffer. The progress of the reaction was followed as described in Materials and Methods. The uninhibited reaction (□) was analyzed using a first-order rate model; the inhibited reaction was analyzed in two portions, (○) 0−400 s and (●) >400 s, using first-order rate models. The solid lines represent the theoretical fits to the data.

As shown in Figure 6, addition of increasing concentrations of U-95,594 resulted in a progressive decrease in the first-order rate constants of CE transfer. The reaction of CE transfer remained first-order at all inhibitor concentrations, and the total mass of lipid transferred at the end of the reaction remained constant, even at the highest concentration of U-95,594 (data not shown). Also, there was no change in the fluorescence background, indicating that the inhibitor did not change the organization of the lipid surface of the donor emulsions which would have affected the self-quenching of the Bodipy lipid. The data on the inhibition of CE transfer by U-95,594 were analyzed in terms of a simple, competitive, hyperbolic inhibition, using a nonlinear least squares procedure. For example, analysis of the data in Figure 6 yielded an apparent K_i of 12.9 ± 2.1 mol % with respect to phospholipid (2.7 ± 0.5 μM); the consistency of the results with this model is shown by the agreement between the experimental data points and the theoretical curve. The kinetics of TG transfer in the presence of U-95,954 were analyzed using eq 19; the apparent first-order rate constants obtained from this analysis showed the same type of decrease as the one observed for CE transfer. As shown in Figure 6, these data also are consistent with a competitive inhibition scheme, with K_i = 10.8 ± 1.3 mol % with respect to phospholipid (2.3 ± 0.3 μM).

We then evaluated U-95,594 as an inhibitor of the transfer of radioactive CE and TG from LDL to HDL in the radioactivity assay. The data in Figure 7 are consistent with eq 5, as evidenced by the agreement between the experimental data points and the theoretical curves. We calculate $K_i \approx$ 1.7 mol % with respect to phospholipid (≈1.2 μM) for inhibition of CE transfer. U-95,594 also readily inhibited the transfer of radioactive TG in our serum-based system. These results are also consistent with eq 5, and we calculate $K_i \approx$ 1.0 mol % with respect to phospholipid (≈0.7 μM). Due to the rather large experimental noise, the K_i values for the two substrates transferred are not significantly different.

Kinetics of Inhibition by U-617. The mercurial cholesterol derivative U-617, identified by screening, was tested for its ability to inhibit the various CETP-mediated lipid transfers. We first examined the full time course of CE transfer in the presence and absence of 0.3 mol % U-617 with respect to phospholipid. As shown in Figure 8, the time-dependent increase in fluorescence for the uninhibited reaction is first-order, whereas that of the inhibited reaction is biphasic, with the apparent first-order rate constant decreasing as the reaction progresses. The time course then was analyzed according to the reaction scheme in eq 21, using eq 34 and a nonlinear least squares algorithm. Experiments at all inhibitor concentrations were found to deviate significantly from a simple first-order model but remained fully consistent with eq 34. From the experiments with varying inhibitor concentrations were obtained the rate constants k_o, k_i, and k_∞. Somewhat unexpectedly, k_o decreased with increasing I, and as shown in Figure 9, the decrease was consistent with pure competitive inhibition at $t = 0$, with $K_{i,o}$ = 0.56 ± 0.12 mol % with respect to phospholipid. The dependence of k_i on I was also unexpected; instead of the expected positive intercept and linear increase (eq 29), we found a saturable

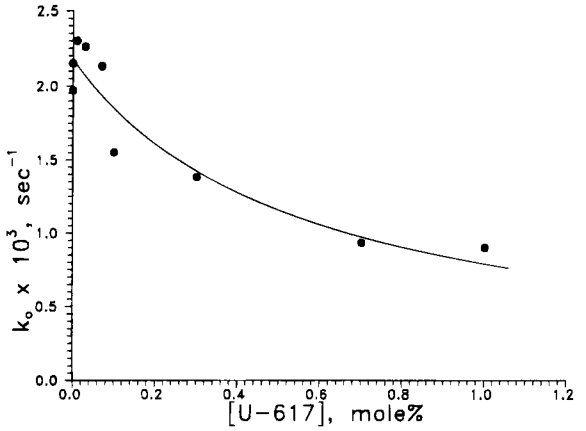


FIGURE 9: Analysis of the kinetics of inhibition by U-617 of CE transfer and dependence of k_o on inhibitor concentration. The time dependencies of fluorescent CE transfer in the presence of various concentrations of U-617, assayed as described for Figure 8, were analyzed using eq 34 in the Appendix. The values of k_o for each inhibitor concentration were then analyzed using a pure competitive inhibition model yielding the constant $K_{i,o}$ as discussed in the text. The solid lines represent the theoretical fit to the experimental data.

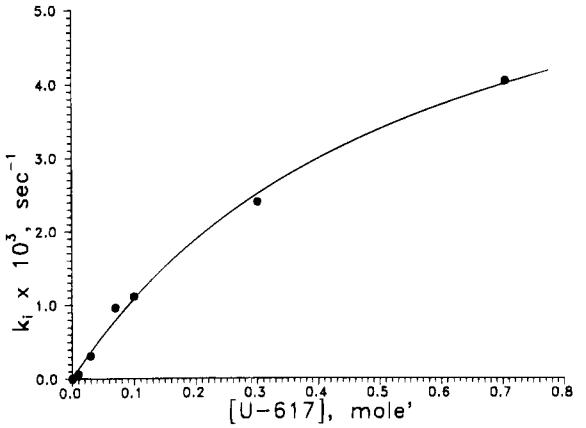


FIGURE 10: Dependence of k_i on inhibitor concentration. Individual k_i values were calculated as described for Figure 9 and then analyzed as a function of inhibitor concentration using an obligatory binding reaction scheme (eq 6) to yield the dissociation constant for the obligatory complex. The solid line represents the theoretical fit to the experimental data.

increase and an unmeasurably low intercept. As shown in Figure 10, the data are consistent with a reaction scheme of obligatory rapid binding followed by a slow reaction. Most importantly, the dissociation constant of the obligatory complex, calculated from these data, 0.57 ± 0.07 mol % with respect to phospholipid, is identical with the $K_{i,o}$ calculated from k_o. Finally, the dependence of k_∞ on I also indicates a reversible, competitive behavior (Figure 11), and analysis of the data yields $K_{i,\infty} = 0.040 \pm 0.010$ mol % with respect to phospholipid. It may be noted that the experimental points at very low inhibitor concentration are rather inaccurate since inhibition is marginal under these conditions. In striking contrast with the above results, the addition of U-617 was without any observable effect on the rate of Bodipy-TG transfer (data not shown).

The above results show that the inhibition of the CETP-catalyzed CE transport is a reversible two-stage process, formally identical with the one described for inhibition of renin by slow-binding peptides (Kati *et al.*, 1987), according to the scheme:

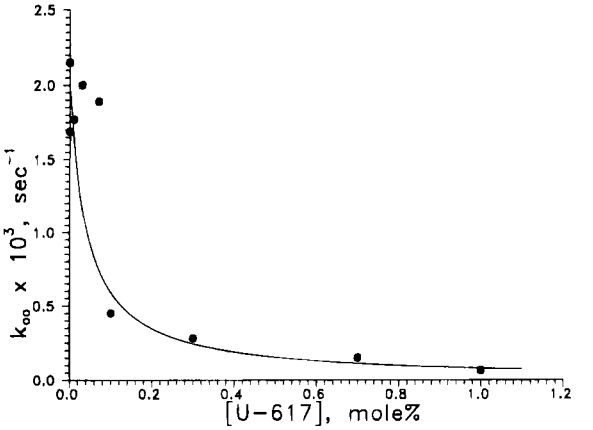


FIGURE 11: Dependence of k_∞ on inhibitor concentration. Individual k_∞ values were calculated as described for Figure 9 and then analyzed as a function of inhibitor concentration using a competitive inhibition model, yielding $K_{i,\infty}$, according to eq 7. The solid line represents the theoretical fit to the experimental data.

$$E + I \overset{K_{i,o}}{\rightleftharpoons} EI_1 \overset{k_r}{\underset{k_f}{\rightleftharpoons}} EI_2 \qquad (6)$$

Thus,

$$K_{i,\infty} = K_{i,o}\frac{k_r}{k_f} \qquad (7)$$

The serum-based assay measures transfer after a time period with respect to which the rate of the "slow" inhibition is rapid. Thus, this assay only measures $K_{i,\infty}$. We found that U-617 does indeed inhibit CE transfer as measured by this assay, and the inhibition appears to be noncompetitive, as shown in Figure 12. The apparent inhibition constant is 1.49 ± 0.36 mol % with respect to phospholipid and the CETP/inhibitor complex, EI_2, appears to be able to catalyze the CE transfer at 14% of the rate of the free enzyme. As was the case with the fluorescence assay, we did not observe any inhibition of TG transfer; rather, we found this transfer to be stimulated by as much as 32% by U-617.

HDL Precipitation and LCAT Inhibition. Since the serum-based assay entails selective lipoprotein precipitation, a spurious inhibition may be obtained if the compound alters the precipitation behavior of the lipoproteins. For this reason, we measured whether U-95,594 or U-617 changes the precipitation behavior of HDL. By our assay, U-95,594 at 100 μM induced a 20% precipitation of HDL and a 2% precipitation at 50 μM. In contrast, U-617 at 2 μM—a concentration far above its K_i—was without effect. Finally, in order to reveal eventual modification of the lipoprotein surface, we also probed the ability of U-617 and U-95,594 to inhibit LCAT. We obtained an $F = 0.05$ for 100 μM U-617 and $F = 1.32$ for 100 μM U-95,594. In other words, the latter compound stimulates LCAT activity.

DISCUSSION

The results presented in this paper support the traditional view that CETP-catalyzed transfer of both CE and TG occurs by a dissociation/diffusion/adsorption/exchange mechanism. The protein has been proposed to be a genuine exchange protein: The lipid transfer appears to be an obligatory exchange, be it between CE and TG or other lipids. In other words, the lipid ligands should compete for the same general

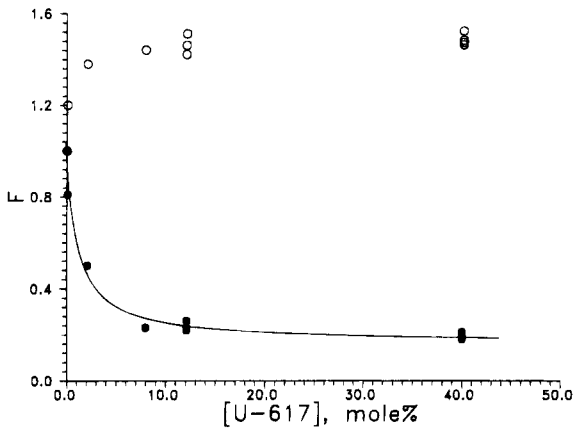


FIGURE 12: Effect of U-617 on CETP-mediated CE and TG transfers as measured by the radioactivity assay. CE and TG transfers were measured by the radioactivity assay as described in Materials and Methods. The data on CE transfer were analyzed using the simple, hyperbolic inhibition model, and the solid line represents the theoretical fit to this model: (●) CE transfer and (○) TG transfer.

Table 1: Nanomoles/Assay of Lipid Associated with LDL and HDL Used in the CETP Radioactivity Assay

lipoprotein	free cholesterol	cholesteryl ester	triglyceride	phospholipid
LDL	7.94	19.68	4.92	16.64
HDL	8.08	18.30	3.30	32.11

Table 2: Dissociation Constants of CETP Complexed with Competitive Inhibitors

	K_i, μM				
				U-617	
	U-95,594			CE	
assay	TG	CE	TG	$t = 0$	$t = \infty$
fluorescent	2.7 ± 0.5	2.3 ± 0.3	∞	0.12 ± 0.03	0.0083 ± 0.0002
radioactive	≈1.2	≈0.7	∞	na	1.05

active site, or if bound to topologically distinct regions, they should, at least, be mutually exclusive. In the latter case, either the two regions partially overlap or an allosteric interaction between the two is operative. The inhibitions observed in this work, summarized in Table 2, shed some light on this problem: The mere fact that U-617 fully inhibits the exchange of labeled CE for TG but not that of labeled TG for the same TG indicates that the binding regions for the two lipids are topologically distinct and that U-617 overlaps only the binding region of CE but not that of TG. In contrast, the inhibition by U-95,594 has all the hallmarks of binding to a single site which is the same for both CE and TG transfer. Thus, the active site-bound U-95,594 overlaps the binding regions for both lipids, or U-95,594 binds to the lipoprotein recognition site which is distinct from the substrate binding sites.

Such a considerable difference in binding locations produced by a seemingly minimal structural change points to a special and specific interaction of the organomercurial function of U-617 with the enzyme. The most likely candidate for such an interaction is a free sulfhydryl group on the protein. In support of this proposal is the fact that all known CETP's contain an odd number of Cys residues and, thus, should contain at least one free SH group. Also, it has been reported that three organomercurials—in the 0.2—2 mM concentration range—inhibited selectively the TG transfer catalyzed by human CETP but were without much effect on the transfer of CE (Morton & Zilversmit, 1982). However, in that same system, neither CE transfer nor TG transfer was inhibited by the sulfhydryl-specific reagent DTNB. The lack of reactivity toward DTNB, the slow formation of the mercaptide, and its easy reversibility could all be due to low accessibility of the sulfhydryl group and a microenvironment unfavorable to the presence of polar compounds.

It appears that CETP has a much higher intrinsic affinity toward CE than TG (Fukasawa *et al.*, 1992; Ko *et al.*, 1994; Morton & Zilversmit, 1983; Morton & Zilversmit, 1982; Morton & Steinbrunner, 1990; Ohnishi *et al.*, 1994). This difference, however, would be unable to account for the observed selected inhibitions since selective inhibition of TG transfer can also be easily achieved without any inhibition of CE transfer (Swenson *et al.*, 1989).

The kinetics of CETP transfer point out the difficulties of identifying the mode of action of compounds that decrease the rate of the CETP-catalyzed reaction. Besides the large number of possible experimental artifacts, true inhibition may be achieved not only by binding to the substrate recognition sites but also by binding to the lipoproteins themselves. When using large acceptor concentrations above their respective K_m's, surface blocking may be distinguished by an expected increase in the rate. Also, changing the organization of the lipid surface should manifest itself by the change in background fluorescence. On the other hand, distinguishing between substrate recognition sites is not possible by these simple kinetic experiments.

The experiments reported in this paper have been designed to be conducive to the simplest data analysis, and yet, they reveal an unusually intricate reaction pathway where one is at a loss even to identify the rate-limiting step of the reaction. It is thus difficult to draw precise mechanistic conclusions from the mere kinetic analysis. The results certainly highlight the complexity of the catalysis of asymmetric exchange and the lack of information concerning the behavior of the system under varying experimental conditions, such as initial concentrations of CE and TG in the donor and acceptor particles, the precise role of apoproteins, and the direct binding of inhibitors to CETP. With all these shortcomings, these results demonstrate, nevertheless, that competitive inhibition of CETP by small molecules can be achieved, but the mode of action of the inhibitor must be defined by elaborate experimentation.

ACKNOWLEDGMENT

We would like to thank Dr. Shinji Yokoyama of the University of Alberta for many helpful discussions.

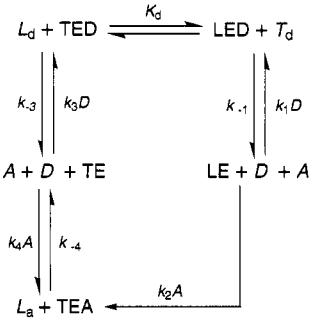
APPENDIX

Kinetics of CETP-Catalyzed CE ÷ TG Exchange. For the following derivation, we posit that (1) the exchange follows a desorption−diffusion−adsorption−exchange mechanism; (2) the concentration of the acceptor particle, A, is much greater than that of the donor, D; (3) the transfer of CE is irreversible under our experimental conditions; (4) CETP only exists as a lipid/protein complex; (5) the exchange of the lipid bound to the adsorbed CETP is instantaneous on the time scale of the experiment; (6) the concentration of

the TG in the acceptor particle, T_a, remains constant throughout the reaction; and (7) the increase in fluorescence is directly proportional to the concentration of the CE in the acceptor particle, L_a, and, thus, the fluorescence of the reaction mixture, f, is given by:

$$f = f_o + \epsilon L_a = f_o + \frac{\epsilon}{R}(L_{d,o} - L_d) = f_\infty - \delta L_d \quad (8)$$

where f_o is the background fluorescence of the donor particle, f_∞ is the fluorescence at the end of the reaction, L_d is the concentration of CE in the donor particle, $L_{d,o}$ the concentration of CE in the donor at the beginning of the experiment, ϵ is the molar emissivity of L_a, R is the ratio of the volumes of the acceptor and donor, and $\delta = \epsilon/R$. Using the symbols T_d for the concentration of TG in the donor particle, LE for the free CETP/CE complex, TE for the free CETP/TG complex, TED for the CETP/TG complex bound to the donor particle, LED for the CETP/CE complex bound to the donor particle, and TEA for the CETP/TG complex bound to the acceptor particle, the reaction pathway is as follows:



(9)

The exchange equilibrium constant within the donor particles, K_d, is defined as:

$$K_d = \frac{\text{LED}\cdot T_d}{\text{TED}\cdot L_d} \quad (10)$$

Using standard kinetic variable elimination methods (Chou & Forsen, 1981; Chou, 1990), one obtains the following rate equation for the decrease of L_d:

$$-\frac{dL_d}{dt} = \frac{\alpha}{\beta + \gamma\dfrac{T_d}{L_d}} \quad (11)$$

where

$$\alpha = k_2k_3E_o\cdot A\cdot D \quad (12)$$

$$\beta = k_3D\left(1 + \frac{k_2A}{k_{-4}}\right) + \frac{k_3D}{k_{-1}}(k_1D + k_2A) + k_2\left(1 + \frac{k_4A}{k_{-4}}\right) \quad (13)$$

and

$$\gamma = \frac{k_1D + k_2A}{K_dk_{-1}}\left(k_3D + k_{-3}\left(1 + \frac{k_4A}{k_{-4}}\right)\right) \quad (14)$$

Since the TG in the donor arises exclusively from exchange for CE in the donor,

$$T_d = L_{d,o} - L_d \quad (15)$$

Substituting for T_d in eq 11, we obtain

$$-\frac{dL_d}{dt} = \frac{\alpha L_d}{(\beta - \gamma)L_d + \gamma L_{d,o}} \quad (16)$$

Separation of the variables yields

$$(\beta - \gamma)\int_{L_{d,o}}^{L_d} dL_d + \gamma L_{d,o}\int_{L_{d,o}}^{L_d}\frac{dL_d}{L_d} = -\alpha\int_0^t dt \quad (17)$$

The integrated form of this equation:

$$-\alpha t = (\beta - \gamma)(L_d - L_{d,o}) + \gamma L_{d,o}\ln\frac{L_d}{L_{d,o}} \quad (18)$$

is transcendental in L_d but gives an explicit solution for t. Substituting $L_d = (f_\infty - f)/\delta$ and $L_{d,o} = (f_\infty - f_o)/\delta$ from eq 8 into eq 18 then gives

$$t = \frac{\beta - \gamma}{\alpha\delta}(f - f_o) + \frac{\gamma}{\alpha\delta}(f_\infty - f_o)\ln\frac{f_\infty - f_o}{f_\infty - f} \quad (19)$$

Thus, the fluorescence as a function of time is described by a first-order term—the logarithmic portion—and a linear term. The experimental data may then be analyzed using eq 19 and a nonlinear least squares-fitting program. The analysis yields—besides f_∞ and f_o—two parameters, namely, the coefficients of the linear and the logarithmic terms. The meaning of the inverse of the latter, equivalent to the first-order rate constant, k_{exp}, becomes apparent after the appropriate substitutions:

$$k_{exp} = \frac{K_d\ k_3}{L_{d,o}k_{-3}}\frac{k_{-1}k_2E_o\cdot D\cdot A}{(k_1D + k_2A)\left(1 + \dfrac{k_3}{k_{-3}}D + \dfrac{k_4}{k_{-4}}A\right)} \quad (20)$$

Thus, the approximate first-order rate constant has the same formal dependence on A and D as found in the symmetrical transfer, cf. eq 1.

Time Course of CE Transfer with Slow, Reversible Inhibition of CETP. For the slow inhibition of CETP, E, by an inhibitor, I, we posit that $I_o \gg E_o$ and that the inhibitor slowly forms an inactive, reversible complex, C, with the protein, according to the reaction scheme:

$$E + I \underset{k_r}{\overset{k_f}{\rightleftharpoons}} C \quad (21)$$

where k_f is the second-order rate constant of the formation of the inhibited complex and k_r is the first-order rate constant of its dissociation. We also assume that the donor and acceptor emulsions do not interfere with either of these reactions. The conservation of mass is expressed as:

$$E_o = E + C = E_\infty + C_\infty \quad (22)$$

where E_∞ and C_∞ are concentrations after equilibrium is reached. The rate of change in uninhibited, active CETP concentration is given by:

$$\frac{dE}{dt} = -k_fI_oE + k_rC = k_rE_o - (k_fI_o + k_r)E_\infty \quad (23)$$

At equilibrium, $dE/dt = 0$, and therefore:

$$k_r E_o = (k_f I_o + k_r)E_\infty \quad (24)$$

and

$$-\frac{dE}{dt} = -\frac{d(E - E_\infty)}{dt} = (k_f I_o + k_r)(E - E_\infty) \quad (25)$$

We define the experimental rate constant of this first-order process as $k_i = k_f I_o + k_r$. Integration of eq 25 yields

$$E = E_\infty + (E_o - E_\infty)e^{-k_i t} \quad (26)$$

The apparent inhibition constant at equilibrium, K_i, is

$$K_i = \frac{I_o E_\infty}{C_\infty} = K_i = \frac{I_o E_\infty}{E_o - E_\infty} = \frac{k_r}{k_f} \quad (27)$$

and by substitution we obtain

$$E = E_\infty\left(1 + \frac{I_o}{K_i}e^{-k_i t}\right) \quad (28)$$

Once K_i is calculated from the experimentally accessible I_o, E_∞, and $(E_o - E_\infty)$, k_f and k_r can be determined from the relationship:

$$k_i = k_f I_o + k_f K_i \quad (29)$$

as

$$k_f = \frac{k_i}{I_o + K_i} \text{ and } k_r = k_f K_i \quad (30)$$

The slow, partial loss of active enzyme may be monitored by measuring the progressive decrease in the CETP-catalyzed lipid transfer. For this purpose, we used our fluorescence-labeled substrate, L, whose rate of transfer is first-order in a good approximation:

$$\frac{dL_a}{dt} = k_e E(L_{a,\infty} - L_a) \quad (31)$$

Substitution of E from eq 26 leads to:

$$\int_0^{L_a} \frac{dL_a}{L_{a,\infty} - L_a} = -k_e E_\infty \int_0^t dt - k_e(E_o - E_\infty)\int_0^t e^{-k_i t} dt \quad (32)$$

which integrates into

$$\ln \frac{L_{a,\infty} - L_a}{L_{a,\infty}} = -k_e E_\infty t + \frac{k_e(E_o - E_\infty)}{k_i}(1 - e^{-k_i t}) \quad (33)$$

Solving for L_a and then substituting from eq 8, we obtain

$$f = f_o + (f_\infty - f_o)\left(1 - e^{\left[-k_\infty t - \frac{(k_o - k_\infty)}{k_i}(1 - e^{-k_i t})\right]}\right) \quad (34)$$

where $k_\infty = k_e E_\infty$ and $k_o = k_e E_o$. This equation may be used to analyze the experimental f vs t curves.

Kinetics of Slow, Irreversible, Partial Inhibition. Mere consistency of the experimental data with eq 34 does not prove that the slow inhibition is due to a reversible complex formation. In the following we show that an f vs t equation indistinguishable from eq 34 may also result from other, simple inhibitory pathways. In particular, we wish to consider a slow, irreversible reaction of the enzyme, E, with the inhibitor, I_o, in great excess to yield a partially active enzyme derivative, X. By defining k_{inh} as the pseudo-first-order rate constant of the inhibition, the time-dependent concentrations of E and X in this process are given by:

$$E = E_o e^{-k_{inh} t}; X = E_o(1 - e^{-k_{inh} t}) \quad (35)$$

Since both forms of the enzyme are active, the rate equation for the appearance of L_a is then composed of two terms:

$$\frac{dL_a}{dt} = (k_e E + k_x X)(L_{a,\infty} - L_a) \quad (36)$$

where k_e is the catalytic pseudo-first-order rate constant characterizing the native enzyme and k_x is that of the enzyme after reaction with the inhibitor. By substitution:

$$\frac{dL_a}{dt} = (L_{a,\infty} - L_a)(k_e E_o e^{-k_{inh} t} + k_x E_o - k_x E_o e^{-k_{inh} t}) \quad (37)$$

By integration, rearrangement, and solving for L_a, we obtain

$$L_a = L_{a,\infty}[1 - e^{-k_x E_o t - E_o \frac{k_e - k_x}{k_{inh}}(1 - e^{-k_{inh} t})}] \quad (38)$$

Substitutions from eq 8 then yield an equation which is formally the same as eq 34, and thus, the two pathways are indistinguishable when only product formation is measured under one given experimental condition.

REFERENCES

Barter, P. J., & Jones, M. E. (1980) *J. Lipid Res. 21*, 238−249.
Bisgaier, C. L., Essenburg, A. D., Minton, L. L., Homan, R., Blankley, C. J., & White, A. (1994) *Lipids 29*, 811−818.
Chou, K.-C. (1990) *Biophys. Chem. 35*, 1−24.
Chou, K.-C., & Forsen, S. (1981) *Can. J. Chem. 59*, 737−755.
Epps, D. E., Harris, J. S., Greenlee, K. A., Fisher, J. F., Marschke, C. K., Castle, C. K., Ulrich, R. G., Moll, T. S., Melchior, G. W., & Kézdy, F. J. (1995) *Chem. Phys. Lipids 77*, 51−63.
Fukasawa, M., Arai, H., & Inoue, K. (1992) *J. Biochem. 111*, 696−698.
Hesler, C. B., Tall, A. R., Swenson, T. L., Weech, P. K., Marcel, Y. L., & Milne, R. W. (1988) *J. Biol. Chem. 263*, 5020−5023.
Ihm, J., Quinn, D. M., Busch, S. J., Chataing, B., & Harmony, J. A. K. (1982) *J. Lipid Res. 23*, 1328−1341.
Kati, W. M., Pals, D. T., & Thaisrivongs, S. (1987) *Biochemistry 26*, 7621−7626.
Ko, K., Ohnishi, T., & Yokoyama, S. (1994) *J. Biol. Chem. 269*, 28206−28213.
Koizumi, J., Mabuchi, H., Yoshimura, A., Michishita, I., Takeda, M., Itoh, H., Sakai, Y., Sakai, T., Ueda, K., & Takeda, R. (1985) *Atherosclerosis 58*, 175−186.
Marotti, K. R., Castle, C. K., Murray, R. W., Rehberg, E. F., Polites, H. G., & Melchior, G. W. (1992) *Arterioscler. Thromb. 12*, 736−744.
Morton, R. E., & Zilversmit, D. B. (1981) *Biochim. Biophys. Acta 663*, 350−355.
Morton, R. E., & Zilversmit, D. B. (1982) *J. Lipid Res. 23*, 1058−1067.
Morton, R. E., & Zilversmit, D. B. (1983) *J. Biol. Chem. 258*, 11751−11757.
Morton, R. E., & Steinbrunner, J. V. (1990) *J. Lipid Res. 31*, 1559−1567.
Nishida, H. I., Arai, H., & Nishida, T. (1993) *J. Biol. Chem. 268*, 16352−16360.
Ohnishi, T., Tan, C., & Yokoyama, S. (1994) *Biochemistry 33*, 4533−4542.

Pape, M. E., Ulrich, R. G., Rea, T. J., Marotti, K. R., & Melchior, G. W. (1991) *Arterioscler. Thromb. 11*, 1759−1771.
Rye, K.-A., & Barte, P. J. (1992) in *Structure and Function of Apolipoproteins* (Rosseneu, M., Ed.) pp 401−426, CRC Press Inc., Boca Raton, FL.
Sarcich, J. L., Fischer, H. D., Babcock, M. S., Leone, J. W., & Tomasselli, A. G. (1994) *J. Protein Sci. 14*, 81−88.
Schoenheimer, R., & Sperry, W. M. (1934) *J. Biol. Chem. 106*, 2745−2760.
Schumaker, V. N., & Puppione, D. L. (1986) *Methods Enzymol. 128*, 155−170.
Sperry, W. M. (1963) *J. Lipid Res. 4*, 221−225.
Stokke, K. T., & Norum, K. R. (1971) *Scand. J. Clin. Lab. Invest. 27*, 21−27.
Swenson, T. L., Hesler, C. B., Brown, M. L., Quinet, E., Trotta, P. P., Haslanger, M. F., Gaeta, F. C. A., Marcel, Y. L., Milne, R. W., & Tall, A. R. (1989) *J. Biol. Chem. 264*, 14318−14326.
van den Besselaar, A. M. H. P., Helmkamp, G. M., Jr., & Wirtz, K. W. A. (1975) *Biochemistry 14*, 1852−1858.
Whitlock, M. E., Swenson, T. L., Ramakrishnan, S., Leonard, M. T., Marcel, Y. L., Milne, R. W., & Tall, A. R. (1989) *J. Clin. Invest. 84*, 129−137.
Yamaoka, K., Tanagawara, Y., Nakagawa, T., & Uno, T. (1981) *J. Pharm. Dyn. 4*, 879−884.
Zilversmit, D. B., Hughes, L. B., & Balmer, J. (1975) *Biochim. Biophys. Acta 409*, 393−398.

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