Cholesterol Esterase: A Cholesterol Transfer Protein†

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ABSTRACT: Rat pancreatic cholesterol esterase was examined for its ability to effect sterol transfer between small unilamellar vesicle (SUV) preparations. Sterol exchange was determined using SUV composed of palmitoyloleoylphosphatidylcholine/sterol (65:35) with or without 10 mol % phosphatidylserine or phosphatidic acid. This recently developed assay does not require separation of donor and acceptor vesicles (Butko et al., 1992). Cholesterol esterase stimulated cholesterol exchange when SUV contained phosphatidylserine and even more so in the presence of phosphatidic acid. Cholesterol esterase increased the initial rate of sterol transfer between phosphatidic acid-containing SUV by approximately 80%. The enzyme increased sterol exchange by significantly decreasing the half-times of sterol transfer and by significantly increasing the initial rates of sterol exchange. In the absence of negatively charged phospholipids, cholesterol esterase was ineffective at increasing sterol transfer. Monolayer studies showed that negatively charged phospholipids seem to play a key role in cholesterol esterase adsorption to lipid interfaces. Finally, a mutant cholesterol esterase lacking a histidine (435) residue essential for esterasic catalysis was found to be equally capable of increasing sterol transfer and binding to charged monolayers. In summary, cholesterol esterase enhances sterol transfer in SUV containing negatively charged phospholipids, independent of esterasic activity.

Bile salt-stimulated cholesterol esterase is synthesized in the pancreas and secreted into the intestine where it catalyzes the hydrolysis of cholesterol esters, phospholipids, and acylglycerols [for a review, see Rudd and Brockman (1984)]. The physiological role of cholesterol esterase appears to be the facilitation of cholesterol ester absorption by producing nonesterified cholesterol that is easily absorbed (Treadwell & Vahouny, 1986; Vahouny & Treadwell, 1964). Removal of the pancreas in the rat dramatically reduces intestinal cholesterol esterase activity and cholesterol absorption, supporting the proposed physiological function (Gallo et al., 1984). Upon infusing pancreatic juice into pancreatized rats, mucosal esterase activity and cholesterol absorption are restored (Gallo et al., 1984). Other investigators have shown that incubation of rat intestinal sacs with cholesterol-containing micelles in the presence of cholesterol esterase resulted in a 3−5-fold increase in cellular cholesterol and cholesterol ester accumulation compared to control (Bhat & Brockman, 1982).

The products of lipolysis form mixed micelles with bile salts and diffuse to the vicinity of the villus membrane (Thompson & Dietschy, 1981). The uptake of free cholesterol into the villus cell has a partial requirement for cholesterol esterase. It was hypothesized that cholesterol esterase is also involved in the esterification of cholesterol within the villus cell (Gallo et al., 1977). This hypothesis was supported by immunological studies (using antisera to purified pancreatic cholesterol esterase) in which it was shown that cholesterol esterase is uniformly distributed within absorptive cells of the rat proximal intestine (Gallo et al., 1980).

If pancreatic cholesterol esterase participates in the intracellular esterification of cholesterol, then both enzyme and sterol must be inside the cell. It is unknown how sterol or enzyme crosses the membrane. On the basis of results accumulated by several groups, Bhat and Brockman (1982) have proposed a mechanism in which cholesterol esterase on both sides of the villus membrane could facilitate transmembrane movement of cholesterol. In this model, luminal cholesterol esterase catalyzes the formation of a small quantity of cholesterol ester. This nonpolar lipid partitions into the bilayer where it readily crosses to the opposite face. Here a cytosolic cholesterol esterase forms free cholesterol (Rudd & Brockman, 1984). Consistent with this model is the observation that newly synthesized fat droplets in villus cells contain almost exclusively free cholesterol of dietary origin (Sjostrand & Borgström, 1967). The model assumes that there is an interaction of cholesterol esterase with either free or membrane-bound cholesterol and that cholesterol esterase enhances the uptake of cholesterol by intestinal epithelial cells. This assumption is supported by recent work showing that cholesterol uptake by Caco-2 cells was greatly enhanced by the addition of cholesterol esterase (Lopez-Candales et al., 1993).

Being virtually insoluble in water, the key question in the uptake of cholesterol from the small intestine remains how cholesterol moves from micelles, through an aqueous environment, to the intestinal membrane. Evidence seems to

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suggest a role for cholesterol esterase in this movement. The purpose of this study is to examine the role of cholesterol esterase in the transfer of membrane-bound sterol. In addition, the differences between enzyme interactions with sterol-free and sterol-containing lipid interfaces are examined.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC),[1] 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (POPA), and L-phosphatidyl-L-serine (PS) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Cholesterol was from Sigma Chemical Co. (St. Louis, MO). Dehydroergosterol was synthesized as previously described (Fischer et al., 1984, 1985). Before use, all sterols were recrystallized in ethanol and their purity was checked by HPLC (Fischer et al., 1985).

Cholesterol Esterase. Normal rat pancreatic cholesterol esterase was purified from recombinant baculovirus-infected Sf9 cells as previously described (DiPersio et al., 1990). A mutant of cholesterol esterase with the $His^{435} \rightarrow Gln^{435}$ substitution was made by site-directed mutagenesis of the cDNA as described (DiPersio et al., 1991). The mutagenized cDNA was subcloned into the baculovirus transfer vector pVL1392 and used to transfect *Spodoptera frigoperda* Sf9 cells in the presence of *Autographa californica* nuclear polyhedrosis virus DNA. Isolation of the recombinant baculovirus and the subsequent expression and purification of the mutant protein were performed exactly as described for purification of nonmutagenized recombinant cholesterol esterase. The purity of the two preparations (nonmutant and mutant cholesterol esterase) is demonstrated in the Figure 1 inset. Bovine pancreatic cholesterol esterase was obtained from Sigma Chemical Co. Monomeric porcine pancreatic cholesterol esterase was purified as described by Rudd et al. (1987).

Cholesterol esterase activity was determined on the basis of hydrolysis of the water-soluble substrate *p*-nitrophenyl butyrate as described (DiPersio et al., 1990). In this assay, 25 ng of purified cholesterol esterase was diluted in 400 μL of buffer containing 0.5 M Tris-HCl, pH 7.4, and 6 mM taurocholate. The assay was initiated by the addition of 200 μL of a freshly prepared *p*-nitrophenyl butyrate solution (100 μg/mL in sodium acetate, pH 5.0). The reaction rate was monitored by the appearance of the product, *p*-nitrophenol, with a Beckman Model 25 spectrophotometer, at 405 nm (Beckman Instruments, Fullerton, CA).

Sterol/Fatty Acid Binding Proteins. Recombinant rat liver fatty acid binding protein (L-FABP) binds both sterol and fatty acid (Nemecz & Schroeder, 1991). It was isolated from *Escherichia coli* expressing the cDNA for rat liver L-FABP according to the methods of Lowe et al. (1984). Recombinant rat intestinal FABP (I-FABP) binds only fatty acids (Nemecz & Schroeder, 1991). It was purified from *E. coli* containing the cDNA for I-FABP according to the methods of Lowe et al. (1987). *E. coli* were generously provided by Dr. J. B. Lowe, Department of Pathology and Laboratory Medicine and Howard Hughes Medical Institute, Medical Center, University of Michigan, Ann Arbor, MI.

SUV Preparation. Small unilamellar vesicles (SUV) were prepared as described earlier (Schroeder et al., 1987, 1988; Nemecz et al., 1988; Schroeder & Nemecz, 1989) with two modifications: (i) SUV preparations were sonicated until optical clarity was achieved. For control SUV (POPC/sterol, 65:35 mol %) and SUV containing PS or POPA [POPC/PS-(POPA)/sterol, 55:10:35 mol %], the sonication times required were approximately 30 and 4 min, respectively. Recovery of phospholipid in SUV, as measured according to Ames (1968), was similar for all SUV. (ii) The buffer (10 mM PIPES/0.2% NaN_3, pH 7.4) in which SUV were sonicated and resuspended was prefiltered with a 0.2 μm filter (Millipore, Bedford, MA).

Sterol Exchange Assay. The exchange of dehydroergosterol for cholesterol between donor and acceptor membranes was monitored at 22 °C in the absence and presence of added protein. The method (Woodford et al., 1993) was the most recent adaptation of the dehydroergosterol fluorescence polarization technique described previously by this laboratory (Nemecz & Schroeder, 1988; Schroeder & Nemecz, 1989; Nemecz et al., 1988). This technique was modified by use of an SLM 4800 spectrofluorometer (SLM Instruments, Champaign, IL) in the T-format (instead of the L-format previously used) and by continuous monitoring of steady-state polarization to obtain 540 data points in 3 h with an IBM or Compaq-PC computer. The excitation source was a 450-W xenon arc lamp (SLM Instruments). Photobleaching with this arc lamp did not occur under the conditions used (Schroeder et al., 1987). The inner-filter effect and light scattering in the samples were made negligible by use of dilute vesicle suspensions (absorbance at the excitation wavelength, 325 nm, was less than 0.1) and by placement of GG-375 cutoff filters (Janos Technology, Townshend, VT) in the emission path.

In brief, donor vesicles containing dehydroergosterol (25 μM total lipid) were added to a 3.0 mL assay cuvette containing buffer. The predetermined amount of protein was then added. Polarization was measured for 2−5 min to stabilize the base line. It is important to note that there was no increase in polarization upon addition of protein to the assay cuvette containing donor vesicles. A 10-fold excess (250 μM total lipid) of acceptor vesicles containing cholesterol instead of dehydroergosterol was then added and the polarization measured for a 3-h period. The ratio of donor to acceptor vesicles in the assay cuvette was 1:10. The total lipid concentration was 275 μM. Dehydroergosterol exchange from donor to acceptor vesicles decreases fluorescence quenching in donor vesicles, resulting in a net increase in polarization. Initial rates were determined by measuring polarization changes in the first 5 min after acceptor addition.

Using a standard curve (Butko et al., 1992), the correlation of polarization change to molar dehydroergosterol transfer between vesicles was calculated. Multiexponential curve-fitting to the 540 data points obtained during exchange was performed on a VAX 8650 computer (Digital Equipment Corp., Maynard, MA) with an NLIN nonlinear least-squares regression procedure (SAS Institute, Inc., Cary, NC). Curve printouts were produced using the SigmaPlot Scientific Graphing System (Jandel Scientific, Corte Madera, CA) on an IBM-PC computer.

Cholesterol Esterase Adsorption to Lipid Surfaces. Studies of enzyme adsorption to phospholipid monolayers were

[1] Abbreviations: CEH, cholesterol esterase; DHE, dehydroergosterol; SCP-2; sterol carrier protein−2; L-FABP, liver fatty acid binding protein; I-FABP, intestinal fatty acid binding protein; SUV, small unilamellar vesicles; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (phosphatidic acid); PS, L-phosphatidyl-L-serine; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

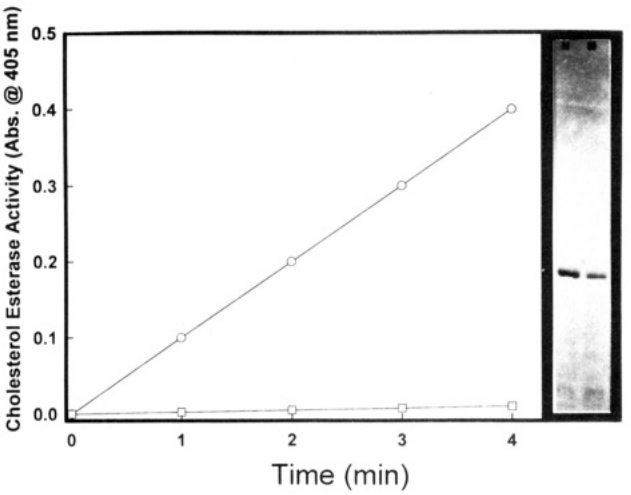

FIGURE 1: Enzymatic activity of native versus mutant rat cholesterol esterase. Normal cholesterol esterase (circles) or mutant cholesterol esterase (squares) with $His^{435} \rightarrow Glu^{435}$ substitution was tested for enzymatic activity using *p*-nitrophenyl butyrate. The inset shows polyacrylamide gel electrophoresis of native cholesterol esterase (N) and histidine^{435}-mutated cholesterol esterase (M).

conducted as previously described (Tsujita & Brockman, 1987). Briefly, an appropriate volume of phospholipid in redistilled petroleum ether/ethanol (95:5) was spread on the aqueous subphase (10 mM PIPES, pH 7.4, 0.02% NaN_3) contained in a two-compartment Teflon trough. Following a delay for solvent evaporation, the liquid was compressed into the circular compartment to give a surface area of 25 cm^2 and surface pressure of 29−30 mN/m. Stirring of the subphase at 100 rpm was begun, and the cholesterol esterase was added by injection of a small volume of concentrated solution into the subphase. After 10 min, the phospholipid monolayer and adherent enzyme were collected using hydrophobic filter paper. The paper was eluted with 5.0 mL of buffer (50 mM potassium phosphate, pH 7.25, 0.1 M NaCl, and 0.1% Brij 35). Aliquots of the eluted monolayer and the subphase were assayed for ^{125}I. The quantity of adsorbed ^{125}I-cholesterol esterase was calculated using the specific activity of the enzyme after correction for monolayer recovery and subphase adherence to the paper.

RESULTS

Esterasic Activity of Normal and Mutant Cholesterol Esterase. Previous studies using a transient expression system in COS cells have shown that the cholesterol esterase with $His^{435} \rightarrow Gln^{435}$ substitution was inactive in hydrolyzing cholesteryl [^{14}C]oleate and *p*-nitrophenyl butyrate (DiPersio et al., 1991). However, it remains possible that the low level of enzyme expression limited the detection of residual enzyme activity in the mutant protein. Therefore, in this experiment, both the normal and mutagenized cholesterol esterase cDNAs were subcloned into baculovirus vectors such that abundant quantities of purified recombinant proteins could be isolated for further analysis. Results suggested that the recombinant cholesterol esterase containing the native sequence was highly efficient in catalyzing the hydrolysis of *p*-nitrophenyl butyrate (Figure 1, circles). In contrast, the mutant protein, with a glutamine instead of histidine at residue 435, was ineffective in hydrolyzing the water-soluble substrate (Figure 1, squares). Hereafter, references to native and mutant cholesterol esterase refer to the two enzymes previously described.

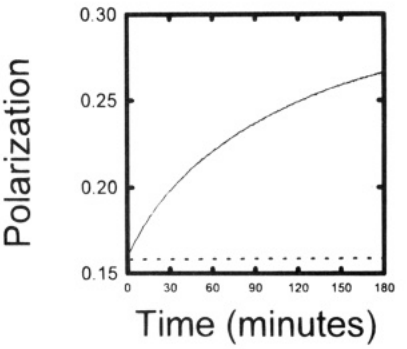

FIGURE 2: Sterol exchange between small unilamellar vesicles containing phosphatidylserine. The change in polarization over a representative 3-h exchange between PS-containing vesicles is shown. In the assay, donor vesicles containing inherently fluorescent dehydroergosterol are mixed with 10-fold more acceptor vesicles containing cholesterol. Upon mixing (shown as time zero here), sterol exchanges between the vesicles, resulting in an increase in polarization. The solid line indicates transfer of sterol between PS-containing vesicles. The dotted line indicates donor vesicles only.

Effect of Cholesterol Esterase on the Initial Rate of Sterol Exchange. The change in polarization of dehydroergosterol fluorescence upon mixing the donor vesicles (POPC/PS/dehydroergosterol, 55:10:35) and acceptor vesicles (POPC/PS/cholesterol, 55:10:35) is shown in Figure 2. The initial rate of polarization is proportional to the initial rate of sterol exchange. The polarization increase was not due to instability of the donor SUV. In the absence of acceptor SUV, the polarization of donor was constant (dotted line, Figure 2).

The effects of cholesterol esterase and several other sterol and fatty acid binding proteins on the initial rate were examined in order to determine the specificity of cholesterol esterase on sterol transfer. Rat pancreatic cholesterol esterase (1.15 μM), which comprises about 4% of total secreted pancreatic juice protein, significantly ($p < 0.05$) increased the initial rate of sterol transfer by approximately 30% over the first 5 min compared to control (Figure 3). At lower concentrations (0.23 μM), rat CEH had a lesser effect on the initial rate, increasing the rate by only about 20%. Mutant rat cholesterol esterase (1.15 μM) increased the initial rate by approximately 35% compared to control. Neither L-FABP, which binds cholesterol (Fischer et al., 1985; Schroeder et al., 1985; Nemecz & Schroeder, 1991), nor I-FABP, which does not bind cholesterol (Nemecz & Schroeder, 1991), enhanced sterol transfer. Thus, both I-FABP and L-FABP did not affect sterol transfer between SUV and were therefore used as negative controls (Figure 3).

Cholesterol Esterase Affects the Sterol Domain Structure of Membranes. Cholesterol has been shown to be nonrandomly distributed in biological (Schroeder et al., 1991, 1994; Woodford et al., 1994) as well as model membranes (Schroeder et al., 1991; Hapala et al., 1994). Membrane cholesterol appears to be organized into structural domains which can be visualized histochemically and described kinetically. These domains seem to play important roles in the modulation of some membrane proteins [reviewed in Gordon et al. (1988)], and are themselves modulated by some lipid transfer proteins (Schroeder et al., 1991; Hapala et al., 1994). By examining the ability of a protein to selectively shift or change one domain or another, one might gain insight into the mechanism of protein-mediated sterol transfer.

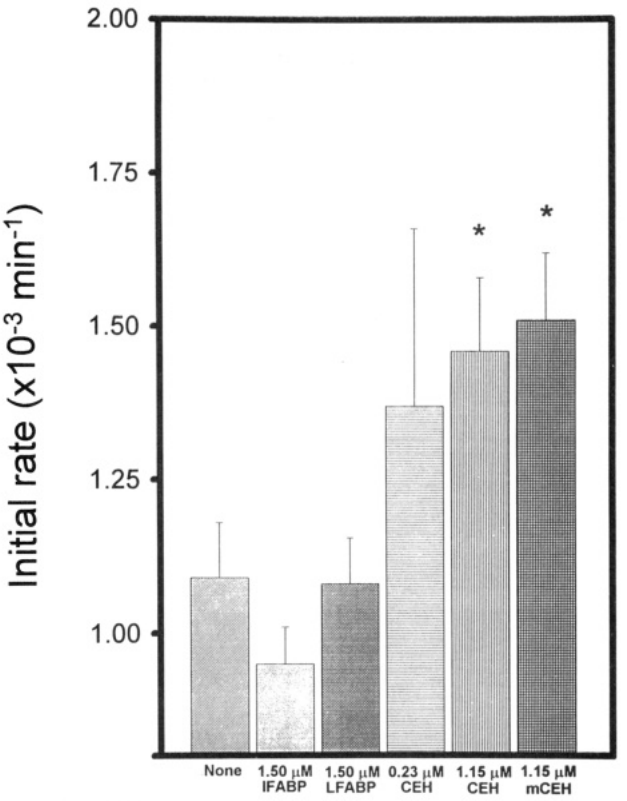

FIGURE 3: Initial rates of sterol exchange for phosphatidylserine-containing vesicles over the first 5 min of exchange. Initial rates of exchange are shown for the first 5 min of the sterol exchange assay. In each case ($n = 3-5$), the total assay volume is 2.0 mL with a vesicle concentration of 275 μM (25 μM donor, 250 μM acceptor). Here a comparison of control (no protein), 1.50 μM I-FABP, 1.50 μM L-FABP, 0.23 μM CEH, 1.15 μM CEH, and 1.15 μM mutant CEH is made. Note that I-FABP neither binds nor transfers sterol and that L-FABP binds sterol but does not transfer. An asterisk indicates a significant difference from "none" ($p < 0.05$) using the Student's t-test. Error bars indicate the standard error of the mean.

Spontaneous sterol transfer (Figure 2) can be resolved into three kinetic sterol domains: fast [fraction 1 (f_1) = 6%], slow (f_2 = 79%), and very slow, or nonexchangeable (f_3 = 15%) domains. Half-times for the fast ($^1t_{1/2}$ = 18.2 min) and slow ($^2t_{1/2}$ = 189.6 min) exchanging domains were determined (Table 1). Rat cholesterol esterase at low concentrations (0.23 μM) had no effect on the half-time for exchange of the fast-exchanging domain ($^1t_{1/2}$) of sterol (data not shown). On the slowly exchanging domain, however, rat cholesterol esterase caused a significant decrease in the half-time ($^2t_{1/2}$). At higher concentrations (1.15 μM), rat cholesterol esterase markedly decreases both $^1t_{1/2}$ and $^2t_{1/2}$ (Table 1). Mutant rat cholesterol esterase mimicked the effects of the native enzyme, significantly ($p < 0.05$) decreasing both $^1t_{1/2}$ and $^2t_{1/2}$ (Table 1).

The mechanism by which cholesterol esterase enhances sterol transfer differs markedly from other sterol binding proteins. Cholesterol esterase had no significant effect on sterol distribution among the sterol domains in SUV, as can be seen in Table 1. L-FABP, which binds cholesterol, also has no effect on cholesterol domains in SUV (Butko et al., 1992). Sterol carrier protein-2, however, selectively shifts sterol from the slow and nonexchangeable pools to the fast-exchangeable sterol pool, in addition to decreasing half-times (Butko et al., 1992). In contrast, rat cholesterol esterase acted to increase the rate of sterol transfer by drawing equally from all three domains, resulting in no net change in the size of any of the three domains.

Anionic Phospholipids Modulate the Effect of Cholesterol Esterase on Intermembrane Sterol Transfer. The effects of cholesterol esterase on sterol transfer were examined for potentiation by acidic phospholipids. The rate of spontaneous exchange of sterol between SUV is slightly higher in SUV containing 10 mol % acidic phospholipids than in SUV containing only neutral phospholipids (Table 2, column 3), with initial rates of sterol transfer of approximately 1.0×10^{-3}, 1.1×10^{-3}, and 1.2×10^{-3} min^{-1} for POPC-, POPS-, and POPA-containing vesicles, respectively. The net charges per molecule for POPC, POPS, and POPA are 0, -1, and -2, respectively. Therefore, the greater the net negative charge of the phospholipid, the greater the increase in the spontaneous rate of sterol transfer compared to neutral phospholipids.

The ability of cholesterol esterase to enhance sterol transfer in SUV was dependent on the presence of negative charge on the surface phospholipid. Cholesterol esterase significantly enhanced the initial rates of transfer in SUV containing acidic phospholipids (Table 2, column 4, and Figure 4). For SUVs containing phosphatidylserine, which has a net charge of -1 per molecule, the initial rates of transfer were increased from 1.14 $\pm$ 0.05 to 1.45 $\pm$ 0.10 (approximately 28% increase) in the presence of CEH compared to controls (Figure 4, middle frame). For SUVs containing phosphatidic acid, which has a net charge of -2 per molecule, the initial rate of transfer increased by approximately 84%, from 1.24 $\pm$ 0.08 to 2.28 $\pm$ 0.14 (Figure 4, top frame). No enhancement of sterol transfer was observed in SUVs containing only the neutral phospholipid phosphatidylcholine (Figure 4, bottom frame).

Cholesterol Esterase Adsorption to Lipid Monolayers. Mutant and native rat cholesterol esterases were compared for their ability to interact with lipid monolayers. As seen in Table 3, negative charge on the monolayer increased adsorption of enzyme to the phospholipid surface. Both mutant and native cholesterol esterases show the same trends, adsorbing approximately 4-fold more enzyme to the negatively charged phospholipid monolayers than to the net neutral charged monolayers.

DISCUSSION

The present study was undertaken to examine the role of cholesterol esterase in sterol transfer. Additionally, the interactions between the enzyme and sterol-free lipid interfaces were explored. A liposomal membrane system using an inherently fluorescent sterol, dehydroergosterol, that does not require separation of donor from acceptor membranes was used for the sterol transfer assays (Butko et al., 1992; Nemecz & Schroeder, 1988; Nemecz et al., 1988). Lipid monolayer studies were utilized to examine the interaction of cholesterol esterase with sterol-free phospholipid interfaces.

The work presented herein addresses four points. First, cholesterol esterase increases sterol transfer. As much as an 80% increase in the initial rate of sterol transfer was observed in the presence of cholesterol esterase. The total increase in sterol transfer is a result of significantly decreased half-times of sterol exchange as well as an increased initial rate of transfer. Second, the increase in sterol transfer is augmented by negatively charged phospholipids. Cholesterol

Table 1: Effect of Native and Mutant Cholesterol Esterase on Sterol Transport and Distribution[a]

sample	$^1t_{1/2}$ (min)	$^2t_{1/2}$ (min)	f_1	f_2	f_3
control (6)[b]	18.2 ± 0.9	189.6 ± 9.0	0.062 ± 0.049	0.785 ± 0.065	0.145 ± 0.045
CEH (5)	13.2 ± 1.3[c]	138.6 ± 3.3[c]	0.047 ± 0.019	0.727 ± 0.013	0.220 ± 0.031
mCEH (3)	13.5 ± 1.2[c]	135.6 ± 5.2[c]	0.055 ± 0.032	0.748 ± 0.029	0.195 ± 0.020

[a] PS-containing vesicles at 22 °C, pH 7.4. Values represent mean ± SE. [b] Numbers in parentheses refer to *n*. [c] Indicates significantly different from control at $p < 0.025$ using the Student's *t*-test.

Table 2: Acidic Phospholipids Increase the Effect of CEH on Sterol Transfer[a]

exchange	net charge	initial rate without protein	initial rate with 1.15 μM CEH
PC (4)[b]	0	0.95 ± 0.04	0.90 ± 0.05
PS (4)	−1	1.14 ± 0.05[c,d]	1.45 ± 0.10[c,d]
PA (4)	−2	1.24 ± 0.08[c,d]	2.28 ± 0.14[c,d]

[a] Phosphatidylserine (PS) or phosphatidic acid (PA) or phosphatidylcholine (PC)/1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine/sterol vesicles at 22 °C, pH 7.4. Values represent mean ± SE. [b] Numbers in parentheses refer to *n*. [c] Significantly ($p < 0.05$) different than PC using the Student's *t*-test. [d] Significantly ($p < 0.05$) different than "without protein" using the Student's *t*-test.

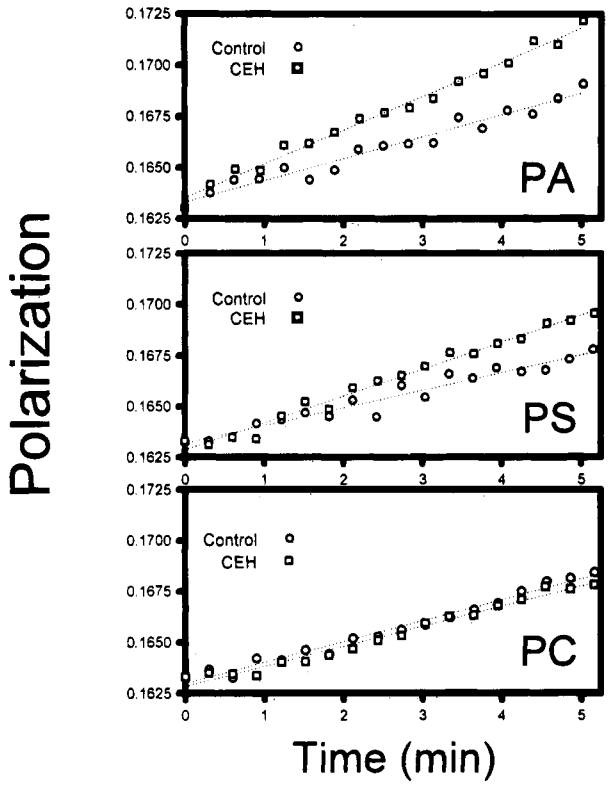

FIGURE 4: Initial rates of exchange for increasingly acidic vesicles with cholesterol esterase. Representative exchanges are shown for the first 5 min of three different exchange assays. Sterol exchange assays were performed in the presence of 1.50 μM CEH for phosphatidylcholine- (PC), phosphatidylserine- (PS), and phosphatidic acid-containing (PA) vesicles. The vesicles were 65/35 mol % POPC/sterol or 55/10/35 mol % POPC/(PS or PA)/sterol. Initial rates are measured for the first 5 min of sterol exchange.

esterase increased transfer in the presence of SUV containing phosphatidylserine and even more so in the presence of phosphatidic acid. Third, esterase activity is not necessary for cholesterol esterase to enhance sterol transfer. A mutant cholesterol esterase lacking a histidine residue essential for catalysis was equally able to enhance sterol transfer. Finally, monolayer studies confirmed the key role of negative charge in the interaction of cholesterol esterase with lipid interfaces. Mutant cholesterol esterase showed the same tendency to

Table 3: Cholesterol Esterase Adsorption to Lipid Monolayers[a]

protein	lipid	measured subphase protein concn (nM)[c]	surface concn (pmol/cm²)	av total surface concn[b] (nmol/cm²) (mean ± SEM)
native CE	POPC	4.78	0.014	15.4 ± 1.40
	POPC	11.10	0.039	
	POPS	4.66	0.070	55.9 ± 14.1
	POPS	10.59	0.095	
	POPA	4.66	0.077	68.5 ± 8.50[d]
	POPA	10.73	0.138	
mutant CE	POPC	7.04	0.019	20.3 ± 1.25
	POPC	11.80	0.039	
	POPS	7.59	0.073	87.5 ± 14.5[e]
	POPS	11.81	0.158	
	POPA	6.19	0.056	54.4 ± 1.65[e]
	POPA	11.75	0.100	

[a] Buffer = 10 mM PIPES, pH 7.4, 0.02% NaN_3. [b] Average total surface concentration is calculated by taking the surface concentration and normalizing the value with the protein concentration used in the assay. Average surface pressure was 29−30 mN/m. [c] Protein concentration is measured immediately before removing the lipid monolayer to assure accurate final representation of protein in the assay. [d] Indicates significantly ($p < 0.05$) different from native CE/POPC. [e] Indicates significantly ($p < 0.05$) different from mutant CE/POPC.

interact with negatively charged monolayers as native cholesterol esterase.

On the basis of these data and those of others described below, it would appear that there may be two distinct lipid binding sites on cholesterol esterase. The first site interacts primarily with phospholipids. It is this site which is influenced by the net charge on the SUV. The second site lies in the catalytic site of cholesterol esterase. This site would bind the sterol moiety of cholesterol esters and free cholesterol upon conclusion of ester hydrolysis. Such a two-site model of lipid interaction for cholesterol esterase is analogous to that proposed for the intracellular lipid transfer protein SCP-2 (Pastuszyn et al., 1987; Hapala et al., 1994; Seedorf et al., 1994).

Several mechanisms may account for CEH enhancement of sterol transfer. The first is direct interaction of CEH with lipid interfaces. Cholesterol esterase readily adsorbs to monomolecular films of many nonphospholipids such as oleate, triolein, and 13,16-docosadienoic acid, and to siliconized glass beads, forming a monolayer of protein molecules at the surface of the lipid film or glass bead (Tsujita & Brockman, 1987; Lombardo & Guy, 1981). The lipid monolayer studies performed suggest that CEH preferentially interacts with negatively charged phospholipid interfaces when compared to neutral phospholipid interfaces. A similar trend is seen with the sterol transfer assays: as the negative charge increased on the SUV, so did the CEH enhancement of sterol transfer. This suggests that the enhancement of sterol transfer caused by cholesterol is in some way controlled by negative charge on the surface of the lipid. However, the interaction is not solely charge−charge. Measurement of surface pressure in the monolayer

studies indicates that the cholesterol esterase is interacting with the lipid interface in such a way that phospholipid molecules are displaced and surface pressure increases. It seems likely that the negative charge initially attracts the CEH to a lipid interface, and upon binding, displacement of phospholipid occurs. Like cholesterol esterase, other sterol transfer proteins have demonstrated a similar charge dependence in their interaction with lipid interfaces (Butko et al., 1990; Hapala et al., 1990, 1994; Schroeder et al., 1990).

The second potential mechanism is through a sterol binding site. Cholesterol esterase may act as a carrier and/or enhance cholesterol desorption from the membrane. Cholesterol esterase has a hypothesized sterol binding site within or near the catalytic site. This is supported by mapping of the catalytic site with transition state analogs (Sutton et al., 1990). Additionally, at the physiological pH found at the luminal surface of the intestine, cholesterol esterase catalyzes the synthesis of cholesterol esters at limited rates (Bhat & Brockman, 1981; Gallo et al., 1977), supporting the idea of a distinct cholesterol binding site. It should therefore be possible for cholesterol esterase to enhance sterol transfer by binding cholesterol in its active site and transferring it to acceptor vesicles. Cholesterol esterase which has been mutated to abolish esterase activity ($His^{435} \rightarrow Gln^{435}$) may have an intact cholesterol binding site and should enhance sterol transfer identically to the native protein. This hypothesis is supported by the results presented here, in which mutant and native cholesterol esterase caused an increase in sterol transfer, similarly increasing initial rates as well as decreasing half-times.

We speculate that under the conditions of our assay, cholesterol esterase adsorbs to the SUV surface in a nondisruptive manner, binds nonesterified sterol, and transfers it out of the membrane. These observations are in support of the proposed mechanism of cholesterol esterase presented by Bhat and Brockman (1982) in which cholesterol esterase in the intestinal lumen catalyzes the esterification of membrane-bound cholesterol to the highly nonpolar cholesterol ester which easily partitions into and across the membrane bilayer. At the cytofacial leaflet of the bilayer, cytoplasmic cholesterol esterase would then hydrolyze the cholesterol ester to free cholesterol and fatty acid.

In conclusion, we have shown that cholesterol esterase can increase sterol transfer between small unilamellar vesicles. The kinetics of that stimulation suggests similarities to and differences from other sterol transfer proteins. Our results suggest more than one type of interaction with the lipid surface for cholesterol esterase. In addition, cholesterol esterase activity is not needed for sterol transfer. Finally, we have shown that membrane phospholipid charge plays a major role in the interaction of cholesterol esterase with lipid interfaces and that increasing negative charge increased sterol transfer.

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