Pancreatic Cholesterol Esterases. 3. Kinetic Characterization of Cholesterol Ester Resynthesis by the Pancreatic Cholesterol Esterases

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Received November 30, 1988; Revised Manuscript Received December 20, 1989

ABSTRACT: The ability of cholesterol esterase to catalyze the synthesis of cholesterol esters has been considered to be of limited physiological significance because of its bile salt requirements for activity, though detailed kinetic studies have not been reported. This study was performed to determine the taurocholate, pH, and substrate requirements for optimal cholesterol ester synthesis catalyzed by various pancreatic lipolytic enzymes, including the bovine 67- and 72-kDa cholesterol esterases, human 100-kDa cholesterol esterase, and human 52-kDa triglyceride lipase. In contrast to current beliefs, cholesterol esterase exhibits a bile salt independent as well as a bile salt dependent synthetic pathway. For the bovine pancreatic 67- and 72-kDa cholesterol esterases, the bile salt independent pathway is optimal at pH 6.0–6.5 and is stimulated by micromolar concentrations of taurocholate. For the bile salt dependent synthetic reaction for the 67-kDa enzyme, increasing the taurocholate concentration from 0 to 1.0 mM results in a progressive shift in the pH optimum from pH 6.0–6.5 to pH 4.5 or lower. In contrast, cholesterol ester hydrolysis by the 67-, 72-, and 100-kDa enzymes was characterized by pH optima from 5.5 to 6.5 at all taurocholate concentrations. Optimum hydrolytic activity for these three enzyme forms occurred with 10 mM taurocholate. Since hydrolysis is minimal at low taurocholate concentrations, the rate of synthesis actually exceeds hydrolysis when the taurocholate concentration is less than 1.0 mM. The 52-kDa enzyme exhibits very low cholesterol ester synthetic and hydrolytic activities, and for this enzyme both activities are bile salt independent. Thus, our data show that cholesterol esterase has both bile salt independent and bile salt dependent cholesterol ester synthetic activities and that it may catalyze the net synthesis of cholesterol esters under physiological conditions.

Abnormal accumulations of cholesterol and cholesterol esters in blood vessels associated with atherogenesis may be due either to defective repression of endogenous synthesis of cholesterol (Norum et al., 1983; Brown & Goldstein, 1986) or to the excessive consumption of cholesterol. Sterol synthesis, a process regulated by 3-hydroxy-3-methylglutaryl-CoA reductase by the concentration of lipoprotein-bound cholesterol delivered to the cell (Norum et al., 1983), has been extensively studied, even at the level of the gene. However, the detailed mechanism of the intestinal absorption of cholesterol remains unknown, and hence, most clinical treatment programs aim at simply reducing its intake.

Cholesterol esters are one of the dietary forms of cholesterol, but intestinal absorption of the sterol can only occur if the ester is first hydrolyzed by pancreatic cholesterol esterase in the presence of 5–10 mM taurocholate (Hyun et al., 1972; Vahouny & Treadwell, 1968). Cholesterol is then thought to diffuse through the lumen to the plasma membrane of the intestinal epithelial cells, and in a poorly understood sequence of events, the sterol appears as reesterified cholesterol esters in the intestinal lymph (Norum et al., 1983; Vahouny & Treadwell, 1968).

The mechanism of cholesterol reesterification in the intestinal cell remains controversial. Some inhibition studies have suggested that cholesterol reesterification is catalyzed by ACAT,[1] a microsomal enzyme that utilizes fatty acyl CoA as a substrate (Heller, 1983; Suckling & Strange, 1984). However, this enzyme has neither been identified nor has its participation in intestinal cell cholesterol esterification been firmly established. Other data have suggested that pancreatic cholesterol esterase itself may be vital to the reesterification of absorbed cholesterol (Gallo et al., 1984, 1977), and its presence within intestinal cells has been detected by specific anti-pancreatic cholesterol esterase antibodies (Gallo et al., 1978). Although cholesterol esterase is known to catalyze the synthesis of cholesterol esters from cholesterol and free fatty acid, the supposed requirement for millimolar taurocholate concentrations for enzymatic activity and the lack of significant concentrations of this bile acid in the intestinal cell cytoplasm have raised doubts about its role in the intracellular esterification process, despite its apparent presence there (Swell & Treadwell, 1962; Lombardo et al., 1980; Hyun et al., 1969).

Three different lipolytic enzymes have been detected from a variety of mammalian pancreases. In all species, there is a 52-kDa triglyceride lipase (Riley et al., 1990). In addition, there is a 67-kDa cholesterol esterase and also a second form in rat, pig, cow, and human, of molecular mass 72, 83, 72, and 100 kDa, respectively. Recently the bovine enzyme cDNA sequence has been reported (Kyger et al., 1989). Because of this heterogeneity, the present study was undertaken to determine the kinetics and cofactor requirements for the synthesis of cholesterol esters by each of these molecular weight forms. We show that the 67-, 72-, and 100-kDa enzymes have both bile salt independent and dependent reaction mechanisms for catalyzing the synthesis of cholesterol esters. From 0 to 100 μM taurocholate, concentrations that may occur intracellularly, these enzymes are active in the synthetic direction, and

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[1] Abbreviations: ACAT, acyl-CoA:cholesterol *O*-acyltransferase; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/90/0429-3853$02.50/0

they are stimulated by micromolar concentrations of taurocholate under conditions where the hydrolytic activity of cholesterol esterase is undetectable. Detailed kinetic studies demonstrate that the rate of hydrolysis exceeds that of synthesis only above 1.0 mM taurocholate. The 52-kDa triglyceride lipase can also synthesize and hydrolyze cholesterol esters but at rates that are 3 orders of magnitude less than those found for the 67-, 72-, and 100-kDa forms, indicating that this enzyme does not contribute significantly to cholesterol metabolism. Cholesterol esterase is therefore capable of playing a much more complete role in the intestinal absorption of cholesterol than was previously thought.

Materials and Methods

Phosphatidylcholine, heparin–agarose, and sodium oleate were purchased from Sigma Chemical Co. [^{14}C]Oleic acid, [^{14}C]taurocholate, and cholesteryl [^{14}C]oleate were purchased from Amersham Corp.

Purification of Pancreatic Cholesterol Esterases. The purification of the bovine 72-kDa and 67-kDa cholesterol esterases is described by Cox et al. (1990), and the purification of the human 52-kDa triglyceride lipase is described by Riley et al. (1990).

Human cholesterol esterase was routinely purified by sequential chromatography over hydroxylapatite, AcA 34, and heparin–agarose. Human pancreatic cytosol was prepared as described (Riley et al., 1990), and the protein was pumped at 40 mL/h onto a hydroxylapatite column (2.6 cm × 10 cm) equilibrated with 50 mM benzamidine and 10 mM phosphate buffer, pH 6.8. All of the cholesteryl [^{14}C]oleate hydrolytic activity was bound. The column was then washed with 50 mM benzamidine and 50 mM phosphate buffer, pH 6.8, and developed with a linear gradient increasing to 50 mM benzamidine and 350 mM phosphate, pH 6.8. Cholesterol esterase activity emerged reproducibly at a conductivity of 20–22 mS/cm with a 5-fold purification.

The enzyme was pooled, concentrated, and then chromatographed at 18 mL/h over AcA 34 (2.6 cm × 90 cm) equilibrated with 500 mM NaCl and 10 mM phosphate buffer, pH 6.0. Active cholesterol esterase emerged with a 157-fold purification. Based on its elution volume, the molecular mass of the active enzyme was 350 kDa; however, our experience with human and other pancreatic cholesterol esterases indicates that the molecular weight of active enzyme determined by gel chromatography is highly dependent on which matrix is used, and the calculated value rarely corresponds to that found on SDS–PAGE (100 kDa) or to simple multiples thereof.

Finally, after dialysis against 10 mM phosphate, pH 6.0, the enzyme was applied to heparin–agarose equilibrated with the same buffer. All the activity was bound, and the resin was then washed with 5–10 column volumes of 50 mM NaCl, 50 mM benzamidine, and 10 mM Tris buffer, pH 7.2, followed by 2 column volumes of 20 mM taurocholate, 30 mM NaCl, 50 mM benzamidine, and 10 mM Tris buffer, pH 7.2. Unlike the bovine 67-kDa pancreatic cholesterol esterase (Cox et al., 1990), the human enzyme is not eluted by bile salt, suggesting a tighter heparin–enzyme complex that cannot be dissociated by taurocholate. Bile salt was removed from the resin by washing with 1 column volume of 50 mM NaCl, 50 mM benzamidine, and 10 mM Tris buffer, pH 7.2, and all the enzyme activity was then removed with 500 mM NaCl, 50 mM benzamidine, and 10 mM Tris buffer, pH 7.2. Immunoblots and SDS–PAGE demonstrated this species to be homogeneous 100-kDa cholesterol esterase.

Enzyme Assays. Cholesterol esterase hydrolytic activity was assayed as described (Cox et al., 1990).

Cholesterol esterase activity in the synthetic direction was assayed by quantitating the formation of cholesteryl [^{14}C]-oleate from cholesterol and [^{14}C]oleic acid (Lange, 1982). Phosphatidylcholine–cholesterol substrate vesicles were prepared by evaporating under a nitrogen stream a chloroform/hexane solution containing 50 μmol of phosphatidylcholine and 31 μmol of cholesterol. The mixture was resuspended in 10 mL of 10 mM NaCl, sonicated, and then centrifuged at 12000*g* for 45 min at 4 °C. Phosphatidylcholine–cholesterol vesicles of varying cholesterol concentrations were prepared by mixing phosphatidylcholine–cholesterol substrate vesicles with phosphatidylcholine vesicles (prepared as described for phosphatidylcholine–cholesterol vesicles, only omitting cholesterol) in the appropriate ratio to give the desired final cholesterol concentration in the assay. These mixtures were sonicated for 3 min on ice with a Branson cell disrupter on setting 2. [^{14}C]Oleate solutions were prepared by sonicating on ice for 5 min a mixture composed of 1.75 mL of 3 mM sodium oleate in 0.1 M NaCl and 20 μCi of [^{14}C]oleic acid.

Cholesterol esterase assays in the synthetic direction contained 70 μL of phosphatidylcholine–cholesterol vesicles (700 μM cholesterol), 90 μL of [^{14}C]oleate vesicles (900 μM oleate, 3000–5000 dpm/nmol), and appropriate amounts of sodium taurocholate (0–10 mM in buffer), enzyme, and 100 mM citrate or 100 mM phosphate buffer to give a total volume of 300 μL. Reactions were conducted at 37 °C for 10 min and stopped by the addition of 1.8 mL of chloroform/methanol (1:2 v/v), 0.5 mL of chloroform, 75 μL of 1 mM cholesteryl oleate in chloroform, and 0.5 mL of H_2O. The mixtures were vortexed and centrifuged, and the entire lower phase was removed and dried under a nitrogen stream. The residues were resuspended in 150 μL of petroleum ether/chloroform/methanol (1:1:1 v/v/v) and spotted on a silica gel TLC plate. Plates were developed with petroleum ether/diethyl ether/acetic acid (75:5:1 v/v/v) and lipids visualized with iodine vapor. Spots with R_f values corresponding to cholesterol oleate standards were scraped and radioactivity was quantitated by liquid scintillation counting.

To verify that this assay was suitable for kinetic studies, a variety of control experiments were performed. First, the linear range of the assay was determined by incubating 700 μM cholesterol and 900 μM oleate with enzyme for various time periods. Similar experiments were performed when different substrate concentrations were used. Second, since assays were performed over a wide range of taurocholate and hydrogen ion concentrations, the stability of the enzyme was determined under these various solvent conditions. Thus, an aliquot of the enzyme was incubated at 37 °C either in the solvent of interest or in 10 mM taurocholate and 10 mM Tris buffer, pH 7.2, a solvent environment in which the enzyme has maximum stability. After 10 min, each enzyme solution was assayed for cholesterol esterase activity. If the rates of hydrolysis were not identical, kinetic studies were not performed under the test solvent condition.

Taurocholate Removal from Cholesterol Esterase. To assess the effect of bile salt on synthetic activity, two methods were devised for the removal of taurocholate. In the first method, taurocholate was removed from cholesterol esterase by treatment with cholestyramine. Three milliliters of enzyme solution (180 μg/mL) were mixed with approximately 15 mg of cholestyramine, and the mixture was allowed to stand on ice for 15–40 min before collection by centrifugation at 2000 rpm for 5 min. This procedure was repeated four times. After the last treatment, residual cholestyramine was removed by filtration through a 0.2-μm filter. All experiments with cho-

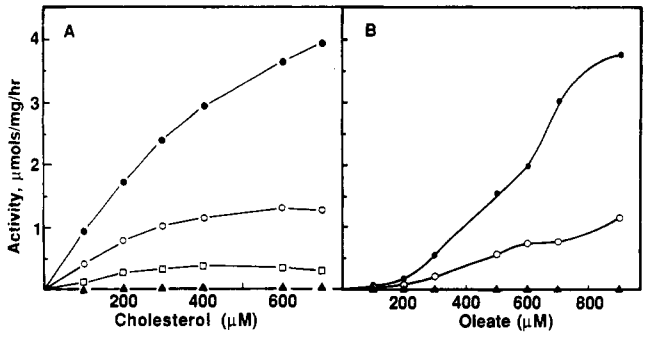

FIGURE 1: Velocity of cholesteryl oleate synthesis by 67-kDa enzyme at pH 4.0 at 0 (▲), 300 (□), 500 (○), and 1000 μM (●) taurocholate. (A) Rates measured at constant 900 μM oleate. (B) Rates measured at constant 700 μM cholesterol.

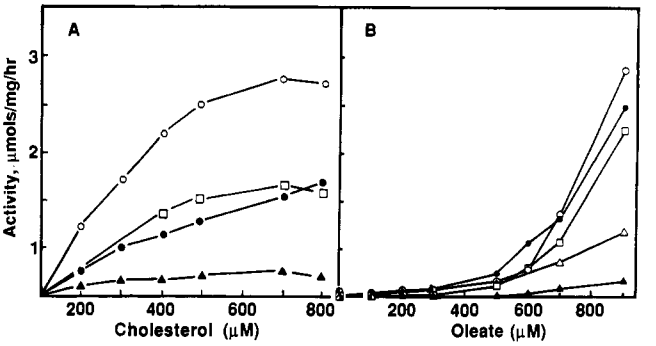

FIGURE 2: Velocity of cholesteryl oleate synthesis by 67-kDa enzyme at pH 6.5 at 0 (▲), 300 (□), 500 (○), and 1000 μM (●) taurocholate. (A) Rates measured at constant 900 μM oleate. (B) Rates measured at constant 700 μM cholesterol.

lestyramine-treated cholesterol esterase were performed within 1 h. In the second method, partially purified bovine pancreatic cholesterol esterase (35 mg) was dissolved in 3 mL of 10 mM phosphate buffer, pH 6.5, and 113 μL of 50 μCi/mL [^{14}C]-taurocholate (1.25×10^7 cpm; 100 nmol of taurocholate) was added. After incubation at 4 °C for 3 h, the mixture was chromatographed over heparin–agarose (1.5 cm × 11 cm) as described elsewhere (Cox et al., 1990). The fractions were then assayed for ^{14}C dpm, protein concentration, and enzymatic activity.

To assess the efficiency with which cholestyramine removed taurocholate from cholesterol esterase, [^{14}C]taurocholate was employed. For example, cholesterol esterase was incubated for 1 h at 4 °C in 25 mM taurocholate (20 μCi/nmol) and then dialyzed against 10 mM Tris and 0.1 M NaCl buffer, pH 7.1. After dialysis, the mixture was treated eight times with 20 mg of cholestyramine, as described above. After the eighth treatment, a 0.5-mL aliquot was removed for liquid scintillation counting as well as for assay of synthetic and/or hydrolytic activity as described above.

Protein Determination. Protein concentrations were determined by the method of Bradford (1976) using Bio-Rad Coomassie blue dye.

Results

Previous studies have indicated that pancreatic cholesterol esterase catalyzes the synthesis of cholesterol esters from cholesterol and free fatty acid, but these investigations have not examined their formation at less than 10 mM taurocholate, the intestinal luminal concentration of bile salt present after eating (Swell & Treadwell, 1962; Lombardo et al., 1980; Hyun et al., 1969). In addition, three forms of pancreatic lipolytic enzymes have now been identified from a variety of mammalian species: 52 kDa, 67 kDa, and a form of variable higher molecular mass, 72 kDa in cow and 100 kDa in human (Riley et al., 1990; Cox et al., 1990). We therefore quantitated the cholesterol esterase catalyzed rates of cholesteryl [^{14}C]oleate formation as a function of the concentration of the following: cholesterol, [^{14}C]oleate, taurocholate, and hydrogen ion. Moreover, these detailed studies were performed with a representative enzyme from each of the three described above.

Cholesterol Ester Synthesis by Bovine 67-kDa Cholesterol Esterase. At pH 4.0, the rates of formation of cholesteryl [^{14}C]oleate at 37 °C catalyzed by bovine 67-kDa cholesterol esterase were determined with a fixed concentration of [^{14}C]oleate, 900 μM, and concentrations of cholesterol varying from 0 to 700 μM cholesterol at taurocholate concentrations of 0, 300, 500, and 1000 μM (Figure 1A). In the absence of taurocholate or enzyme, no detectable product was formed. On the other hand, in the presence of 300 μM taurocholate, the enzyme catalyzed the synthesis of cholesteryl [^{14}C]oleate at measurable rates, varying from 0.125 μmol mg^{-1} h^{-1} at 100 μM cholesterol to the saturation value of 0.375 μmol mg^{-1} h^{-1} at 700 μM cholesterol. As the taurocholate concentration increased, saturation rates of cholesteryl [^{14}C]oleate formation increased markedly so that at 1000 μM taurocholate and 700 μM cholesterol, a rate of 3.9 μmol mg^{-1} h^{-1} was found. Above 1000 μM taurocholate, rates of cholesterol [^{14}C]oleate formation increased minimally.

Over a similar range of taurocholate concentrations, the rates of cholesteryl [^{14}C]oleate synthesis were determined at pH 4.0, but this time the concentration of cholesterol was fixed at 700 μM and the [^{14}C]oleate concentration was varied from 0 to 900 μM (Figure 1B). Again, in the absence of taurocholate, no cholesteryl [^{14}C]oleate was formed. At 500 and 1000 μM taurocholate, the rates were 1.2 μmol mg^{-1} h^{-1} and 3.75 μmol mg^{-1} h^{-1}, respectively, but in this case saturation kinetics may not have been achieved. Therefore, all these results indicate that at pH 4.0 cholesterol ester synthesis has an absolute taurocholate requirement.

Because the pH of the intestinal lumen is 6.0–6.5, the same experiments were repeated at this pH. Thus, as described above, rates of ester synthesis were determined at 900 μM [^{14}C]oleate and at varying concentrations of cholesterol and taurocholate. Importantly, at this pH the enzyme catalyzes the formation of cholesteryl [^{14}C]oleate in the absence of taurocholate (Figure 2A), with saturation kinetics and a maximum rate of 0.27 μmol mg^{-1} h^{-1}. At pH 6.0, the saturation rates of cholesteryl [^{14}C]oleate formation increase with increasing bile salt to 2.23 μmol mg^{-1} h^{-1} at 500 μM taurocholate; however, above this bile salt concentration, rates actually decrease. Thus, new features of cholesterol ester synthesis are discernible at physiological pH. First, a taurocholate-independent reaction pathway exists whose rate of flux is enhanced approximately 9-fold by 500 μM taurocholate. Second, high concentrations of taurocholate actually inhibit cholesterol ester synthesis.

In the presence of a constant concentration of cholesterol, 700 μM, the rates of cholesteryl [^{14}C]oleate formation were determined at variable [^{14}C]oleate and taurocholate concentrations at pH 6.0 (Figure 2B). Once again, the taurocholate-independent pathway was observed and also significant taurocholate inhibition at higher bile salt concentrations.

Cholesterol Ester Synthesis by Bovine 72-kDa Cholesterol Esterase. The synthesis of cholesteryl oleate catalyzed by 72-kDa cholesterol esterase was studied as a function of each substrate, over the same concentration ranges as those reported above for the 67-kDa enzyme. However, since this enzyme has more limited stability (see above), only pH 6.0 was chosen for study. At this pH value, the same kinetic features were

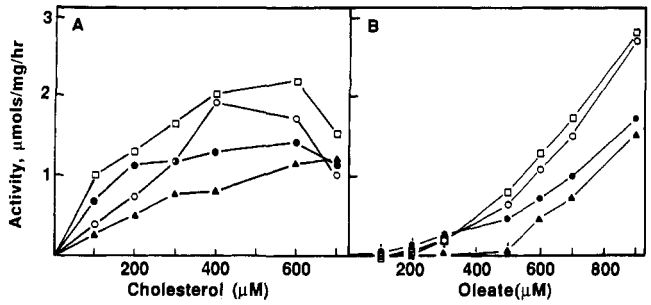

FIGURE 3: Velocity of cholesteryl oleate synthesis by 72-kDa enzyme at pH 6.0 at 0 (▲), 300 μM (□), 500 μM (○), and 10 mM (●) taurocholate. (A) Rates measured at constant 900 μM oleate. (B) Rates measured at constant 700 μM cholesterol.

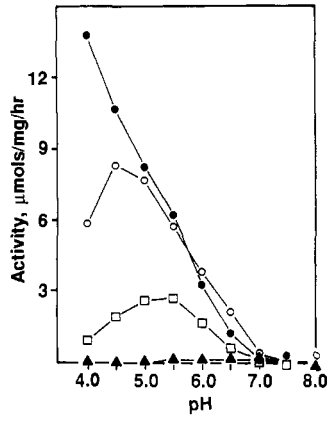

FIGURE 4: Velocity of cholesteryl oleate synthesis by bovine 67-kDa enzyme as a function of pH at 0 (▲), 0.3 (□), 0.5 (○), and 1.0 mM (●) taurocholate concentrations. The rates were measured at constant 700 μM cholesterol and 900 μM oleate.

observed with the 72-kDa enzyme as those found for the 67-kDa form when cholesterol concentration was varied at fixed oleate or when oleate concentration was varied at fixed cholesterol (Figure 3). Moreover, a taurocholate-independent pathway was found with a rate of 1.6 μmol mg^{-1} h^{-1}, a value 6 times greater than that found for the 67-kDa enzyme. As found with the 67-kDa enzyme, at higher taurocholate concentrations, cholesteryl oleate synthesis was inhibited.

Effects of pH and Taurocholate Concentration on Cholesteryl Oleate Synthesis. The effects of pH and taurocholate concentration on the synthesis of cholesteryl [^{14}C]oleate catalyzed by the bovine 67-kDa cholesterol esterase were determined from pH 4.0 to 8.0 at 0, 0.3, 0.5, and 1.0 mM taurocholate (Figure 4). In the absence of taurocholate, optimal synthetic activity is observed at pH 6.0–6.5. Taurocholate addition stimulates the activity and also shifts the pH optimum in the acidic direction. Thus, increasing the bile salt concentration to 0.5 mM shifts the pH optimum to 4.5, where cholesterol ester synthesis by cholesterol esterase has an absolute requirement for taurocholate (see above). At 1.0 mM taurocholate the activity is stimulated further, but since the enzyme is unstable below pH 4.0, it was not possible to determine the activity optimum.

Similar profiles for the bovine 72-kDa cholesterol esterase and human 100-kDa cholesterol esterase were also found, although the studies were performed no lower than pH 5.0 because of the instability of these two enzymes.

Effects of pH and Taurocholate Concentration on Cholesteryl Oleate Hydrolysis. Cholesterol esterase is also a hydrolytic enzyme; therefore, the influence of pH and taurocholate concentration on the rate of hydrolysis of cholesteryl oleate was determined for the 67-, 72-, and 100-kDa cholesterol

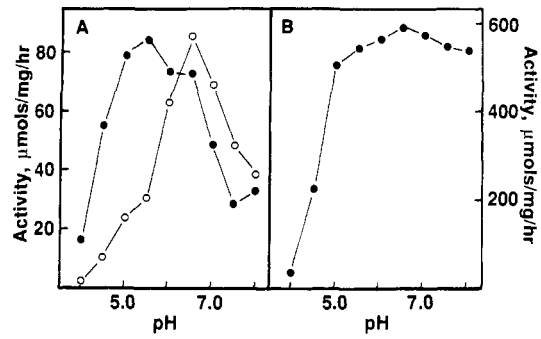

FIGURE 5: Velocity of cholesteryl oleate hydrolysis as a function of pH in the presence of 10 mM taurocholate. (A) Bovine 67-kDa enzyme (●) and human 100-kDa enzyme (○). (B) Bovine 72-kDa enzyme (●).

esterases. In the absence of bile salt, all three enzymes were catalytically inactive regardless of pH, and hydrolytic activity became detectable only when the bile salt concentration was increased from 0.5 to 1.0 mM. Maximum activity was found with 10 mM sodium taurocholate; the pH–rate profiles are shown in Figure 5 for the three enzyme forms. The human 100-kDa cholesterol esterase and the 67-kDa cholesterol esterase have the same maximum activity, 85 μmol mg^{-1} h^{-1}, and well-defined pH optima at pH 5.5 (67 kDa) and pH 6.5 (100 kDa). The bovine 72-kDa enzyme is a more active hydrolytic enzyme with a maximum activity of 600 μmol mg^{-1} h^{-1}, about 7 times greater than that found for the other enzyme forms. Its activity increases sharply above pH 4.5 and remains at a constant level from pH 5.0 to pH 8.0 so that there is no narrow pH optimum.

Human 52-kDa Cholesterol Esterase Activity versus pH and Taurocholate Concentration. Although the 52-kDa enzyme exhibits primarily fatty acid ethyl ester synthase activity (Riley et al., 1990), it is active as a cholesterol esterase, possessing both hydrolytic and synthetic activities. However, the details of its kinetic behavior are markedly different when compared to those for either the 67- and 72-kDa enzymes or the 100-kDa enzyme. First, regardless of pH or taurocholate concentration, both the hydrolytic and synthetic activities are much lower than those found for the other three enzymes. For example, in the presence of 10 mM taurocholate at pH 6.0, the 52-kDa enzyme hydrolyzes and synthesizes cholesterol oleate at a rate of approximately 0.002 μmol mg^{-1} h^{-1}, 3–4 orders of magnitude less than those found for the other three enzymes. Second, although the rate is small, the 52-kDa cholesterol esterase can hydrolyze cholesterol esters in the absence of taurocholate. Finally, bile salt has a much smaller effect on both activities, increasing rates, at most, 2-fold.

Ratio of Synthetic Rate to Hydrolytic Rate for 67-, 72-, 100-, and 52-kDa Cholesterol Esterases. Taken together, these data demonstrate the critical roles played by taurocholate, pH, and the enzyme form in determining the absolute rates of synthesis and hydrolysis of cholesterol esters. However, from a physiological point of view, the important parameter is the rate of synthesis relative to the rate of hydrolysis. Therefore, the ratio of the synthetic activity to hydrolytic activity was determined for each enzyme form at pH 6.0 from 0 to 10 mM taurocholate. As shown in Table I, at lower taurocholate concentrations, a condition that could pertain at the intestinal membrane or in the intestinal cell, net synthesis is favored. On the other hand, in the intestinal lumen where the taurocholate concentration may approach 10 mM, hydrolysis becomes more favorable. The relative activities of the 52-kDa enzyme are insensitive to taurocholate, but as shown above, the absolute rates are so low that they probably do not

Table I: Activity of Cholesterol Esterase at pH 6.0[a]

	[taurocholate]			
enzyme form	0 mM	0.1 mM	1.0 mM	10.0 mM
67 kDa				
synthetic	0.83	14.2	32.5	78.3
hydrolytic	0	0	12.0	73.5
ratio[b]			2.7	1.1
72 kDa				
synthetic	1.6		1.3	15.5
hydrolytic	0		4.5	565.0
ratio[b]			0.30	0.03
100 kDa				
synthetic	0.3	0.8	0.43	2.8
hydrolytic	0	0	1.5	63.0
ratio[b]			0.30	0.04
52 kDa				
synthetic	0.0044	0.0039	0.0023	0.0021
hydrolytic	0.0022	0.0036	0.0038	0.0015
ratio[b]	2	1.1	0.6	1.4

[a] Activities are expressed as μmol mg^{-1} h^{-1}. [b] Ratios are expressed as synthetic rate/hydrolytic rate.

Table II: Addition of Taurocholate to Stripped Cholesterol Esterase

[TC] (μM)	synthetic activity[a] (μmol mg^{-1} h^{-1})
0	0.16
10	0.24
30	0.27
50	0.33
100	0.42

[a] Synthetic activity measured at 700 μM cholesterol and 900 μM oleate at pH 6.5.

contribute in a meaningful way to cholesterol homeostasis in the intestine.

Lack of a Stoichiometric Taurocholate Requirement for Cholesterol Esterase Catalyzed Synthesis of Cholesterol Esters. To evaluate the possibility that stoichiometric amounts of taurocholate are an integral part of the active site of cholesterol esterase in the synthetic direction, homogeneous bovine 67-kDa cholesterol esterase was incubated with 25 mM [^{14}C]taurocholate (20 μCi/nmol) and then dialyzed against 10 mM Tris buffer, pH 7.1. Affinity chromatography over heparin–agarose was then employed as described under Materials and Methods. After this treatment, the ratio of taurocholate to enzyme was 6×10^{-5}. Even though the preparation was free of taurocholate, at pH 6.0 the enzyme still possessed 5% of maximum synthetic activity but no hydrolytic activity. Readdition of small increments of taurocholate from 1 to 100 μM produced progressively more synthetic activity without any detectable hydrolytic activity (Table II). These results indicate that micromolar concentrations of taurocholate are effective in stimulating the cholesterol esterase catalyzed synthesis of cholesterol esters but that no tightly bound, stoichiometric quantity of taurocholate is necessary for this activity.

Discussion

Pancreas contains three distinct enzymes that hydrolyze neutral lipid substrates. All mammalian species contain a 52-kDa protein (triglyceride lipase), a 67-kDa protein, and a third protein varying in molecular mass from 72 to 100 kDa, depending on the species. Cholesterol esterase can catalyze the synthesis or hydrolysis of cholesteryl oleate, and both of these reactions are used here to define the kinetic properties of each of these enzyme forms.

In the presence of 10 mM taurocholate, all three enzyme forms can catalyze the hydrolysis of cholesteryl oleate. The higher molecular weight form, bovine 72 kDa or human 100 kDa, has a pH optimum of 6.5, the pH of the intestinal lumen, indicating that this high molecular weight species is probably responsible for hydrolyzing cholesterol ester in the gut. The 67-kDa enzyme has a more acidic activity optimum, pH 5.5, and it is present in much smaller quantities in the pancreas (Cox et al., 1990), suggesting that this enzyme form may not be involved in cholesterol ester hydrolysis. Finally, the 52-kDa peptide, in contrast to the 100-, 72-, and 67-kDa peptides, has very little cholesterol esterase hydrolytic activity, even though it has high triglyceride lipase activity and fatty acid ethyl ester synthase activity (Riley et al., 1990). Therefore, it has a minimal role in intestinal cholesterol uptake.

The cholesterol esterase catalyzed synthesis of cholesterol esters from sterol and free fatty acid provides yet another way to discriminate between these forms. The most striking feature of cholesterol ester synthesis is the complex role played by taurocholate, regardless of enzyme form. Thus, in the absence of bile salt, all enzyme forms synthesize cholesterol esters with a pH optimum of 6.5. Addition of taurocholate enhances the activity and shifts the pH optimum to acidic pH. These results have two functional consequences. First, in the presence of greater than 1 mM taurocholate, cholesterol ester hydrolysis will be favored in the intestinal lumen. This is accomplished by shifting the pH optimum of the cholesterol esterase catalyzed synthetic reaction to very acidic values, such that the luminal pH is two units higher than the optimum for the synthetic reaction. Second, unlike the hydrolytic reaction, which has an absolute requirement for bile salt, synthesis of cholesterol esters can occur at measurable rates in the absence of bile salt, and moreover, the pH optimum of the synthetic reaction under this condition occurs near neutrality where it may be physiologically relevant.

At the present time, a controversy exists concerning the mechanism of cholesterol reesterification in the intestinal cell, especially as it relates to ACAT or cholesterol esterase dependent processes. While the kinetic results described above do not address the role of ACAT in cholesterol esterification, they are relevant to observations reported earlier. Pancreatic cholesterol esterase apparently is internalized into the intestinal cell (Gallo et al., 1980, 1978, Bhat & Brockman, 1982). If this internalization process does occur, the intracellular concentrations of absorbed taurocholate and glycocholate could be in the micromolar range, which may be sufficient to support high levels of cholesterol esterase synthetic activity while significantly inhibiting ACAT activity (Norum et al., 1981). There could be at least two advantages to this pathway for cholesterol reesterification. First, cholesterol esterase can be switched from a synthetic to a hydrolytic enzyme by regulating the bile salt and/or proton concentrations. Second, unlike ACAT, cholesterol esterase does not utilize ATP for its driving force in catalyzing synthesis of cholesterol esters (Mogelson et al., 1984). Because cholesterol is not used as a fuel source but rather as a structural component of membranes or as a precursor for synthesis of more complex metabolites, the expenditure of ATP in the ACAT reesterification of cholesterol may not be energetically efficient. Thus, cholesterol esterase catalyzed reesterification may provide the body with an essential structural element merely by equilibrating cholesterol across a membrane down a taurocholate gradient.

Other factors involved in the driving force of the cholesterol esterase catalyzed synthetic reaction are also undoubtedly relevant. Fatty acid substrate concentrations are locally modulated by cholesterol esterase hydrolytic activity as well as by other hydrolytic enzymes to produce free fatty acid from other sources, such as triglyceride. In biopsy samples from

human intestinal mucosa, unesterified fatty acid concentrations were approximately 2.8 μmol/g of tissue, more than enough to support reasonable levels of enzymatic activity (Gangl & Renner, 1978).

The mechanism of cholesterol esterase activation in the synthetic direction of taurocholate is undoubtedly complex. While it is difficult to describe lipid-utilizing enzymes with Michaelis–Menten kinetics, the data presented in Figures 1–3 suggest that bile salt increases the synthetic rate by enhancing the catalytic step, as indicated by the increase in apparent V_{max} with increasing taurocholate concentrations. Furthermore, bile salt appears to have little influence on substrate binding, since the concentration where half-maximum activity occurs varies little with taurocholate concentration. For the bile salt independent pathway, stimulation occurs at subcritical micelle concentrations, and therefore, the bile salt may represent a classical effector influencing the active site. However, taurocholate is not a catalytically essential cofactor since enzyme stripped of all bile salt is still an active catalyst, retaining 5% of maximum synthetic activity (Table II).

Taurocholate causes the pH optimum of the synthetic reaction to become more acidic. This shift with increasing taurocholate could be due either to a decrease in the pK_a of an active-site amino acid residue or to an increase in the reactivity of a catalytically essential residue. In this regard, irreversible inhibition of cholesterol esterase by phenylmethanesulfonyl fluoride is greatly augmented in the presence of bile salt (Hyun et al., 1969), indicating that taurocholate can increase the reactivity of an active-site residue. For the taurocholate-dependent pathway, as the bile salt concentration approaches the critical micelle concentration, activation occurs with sigmoid kinetics (data not shown). Accordingly, bile salt may provide a more favorable interface for binding of substrate, enzyme, or both and thus promote catalysis.

In summary, our findings demonstrate that under physiological conditions pancreatic cholesterol esterase can catalyze the net synthesis of cholesterol esters from free fatty acid and cholesterol in the absence of the bile salt taurocholate. Of the four lipolytic enzymes described, the 100-, 72-, and 67-kDa enzymes are much more efficient than the 52-kDa enzyme. Therefore, the kinetic data presented here suggest that cholesterol esterase is capable of promoting cholesterol uptake from the intestinal tract in a much more complex manner than previously recognized.

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