Identification of a Species Specific Regulatory Site in Human Pancreatic Cholesterol Esterase

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ABSTRACT: All mammalian pancreatic cholesterol esterases (CEase) bind to membrane-associated heparin at a single site on the intestinal brush border membrane with a dissociation constant of 100 nM. While the enzyme is bound to the membrane, the activity of the human and bovine enzymes is enhanced 2-fold when compared to the activity of the enzyme in solution. On the other hand, soluble heparin potently inhibits the human CEase-catalyzed hydrolysis of cholesterol oleate with an IC_{50} of 2×10^{-4} mg/mL, a value that is about 10^4 times more potent than that found with the bovine enzyme. The C-terminal portion of the human enzyme contains 16 proline-rich repeats of 11 amino acids each, while that from other species contains only a few of these repeat units. To determine if the unique human C-terminus is responsible for this inhibition, two chimeras containing either the human N-terminus (residues 1−445) and the bovine C-terminus (residues 446−557), HB, or the bovine N-terminus (residues 1−445) and the human C-terminus (residues 446−722), BH, were prepared. The cholesterol oleate hydrolytic activity of these chimeras was similar to that for the recombinant human and bovine enzymes. Importantly, each chimera was inhibited by heparin with IC_{50} values of 0.03 and 0.1 mg/mL for HB and BH, respectively. These intermediate IC_{50} values indicate that human CEase has two structural regions that contribute to its unique inhibition by this sulfated glycosaminoglycan, and these could regulate cholesterol uptake in humans.

The molecular mechanism describing intestinal cholesterol absorption is not understood. The unstirred water layer has been characterized as a physical barrier for cholesterol transport from the intestinal lumen into the enterocyte, but other observations have suggested that particular proteins are also involved (Thomson, 1980; Thurnhofer & Hauser, 1990; Bosner et al., 1988). For example, sterol absorption is heterogeneous in humans, varying from 15 to 90% (Kern, 1991). It would be unusual to attribute such heterogeneity to the physical properties of water, because most other nutrients, including fatty acids, are absorbed uniformly. Most likely, genetic heterogeneity of a protein(s) responsible for cholesterol absorption influences the rate-limiting step.

Recent studies have indicated that secreted pancreatic proteins may mediate absorption and be responsible for the heterogeneity of this process (Bosner et al., 1988). Elimination of pancreatic exocrine products from the intestine markedly decreases cholesterol absorption and serum cholesterol levels (Bell & Swell, 1968). One of these, pancreatic cholesterol esterase (CEase),[1] plays a dual role. First, it hydrolyzes the small amount of intestinal sterol that is present in the esterified form. Second, and more importantly, it is a transport protein that binds to intestinal brush border membrane heparin, and by doing so, it functions within the unstirred water layer to promote the absorption of non-esterified cholesterol (Bosner et al., 1988; Lopez-Candales et al., 1993). In the absence of this second activity, sterol does not enter the metabolically active pool of cholesterol destined for esterification.

To understand the molecular basis for this cholesterol transport property, cDNA for CEase from rabbit, rat, cow, and human has been cloned and sequenced, and the catalytically essential residues have been identified (Kissel et al., 1989; Kyger et al., 1989; Colwell et al., 1993; Nilsson et al., 1990; DiPersio et al., 1990, 1991, 1993). While there is high sequence homology between species, the human CEase gene has a unique exon 11, encoding a large COOH terminus (Kumar et al., 1992). Thus, the C-terminal portion of the human enzyme contains 16 proline-rich repeats of 11 amino acids each, while that from other species contains only a few of these repeat units. In this work, we show that the human enzyme is also catalytically unique because it contains a heparin inhibitory site(s), a potential regulatory locus, which has little or no activity in other mammalian species.

METHODS

Purification and Characterization of Native CEase. CEase was purified to homogeneity from cow, rabbit, rat, dog, and mouse pancreas. To minimize degradation by pancreatic proteases, the tissue was immediately plunged into liquid nitrogen, lyophilized, and then stored in liquid nitrogen. The human enzyme was isolated from pancreatic acinar cells, which were received from Washington University School

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[1] Abbreviations: CEase, cholesterol esterase; BSSL, bile salt-stimulated lipase; HH, recombinant human CEase; BB, recombinant bovine CEase; HB, CEase chimera formed from the human N-terminus and the bovine C-terminus; BH, CEase chimera formed from the bovine N-terminus and the human C-terminus; TC, sodium taurocholate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; pNPB, *p*-nitrophenyl butyrate; Caco-2, colon adenocarcinoma cell line.

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of Medicine and stored frozen at −80 °C. In a typical purification, 10 g of freeze-dried material was homogenized in 100 mL of cold 25 mM sodium acetate (pH 5.1), centrifuged at 100000*g* for 1 h, and then filtered through a 0.8 μm filter. Human BSSL was isolated from breast milk, which was stored at −20 °C after collection. For chromatography, the pH of the thawed breast milk was adjusted to 5.1 with concentrated acetic acid, centrifuged (45 min at 12000*g*), and filtered through cheesecloth and then through a 0.8 μm filter.

The general purification protocol for bovine pancreatic CEase was used, regardless of species or source, to purify enzyme to homogeneity at 4 °C. Pancreatic extract was pumped onto an S-Sepharose column (2.5 × 15 cm) equilibrated with 25 mM sodium acetate (pH 5.1) (buffer A), and the resin was washed with 500 mL of buffer A containing 200 mM NaCl. Application of a 300 mL gradient increasing in salt concentration to 800 mM NaCl removed the CEase activity as a single peak, which was pooled and dialyzed against buffer A. The enzyme solution was then applied to an SP-Sephadex C25 column (1.6 × 8 cm) equilibrated with buffer A. After the resin was washed with 50 mL of buffer A, followed by 75 mL of buffer A containing 25 mM NaCl, bovine pancreatic CEase was eluted with a 90 mL salt gradient increasing in concentration to 300 mM NaCl in buffer A.

Occasionally, an additional chromatographic step was required to effect complete purification. In that case, active fractions from the SP-Sephadex column were pooled and dialyzed against buffer A containing 10 mM NaCl. After dialysis, the sample was loaded at a flow rate of 1.0 mL/min onto a Waters Advanced Protein Purification System utilizing a Waters SP-15HR column (1.0 × 10 cm) equilibrated with buffer A. The column was washed with 10 mL of buffer A, then the salt concentration was increased linearly to 150 mM NaCl in buffer A, and the column was washed with 30 mL of this salt buffer. Homogeneous enzyme was then eluted with a linear 110 mL salt gradient increasing to 350 mM NaCl in buffer A.

Active fractions were analyzed using 8% SDS−PAGE in a NOVEX X Cell II MINI-Cell by loading 500 ng per lane. Those samples which contained a single band were pooled and stored frozen at −80 °C.

Purification and Characterization of Recombinant CEase. Recombinant CEases and chimeras were purified to homogeneity by employing the chromatographic and characterization procedures used for the native enzymes.

Western Blotting. Two 8% SDS−polyacrylamide gels were run; one gel contained 6 ng per lane of each CEase species, and the other contained 20 ng per lane. After electrophoresis, the protein was transferred to a nitrocellulose membrane in a NOVEX blot module. The blots were then developed following an established procedure (Sambrook et al., 1989) using anti-bovine CEase for the membrane containing 20 ng per lane and anti-human BSSL for the membrane containing 6 ng per lane. The antibodies to purified bovine CEase and human BSSL were raised in rabbits following the procedure described in Cox et al. (1990).

Enzyme Assays. CEase hydrolytic activity toward neutral lipid was determined in two ways. In the first method, the release of [^{14}C]oleic acid from vesicles containing cholesteryl [1-^{14}C]oleate was measured in 8 mM TC and 150 mM tris(hydroxymethyl)aminomethane (pH 7.5) (Cox et al., 1990). The second method was performed under the same conditions, only this time the substrate was [^{14}C]triolein (Riley et al., 1990).

The esterolytic activity of CEase was measured by monitoring, at 405 nm, the release of *p*-nitrophenol from pNPB (Hosie et al., 1987) at 25 °C in 25 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.5) with or without 2.0 mM TC. Unlike lipid substrates, hydrolysis of this water soluble ester can be analyzed using conventional Michaelis−Menten kinetics. Therefore, hydrolysis was monitored from 0.15 mM pNPB to 1.0 mM pNPB, and kinetic constants were determined using the kinetics package on a Shimadzu spectrophotometer (Model CPS-260).

CEase activity in the synthetic direction was assayed by quantitating the formation of cholesteryl [^{14}C]oleate from cholesterol and [^{14}C]oleic acid (Kyger et al., 1990).

Phospholipase activity was measured using [1-^{14}C]palmitoyl lysophosphatidylcholine (lyso-PC) as substrate (Van Den Bosch et al., 1981).

Vesicle Binding Studies. Small intestinal vesicles were prepared from male New Zealand white rabbits, and binding of [^{125}I]CEase was quantitated as described (Bosner et al., 1988).

CEase Hydrolytic Activity in the Presence of Caco-2 Cells. To determine the effect of membrane-bound heparin on CEase activity, hydrolysis of cholesteryl [^{14}C]oleate was measured in the presence or absence of Caco-2 cells. In a typical experiment, cells were grown in 24-well Falcon plates and, when confluent, they were washed with 500 μL of phosphate-buffered saline. The medium was replaced with a mixture of 170 μL of phosphate-buffered saline, 75 μL of cholesteryl [^{14}C]oleate vesicles, and 25 μL of 100 mM TC in phosphate-buffered saline. The same mixture was placed in wells which contained no Caco-2 cells. The plates were placed in a 37 °C water bath, and the reaction was initiated by addition of 30 μL of CEase to each well. After 10 min, the plates were immersed in an ice−water bath, and the reaction was quenched by the addition of 600 μL of 0.3 M NaOH. The assay was completed as described elsewhere (Cox et al., 1990). To assess intrinsic CEase activity, identical assays were done in triplicate utilizing equal aliquots of Caco-2 cells.

Effect of Heparin on CEase Enzymatic Activity. Porcine intestinal heparin (Sigma) in 150 mM tris(hydroxymethyl)-aminomethane (pH 7.2) was added to CEase assays as described above to give a final concentration of 1×10^{-4} to 20 mg/mL. The activity in the presence of the glycosaminoglycan was compared to that of a control which contained no heparin.

Construction of CEase Chimeras. To construct chimeras between bovine and human CEase, the full-length cDNA from both species was cleaved at the internal XmnI site, which cuts at the codon corresponding to amino acid 445 in both sequences. The 5′ half of the bovine sequence was then ligated to the 3′ half of the human sequence with T4 DNA ligase, and the 5′ half of the human sequence was ligated to the 3′ half of the bovine sequence.

Transfection and Expression. To express the various constructs, the cDNA was subcloned into expression vector pCEP4 (Invitrogen, San Diego, CA). The recombinant DNA was then introduced into 293-EBNA cells (Invitrogen) using LipofectAmine (Gibco-BRL, Gaithersburg, MD) following

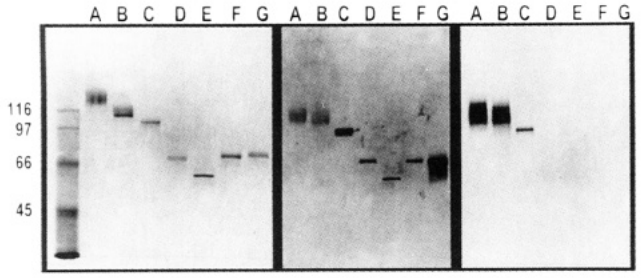

FIGURE 1: (left) SDS−PAGE of purified CEase from (A) BSSL, (B) human acinar cells, (C) dog pancreas, (D) rat pancreas, (E) rabbit pancreas, (F) mouse pancreas, and (G) bovine pancreas. (center) Corresponding Western blot of SDS−PAGE with antibody to the bovine enzyme. (right) Corresponding Western blot of SDS−PAGE with antibody to the human pancreatic enzyme.

the manufacturer's instructions. Forty-eight hours after transfection, the cells were split 1:10 and plated in medium containing 250 μg/mL G418 (Gibco-BRL) and 400 μg/mL hygromycin B (Sigma Chemical, St. Louis, MO). After the cells reached confluence, they were passaged the same way as normal adherent cells, with the selection pressure of G418 and hygromycin B constant applied.

For large scale expression, the cells were grown to confluence in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum, 250 μg/mL G418, and 400 μg/mL hygromycin B. The cells were maintained for an additional 3 days, and the medium was harvested for protein purification.

RESULTS

Purification of Mammalian CEases. Mammalian pancreatic CEases have a high degree of sequence homology except in the C-terminal region. For example, the human enzyme has 16 similar repeating units composed of 11 amino acids each, while the enzyme from cow and rat has two and four units, respectively. To determine the functional consequences of this varying structural motif, pancreatic CEase was isolated from cow, dog, human, mouse, rabbit, and rat. Since human breast milk BSSL was recently found to be identical to human pancreatic CEase, this protein was also purified (Nilsson et al., 1990). As shown in Figure 1 (left panel), mammalian CEase is characterized by a wide range of molecular masses, as shown by the following molecular mass values: human (pancreas), 118 kDa; human BSSL, 122 kDa; dog, 98 kDa; rat, 70 kDa; rabbit, 57 kDa; mouse, 68 kDa; and cow, 73 kDa.

Immunological Properties of Mammalian CEases. Polyclonal antibodies were raised in rabbits against either homogeneous bovine pancreatic CEase or human BSSL, and they were used in Western blots of the purified enzymes shown in Figure 1 (left panel). When CEases from all the species were exposed to bovine antibody, they all cross-reacted, although those from human and mouse interacted weakly with this antibody (Figure 1, center panel). On the other hand, the rabbit anti-human CEase antibody recognizes only the human enzyme, with some cross-reactivity seen with the dog enzyme (Figure 1, right panel). These data indicate that the human enzyme contains a structural element that is strongly immunogenic and is not present in CEases from the other species.

Enzymatic Activities of Mammalian CEases. To determine if the unique structural feature found in the human enzyme affected catalytic properties, the enzymatic activities for all the purified CEases were determined. For many years, CEase was thought to be only a simple esterase, catalyzing the hydrolysis of an ester bond, such as that found in cholesteryl oleate, triolein, and pNPB; however, it was recently shown that the lysophospholipase activity found in pancreas actually resides in CEase, and in addition, under conditions that may pertain in the cell, the enzyme can also synthesize cholesterol ester (Kyger et al., 1989, 1990). Finally, while bound to heparin at a specific site on the enterocyte, the enzyme functions as a transport protein, catalyzing the uptake of free, unesterified cholesterol into the intestinal cell (Lopez-Candales et al., 1993).

Table 1: Enzymatic Activities of CEases

	activity, μmol (mg of CEase)$^{-1}$ h^{-1}					
		hydrolytic				
	synthetic				pNPB[d]	
species	cholesterol oleate[a]	cholesterol oleate[b]	triolein[b]	lyso-PC[c]	K_m, mM	V_{max} ($\times 10^{-3}$)
cow	5.0	460	190	0.14	1.5	6.0
dog	2.1	270	80	0.03	0.9	1.2
mouse	3.6	280	200	0.02	1.5	3.6
rabbit	2.5	920	160	0.07	0.8	13.8
rat	17.0	230	60	0.01	1.4	3.6
BSSL	0.30	110	93	0.01	0.8	2.4
man	0.20	180	85	0.01	0.9	2.4

[a] 200 mM citrate, pH 5.0, 0.3 mM TC, 37 °C. [b] 150 mM tris(hydroxymethyl)aminomethane, pH 7.5, 8.0 mM TC, 37 °C. [c] 150 mM tris(hydroxymethyl)aminomethane, pH 7.5, 37 °C. [d] 25 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.5, 25 °C.

These activities were examined under standard assay conditions with purified, homogeneous enzymes, and the results are tabulated in Table 1. To determine if differences in activity can be attributed exclusively to binding or catalysis, without regard to interfacial interactions, the esterase activity of the nonphysiological substrate, pNPB, was studied in the absence of TC. The K_m for substrate binding (~1 mM) is virtually identical for all species, but the V_{max} for hydrolysis varies about 12-fold from 1.2 mmol mg h^{-1} for dog CEase to 13.8 mmol mg h^{-1} for the rabbit enzyme. Addition of 2 mM TC increased V_{max} by about 4-fold and decreased K_m by about 3-fold for all species, except for rabbit CEase which had a slight decrease in V_{max} (data not shown). Importantly, the observed activities for the human enzyme, which contains the unique C-terminal tail, do not differ significantly from those of the other species, indicating that this structural feature does not play a major role in catalysis. For all four lipid substrates examined, the specific activity is similar from species to species.

Saturable Binding of Mammalian CEases to Small Intestine Vesicles. To determine how the C-terminal tail might affect the interaction of CEase with membrane-bound heparin, the equilibrium binding constant of three mammalian enzymes to small intestine vesicles was measured. Bovine and rat CEase were selected since they have two and four repeat units, respectively, and these results were compared to that for the human enzyme, which contains 16 repeats. Using [^{125}I]-radiolabeled homogeneous mammalian CEase, total binding of [^{125}I]-labeled enzyme to small intestine vesicles increased in a concentration dependent and saturable manner. Nonspecific binding, determined in the presence of a 100-fold molar excess of unlabeled enzyme, increased

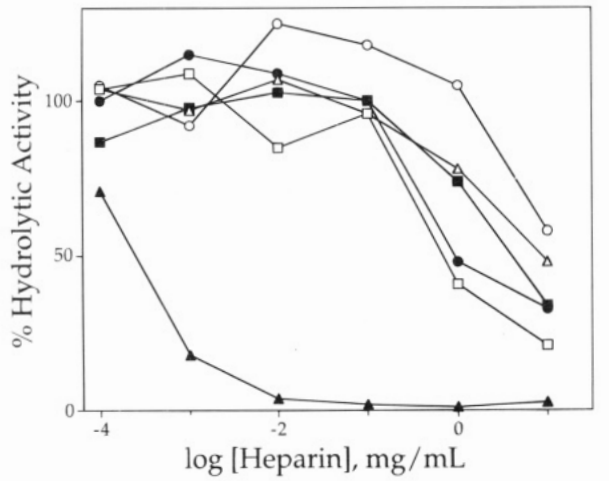

FIGURE 2: Heparin inhibition of cholesteryl [^{14}C]oleate hydrolysis by CEase from human (▲), dog (□), bovine (○), rabbit (△), rat (■), and mouse (●).

in a linear fashion, but it represented less than 20% of total binding. Scatchard analyses for all the specific binding curves produced linear plots (Bosner et al., 1988). For the bovine enzyme, K_D was 86 nM and B_{max} was 84 pmol/mg; for the rat enzyme, K_D was 100 nM and B_{max} was 24 pmol/mg; and for human CEase, K_D was 114 nM and B_{max} was 79 pmol/mg. Therefore, each of these pancreatic CEases binds to the brush border at a single saturable site, with nearly the same dissociation constant of approximately 100 nM, indicating that the C-terminal tail does not play a role in this binding interaction.

Binding of Mammalian CEases to Caco-2 Cells: Kinetic Consequences. The hydrolytic activity of CEase was determined while the enzyme was bound to Caco-2 cells. An aliquot of either bovine or human CEase was added to either confluent cells or buffer, and the rate of hydrolysis of cholesterol [^{14}C]oleate was measured using standard assay conditions. The observed activity increased as the enzyme concentration increased in the assay mixture; however, in the presence of Caco-2 cells, the activity was twice that in buffer alone (data not shown). Importantly, this enhanced activity was not due to intrinsic activity of the Caco-2 cells since no cholesterol ester hydrolytic activity was noted when the cells alone were assayed. Therefore, CEase has greater activity when bound to membrane-associated heparin.

Heparin Inhibition Differentiates Human CEase from Other Mammalian CEases. While immobilized heparin enhances the activity of CEases from all species, soluble heparin is a unique and potent inhibitor of human pancreatic CEase activity. Using homogeneous CEase from human, rat, cow, dog, mouse, or rabbit, the rate of hydrolysis of cholesterol [^{14}C]oleate was quantitated under standard assay conditions over a 20000-fold range of soluble heparin concentration (10^{-4} to 20 mg/mL). Rat, bovine, dog, mouse, and rabbit pancreatic CEases were inhibited weakly by heparin with IC_{50} values greater than 0.5 mg/mL; however, the human pancreatic enzyme or human BSSL was markedly inhibited in a concentration dependent manner with an IC_{50} of 2×10^{-4} mg/mL, or 2500-fold lower than that found for CEases from all the other species (Figure 2).

Preparation of CEase Chimeras. To determine the locus of the heparin inhibitory site(s) in human CEase, chimeras which contained amino acid sequences common to the bovine and human enzymes were prepared (Figure 3). Thus, the human C-terminus (residues 446−722) was added to the bovine enzyme at residue 445 (BH), and the bovine C-terminus (residues 446−557) was added to the human enzyme at residue 445 (HB). When expressed and purified to homogeneity (Figure 4, left panel), the chimeras and the corresponding wild type CEases (BB and HH) had the following molecular masses: 90 kDa (BH), 74 kDa (HB), 90 kDa (HH), and 77 kDa (BB). In preliminary experiments, it was found that the cell type used to express the wild type enzyme can lead to a different pattern of glycosylation than that which occurs in the pancreas, explaining the apparent discrepancy between the molecular masses of the wild type and native enzymes. Because of this different glycosylation pattern between native and wild type CEase, the chimeras and wild type enzymes were expressed, isolated, and purified from the same K293 cell line. In addition, a number of unglycosylated mutant species were prepared, and they were inhibited with the same IC_{50} as that found for the native species. For example, human CEase truncated at position 535 has no O-glycosylation, but its IC_{50} remained unchanged; inhibition of the bovine enzyme by heparin was unaffected when this protein was mutated at its N-glycosylation sites, residues 187 and 361 (data not shown).

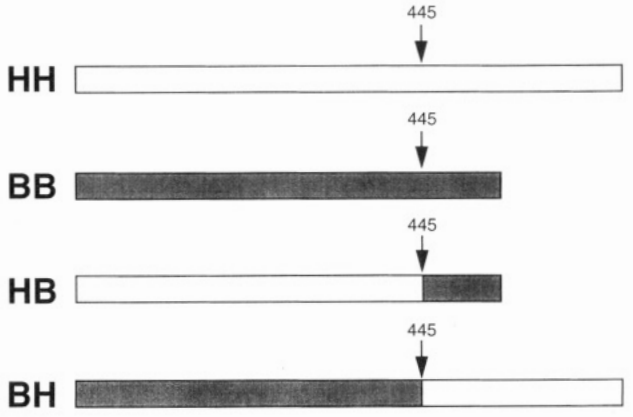

FIGURE 3: Schematic representation of the construction of the HB and BH chimeras.

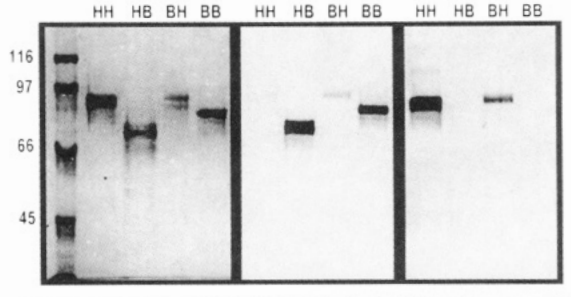

FIGURE 4: (left) SDS−PAGE of purified recombinant human CEase (HH), the chimera constructed from the human N-terminus and the bovine C-terminus (HB), the chimera constructed from the bovine N-terminus and the human C-terminus (BH), and purified recombinant bovine CEase (BB). (center) Corresponding Western blot of SDS−PAGE with antibody to the bovine enzyme. (right) Corresponding Western blot of SDS−PAGE with antibody to the human pancreatic enzyme.

When analyzed by Western blot using rabbit anti-bovine CEase antibody (Figure 4, center panel), BB and HB strongly cross-reacted, while the interaction between this antibody and either HH or BH was barely visible. Similarly, when antibody to BSSL was used (Figure 4, right panel), HH and BH strongly cross-reacted, but under the conditions used

Table 2: Kinetic Properties of rCEase and Chimerase

species	cholesterol oleate hydrolysis[a]	heparin inhibition, IC_{50}[b]
HH	102	0.0002
HB	180	0.03
BB	560	9.0
BH	380	0.1

[a] μmol (mg of CEase)$^{-1}$ h^{-1}. [b] mg/mL.

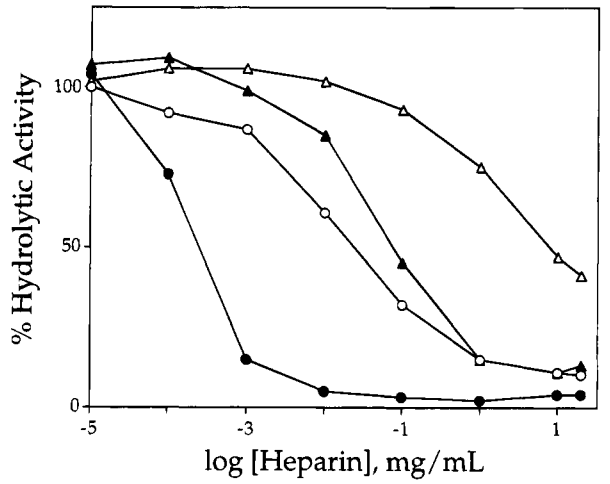

FIGURE 5: Heparin inhibition of cholesterol oleate hydrolysis of BB (△), HH (●), BH (▲), and HB (○).

here, there was little or no interaction between this antibody and either the BB or HB. These data indicate that the C-terminus contains a primary antigenic determinant for CEase, a similar finding reported by Hansson et al. (1993).

Kinetic Characterization of CEase Chimeras. The purified chimeras were then characterized kinetically by determining the rate of hydrolysis and the rate of synthesis of cholesterol ester. These values were then compared to those for the wild type human and wild type bovine enzymes. As shown in Table 2, HB's hydrolytic activity, 180 μmol mg^{-1} h^{-1}, is very similar to that for the wild type HH, 100 μmol mg^{-1} h^{-1}. Similarly, BH's hydrolytic activity, 380 μmol mg^{-1} h^{-1}, is similar to that for the wild type BB, 560 μmol mg^{-1} h^{-1}. These data indicate that hydrolytic activity is largely determined by the N-terminus, a property that is also seen for synthetic activity (data not shown).

Using purified HH and BB and the HB and BH chimeras, the rate of hydrolysis of cholesterol [^{14}C]oleate was quantitated in the presence of heparin, increasing in concentration from 10^{-5} to 20 mg/mL (Figure 5; Table 2). Similar to the native enzyme, wild type recombinant bovine CEase was inhibited weakly by heparin with an IC_{50} value of 9.0 mg/mL. Likewise, the human wild type recombinant and native enzymes displayed potent inhibition by heparin, with an IC_{50} of 2.0 $\times$ 10^{-4} mg/mL. However, replacement of the C-terminus of one species by that of the other markedly altered the pattern of inhibition. Thus, the IC_{50} for BH is 0.1 mg/mL which is 90 times less than that for BB, indicating that replacing the bovine C-terminus with the human C-terminus enhances heparin inhibition. On the other hand, when the human C-terminus is replaced with that for the bovine (HB), the IC_{50} value increases 100-fold from 2.0 $\times$ 10^{-4} to 3.0 $\times$ 10^{-2} mg/mL. However, this IC_{50} value is still over 100-fold less than that for the recombinant bovine enzyme (BB), demonstrating that the human N-terminus also contributes to the overall potent inhibition. Taken together, these data indicate that there are regions in both the C-terminus and N-terminus that contribute to the unique heparin inhibition displayed by the human enzyme.

DISCUSSION

Molecular events responsible for human cholesterol absorption have not been extensively investigated. A key step in intestinal cholesterol uptake and its subsequent esterification is the specific binding of pancreatic CEase to the epithelial cell through membrane-associated heparin (Bosner et al., 1988; Lopez-Candales, 1993). Data shown here reveal that this binding step is common to all mammalian species examined, suggesting that this mechanism is conserved as a common pathway for intestinal lipid absorption and, moreover, that the sulfated polysaccharide, heparin, plays a central role in lipid metabolism. Once the enzyme is bound to the cell, its specific activity increases slightly, indicating that CEase is fully functional in this partially immobilized state.

While CEases from all species bind to the cell membrane and are fully functional at the cell surface, the human enzyme responds very differently to soluble heparin. Thus, the hydrolytic activity of CEase from cow, pig, mouse, rabbit, and rat is unaffected by soluble heparin, even when the concentration of the glycosaminoglycan is greater than 0.5 mg/mL (Figure 2). In this regard, they resemble lipoprotein lipase, another lipolytic enzyme which binds to immobilized heparin, but which is not inhibited by soluble heparin (Patten & Hollenberg, 1969). However, human CEase is strikingly different since soluble heparin inhibits its enzymatic activity with an IC_{50} of 2 $\times$ 10^{-4} mg/mL (Figure 2), suggesting that human CEase has a unique heparin regulatory site(s), apart from the membrane binding site present in all species.

The function of the unusual C-terminus found in human CEase has been the subject of much speculation, and therefore, this structural feature was the focus for the investigation of the molecular basis for the unique inhibition observed with soluble heparin (Hansson et al., 1993; Downs et al., 1994; DiPersio et al., 1994). In this work, two observations indicate that the tail region does not have a profound effect on catalytic efficiency. First, for any given substrate, CEase has a similar catalytic efficiency from species to species (Table 1). However, since detailed sequences are not known for CEase from many of these species, it can only be inferred from their different molecular masses that their C-terminal structures are different, and hence, it is difficult to conclude from these comparative kinetic studies what role, if any, the tail region plays in catalysis. Second, a more definitive answer was provided by the construction of the two chimeras, BH and HB, which could be isolated, purified, and characterized kinetically. The hydrolytic activity of all the chimeras is within a factor of 5 of that of either the wild type human or bovine enzymes. This finding is similar to other work in which the truncated, recombinant human enzyme, formed by the removal of this sequence, had similar activity and bile salt dependence as that found in the native enzyme (Hansson et al., 1993; Downs et al., 1994). Taken together, these results indicate that the C-terminus does not interact with the charge relay system which is located in the N-terminal part of the protein (DiPersio et al., 1990, 1991, 1993).

While the C-terminal tail plays little role from species to species in determining catalytic efficiency, it does explain,

at least in part, the effects of soluble heparin on the activity of the human enzyme. Thus, replacement of the bovine C-terminus with that from the human enzyme converts a poorly inhibited CEase (IC_{50} = 9 mg/mL) into a new species whose IC_{50} is about 90-fold less than that for the recombinant bovine enzyme, but still 400-fold greater than that for the native human enzyme. These data indicate that the observed inhibition is not due to a single structural element in the human C-terminal tail, an observation that is confirmed by the inhibition pattern of the HB chimera. In this case, the IC_{50} increases over 100-fold from 2×10^{-4} mg/mL for the recombinant human enzyme to 3×10^{-2} mg/mL for HB, a striking increase but one which produces an IC_{50} value that is still far less than that found in the recombinant bovine enzyme. All these observations are consistent with the existence of two human CEase protein structural elements, one in the N-terminal section (residues 1−445) and one in the C-terminal section (residues 446−722), that interact with heparin to produce the observed potent inhibition.

A detailed comparison of the IC_{50} values permits quantitation of the contributions of each region to the observed inhibition. To determine the contribution of the human N-terminal region to heparin inhibition, the ratio IC_{50} (BH)/IC_{50}(HH) was calculated to be 1×10^{-1} mg/mL/2×10^{-4} mg/mL or 500. Therefore, a structural domain in the human N-terminus enhances heparin inhibition 500-fold over that of the same domain in the bovine N-terminus. Similarly, the enhancement factor provided by the human C-terminus is given by IC_{50}(HB)/IC_{50}(HH) or 3×10^{-2} mg/mL/2×10^{-4} mg/mL or 150. These calculations indicate that structural domains in the N-terminus (residues 1−445) and C-terminus (residues 446−722) of the human enzyme contribute about equally to the novel heparin inhibition.

The importance of at least two domains for heparin binding is a recurring theme in other studies in which this glycosaminoglycan interacts strongly and specifically with a protein (Kuhn et al., 1990; Mellet et al., 1995). For example, using chemical modification studies and the X-ray crystal structure, the mucus proteinase inhibitor was shown to have two lysine residues, one in the N-terminus and one in the C-terminus, that extended above the surface of the molecule to serve as potential anchoring points for the negatively charged heparin (Mellet et al., 1995). It is speculated that this kind of interaction can "freeze" the protein into a certain conformation. This kind of argument may be especially pertinent for lipases since their mechanism of action depends critically on the movement of a polypeptide "lid" which allows a lipid substrate to enter a deep hydrophobic crevice where the catalytic triad is located (Winkler et al., 1990; Van Tilbeurgh et al., 1993). Although the crystal structure of a mammalian CEase has not been reported, the enzyme is a homologue of *Candida rugosa* lipase, a protein in which this type of rearrangement plays a critical role (Cygler et al., 1993; Grochulski et al., 1994). Binding of heparin to the human enzyme may interfere with the movement of this lid, preventing substrate from interacting with the enzyme's catalytic machinery.

The existence of this unique heparin interaction and its relationship to cellular cholesterol uptake may bear importantly on the various conflicting clinical studies on dietary lipid absorption. Epidemiological investigations have shown that dietary cholesterol is an independent risk factor for coronary artery disease (Shekelle & Stamler, 1989). However, mechanisms relating dietary lipids and atherosclerosis have not been adequately investigated, and they have often been studied in nonhuman models. As we have shown here, CEase specific binding to intestinal membranes is common in mammalian species, and this interaction is the first step in the intestinal uptake of cholesterol and its subsequent esterification (Bosner et al., 1988; Lopez-Candales et al., 1993). Since the human enzyme interacts with soluble heparin in a unique fashion to inhibit enzymatic activity, the absorptive events facilitated by the human enzyme may be fundamentally different compared to those of other commonly studied species.

Previous animal models developed to investigate intestinal lipid absorption may therefore have limitations when results are extrapolated to understanding human cholesterol metabolism. In this regard, recent findings with acyl coenzyme A:cholesterol acyl transferase, another enzyme implicated in intestinal cholesterol absorption, are instructive. Specific acyl coenzyme A:cholesterol acyl transferase inhibitors have been developed, and initial animal studies suggested that serum cholesterol levels were significantly reduced in the presence of these inhibitors (Largis et al., 1989). However, data for human phase I investigations have shown no significant effect of these compounds in man (Harris et al., 1990). This suggests the necessity for the development and investigation of more precise models of lipid absorption in man since biochemical pathways of lipid absorption appear to vary in a species dependent manner, with both acyl coenzyme A:cholesterol acyl transferase and CEase being functionally quite different in humans than in currently studied laboratory animals.

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