Purification and properties of a cholesteryl ester hydrolase from rat liver microsomes

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Abstract **This report describes a purification procedure for a cholesteryl ester hydrolase (CEH) from female rat liver microsomes, and some structural, immunological, kinetic, and regulatory properties of the enzyme that distinguish the microsomal CEH from other hepatic cholesteryl ester-splitting enzymes. CEH was purified 12.4-fold from re-isolated microsomes using sequential solubilization by sonication, polyethylene glycol precipitation, fractionation with hydroxyapatite, anion exchange chromatography, and chromatography on hydroxyapatite, with an overall yield of 3.2%. CEH activity was purified 141-fold over nonspecific esterase activity and 56-fold over triacylglycerol lipase activity. In sharp contrast with most esterases and lipases, CEH did not bind to concanavalin A-Sepharose and heparin-Sepharose. After polyacrylamide gel electrophoresis, the purified enzyme exhibited two silver-stained bands, but only the protein electroeluted from the low mobility band had CEH activity. Affinity-purified polyclonal antibodies raised to electroeluted CEH inhibited 90% of the activity of liver microsomal CEH and reacted with a 106 kDa protein band on Western blot analysis. This 106 kDa CEH contains a unique N-terminal amino acid sequence. The purified enzyme had optimal activity at pH 6 and no taurocholate requirements, and was inhibited by the serine active site inhibitor phenylmethylsulfonyl fluoride and by free sulfhydryl specific reagents. It hydrolyzed cholesteryl oleate much more efficiently than trioleine, and hydrolytic activity with *p*-nitrophenyl acetate was higher than with *p*-nitrophenyl butyrate. These results indicate that rat liver microsomes contain a bile salt-independent catalytic protein that is relatively specific for cholesteryl ester hydrolysis.**—Cristóbal, S., B. Ochoa, and O. Fresnedo. **Purification and properties of a cholesteryl ester hydrolase from rat liver microsomes.** ***J. Lipid Res.*** **1999.** 40: **715–725.**

Supplementary key words bile salt-independent cholesteryl esterase • lipase • rat liver endoplasmic reticulum

Cholesteryl ester hydrolases (CEH) are found ubiquitously in many tissues, and it is generally assumed that their function, activity, and regulation depend on the enzyme location. Whereas the CEH from pancreas is a prerequisite for full absorption of dietary cholesterol and vitamins, isoenzymes in adrenals and gonads provide free cholesterol for steroid hormone synthesis. One of the best known cholesteryl esterases is the pancreatic CEH, bile salt-stimulated lipase or carboxyl ester lipase (for a review, see ref. 1), which is considered a model lipase. Most lipases are glycoproteins; they contain a catalytic triad of Ser / His / Asp or Glu, and have broad substrate specificities (1–3); i.e., the pancreatic CEH hydrolyzes, in addition to cholesteryl esters, tri-, di-, monoacylglycerols and phospholipids (3). In the liver, the central organ in maintaining body cholesterol homeostasis, cholesterol exists both in a free metabolically active form and as cholesteryl esters that may be stored, hydrolyzed, or secreted as lipoprotein core components. Cholesteryl ester hydrolase activities have been reported in the lysosomal, cytosolic, and microsomal fractions of the liver. Lysosomal acid lipase is involved in the hydrolysis of cholesteryl esters and triacylglycerols delivered to the hepatocytes via receptor-mediated endocytosis of lipoproteins (4). The acid lipase from several species, including the human liver (5), has been purified, characterized, and cloned. Ghosh and Grogan (6) reported the purification and characterization of the major cytosolic CEH from rat liver. The enzyme is different from the pancreatic esterase in biochemical and immunological properties (6). The cDNA for this neutral cytosolic CEH has been cloned, sequenced, and expressed (7), and found to have higher homology with rat liver microsomal pI 6.1 esterase (8) than with pancreatic CEH (9). Increasing evidence suggests that CEH activity in liver cytosol is involved in maintaining the pool of metabolically active free cholesterol. Several reports have documented the fact that microsomes display high CEH spe-

Abbreviations: CEH, cholesteryl ester hydrolase; ConA, concanavalin A; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic) acid; DTT, 1,4-dithiothreitol; IAA, iodoacetic acid; NE, nonspecific esterase; NEM, N-ethylmaleimide; PEG, polyethylene glycol 6000; pHMB, *p*-hydroxy-mercuribenzoic acid; PMSF, phenylmethylsulfonyl fluoride; PNP, *p*-nitrophenyl; PVDF, polyvinylidene difluoride; TGL, triacylglycerol lipase. Enzymes: Carboxylesterase (EC 3.1.1.1); triacylglycerol lipase (EC 3.1.1.3); cholesteryl ester hydrolase (EC 3.1.1.13); retinyl ester hydrolase (EC 3.1.1.21); hepatic lipase (EC 3.1.1.32).

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cific activity in the absence of bile salts (10–12). However, whether one or more proteins are responsible for this activity and the physiological importance of the microsomal CEH in the complex cholesteryl ester synthesis–hydrolysis cycle remain to be answered. Until now, most studies have focused on the characterization of the neutral CEH activity in microsomal vesicles. CEH was reported to be mainly embedded in RNA-rich microsomal membranes (13), and to be regulated by postnatal development (14), circadian rhythm (15), and several activators of the cAMP-dependent protein kinase (16, 17), the protein kinase C (17) and the AMP-activated protein kinase (18).

Hepatic carboxylesterases are involved in the biotransformation of xenobiotics and natural substrates by hydrolyzing compounds containing an ester, thioester, or amide group (19). Several investigators have demonstrated that liver endoplasmic reticulum contains multiple isoforms of carboxylesterases with broad and overlapping substrate specificities (20–24). Substrates include acylcarnitines, palmitoyl-CoA, mono- and diacylglycerols (reviewed in ref. 24) and triacylglycerols (25).

The goal of our research was to elucidate whether in rat liver microsomes there is an enzyme with dominant hydrolytic activity on cholesteryl esters or whether the CEH activity is the convergence of secondary activities from a number of enzymes located there exhibiting nonspecific esterase or lipase activities. Described below is a purification strategy for a cholesteryl ester hydrolase from rat liver microsomes using conventional protein purification procedures, preparation of neutralizing antibodies, and characterization of some properties of this enzyme. On the basis of differential purification, and structural, immunological, kinetic and regulatory features, the purified CEH can be clearly differentiated from the cytosolic CEH, the lysosomal CEH, the hepatic lipase, the retinyl ester hydrolase, the triacylglycerol lipase, and nonspecific microsomal carboxylesterases.

This work provides the basis for determining the metabolic functions of this enzyme.

MATERIALS AND METHODS

Materials

Chromatography matrices and columns, including HiTrap Q, HiTrap NH-Sepharose, heparin-Sepharose, and concanavalin A-Sepharose (ConA-Sepharose) were purchased from Pharmacia. Ceramic hydroxyapatite cartridges, type Econo-Pac CHT-II, were from Bio-Rad. Polyethylene glycol 6000 (PEG), type III hydroxyapatite, cholesteryl oleate, trioleine, *p*-nitrophenyl (PNP)-acetate, PNP-butyrate, CEH from bovine pancreas, goat anti-rabbit IgG, α-naphthylacetate and molecular weight markers were from Sigma. Cholesteryl [1-^{14}C]oleate (55 mCi/mmol) and [1-^{14}C] oleic acid (57.4 mCi/mmol) were obtained from Amersham, and [carboxyl-^{14}C]triolein (110 mCi/mmol) was from DuPont. Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore, egg lecithin from Lipid Products, and acrylamide and *bis*-acrylamide from BDH. Unless specified, other reagents were of the highest quality obtained from either Sigma or Boehringer Mannheim.

Measurement of cholesteryl esterase, triacylglycerol lipase, and nonspecific esterase activity

Enzyme assays were performed with protein concentrations within the linear range of the assay. They were in the range of 10–300 μg for CEH, of 40–200 μg for triacylglycerol lipase (TGL), and of 5–20 μg for nonspecific esterase (NE); the given values correspond to purified and microsomal protein. The radiometric assay of neutral CEH activity was carried out essentially as described previously in detail (11). Briefly, protein was incubated in 0.2 ml solution containing 100 mm potassium phosphate buffer, pH 6.0, 30 nmol cholesteryl [1-^{14}C]oleate (12 mCi/mmol) incorporated in mixed micelles with lecithin and taurocholate in a 10:47:25 molar ratio and 15 mm hydroxypropyl β-cyclodextrin. Solutions of the tested compounds were prepared as indicated previously (26) and appropriate controls were performed. CEH activity is expressed as pmol of cholesteryl oleate hydrolyzed per min and mg of protein. In order to determine TGL activity, the enzyme was incubated at 37°C for 30 min in a final 0.2 ml volume of 100 mm Tris-HCl buffer, pH 8.0, with 15 mm hydroxypropyl β-cyclodextrin and 150 nmol [carboxyl-^{14}C]triolein (3.6 mCi/mmol) incorporated in micelles with lecithin and taurocholate in a 10:17:8 molar ratio. The released labeled fatty acids were extracted and processed as described previously (11). TGL activity is expressed as pmol of triolein hydrolyzed per min and mg of protein. PNP-acetate is a common substrate for the determination of carboxylesterase activity. Nonspecific esterase activity was thus determined by monitoring the rate of *p*-nitrophenol formation (nmol/min × mg of protein) at 37°C from 250 nmol PNP-acetate in 500 μl of 50 nm Tris-maleate buffer, 15 mm cholate, pH 8.0, according to the procedure of Harrison, Smith, and Goodman (27). Protein was determined by the dye-binding method of Bradford (28) with BSA as the standard.

Purification of CEH from rat liver microsomes

The CEH purification procedure from the livers of three animals is summarized in Table 1. All steps were carried out at 4°C except the chromatographies that were done at 8°C.

Steps 1 and 2: Preparation of washed microsomal fraction. We used female Sprague-Dawley rats (200 g) maintained under controlled lighting (lights on from 08:00 to 20:00), and fasted for 24 h before starting the experiments at 08:00. With these conditions, the potential contribution of lysosomal CEH activity is minimized (15). Livers (24 g) were homogenized in 3 vol of 250 mm sucrose solution containing 0.5 mm DTT and 0.5 mm EDTA, pH 6.8. Microsomes were obtained by differential centrifugation (1,000 *g*, 10 min; 22,000 *g*, 10 min; 105,000 *g*, 60 min). To remove adhered cytosolic proteins, the membrane pellet was washed at pH 8.0 in 150 mm Tris-HCl buffer, 0.5 mm DTT, and 0.5 mm EDTA.

Steps 3 and 4: Solubilization by sonication and PEG precipitation. The reisolated microsomes were resuspended at 2 mg/ml of protein in buffer A, consisting of 20 mm Tris-HCl, 20% (w/v) glycerol, 0.5 mm DTT, and 0.5 mm EDTA, pH 8.0. The suspension, in 50-ml portions, was sonicated at a probe amplitude of 8 microns with 30 cycles of 15 s on/15 s off (MSE Soniprep 150 Ultrasonic Disintegrator, 9.5 mm tip diameter probe) and then centrifugated at 105,000 *g* for 60 min. The supernatant retained 95% of the original microsomal CEH activity, but only one-third of the protein of microsomes. Solubilized CEH can be stored at −80°C for 1 month without loss of activity (29). The CEH protein was precipitated by adding to solubilizate 1 vol of 20 mm Tris-HCl, 0.5 mm DTT, 5 mm urea, 14% (w/v) polyethylene glycol 6000, and 150 mm KC1, pH 8.0. After a 60-min equilibration period with shaking, the mixture was centrifuged (22,000 *g*, 20 min). The pellet was redissolved in buffer A at 2 mg/ml of protein and resolubilized by sonication as described above.

Step 5: Fractionation with hydroxyapatite. Hydroxyapatite was added to the solubilized PEG precipitate to give a final concentration of 0.1% (w/v). The mixture was gently shaken for 30 min and then centrifuged (10,000 *g*, 10 min), and the supernatant was collected and passed through a 0.22-μm pore filter.

Steps 6 and 7: HiTrap Q and hydroxyapatite chromatography. SMART micropurification equipment (Pharmacia Biotech) was used for the chromatographies. The filtrate was applied to a 5-ml HiTrap Q column equilibrated with buffer B (20 mm Tris-HCl, 5 mm urea, and 0.5 mm DTT, pH 8.0), and eluted with a stepwise gradient of 0–25% and 0–50% of buffer C (buffer B + 1 m KCl) at a flow rate of 700 μl/min. The proteins containing the CEH activity were eluted with 50% buffer. The sample, after dilution to 200 mm KCl, was chromatographed on an hydroxyapatite cartridge Econo-Pac CHT-II (1 ml) equilibrated with 10 mm potassium phosphate buffer, containing 5 mm urea, 0.5 mm DTT, and 200 mm KCl, pH 8.0. Successive elution was carried out with 50% and 100% of 200 mm potassium phosphate buffer, 5 mm urea, 0.5 mm DTT, 1 m KCl, pH 8.0, at a flow rate of 500 μl/min. The purified protein eluted with approximately 100 mm potassium phosphate buffer, 600 mm KCl, 5 mm urea, 0.5 mm DTT.

Purified CEH was unstable in storage at 4°C, −20°C or −80°C; however, 80–50% of activity was retained for 2–7 days with storage at −20°C in the eluting buffer (pH 8.0) supplemented with 0.5 % (w/v) BSA.

Production of affinity-purified polyclonal antibodies

For the production of polyclonal antibodies against bovine pancreatic CEH, New Zealand white rabbits (2.5 kg) were injected subcutaneously with 30 μg of pure protein emulsified with complete (first injection) or incomplete (subsequent injections) adjuvant at 2-week intervals. For the production of antibodies against the rat liver microsomal CEH, rabbits were injected twice with 200 μg of the CEH purified as described above. The fraction from the last step of purification was subjected to PAGE and the protein from the slice with CEH activity was electroeluted as described below and used for the second and subsequent booster immunization (20 μg protein). One week after the second booster, the rabbits were bled by ear puncture to collect serum, and the serum was screened for its ability to detect the protein in a Western blot. A booster was given in incomplete adjuvant 10 days prior to subsequent bleedings. Antibodies were purified from antiserum by immunoaffinity chromatography on a Hitrap NH-Sepharose column that had been coupled on the pure protein according to the manufacturer's intructions.

Electrophoretic techniques and immunoblotting

To identify protein bands with nonspecific carboxylesterase reactivity, proteins were separated by PAGE under nondenaturing conditions on a 5–10% polyacrylamide gradient (30) and gels incubated with the esterase substrate α-nafthylacetate (31). To identify protein bands with CEH activity, duplicate samples of protein were separated by PAGE as above, and a part of the gel was stained with silver nitrate (0.2%, w/v). From the lanes of an unstained gel, six 1-cm slices were excised and placed in separate tubes. Protein was electroeluted (Bio-Rad Electroeluter) from the slices at 250 mV and 4°C for 12 h into 25 mm Tris, 0.2 m glycine, pH 8.3. The CEH activity in eluates was measured following the standard assay except that incubation time was 60 min and cholesteryl [^{14}C]oleate specific activity was 24 mCi/mmol. For Western blot analysis, marker proteins and the purified protein, along with pancreatic CEH, were subjected to 10% SDS-PAGE under reduced denaturing conditions (30), and either the gels were stained with 0.1% (w/v) Coomassie Blue R-250 or the proteins were transblotted on PVDF membranes at constant voltage (15 V) (Bio-Rad Mini Trans Blot Cell). Dilutions of 1:1000 (v/v) of purified rabbit IgG for either the purified protein or pancreatic CEH and 1:2000 of horseradish peroxidase-conjugated goat anti-rabbit IgG were used. The blots were developed with freshly prepared peroxidase substrates, hydrogen peroxide and 4-chloro-1-naphthol.

Amino acid composition and N-terminal sequence analysis

The protein band corresponding to Coomassie-stained 106 kDa was cut from the PVDF membrane and used to determine the amino acid composition. For N-terminal amino acid sequencing, the purified CEH was electrophoresed on a 10% SDS-polyacrylamide gel under reduced denaturing conditions, and the 106 kDa protein was electroeluted into 25 mm Tris, 192 mm glycine, 0.1% SDS (60 min, 80 V, Bio-Rad Mini Whole Gel Eluter), precipitated with 10 vol. of cold ethanol–diethyl ether 1:1 (by vol) and dissolved in 100 μl of acetic acid–acetonitrile–trifluoroacetic acid–H_2O 20:32:0.04:47.6 (by vol). The N-terminal sequence was determined after blotting on a PVDF membrane by automated Edman degradation using an Applied Biosystems gas-phase microsequencer with on-line phenylthiohydantoin analyzer.

Immunoinhibition of CEH activity

Affinity-purified anti-microsomal CEH antibodies (2–7.5 μg) were incubated with rat liver microsomes (10 μg of protein) or the purified protein (4 μg) at 37°C with vigorous shaking for 15 min in 100 μl of 10 mm Tris, 150 mm NaCl buffer, pH 7.4. The volume was then brought to 200 μl with 100 mm potassium phosphate buffer, pH 6.0, and substrate was added for the assay of CEH activity as described above. Affinity-purified anti-pancreatic CEH antibodies (2–7.5 μg) served as negative controls.

RESULTS

Purification of CEH from rat liver microsomes

The differential CEH purification was assessed by testing cholesteryl esterase, triacylglycerol lipase, and nonspecific esterase activity. A summary of the results of the purification of microsomal CEH from the livers of three rats, described in Materials and Methods, is shown in **Table 1**. To date, 20 preparations of the enzyme have been carried out with results very comparable to those shown. The purification scheme includes sequential solubilization by sonication, polyethylene glycol precipitation, fractionation with hydroxyapatite, anion exchange chromatography, and chromatography on hydroxyapatite.

In an earlier work we reported poor resolution when attempting to solubilize CEH from microsomes with several ionic and nonionic detergents, with the exception of the nonionic detergent *n*-octyl β-d-glucopyranoside, which yielded total solubilization and recovery of CEH activity (29). Nevertheless, we have adopted here solubilization of CEH by disrupting microsomes by sonication, as this allows us to recover in less than 8 min essentially all CEH activity from 100 mg of microsomal protein in a solubilization agent-free solution.

Precipitation of CEH activity with organic solvents or ammonium sulfate rendered only very little activity and the pellets were hardly retrosolubilized (data not shown). This suggests a highly hydrophobic character for the CEH protein when separated from lipid components. Precipitation with 7% (w/v) PEG yielded 15.7% of microsomal pro-

TABLE 1. Purification of cholesteryl ester hydrolase from rat liver microsomes

Step	Protein	Specific Activity			Purification	Yield	CEH/TGL	CEH/NE
		CEH	TGL	NE				
	mg	*pmol/min × mg*			*fold*	*%*	*fold*	*fold*
Microsomes	154	80.5	521.3	2501	1	100	1	1
Washed microsomes	114	114.0	371.2	2065	1.41	105	2.0	1.7
Sonication supernatant	52.7	224.5	900.8	2044	2.78	95.4	1.6	3.4
PEG 6000	24.2	230.5	973.5	582	2.86	45.1	1.5	12.3
Hydroxyapatite fractionation	16.7	265.2	851.5	477	3.29	35.8	2.0	17.3
HiTrap Q	1.78	676.6	554.4	315	8.40	9.69	7.9	66.7
Hydroxyapatite	0.39	1000.6	116.4	220	12.4	3.21	55.8	141.3

The values for the ratios CEH/TGL and CEH/NE represent fold- the corresponding ratio in microsomes, which were 0.154 and 0.032, respectively.

tein with 45% of CEH activity recovered and CEH activity was enriched 12-fold versus NE activity. We believed that the sample obtained in this step, the PEG fraction, fulfilled the requirements for being chromatographed. It was soluble, membrane debris-free, with a pH of 8.0, which is suitable for an anion-exchange chromatography, and CEH activity was retained for 1 week in storage at −20°C or −80°C. However, even after drastic washes, many contaminants remained strongly adsorbed to chromatography supports, and columns became useless with one passage. To remove contaminants, the PEG fraction was treated with 0.1% (w/v) hydroxyapatite for 30 min. Equilibration time and hydroxyapatite amount were found to be essential factors to balance impurities retained by and CEH protein adsorbed on hydroxyapatite. This unusual step, though it did not render a substantial increase in CEH specific activity, was crucial for CEH purification, as it rendered a sample suitable for chromatography and columns turned out to be reusable tools after appropriate regeneration processes.

From the anion exchange column of HiTrap Q Sepharose, the highest specific activity CEH was recovered in the 50% elution buffer, whereas most proteins with the highest activity NE eluted with 25% buffer. This step yielded 1.2% of microsomal protein with 9.7% of CEH activity, which was purified 66.7-fold regarding NE activity. The sample should be maintained in a high salt concentration solution to keep CEH fully active at 4°C until the next chromatography. After application to ceramic hydroxyapatite and further elution with 100 mm potassium phosphate/600 mm KCl, the CEH protein was purified 12.4-fold with an overall yield of 3.2%, and CEH activity was purified 56-fold versus TGL activity and 141-fold versus NE activity. Attempts for further purification were frustrated by the instability of catalytic activity.

During the development of the purification scheme for rat liver microsomal CEH, samples at several stages of purification were analyzed by affinity chromatography on heparin-Sepharose and ConA-Sepharose. In experiments not shown, and conducted essentially as reported by Robbi and Beaufay (21), CEH was found not to absorb ConA, and the run-through fractions contained about 80–90% of both the loaded protein and the CEH activity. Also, CEH behaved as a heparin-unreactive protein, in sharp contrast to several secretion lipases, such as the hepatic lipase (32), and resident lipases, such as the triacylglycerol lipase (25, 33) and the retinyl ester hydrolase (34).

CEH activity displayed extreme and increasing instability throughout the purification steps, either by dilution, desalting, or even by the physical process itself of passing through a chromatography matrice. Moreover, attempts to subject the purified protein to gel permeation chromatography resulted in a total loss of activity. In experiments not shown, purified protein was found to be unstable with storage. At 4°C for 24 h in 100 mm phosphate buffer, 5 mm urea, 0.5 mm DTT, and 500 mm KCl, pH 8.0, purified CEH retained 40% of its original activity, and more than 80% of activity was lost after 4 days at 4°C, −20°C, or −80°C. Addition of 0.5% (w/v) BSA to the purified protein resulted in moderate stabilization of CEH, and 80% of its original activity was retained after 2 days and 50% after 1 week at −20°C. This was used for standard storage. Other potential stabilizers, such as glycerol, EDTA, urea, and KCl, were tested but none of them maintained enzyme activity.

Electrophoretical and immunological characterization

The key-step in the separation of cholesteryl esterase from nonspecific esterase activity was the anion exchange chromatography on HiTrap Q Sepharose. In the biochemical analysis, most CEH activity eluted with 50% and most NE activity eluted with 25% of the elution buffer. This was confirmed by staining for esterase activity the samples representing steps in the purification of CEH after protein separation by PAGE on a 5–10% polyacrylamide gel (**Fig. 1**). Little esterase activity was detected in the HiTrap Q eluate and none in the purified protein, even though 15 μg (lane 4) and 20 μg (lane 5) protein were loaded into gels, whereas a great number of reactive bands were found in previous stages of purification; i.e., 3 μg protein of the solubilization supernatant was loaded in lane 1. When the active fraction was subjected to PAGE in the absence of SDS, two major silver-stained protein bands with different intensity were obtained (**Fig. 2**, lane). To elucidate which band represented the CEH activity, slices were excised from lanes of an unstained gel, and the proteins were electroeluted from slices and assayed for enzyme activity. Only the protein from slice 2, that corresponded to the band with low mobility and low intensity, was catalytically competent for CEH activity (Fig. 2, bar graph). It was

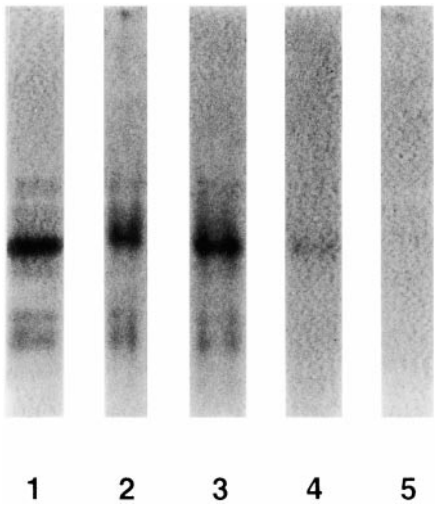

Fig. 1. Representative carboxylesterase stain of various steps of purification in the rat liver microsomal CEH purification procedure. Aliquots representing steps in the purification of CEH from rat liver microsomes were electrophoresed on a 5–10% polyacrylamide gel under non-denaturing conditions and the gels were then incubated with α-naphthylacetate as described in the text. Lane 1, 3 μg solubilization extracted supernatant; lane 2, 10 μg hydroxyapatite fractionation; lane 3, 10 μg HiTrap Q 25% eluate; lane 4, 15 μg HiTrap Q 50% eluate; lane 5, 20 μg purified protein.

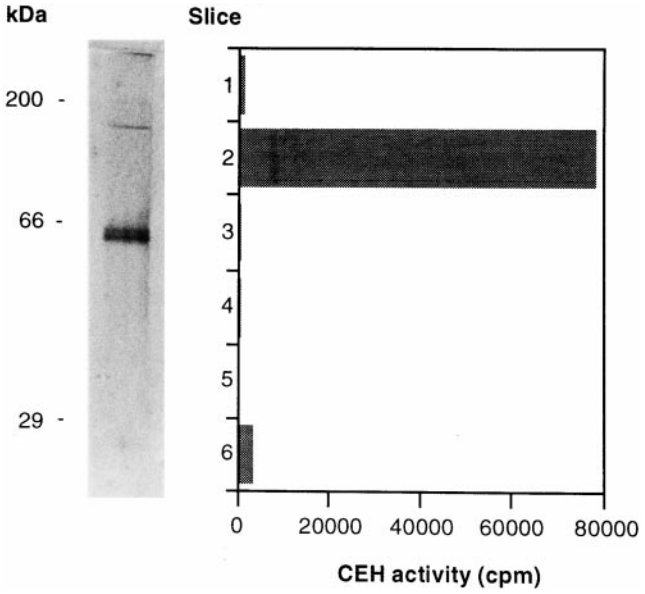

Fig. 2. PAGE analysis of the purified rat liver microsomal CEH and recovery of CEH activity. Purified CEH samples (5 μg) were subjected to electrophoresis on a 5–10% polyacrylamide gel under non-denaturing conditions. A part of the gel was stained with silver nitrate. The bar graph shows CEH activity, (as [^{14}C]oleate cpm released in 60 min) in protein recovered from slices excised from unstained lanes, plotted as a function of the slice number from the top of the gel. This is aligned with the stained gel. Molecular mass standards in kDa are listed on the left: α-amylase, 200 kDa; bovine serum albumin, 66 kDa; carbonic anhydrase, 29 kDa.

notable that the band with higher reactivity to silver nitrate did not contain CEH activity. Taking into account that about 6 and 8% of the protein loaded was recovered in the eluates of slices 2 and 3, the eluate from the slice 2 was at least enriched 600-fold in specific activity CEH. In a similar experiment, *p*-nitrophenylesterase activity of the eluates was also determined, and we found that the eluate from the slice 3 had an esterase activity slightly higher (about 35%) than that of the eluate of the slice 2 (data not shown).

The purified fraction was examined for its reactivity with affinity-purified rabbit polyclonal antibodies raised against the electroeluted CEH protein on Western blots. The antibody specifically reacted with one protein band of 106 kDa in microsomes and the purified CEH preparation (**Fig. 3**, lane 2), and exhibited no cross-reactivity with proteins from rat liver cytosol or ammonium sulfate precipitates of cytosol (not shown). These results are consistent with monospecificity of the polyclonal antibodies for the CEH protein from rat liver microsomes, and suggest that this enzyme is immunologically different from other proteins in rat liver microsomes and cytosol. Moreover, antibodies to pancreatic CEH did not cross-react with proteins in the purified preparation (Fig. 3, lane 3), thus supporting the view by Ghosh and Grogan (6) that the major neutral CEH isoforms of liver and pancreas are different enzymes.

The capacity of antibodies against the eluted CEH protein to inhibit microsomal CEH activity was then examined and found to neutralize up to 90% CEH activity in rat liver microsomes and 100% CEH activity in the purified preparation (**Fig. 4**). Nonspecific inhibition was tested with antibodies to pancreatic CEH. Under identical conditions, these antibodies had little inhibitory effect on liver microsomal CEH activity, 70–80% activity remaining (data not shown). This strongly suggests that the CEH enzyme purified by us is the predominant activity present in rat liver microsomes accounting for at least 70% of the CEH activity, and relates CEH activity to a 106 kDa microsomal protein.

Amino acid composition and N-terminal sequencing analysis

The amino acid composition of the 106 kDa CEH is given in **Table 2**. A remarkable feature is the existence of a considerable number of serine and cysteine residues, 63 and 30 out of the 860 amino acid residues estimated in the protein, respectively. The molecular identity of the microsomal 106 kDa protein was determined by performing amino acid sequencing of the N-terminal region (Fig. 3, right). By searching the available databases at the National Center for Biotechnology Information with BLAST, we found no proteins that contain amino terminal or downstream sequences identical or very similar to the microsomal MVPHGA. The N-terminus of the here-described rat liver microsomal CEH particularly differs from the sequences MRLYPL, unique for the rat liver cytosolic CEH (7), MKMRFL, unique for the human lysosomal acid lipase (5), AKLGXV, shared by pancreatic cholesterol esterases (i.e., see refs. 3, 9), and GXPXSP, shared by a number of hepatic nonspecific carboxylesterases (i.e., see refs. 8, 21) and retinyl ester hydrolases (35).

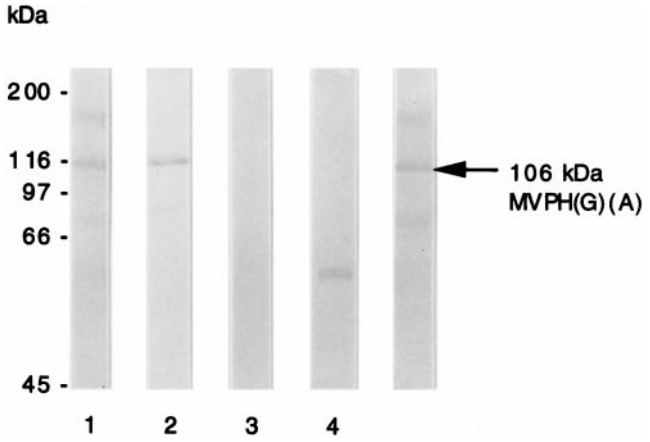

Fig. 3. Western blot analysis and N-terminal amino acid sequencing of the purified rat liver microsomal CEH. The purified protein (20 μg) was resolved on a 10% polyacrylamide gel, and either the gel was stained with Coomassie Blue (lanes 1 and 5) or proteins were transferred to PVDF membranes and incubated with affinity-purified rabbit anti-rat liver microsomal CEH IgG (lane 2) or anti-bovine pancreatic CEH IgG (lane 3). Lane 4, immunoblot of bovine pancreatic CEH (2 μg) with anti-pancreatic CEH IgG. Immunoreactive bands were visualized by incubating the blots with goat anti-rabbit IgG conjugated to peroxidase and developed as described in the text. Molecular mass standards in kDa are listed on the left. The N-terminal sequence of the 106 kDa protein band is shown on the right-hand side. The standard single-letter code for amino acids is used. Amino acids in parentheses indicate ambiguous calls on the sequencer run.

TABLE 2. Amino acid composition of the rat liver microsomal CEH

Amino Acid	Mol (%)	Amino Acid	Mol (%)
Ala	8.04	Lys	4.68
Arg	4.68	Met	2.79
Asx[a]	10.22	Phe	3.59
Cys	3.75	Pro	3.08
Glx[b]	13.24	Ser	7.76
Gly	13.97	Thr	4.85
His	2.17	Trp	ND
Ile	2.97	Tyr	1.59
Leu	6.73	Val	5.87

The data represent the average of two separate experiments; ND, not determined.
[a] Asp + Asn residues.
[b] Glu + Gln residues.

pH optimum, kinetic properties, and taurocholate effect

The activity of purified CEH was measured in buffers ranging from pH 4.2 to 8.8. As shown in **Fig. 5**, this enzyme exhibited a single broad peak of activity over the range pH 5.4–6.4, with optimal activity at pH 6, which was used in the standard assay. The physiological meaning of this slightly acidic optimum pH for the purified enzyme is unkown. It is likely that when the enzyme is separated from lipid components, it may show kinetic characteristics different from those shown by the native enzyme. In fact, the pH profile for CEH activity in rat liver microsomes showed a smaller peak of activity in the neutral pH range (6–8) (10), and most routine assays are performed at pH 6.8–7.4 (13–18).

Hydrolytic activity was monitored as a function of concentration of the defining substrate cholesteryl oleate, and of triolein, PNP-acetate and PNP-butyrate. Results are illustrated in **Fig. 6**. The purified CEH hydrolyzed cholesteryl oleate much more efficiently than triolein. Cholesteryl oleate hydrolysis increased rapidly up to 50 μm substrate with saturation at 100–150 μm, thus giving a hyperbolic curve to which Michaelis kinetics could be applied. The kinetic parameters of CEH with cholesteryl oleate varied moderately from preparation to preparation. The maximal reaction velocity averaged 1200 pmol of cholesteryl oleate hydrolyzed × min^{-1} × mg $protein^{-1}$, with an apparent K_m that averaged 10 μm. Triolein was hydrolyzed at a very low rate unless substrate was added at concentrations in the millimolar range, and saturation kinetics may not have been achieved. At 100–150 μm, concentrations of substrate commonly used, the hydrolysis rate of cholesteryl oleate is higher than 100-fold that of tri-

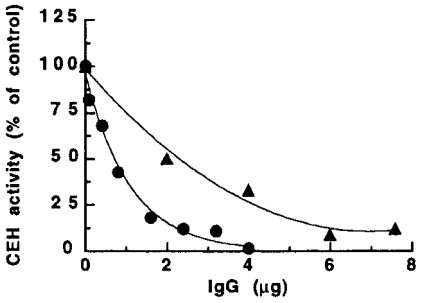

Fig. 4. Inhibition of CEH activity in rat liver microsomes and the purified protein by rabbit anti-microsomal CEH antibodies. Microsomes (10 μg of protein, ▲) or the purified protein (4 μg, ●) were incubated with affinity-purified anti-microsomal CEH antibodies for 15 min before measuring CEH activity as described in Materials and Methods. Data points represent means of duplicates, and the experiment was repeated three times with essentially the same results. The 100% control activity in microsomes and the purified protein averaged 85 and 335 pmol of cholesteryl oleate hydrolyzed × min^{-1} × mg $protein^{-1}$, respectively.

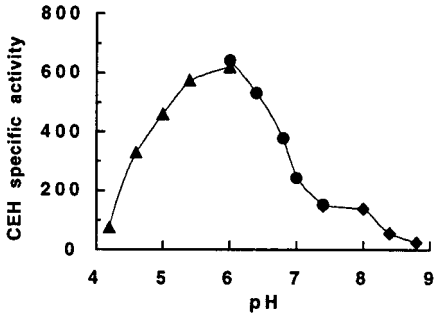

Fig. 5. pH dependence of the purified rat liver microsomal CEH. Buffers used at a final concentration of 100 mm were: acetate (▲), for pH 4.2–6; phosphate (●), for pH 6–8; and Tris-HCl (◆), for pH 8–8.8. Except for that, standard enzyme assays were conducted, and CEH activity is expressed as pmol of cholesteryl oleate hydrolyzed × min^{-1} × mg $protein^{-1}$. The data represent the average of duplicate determinations and are representative of three separate experiments.

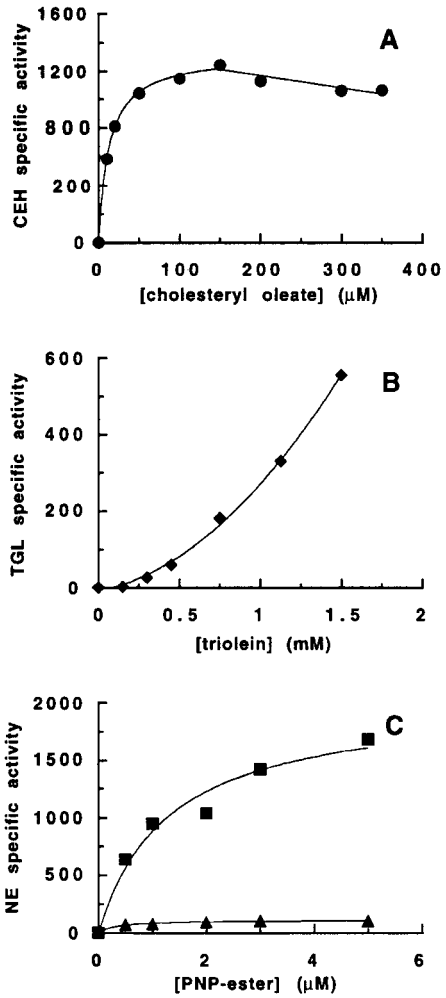

Fig. 6. Concentration dependence of cholesteryl oleate, triolein, PNP-acetate, and PNP-butyrate hydrolysis by the purified rat liver microsomal CEH. The enzyme was assayed with indicated concentrations of cholesteryl oleate (panel A), triolein (panel B), PNP-acetate (■), and PNP-butyrate (▲) (panel C) as described in the text. Activities are expressed as A) pmol, B) pmol, and C) nmol of substrate hydrolyzed × min^{-1} × mg $protein^{-1}$. The data represent the average of duplicate determinations and are representative of three separate experiments.

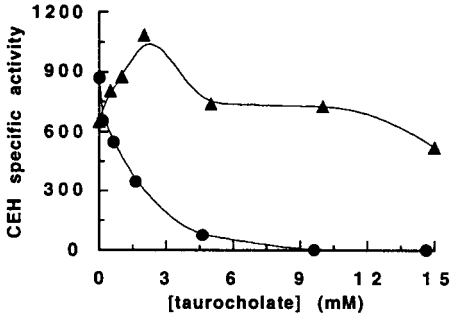

Fig. 7. Effect of taurocholate on the activity of the purified rat liver microsomal CEH. The enzyme was treated with the indicated concentration of sodium taurocholate for 15 min at 37°C prior to assaying CEH activity with the substrate cholesteryl oleate added either in standard micelles (●) or in 0.05% Triton X-100 (▲). Enzyme activity is expressed as pmol of cholesteryl oleate hydrolyzed × min^{-1} × mg $protein^{-1}$. The data represent the average of duplicate determinations and are representative of three separate experiments.

olein. In experiments not shown, assessing the effect of the amount of purified CEH and the time dependence of purified activity on hydrolysis of cholesteryl oleate and triolein, the activity was linear in the range of 2–20 μg for cholesteryl oleate and of 5–25 μg for triolein, and hydrolysis for both cholesteryl oleate and triolein were linear for up to 60 min. Hydrolysis of triolein by the purified fraction was undetectable when estimated by the methods followed previously (32, 33) (data not shown).

Hydrolytic activity of purified CEH was also determined for PNP-acetate and PNP-butyrate. In contrast to cytosolic CEH (36), more activity was measured with PNP-acetate than with PNP-butyrate. Both yielded hyperbolic saturable profiles of activity, to which Michaelis kinetics could be applied. The catalytic efficiency, as measured by V_{max}/K_m, for PNP-acetate (ca. 900) was 3-fold that for PNP-butyrate (ca. 300).

In an earlier work we showed that the CEH specific activity in microsomes was higher than previously reported when the substrate cholesteryl oleate is presented to the enzyme included in mixed micelles of lecithin and taurocholate, and then hydroxypropyl β-cyclodextrin is added to the assay (11). As the standard CEH assay used in this study was based on it, taurocholate concentration in the assay was 0.375 mm. In experiments to assess taurocholate dependence of the purified activity, protein was preincubated with exogenous taurocholate (0.25–15 mm) and CEH activity was measured with the substrate cholesteryl oleate added either in the standard micelles or emulsified in Triton X-100 (**Fig. 7**). Values for control CEH activity were comparable with both assays. However, addition of taurocholate had mild or no activation effect on hydrolysis of cholesteryl oleate in Triton X-100, whereas it dramatically inhibited the hydrolysis of cholesteryl oleate in cholesteryl oleate/bile salt/lecithin micelles. This, together with the fact that similar profiles of activity were obtained for deoxycholate (data not shown), strongly supports the idea that the protein we have purified is a bile salt-independent cholesteryl ester hydrolase and that, although its activity is not sensitive to exogenous taurocholate concentration, it is highly sensitive to the physico-chemical properties of the substrate.

Effect of PMSF, sulfhydryl reagents, and cations

For comparison with other esterases and to indicate potentially important amino acid residues, the purified protein was probed with the serine specific reagent PMSF and the free sulfhydryl-reacting reagents *p*-hydroxy-mercuribenzoic acid (PHMB), N-ethylmaleimide (NEM), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and iodoacetic acid (IAA) (**Fig. 8**). One of the identifying features of the lipases and esterases family is the existence of a consensus active site with serine (2, 37, 38). Consistent with this, CEH activity was markedly inhibited by relatively low concentrations of

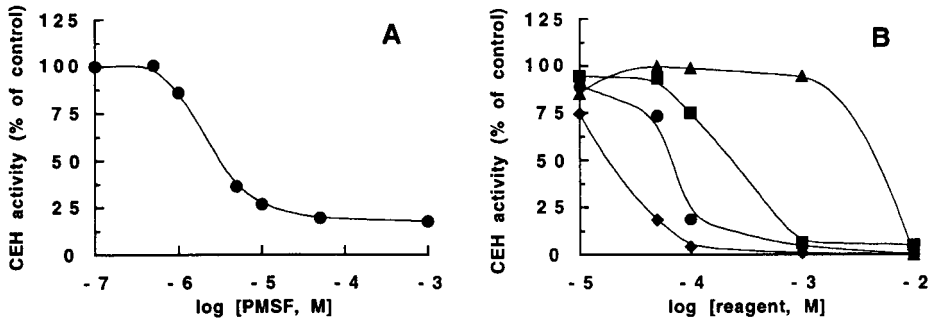

Fig. 8. Concentration-dependent inactivation of the purified rat liver microsomal CEH by PMSF and thiol-reacting compounds. The enzyme was treated with the indicated concentration of PMSF (panel A), pHMB (◆), DTNB (●), NEM (■), or IAA (▲) (panel B), for 15 min at 37°C prior to assaying CEH activity as reported in the text. The 100% control activity in panels A and B averaged 1227 and 1038 pmol of cholesteryl oleate hydrolyzed × min^{-1} × mg $protein^{-1}$, respectively. The data represent the average of duplicate determinations and are representative of two separate experiments.

PMSF, with an IC_{50} of about 2 μm. The four sulfhydryl-reacting compounds, tested in a 10 μm–10 mm range, completely inhibited CEH activity in a dose-dependent manner. Both alkylation and oxidation of thiols resulted in a total loss of enzyme activity. The order of inhibitory potency pHMB > DTNB > NEM > IAA coincides with what we reported for the native and solubilized microsomal CEH (39).

In view of the inhibitory effect of some divalent cations on liver cytosolic (36, 40) and lysosomal CEH (40–42), the effect of a 1 mm concentration of various cations on the purified CEH activity was studied (**Table 3**). Of the divalent cations tested, only $NiCl_2$ mildly activated CEH activity. $MgSO_4$ and the chloride salts of Ca^{2+}, Mn^{2+}, and Na^+ had no effect on the enzyme activity. $CoCl_2$ caused a moderate decrease in activity, and $CuCl_2$ and $ZnCl_2$ totally inhibited CEH activity. Also, 1 mm Fe^{3+} inhibited enzyme activity by 50%. The effect of 1 m NaCl was also tested as Jensen, Daggy, and Bensadoun (32) reported that rat hepatic lipase is resistant to 1 m NaCl. The CEH activity purified from rat liver microsomes was, however, completely abolished by the presence of 1 m NaCl.

TABLE 3. Effect of cations on the activity of the purified rat liver microsomal CEH

Cation	Concentration	% of Control CEH Activity
Ni^{2+}	1 mm	127
Ca^{2+}	1 mm	100
Mg^{2+}	1 mm	98
Mn^{2+}	1 mm	105
Na^+	1 mm	100
Fe^{3+}	1 mm	50
Co^{2+}	1 mm	66
Cu^{2+}	1 mm	10
Zn^{2+}	1 mm	7
Na^+	1 m	4

The enzyme was treated with the indicated concentration of each compound for 15 min at 37°C prior to assaying CEH activity as described in the text. The control activity, measured in the absence of cation addition, was 1308 pmol of cholesteryl oleate hydrolyzed × min^{-1} × mg $protein^{-1}$. The data represent the average of duplicate determinations and are representative of three separate experiments.

DISCUSSION

In the current report, we provide the first evidence to indicate that female rat liver microsomes contain a bile salt-independent esterase that hydrolyzes preferentially cholesteryl esters. A number of properties, including differential purification, N-terminal amino acid sequence, no affinity for heparin and ConA, kinetic and immunological properties, and sensitivity to metal ions and to serine and sulfhydryl specific reagents, distinguish the enzyme described in this study from the microsome resident proteins triacylglycerol lipase, nonspecific carboxylesterase, and retinyl palmitate hydrolase, from the hepatic lipase that transits the secretory pathway and from the homologous cholesterol ester hydrolases located in cytosol and lysosomes.

The 7-step purification procedure described here yielded a protein preparation associated with a 12.4-fold increase in CEH activity and with two silver-stained bands on PAGE. Electroelution of proteins confirmed the identity of the low mobility protein as the cholesteryl ester hydrolase. The molecular mass of rat liver microsomal CEH (106 kDa) determined by SDS-PAGE is much greater than those of the isoforms purified from rat liver cytosol (66 kDa) (6) and lysosomes (60 kDa) (41). Moderate yield (3.2%) and relatively low purification might reduce confidence that the purified enzyme makes a major contribution to liver microsomal CEH in vivo. However, using monospecific antibodies raised to the electroeluted 106 kDa CEH protein we demonstrate that the enzyme we have purified accounts for at least 70% of total CEH activity in female rat liver microsomes. This percentage could vary according to the enzyme assay conditions used. It is thus likely that another microsomal CEH isozyme may exist, i.e., a 66 kDa protein band immunoreactive with IgG anti rat liver cytosolic CEH (14). The extreme instability

of the microsomal CEH once it is separated from lipid components is likely to be the major factor responsible for low purification. This may be furthermore stressed by the complexity and duration of the purification procedure. Serine and cysteine protease inhibitors were not included in the buffers used for purification because of concomitant CEH inactivation (data not shown), whereby some proteolysis might also have occurred. In view of the fact that some proteasomes, the major non-lysosomal degradative machinery of eukaryotic cells, appear to be closely associated with the endoplasmic reticulum (43), proteolysis might have occurred not only in the isolation of microsomes, but in further purification steps.

Lipases and carboxylesterases form a protein superfamily with a common structural fold and a consensus active site with serine (2, 37, 38). However, members of this family differ in loop structures formed by disulfide bridges, which confer substrate binding specificity, and by the presence of additional Cys residues and heparin-binding motifs (37, 38, 44). Chemical modification studies of the purified microsomal CEH indicated that serine residue and free thiol(s) are essential for catalytic activity. CEH activity was highly sensitive to irreversible sulfonylation of the active site serine residue by PMSF. Inhibition was 50% at 2 μm PMSF, this value being much lower than those reported for a group of nonspecific microsomal esterases (100 μm) (24) and for the rat liver cytosolic (100 μm) (36) and lysosomal (1 mm) CEH (41). The free sulfhydryl-reacting compounds PHMB, DTNB, NEM, and IAA completely inactivated CEH with an order of inhibitory potency identical to that observed in an earlier work that we conducted with sealed and solubilized microsomes (39). Together, these findings suggest that one or more sulfhydryl groups are at the active site or sufficiently near to interfere with the catalysis when they are reacted, and that the referred groups are not altered or masked by changes in the lipid environment of the protein. Regarding thiol dependence, the microsomal CEH is similar to the lysosomal CEH (41), but it is different from the cytosolic CEH, which was modestly activated by iodoacetamide and NEM and inhibited by Hg^{2+} (36). Interestingly, carboxylesterases (24) and triacylglycerol lipase (25) from rat liver are devoid of free sulfhydryl groups, because *p*-chloromercuribenzoate did not change enzyme activities. In addition to this, triacylglycerol lipase can be clearly differentiated from the enzyme described in this work on the basis of differential purification, heparin-binding ability, and hydrolysis rate of triolein.

For kinetic characterization and as an indication of its physiological role, the hydrolytic capacity of the purified CEH was monitored with cholesteryl oleate and triolein as substrates. Affinity toward cholesteryl oleate (K_m, 10 μm), was comparable to that found by Natarajan, Ghosh, and Grogan (36) for the cytosolic isoenzyme. The hydrolysis rate of the triolein concentrations that one can expect in the cell was so low that it seems unlikely that CEH, at the time that it hydrolyzes cholesteryl esters, may aid in the hydrolysis of some triacylglycerols, unless they accumulate to a great extent in the endoplasmic reticulum before starting the assembly of very low density lipoproteins. It is, of course, possible that in the intact cell there are cofactors that alter the affinity of the enzyme for these substrates. The substrate specificity is a property that differentiates the purified microsomal CEH from TGL (25) and the heparin-releasable hepatic lipase (32), whose primary function is the degradation of triacylglycerols, and from the lysosomal acid CEH (41) and the cytosolic neutral CEH (36), both of them hydrolyzing cholesteryl esters at rates moderately superior to triacylglycerols. As far as taurocholate effect is concerned, the purified microsomal CEH does not require taurocholate to be active, although the detergent properties of the bile salt seem to modify the physical state of the substrate. The microsomal CEH may be thus defined as a bile salt-independent cholesteryl esterase, the same as the isoenzymes from cytosol (6, 36) and lysosomes (5). The purified cytosolic CEH, however, requires a minimal level of taurocholate to prevent aggregation of the enzyme into an inactive state (6). Other properties in common among the three isoenzymes are their response to metal ions and the absence of cross-reactivity with antibodies to pancreatic CEH. From an overall consideration, the microsomal, cytosolic (36, 40) and lysosomal (41–42) cholesteryl esterases are little or not affected by Ca^{2+}, Mg^{2+}, and Mn^{2+}, but are drastically inhibited by Zn^{2+} and Cu^{2+}.

One remarkable and unexpected finding was that CEH protein did not react with ConA. In the present study we analyzed preparations of CEH at several purification steps by fractionation on an immobilized ConA column. ConA-Sepharose is known to bind Asn-linked oligosaccharides of biantennary complex-type and high mannose-type, but it does not bind complex triantennary and tetraantennary structures (45, 46). Unlike the mature molecules of most nonspecific microsomal esterases that are glycoproteins of the high mannose-type (21, 22), CEH was ConA-unreactive. Hepatic lipase, TGL, and pancreatic CEH are also glycoproteins (32–34, 47). Whether the purified protein is a glycoprotein with a complex N-linked oligosaccharide structure or a non-glycosylated protein is not known presently and further studies on the CEH molecule are required to clarify this.

Here we report that the purified CEH also has *p*-nitrophenylesterase activity, with higher affinity toward PNP-acetate than toward PNP-butyrate, as most microsomal carboxylesterases (22–24). In contrast, the cytosolic CEH, which exhibits comparable *p*-nitrophenylesterase activity, hydrolyzes PNP-butyrate much more actively than PNP-acetate (36). According to the criteria of Morgan et al. (24), the enzymatic hydrolysis of PNP-acetate by rat liver microsomes is predominantly catalyzed by hydrolases A and B. Hydrolase A is a PMSF-sensitive esterase (IC_{50}, 100 nm) with high substrate affinity (K_m, 25 μm) and V_{max} of 150 μmol/min × mg. Hydrolase B is a PMSF-insensitive esterase (IC_{50}, 100 μm) with low substrate affinity (K_m, 400 μm) and V_{max} of 330 μmol/min × mg. The microsomal CEH protein purified by us has none of the properties of these esterases. In addition to differences in V_{max} and K_m for PNP-acetate and CEH sensitivity to PMSF, the

purified CEH protein does not have the properties of a class A-esterase; which binds avidly to ConA and is not inhibited by the alkylating *p*-chloromercuribenzoate (24).

Finally, we would like to point out the contribution of microsomal CEH activity to liver CEH activity. Using polyclonal antibodies against rat liver cytosolic CEH, Ghosh and Grogan (48) found that liver CEH activity is predominantly cytosolic and accounts for at least 75% of total cellular CEH activity in male rats. However, in adult female rat liver microsomes, a greater proportion of protein reactive with IgG anti-cytosolic CEH remained associated with washed microsomes (14), suggesting that microsomal CEH contributed a greater fraction of bulk CEH activity in female than in male rat liver. Work on this is in progress at present. The CEH enzyme is known to be mainly inserted in the RNA-rich endoplasmic reticulum facing the luminal side (13). The peculiar location of the microsomal CEH in a place of the endoplasmic reticulum in which the assembling of very low density lipoproteins begins suggests that this enzyme may play a role in controlling the assembling itself and the amount of cholesteryl esters that is packed together with triacylglycerides in the very low density lipoprotein core for further secretion. The current study provides the basis for determining the metabolic function of the microsomal cholesterol ester hydrolase.

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