Purification and Characterization of a Microsomal Bile Acid β-Glucosidase from Human Liver

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A human liver microsomal β-glucosidase has been purified to apparent homogeneity in sodium dodecyl sulfate-polyacrylamide gel electrophoresis where a single protein band of M, 100,000 was obtained under reducing conditions. The enzyme was enriched about 73,000-fold over starting microsomal membranes by polyethylene glycol fractionation, anion exchange chromatographies on DEAE-Trisacryl, and Mono Q followed by affinity chromatography on N-(9-carboxyxy)-1-deoxynojirimycin-AH-Sepharose 4B. The purified enzyme had a pH optimum between 5.0 and 6.4, was activated by divalent metal ions, and required phospholipids for exhibition of activity. The enzyme catalyzed the hydrolysis of 3β-O-glucosido-lithocholic and 3β-O-glucosido-chenodeoxycholic acids with high affinity (Km, 1.7 and 6.2 μM, respectively) and of the β-D-glucose (Km, 210 μM) and the β-D-galactoside of 4-methylumbelliferone. The ratio of relative reaction rates for these substrates was about 6:3:1:1. No activity was detectable toward 6β-O-glucosido-hyodeoxycholic, glucocerebrosides, and the following glucosides of 4-methylumbelliferone: α-D-glucoside, α-D-arabinoside, β-D-fucoside or β-D-xylidoside. Immunoinhibition and immunoprecipitation studies using antibodies prepared against lysosomal glucocerebrosidase showed no cross-reactivity with microsomal β-glucosidase suggesting that these two enzymes are antigenically unrelated.

Bile acid glucosides have been shown to be formed in human liver microsomes by a glucosyltransferase that is sugar nucleotide-independent and utilizes dolichol phosphoglucose as natural cosubstrate (Ref. 1, for recent reviews on glucosidic conjugation of bile acids, see Refs. 2 and 3). The position of the glucose moiety in these bile acid glucosides has been determined from enzymatically synthesized bile acid glucosides to be the 3-position (4) with a β-glucosidic linkage (1). The physiological significance of bile acid 3-O-glucosidation is suggested by the identification of glucosides of unconjugated as well as glycine- and taurine-conjugated bile acids in urine from healthy humans (5) and patients with extrahepatic cholestasis (6). In addition to bile acid 3-O-glucosidation, the synthesis of bile acid 6-O-glucosides was recently described in human liver microsomes by a UDP-sugar-dependent mechanism (7).

Bile acid 3-O-glucosides have been recently shown to be hydrolyzed in vitro by a human liver microsomal β-glucosidase (8). This β-glucosidase is localized predominantly in the microsomal fraction of human liver (8) where it is confined to the smooth endoplasmic reticulum (9). Hydrolytic activity toward bile acid 3-O-glucosides thus occurs in the same subcellular compartment as synthesis of these bile acid conjugates, which has been shown to be localized in the smooth endoplasmic reticulum of human liver (10).

Preliminary studies gave evidence (8) that hydrolytic activity toward bile acid 3-O-glucosides differs from the two known prominent β-glucosidases in human tissues, the lysosomal membrane-bound enzyme glucocerebrosidase (N-acylsphingosyl-1-O-β-D-glucoside:glucohydrolase) (11) and a cytosolic broad specificity β-glucosidase (12). The following study will show that β-glucosidase activity toward bile acid 3-O-glucosides is a novel microsomal β-glucosidase that is not identical to the known β-glucosidases described before. The present report describes properties and a method for the purification of human liver microsomal β-glucosidase activity toward bile acid 3-O-glucosides to apparent homogeneity in SDS-polyacrylamide gel electrophoresis. According to its natural substrates the isolated enzyme will be named bile acid β-glucosidase (3β-D-glucosido-bile acid:glucohydrolase).

**EXPERIMENTAL PROCEDURES**

Sources of chemicals (1, 8, 13) and of human liver (13) were the same as described in previous papers. The following materials were obtained from Sigma, Munich: various 4-methylumbelliferyl glycosides, phospholipids, and sheep anti-rabbit IgG agarose. The following compounds were synthesized enzymatically with human liver microsomes as previously published: [24-14C]chondrocyte acid glucoside (8 μCi/μmol) (5) and hyodeoxycholic acid [U-14C]glucoside (1.6 μCi/μmol) from UDP-[U-14C]glucose and hyodeoxycholic acid (7). The following samples were generous gifts: lithocholic acid [U-14C]glucoside (17 μCi/μmol) from Prof. Dr. F. Dallacker, Department of Organic Chemistry, Aachen University of Technology, Germany; dNM from Dr. D. Schmidt, Bayer AG, Wuppertal, Germany, and rabbit immune serum prepared against human placental glucocerebrosidase from Dr. C. Incerti, Genzyme Therapeutics, Modena, Italy.

The abbreviations and trivial names used are: CBE, conduritol B epoxide; Br-CBE, bromoconduritol B epoxide; dNM, N-deoxynojirimycin; carboxonyl-dNM and dodecyl-dNM (15). The following bile acid glucosides were synthesized enzymatically with human liver microsomes as previously published: [24-14C]chondrocyte acid glucoside (8 μCi/μmol) (5) and hyodeoxycholic acid [U-14C]glucoside (1.6 μCi/μmol) from UDP-[U-14C]glucose and hyodeoxycholic acid (7). The following samples were generous gifts: lithocholic acid [U-14C]glucoside (17 μCi/μmol) from Prof. Dr. F. Dallacker, Department of Organic Chemistry, Aachen University of Technology, Germany; dNM from Dr. D. Schmidt, Bayer AG, Wuppertal, Germany, and rabbit immune serum prepared against human placental glucocerebrosidase from Dr. C. Incerti, Genzyme Therapeutics, Modena, Italy.

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β-Glucosidase activity toward bile acid glucosides was estimated as described for \([1^{4}\text{C}]\)chenedoxygenyloxy acid glucoside as substrate (8) with the following modifications: hydrolysis of hydroxyoxygenyloxy acid \([1^{4}\text{C}]\)gluco-
side was estimated with a substrate concentration of 106 μM; the assay mixture with lithocholic acid \([1^{4}\text{C}]\)glucoside (9 μM), containing maximally 4 μg of protein in 30 μl, was stopped with 100 μl of 0.5 mM glycine HCl, pH 2.0. The water phase was extracted for 5 min with 300 μl of chloroform/n-butyl alcohol (9:1, v/v) resulting in transfer of >99% of unreacted lithocholic acid \([1^{4}\text{C}]\)glucoside into the organic phase. The labeled reaction product \((1^{4}\text{C})\)glucose remained in the water phase with a yield of about 97%. A 100-μl aliquot of the upper aqueous phase was counted for radioactivity in 5 ml of Rotiszint Eco-Plas (Rotis, Karlsruhe) scintillation mixture. For identification of \([1^{4}\text{C}]\)glucose as reaction product, thin layer chromatography was performed as described (16) with (U-\([^{12}\text{C}]\)glucose (DuPont NEN) as standard.

Gluconeogenesis was assayed fluorometrically with a mixture of glucosylceramide and the synthetic analog NBD-glucosylceramide as described (17). Hydrolytic activity toward various glucosides of 4-methylumbelliferyl was estimated fluorometrically according to a published procedure (18) with the following modifications. The assay mixtures contained 0.1 mM sodium acetate, pH 5.5, as buffer, 5 mM MgCl₂, and a glycode of 4-methylumbelliferyl, each 1 mM.

For reconstitution of enzyme activities prior to assays aliquots of enzyme samples from column fractions were preincubated in the presence of 0.1 mg/ml 1,1′-phospho-di-litol from bovine liver and 83 mg/ml human serum albumin for 30 min on ice. Aliquots of these preincubated mixtures were added to buffer mixtures which additionally contained 0.7 mg/ml CHAPS. Phospholipids were freshly prepared as dispersions in the respective sample buffers as described (13).

Protein was assayed by the dye-binding method of Bio-Rad using bovine serum albumin as standard. After the final purification step the concentration of protein was too low to be measured by a common method (about 0.05 mg/ml total protein). Therefore, the amount of protein in the eluate after affinity chromatography was estimated from the density of the silver-stained protein band that was visible in the final enzyme preparation after SDS-polyacrylamide gel electrophoresis (see below). Quantitation of density was performed by comparison to the density of bands from a bovine serum albumin standard using a laser densitometer (Ultrascan XL, LKB, Bromma, Sweden).

Analytical Methods

**β-Glucosidase**

**General Procedures**—If not stated otherwise, all steps were carried out at 4 °C. Standard buffer was 20 mM Bis-Tris propane HCl, pH 6.5, containing 1 mM dithioerythritol and 20% (v/v) glycerol except for step 4 and chromatofocusing where 10% (v/v) glycerol was used. The following protease inhibitors were added to buffers where indicated: aprotin, chymotatin, leupeptin, and pepstatin, 0.1 μg/ml each, and phenylmethylsulfonyl fluoride, 10 μM final concentration.

**Step 1: Preparation of a Solubilized Microsomal Extract**—Microsomes were prepared from 40 g of human liver and washed with 0.15 M KCl as described previously (20). The washed microsomes were suspended in 60 ml of 0.25 M sucrose containing 5 mM Tris/HCl, pH 7.4, and protease inhibitors. Aliquots of this suspension could be stored at −70 °C for at least 3 months with no apparent loss of enzyme activity. For solubilization an equal volume of 40 mM Bis-Tris propane HCl, pH 7.2, containing 40% (v/v) glycerol, 20% (v/v) acetonitrile, 2 mM dithio-
erythritol, 0.4% (w/v) SB12, 0.44% (w/v) octyl α-glucoside, and protease inhibitors was added to the microsomal suspension. After 30 min the mixture was centrifuged at 100,000 × g for 60 min.

**Step 2: Polyethylene Glycol Fractionation**—Fractionation with polyethylene glycol 6000 was carried out as described previously (20) except that the protein precipitating between 5 and 7% (w/v) of polyethylene glycol was used for subsequent purification steps. The precipitate was dissolved in standard buffer containing 10% (v/v) acetonitrile, 0.2% (w/v) SB12, 0.05% (w/v) octyl β-glucoside, and protease inhibitors.

**Step 3: DEAE-Trisacyrul Chromatography**—The protein fraction of step 2 was centrifuged for 35 min at 100,000 × g. The clear supernatant was applied to a DEAE-Trisacyrul column (1.6 × 5 cm; flow rate, 50 ml/h) equilibrated with standard buffer containing 10% (v/v) acetonitrile, 0.2% (w/v) SB12, and 0.05% (w/v) octyl β-glucoside. After the column was washed with 30 ml of equilibration buffer followed by washing with 30 ml of equilibration buffer to which the additional protease inhibitors, a linear gradient from 0 to 0.16 M NaCl in the same buffer was applied (total volume, 90 ml; flow rate, 20 ml/h). Bile acid β-glucosidase activity emerged at approximately 0.06 M NaCl.

**Step 4: Mono Q Chromatography**—The active fractions of step 3 were diluted by an equal volume of standard buffer containing 0.2% (w/v) SB12 and 0.2% (w/v) octyl β-glucoside and were subjected to ion-exchange chromatography on a Mono Q column using a Pharmacia FPLC system (flow rate, 1 ml/min; pressure, 2.5 megapascal). After application of the sample the column, previously equilibrated with standard buffer containing 0.2% (w/v) SB12 and 0.1% (w/v) CHAPS, was washed with 5 ml of the same buffer and then subjected to elution with two linear NaCl gradients in equilibration buffer, first from 0 to 0.07 M NaCl (total volume, 7 ml) and then from 0.07 to 0.2 M NaCl (total volume, 48 ml). Enzyme activity was eluted at about 0.1 M NaCl.

**Step 5: Affinity Chromatography**—To the active fractions of step 4 MgCl₂ and NaCl were added to a final concentration of 5 mM and 0.3 M, respectively. This protein solution was treated batchwise with 150 μl of affinity gel which was prepared by coupling carboxyphosphonyl-dNM to AH-Sepharose 4B according to the instructions given by Pharmacia (ligand concentration, 2.16 μmol/ml settled gel). The affinity column was washed with 3 M NaCl; 10 times with 0.25 ml of 10 mM Bis-Tris propane HCl, pH 6.5, containing 0.9% (w/v) ethylene glycol and 1 mM dithioerythritol. After these successive washings bile acid β-glucosidase was eluted from the gel by repeated suspension and centrifugation of the gel in 0.25 ml of the last washing buffer with 90% ethylene glycol saturated with NaCl (15 times). For enzyme assays aliquots of these fractions were diluted with 10 mM Bis-Tris propane HCl, pH 6.5, containing 1 mM dithioerythritol to reduce the ethylene glycol to a final concentration of 30%; 0.05% (w/v) SB12 and 0.1% (w/v) CHAPS were added (final concentrations), and samples were reconstituted with L-α-phosphatidylcholines and human serum albumin (see above).

**Chromatofocusing**—For enzyme characterization an enzyme preparation after Mono Q chromatography (step 4 of the purification procedure) was further purified by chromatofocusing on a Mono P column according to the manufacturer's instructions. For hydrolases activities toward lithocholic acid glucoside or glucoscerbroside were measured in the respective fractions as described above.

**Purification of Bile Acid β-Glucosidase**

**RESULTS**

**Purification of β-Glucosidase**—The purification of human liver bile acid β-glucosidase was achieved from a solubilized microsomal preparation by polyethylene glycol fractionation,
followed by anion-exchange chromatographies on DEAE-Trisacryl and on Mono Q and affinity chromatography on carboxyoxynyl-dNM-AH-Sepharose (Table I). The purification was about 73,000-fold with respect to the solubilized microsomal preparation; the yield was 1.4%.

The supernatant of the solubilized microsomal preparation contained 39% of the microsomal protein and 56% of the bile acid β-glucosidase activity toward lithoholic acid glucoside present in microsomes before solubilization. The loss of activity of about 40% can be attributed to the addition of SB12 and acetonitrile to microsomes, whereas octyl-α-glucoside exerted a stabilizing effect on enzyme activity and prevented a greater loss of active enzyme during solubilization. Other detergents, e.g. CHAPS (0.3%, w/v) or Triton X-100 (0.2%, v/v), were also suitable for extraction of bile acid β-glucosidase from the microsomes with yields of 74 and 51%, respectively. However, after solubilization of microsomal protein with these detergents, the formation of aggregates of variable composition with protein, detergent, and phospholipids appeared to be favored, so that separation of bile acid β-glucosidase from contaminating proteins could not be achieved in subsequent chromatographic procedures. Following solubilization of microsomes an initial polyethylene glycol fractionation proved to be favorable for further purification of the β-glucosidase. When polyethylene glycol fractionation was omitted, purification of bile acid β-glucosidase was only about 23-fold after DEAE-Trisacryl and Mono Q chromatographies in contrast to about 600-fold purification with the method used for sample concentration: in lane 7, sample buffer without protein was concentrated in a dialysis bag against solid polyethylene glycol 20,000; in lane 8, sample buffer without protein was concentrated by a phenol-ether extraction method as described (21). These bands were also visible in the final enzyme preparation (lane 9, concentration of the sample with polyethylene glycol) and were described by other authors as an artifact of silver staining resulting from the presence of 2-mercaptoethanol (22, 23).

Affinity purification of the β-glucosidase was carried out as the final step of the purification procedure. Under the conditions described, the enzyme was nearly quantitatively bound to carboxyoxynyl-dNM as ligand of the affinity gel. In the presence of the β-glucosidase inhibitor dNM (1 mM), no binding of the enzyme to the affinity gel was observed suggesting that interaction of the β-glucosidase with the gel was of specific nature. Elution of the enzyme from the affinity gel was possible with 90% ethylene glycol saturated with NaCl. Under this condition about 10% of bile acid β-glucosidase activity applied to the affinity gel could be recovered, whereas with 3 mM NaCl or 90% ethylene glycol the recovery of enzyme activity from the affinity gel was less than 0.1%. The enzyme was present in the eluate in an inactive form which could be reconstituted with phospholipids (see below). However, the enzyme preparation rapidly lost the potential for reconstitution of activity when stored in the final elution buffer at 4 °C or −70 °C (half-life, <2 h). In the reconstituted form, the enzyme could be stored for 40 h at 4 °C (half-life, 14 days) and for at least 2 months at −70 °C with minimal loss of activity (<20% of the initial value). The instability of the enzyme prior to reconstitution may be the reason for the low recovery of enzyme activity in the last step of the purification procedure.

As shown in Fig. 1, the final enzyme preparation exhibited a single protein band of Mr 100,000 in SDS-polyacrylamide gel electrophoresis under reducing conditions. A protein band of Mr 100,000 was also obtained after treatment of the affinity gel with 1 mM dodecyl-dNM and analysis of this eluate by SDS-polyacrylamide gel electrophoresis (not shown). However, no β-glucosidase activity was detectable in the eluate with dodecyl-dNM, which had been subjected to DEAE-Trisacryl chromatography for removal of this inhibitory dNM derivative. Therefore, bile acid β-glucosidase appears to have been eluted by dodecyl-dNM but could not be reactivated due to either its lability or its strong binding property to this inhibitor (Table III) which remained bound to the enzyme during DEAE-Trisacryl chromatography. That β-glucosidase was specifically eluted from the affinity gel by dodecyl-dNM is further supported by the fact that a successive elution with 90% ethylene glycol saturated with NaCl resulted in less than 0.5% of the applied β-glucosidase activity being detected. In comparison a yield of enzyme activity of about 10% was obtained in this elution step without pretreatment of the affinity gel with dodecyl-dNM. In contrast to dodecyl-dNM the parent compound dNM (1 mM) was not able to elute the enzyme from the affinity gel.
which may be explained by the weak inhibitory potencies of dNM (Table III).

**Properties of β-Glucosidase**

**Substrate Specificity**—As shown in Table II the pure bile acid β-glucosidase hydrolyzes 3β-glucosides of the bile acids lithocholic and chenodeoxycholic acids, whereas the 6β-glucoside of the bile acid hyodeoxycholic acid is not a substrate of the enzyme. Hydrolysis of 6β-D-glucosido-hyodeoxycholic acid was also not detectable with a partially purified enzyme preparation after Mono Q chromatography or with microsomes suggesting that bile acid 6-O-glucosides are not subject to enzymatic hydrolysis in human liver in contrast to bile acid 3-O-glucosides. As may be seen from Table II glucocerebrosidase, which is the natural substrate of glucocerebrosidase, showed no reaction with bile acid β-glucosidase. Activity toward glucocerebroside was detectable in human liver microsomes (8) but was separated from the activity toward bile acid glucosides by DEAE-Trisacryl chromatography where glucocerebrosidase activity appeared in the flow-through, whereas bile acid β-glucosidase was eluted within a salt gradient (results not shown). Of the glycosides of 4-methylumbelliferone tested as artificial substrates of the pure β-glucosidase, the β-d-glucoside supported a high activity, whereas the β-d-galactoside was a poor substrate of the enzyme (Table II). No reaction was observed with the following glycosides of 4-methylumbelliferone: α-d-glucoside, α-l-arabinofuranoside, β-d-fucoside, and β-d-xyloside (limit of detection, 0.5 μmol per min per mg ofpure enzyme protein).

The apparent Km values given in Table II were obtained from double-reciprocal plots of initial rates of enzyme activity as a function of varying substrate concentrations yielding straight lines. From the ratio of the $k_{cat}/K_m$ values it may be seen that the enzyme exhibited about 10–70-fold higher catalytic efficiency with bile acid glucosides as compared with the β-glucoside of 4-methylumbelliferone (Table II).

**Catalytic Properties**—The purified enzyme showed a broad pH optimum for hydrolysis of lithocholic acid glucoside between pH 5.0 and 6.4. Activity was 3-fold higher at pH 6.4 compared with pH 5.0. A difference in pH dependence for hydrolysis of bile acid glucosides was observed in the same buffer system with the crude microsomal enzyme showing a sharp optimum close to pH 5.0 as described in a previous report (8).

As shown in Fig. 2 the purified β-glucosidase could be stimulated about 6-fold by the addition of divalent metal ions such as Mn²⁺, Co²⁺, or Mg²⁺ compared with a control without metal ions and with 1 mM EDTA. Ni²⁺, Ca²⁺, or Zn²⁺ were less effective in activation of bile acid β-glucosidase. Ba²⁺ had no significant effect on enzyme activity (Fig. 2). In the absence of metal ions EDTA (1 mM) produced a decrease in activity of the purified enzyme by about 20%. The purified enzyme showed a similar metal ion dependence for hydrolysis of the β-glucoside of 4-methylumbelliferone as observed for bile acid glucoside hydrolysis (not shown).

**Effect of Inhibitors**—The microsomal β-glucosidase had already been shown in a previous report to be sensitive to inhibition by various glucosidase inhibitors such as 1-deoxynojirimycin and natural or synthetic glucosides (8). In addition to these compounds N-alkyl derivatives of 1-deoxynojirimycin and the catalytic site-directed covalent inhibitors conduritol B epoxide (CBE) and bromoconduritol B epoxide (Br-CBE) (14) have been studied as effectors of the purified bile acid β-glucosidase. As shown in Table III, N-alkylation of 1-deoxynojirimycin increased the inhibitory potential of the parent structure by several orders of magnitude. Thus, dodecyl-dNM produced about 50% inhibition of the enzyme with a concentration of 0.002 μM, whereas for 1-deoxynojirimycin a concentration of 50 μM was necessary to achieve a similar inhibitory effect.

Whereas bromoconduritol A/B reacted as an inhibitor of the purified β-glucosidase as already described for the microsomal enzyme (8), CBE and Br-CBE showed only a marked inhibitory effect on the enzyme in microsomes (Table III). The purified β-glucosidase was not significantly affected by these compounds in concentrations of 2 mM even after preincubation of the enzyme with the inhibitors for 12 h at 4 °C with or without pretreatment of the enzyme with phospholipids (see below). A marked loss in sensitivity of the enzyme to CBE and Br-CBE was already observed when microsomes were solubilized as in step 1 of the purification procedure. Whereas enzyme activity in untreated microsomes was inhibited by about 50% in the presence of 20 μM CBE or 1 μM Br-CBE (Table III), a similar inhibitory effect on the enzyme from solubilized microsomes required 2 mM concentrations of these epoxide inhibitors (not shown). This decrease in sensitivity of the enzyme from solubilized microsomes to CBE and Br-CBE was already observed before insoluble microsomal membrane components were removed by high speed centrifugation. The yield of bile acid

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**Table II**

**Substrate specificity and apparent kinetic constants of bile acid β-glucosidase**

| Substrate                        | Specific activity | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|----------------------------------|------------------|----------|-------|---------------|
| 3β-D-Glucosido-lithocholic acid   | 25               | 2500     | 1.7   | 1471          |
| 3β-D-Glucosido-chenodeoxycholic acid| 13               | 1300     | 6.2   | 210           |
| 6β-D-Glucosido-hyodeoxycholic acid| ND               |          |       |               |
| Glucocerebroside                 | ND               |          |       |               |
| 4-Methylumbelliferyl-β-D-glucose  | 47               | 4700     | 210   | 22            |
| 4-Methylumbelliferyl-β-D-galactose| 4.3              | 430      |       |               |

**Fig. 2. Effect of divalent metal ions on β-glucosidase activity.** A partially purified enzyme preparation after Mono Q chromatography (specific activity, 0.2 μmol per min per mg of protein) was assayed with lithocholic acid glucoside as substrate in the presence of the indicated concentrations of various divalent metal ions. Control, enzyme activity without metal ion and with 1 mM EDTA.

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**Human Liver Microsomal Bile Acid β-Glucosidase**

 Specific activities were estimated with the pure enzyme after reconstitution with phospholipids as described under "Experimental Procedures." Apparent $K_m$ values were determined from a partially purified preparation after Mono Q chromatography and are given as means ± S.D. with the number of determinations in parentheses. For determination, substrate concentrations varied between 0.5 and 10 μM. $K_m$ values were determined from a partially purified preparation after Mono Q chromatography and are given as means ± S.D. with the number of determinations in parentheses. For determination, substrate concentrations varied between 0.5 and 10 μM. 40 and 400 μM for the glucosides of lithocholic and chenodeoxycholic acids and methylumbelliflorone, respectively, ND, not detectable.
**TABLE III**

*Effect of various inhibitors on bile acid β-glucosidase*

A partially purified enzyme preparation after Mono Q chromatography was used as an enzyme source except for assay mixtures with CBE* and Br-CBE*, which contained crude microsomes instead of the purified enzyme. Prior to the addition of lithocholic acid glucoside as substrate (9 μM, final concentration) and start of the enzyme reaction, a preincubation of crude or purified enzyme with inhibitors was performed in assay mixtures for 30 min at 0°C. N-Alkyl-dNM derivatives were dissolved in dimethyl sulfoxide (final concentration in assay mixtures, 1%), which had no effect on enzyme activity in control experiments. Control activity represents enzyme activity determined in the absence of additions to the assay.

| Additions            | μM | β-Glucosidase activity % control |
|----------------------|----|---------------------------------|
| 1-Decoxynoririmycin  | 50 | 50.3                            |
| Carboxymethyl-dNM    | 0.02 | 49.2                         |
| Dodecyl-dNM          | 0.002 | 49.6                         |
| Bromoconduritol A/B  | 50 | 42.8                            |
| CBE*                 | 20 | 47.8                            |
| CBE                  | 2000 | 96.1                         |
| Br-CBE*              | 1 | 50.4                            |
| Br-CBE               | 2000 | 92.5                         |

β-glucosidase activity in these solubilized microsomes using a small scale preparation was about 80% as compared with the activity in untreated microsomes. Therefore, the possibility can be excluded that two forms of microsomal bile acid β-glucosidase may have been separated which differ in sensitivity to CBE and Br-CBE. After polyethylene glycol fractionation (step 2 of the purification procedure) the enzyme was as resistant against CBE and Br-CBE as the enzyme preparation after Mono Q chromatography (shown in Table III). No separation of two different forms of bile acid β-glucosidase was observed during polyethylene glycol fractionation. All fractions obtained in this procedure exhibited only bile acid β-glucosidase activity with low sensitivity to CBE and Br-CBE.

All other compounds tested as inhibitors of microsomal bile acid β-glucosidase (8) retained their inhibitory potency for the purified enzyme with the exception of the inhibitors castanospermine and octyl α-glucoside. These compounds affected only weakly the microsomal enzyme (8) and showed no reaction with the purified enzyme in concentrations of 3 and 5 mM, respectively.

**Phospholipid and Detergent Dependence**—The isolated bile acid β-glucosidase was dependent on the presence of phospholipids for exhibition of enzyme activity. Without the addition of phospholipids the pure enzyme was inactive suggesting that the β-glucosidase was obtained in a delipidated form from the final column step and that phospholipids are necessary to maintain the enzyme in an active conformation. Whereas phospholipids did not increase enzyme activity in the first three steps of the purification procedure, a first activatory effect of about 2-fold could be observed after Mono Q chromatography. With a more purified and therefore more delipidated enzyme preparation after chromatofocusing (purification as compared with solubilized microsomes, 6400-fold), the effect of naturally occurring phospholipids on enzyme activity was studied. As shown in Fig. 3, treatment of the enzyme with various concentrations of phosphatidylcholine, phosphatidylethanolamine, or lysophosphatidylethanolamine resulted in a maximal activation about 3.7-, 2.9-, 2.7-, or 2.2-fold, respectively. Phosphatidylethanolamine and sphingomyelin were less potent activators of the enzyme (Fig. 3). All of these compounds were inhibitory in higher concentrations.

Since detergents may act as phospholipid substitute on the activity of membrane bound enzymes, the effect of various detergents on a partially purified preparation of bile acid β-glucosidase activity was studied. As shown in Fig. 4, addition of CHAPS, taurocholate, or Triton X-100 led to a maximal activation of enzyme activity of about 2.5-, 2-, or 1.4-fold, respectively. Brij 58 was only inhibitory to the enzyme. Whereas in microsomes, detergents such as taurocholate or Triton X-100 had no effect or led to inhibition of enzyme activity in higher concentrations (8), in the partially purified state, e.g. after Mono Q chromatography, detergents could replace phospholipids to produce a maximally activated form of the enzyme. Very dilute preparations after chromatofocusing (protein, 0.7 μg/ml) additionally required the presence of 0.5–1% (w/v) human serum albumin for enzyme reconstitution. The pure enzyme, however, could not be activated by the addition of detergents and albumin. Only after reconstitution in the presence of phospholipids the pure enzyme exhibited hydrolytic activity.

**Immunochemical Cross-reactivity**—In an effort to explore the possible structural relationship between bile acid β-glucosidase and

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**Fig. 3.** Effect of phospholipids on β-glucosidase activity. A partially purified enzyme preparation after chromatofocusing as described under “Experimental Procedures” (specific activity, 2.2 μmol per min per mg of protein) was added in aliquots either to dispersions of phospholipids in sample buffer at the indicated phospholipid concentrations or to sample buffer as the control at a final protein concentration of 7.3 μg/ml and incubated for 60 min at 0°C. Aliquots of these mixtures were then added to an otherwise complete assay with lithocholic acid glucoside as substrate. PtdIns, 1-α-phosphatidylinositol from bovine liver; PtdSer, 1-α-phosphatidylserine from bovine brain; PtdCho, 1-α-phosphatidylcholine; LysoPtdCho, 1-α-lysophosphatidylcholine; PtdEtn, 1-α-phosphatidylethanolamine; Sph, sphingomyelin, the latter all from egg yolk.

**Fig. 4.** Effect of detergents on β-glucosidase activity. A partially purified enzyme preparation after Mono Q chromatography (specific activity, 0.2 μmol per min per mg of protein) was assayed with lithocholic acid glucoside as substrate. CHAPS, taurocholate, or Triton X-100 led to a maximal activation of enzyme activity of about 2.5-, 2-, or 1.4-fold, respectively. Brij 58 was only inhibitory to the enzyme. Whereas in microsomes, detergents such as taurocholate or Triton X-100 had no effect or led to inhibition of enzyme activity in higher concentrations (8), in the partially purified state, e.g. after Mono Q chromatography, detergents could replace phospholipids to produce a maximally activated form of the enzyme. Very dilute preparations after chromatofocusing (protein, 0.7 μg/ml) additionally required the presence of 0.5–1% (w/v) human serum albumin for enzyme reconstitution. The pure enzyme, however, could not be activated by the addition of detergents and albumin. Only after reconstitution in the presence of phospholipids the pure enzyme exhibited hydrolytic activity.

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FIG. 5. Inhibition (●) and precipitation (○, □) of human liver $\beta$-glucosidase activities by rabbit antiserum against human placental glucocerebrosidase. Assays with lysosomal glucocerebrosidase (closed symbols; specific activity, 11.3 nmol per min per mg of protein) and with microsomal bile acid $\beta$-glucosidase activity toward lithocholic acid glucoside (open symbols: specific activity, 0.27 nmol per min per mg of protein) were performed as described under “Experimental Procedures.” Control, activities after pretreatment with nonimmune rabbit serum.

As shown in Fig. 5, at the highest antibody concentration used, glucocerebrosidase was inhibited by about 70% and could be quantitatively immunoprecipitated, whereas under these conditions the antibodies were incapable of precipitating or inactivating bile acid $\beta$-glucosidase (shown in Fig. 5 only for immunoprecipitation of the enzyme). The same result was obtained with a partially purified preparation of bile acid $\beta$-glucosidase after Mono Q chromatography. These results suggest that glucocerebrosidase and bile acid $\beta$-glucosidase are antigenically unrelated, whereas human liver lysosomal glucocerebrosidase is recognized by antibodies to the placental form of the enzyme.

DISCUSSION

The present study describes the isolation of a microsomal human liver bile acid $\beta$-glucosidase to apparent electrophoretic homogeneity using carboxyxyxynyl-dNM-AH-Sepharose for affinity purification of the enzyme. N-$\omega$-Carboxyalkyl derivatives of dNM and its $\alpha$-manno analog have already been used for the purification of various glycosidases (24, 25). Thus, the carboxyxyxynyl-dNM support used in the present study was also suitable for the isolation of a cystolic calf liver $\beta$-glucosidase (15) and of lysosomal human placental glucocerebrosidase (26). Whereas, however, the cystolic and the lysosomal enzyme eluted under mild conditions from the affinity support, with 1 mM octyl $\beta$-glucoside (15) or 40–60% ethylene glycol (26), respectively, bile acid $\beta$-glucosidase activity could not be recovered under these conditions but emerged from the column with 90% ethylene glycol saturated with NaCl.

In addition to conditions for elution from the affinity column, further properties of bile acid $\beta$-glucosidase show that the enzyme is distinct from the previously described $\beta$-glucosidases, e.g. the effect of lipids on enzyme activities. Whereas bile acid $\beta$-glucosidase was activated by taurocholate, acidic, and neutral phospholipids (Figs. 3 and 4), these compounds were inhibitory to the cystolic $\beta$-glucosidase (12). Glucocerebrosidase was only activated by taurocholate and acidic phospholipids, e.g. phosphatidylserine or phosphatidylinositol (27), whereas the neutral phospholipids phosphatidylcholine or phosphatidylethanolamine were without effect on enzyme activity (28). In contrast to the pure form of glucocerebrosidase, which was also active without the addition of phospholipids (29), bile acid $\beta$-glucosidase showed an absolute phospholipid requirement for exhibition of activity in the pure state. Furthermore, bile acid $\beta$-glucosidase is the only glucosidase described at present that is metal ion-dependent for expression of full activity (Fig. 2). However, various a-mannosidases have been shown to require divalent metal ions for exhibition of activity (25, 30).

In addition to the membrane-bound lysosomal glucocerebrosidase a second membrane-bound $\beta$-glucosidase has been described from a crude membrane fraction of human spleen and has been termed “nonspecific $\beta$-glucosidase” since it was active toward the artificial substrate 4-methylumbelliferyl-$\beta$-D-glucoside (31, 32). The subcellular location of this enzyme has not been determined. To evaluate the relationship between the bile acid $\beta$-glucosidase and the “nonspecific” membrane-bound $\beta$-glucosidase more information is needed on the latter enzyme.

A comparison of the properties of the purified bile acid $\beta$-glucosidase with those of the corresponding microsomal-bound enzyme shows that some marked differences in characteristics between the purified enzyme and its membrane-bound form are apparent. The purified, soluble form of enzyme exhibited a broad pH optimum of activity between pH 5.0 and 6.4, whereas the membrane-bound form showed a sharp optimum of activity close to pH 5.0 (8). Furthermore, the membrane-bound enzyme was highly sensitive to inhibition by the active site-directed inhibitors CBE and Br-CBE, whereas these compounds did not significantly affect the soluble purified enzyme even after reconstitution with phospholipids (Table III). Changes in properties between membrane-bound enzymes and the corresponding soluble forms have been described for a variety of enzyme activities (33, 34). As a first example, the membrane-bound form of mitochondrial ATPase has been described to be sensitive to inhibition by oligomycin, whereas in its soluble form the enzyme was oligomycin-resistant (34). This phenomenon has been termed allotropy (34) and may be applied to bile acid $\beta$-glucosidase. Membrane-bound proteins that show the phenomenon of allotropy are dependent on lipid-protein interactions for the exhibition of activity. After removal of the natural membrane environment an artificial lipid support has to be provided which may lead to changes in properties of the respective enzyme as observed in the present report for bile acid $\beta$-glucosidase.

Even though the enzyme studied here resembled most other $\beta$-glucosidases in being strongly inhibited by the basic analog of $\alpha$-glucose dNM and its $\alpha$-alkyl derivatives, it was not inhibited by castanospermine, a rigid, bicyclic analog of dNM which inhibits other $\beta$-glucosidases with $K_v$ values in the micromolar range (see Ref. 35 for reviews). This feature and the exceptional resistance of the enzyme against CBE and Br-CBE sets it aside from most other $\beta$-glucosidases and might point to details of the catalytic mechanism that differ from the generally accepted model (35).

The isolated enzyme appears to be highly specific for bile acid 3-O-$\beta$-$D$-glucosides since bile acid 6-O-$\beta$-$D$-glucosides were not hydrolyzed by the enzyme. Furthermore, of the various glycosides of 4-methylumbelliferone tested only the $\beta$-glucoside and the $\beta$-$D$-galactoside showed a reaction with the enzyme with a ratio of relative reaction rates of 11:1. Thus, bile acid $\beta$-glucosidase cannot be classified as a broad specificity $\beta$-glucosidase in contrast to the cystolic $\beta$-glucosidase. This enzyme was shown to hydrolyze not only the $\beta$-$D$-glucoside or the $\beta$-$D$-galactoside but also the $\alpha$-$L$-arabinoside, the $\beta$-$L$-fucoside, or the $\beta$-$D$-xyloside of 4-methylumbelliferone with high activity (12). Bile acid 3-O-glucosides are at present the only natural compounds that could be identified as substrates of bile acid
β-glucosidase. The physiological role of bile acid glucoside hydrolysis is, however, unknown at present and has to be explored by further studies.

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REFERENCES
1. Matern, H., Matern S., and Gerok, W. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 7036–7040
2. Radominska, A., Tread, S., and Little, J. (1993) Semin. Liver Dis. 13, 219–234
3. Matern, S., Marschall, H.-U., Heinemann, H., Gartung, C., Wietholtz, H., Matern, H., and Sjövall, J. (1994) in Cholestatic Liver Diseases. New Strategies for Prevention and Treatment of Hepatobiliary and Cholestatic Liver Diseases (van Berge Henegouwen, G. P., van Hoek, B., de Groote, J., Matern, S., and Stockburger, R. W., eds) pp. 62–68, Kluwer Academic Publishers, Dordrecht, Netherlands
4. Marschall, H.-U., Griffiths, W. J., Zhang, J., Wietholtz, H., Matern, H., Matern, S., and Sjövall, J. (1994) J. Lipid Res. 35, 1599–1610
5. Marschall, H.-U., Egestad, B., Matern, H., Matern, S., and Sjövall, J. (1987) FEBS Lett. 213, 411–414
6. Wietholtz, H., Marschall, H.-U., Reuschelbach, R., Matern, H., and Matern, S. (1991) Hepatology 13, 656–662
7. Radominska, A., Little, J., Pyrek, J. S., Drake, R. R., Igari, Y., Fournel-Gigleux, S., Magdalou, J., Burchell, B., Elbein, A. D., Siest, G., and Lester, R. (1993) J. Biol. Chem. 268, 1527–1535
8. Matern, H., Gartzen, R., and Matern, S. (1992) FEBS Lett. 314, 183–186
9. Gartung, C., Matern, S., and Matern, H. (1993) Gastroenterology 104, A905
10. Gartung, C., Matern, S., and Matern, H. (1994) J. Hepatol. 20, 32–40
11. Pentchev, P. G., Brady, R. O., Hibbert, S. R., Gal, A. E., and Shapiro, D. (1973) J. Biol. Chem. 248, 5256–5261
12. Daniels, L. R., Coyle, P. J., Chiao, Y.-B., Glew, R. H., and Labow, R. S. (1981) J. Biol. Chem. 256, 13004–13013
13. Matern, H., Bolz, R., and Matern, S. (1990) Eur. J. Biochem. 190, 99–105
14. Legler, G. (1977) Methods Enzymol. 46, 388–381
15. Legler, G. and Bieberich, E. (1988) Arch. Biochem. Biophys. 260, 427–436
16. Matern, H., Bolz, R., Marschall, H.-U., Sjövall, J., and Matern, S. (1990) FEBS Lett. 270, 11–14
17. Dinur, T., Grabowski, G. A., Desnick, R. J., and Gatt, S. (1984) Anal. Biochem. 136, 223–234
18. Chester, M. A., Hultberg, B., and Öckerman, P.-A., (1976) Biochim. Biophys. Acta 429, 517–536
19. Laemmli, U. K. (1970) Nature 227, 680–685
20. Matern, H., Matern, S., and Gerok, W. (1982) J. Biol. Chem. 257, 7422–7429
21. Sauvé, D. M., Ho, D. T., and Robert, M. (1990) Anal. Biochem. 226, 382–383
22. Tashova, B., and Dossev, G. (1983) Anal. Biochem. 129, 98–102
23. Suresh-Kumar, T. K., Gopalakrishna, K., Prasad, V. V. H., and Pandit, M. W. (1993) Anal. Biochem. 213, 226–228
24. Hettikamp, H., Legler, G., and Bause, E. (1984) Eur. J. Biochem. 142, 85–90
25. Schweden, J., Legler, G., and Bause, E. (1986) Eur. J. Biochem. 157, 563–570
26. Osiecki-Newman, K. M., Fabbro, D., Dinur, T., Boas, S., Gatt, S., Legler, G., Desnick, R. J., and Grahowski, G. A. (1986) Enzyme (Basel) 35, 147–153
27. Grahowski, G. A., Gatt, S., Kruse, J., and Desnick, R. J. (1984) Arch. Biochem. Biophys. 251, 144–157
28. Ho, M. W., and Light, N. D. (1973) Biochem. J. 136, 821–823
29. Aerts, J. M. F. G., Sa Miranda, M. C., Brouwer-Kelder, E. M., Van Weely, S., Barranger, J. A., and Tager, J. M. (1990) Biochem. Biophys. Acta 1041, 55–63
30. Schutzbach, J. S., and Forsee, W. T. (1990) J. Biol. Chem. 265, 2546–2549
31. Yaqoob, M., and Carroll, M. (1980) Biochem. J. 185, 541–543
32. Mareet, A., Salvayre, R., Negre, A., and Deuste-Blazy, L. (1981) Eur. J. Biochem. 115, 455–461
33. Coleman, R. (1973) Biochim. Biophys. Acta 300, 1–30
34. Racker, E. (1967) Fed. Proc. 26, 1335–1340
35. Legler, G. (1990) Adv. Carbohydr. Chem. Biochem. 48, 319–384