Formation of bile acid glucosides by a sugar nucleotide-independent glucosyltransferase isolated from human liver microsomes

(hepatic metabolism/steroids/conjugation)

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ABSTRACT A heat-labile protein has been detected in microsomes from human liver which catalyzes the formation of glucosides of the bile acids chenodeoxycholic, deoxycholic, and Ursodeoxycholic acids. This glucosyltransferase activity has been purified about 900-fold from human liver microsomes, resulting in homogeneity as determined by sodium dodecyl sulfate gel electrophoresis. The subunit molecular weight was calculated to be about 56,000. The enzyme was separated from bile acid UDP-glucuronosyltransferase [UDP-glucuronate β-D-glucuronosyltransferase (acceptor-unspecific), EC 2.4.1.17] during purification and does not catalyze the formation of bile acid glucuronides. The purified glucosyltransferase utilizes lipophilic alkyld-glucopyranosides as artificial donor substrates and dolichyl phosphoglycoside as natural donor for the transfer of glucose to bile acids and does not exhibit bile acid conjugating activity in the presence of sugar nucleotides such as UDP-glucose. The apparent K\textsubscript{m} values estimated for various alkyl β-D-glucopyranosides decreased with increasing alkyl chain length from 680 × 10^{-6} M for hexyl β-D-glucopyranoside to 20 × 10^{-6} M as estimated for decyl and dodecyl β-D-glucopyranoside. The results suggest that a glucoside-conjugation pathway of bile acids exists in humans. This conjugation is catalyzed by a sugar nucleotide-independent glucosyltransferase and is therefore distinct from the known mechanisms of glycoside conjugation.

Conjugation reactions play an important role in bile acid metabolism, facilitating elimination of potentially hepatotoxic bile acids (1, 2). Three conjugation mechanisms are known at present in bile acid metabolism: conjugation with amino acids such as glycine or taurine (3), sulfation (4), and glucuronidation (5, 6). The present paper shows that glucosidation is an additional conjugation mechanism of bile acids and is catalyzed by a human liver microsomal enzyme. This microsomal glucosyltransferase does not depend on sugar nucleotides as donor substrates for the transfer of glucose to bile acids but utilizes a lipophilic glucosyl donor. The isolation and further properties of the bile acid glucosyltransferase will be described.

EXPERIMENTAL PROCEDURES

Materials. Hexyl, heptyl, octyl, nonyl, and decyl β-D-glucopyranoside and dodecyl β-D-maltoside were purchased from Calbiochem (Freiburg, F.R.G.). Dodecyl β-D-glucopyranoside, β-D-glucosidase from almonds, and α-glucosidase from yeast were purchased from Boehringer (Mannheim, F.R.G.). Sugar nucleotides were from Sigma (Munich, F.R.G.). Dolichyl phospho[6-3H(N)]glucose (6.5 Ci/mmole; 1 Ci = 37 GBq) was obtained from New England Nuclear (Dréieich, F.R.G.). n-[1-14C]Octyl β-D-glucoside was obtained from the Commissariat à l’Energie Atomique (Gif-Sur-Yvette, France). DEAE-Trisacryl was from LKB (Munich, F.R.G.). Sep-Pak C\textsubscript{18} cartridges were from Waters Associates (Milford, MA). The prepacked columns Mono Q HR 5/5 and Mono P HR 5/20 and the FPLC (fast protein, peptide, and polynucleotide liquid chromatography) system were obtained from Pharmacia (Freiburg, F.R.G.). Ethylagarose was from Miles (Frankfurt). Sources of other chemicals were the same as described in previous papers (7, 8).

Human liver autopsy samples were collected from organ donors within 30 min after cessation of life support, perfused with cold 0.9% NaCl, and frozen at −70°C. Samples from the following subjects were used: 8, 17-year-old woman, death due to intracranial hemorrhage; 32-year-old woman and 25-year-old man, death due to head injury. The liver samples were macroscopically normal.

Purification of Glucosyltransferase Activity Toward Chenodeoxycholic Acid. All operations were carried out at 4°C, except where stated otherwise. Standard buffer was 25 mM imidazole·HCl, pH 6.5, containing 10% (vol/vol) glycerol, 1 mM dithioerythritol, and 0.5% (wt/vol) octyl glucoside.

Step 1. Preparation and solubilization of human liver microsomes. Microsomes were prepared from 30 g of human liver, washed and suspended in 0.1 M potassium phosphate, pH 7.4, as previously described (7). The microsomal suspension was solubilized and centrifuged as described elsewhere (7) except that the final concentration of octyl glucoside was 1.3% (wt/vol).

Step 2. Polyethylene glycol fractionation. Fractionation with polyethylene glycol 6000 was carried out as previously described (7) except that the protein precipitating between 5% and 10% (wt/vol) of polyethylene glycol was used for subsequent purification steps. The precipitate was dissolved in standard buffer containing 0.1% Zwittergent 3-12.

Step 3. DEAE-Trisacryl chromatography. The protein fraction from step 2 was applied to a DEAE-Trisacryl column (2.6 × 15 cm) equilibrated with standard buffer containing 0.1% Zwittergent 3-12. After washing with 400 ml of equilibration buffer (flow rate, 80 ml/hr) a linear gradient from 0 to 0.15 M NaCl in the same buffer was applied (total volume, 600 ml; flow rate, 40 ml/hr). Glucosyltransferase activity toward chenodeoxycholic acid emerged at approximately 0.08 M NaCl.

Step 4. Mono Q chromatography. The fractions containing enzyme activity as obtained from step 3 were diluted by an equal volume of standard buffer and subjected to anion-exchange chromatography on a Mono Q column using a Pharmacia FPLC system (flow rate, 1.5 ml/min; pressure, 3.0 MPa). After application of the sample the column, previously equilibrated with standard buffer containing 0.05% Zwittergent 3-12, 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 0.08 M NaCl (total volume, 15 ml) and then from 0.08 to 0.16 M NaCl (total volume, 27 ml). Enzyme activity was eluted at 0.12 M NaCl.

Step 5. Mono P chromatography. The active fractions of step 4 were diluted by 2 vol of standard buffer and subjected
to chromatofocusing on a Mono P column equilibrated with standard buffer containing 0.05% Zwittergent 3-12. Chromatography was performed with a Pharmacia FPLC system (flow rate 0.8 ml/min pressure 0.5 MPa). Two pH gradients, first pH 6.5 to 5.0 and then from 5.0 to 4.5, were developed on the column with two aqueous solutions of 10% (vol/vol) Polybuffer 74 (Pharmacia) containing 10% glycerol, 1 mM dihioeithrythrol, 0.5% octyl glucoside, and 0.05% Zwittergent 3-12, adjusted with HCl to pH 5.0 (elucent volume, 40 ml) and to pH 4.5 (elucent volume, 30 ml). Two peaks of glucosyltransferase activity were eluted from the column. The first activity peak was observed at pH 5.0 at the end of the pH gradient. The second activity peak appeared at pH 4.8 within the second pH gradient.

**Step 6. Ethylagarose chromatography.** The active fractions from step 5 eluting at pH 5.0 or at pH 4.8 from the Mono P column were pooled separately and applied to separate columns of ethylagarose (1 × 12 cm) equilibrated with standard buffer containing 0.05% Zwittergent 3-12. After washing with 50 ml of equilibration buffer a linear gradient from 0 to 0.3 M NaCl in the same buffer was applied (total volume 100 ml, gradient rate 10 ml/hr). Glucosyltransferase activity toward chenodeoxycholic acid from both activity peaks of step 5 was eluted identically at approximately 0.1 M NaCl, pooled separately, and stored at −20°C. Under these conditions the enzyme appeared to be stable for at least 1 month.

**Analytical Methods. Enzyme assays.** For the determination of bile acid glucosyltransferase activity standard incubations were performed at 37°C for 30 min in the presence of 0.1 mM [14C]chenodeoxycholic acid (4 μCi/μmol), 1 mM octyl β-D-glucopyranoside (octyl glucoside) as donor substrate, 15 mM MgCl2, and 0.1 M imidazole-HCl, pH 6.4. The incubations were terminated, and the products were extracted and analyzed as described previously for the assay of bile acid UDP-glucuronosyltransferase (8). For estimation of bile acid glucosyltransferase with dolichyl phosphop(1H)glucose (0.15 μM, 6.5 Ci/mmol) instead of octyl glucoside as donor substrate the dolichyl derivative dissolved in chloroform/methanol (2:1, vol/vol) was first added to the assay tube. The organic solvent was evaporated under a stream of nitrogen, and 0.3 mM chenodeoxycholic acid and 15 mM MgCl2 in 0.1 M imidazole-HCl (pH 6.4) were added. After sonication of the reaction mixture for 15 sec (4°C, 50 W) the reaction was initiated by the addition of enzyme preparation (see legend to Fig. 2) and stopped by mixing with 10 vol of chloroform after incubation for 30 min at 37°C. The aqueous phase, containing the reaction product, was desalted on a Sep-Pak C18 cartridge as described (9), and then the reaction product was separated from residual dolichyl phosphop(1H)glucose on silica gel 60 plates in 1-butanol/acidic acid/water (50:5:10, vol/vol) (RF of reaction product, 0.69; RF of dolichyl phosphogluco, 0.48). The radioactive spot corresponding to the reaction product was eluted with methanol and radioactivity was measured after evaporation of the organic solvent as described (8). Glucuronic acid conjugation of chenodeoxycholic acid was estimated to a published procedure (8). All samples for assays (except microsomual preparations) were reconstituted for 10 min at 4°C with sonicated dispersions of 50 μg of egg yolk l-α-phosphatidylcholine per assay mixture prepared as described elsewhere (7). Units of enzyme activity are expressed as nmol of aglycone substrate conjugated per min.

**Identification of Chenodeoxycholic Acid Glucoside.** To identify the product of the reaction of chenodeoxycholic acid and octyl glucoside, 0.1 unit of the pure enzyme (step 6, Table 1) was incubated in a reaction mixture containing 0.3 mM [14C]chenodeoxycholic acid (1.3 μCi/μmol) and 1 mM octyl glucoside. After 5 hr at 37°C unreacted chenodeoxycholic acid was extracted three times with chloroform. The aqueous phase containing the reaction product was desalted on a Sep-Pak C18 cartridge (9) and shown by TLC on silica gel 60 in 1-butanol/acidic acid/water (50:5:10, vol/vol) not to be contaminated with free chenodeoxycholic acid. Two peaks of glucosyltransferase activity from the reaction product from unreacted octyl glucoside the eluate from Sep-Pak C18 was chromatographed twice on silica gel 60 plates in chloroform/ethanol/28% aqueous ammonia (25:35:1, vol/vol) (10). A single radioactive spot representing the reaction product was detectable at RF 0.05 by a TLC scanner, and this differed in mobility from reference [14C]octyl glucoside (RF 0.66). The reaction product obtained by elution of the radioactive spot with methanol and evaporation to dryness was characterized as follows.

For identification of the steroid moiety, the purified reaction product was treated with β-glucosidase at 100 units/ml in 50 mM sodium acetate, pH 5.0, in the presence and absence of the β-glucosidase inhibitor glucono-1,5-lactone at 10 mM. After 5 hr at 25°C the incubation mixtures were deproteinized with 9 vol of ethanol, and the precipitated protein was washed three times with ethanol. After concentration of the ethanol extracts by evaporation under a stream of nitrogen, TLC was performed with the following buffer (50:5:10, vol/vol): RF 0.69, the sample without the addition of glucono-1,5-lactone was susceptible to β-glucosidase, giving a single radioactive spot with the same mobility as reference [14C]chenodeoxycholic acid, whereas the sample with the addition of glyco-1,5-lactone was stable to β-glucosidase treatment, giving a single radioactive spot at RF 0.69, which was identical with that of the untreated purified reaction product. When the purified radioactive reaction product was treated either with bovine liver β-glucuronidase as described previously for the hydrolysis of bile acid glucuronides (8) or with α-glucosidase at 100 units/ml in 50 mM potassium phosphate, pH 6.0, for 5 hr at 25°C no cleavage of the reaction product was observed after analysis by TLC. For identification of the glucose moiety the purified reaction product was subjected to mild acid hydrolysis as described by Williamson et al. (11). After extraction with chloroform the aqueous phase was evaporated to dryness and the residue was taken up in ethanol/water (7:3, vol/vol) and examined on silica gel 60 plates in 1-propanol/water (7:1, vol/vol) (12) or methyl acetate/2-propanol/water (18:1:1, vol/vol) (12). After spraying with a silver nitrate reagent (12) each chromatogram yielded a single spot with the same mobility as authentic glucosyltransferase.

The reaction product obtained from incubations of enzyme preparation (see legend to Fig. 2) with 0.15 μM dolichyl phosphol[6-3H]glucose and 0.3 mM [14C]chenodeoxycholic acid (1.3 μCi/μmol) for 120 min at 37°C was identified by TLC in 1-butanol/acidic acid/water (50:5:10, vol/vol) exhibiting the same RF as chenodeoxycholic acid glucoside that was enzymatically synthesized from incubations with octyl glucoside as the donor substrate. Furthermore, treatment of the purified reaction product with β-glucosidase as described above yielded free [14C]chenodeoxycholic acid and [14C]glucose, which were identified by TLC in 1-butanol/acidic acid/water (50:5:10, vol/vol) (RF for glucose, 0.27; RF for chenodeoxycholic acid, 0.93). No cleavage of the reaction product was observed after treatment with α-glucosidase or with β-glucosidase in the presence of the β-glucosidase inhibitor glucono-1,5-lactone at 10 mM.

**Other analytical methods.** Protein concentrations were determined as described previously (7). Due to interference of octyl glucoside and Zwittergent 3-12 in concentrations greater than 0.1% with the protein assay the protein content from dilute solutions (<5 μg/ml) was estimated after quantitative precipitation as described by Polacheck and Cabib (13) with the following modifications: The dissolved precipitate was neutralized with 0.5 M HCl, after which protein was
measured as described elsewhere (7).

Disc electrophoresis in the presence of sodium dodecyl sulfate was performed according to Laemmli (14) with 7.5% polyacrylamide gels. The standard proteins used in this method were the same as previously described (7).

RESULTS

Purification of Glucosyltransferase Activity Toward Cheno-

dodeoxycholic Acid. The present study describes the isolation of a human liver microsomal glucosyltransferase catalyzing glucosyl transfer from octyl glucoside to chenodeoxycholic acid. The results of the purification procedure are summarized in Table 1. The procedure involves a polyethylene glycol fractionation followed by anion-exchange chromatography on DEAE-Trisacryl and on Mono Q, chromatofocusing on Mono P, and hydrophobic chromatography on ethylagarose, resulting in an about 900-fold purification of glucosyltransferase activity toward chenodeoxycholic acid from the solubilized microsomal preparation.

After solubilization of the microsomal suspension about 90% of the protein and of the enzyme activities conjugating chenodeoxycholic acid with glucose or glucuronic acid were recovered in the supernatant fluid after high-speed centrifugation. In this solubilized microsomal preparation the ratio of relative activities conjugating chenodeoxycholic acid with glucuronic acid and glucose was 1:0.7 when estimated at a concentration of the bile acid of 0.3 mM. Both enzymes catalyzing glycoside conjugation of chenodeoxycholic acid co-purified by fractionation with polyethylene glycol. However, glucose and glucuronic acid conjugating activities were separated on the DEAE-Trisacryl column. Whereas bile acid UDP-glucuronosyltransferase (EC 2.4.1.17) was not retained on the column and was eluted exclusively before the NaCl gradient was applied, bile acid glucosyltransferase was eluted exclusively at about 0.08 M NaCl. Glucosyltransferase activity toward chenodeoxycholic acid was further purified and could be resolved into two peaks by chromatofocusing, eluting at pH 5.0 and at pH 4.8 with a ratio of total units of about 1:1. The enzyme activities from the two peaks exhibited identical behaviors on hydrophobic chromatography. After this purification step both preparations gave a single band of identical mobility in sodium dodecyl sulfate gel electrophoresis (Fig. 1). A plot of the relative mobility versus the logarithm of the molecular weight of protein standards (data not shown) indicated a subunit molecular weight of the glucosyltransferase of about 56,000. All properties of the pure glucosyltransferase described below were determined for both enzyme preparations purified from the two activity peaks of the Mono P column; the two preparations yielded the same results.

Substrate Specificity. In addition to octyl glucoside, other alkyl glucosides of hydrocarbon chain lengths of 6 to 12 carbon atoms have been investigated to act as glucosyl donor in the formation of chenodeoxycholic acid glucoside. Kinetic analysis of these reactions yielded the Lineweaver–Burk plots shown in Fig. 2. Whereas the V_max values were identical for the various alkyl glucosides studied, the K_m values for these substrates decreased with increasing alkyl chain length, reaching the minimal apparent K_m of 2.0 x 10^-3 M for decyl β-D-glucopyranoside. The same apparent K_m was estimated for dodecyl β-D-glucopyranoside (Fig. 2). Replacement of alkyl glucosides by dodecyl β-D-maltoside (1 mM), or 10 mM UDP-glucuronic acid, UDP-glucose, ADP-glucose, GDP-glucose, GDP-glucose, TDP-glucose, or free glucose did not cause measurable glycoside conjugation of chenodeoxycholic acid by the partially purified glucosyltransferase (see legend to Fig. 2). However, incubation of the partially purified enzyme (see legend to Fig. 2) with doli-
chyl phosphol[3H]glucose and chenodeoxycholic acid resulted in the formation of chenodeoxycholic acid glucoside. The reaction rate was estimated to be 0.02 nmol of bile acid glucoside synthesized per min per mg of protein.

Studies on the bile acid substrate specificity of the pure glucosyltransferase showed that the enzyme was able to cata-
lyze the transfer of glucose from octyl glucoside to deoxy-
cholic or ursodeoxycholic acids in addition to chenodeoxy-
cholic acid. In the presence of 50 μM of these bile acids and 1 mM octyl glucoside the pure enzyme conjugated 30 nmol of chenodeoxycholic acid, 25 nmol of ursodeoxycholic acid, and 68 nmol of deoxycholic acid per min per mg of protein, respectively.

Catalytic Properties. The influence of the pH upon gluco-
syl transfer from octyl glucoside to chenodeoxycholic acid (final concentration, 10 μM) as catalyzed by the pure en-
zyme was investigated at pH 5.5–8.0 in a mixture of 0.1 M concentrations of morpholinoethanesulfonic acid (Mes), N-

Table 1. Purification of glucosyltransferase activity toward chenodeoxycholic acid

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein, mg</th>
<th>Specific activity, units/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Solubilized microsomes</td>
<td>551</td>
<td>0.053</td>
</tr>
<tr>
<td>2. Polyethylene glycol fractionation</td>
<td>129</td>
<td>0.108</td>
</tr>
<tr>
<td>3. DEAE-Trisacryl</td>
<td>8.6</td>
<td>0.97</td>
</tr>
<tr>
<td>4. Mono Q FPLC</td>
<td>0.570</td>
<td>11.7</td>
</tr>
<tr>
<td>5. Mono P FPLC*</td>
<td>0.047</td>
<td>38.3</td>
</tr>
<tr>
<td>6. Ethylagarose*</td>
<td>0.012</td>
<td>48.3</td>
</tr>
</tbody>
</table>

*Data are given for the activity peak eluting at pH 5.0 of Mono P chromatography. Enzyme activity was determined with octyl glucoside as donor substrate.
The enzyme was purified and obtained by elution of a DEAE-Trisacryl column as described in Experimental Procedures except that the buffer used contained 0.2% 3-[3-cholamidopropyl]-dimethylammoniomio)-1-propanesulfonate (CHAPS) instead of octyl glucoside. Units per mg of protein, β-D-Glucopyranosides are as follows: C6, hexyl; C7, heptyl; C8, octyl; C9, nonyl; C10, decyl; and C12 (ο), dodecyl.

(2-acetamido)-2-aminoethanesulfonic acid (Aces), and N-2-hydroxyethylpiperazine-N'-propanesulfonic acid (Hepps) as buffers. The activity of the glucosyltransferase was optimal between pH 6.0 and 6.5 and declined steeply toward pH 5.5 and 8.0.

As shown in Table 2, the activity of the pure glucosyltransferase was influenced by the presence of divalent metal ions. The enzyme was maximally stimulated about 2-fold by 15 mM Mg²⁺. No further increase or decrease in enzyme activity was observed when the concentration of Mg²⁺ was raised from 15 mM to 50 mM (not shown). Mn²⁺ and Ni²⁺ could replace Mg²⁺ in the activation of the glucosyltransferase, whereas Co²⁺ and Ba²⁺ were less stimulatory than Mg²⁺. No significant effect on enzyme activity was observed in the presence of Ca²⁺ or Fe²⁺, whereas Zn²⁺ was inhibitory. Furthermore, enzyme activity was inhibited by 10 mM EDTA.

Other Properties. The isolated protein showed the features of a thermolabile, membrane-bound enzyme. Incubation of the pure glucosyltransferase for 10 min at 56°C led to a loss of enzyme activity toward chenodeoxycholic acid and octyl glucoside by 85% of the initial value. As observed for other membrane-bound lipid-requiring enzymes (15), glucosyltransferase activity was enhanced by the presence of lecithin. The transfer of glucose from octyl glucoside to chenodeoxycholic acid as catalyzed by the pure enzyme was maximally increased about 2-fold by the addition of 50 μg of egg yolk 1-α-phosphatidylcholine per assay mixture.

**DISCUSSION**

The present paper shows that, in addition to the known mechanisms of bile acid conjugation such as amino acid conjugation (3), sulfation (4), and glucuronidation (5, 6), a bile acid-glucosylating pathway may occur in humans. The existence of a glucosyltransfer mechanism in human hepatic bile acid metabolism may be postulated from the demonstration of an enzyme, isolated in the present study from human liver microsomes to a homogeneous state (Fig. 1, Table 1), catalyzing the formation of glucosides of chenodeoxycholic, ursodeoxycholic, and deoxycholic acids. This bile acid glucosyltransferase is physically distinct from the previously described bile acid UDP-glucuronosyltransferase from human liver (6), since the two enzyme forms were separated on a
DEAE-Trisacryl column (see Results). Whereas the physiological significance of bile acid glucuronidation has been demonstrated by the finding of bile acid glucuronides—e.g., in human urine (5, 16)—the biological importance of the formation of bile acid glucosides remains to be explored.

Glucosidation of xenobiotics (17, 18) or endogenous compounds such as estrogens (19, 20) and bilirubin (21) has been shown to be catalyzed by crude homogenates or microsomal preparations of various tissues derived from higher organisms such as fish (17) or mammalian species (18–21), including humans (20, 21). The only glucosyl donor implicated as the source of the glucose for these previously described conjugation reactions was UDP-glucose (17–21). Some formation of estrogen glucosides, however, has been reported by Williamson et al. (22) to be catalyzed in the absence of UDP-glucose by washed rabbit liver microsomes. These authors suggested the presence of a lipid-soluble microsomal sugar donor reacting as intermediate in glucose transfer from UDP-glucose to an aglycone (22). The hypothesis of a lipid intermediate in sugar transfer was also put forward for glucuronic acid conjugation of aglycones (23). However, attempts to demonstrate a glycolipid capable of glycoside conjugation failed (23).

The present study shows the existence of a sugar nucleotide-independent glucosyltransferase in human liver microsomes catalyzing the transfer of glucose from lipophilic alkyl glucosides as artificial donor substrates or from dolichyl phosphoglucose as natural glucosyl donor to bile acids. Therefore, dolichyl phosphoglucose is involved not only as a lipid intermediate in glycoprotein biosynthesis as previously described (24) but also in glucoside conjugation. Lipophilic alkyl glucosides acting as artificial donor substrates in glycosyl transfer to bile acids as shown in the present paper may facilitate further studies on this lipid intermediate-dependent glucoside conjugation.

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