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β-Glucosidase activity towards a bile acid glucoside in human liver

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Human liver contains hydrolytic activity toward 3β-glucosido-chenodeoxycholic acid This β-glucosidase activity, localized predominantly in the microsomal fraction, was optimally active in the presence of divalent metal ions close to pH 5.0 and was inhibited by EDTA. Kinetic parameters and other catalytic properties of hydrolytic activity towards 3β-glucosido-chenodeoxycholic acid from human liver microsomes are described.

Bile acid; Glucoside; Glucosidase

1. INTRODUCTION

Bile acid glucosides have been shown to be formed in human liver microsomes by a glucosyltransferase [1,2] and to occur as constituents of human urine in health [3] and disease [4]. In the present report hydrolytic activity toward the glucoside of the main bile acid chenodeoxycholic acid is described from human liver. This β-glucosidase activity has been shown to have properties distinct from the two β-glucosidases known in human liver and many other mammalian tissues: a lysosomal β-glucosidase, termed glucocerebrosidase (*N*-acylsphingosyl-1-*O*-β-D-glucoside: glucohydrolase) according to its natural substrate glucosylceramide [5], and a broad specificity β-glucosidase located in the cytosol [6–8].

2. MATERIALS AND METHODS

2.1. *Materials*

Sources of chemicals [1,2] and of human liver [9] were the same as described in previous papers. The following materials were obtained from Sigma, Munich: deoxycorticosterone 21-glucoside, octyl α-D-glucopyranoside, glucosylsphingosine (sphingosyl-1-*O*-β-D-glucoside), galactosylsphingosine(sphingosyl-1-*O*-β-D-galactoside), glucosylceramide (glucocerebroside, *N*-acylsphingosyl-1-*O*-β-D-glucoside) from human spleen, NBD (12-[*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)] aminododecanoic acid)-glucosylceramide, L-α-phosphatidyl-L-serine from bovine brain and bromoconduritol A/B. Castanospermine was from Boehringer, Mannheim. 1-Deoxynojirimycin was a generous gift from Drs. G. Scangos and D. Schmidt, Bayer AG, Wuppertal.

[24-^{14}C]Chenodeoxycholic acid glucoside (8 μCi/μmol) was synthesized by enzymatic conjugation of [24-^{14}C]chenodeoxycholic acid with human liver microsomes as enzyme source and octyl β-D-glucopyranoside as glucose donor as described [3]. The enzymatically synthesized chenodeoxycholic acid glucoside has been characterized to be the 3-β-glucoside of the bile acid [1,10] and analyzed by fast atom bombardment mass spectrometry [3].

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2.2. *Tissue preparation*

Liver specimens could be stored frozen at −30°C for at least 2 months without loss of β-glucosidase activity toward chenodeoxycholic acid glucoside. For tissue fractionation, however, only fresh liver samples were used. Liver homogenates were obtained and subjected to differential centrifugation into 4 fractions by a modification of the method described by Amar-Costesec et al. [11]. The tissues were homogenized in 0.25 M sucrose/5 mM Tris-HCl (pH 7.4) using a glass homogenizer with a teflon pestle (Braun-Melsungen). The nuclear fraction was obtained by centrifugation of the homogenate at 1,000 × *g* for 10 min; large granules were sedimented at 25,000 × *g* for 10 min, the microsomal fraction was separated from the final supernatant at 100,000 × *g* for 60 min. All pellets were washed by resuspension in homogenization medium as described [11] followed by recentrifugation at the same force used for the respective fractionation step. The washings were added to the corresponding supernatants before further treatment, and the final pellets were suspended in homogenization medium. All centrifugal forces (*g*) are calculated from average values of radii in rotors.

2.3. *Enzyme assays*

Unless otherwise stated β-glucosidase activity toward chenodeoxycholic acid glucoside was determined as follows: Approximately 10–30 μg of protein was incubated in a reaction mixture which contained (in a total volume of 30 μl) 0.7 nmol [^{14}C]chenodeoxycholic acid glucoside, 0.1 M sodium acetate, pH 5.0, 10 mM $MnCl_2$, 0.1 mM EDTA and 1 mM dithioerythritol. After 20 min at 37°C the reaction was stopped by addition of 30 μl ethanol containing 0.2 nmol unlabeled chenodeoxycholic acid; 50 μl of the mixture was directly applied to the preadsorbent layer of 19-channeled silica gel thin-layer plates (Baker, Phillipsburg, USA), which were developed with chloroform/isopropanol/isobutanol/acetic acid/water (30:20:10:2:1, v/v) and analyzed in a thin-layer chromatogram scanner with a data system (Berthold, Wildbad). In the presence of β-glucosidase activity a radioactively labeled reaction product was detectable which was separated from unreacted [^{14}C]chenodeoxycholic acid glucoside (R_f 0.50) and was identical to the position of authentic [^{14}C]chenodeoxycholic acid (R_f 0.89).

Glucocerebrosidase was assayed fluorimetrically with a mixture of glucosylceramide and the synthetic analogue NBD-glucosylceramide as described [12]. The following marker enzymes were assayed according to published procedures: succinate-INT (2(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium) reductase [13] and glucose-6-phosphatase [14] Acid phosphatase was estimated with *p*-nitrophenylphosphate according to the instructions by Boehringer, Mannheim.

Protein was determined as described in a previous report [1].

3. RESULTS AND DISCUSSION

3.1. *Subcellular distribution*

The distribution of β-glucosidase activity toward chenodeoxycholic acid glucoside in subcellular fractions of liver homogenates in comparison to the distribution pattern of the known membrane-associated β-glucosidase glucocerebrosidase and of various marker enzymes is shown in Table I. According to the distribution of succinate-INT reductase and acid phosphatase as markers of the mitochondrial–lysosomal fraction and of glucose-6-phosphatase as marker enzyme of microsomes [11,13], the pellets sedimenting at 25,000 × *g* and 100,000 × *g* are enriched in mitochondria–lysosomes and microsomes, respectively. Hydrolytic activity toward chenodeoxycholic acid glucoside was found to be mainly recovered in the 100,000 × *g* pellet which showed the highest relative specific activity of 3.5 and therefore the highest enrichment of the enzyme activity. In contrast, however, to the microsomal marker enzyme glucose-6-phosphatase, which was exclusively enriched in the 100,000 × *g* pellet, β-glucosidase activity toward chenodeoxycholic acid glucoside showed an additional minor enrichment of about 1.5-fold as compared to the homogenate in the 25,000 × *g* pellet. Only about 3% of the total hydrolytic activity toward chenodeoxycholic acid glucoside present before fractionation was detectable in the 100,000 × *g* supernatant, indicating the membrane-associated nature of the enzyme activity. The low recovery and lack of enrichment of hydrolytic activity toward chenodeoxycholic acid glucoside in the final supernatant indicates that the enzyme activity is not identical with a broad specificity human liver β-glucosidase which has been localized in the cytosol [7]. The results of Table I suggest that β-glucosidase activity toward chenodeoxycholic acid glucoside is mainly located in the microsomal fraction in addition to a minor association with the 25,000 × *g* pellet. The apparently bimodal distribution of this enzyme activity in human liver should be more precisely clarified when the purified enzyme will be available, e.g. using immunohistochemical methods.

As shown in Table I glucocerebrosidase differed in its subcellular distribution from β-glucosidase activity toward chenodeoxycholic acid glucoside. Enzyme activity toward glucosylceramide was mainly enriched in the 25,000 × *g* pellet and showed a minor association with the 100,000 × *g* pellet as judged from the relative specific activities of glucocerebrosidase in these pellets of 3.0 and 1.3, respectively. The main recovery of glucocerebrosidase in the 25,000 × *g* pellet (Table I) is in accordance with the known lysosomal localization of this enzyme [15]. The different subcellular distribution of activities toward chenodeoxycholic acid glucoside and glucosylceramide indicates the nonidentity of both enzyme activities.

3.2. *Characteristics of β-glucosidase*

The following characteristics of β-glucosidase activity toward chenodeoxycholic acid glucoside have been obtained with human liver microsomes as enzyme source according to the main localization of the enzyme activity in the microsomal fraction (Table I).

The influence of pH on the activity of β-glucosidase toward chenodeoxycholic acid glucoside was investigated at pH 3.5–8.0 in a mixture of sodium acetate, MES and Bis-Tris-Propane, each component 50 mM. Enzyme activity showed a sharp optimum close to pH 5.0 and declined steeply toward pH 3.5 and 8.0, with half maximal activities at pH 4.2 and 6.0 (not shown).

As may be seen from Table II hydrolysis of chenodeoxycholic acid glucoside was activated by divalent metal ions. At pH 6.5, enzyme activity was inhibited about 90% by 2.5 mM EDTA and was completely or partially restored by the addition of Mn^{2+}, Ni^{2+} or Zn^{2+} (5 mM, in the order of decreasing efficiency, Table II). Other

Table I

Quantitative distribution of β-glucosidase activity toward chenodeoxycholic acid glucoside and glucocerebrosidase in relation to marker enzymes and protein in human liver after differential centrifugation

Constituent	Enzyme activities and protein in subcellular fractions (% of homogenate)				
	1,000 × *g* pellet	25,000 × *g* pellet	100,000 × *g* pellet	100,000 × *g* supernatant	Recovery
Enzyme activity toward bile acid glucoside	22.1 ± 5.5 (0.9)	21 8 ± 6.2 (1.5)	46.0 ± 7.6 (3.5)	2.9 ± 1.6 (0.1)	92.8
Glucocerebrosidase	13.5 ± 5.7 (0.5)	41.8 ± 4.3 (3.0)	16.8 ± 3.7 (1.3)	26.1 ± 6.5 (0.6)	98.2
Acid phosphatase	19.8 ± 4 9 (0 8)	30.6 ± 2 8 (2.2)	9.0 ± 1.3 (0.7)	26.3 ± 5.3 (0.6)	85.7
Succinate-INT reductase	25.4 ± 5.1 (1.0)	55.3 ± 5.7 (3.9)	13.5 ± 1.2 (1 0)	4 8 ± 1.2 (0.1)	99 0
Glucose-6-phosphatase	21.4 ± 4.8 (0.9)	8.5 ± 1.3 (0.6)	57 1 ± 5.9 (4.4)	2.8 ± 1.1 (0.1)	89.8
Protein	24.9 ± 4.4	14.1 ± 1.2	13.0 ± 1.8	46.6 ± 7.4	98.6

Values are the mean ± S.D. of 4 experiments. Values in parentheses represent relative specific activities expressed as percent activity/percent protein Specific activities of enzymes in homogenates: (i) in nmol per min per mg protein, hydrolysis of chenodeoxycholic acid glucoside 0.054 ± 0.02, glucocerebrosidase 1.97 ± 0.7. (ii) in μmol per min per mg of protein acid phosphatase 0.03 ± 0.01, succinate-INT reductase 0.044 ± 0 008, glucose-6-phosphatase 0.09 ± 0.02. Total protein in homogenate, 185 ± 24 mg, corresponding to 2 g of fresh liver.

divalent metal ions such as Mg^{2+}, Ca^{2+} or Co^{2+} caused a comparable activation of enzyme activity as described in Table II for Mn^{2+} (not shown). At pH 5.0, metal ions had a similar effect on hydrolysis of chenodeoxycholic acid glucoside as observed at pH 6.5. Enzyme activity at pH 5.0 was, however, less sensitive to inhibition by EDTA, with 50% inhibition occurring at an EDTA concentration of 10 mM. The metal ion requirement observed for β-glucosidase activity toward chenodeoxycholic acid glucoside is in contrast to other α- or β-glucosidase activities, which have not been described to be metal ion dependent or affected by metal ion chelating agents such as EDTA [5,8,16]. However, various α-mannosidases have been shown to require divalent metal ions for activity and are inhibited by EDTA [17–19].

Since the membrane-associated lysosomal β-glucosidase glucocerebrosidase was shown to require detergents and/or negatively charged lipids for optimal hydrolysis of substrates [20] the effect of Triton X-100 (0.001–0.25%, v/v), taurocholate (0.1–10 mM) or phosphatidylserine (0.02–2 mM) was studied on microsomal β-glucosidase activity toward chenodeoxycholic acid glucoside. In the presence of these compounds hydrolysis of chenodeoxycholic acid glucoside was not increased but inhibited with increasing concentrations of Triton X-100 (> 0.005%), taurocholate (> 2 mM) or phosphatidylserine (> 0.5 mM). Since these compounds were shown to be activators of glucocerebrosidase [20] these results show that β-glucosidase activities toward chenodeoxycholic acid glucoside and glucosylceramide exhibit marked differences in properties.

Double-reciprocal plots of initial rates of β-glucosidase activity as a function of varying concentrations of chenodeoxycholic acid glucoside (3–26 μM) yielded straight lines, from which the apparent K_m was calculated to be 15.4 ± 7.1 μM (not shown). The V_{max} was 0.73 ± 0.3 nmol/min/mg protein (all values given as mean ± S.D., which were obtained from 4 different liver samples).

Table II

Metal ion dependence of β-glucosidase

Metal ion (5 mM)	EDTA (2.5 mM)	β-Glucosidase activity (% of control)
None	–	100
None	+	13
Mn^{2+}	–	187
Mn^{2+}	+	210
Ni^{2+}	+	150
Zn^{2+}	+	91

The assay for β-glucosidase activity toward chenodeoxycholic acid glucoside was similar to the standard assay except that the buffer was 50 mM MES, pH 6.5, and additions of metal ion and/or EDTA were as indicated. Control activity represents enzyme activity without the addition of EDTA and metal ions.

As shown in Table III hydrolysis of chenodeoxycholic acid glucoside was highly sensitive to inhibition by compounds known as inhibitors of α- and β-glucosidases, such as D-(1,5)-gluconolactone, the basic glucose analogue, 1-deoxynojirimycin, bromoconduritol A/B [21] and the alkaloid, castanospermine. Furthermore, β-glucosidase activity toward chenodeoxycholic acid glucoside was inhibited by natural or synthetic glycosides, such as glucosylsphingosine, galactosylsphingosine, deoxycorticosterone 21-glucoside or octyl β-D-glucopyranoside and its α-anomer. Most of these compounds have also been described to cause inhibition of cytosolic β-glucosidase [7,8] and of glucocerebrosidase [20]. Hydrolysis of chenodeoxycholic acid glucoside was not inhibited by glucosylceramide (0.01–0.5 mM), which is the natural substrate of glucocerebrosidase. A comparison of the inhibitory effects of glucose and galactose or of their glycosidic derivatives, glucosylsphingosine and galactosylsphingosine, shows a higher inhibitory potency for the gluco-compounds. Comparing the effects of α- and β-linked octyl D-glucopyranosides on enzyme activity, the β-anomer showed a similar inhibition as the α-anomer at a 50-fold lower concentration (Table III). Whether or not the glycosidic inhibitors of β-glucosidase indicated in Table III also act as substrates of the enzyme activity should be clarified when the purified enzyme becomes available.

The results of the present study show the existence of a human liver β-glucosidase activity that is mainly present in the microsomal fraction and is able to hydrolyze chenodeoxycholic acid glucoside as endogenous substrate. Since in human liver microsomes bile acid glucosides can also be synthesized by a glucosyltransferase [1], futile cycling of bile acid glucosides may occur via hydrolysis and re-synthesis in human liver. Futile cy-

Table III

Inhibition of β-glucosidase

Additions (mM)		β-Glucosidase activity (% of control)
D-(1,5)-Gluconolactone	0.2	48
1-Deoxynojirimycin	0.001	57
Bromoconduritol A/B	0.3	53
Castanospermine	2.0	50
Glucosylsphingosine	0.02	46
Galactosylsphingosine	0.1	42
Glucosylceramide	0.5	105
Deoxycorticosterone 21-glucoside	0.1	57
Octyl β-D-glucopyranoside	0.1	46
Octyl α-D-glucopyranoside	5.0	53
Glucose	100	48
Galactose	100	91

Hydrolytic activity toward chenodeoxycholic acid glucoside was measured in the presence or absence of the indicated additions using the assay described in Materials and Methods. Control activity represents enzyme activity determined in the absence of additions to the assay.

cling has been considered to be of biological importance in the regulation of sulfate conjugates by the action of specific transferases and sulfatases in the liver [22]. Whether or not futile cycling of bile acid glucosides plays a physiological role in bile acid metabolism has to be clarified by further studies.

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