Bilirubin mono- and di-glucuronide formation by purified rat liver microsomal bilirubin UDP-glucuronyltransferase

Brian BURCHELL* and Norbert BLANCKAERT†‡

*Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland, U.K., and †Laboratory of Hepatology, Catholic University of Leuven, B-3000, Leuven, Belgium

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Highly purified bilirubin UDP-glucuronyltransferase from Wistar-rat liver, when reconstituted with Gunn-rat liver microsomes (microsomal fraction), was able to catalyse the conversion of unesterified bilirubin into both bilirubin monoglucuronide and diglucuronide. Under zero-order kinetic conditions for monoglucuronide formation, the fraction of bilirubin diglucuronide formed by incubation of bilirubin with the reconstituted highly purified transferase accounted for 18% of total bilirubin glucuronides, which was only slightly lower than the fraction of diglucuronides (23% of total bilirubin glucuronides) formed by incubation with hepatic microsomes in the presence of UDP-*N*-acetylglucosamine or Lubrol. The reconstituted purified enzyme also catalysed the UDP-glucuronic acid-dependent conversion of bilirubin monoglucuronide into diglucuronide and, when bilirubin was incubated with UDP-glucose or UDP-xylose, the formation of bilirubin glucosides and xylosides respectively. These results suggest that a single microsomal bilirubin UDP-glycosyltransferase may be responsible for the formation of bilirubin mono- and di-glycosides.

Bilirubin, the principal breakdown product of haem in mammals, is excreted in bile largely in the form of glycosidic conjugates (Blanckaert & Schmid, 1982). In man and most mammals, the major polar bilirubin conjugate excreted is bilirubin diglucuronide (Gordon *et al.*, 1976; Fevery *et al.*, 1977; Compennolle *et al.*, 1978). Formation of bilirubin monoglucuronide (BMG) is known to be catalysed by bilirubin UDP-glucuronyltransferase (see Dutton, 1980), one of a family of hepatic microsomal UDP-glucuronyltransferases (EC 2.4.1.17; Burchell, 1981a). It has been proposed that 1 mol of bilirubin diglucuronide (BDG) can be formed by dismutation of 2 mol of BMG catalysed by a separate plasma-membrane enzyme (Jansen *et al.*, 1977; Chowdhury *et al.*, 1978). This proposal has been challenged on the basis of studies of the metabolism of BMG in intact rats (Blanckaert *et*

al., 1980; Sieg *et al.*, 1982), and UDP-glucuronic acid-dependent formation of BDG by digitonin-treated hepatic microsomes (microsomal fraction) has been demonstrated (Blanckaert *et al.*, 1979; Gordon & Goresky, 1980; Gordon *et al.*, 1983). Further indirect support for the thesis that both mono- and di-glucuronides are formed by a microsomal UDP-glucuronyltransferase system came from the observation that conversion of BMG *in vivo* and *in vitro* into the diglucuronide is totally absent in the homozygous Gunn rat, a mutant rat strain unable to esterify bilirubin because it lacks hepatic bilirubin UDP-glucuronyltransferase activity (Blanckaert *et al.*, 1979). Sieg *et al.* (1982) have concluded that conversion of BMG into BDG in the presence of plasma-membrane preparations was a non-enzymic phenomenon involving disproportionation of BMG.

We report here that formation of both BMG and BDG appears to be catalysed by a single microsomal bilirubin UDP-glucuronyltransferase purified to apparent homogeneity. The substrate specificity of this purified enzyme has been further examined with UDP-glucose and UDP-xylose as potential sugar donors.

Abbreviations used: BMG, bilirubin monoglucuronide; BDG, bilirubin diglucuronide; h.p.l.c., high-pressure liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

‡ Present address: Department of Laboratory Medicine, L-518, University of California, San Francisco, CA 94143, U.S.A.

Materials and methods

Chemicals and reagents were as described elsewhere (Burchell, 1977, 1980, 1981*b*; the preceding paper, Vermeir *et al.*, 1984), except when stated otherwise in the text. The UDP-sugars were purchased from Sigma (St. Louis, MO, U.S.A.).

Microsomal and purified bilirubin UDP-glucuronyltransferase were prepared from liver of male Wistar rats pretreated with 2g of phenobarbital/litre in the drinking water for 7 days, by methods previously described (Burchell, 1977, 1980, 1981*b*). Purified enzyme fractions were reconstituted with Gunn-rat liver microsomes before assay (Burchell, 1982).

Total bilirubin UDP-glycosyltransferase (glucuronyl-, glucosyl- or xylosyl-transferase) activity was measured by the ethyl anthranilate diazo-assay, as described by Heirwegh *et al.* (1972) and Fevery *et al.* (1972). Radioassay of initial rates of formation of the individual C-8 and C-12 BMGs and BDG was performed with [¹⁴C]bilirubin as substrate and with alkaline methanolysis combined with t.l.c. for analysis of the reaction products (Vermeir *et al.*, 1984). Incubation mixtures contained 10 or 50 μ M-bilirubin, 10mM-UDP-glucuronic acid, 2mM-UDP-*N*-acetylglucosamine and 1mg of microsomal protein/ml. Portions of the reaction mixtures were removed for analysis at various time intervals during an incubation period of up to 8min to enable initial rates of reaction to be determined. Computed initial velocities were based on linear portions of the product-formation curve and defined by at least three time points.

Assay of conversion of unlabelled BMG into BDG was performed by the alkaline-methanolysis method combined with reverse-phase h.p.l.c. (Muraca & Blanckaert, 1983). The BMG substrate was purified from rat bile (Blanckaert *et al.*, 1979) and contained 83% authentic BMG, 12% BDG and 5% unconjugated pigment. BMG dissolved in 0.140M-Hepes buffer, pH7.7, containing 5mM-sodium ascorbate to inhibit disproportionation (Sieg *et al.*, 1982), was added to the incubation mixture, and absence of disproportionation of BMG during incubation was verified by analysis of the III α and XIII α isomers of bilirubin prepared by saponification of the incubated pigment as described elsewhere (Blanckaert *et al.*, 1979).

Protein concentrations greater than 1mg/ml were determined by a modification of the Lowry method eliminating the interference by detergents as described by Dulley & Grieve (1975). Protein concentrations of less than 1mg/ml were determined by the method of Bradford (1976). SDS/polyacrylamide-gel electrophoresis was performed using a slab gradient [7–9% (w/v) polyacrylamide gel by the method of Laemmli (1970).

Results and discussion

Enzyme purification and bilirubin glucuronidation

Wistar-rat liver microsomal fractions, the initial purification stage, were assayed in the presence of UDP-*N*-acetylglucosamine or Lubrol. The results obtained show that these preparations catalyse the formation of both BMGs and BDG. Under

Table 1. *Formation of bilirubin monoglucuronides and bilirubin diglucuronide by hepatic bilirubin UDP-glucuronyltransferase at various stages of purification*

Initial rates of formation of individual bilirubin glucuronides by the enzyme preparation were determined by radioassay. Incubation mixtures contained 50 (expt. A) or 10 (expt. B) μ M-bilirubin, 10mM-UDP-glucuronic acid and 1mg of protein/ml (including Gunn-rat microsomal protein in reconstituted preparations; see the Materials and methods section). Enzyme activities of microsomes were assayed in the presence of 2mM-UDP-*N*-acetylglucosamine and 0.1% (w/v) Lubrol in expts. A and B respectively. The other purified enzyme preparations were reconstituted with Gunn-rat microsomes before assay of transferase activity. Fraction 1, 25–60% satd.-(NH₄)₂SO₄ precipitate; fraction 2, DEAE-cellulose fraction; fraction 3, UDP-glucuronic acid eluate from UDP-hexanolamine affinity column. Abbreviations used: C-8 and C-12 BMG, bilirubin C-8 and C-12 monoglucuronides.

Expt.	Purification step	Specific activity (nmol/min per mg of protein)			Purification of transferase activity (fold)	Composition of formed glucuronides (as ratio)	
		C-8 BMG	C-12 BMG	BDG		C-8 BMG	BMG
						C-12 BMG	BDG
A	Microsomes	0.43	0.18	0.18	1.0	2.4	3.4
	Fraction 1	0.36	0.16	0.29	1.0	2.3	1.8
	2	0.63	0.25	0.22	1.4	2.5	3.9
	3	24.57	10.57	7.71	55	2.3	4.6
B	Microsomes	0.37	0.27	0.18	1.0	1.4	3.5
	Fraction 1	0.57	0.30	0.16	1.3	1.9	5.2
	3	14.79	8.58	5.0	35.3	1.8	4.8

zero-order kinetic conditions the diglucuronide amounted to 22–23% of the rate of formation of the total BMG (Table 1). These results are in agreement with those obtained in previous work (Blanckaert *et al.*, 1979; Gordon *et al.*, 1983; Vermeir *et al.*, 1984). Further, the initial rates of formation of the two isomeric (C-8, C-12) BMGs by these microsomal preparations indicated that the C-8 isomer was produced approximately twice as rapidly as the C-12 isomer (Table 1), confirming previous work of Blanckaert *et al.* (1979).

Gunn-rat liver microsomes, used to reconstitute purified transferase preparations (Burchell, 1982), were also examined for their ability to catalyse the formation of bilirubin glucuronides. Gunn-rat liver microsomes alone, incubated in the presence or absence of 0.1% (w/v) Lubrol, were unable to catalyse the formation of BMG and the conversion of BMG into BDG, confirming the results obtained by Blanckaert *et al.* (1979) and Sieg *et al.* (1982).

Bilirubin UDP-glucuronyltransferase was then purified 55-fold [over the $(\text{NH}_4)_2\text{SO}_4$ fraction of Wistar-rat liver microsomes previously described by Burchell (1982)], and the results are summarized in Table 1.

Separation of microsomal UDP-glucuronyltransferases by DEAE-cellulose chromatography (see Burchell, 1981a) facilitates the isolation of bilirubin UDP-glucuronyltransferase from the other isoenzymes (Burchell, 1980). Various fractions from the DEAE-cellulose column in which bilirubin UDP-glucuronyltransferase activity was detected by the diazo-assay were 'pooled' and concentrated as previously described (Burchell, 1980). The initial rates of formation of the individual BMGs and BDG at each stage of the purification were determined by radioassay (Table 1). The ratio of initial formation rates of the two isomeric (C-8, C-12) monoglucuronides remained relatively constant at each stage of the enzyme purification such that the C-8 isomer was produced approximately twice as rapidly as the C-12 isomer, indicating that purification does not alter the specificity of production of monoglucuronides. Further, the percentage of diglucuronide formation was not affected to a major extent by purification of the enzyme system by ion-exchange chromatography (Table 1). Thus no obvious enrichment of BDG-forming-enzyme activity over BMG-forming-enzyme activity occurred during separation of UDP-glucuronyltransferase isoenzymes by DEAE-cellulose chromatography, indicating that these two enzyme activities are apparently co-purified.

The DEAE-cellulose fraction exhibiting the highest bilirubin UDP-glucuronyltransferase activity was applied to a UDP-hexanolamine-

Sephacryl column and the transferase was further purified some 40-fold to near-homogeneity. Only one major protein (Coomassie Blue-staining band, M_r 53000) was visible after SDS/polyacrylamide-gel electrophoresis analysis of the purified enzyme (Fig. 1). Bilirubin UDP-glucuronyltransferase activity was 40–50 nmol of total bilirubin glucuronides formed/min per mg of protein when the purified enzyme was assayed in the presence of Gunn-rat liver microsomes by the diazo-method or the radioassay. The final specific activity of the purified enzyme could be higher (Burchell, 1982),

Fig. 1. SDS/polyacrylamide-gel electrophoresis of purified bilirubin UDP-glucuronyltransferase

Slab-gel electrophoresis was performed in a linear 7–9%-(w/v)-acrylamide separating gel (15 cm × 15 cm) and a 3%-(w/v)-polyacrylamide stacking gel (2 cm/15 cm) in the presence of 0.1% SDS (see the Materials and methods section). Track 1, purified bilirubin UDP-glucuronyltransferase (2 μg of protein); tracks 2 and 3, not used; track 4, protein standards [albumin (M_r 68000), pyruvate kinase (57000), fumarate (49000) and aldolase (40000)]. Gels were stained with Coomassie Brilliant Blue for 60 min and destained with acetic acid/methanol/water (7:5:43, by vol.). The direction of migration was from the top to the bottom.

but reflects the instability of the purified transferase. However, the highly purified bilirubin UDP-glucuronyltransferase was still able to catalyse the formation of BDG and the initial rates of diglucuronide formation, expressed as a percentage of initial rates of total BMG formation, was still comparable with the ratio observed in incubations with microsomal enzyme (i.e. 17 and 18% with highly purified enzyme, and 22 and 23% with the microsomal enzyme respectively; see Table 1). These results suggest that both BMGs and BDG are apparently formed by a single hepatic microsomal enzyme.

To exclude the possibility that the diglucuronide obtained during incubation with the highly purified transferase was formed non-enzymically, by disproportionation of enzymically formed BMG, purified BMG was substituted for [^{14}C]bilirubin as substrate. At an initial substrate concentration of $20\text{ }\mu\text{M}$ -BMG, the initial velocity of diglucuronide formation was $3.34\text{ nmol/min per mg}$ of purified enzyme protein (Fig. 2), which is comparable with the rate of diglucuronide formation with the same enzyme preparation incubated with [^{14}C]bilirubin (Table 1). Conversion of BMG into diglucuronide was absent when UDP-glucuronic acid was omitted from the incubation medium. Collectively, these results demonstrate that diglucuronide formation was catalysed by the highly purified UDP-glucuronyltransferase and are consistent with the hypothesis that both BMG and BDG are formed by a single hepatic microsomal UDP-glucuronyltransferase.

Esterification of bilirubin with glucose and xylose

A combined hereditary defect of conjugation *in vivo* and *in vitro* of bilirubin with glucuronic acid, glucose and xylose in the homozygous Gunn rat has been well documented (Feverly *et al.*, 1972; Blanckaert *et al.*, 1977). These observations are consistent with the idea that conjugation of bilirubin with these three sugars is catalysed by a single microsomal UDP-glycosyltransferase. Thus we have compared the activities of reconstituted highly purified bilirubin UDP-glucuronyltransferase towards the three sugar-nucleotide substrates (Table 2). Disruption of hepatic microsomes with Lubrol considerably decreased the UDP-glycosyltransferase activity toward UDP-glucose and UDP-xylose. However, the highly purified bilirubin UDP-glucuronyltransferase was able to accept UDP-xylose and UDP-glucose as substrate in the formation of bilirubin glycosides, although we have not determined whether a second glycosyltransferase had been removed during the purification of the bilirubin UDP-glucuronyltransferase activity.

A preliminary search for other aglycone sub-

Fig. 2. Conversion of purified BMG into BDG by highly purified bilirubin UDP-glucuronyltransferase. Purified BMG ($20\text{ }\mu\text{M}$) was incubated with 5 mM -UDP-glucuronic acid and 0.2 mg of highly purified bilirubin UDP-glucuronyltransferase (fraction 3 of expt. B, Table 1)/ml of incubation medium. The enzyme was reconstituted with Gunn-rat microsomes (see the Materials and methods section). After various periods of incubation, samples were removed and the composition of bilirubins was analysed by the h.p.l.c. method of Muraca & Blanckaert (1983). The curves show the formation of diglucuronide (▲) and the disappearance of BMG (●). The open symbols give the composition after 10 min of incubation in the absence of added UDP-glucuronic acid.

strates for the purified bilirubin UDP-glucuronyltransferase indicates that the enzyme does not simply accept substrates that contain a free carboxylic acid group and form ester glucuronides. For example, the hypolipidaemic carboxylic acid drug clofibrate is an inducer of bilirubin UDP-glucuronyltransferase and is glucuronidated by microsomes of Wistar-rat liver (Odum & Orton, 1983). However, clofibrate UDP-glucuronyltransferase activity was not detected in partially purified bilirubin UDP-glucuronyltransferase, but

Table 2. Activity of microsomal and purified bilirubin UDP-glucuronyltransferase towards UDP-sugar substrates. Bilirubin conjugation was measured by the ethyl anthranilate diazo-assay in incubation mixtures containing 1 mg of protein/ml, 100 μ M-bilirubin, and 5 mM-UDP-glucuronic acid (UDP-GlcA), 5 mM-UDP-glucose (UDP-Glc) or 5 mM-UDP-xylose (UDP-Xyl). Results shown are mean values for four experiments. For details of fraction 3, see Table 1 and the Materials and methods section.

Enzyme preparation	Bilirubin UDP-glucosyltransferase activity			
	Specific activity using UDP-GlcA (nmol/min per mg of protein)	Relative rates of bilirubin conjugation		
		UDP-GlcA	UDP-Glc	UDP-Xyl
Untreated microsomes	0.60	1.0	0.78	0.28
Microsomes + 0.1% Lubrol	1.62	1.0	0.18	0.05
Fraction 3	41.50	1.0	0.35	0.21

was found in a separate fraction corresponding with purified 4-nitrophenol UDP-glucuronyltransferase (B. Burchell & J. Odum, unpublished work). Further, glucuronidation of clofibric acid was catalysed by Gunn-rat liver microsomes (B. Burchell & J. Odum, unpublished work). These results indicate that clofibric acid is not a substrate-related inducer of bilirubin UDP-glucuronyltransferase.

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