REACTIVATION OF A PURE DEFECTIVE UDP-GLUCURONYLTRANSFERASE FROM HOMOZYGOUS GUNN RAT LIVER

Philip J. WEATHERILL and Brian BURCHELL
Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee, DD1 4HN, Scotland

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1. Introduction

The conjugation of endogenous compounds and xenobiotics with glucuronic acid is catalysed by hepatic microsomal UDP-glucuronyltransferase [EC 2.4.1.17] [1]. The purification of this pharmacologically important enzyme from Wistar rats has already been reported [2,3].

Gunn (1938) described a mutant strain of Wistar rat which has hereditary hyperbilirubinemia [4]. More recent work has shown this mutant strain to be a good animal model for the study of the human Crigler-Najjar syndrome [5]. The hereditary defect in Gunn rat affects the rate of glucuronidation of various substrates and the rate of glucuronidation appears to depend upon the type of aglycone substrate used for assay [6]. Purification of a defective UDP-glucuronyltransferase from the Gunn rat should provide an insight into the biochemistry of inherited UDP-glucuronyltransferase deficiency diseases.

Diethylnitrosamine has been shown to cause a remarkable stimulation of the abnormally low UDP-glucuronyltransferase activity of Gunn rat liver preparations towards 2-aminophenol and paracetamol, measured in vitro [7]. More recently this hepatocarcinogen has been used to activate UDP-glucuronyltransferase towards several other substrates and in liver preparations from other species (referenced [6]).

We have used diethylnitrosamine to facilitate the isolation of a pure defective UDP-glucuronyltransferase from Gunn rat liver. The activity of the pure enzyme towards 2-aminophenol and 4-nitrophenol was not detectable. However enzyme activity could be reactivated by the addition of diethylnitrosamine, but not phospholipids.

2. Materials and methods

Lubrol 12A9 (a condensate of dodecyl alcohol with approx. 9.5 mol ethylene oxide/mol) was a gift from ICI Organics Division, Manchester.

UDP-Hexanolamine–agarose (6–7 μmol UDP hexanolamine/g Sepharose 4B) was synthesized in this laboratory as in [8].

UDP-Glucuronic acid (triammonium salt), 4-nitrophenol, bilirubin, bovine serum albumin and Coomassie brilliant blue protein stain were all from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey. 2-aminophenol was from B.D.H. Chemicals Ltd., Poole, Dorset and twice resublimed before use. Diethylnitrosamine was from Eastman Kodak Co., Rochester, NY and 1-[1-^{14}C]naphthol from the Radiochemical Centre, Amersham. All other chemicals were analytical-reagent grade, where available.

The initial stock of Gunn rats was a kind gift from Professor B. H. Billing, Royal Free Hospital, London and a colony has been successfully bred from this stock in the Institute animal unit. Wistar rats were from the colony maintained in the Institute.

2.1. *Enzyme assays*

UDP-glucuronyltransferase activity towards various substrates was assayed by the following methods: 2-aminophenol and 4-nitrophenol [9], bilirubin [10] and 1-naphthol [11]. Protein concentrations were measured by the Biuret method [12] and the method in [13] with bovine serum albumin as standard.

2.2. *Gel electrophoresis*

Gel electrophoresis was performed using 7.5% polyacrylamide gels in the presence of 0.1% sodium

dodecyl sulphate at 20°C, pH 7.2, at a constant 60 V [14] and under non-denaturing conditions using 7.3% polyacrylamide gels at 20°C, pH 8.9, at a constant 100 V [15]. The proteins were stained with Coomassie brilliant blue as in [2].

2.3. *Purification of UDP-glucuronyltransferase*

Individual male Gunn rat livers were homogenised in 3 vol. ice-cold 0.25 M sucrose and immediately assayed for UDP-glucuronyltransferase activity using bilirubin as aglycone substrate. Non-detectable enzyme activity towards bilirubin in the 25% (w/v) homogenates was taken as confirmation that the animals were of the Gunn strain. Four individual homogenates were then pooled and used as the enzyme source for purification of UDP-glucuronyltransferase as in [2,3].

3. Results and discussion

3.1. *Comparison of the activity of unpurified UDP-glucuronyltransferase from Gunn and Wistar rat liver preparations towards various substrates*

UDP-glucuronyltransferase activity of male Gunn or Wistar rat liver preparations were assayed using various aglycone substrates. The enzyme activities of the 25% (w/v) homogenates, centrifugation fractions and microsomes activated by Lubrol were compared. Enzyme activity towards bilirubin was not detectable in any of these Gunn rat liver preparations even after solubilisation with Lubrol.

UDP-glucuronyltransferase activity of Gunn rat liver microsomal preparations towards 2-aminophenol, 4-nitrophenol and 1-naphthol were only 8.5%. 43.6% and 21.2%, respectively, of the activity of the equivalent Wistar liver preparations. These differences were also observed in the crude homogenate and the 10 000 × *g* supernatant. The reduced levels of enzyme activity towards 4-nitrophenol and 2-aminophenol observed in Gunn rat liver preparations are similar to values in [16]; (referenced [6].)

UDP-glucuronyltransferase activity of Gunn rat liver microsomal fractions was not activated towards 2-aminophenol by Lubrol, although the activity of the comparable Wistar rat liver microsomal fraction was increased 4.5-fold. However, enzyme activities towards 4-nitrophenol and 1-naphthol were increased 1.7-fold and 6.7-fold, respectively, by addition of the detergent, although these activation values are much lower than the 15–20-fold observed using Wistar rat liver microsomes. Resistance to activation may depend on the concentration and type of activator used (referenced [6]). In fact UDP-glucuronyltransferase activity of Gunn or Wistar rat liver preparations was increased to a similar extent towards testosterone or 4-nitrophenol by optimal concentrations of digitonin, reported [17].

3.2. *Purification of UDP-glucuronyltransferase*

UDP-glucuronyltransferase activity was solubilized from Gunn rat liver using Lubrol 12A9 as in [2]. Microsomal pellets were resuspended in 1% Lubrol/0.2 M potassium phosphate buffer pH 7.0 and then centrifuged at 105 000 × *g* for 60 min. This procedure resulted in 95% of the enzyme activity towards 4-nitrophenol, 79% of that towards 2-aminophenol and 75% of the activity towards 1-naphthol present in the Lubrol suspension, remaining in the 105 000 × *g* Lubrol-soluble supernatant, values in good agreement with those obtained using Wistar rat liver microsomal pellets [2]. As 85% of the original microsomal protein was also contained in this fraction, only a slight purification was achieved, and this was ignored in the estimation of enzyme purification.

The specific activity of UDP-glucuronyltransferase from Gunn rat liver increased from 0.05–588.7 units/mg protein towards 1-naphthol as substrate (table 1). Thus the enzyme has been purified 1512-fold over the 10 000 × *g* supernatant fraction, if enzyme activation during Lubrol solubilisation was excluded from the calculation. This purification value was comparable to that reported using untreated Wistar rat liver as the enzyme source [3]. The specific activity of the enzyme in the final preparation was 30% of the value observed in a comparable preparation from phenobarbital-treated Wistar rats, obtained at the same time using similar chromatography materials.

Purification of Gunn rat liver UDP-glucuronyltransferase using affinity chromatography works as efficiently as the purification of the enzyme from Wistar rat liver. Approx. 30–40% yield of enzyme activity was obtained at this stage in both cases. This result suggests that a defect of the UDP-binding site, as proposed on kinetic evidence [16] is unlikely to exist, as binding to immobilised UDP and inhibition

Table 1
Purification of Gunn rat liver UDP-glucuronyltransferase activity towards 1-naphthol as substrate

Purification step	Total protein (mg)	Specific activity (units/mg protein)	Relative purification	Total activity[a] (units)	Yield (%)
1. 10 000 × *g* supernatant	9020	(0.05)	1	–	–
2. Microsomal fraction	–	(0.28)	5.6	–	–
3. Lubrol-soluble supernatant	1842	2.18	5.6	4016	100
4. 25–60% satn. $(NH_4)_2SO_4$ precipitate	900	1.79	4.6	1611	40.1
5. DEAE-cellulose eluate	185	3.17	8.1	587	14.6
6. CM-cellulose eluate / DEAE–Sephadex eluate	15.8	14.9	38.3	235	5.9
7. UDP-Hexanolamine agarose/ UDP-Glucuronic acid eluate	0.168	588.7	1512	98.9	2.5

[a] 1 unit activity represents 1 nmol glucuronide formed/min. Figures in parentheses represent enzyme activity measured in the absence of detergent. Results shown were obtained from 4 male Gunn rat livers

by free UDP [16] appear to be similar for the UDP-glucuronyltransferase from both Wistar and Gunn rat liver.

3.3. *Criteria for purity of Gunn rat liver UDP-glucuronyltransferase*

Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate was used to assess the purity of the UDP-glucuronyltransferase preparation. Figure 1 is a photograph of the stained polypeptides visible after gel electrophoresis. Gel C shows that two major polypeptides are present in the DEAE–Sephadex eluate which was applied to the UDP-hexanolamine–Sepharose column. In comparison only one staining band was observed on gel D, which shows the enzyme protein eluted by UDP-glucuronic acid from the UDP-hexanolamine Sepharose column. Gels A and B show the staining polypeptides obtained at the same stages of a recent UDP-glucuronyltransferase preparation from phenobarbital-treated Wistar rat liver. Comparison of gels D and B shows that the enzyme proteins from the two liver sources exhibit a similar molecular size and mobility. In order to

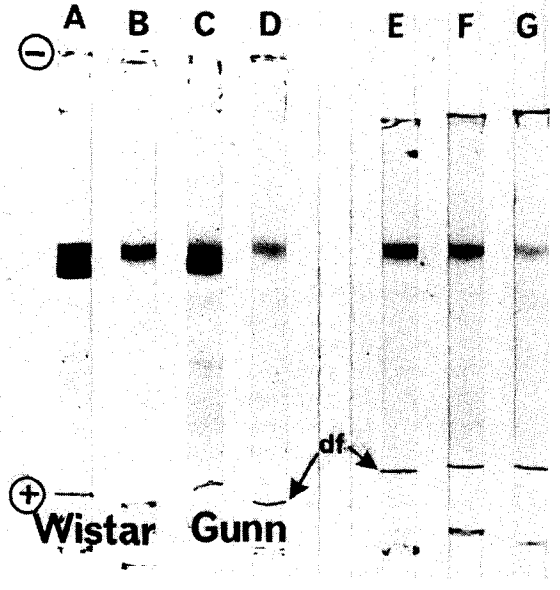

Fig.1. Sodium dodecyl sulphate gel electrophoretograms of purified UDP-glucuronyltransferase. Disc electrophoresis was performed with 7.5% cylindrical gels (7.0 × 0.6 cm) in the presence of 0.1% sodium dodecyl sulphate (see section 2): Gel A, DEAE–Sephadex eluate (15.4 μg protein); Gel B the UDP-glucuronic acid eluate from the UDP-hexanolamine agarose column (1.6 μg protein); from Wistar rat liver; Gel C, DEAE–Sephadex eluate (9.2 μg protein); Gel D, the UDP-glucuronic acid eluate from the UDP-hexanolamine agarose column (0.9 μg protein) from Gunn rat liver. Mixtures of pure enzymes were analyzed using cylindrical gels (6.0 × 0.6 cm). Gels E and G are as described for gels B and D, respectively. Gel F is an equal volume mixture of pure proteins from Wistar and Gunn rat liver (0.8 μg Wistar enzyme, 0.45 μg Gunn enzyme). Gels were stained with 0.25% Coomassie brilliant blue for 60 min and destained with acetic acid/methanol/water (7:5:43, by vol.). The direction of migration is from the top to the bottom. (df) Dye front.

directly compare their molecular size, equal volume samples of pure enzyme from the two sources were mixed and then analysed by sodium dodecyl sulphate gel electrophoresis. Gel F (fig.1) shows the single staining band obtained using this mixture. This band has the same mobility as the two unmixed pure proteins illustrated by gels E and G run at the same time. Thus pure UDP-glucuronyltransferase from Gunn rat liver exhibited the same molecular weight, possibly subunit molecular weight, as the pure enzyme from Wistar rat liver.

To determine the nature of the structural abnormality which presumably exists in the pure protein from Gunn rat liver, we compared mobilities of the enzyme from both strains by polyacrylamide gel electrophoresis using non-denaturing conditions. A single protein-staining band was observed in the preparations from both strains, both bands exhibiting the same mobility. This result suggests that the two proteins display a similar charge at pH 8.9, and that any existing enzyme defect is not detectable by this procedure.

UDP-glucuronyltransferase in the crude Lubrol-soluble supernatant fractions from Gunn and Wistar rat livers was examined by Ouchterlony double-diffusion analysis [18] using antiserum produced in rabbits the pure Wistar rat enzyme [19]. A single sharp continuous immunoprecipitin line with no spur formation was observed, suggesting that the enzyme from the two liver sources was immunologically identical.

3.4. *Reactivation of pure UDP-glucoronyltransferase by diethylnitrosamine*

Only extremely low levels of UDP-glucoronyltransferase activity towards 2-aminophenol and 4-nitrophenol were detectable in Gunn rat liver purification fractions throughout the isolation procedure (table 2). No enzyme activity towards these substrates was detectable in the final pure preparation. Diethylnitrosamine stimulated the activity of UDP-glucuronyltransferase in Gunn or Wistar rat liver preparations towards 4-nitrophenol and 2-aminophenol, but enzyme activity towards 1-naphthol or bilirubin was either slightly inhibited or unchanged. If 10 mM diethylnitrosamine was added to Gunn rat liver purification fractions prior to assay, UDP-glucuronyltransferase activity towards 2-aminophenol and 4-nitrophenol substrates was easily detectable at all stages of the enzyme purification. Indeed the pure UDP-glucuronyltransferase preparation was reactivated

Table 2
Activation of UDP-glucuronyltransferase from Gunn rat liver by diethylnitrosamine (DENA) towards 4-nitrophenol and 2-aminophenol as substrate

	4-Nitrophenol			2-Aminophenol		
	Specific activity			Specific activity		
Purification step	−DENA	+DENA	Purification[a]	−DENA	+DENA	Purification[a]
1. Lubrol-soluble supernatant	0.92	2.1	1	n.a.[b]	0.60	1
2. 25–60% satn. $(NH_4)_2SO_4$ precipitate	0.26	5.8	2.8	0.01	0.52	0.87
3. DEAE–cellulose eluate	1.5	7.2	3.5	0.03	0.38	0.63
4. CM-cellulose eluate	0.83	6.2	3.0	0.02	0.25	0.42
5. DEAE–Sephadex eluate	4.4	25.4	12.2	0.08	2.1	3.6
6. UDP-Hexanolamine agarose/ UDP-Glucuronic acid eluate	n.d.[c]	574	276	n.d.[c]	91.3	152

[a] Purification values were calculated from enzyme activities measured in the presence of diethylnitrosamine
[b] n.a., not available
[c] n.d., not detectable by the routine assay procedure

Results shown were obtained from 4 male Gunn rat livers. Specific activity units are nmol glucuronide formed/min/mg protein. The final concentration of diethylnitrosamine used in the assay was 10 mM

from non-detectable to high levels of enzyme activity (table 2).

The activation of the UDP-glucuronyltransferase activity caused by diethylnitrosamine progressively increased during purification. Thus a loss of functional activity occurs during purification, creating an effect similar to the mechanism observed in the inherited deficiency of the enzyme. As UDP-glucuronyltransferase was purified, the functional instability of the defective enzyme increased.

The activity of UDP-glucuronyltransferase towards 4-nitrophenol and 2-aminophenol assayed in the presence of diethylnitrosamine, in various purification fractions, displayed increases comparable to those obtained when using 1-naphthol as substrate in the absence of diethylnitrosamine. Under these conditions the relative specific activity values using 4-nitrophenol, 2-aminophenol and 1-naphthol as substrates increased 276-, 152- and 270-fold, respectively, over the Lubrol-soluble supernatant fraction, confirming previous work showing that 4-nitrophenol, 2-aminophenol and 1-naphthol are glucuronidated by the same enzyme protein [3].

Attempts to reactivate the pure UDP-glucuronyltransferase preparation from Gunn rat liver using phospholipid mixtures that stimulated pure UDP-glucuronyltransferase activity from Wistar rat liver [20], were not successful. Further, the pure enzyme obtained from Wistar rat liver contains very little, if any, bound phospholipids and the enzyme activity appears to be dependent on the presence of the detergent, Lubrol [20]. Therefore the results presented here suggest that diethylnitrosamine stimulates UDP-glucuronyltransferase activity, possibly by interference with the protein–phospholipid (or protein–Lubrol) interaction [21] or by direct interaction with the enzyme protein. Thus an intramolecular conformational change of UDP-glucuronyltransferase structure could occur and allow facilitated access of 2-aminophenol and 4-nitrophenol to the active site.

In conclusion, we have successfully purified a defective UDP-glucuronyltransferase from homozygous Gunn rat liver to homogeneity, judged by electrophoretic and immunochemical criteria. This enzyme protein was also similar in molecular size, charge and immunological character to the pure enzyme from Wistar rat liver. Enzyme activity, not detectable towards 2-aminophenol and 4-nitrophenol was reactivated by addition of diethylnitrosamine.

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