MULTIPLE FORMS AND A DEFICIENCY OF URIDINE DIPHOSPHATE-GLUCURONOSYLTRANSFERASES IN WISTAR RATS

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Uridine diphosphate (UDP)-glucuronosyltransferase (GT) active on androsterone (AD) and 4-nitrophenol (NP) was solubilized from male rat liver microsomes of the Wistar strain. The precipitate obtained in the 60%-satd. ammonium sulfate was purified by diethylaminoethyl (DEAE)-cellulose chromatography and affinity chromatography on UDP-hexanolamine Sepharose 4B. DEAE-cellulose chromatography showed the existence of two peaks of GT active on AD and NP. Peak I was found in rats with the high-activity and low-activity phenotypes in terms of AD glucuronidation and had high NP-GT and low AD-GT activities. In contrast, peak II was found only in rats with the high-activity phenotype, corresponded to high AD-GT activity and had comparatively low NP-GT activities. The corresponding peak in rats with the low-activity phenotype had only NP-GT activity. Comparison of K_m values for AD obtained from microsomes and purified enzymes provides evidence that AD-GT isoenzyme should be deficient in Wistar rats with the low-activity phenotype and that AD glucuronidation should be catalyzed poorly by other GT isoenzyme in these rats.

Keywords — Wistar rat; liver uridine diphosphate-glucuronosyltransferase; isoenzyme; androsterone; 4-nitrophenol; deficiency; multiplicity

INTRODUCTION

Hepatic uridine diphosphate (UDP)-glucuronosyltransferase (EC 2. 4. 1. 17, GT) catalyzes the glucuronidation of endogenous and exogenous compounds, furnishing them water soluble and accelerating their excretion into urine or bile. Glucuronide formation of wide variety of compounds has suggested the existence of multiple forms of GT. At least two or more forms of GT having overlapping substrate specificities have been purified in the rat.¹⁻⁶⁾

The hereditary deficiency of bilirubin (BL) GT isoenzyme is known in Gunn rats, a mutant strain of Wistar rats.⁵⁻⁷⁾ Previous studies from this laboratory showed discontinuous variation in hepatic GT activity toward androsterone (AD), the high-activity to low-activity ratios being approximately 16:1.⁸⁾ In contrast, such a striking individual difference was not found in GT activities toward 4-nitrophenol (NP), phenolphthalein (PH), BL and testosterone (TS).⁸⁾

Classification and breeding experiments revealed dominance of the high-activity phenotype.⁹⁾ These results suggest that our colony of Wistar rats with the low-activity phenotype appears to be another example of GT isoenzyme deficiency of genetic origin. The current study presents the separation of AD-GT isoenzyme from Wistar rats with the high-activity and low-activity phenotypes by diethylaminoethyl (DEAE)-cellulose and UDP-hexanolamine affinity chromatography.

MATERIALS AND METHODS

Chemicals — [1, 2-³H] AD (40.8 Ci/mmol) was purchased from New England Nuclear Corp., Boston, MA, U.S.A. AD, phosphatidylcholine (egg yolk, type IIIE), 6-amino-1-hexanol and uridine-5'-monophosphoric acid sodium salt were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. S-Ethyl thiotrifluoroacetate and *N, N'*-carbonyldiimidazole were purchased

from Pierce Chemical Co., Rockford, ILL, U.S.A. and Aldrich Chemical Co., Milwaukee, WIS, U.S.A. respectively. UDP-glucuronic acid disodium salt (UDPGA) was obtained from Boehringer, Mannheim, Germany. Octaethylene glycol mono-*n*-dodecyl ether (OGDE) and NP were from Tokyo Kasei Kogyo Co., Tokyo, Japan and Wako Pure Chemical Ind. Ltd., Tokyo, Japan respectively. DEAE-cellulose (DE-52) and cyanogen bromide-activated Sepharose 4B were obtained from Whatman Biochemicals, Clinton, New Jersey, U.S.A. and Pharmacia Fine Chemicals, Uppsala, Sweden respectively. All other reagents were of analytical grade. UDP-hexanolamine was synthesized according to Barker *et al.*,¹⁰ by reaction of 6-amino-1-hexanol with phosphoric acid, *S*-ethyl thiotrifluoroacetate, *N*, *N'*-carbonyldiimidazole and uridine-5'-monophosphoric acid. UDP-hexanolamine Sepharose 4B was prepared by coupling of UDP-hexanolamine with cyanogen bromide-activated Sepharose 4B.¹¹ UDP-hexanolamine Sepharose 4B contained 5.8–6.5 μmol of UDP per ml of settled gel.

Animals and Preparation of Microsomal Fractions — Wistar rats were classified into homozygous high-activity (genotype HH) and low-activity (hh) groups in terms of hepatic GT activity toward AD, as described previously.¹² In this study, the offspring (HH) from crosses between homozygous dominant rats were used as rats with the high-activity phenotype, and the offspring (hh) from matings of homozygous recessive rats were used as rats with the low-activity phenotype. The animals were fed on commercial diets, given water *ad libitum* and were housed in air-conditioned (23 °C) and light-controlled (12 h-light/12 h-dark cycle) animal rooms.

Four male rats (350–500 g) were decapitated and a 20% (w/v) liver homogenate was prepared in ice-cold 0.25 M sucrose–0.1 M Tris-HCl buffer, pH 7.4 (buffer A), with a Teflon-glass homogenizer. All procedures for the isolation and purification of GT were done at 0–4 °C. Microsomal fractions were obtained by differen-

tial centrifugation ($2000 \times g$ for 10 min, $16000 \times g$ for 45 min and $105000 \times g$ for 60 min). The microsomal pellets were resuspended in buffer A, and stored at –80 °C. The microsomal fractions could be stored at least for 2 months without apparent loss of glucuronidation activity.

Assay Procedures — GT activities toward AD and NP were determined by a modification of the method described previously.¹² AD-GT activity was assayed in 0.1 M Tris-HCl buffer, pH 7.4 or 8.0, while NP-GT activity was measured in 0.1 M Tris-HCl buffer, pH 7.4. The incubation medium contained 2 mM UDPGA, 10 mM MgCl_2 , 40 μM ethylenediaminetetra acetic acid (EDTA), and 0.17 mM [^3H] AD (0.023 μCi) or 0.36 mM NP in a total volume of 1.0 ml. The incubation was performed at 37 °C for 20–30 min.

Protein concentrations were determined by the method of Lowry *et al.*¹³ and the method of Bradford,¹⁴ with bovine serum albumin as standard.

Separation and Purification of GT — Frozen microsomal fractions from four rat livers were thawed and centrifuged for 45 min at $105000 \times g$. The pellets were then suspended in 100 ml of buffer A containing 1% OGDE, gently stirred for 30 min, and centrifuged at $105000 \times g$ for 60 min. The supernatant contained major portion of GT activities toward AD and NP. The solubilized GT was purified by a modification of the method described previously.^{2,15}

Finely ground solid ammonium sulfate was slowly added to the supernatant liquid to 60% saturation. The mixture was stirred for 20 min and centrifuged at $15000 \times g$ for 15 min. The precipitate was redissolved in 25 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 0.05% OGDE, 1 mM EDTA, 0.1 mM dithiothreitol and 20% glycerol (buffer B), and dialyzed against 80 volumes of the same buffer overnight.

The clear ammonium sulfate fraction was applied to a DEAE-cellulose column (2.6 cm $\times$ 40 cm) equilibrated with buffer B. The column was

washed with 160 ml of buffer B and eluted with a linear KCl-gradient (0–0.3 M, 500 ml each) in buffer B.

Further purification of GT activity was performed by affinity chromatography on UDP-hexanolamine Sepharose 4B. To fractions containing GT activities toward AD and NP was added MgCl_2 (5 mM, final concentration) and the preparations were applied to an affinity column (1.5 cm $\times$ 20 cm). The column was washed with 200 ml of buffer B containing 50 mM KCl and phosphatidylcholine (25 $\mu\text{g}/\text{ml}$), and then eluted with 50 ml of buffer B containing 5 mM UDPGA, 50 mM KCl and phosphatidylcholine (25 $\mu\text{g}/\text{ml}$).

Gel Electrophoresis — Polyacrylamide gel electrophoresis was performed on a 10% po-

lyacrylamide slab gel in the presence of 0.1% sodium dodecyl sulfate (SDS). The standard proteins used in gel electrophoresis were obtained from Sigma Chemical Co., Rockford, St. Louis, MO, U.S.A.

RESULTS

Solubilization and Effect of pH on Rat Liver GT Activities toward AD and NP

A nonionic detergent OGDE optimally activated GT activities toward AD and NP by 5–7-fold at the detergent concentrations of 0.02 and 0.01% (w/v) respectively.⁹⁾ OGDE concentration above this level resulted in progressive inactivation of GT activities. At 1% (w/v) OGDE concentration, GT activities toward AD and NP were about 1–2-fold higher than native

FIG. 1. DEAE-Cellulose Column Chromatography of Hepatic GT Activities toward AD and NP from Wistar Rats with the High-activity Phenotype

The column procedure is described in the Materials and Methods section. An $(\text{NH}_4)_2\text{SO}_4$ fraction was dialyzed and applied to a DEAE-cellulose column. Buffer B (160 ml) was passed through the column before application of a linear 0–0.3 M KCl gradient (— —). Fractions (9 ml) were collected and assayed for GT activities toward AD (○) and NP (△). GT activity is expressed as nmol/min per ml fraction. Protein (■) was determined at 280 nm.

microsomes. Though 10–15% of microsomal AD-GT and NP-GT activities were solubilized with 0.02% (w/v) OGDE, approximately 90 and 80% of microsomal AD-GT and NP-GT activities were solubilized with 0.5–1% (w/v) OGDE respectively (data not shown).

Microsomal and solubilized AD-GT exhibited a broad pH profile in the range of pH 7.2–8.0, whereas NP-GT showed a marked loss of activity in alkaline pH (Table I). Based on these results, AD-GT activity was determined at pH 7.4 or 8.0, while NP-GT activity was assayed at pH 7.4.

Separation and Purification of Rat Liver GT

Hepatic microsomal pellets from male Wistar rats, who had high-activity and low-activity phenotypes in terms of AD glucuronidation,

were solubilized in 1% OGDE-buffer A and the resultant supernatant was fractionated further with solid ammonium sulfate. After dialysis of the ammonium sulfate precipitate in buffer B, the fraction was passed through a DEAE-cellulose column.

When rat livers from the high-activity phenotype were used as enzyme source, two GT forms with different specificities toward AD and NP were separated by DEAE-cellulose chromatography (Fig. 1). Peak I (fraction No. 12–15) was eluted in buffer B and contained high NP-GT and low AD-GT activities, whereas peak II (fraction No. 54–67) was eluted at approximately 0.05–0.09 M KCl in buffer B and showed high AD-GT and comparatively low NP-GT activities. With the enzyme prepa-

FIG. 2. DEAE-Cellulose Column Chromatography of Hepatic GT Activities toward AD and NP from Wistar Rats with the Low-activity Phenotype

The column procedure is described in the Materials and Methods section. An $(\text{NH}_4)_2\text{SO}_4$ fraction was dialyzed and applied to a DEAE-cellulose column. Buffer B (160 ml) was passed through the column before application of a linear 0–0.3 M KCl gradient (— —). Fractions (9 ml) were collected and assayed for GT activities toward AD (○) and NP (△). GT activity is expressed as nmol/min per ml fraction. Protein (■) was determined at 280 nm.

rations from rats with the low-activity phenotype, peak I (fraction No. 13–18) contained high NP-GT and low AD-GT activities as in the case of the high-activity phenotype (Fig. 2). In contrast, the corresponding peak II (fraction No. 57–65) had comparatively low NP-GT activity and was free of AD-GT activity.

Further purification of GT activities were performed by affinity chromatography on UDP-hexanolamine Sepharose 4B. After application of peaks I and II, GT activities were eluted from the affinity column with 5 mM UDPGA in buffer B. The results of a typical purification ex-

periment from rat livers of the high-activity phenotype is shown in Table II. Application of peak I to the affinity column resulted in 41-fold purification of NP-GT activity from OGDE-soluble fraction (fraction A). In contrast, purification of AD-GT activity was only 1.5-fold, because most of AD-GT activity present in OGDE-soluble fraction appeared in peak II. When peak II was applied to the affinity column, GT activities toward AD and NP were copurified 43–49-fold from OGDE-soluble fraction (fraction B). Table III shows a typical purification experiment of GT activities toward

TABLE I. *Effect of pH on Hepatic GT Activities toward AD and NP*

	GT activity (nmol/min per mg protein)					
	Microsomes			Solubilized microsomes		
	pH 7.2	pH 7.4	pH 8.0	pH 7.2	pH 7.4	pH 8.0
AD	3.20	3.20	3.69	2.81	2.76	3.24
NP	8.40	5.50	1.90	7.25	5.54	1.62

Microsomes and OGDE-solubilized microsomes were obtained from male Wistar rats with the high-activity phenotype in terms of AD glucuronidation and incubated with AD or NP in 0.1 M Tris-HCl buffer, pH 7.2, 7.4 and 8.0 as described in the Materials and Methods section.

TABLE II. *Purification of Hepatic GT Activities toward AD and NP from Wistar Rats with the High-activity Phenotype*

Purification step	Protein (mg)	AD			NP		
		SA	TA	RP	SA	TA	RP
Microsomes	954	2.27	2170		1.88	1790	
Solubilized microsomes	614	3.53	2170	1	4.41	2710	1
(NH ₄) ₂ SO ₄ precipitate	346	1.98	685	0.6	1.94	670	0.4
DEAE-cellulose chromatography							
Peak I	19.6	1.69	33.0	0.5	42.7	838	9.7
Peak II	44.5	16.7	743	4.7	12.5	555	2.8
UDP-hexanolamine Sepharose 4B chromatography							
Fraction A	2.7	5.2	14.0	1.5	182	496	41
Fraction B	1.5	171	258	49	190	286	43

SA is the specific activity in nmol/min per mg protein. TA is the total activity in nmol/min. RP is the relative purification. GT activities toward AD and NP were assayed as described in the Materials and Methods section.

AD and NP from rat livers of the low-activity phenotype. Application of peak I to the affinity column provided 42- and 75-fold purification of AD-GT and NP-GT activities from OGDE-soluble fraction respectively (fraction A), whereas peak II was devoid of AD-GT activity and resulted in comparatively low purification of NP-GT activity (fraction B).

SDS-Polyacrylamide Gel Electrophoresis of Purified GT

SDS-polyacrylamide gel electrophoresis of the purified enzymes showed prominent polypeptide bands with subunit molecular weights of 50000 and 53000 (fraction A, the high-activity and low-activity phenotypes), 55000 (fraction B, the high-activity phenotype), and 56000 (fraction B, the low-activity phenotype) (Fig. 3). Though very minor higher molecular weight contaminants were present in the purified enzymes, these values are similar to those obtained for apparently homogeneous GTs from rat livers (50000–59000).^{1–6)}

Kinetic Studies on Glucuronidation

Double-reciprocal plots of initial velocity against variable concentrations of AD at 2 mM UDPGA were determined in the purified

enzyme preparations (Table IV). The apparent K_m values of the enzymes present in fraction A were 96 and 108 μM in the high-activity and low-activity phenotypes respectively, while the K_m value of the enzyme in fraction B was 20 μM in the high-activity phenotype. Interestingly, the K_m values for AD obtained from microsomes of the high-activity and low-activity phenotypes were 24 and 97 μM respectively.¹⁶⁾

In contrast, the apparent K_m values for NP were quite similar in these purified enzymes. The K_m values of the enzymes present in fraction A were 460 and 450 μM in the high-activity and low-activity phenotypes respectively. The corresponding values of the enzymes in fraction B were 400 and 270 μM respectively.

DISCUSSION

The present paper describes separation and purification of GT active on AD and NP from Wistar rats, who had the high-activity and low-activity phenotypes in terms of AD glucuronidation. DEAE-cellulose chromatography separated two GT peaks with different specificities toward AD and NP. Peak I had high NP-GT and low AD-GT activities and was present in

TABLE III. *Purification of Hepatic GT Activities toward AD and NP from Wistar Rats with the Low-activity Phenotype*

Purification step	Protein (mg)	AD			NP		
		SA	TA	RP	SA	TA	RP
Microsomes	1240	0.10	127		0.84	1040	
Solubilized microsomes	733	0.11	83	1	3.71	2720	1
(NH ₄) ₂ SO ₄ precipitate	446	0.07	33.2	0.7	3.25	1450	0.9
DEAE-cellulose chromatography							
Peak I	50.7	0.06	2.88	0.5	6.74	342	1.8
Peak II	25.6	ND	—	—	6.07	156	1.6
UDP-hexanolamine Sepharose 4B chromatography							
Fraction A	0.63	4.8	2.98	42	278	173	75
Fraction B	0.68	ND	—	—	50.7	34.6	14

SA is the specific activity in nmol/min per mg protein. TA is the total activity in nmol/min. RP is the relative purification. ND means that GT activity is not detectable by assay procedure. GT activities toward AD and NP were assayed as described in the Materials and Methods section.

both high-activity and low-activity phenotypes. Peak II had high AD-GT and comparatively low NP-GT activities and was found only in rats

FIG. 3. *SDS-Polyacrylamide Gel Electrophoresis of the Purified GTs after UDP-Hexanolamine Sepharose 4B Chromatography and Commercial Standards*

A, fraction A from the high-activity phenotype; B, Fraction B from the high-activity phenotype; C, fraction A from the low-activity phenotype; D, fraction B from the low-activity phenotype; E, β -galactosidase (116000), phosphorylase b (97000), bovine albumin (66000), egg albumin (45000), and carbonic anhydrase (29000). Migration is from top to bottom.

with the high-activity phenotype. The corresponding peak in the low-activity phenotype had only NP-GT activity. Peaks I and II were further purified by affinity chromatography on UDP-hexanolamine Sepharose 4B to afford purified enzymes of fraction A and B respectively. Comparison of K_m values for AD between microsome-bound and purified enzymes revealed an interesting aspect. The K_m value of the microsome-bound enzyme was similar to that of the purified enzyme present in fraction B in rats with the high-activity phenotype. In rats with the low-activity phenotype, the microsome-bound enzyme had the similar K_m value to the purified enzyme in fraction A. These results indicate that AD glucuronidation should be mainly catalyzed by GT isoenzymes present in fraction A and B in the low-activity and high-activity phenotypes respectively. If GT isoenzyme is named after a major substrate,¹⁷⁾ AD-GT isoenzyme should be deficient in Wistar rats with the low-activity phenotype. AD glucuronidation in consequence should be catalyzed poorly by NP-GT isoenzyme in these rats.

Several authors have reported the purification of two or more forms of GT having partially overlapping substrate specificities.¹⁻⁶⁾ However, there have been several discrepancies between their subunit molecular weights and substrate specificities. It was reported that highly purified

TABLE IV. *Apparent K_m Values of Purified GT Activities toward AD and NP in Wistar Rats*

Phenotype	Enzyme fraction	K_m (μ M)	
		AD	NP
High-activity	Fraction A	96	460
	Fraction B	20	400
Low-activity	Fraction A	108	450
	Fraction B	—	270

Wistar rats were classified into high-activity and low-activity phenotypes in terms of hepatic GT activity toward AD. Apparent K_m values for AD and NP were determined by Lineweaver-Burk plots against variable concentrations of AD and NP at 2 mM UDPGA. Enzyme fraction A and B were obtained by UDP-hexanolamine Sepharose 4B chromatography as indicated in Table II and III.

rat liver NP-GT had subunit molecular weights of 54000–59000.^{3,5)} Recently Tephly and his coworkers^{3,4)} purified AD-GT isoenzyme with a subunit molecular weight of 52000 in the pH 7.8 fraction by using chromatofocusing and affinity chromatography. These authors also isolated GT active on AD and TS in the pH 8.1 fraction, which had subunit molecular weights of 50000 and 52000. In our study, high NP-GT or AD-GT activity had subunit molecular weights of 50000 and 53000 or 55000 respectively. Differences in subunit molecular size estimation might be ascribable to the limited accuracy of the SDS-gel technique and to the different detergent systems used to solubilize the microsomal enzymes. On the other hand, differences in substrate specificities might suggest the contamination of other GT isoenzyme or the reconstitution of different GT isoenzyme subunits *in vivo* or during purification procedures. It is hoped that further study under way may clarify these apparent discrepancies.

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