Genetic deficiency of androsterone UDP-glucuronosyltransferase activity in Wistar rats is due to the loss of enzyme protein

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Hepatic microsomal UDP-glucuronosyltransferases towards androsterone and testosterone were purified by chromatofocusing and UDP-hexanolamine affinity chromatography in Wistar rats which had genetic deficiency of androsterone UDP-glucuronosyltransferase activity. In rats with the high-activity phenotype, androsterone (the 3-hydroxy androgen) UDP-glucuronosyltransferase was eluted at about pH 7.4 and had a subunit M_r of 52000, whereas testosterone (the 17-hydroxy steroid) UDP-glucuronosyltransferase was eluted at about pH 8.4 and had a subunit M_r of 50000. The transferase that conjugates both androsterone and testosterone was eluted at about pH 8.0, had subunit M_r values of 50000 and 52000, and appeared to be an aggregate or hybrid of androsterone and testosterone UDP-glucuronosyltransferases. In rats with the low-activity phenotype, androsterone UDP-glucuronosyltransferase was absent, whereas testosterone UDP-glucuronosyltransferase was eluted at around pH 8.5, with a subunit M_r of 50000.

INTRODUCTION

Hepatic microsomal UDP-glucuronosyltransferase (GT) catalyses the glucuronidation of endogenous and exogenous compounds. The heterogeneity of GT has been established by its substrate specificity, perinatal development, inducibility by various compounds, and chromatographic purification (Dutton, 1980; Burchell, 1981). At least two or more forms of GT with partially overlapping substrate specificities have been purified in the rat (Bock *et al.*, 1979; Weatherill & Burchell, 1980; Matern *et al.*, 1982; Falany & Tephly, 1983; Mackenzie *et al.*, 1984).

The hereditary deficiency of bilirubin GT isoenzyme is known in Gunn rats, a mutant strain of Wistar rats (Burchell, 1981; Chowdhury *et al.*, 1984). Previous studies from our laboratory showed discontinuous variation in rat liver GT activity towards androsterone, but not towards bilirubin, testosterone, 4-nitrophenol and phenolphthalein (Matsui & Hakozaiki, 1979; Matsui *et al.*, 1979). Wistar, Wistar King and Donryu rats showed the discontinuous variation; however, Long Evans and Sprague–Dawley rats did not exhibit such diversity (Matsui *et al.*, 1979). Subsequent studies demonstrated that the genetic expression of the high-activity phenotype is inherited as a single autosomal dominant trait (Matsui & Watanabe, 1982). Comparison of the purified enzyme obtained by DEAE-cellulose and UDP-hexanolamine–Sephacrose 4B chromatography revealed the defective nature of the androsterone GT isoenzyme in the low-activity phenotype (Matsui & Nagai, 1985).

Rat liver 3-hydroxy androgen (androsterone) and 17-hydroxy steroid (testosterone) GT isoenzymes have been purified by using chromatofocusing and UDP-hexanolamine affinity chromatography (Falany &

Tephly, 1983; Kirkpatrick *et al.*, 1984). The current study describes the separation of androsterone and testosterone GT isoenzymes from Wistar rats with the high-activity and low-activity phenotypes by chromatofocusing and UDP-hexanolamine affinity chromatography, and their properties.

MATERIALS AND METHODS

Materials

[1,2-³H]Androsterone (sp. radioactivity 40.8 Ci/mmol) and [1,2-³H]testosterone (sp. radioactivity 49.0 Ci/mmol) were obtained from New England Nuclear Corp., Boston, MA, U.S.A. Androsterone, testosterone and phosphatidylcholine (egg yolk; type III) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. UDP-glucuronic acid (disodium salt) was obtained from Boehringer, Mannheim, Germany. Emulgen 911 and octaethylene glycol mono-n-dodecyl ether were from Kao Atlas Ltd., Tokyo, Japan, and Tokyo Kasei Kogyo Co., Tokyo, Japan, respectively. Materials for chromatofocusing and CNBr-activated Sepharose 4B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. UDP-hexanolamine was synthesized and coupled with CNBr-activated Sepharose 4B by the methods of Barker *et al.* (1972) and Burchell & Weatherill (1981) as described previously (Matsui & Nagai, 1985). UDP-hexanolamine–Sephacrose 4B contained 5.8–6.5 μ mol of UDP/ml of settled gel. All other reagents were of analytical grade.

Animals and preparation of microsomal fractions

Wistar rats were classified into homozygous high-activity and low-activity groups in terms of hepatic GT activity towards androsterone, as described previously

Abbreviation used: GT, UDP-glucuronosyltransferase (EC 2.4.1.17).

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(Matsui & Watanabe, 1982). The offspring from crosses between homozygous-dominant rats were used as rats with the high-activity phenotype, and the offspring from matings of homozygous-recessive rats were used as rats with the low-activity phenotype.

A female rat (250–280 g) was decapitated and a 10% (w/v) liver homogenate was prepared in ice-cold 1.15% (w/v) KCl, with a Teflon/glass homogenizer. All procedures for isolation and purification of GT were done at 0–4 °C. Microsomal fractions were obtained by differential centrifugation (2000 *g* for 10 min, 16000 *g* for 45 min and 105000 *g* for 60 min). The microsomal pellets were resuspended in 1.15% KCl and stored at –80 °C.

Assay procedures

GT activities towards androsterone and testosterone were determined by a modification of the method described previously (Matsui *et al.*, 1979). The standard incubation medium contained 0.17 mM-[³H]androsterone (0.023 μ Ci) or 0.30 mM-[³H]testosterone (0.031 μ Ci), 2 mM-UDP-glucuronic acid, 10 mM-MgCl₂ and 20 μ M-EDTA in a total volume of 1.0 ml of 0.1 M-Tris/HCl buffer, pH 8.0. When activated microsomal fractions were assayed, 0.02% octaethylene glycol mono-n-dodecyl ether was added to the assay medium (Matsui & Watanabe, 1982). The incubation was performed at 37 °C for 20–30 min. For kinetic studies, the assay was identical with the standard incubation mixture, except that different sets of substrate concentrations were employed as described previously (Matsui & Hakozaiki, 1979).

Protein concentrations were determined by the method of Lowry *et al.* (1951) and the method of Bradford (1976), with bovine serum albumin as standard.

Separation and purification of GT activity

Frozen microsomal fractions were thawed and centrifuged at 105000 *g* for 45 min. The pellets were then suspended in 25 mM-ethanolamine/HCl buffer, pH 9.4, containing 20% (v/v) glycerol, 0.1 mM-dithiothreitol and 0.05% Emulgen 911 to give a 10% (w/v) suspension. The suspension was solubilized by further addition of 0.5 mg of Emulgen 911/mg of protein, stirred for 30 min, and then centrifuged at 105000 *g* for 30 min. The solubilized GT was purified as described by Kirkpatrick *et al.* (1984). Solubilized GT fraction was applied to a PBE94 chromatofocusing column (1 cm $\times$ 45 cm) equilibrated with 25 mM-ethanolamine/HCl, pH 9.4. The column was eluted with 500 ml of Polybuffer 96/HCl, pH 7.0,

containing 20% glycerol, 0.1 mM-dithiothreitol and 0.05% Emulgen 911.

Further purification of GT activity was performed by affinity chromatography on UDP-hexanolamine-Sepharose 4B. To fractions containing GT activities towards androsterone and testosterone was added MgCl₂ (5 mM final concn.), and the preparations were applied to an affinity column (1.5 cm $\times$ 20 cm) equilibrated with 20 mM-Tris/HCl, pH 8.0, containing 20% glycerol, 0.1 mM-dithiothreitol, 1 mM-EDTA and 0.05% Emulgen 911. The column was washed with 300 ml of the equilibrating buffer containing 50 mM-KCl and phosphatidylcholine (50 μ g/ml). The column was eluted with 70 ml of the same buffer containing 0.2 mM-UDP-glucuronic acid and subsequently with 30 ml of the same buffer containing 5 mM-UDP-glucuronic acid. GT activities were eluted in the buffer containing 0.2 mM-UDP-glucuronic acid.

Gel electrophoresis

Polyacrylamide-gel electrophoresis was performed on a 10%-polyacrylamide slab gel in the presence of 0.1% SDS. The standard proteins used in gel electrophoresis were obtained from Sigma, and subunit *M_r* values were estimated as described previously (Matsui & Nagai, 1985).

RESULTS

Hepatic microsomal GT activities towards androsterone and testosterone in Wistar rats

The hepatic GT activities towards androsterone and testosterone in fresh and octaethylene glycol mono-n-dodecyl ether-activated microsomal fractions are shown in Table 1. A distinctive feature is the existence of discontinuous variation in GT activity towards androsterone. Rats with the high-activity phenotype showed about 10–16-fold higher activity than did rats with the low-activity phenotype in fresh and detergent-activated microsomal fractions respectively. In contrast, no significant difference was observed in the rate of glucuronidation with testosterone between the high-activity and low-activity groups.

Separation and purification of GT activities towards androsterone and testosterone

Hepatic microsomal pellets obtained from the high-activity or low-activity phenotype were solubilized in Emulgen 911, and the resultant supernatant was separated by chromatofocusing.

Table 1. Hepatic microsomal UDP-glucuronosyltransferase activities in Wistar rats

Microsomal fractions were obtained from female rats with the high-activity and low-activity phenotypes in terms of androsterone glucuronidation, and were incubated with androsterone and testosterone in the presence or in the absence of the detergent as described in the Materials and methods section. Enzyme activity is expressed as nmol of glucuronide/min per mg of protein. Each value represents the mean $\pm$ S.D. for five animals.

Substrate	Phenotype . . . Microsomes . . .	UDP-glucuronosyltransferase			
		High-activity		Low-activity	
		Fresh	Activated	Fresh	Activated
Androsterone		0.74 $\pm$ 0.21	3.38 $\pm$ 0.56	0.08 $\pm$ 0.01	0.21 $\pm$ 0.03
Testosterone		0.86 $\pm$ 0.16	3.96 $\pm$ 0.64	0.81 $\pm$ 0.15	3.67 $\pm$ 0.42

Fig. 1. Chromatofocusing of hepatic UDP-glucuronosyltransferase activities towards androsterone and testosterone from Wistar rats with the high-activity phenotype

Chromatofocusing was performed as described in the Materials and methods section, generating a pH gradient of 9.4–7 (■). Fractions (6.8 ml) were collected and assayed for transferase activities towards androsterone (○) and testosterone (△). Enzyme activity is expressed as nmol of glucuronide/min per ml of fraction.

When the rat liver from the high-activity phenotype was used as enzyme source, four fractions of GT activities with different specificities towards androsterone and testosterone were separated from the column (Fig. 1). The first fraction was eluted at pH 9.3 (void volume) and contained high GT activities towards androsterone and testosterone. This fraction was not studied further in this study. The second fraction was eluted at about pH 8.4 and had high GT activity towards testosterone and low GT activity towards androsterone. The third fraction was eluted at approx. pH 8.0 and showed high GT activities towards androsterone and testosterone. The fourth fraction appeared at about pH 7.4 and had high GT activity towards androsterone and low GT activity towards testosterone. After UDP-hexanolamine-Sepharose 4B chromatography and gel electrophoresis, a single band with a subunit M_r of 50000 was observed in the pH 8.4 (fractions 40–46) fraction (Fig. 2). The pH 7.4 (fractions 96–106) fraction displayed a single band with a subunit M_r of 52000 (Fig. 2). The pH 7.4 and pH 8.4 fractions correspond to the 3-hydroxy androgen and 17-hydroxy steroid GT isoenzymes respectively (Falany & Tephly, 1983), and the pH 8.0 fraction (fractions 70–82) contained two bands with subunit M_r values of 50000 and 52000 (Fig. 2).

Double-reciprocal plots of initial velocity against concentrations of androsterone and testosterone at 2 mM-UDP-glucuronic acid were determined in the purified enzyme preparations. The apparent K_m and V_{max} (expressed as nmol/min per mg of protein) values for androsterone were 8.0 μ M and 43, and 5.2 μ M and 36, in the pH 8.0 and pH 7.4 fractions respectively. The apparent K_m and V_{max} values for testosterone were 120 μ M and 20, and 110 μ M and 50, in the pH 8.4 and pH 8.0 fractions respectively.

When the rat liver from the low-activity phenotype was used as enzyme source, the chromatographic profile was quite different from that of the high-activity phenotype (Fig. 3). Androsterone GT activity was almost negligible

(a) (b) (c) (d) (e)

Fig. 2. SDS/polyacrylamide-gel electrophoresis of rat liver UDP-glucuronosyltransferases after purification by chromatofocusing and UDP-hexanolamine-Sepharose 4B

(a) The pH 8.5 fraction from the low-activity phenotype; (b) M_r standards: β -galactosidase (116000), phosphorylase b (97000), bovine albumin (66000) and egg albumin (45000); (c) the pH 7.4 fraction from the high-activity phenotype; (d) the pH 8.0 fraction from the high-activity phenotype; (e) the pH 8.4 fraction from the high-activity phenotype. Migration is from top to bottom.

and appeared at about pH 8.5, overlapped with high testosterone GT activity. There were no GT activities towards androsterone at around pH 8.0 and 7.4, indicating the loss of androsterone GT isoenzyme. Low testosterone GT activity was observed at approx. pH 9.3 (void volume), and high GT activity towards testosterone was eluted at about pH 8.5. After UDP-hexanolamine affinity chromatography and gel electrophoresis, the pH 8.5 fraction (fractions 30–36) displayed a single band with a subunit M_r of 50000 (Fig. 2). Testosterone GT

Fig. 3. Chromatofocusing of hepatic UDP-glucuronosyltransferase activities towards androsterone and testosterone from Wistar rats with the low-activity phenotype

Chromatofocusing was performed as described in the Materials and methods section, generating a pH gradient of 9.4–7 (■). Fractions (7.4 ml) were collected and assayed for transferase activities towards androsterone (○) and testosterone (△). Enzyme activity is expressed as nmol of glucuronide/min per ml of fraction.

Table 2. Purification of androsterone UDP-glucuronosyltransferases from Wistar rats with the high-activity phenotype

UDP-glucuronosyltransferase activities were solubilized from microsomal fractions, purified by chromatofocusing and UDP-hexanolamine affinity chromatography, and assayed as described in the Materials and methods section. Specific activity is expressed as nmol of glucuronide/min per mg of protein. Total activity is expressed as nmol of glucuronide/min. Abbreviations used: SA, specific activity; TA, total activity; RP, relative purification.

Fraction	Total protein (mg)	UDP-glucuronosyltransferase					
		Androsterone			Testosterone		
		SA	TA	RP	SA	TA	RP
Solubilized fraction	202.7	4.60	932	1	2.87	581	1
Chromatofocusing and UDP-hexanolamine chromatography							
pH 7.4 fraction	0.99	51.5	51.5	11	16.1	15.9	5.6
pH 8.0 fraction	1.05	59.1	62.2	13	56.9	59.9	20

eluted at about pH 8.5 corresponds to the 17-hydroxy steroid GT. In contrast with the high-activity phenotype, there was no testosterone GT activity at around pH 8.0.

The results of a typical purification of androsterone GT isoenzymes from the high-activity phenotype are shown in Table 2. After purification by UDP-hexanolamine affinity chromatography, GT activities towards androsterone were increased only 11–13-fold compared with the solubilized microsomal fractions, even though the 3-hydroxy androgen GT was purified to apparent homogeneity.

Inhibition of androsterone glucuronidation by testosterone in rats with the high-activity phenotype

Falany & Tephly (1983) described that the 3-hydroxy androgen GT did not react with testosterone and that testosterone (150 μ M) did not exert any inhibitory influence on the enzyme activity. In a previous paper, we demonstrated that GT activity towards androsterone was

competitively inhibited by testosterone (173 μ M) in microsomal fractions obtained from the high-activity rats (Matsui & Hakozaki, 1979). The present study also indicates that testosterone was glucuronidated by the purified androsterone GT. In order to study these in detail, androsterone glucuronidation was assayed with the purified androsterone GTs eluted at about pH 7.4 and 8.0 in the presence or in the absence of testosterone, and double-reciprocal plots were made of the data (Figs. 4a and 4b). The results indicate that testosterone competitively inhibited androsterone glucuronidation, with an apparent K_i of 43 μ M for the pH 7.4 fraction and a K_i of 61 μ M for the pH 8.0 fraction.

DISCUSSION

Our laboratory previously reported the separation and purification of androsterone GT isoenzyme from Wistar rats, by utilizing DEAE-cellulose and UDP-hexanolamine

Fig. 4. Double-reciprocal plots of androsterone UDP-glucuronosyltransferase activity in the pH 7.4 and 8.0 fractions

Double-reciprocal plots of initial velocity (v) against androsterone concentrations at 2 mM-UDP-glucuronic acid were determined in the purified enzymes eluted at pH 7.4 (a) and pH 8.0 (b) obtained from the high-activity phenotype in the absence (○) or presence (Δ) of 200 μ M-testosterone. Enzyme activity is expressed as nmol of glucuronide/min per mg of protein.

affinity chromatography (Matsui & Nagai, 1985). In the high-activity phenotype, androsterone GT exhibited a subunit M_r of 55000 and had high activity towards androsterone and comparatively low activity towards 4-nitrophenol. In the low-activity phenotype, the corresponding fraction was devoid of androsterone GT activity. Another GT with high 4-nitrophenol and low androsterone GT activities was eluted in earlier fractions from the DEAE-cellulose column and was found in both high-activity and low-activity phenotypes. If a GT isoenzyme is named after a major substrate (Bock *et al.*, 1983), androsterone GT isoenzyme should be deficient in Wistar rats with the low-activity phenotype. Androsterone glucuronidation appears to be catalysed poorly by 4-nitrophenol GT isoenzyme in these rats. In the present study, we employed different chromatographic procedures and confirmed a deficiency of androsterone GT protein in the low-activity phenotype, though the molecular mechanism of the defective enzyme is yet to be clarified. A difference in estimated subunit M_r between two studies might be ascribable to the different detergent systems used to solubilize and purify the microsomal enzymes.

Comparison of K_m values for androsterone or testosterone between microsomal-bound and purified enzymes displayed comparatively consistent results in the high-activity rats. The microsomal-bound enzyme had apparent K_m values of 20–33 μ M and 133–192 μ M for androsterone and testosterone respectively (Matsui & Hakozaiki, 1979). The purified androsterone GT obtained by DEAE-cellulose and UDP-hexanolamine chromatography gave an apparent K_m of 20 μ M for androsterone (Matsui & Nagai, 1985). These data appear to be compatible with the present study, which obtained apparent K_m values of 5–8 μ M and 110–120 μ M for

androsterone and testosterone in the purified enzymes respectively.

The 3-hydroxy androgen (androsterone) and 17-hydroxy steroid (testosterone) GT isoenzymes have been purified in Sprague-Dawley rats by using chromatofocusing and UDP-hexanolamine affinity chromatography, and displayed subunit M_r values of 52000 (the pH 7.8 fraction) and 50000 (the pH 8.5 fraction) respectively (Falany & Tephly, 1983). These authors also isolated GT active on both androsterone and testosterone in the pH 8.1 fraction, which had subunit M_r values of 50000 and 52000. The 3-hydroxy and 17-hydroxy steroid GT isoenzymes had the same K_m value of 10 μ M for androsterone and testosterone respectively. When a similar purification procedure was used, Wistar rats with the high-activity phenotype provided analogous chromatographic profiles and similar subunit M_r values, except for the slightly lower pH of elution of the 3-hydroxy androgen GT (the pH 7.4 fraction) and the much higher apparent K_m for testosterone in the 17-hydroxy steroid GT. The low purification factor of androsterone GT suggests that GT activity might have been partially denatured or some essential cofactors might have been removed during purification. Mackenzie & Owens (1983) purified mouse liver GT isoenzymes, which glucuronidated androsterone, testosterone and other steroids, by utilizing chromatofocusing and UDP-hexanolamine affinity chromatography, and observed similar low purification factors of GT isoenzymes. However, Kirkpatrick *et al.* (1984) reported approx. 37-fold purification of the 3-hydroxy androgen GT isoenzyme from solubilized liver microsomal fractions in Sprague-Dawley rats.

Other important differences are formation of testosterone glucuronide and competitive inhibition of androsterone conjugation by testosterone in our purified enzymes. Sprague-Dawley rats appear to have much higher androsterone GT activity than do Wistar rats, and do not appear to have a defective androsterone GT isoenzyme (Matsui *et al.*, 1979). Although direct comparison has not yet been made between Wistar and Sprague-Dawley rats, it is possible that strain difference may be responsible for different results.

An interesting aspect of the chromatographic profile in the low-activity phenotype is the absence of testosterone GT activity eluted at around pH 8.0, as well as the defect of androsterone GT. Before purification, the microsomal-bound enzyme did not show any significant difference in testosterone GT activity between the high-activity and low-activity phenotypes. In a previous paper, we described that K_m values for testosterone were similar in the microsomal-bound enzymes obtained from the high-activity and low-activity rats (Matsui & Hakozaiki, 1979). In the high-activity phenotype, the pH 8.0 fraction contained high androsterone and testosterone GT activities and displayed two protein bands, whose subunit M_r values correspond to those of androsterone and testosterone GT isoenzymes. Furthermore, GT isoenzymes in the pH 8.0 and 7.4 or pH 8.4 and 8.0 fractions had similar K_m values for androsterone or testosterone respectively. Peters *et al.* (1984) estimated M_r values of several GT isoenzymes by radiation analysis, compared their molecular masses with those obtained by gel-filtration techniques, and pointed out that enzyme aggregation might cause overestimation of their molecular masses. Singh *et al.* (1984) hybridized two distinct types of subunits of rat lung glutathione *S*-transferases *in vitro* and

obtained three enzymes, with pI 9.4 (homodimer), 7.2 (heterodimer) and 4.8 (homodimer). The pI of the heterodimer is almost midway between those of the two homodimers. The theory of protein separation according to charge heterogeneity indicates that proteins are eluted from a chromatofocusing column at a pH just below their pI value. If GT isoenzymes are eluted from the column near their pI, the GT isoenzyme eluted at pH 8.0 appears to occupy a good place as a hybrid or aggregate of the isoenzymes eluted at pH 7.4 and 8.4. These data appear to indicate that the GT isoenzyme eluted at about pH 8.0 might be yielded by aggregation or reconstitution of androsterone and testosterone GT isoenzymes *in vivo* or during purification, but still maintaining their ability for glucuronidation. This hypothesis clearly explains why the single peak of testosterone GT activity was observed in the low-activity phenotype. Another possibility may be that rats with the low-activity phenotype have at least one other defective GT which is capable of conjugating androsterone and testosterone. However, further study is required to clarify these aberrant chromatographic profiles.

Several authors have published the purification of GT isoenzymes having partially overlapping substrate specificities. However, there have been several discrepancies between the reported substrate specificities and enzyme properties. The present study may provide a clue for further understanding of the heterogeneity and overlapping substrate specificity of GT isoenzymes.

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