Multiplicity of UDP-glucuronosyltransferases in fish

Purification and characterization of a phenol UDP-glucuronosyltransferase from the liver of a marine teleost, *Pleuronectes platessa*

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The aim of this work was to determine if a non-mammalian species had multiple UDP-glucuronosyltransferase (UDPGT) isoforms. At least six highly purified UDPGT isoenzymes were partially resolved by anion-exchange chromatography and UDP-hexanolamine–Sephacrose 4B affinity chromatography from liver microsomes of a fish, the plaice. Q-Sepharose FF, chromatofocusing and affinity-chromatographic procedures were employed to separate and purify the phenol UDPGT isoform to apparent homogeneity. The purified enzyme conjugated 1-naphthol, but not bilirubin or steroids, and displayed a pI of 7.0 and a subunit molecular mass of 55 kDa. Bilirubin and testosterone UDPGT activities were more labile and, although purified over 200-fold, these preparations also contained the phenol UDPGT and had multiple polypeptides with molecular masses of 52–57 kDa. Antisera to rat bilirubin/phenol UDPGT and testosterone/phenol UDPGT isoforms cross-reacted strongly with the partially purified plaice UDPGT isoforms of molecular masses 52, 53 and 57 kDa and less strongly with phenol UDPGT 54 kDa and 56 kDa isoforms. Fish and mammalian UDPGTs therefore apparently possess a high degree of evolutionary conservation.

INTRODUCTION

Glucuronidation is a major pathway for the metabolism and excretion of both endogenous and exogenous compounds in vertebrates [1]. Transfer of the glucuronide moiety from UDP-glucuronic acid (UDPGA) to the aglycone acceptor compound is catalysed by UDP-glucuronosyltransferases (UDPGTs, EC 2.4.1.17), a family of enzymes present in the endoplasmic reticulum. Addition of the glucuronide moiety increases the polarity of the acceptor compound, and thus enhances its excretion in either urine or bile. From studies of substrate specificity, differential ontogeny, differential induction, purification, cDNA cloning and strain differences between animals, at least nine isoforms have been identified in rats, some of which appear to display a fairly strict substrate specificity, whereas others exhibit overlapping specificities (reviewed in [2] and [3]).

To date there has been no report on the multiplicity of UDPGTs in non-mammalian species. Such comparative investigations may help to delineate the phylogenetic evolution of this important family of enzymes. In this context, fish are of particular interest because they are the most primitive of species to utilize glucuronidation as a biotransformation pathway [1,4]. Study of detoxication enzymes in these animals is also pertinent, as the environment they live in is being increasingly threatened by an alarming number of man-made pollutants.

Glucuronidation of endogenous compounds is important in fish species for chemical signalling (dihydroprogesterone glucuronides are the sex pheromones [5–7]), cessation of hormone action via excretion (steroid and thyroid hormones [8,9]) and in biliary elimination of bilirubin [10]. Glucuronide conjugates of a wide range of structurally diverse xenobiotic compounds have also been detected in bile, urine and tissues in fish, thus demonstrating the importance of glucuronidation in their detoxication and elimination (recently reviewed in [11]). In preliminary

studies we have established that liver microsomes prepared from a marine teleost flatfish, the plaice *Pleuronectes platessa*, display very high conjugating activity towards planar phenolic compounds such as 1-naphthol and 4-nitrophenol, have low but measurable activities with bilirubin and hydroxysteroid substrates, and contain multiple weakly immunoreactive polypeptides on immunoblot analysis with anti-(rat UDPGT) sera [12]. Thus the broad substrate specificity and immunochemical evidence would suggest that fish also possess multiple UDPGT isoforms.

In this study we report purification of multiple UDPGT isoforms displaying immunochemical similarity to rodent enzymes, and purification of the important phenol-conjugating UDPGT from the liver of the plaice. Preliminary data were reported previously [13].

MATERIALS AND METHODS

Materials

Androsterone, 2-aminophenol, bilirubin, Lubrol PX, 4-nitrophenol, 1-naphthol, oestrone, β -oestradiol, testosterone, UDPGA (ammonium salt), UDP-hexanolamine, Nitro Blue Tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, Ponceau S and alkaline phosphatase-conjugated rabbit anti-goat IgG were all obtained from Sigma (Poole, Dorset, U.K.). 2-Aminophenol was from Aldrich Chemical Co. (Gillingham, Dorset, U.K.) and was twice resublimed before use; ethyl anthranilate was from Kodak (Rochester, NY, U.S.A.). Nitrocellulose was obtained from Anderman (Kingston-upon-Thames, Surrey, U.K.). BSA fraction V was from Boehringer Mannheim (Lewes, Sussex, U.K.).

1-[14 C]Naphthol, 4-[14 C]testosterone, [2,4,6,7- 3 H]oestradiol and [4- 14 C]oestrone were from Amersham International (Amersham, Bucks., U.K.). [9,11- 3 H]Androsterone was from

Abbreviations used: UDPGA, UDP-glucuronic acid; UDPGT, UDP-glucuronosyltransferase; PC, phosphatidylcholine; RAL1, anti-(rat testosterone/phenol UDPGT) antibody; RAK1, anti-(rat bilirubin/phenol UDPGT) antibody; PAHs, polycyclic aromatic hydrocarbons.

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New England Nuclear (Stevenage, Herts., U.K.). Egg-yolk phosphatidylcholine (PC) was from Lipid Products (South Nutfield, Surrey, U.K.). Lubrol 12A9 was from ICI Organics Division (Manchester, U.K.). Ultrafiltration apparatus was from Amicon (Stonehouse, Glos., U.K.). CNBr-activated Sepharose 4B, Mono P HR5/20 columns, Polybuffer 96, Q-Sepharose fast flow, Sephacryl S-300, Sephadex G25M PD10 columns and f.p.l.c. system were from Pharmacia (Milton Keynes, U.K.). All other chemicals were of analytical grade from BDH (Poole, Dorset, U.K.).

Animals

Sexually immature plaice (*Pleuronectes platessa*) of 200–350 g were obtained by a commercial seine boat off the coast of Kincardineshire, Scotland, and were transported live to the laboratory before being killed. For enzyme purification, livers were removed and stored at -80°C until use (within 1 month).

Preparation of microsomal fractions from fresh liver

Livers were removed, chopped finely, and a 20% (w/v) homogenate was prepared in ice-cold 0.25 M-sucrose with a Teflon/glass homogenizer. Homogenates were centrifuged at 10000 g for 15 min, and the resulting supernatant was centrifuged at 105000 g for 1 h to sediment microsomes. The microsomal pellets were resuspended in 0.25 M-sucrose at a protein concentration of 20–30 mg/ml and stored at -80°C until use (within 1 month).

Enzyme assays

Microsomes were preincubated for 20 min on ice with an optimally activating concentration of the non-ionic detergent Lubrol PX determined for each sample before assay to allow the measurement of the full glucuronidation capacity of the preparation. UDPGT assays were carried out at 37°C in 0.1 M-Tris/maleate buffer, pH 7.0, in the presence of 30 mM-MgCl₂ (see the Results section).

UDPGT assays using various substrates were carried out with 4 mM-UDPGA in assays according to previously described methods: 1-naphthol [14], 2-aminophenol [15], 4-nitrophenol [16], bilirubin [17]. Testosterone, oestrone and β -oestradiol UDPGT assays were carried out as described by Rao *et al.* [18,19], except that all steroids were present at 0.6 mM. Androstosterone UDPGT assay was based on that described by Matsui & Hakozaki [20], except that the steroid was present at 0.1 mM instead of 0.172 mM. Free steroid and glucuronide fractions were separated as described by Rao *et al.* [18].

Protein determination

Protein content of microsomal samples was determined by the method of Lowry *et al.* [21], with BSA as standard. Protein measurement in purified fractions was by the procedure of Bradford [22], with a Bio-Rad assay kit (Bio-Rad, Hemel Hempstead, Herts., U.K.).

Anti-UDPGT antibodies

The production and characterization of anti-(rat liver testosterone/4-nitrophenol UDPGT) (RAL1) and anti-(rat kidney bilirubin/1-naphthol UDPGT) (RAK1) antibodies have been described elsewhere [23,24].

Electrophoresis and immunoblot analysis

Liver microsomal proteins were resolved electrophoretically by the method of Laemmli [25], on 7% polyacrylamide gels in the presence of 0.1% SDS. Gels were stained by a combined Coomassie Blue G250/silver stain as described by De Moreno *et al.* [26]. Electroblothing of the resolved proteins on to nitro-

cellulose (Schleicher and Schuell) was performed essentially as described by Towbin *et al.* [27]. Chromogenic detection of immune complexes was by an alkaline-phosphatase-linked system [28]. Staining of total protein on nitrocellulose was performed by immersing the nitrocellulose in 0.15% (w/v) Ponceau S/3% (w/v) trichloroacetic acid for 5 min. Excess stain not bound to proteins was eluted with a few brief washes of distilled water.

Separation and purification of UDPGT activities

All procedures were carried out at 5°C unless otherwise stated. Purification of enzyme activity was monitored throughout the purification procedure with 1-naphthol, bilirubin and testosterone as acceptor substrates and addition of PC liposomes to the assays. Microsomal pellets were prepared from livers (~100 g) of 50–60 fish as described above. The microsomal pellet was then resuspended and solubilized by hand homogenization in 120 ml of buffer A [200 mM-potassium phosphate, 1% (w/v) Lubrol 12A9, 5 mM-2-mercaptoethanol, pH 7.0] to give 0.5 mg of detergent/mg of protein and stirred for a further 30 min. Insoluble material was removed by re-centrifugation at 105000 g for 60 min. Finely ground (NH₄)₂SO₄ was then slowly added with stirring to the supernatant fraction to a concentration of 134 g/l (25% saturation). After stirring for a further 20 min, precipitated proteins were removed by centrifugation at 15000 g for 15 min. The procedure was then repeated at 60% saturation [336 g of (NH₄)₂SO₄/l] to precipitate an active UDPGT fraction. This pellet was resuspended in approx. 20 ml of buffer B [20 mM-Tris/HCl, 0.05% Lubrol 12A9, 5% (v/v) glycerol, 5 mM-2-mercaptoethanol], and after dialysis in this buffer (three changes of 100 vol. over 24 h) undissolved material was removed by centrifugation at 15000 g for 15 min. The supernatant was applied at 80 ml/h to a column (2.5 cm $\times$ 55 cm) of Q-Sepharose fast flow previously equilibrated in buffer B. Unbound protein was eluted with buffer B, and protein retained on the column was eluted with a linear gradient of 0–0.5 M-KCl in buffer B (500 ml). Fractions containing UDPGT activity were pooled as three separate pools designated A, B and C, which were concentrated to 4–5 ml by ultrafiltration using an Amicon YM30 membrane. The active concentrates were dialysed against three changes of 100 vol. of buffer C (buffer B containing 5 mM-MgCl₂, pH 8.0) over 24 h and applied to a 15 ml UDP-hexanolamine-Sepharose 4B affinity column (synthesized as described by Burchell & Weatherill [29]) equilibrated with buffer C. After application, the flow was stopped for 60 min to facilitate binding of UDPGTs to the UDP-ligand. Unbound material was then eluted by extensive washing with buffer C (at 20 ml/h), and non-specifically bound material was removed by elution with 10 ml of 50 mM-KCl in buffer C. Bound UDPGT was eluted by sequential applications of 5 ml of 50 μM -, 100 μM - and 500 μM -UDPGA in buffer C, and fractions exhibiting high specific activity were pooled and stored on ice.

Purification of phenol UDPGT

Phenol UDPGT was purified to apparent homogeneity by inclusion of a chromatofocusing step in the procedure above. Pooled fractions from the leading portion of the active peak eluted by 50–75 mM-KCl from the Q-Sepharose chromatography, which contained phenol UDPGT activity and little bilirubin or testosterone UDPGT activities, were passed through a Sephadex G25 column (PD10; 9.1 ml bed vol.) equilibrated with buffer D [75 mM-Tris acetate, 0.05% Lubrol 12A9, 5% glycerol, 5 mM-2-mercaptoethanol, pH 9.3] for buffer exchange. Then 10–20 mg of protein of the resulting sample (in less than 2 ml of buffer) was applied at room temperature to a Mono P HR5/20 column fitted to a Pharmacia f.p.l.c. system previously equilibrated with buffer D, and the column was washed with 2 column vol. of

buffer D at a flow rate of 1 ml/min, followed by 35 ml of buffer E [10% (v/v) Polybuffer 96, 0.05% Lubrol 12A9, 5% glycerol, 5 mM-2-mercaptoethanol, adjusted to pH 6.0 with acetic acid] to generate a pH gradient from pH 9.3 to 6.0 over 30 ml. Any material still bound to the column was then removed by elution with 1 M-NaCl. Fractions with high 1-naphthol UDPGT activity were pooled, concentrated to approx. 2 ml and passed through a Sephacryl S-300 column (1 cm × 10 cm) equilibrated with buffer F [buffer C containing 200 µg of PC/ml] for buffer exchange. The resultant protein-containing fractions were then purified by affinity chromatography on UDP-hexanolamine-Sepharose as above, but with inclusion of 200 µg of PC liposomes/ml in the buffers.

Effect of phospholipid on glucuronidation by purified UDPGT

PC liposomes were prepared by addition of egg-yolk PC to ice-cold assay buffer and ultrasonication until the solution clarified as described by Cater *et al.* [30]. Before addition of substrates and cofactors, purified UDPGT fractions were equilibrated on ice with the liposomes suspensions for 10 min. Assays were then performed in the standard manner as above.

RESULTS

Glucuronidation by plaice liver microsomes

Plaice microsomes are capable of glucuronidation of a variety of substrates (Table 1). Optimal rates were obtained at 37 °C, pH 7.0 and a final Mg²⁺ concentration in the assays of 30 mM (Fig. 1). The characteristic latency of UDPGTs was abolished by the disruption of microsomal vesicles with Lubrol PX at detergent concentrations of 0.1–0.3 mg/mg of microsomal protein, depending on the substrate. Rates of glucuronidation of planar phenols (1-naphthol and 4-nitrophenol) were high, whereas those

for endogenous substrates (bilirubin and steroids) were low (Table 1). From the substrate specificities of mammalian UDPGTs, this would imply the presence of at least three isoforms in plaice, conjugating phenols, bilirubin and hydroxy steroids respectively. This question was addressed directly by enzyme purification studies in which activities towards the three classes of substrate were measured.

Partial resolution and immunoblot analysis of multiple UDPGT isoforms

Microsomes solubilized with Lubrol 12A9 were initially fractionated with (NH₄)₂SO₄, and different UDPGT activities were partially resolved by anion-exchange chromatography (Fig. 2). Three broad peaks of UDPGT activity, accounting for more

Table 1. Substrate specificity of glucuronidation in plaice liver microsomes

UDPGT activities were measured at 37 °C with 0.1–0.6 mM substrates, 4 mM-UDPGA and optimally activating concentrations of Lubrol PX (determined for each substrate) in the incubation mixture. Enzyme activities are expressed as means ± S.D. for at least three determinations.

Substrate	Activity (nmol/min per mg)
4-Nitrophenol	56.3 ± 18.5
1-Naphthol	14.3 ± 5.4
2-Aminophenol	0.15 ± 0.05
Bilirubin	0.11 ± 0.03
Androsterone	0.03 ± 0.01
Testosterone	0.18 ± 0.08
Oestrone	0.16 ± 0.06
17β-Oestradiol	0.17 ± 0.05

Fig. 1. Optimal assay conditions for plaice 1-naphthol UDPGT

(a) pH-dependence: pH was varied with a range of Tris/maleate buffers. (b) Temperature-dependence; 10 min incubation time. (c) Mg²⁺-dependence. (d) Effect of varying the Lubrol PX concentration; samples were preincubated with detergent for 20 min on ice. Assays were carried out as described in the Materials and methods section; results are means of at least three separate determinations.

Fig. 2. Separation of plaice liver UDPGT activities by anion-exchange chromatography

After Lubrol solubilization and $(\text{NH}_4)_2\text{SO}_4$ fractionation, 400 mg of protein was applied to a Q-Sepharose column (2.5 cm $\times$ 55 cm) and eluted with a salt gradient as described in the Materials and methods section. Fractions (5 ml) were monitored for A_{280} (■—■), KCl concentration (□---□; by conductivity), and assayed for UDPGT activities (●, 1-naphthol UDPGT; ○, bilirubin UDPGT; △, testosterone UDPGT) in the presence of 200 μg of PC/ml of assay mixture. Fractions were pooled from different regions of this profile and were designated A (fractions 13–19), B (fractions 20–29) and C (fractions 30–40).

than 75 % of the applied activity, were eluted from a Q-Sepharose column by a salt gradient from 0 to 0.5 M-KCl, with maximal activities eluted at 100 mM-, 110 mM- and 200 mM-KCl for 1-naphthol, testosterone and bilirubin UDPGT activities respectively. Use of buffers over a range from pH 9.0 to 8.0 failed to resolve these activities completely. By selection of fractions from different regions of the active peak after anion-exchange chromatography (pools A, B and C in Fig. 2) and subsequent affinity chromatography on a UDP-hexanolamine affinity matrix, purified preparations which were selectively enriched in 1-naphthol, testosterone and bilirubin UDPGT activity (designated UDPGT pools A, B and C respectively) were obtained which exhibited enrichments of greater than 200-fold over the solubilized microsomal preparation (Table 2). All three preparations contained a major polypeptide of molecular mass 55 kDa displayed by silver staining after SDS/PAGE; however, at least five other polypeptides with molecular masses of 52–57 kDa were present (Fig. 3). Preparations with a relatively greater enrichment of bilirubin UDPGT activity (B and C) appeared to contain greater amounts of the 57 kDa component. In previous studies we have reported the preparation and characterization of two anti-(rat UDPGT) polyclonal antibodies [23,24]. RAL1 has a broad specificity and reacts with phenol, bilirubin and steroid UDPGT isoforms, whereas RAK1 has a more restricted specificity for phenol and bilirubin UDPGT isoforms. Analysis by SDS/PAGE and immunoblotting with the two anti-(rat UDPGT) sera revealed that all the polypeptides were immunoreactive with

Table 2. Purification of multiple UDPGTs from plaice liver microsomes

UDPGTs were purified from Lubrol-solubilized microsomes by $(\text{NH}_4)_2\text{SO}_4$ fractionation, anion-exchange and affinity chromatography of selected fractions (detailed in Fig. 2) as described in the Materials and methods section. Assays were carried out in the presence of 200 μg of PC liposomes/ml. Data from a single representative experiment are shown. Values in parentheses are for purification (fold).

Fraction	UDPGT activity (nmol/min per mg of protein)		
	1-Naphthol	Testosterone	Bilirubin
Solubilized microsomes	8.4 (1)	0.03 (1)	0.06 (1)
25–60 %-satn.- $(\text{NH}_4)_2\text{SO}_4$ ppt.	9.5 (1.1)	0.06 (2.0)	0.11 (1.8)
Q-Sepharose eluates			
A	33.4 (4.0)	0.17 (5.7)	0.04 (0.7)
B	56.4 (6.7)	0.40 (13.3)	0.38 (6.3)
C	23.1 (2.8)	0.23 (7.7)	0.54 (9.0)
UDP-hexanolamine- Sepharose UDPGA eluates			
A	1851 (220.4)	6.88 (229.3)	1.00 (16.7)
B	1148 (136.7)	8.58 (286.0)	3.57 (59.5)
C	874 (104.0)	8.71 (290.3)	12.9 (215.0)

Fig. 3. SDS/PAGE and immunoblot analysis of partially resolved plaice liver UDPGTs

Affinity-purified UDPGTs from pools A, B and C on anion-exchange chromatography (Fig. 2) were electrophoresed on an SDS/7.0 % polyacrylamide gel. Proteins were detected by silver staining, or were transferred and immunoblotted with anti-rat UDPGT antisera. Lanes 1, 2 and 3 represent UDPGT eluted fractions derived from affinity chromatography of Q-Sepharose pools A, B and C respectively. Protein loadings were 2 μg of pool A, and 3 μg of pools B and C. M denotes molecular-mass markers (2 μg of each): albumin, 68 kDa; pyruvate kinase, 57 kDa; fumarase, 49 kDa; aldolase, 40 kDa. Panel (a), Coomassie Blue/silver-stained; panel (b), immunostained with RAL1 antiserum; panel (c), immunostained with RAK1 antiserum; panel (d), stained with Ponceau S after transfer to nitrocellulose. Molecular masses of marker proteins in kDa are shown on the right.

both antisera to varying degrees (Fig. 3), indicating that common epitopes exist between the fish and mammalian enzymes. The two anti-(rat UDPGT) antibodies displayed different reactivities with particular polypeptides in the purified preparations. The 55 kDa component was not the major immunoreactive polypeptide; the 52, 53 and 57 kDa components reacted most strongly, which may reflect some degree of specificity in the

Fig. 4. Effect of PC liposomes on activities of co-purified plaice liver UDPGTs

The effect of varied concentrations of exogenous PC liposomes on the activity of affinity-purified UDPGTs was examined: 1.5 µg and 5 µg of affinity-purified UDPGT was used in the reconstitution assays towards 1-naphthol (●) and exogenous substrates (○, bilirubin; △, testosterone) respectively. The results are representative of experiments performed with one purified preparation.

Table 3. Purification of phenol UDPGT from plaice liver microsomes

Phenol UDPGT was purified from Lubrol-solubilized microsomes by $(\text{NH}_4)_2\text{SO}_4$ fractionation, anion exchange, chromatofocusing and affinity chromatography as described in the Materials and methods section. Assays were carried out in the presence of 200 µg of PC liposomes/ml. Data from a single representative experiment are shown (N.D., not detectable). Values in parentheses are percentage yields.

Fraction	UDPGT activity (nmol/min per mg of protein)		
	1-Naphthol	Testosterone	Bilirubin
Solubilized microsomes	8.8 (100)	0.04 (100)	0.06 (100)
$(\text{NH}_4)_2\text{SO}_4$ fractionated	12.1 (42.3)	0.08 (63.3)	0.14 (64.7)
Q-Sepharose FF	37.8 (4.7)	0.12 (0.4)	0.02 (3.6)
Chromatofocusing	695 (2.7)	N.D.	N.D.
UDP-hexanolamine-Sepharose 4B	3330 (1.2)	N.D.	N.D.

antisera which were raised against rat liver testosterone/1-naphthol UDPGT and rat kidney bilirubin/1-naphthol UDPGT isoforms (RAL1 and RAK1 respectively). In particular, the 57 kDa polypeptide, which was more abundant in the pools enriched in bilirubin UDPGT, was the most antigenic to the RAK1 antisera.

Effect of PC on activity of co-purified plaice UDPGTs

For comparative studies of phospholipid dependence, the purified UDPGT preparation B which contained all the UDPGT isoenzymes identified (Table 2, Fig. 3) was used. On addition of PC to the co-purified UDPGT preparation, rates of glucuron-

Fig. 5. Chromatofocusing of partially purified 1-naphthol UDPGT glucuronosyltransferase from plaice liver

Selected fractions eluted by low salt concentrations from a Q-Sepharose column with a high ratio of 1-naphthol UDPGT activity relative to other UDPGT activities were pooled and applied to a chromatofocusing column (Mono P HR5/20) fitted to a Pharmacia f.p.l.c. system at pH 9.2. After elution of unbound protein, proteins were eluted with Polybuffer 96/acetate, pH 6.0, to generate a pH gradient. A_{280} (—) and pH (---) were monitored continuously with on-line detectors. Eluted fractions were assayed for UDPGT activities towards 1-naphthol, testosterone and bilirubin after reconstitution with PC liposomes (200 µg/ml).

dation were enhanced, maximal rates being obtained at PC concentrations of 200–300 µg/ml (Fig. 4). The degree of enhancement of activities varied according to aglycone substrate, the activities with the two endogenous substrates being enhanced greatest.

Purification of phenol UDPGT to apparent homogeneity

By selection of the leading fractions of the activity peak on anion-exchange chromatography which contained activity towards 1-naphthol and very little or no activity with the other substrates, an apparently homogeneous preparation of phenol UDPGT was obtained by chromatofocusing and affinity chromatography. On chromatofocusing, multiple protein peaks were eluted; one, of pI 7.0, corresponded to the peak of 1-naphthol UDPGT activity (Fig. 5). No bilirubin or testosterone UDPGT activities were detected in the column eluate. The phenol UDPGT was concentrated and subjected to affinity chromatography as described in the Materials and methods section. The purified product eluted by 50 µM-UDPGA had a specific activity towards 1-naphthol of 2.72–3.33 µmol conjugated/min per mg of protein (3 preparations), representing a purification of some 380-fold over solubilized microsomes (Table 3). The purified plaice liver phenol UDPGT conjugated 1-naphthol and displayed no de-

Fig. 6. SDS/PAGE of purified phenol UDPGT from plaice liver

Purified plaice liver phenol UDPGT was subjected to SDS/PAGE (7%-acrylamide gel) and stained with a combined Coomassie Blue/silver stain as outlined in the Materials and methods section. Lanes: 1, 50 μ g of plaice hepatic microsomes; lane 2, molecular-mass standards as in Fig. 3; 3, 2 μ g of phenol UDPGT. Molecular masses of marker proteins in kDa are shown on the right.

tectable activity (<0.01 mmol/min per mg) towards androstosterone, bilirubin, oestrone, 17β -oestradiol or testosterone.

A single polypeptide of molecular mass 55 kDa was present on SDS/PAGE (Fig. 6) which was immunoreactive on immunoblot analysis with both anti-(rat UDPGT) antisera preparations (results not shown), indicating that the homologue of one of the rat phenol UDPGT isoenzymes has been isolated.

DISCUSSION

Glucuronidation is a major detoxication pathway in all vertebrates, whereas invertebrates utilize the analogous glucosidation reaction for such a function [1,4]. The presence of multiple forms of UDPGTs in rat, rabbit and human has been well established [3], but no direct information on the multiplicity of UDPGTs in more primitive phyla exists. Fish are the most ancient vertebrate phylum, which account for over 40% of all living vertebrate species. In this paper the resolution of at least six UDPGTs and the characteristics of a highly active purified enzyme conjugating a planar phenol from the liver of a teleost fish are reported.

The optimal reaction parameters for glucuronidation of aglycone substrates by plaice liver microsomes are very similar to those found in rats: maximal activity after detergent disruption of the membranes, a temperature optimum around 37 °C and maximal activity around neutral pH in the presence of Mg^{2+} (see Fig. 1). The high rates of glucuronidation of 1-naphthol and

similar planar phenolic compounds observed in plaice liver microsomes (see Table 1) may reflect the need for an active detoxication system for potentially carcinogenic phenolic metabolites, for example benzo[a]pyrene 7,8-dihydrodiols, generated by the highly active cytochrome *P*-450 1A in this species [31,32]. Rates of glucuronidation for the endogenous UDPGT substrates, steroid hormones and bilirubin were at least an order of magnitude lower in plaice liver microsomes than those observed in rats [33]. This may be a reflection of the lower metabolic rates at the lower body temperature in these poikilotherms (4–14 °C) as well as physiological differences. In common with avians, fish also have nucleated red blood cells, resulting in lower turnover rates of haem; thus the capacity for bilirubin glucuronidation may be lower. Biliary excretion of androgen and oestrogen glucuronides has been reported in a number of fish species [6,34]; however, glucuronidation of steroids may be quantitatively more important in reproductive tissue of fish, where relatively high levels of steroid glucuronides have been measured (reviewed in [11]).

Toxicologically, it is important to know whether xenobiotic pollutants, for example polycyclic aromatic hydrocarbons (PAHs), might compete with steroids or bilirubin for the same active site(s) on fish UDPGT, which might result in serious physiological consequences, such as aberrations in reproductive function or jaundice. Indeed, we have recently reported that Atlantic salmon suffering from a multiple pollutant (including PAHs)-induced jaundice have an apparent decrease in bilirubin UDPGT activity [35], which may indicate such a competition.

Other studies in our laboratories on the fish glucuronidation system have demonstrated that phenol UDPGT was selectively induced by 3-methylcholanthrene and Aroclor 1254, and has a more widespread tissue distribution than for bilirubin and testosterone UDPGT activities (D. J. Clarke, B. Burchell & S. G. George, unpublished work). This appears to indicate that, as in mammals, a number of isoforms exist in fish species; however, such evidence is indirect. The most direct approach to determining if multiple isoforms exist in a species requires physical separation of different enzyme forms. Thus in the present study we have used protein purification to assess whether fish have multiple UDPGT isoenzymes.

Multiple UDPGT activities were partially resolved by ion-exchange chromatography, and, after affinity chromatography of these co-purified activities, a major polypeptide of subunit molecular mass 55 kDa was present on SDS/PAGE. At least five other polypeptides with molecular masses of 52–57 kDa were also identified, and appeared more abundant in preparations displaying relative enrichments of bilirubin and testosterone UDPGT activities. These components also reacted more strongly with the UDPGT-isoform-non-specific RAL1 and phenol/bilirubin-UDPGT RAK1 rat UDPGT antisera, which may indicate that they are homologues of the rat 17β -hydroxy steroid and bilirubin UDPGTs. Interestingly, as with the corresponding purification of UDPGTs from rats [36], 1-naphthol UDPGT activity was eluted from the anion-exchange matrix earlier than the bilirubin UDPGT activity, which may be another indication of a degree of evolutionary structural conservation.

In rats the 17β -hydroxysteroid UDPGT isoenzyme also conjugates simple phenols such as 1-naphthol [37]. Plaice liver 1-naphthol UDPGT activity was not eluted in a symmetrical peak from an anion-exchange column; there was an indication of a shoulder corresponding to the peak of testosterone UDPGT activity and SDS/PAGE/immunoblots showed the presence of several additional UDPGTs in this region of the chromatogram. These data may indicate that there is also a fish homologue of the enzyme which conjugates 17β -hydroxysteroids as well as 1-naphthol.

Plaice microsomal bilirubin UDPGT and testosterone UDPGT activities were low, and these activities were labile on chromatofocusing. Consequently, although highly purified preparations showing enrichments some 215- and 290-fold respectively from solubilized plaice microsomes were obtained by anion-exchange and affinity chromatography, it was not possible to purify these isoforms to homogeneity. The specific activities of these highly purified obtained fractions were approx. 4-fold and 20-fold lower than for reportedly homogeneous preparations of rat liver bilirubin UDPGT and 17 β -hydroxysteroid UDPGT respectively [37,38], and they contained multiple immunoreactive polypeptides; thus assignment of their identities and substrate specificities was not possible. The purified plaice UDPGTs also required exogenous PC for maximal expression of activity, and the degree of activation appeared to vary according to the aglycone acceptor substrate, as reported for purified mammalian UDPGTs [39–41].

In studies reported elsewhere, we have shown that phenol UDPGT and cytochrome *P*-450 1A are induced pleiotropically in fish by 3-methylcholanthrene [42,43] and that glucuronidation is an important pathway for the metabolism of the carcinogen benzo[*a*]pyrene [32]. In the present study we have reported the first data on the separation and isolation of a the 3-methylcholanthrene-inducible phenol UDPGT from fish liver, which is presumably the isoform involved in the metabolism of PAHs as the analogous form is in mammals [44,45]. By extremely selective pooling of fractions from the leading edge of the 1-naphthol UDPGT activity peak after anion-exchange chromatography, a phenol UDPGT could be purified to apparent homogeneity by chromatofocusing and affinity chromatography. This enzyme, purified some 400-fold from plaice microsomes, had an apparent *pI* of 7.0, a subunit molecular mass of 55 kDa, a specific activity of 2.72–3.33 μ mol/min per mg of protein and no detectable activity towards bilirubin or steroids, which indicates that this enzyme is orthologous with the rat liver 3-methylcholanthrene-inducible phenol UDPGT [37,45] and the phenol UDPGT isolated from the chick [46].

In conclusion, the present study demonstrates for the first time that a non-mammalian species, the fish, contains multiple UDPGT isoforms with similar functional and structural properties to mammalian UDPGTs, thus demonstrating a strong evolutionary conservation between phyla which diverged over 350 million years ago. It will be interesting to examine the substrate specificity of the fish phenol-metabolizing isoform towards PAHs.

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