RESEARCH COMMUNICATION

A critical amino acid residue, Asp^{446}, in UDP-glucuronosyltransferase

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An amino acid residue, Asp^{446}, was found to be essential for the enzymic activity of UDP-glucuronosyltransferase (UGT). We obtained a rat phenol UGT (UGT1*06) cDNA (named *Ysh*) from male rat liver by reverse-transcription (RT)-PCR using *pfu* polymerase. A mutant *Ysh* having two different bases, A^{1337}G and G^{1384}A (named *Ysh A^{1337}GC^{1384}A*), that result in two amino acid substitutions, D^{446}G and V^{462}M, was obtained by RT-PCR using *Taq* polymerase. *Ysh* was expressed functionally in microsomes of *Saccharomyces cerevisiae* strain AH22. However, the expressed protein from *Ysh A^{1337}GG^{1384}A* had no transferase activity. Two other mutant cDNAs with *Ysh A^{1337}G* having one changed base, A^{1337}G, resulting in one amino acid substitution, D^{446}G, and *Ysh G^{1384}A* having a changed base, G^{1384}A, resulting in an amino acid substitution, V^{462}M, were constructed and expressed in the yeast. The expressed protein from *Ysh G^{1384}A* (named Ysh V^{462}M) exhibited enzymic activity, but the one from *Ysh A^{1337}G* (named Ysh D^{446}G) did not show any activity at all. Asp^{446} was conserved in all UGTs and UDP-galactose:ceramide galactosyltransferases reported, suggesting that Asp^{446} plays a critical role in each enzyme.

INTRODUCTION

UDP-glucuronosyltransferase (UGT; EC 2.4.1.17 is an intracellular membrane protein that catalyses glucuronidation of bilirubin and steroids as well as xenobiotic compounds, including carcinogens [1,2]. UGTs have been classified into two major subfamilies (*UGT1* and *UGT2*) on the basis of amino-acid-sequence similarities [3]. All of these proteins are closely related at the C-terminus, whereas considerable variation in amino acid sequence occurs in the N-termini of the UGTs in different subfamilies [3]. Recent reports on human [4] and rat [5] UGT1 genes have shown that there are several unique first exons coding for 5′ portions of each isoform and are followed by only one set of commonly used exons (exons **II**, **III**, **IV** and **V**) encoding the C-terminal portion, suggesting that the C-terminal portion has a common function in UGT, such as the interaction with a donor substrate, UDP-glucuronic acid. A consensus region has been drawn between residues 350 and 400 for the UGTs and various UDP-glycosyltransferases [6]. Ciotti et al. [7] found that the expressed proteins having mutations in Gly^{276}, Gly^{277} and Pro^{270} were inactive in bilirubin UGT. The UDP-binding site was reported to be located in the C-terminus (amino acids 299–446) of the human liver bilirubin UGT by Pillot et al. [8]. In the present study we found a critical residue, Asp^{446}, in the C-terminal portion of UGT.

MATERIALS AND METHODS

Materials

Restriction endonucleases, other DNA-modifying enzymes and enzymes and reagent kits were purchased from Takara Shuzo (Kyoto, Japan), Toyobo (Osaka, Japan), New England Biolabs (Beverly, MA, U.S.A) and Boehringer (Mannheim, Germany). Peroxidase-conjugated anti-rabbit IgG antibody was obtained from Jackson Immuno Research Laboratories (West Grove, U.S.A.). Nitrocellulose membranes were purchased from Advantec Toyo (Tokyo, Japan) and Schleicher and Schuell (Dassel, Germany). Other reagents were of the highest grade available.

Preparation of a UGT cDNA by RT-PCR

A rat phenol UGT cDNA was obtained by reverse-transcription (RT)-PCR. Total RNA was prepared from the liver of 3-methylcholanthrene-treated male Wister rats as previously reported [9]. A single-stranded cDNA was synthesized from the total RNA by Super Script II reverse transcriptase. The coding region of UGT cDNA was amplified by PCR with oligonucleotide primers (containing an *Spe*I site), which are designed by reference to the sequence of RATUGT1A1A gene reported by Emi et al. [5]. The forward primer was 5′-CGAACTAGTATGGCTTG-CCTTCTTCCTG-3′ and the reverse primer was 5′-GGGA-CTAGTTCAGTGGGTCTTGGATTTG-3′. A 100 μl portion of PCR mixture, containing 0.2 mM dNTPs, 0.2 mM of each primer and 25 units/ml *Pfu* DNA polymerase (Stratagene) or LA *Taq* polymerase (Takara shuzo), was subjected to 30 cycles of amplification; 1 min at 94 °C, 2 min at 45 °C and 4 min at 72 °C. The two amplified cDNAs were subcloned into the pUC119 vector and sequenced by an Applied Biosystems model 377 sequencer. The cDNA amplified by *Pfu* polymerase was named *Ysh* and the other cDNA, which was shown to have two substitutions (A^{1337}G and G^{1384}A), was amplified by *Taq* polymerase and named *Ysh A^{1337}GG^{1384}A*.

Construction of *Ysh A^{1337}G* and *Ysh G^{1384}A*

For preparation of *Ysh A^{1337}G*, a fragment containing a mutation A^{1337}G and corresponding to the sequence from A^{1190} to C^{1417} of

Abbreviations used: UGT, UDP-glucuronosyltransferase; RT-PCR, reverse-transcription PCR; *Ysh*, a cDNA obtained from 3-methylcholanthrene-treated male Wister rat liver by RT-PCR in the present study; 5-HT, 5-hydroxytryptamine; X^{n}Y, substitution of amino acid (or base) X by Y at position *n*; CGT, UDP-galactose:ceramide galactosyltransferase.

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The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank/EMBL Nucleotide Sequence Databases under the accession number D83796.

Ysh, was amplified by PCR using oligonucleotide primers (5′-ACGCCAAGCGCATGGAAACTCG-3′ and 5′-GGCGCAG-GTGTGGCGCCCCCTTGTGCCTCATCACGTAC-3′), *Pfu* polymerase and *Ysh* $A^{1337}GG^{1384}A$ as a template. This cDNA fragment was inserted into the *Bst*1–*Bsa*A1 site of *Ysh*. Another mutant, *Ysh* $G^{1384}A$, was reconstituted with a 3′-region fragment from the *Eco*R1 site to the end of the coding region containing a single mutation, $G^{1384}A$. Because there are no suitable restriction-enzyme sites located in the region from A^{1337} to $G^{1384}A$, mutagenesis of this fragment was performed by a two-step PCR reported by Landt et al. [10]. In the first step, a 5′-universal primer (5′-GGGTTTTCCCAGTCACGACG) and 3′-mutagenic primer (5′-TGGAGTACATGATGAGGC) generate the intermediate mutagenic fragment using a pUC18 vector having an insert from the *Eco*R1 site to the end of *Ysh* as a template. The amplified fragment was purified and used as a 5′-mutagenic primer in the second step of PCR, together with 3′-universal primer (5′-GGAAACAGCTATGACCATG) and the same template as that of the first step of PCR. The resulting fragment, having a single mutation, $A^{1386}G$, was inserted into the pUC19 vector, and the 5′-region fragment from the *Eco*R1 site of *Ysh* was inserted into the *Eco*R1 site of the fragment. The constructed *Ysh* $A^{1337}G$ and *Ysh* $G^{1384}A$ were sequenced to ensure that no more mutations in the respective cDNA occurred.

Expression of the cDNA

Ysh in pUC119 was digested with *Spe*I and both ends of the fragment were partially filled by a klenow fragment with dCTP and dTTP. The yeast expression vector, pAAH5, previously digested with *Hin*dIII, was also partially filled with dGTP and dATP. The *Ysh* fragment was inserted into the pAAH5 vector. Yeast AH22 (a, *leu* 2-3, *leu* 2-112, *his* 4-519, *can* 1, [cir$^+$]) cells were transformed by the expression plasmid or the vector using the method with lithium acetate [11]. Transformants were selected by complementation of *leu*2 auxotrophy.

Preparation of microsomes and antibodies

Treatment of rats, preparation of liver microsomes, purification of rat phenol UGT and preparation of antibodies against the phenol UGT were performed by the methods previously described [12–14]. Yeast transformants were cultivated in a synthetic minimal medium (0.67% yeast nitrogen base without amino acids/2% dextrose) supplemented with 20 mg/l histidine, and finally in YPD medium (1% yeast extract/2% peptone/2% dextrose). Yeast microsomes were prepared by the method of Oeda et al. [15].

Immunoblot analysis

Microsomes and other protein samples were subjected to SDS/PAGE. The polypeptide bands thus separated were transferred to a nitrocellulose membrane and immunoreactive bands were detected using the polyclonal antibodies against the purified UGT, as described by Howe and Hershey [16], with a slight modification [13].

Enzyme assay methods

UGT activities toward various substrates were assayed at the following concentrations of aglycone by the methods described in the respective references: 1-naphthol (0.05 mM) [17], 4-nitrophenol (5.0 mM) [18], 4-methylumbelliferone (1.0 mM) [19], 5-hydroxytryptamine (1.0 mM) [20], 4-hydroxybiphenyl (0.5 mM) [21], and bilirubin (1.0 mM) [22]. Protein was determined by the method of Lowry et al. [23], using BSA as a standard.

HPLC

Enzyme reaction products (1-naphthol glucuronides) were filtered on a disposable-disc filter (HPLC-DISK™3; Kanto Co., Tokyo, Japan) and analysed on an HPLC system consisting of a Tosoh TSKgel 80TM reversed-phase column (7.8 mm × 30 cm). Samples were injected and eluted with acetonitrile/water/acetic acid (350:650:1, by vol.).

RESULTS

Sequence of obtained UGT cDNAs

A UGT1*06 cDNA encoding a phenol UGT [3] was obtained from mRNA in the liver of 3-methylcholanthrene-treated male Wister rats by RT-PCR using *Pfu* polymerase and was named *Ysh*. We could isolate UGT1*06 cDNA from male rat liver cDNA library by using polyclonal antibodies against phenol UGT and confirmed that the sequence was completely identical with the cDNA sequence obtained by the PCR. The cDNA sequence was identical with the exon sequence of a UGT gene, RATUGT1A1A, recently reported by Emi et al. [5], although the cDNA had one base change, $G^{499}A$, which resulted in an amino acid substitution, $V^{167}I$. However, the cDNA sequence was clearly different from that of 4NP-UGT cDNA [24], which was isolated from the cDNA library of the female rat liver, in two base changes, $A^{258}G$ and $C^{988}G$, resulting in two different amino acids, $I^{180}L$ and $I^{329}V$, and three base additions resulting in one amino acid addition, M^{180}. A mutant *Ysh* clone having two substitutions, $A^{1337}G$ and $G^{1384}A$, was obtained from the same liver mRNA by RT-PCR using *Taq* polymerase. This clone was named *Ysh* $A^{1337}GG^{1384}A$. Other mutant clones, such as *Ysh* $A^{1337}G$, having only a single base change, $A^{1337}G$, and *Ysh* $G^{1384}A$, having only a single base change, $G^{1384}A$, were constructed as described in the Materials and methods section.

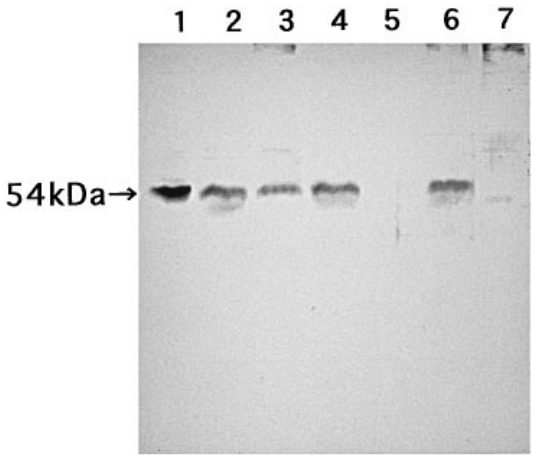

Figure 1 Western blotting of microsomal protein from rat liver and yeast AH22 cells with the antibodies against the rat phenol UGTs

Microsomes were prepared from rat liver (lane 1, 10 μg), AH22 cells expressing *Ysh* (lane 2, 40 μg), AH22 cells expressing *Ysh* $A^{1337}GG^{1384}A$ (lane 3, Ysh $D^{446}GV^{462}M$, 40 μg), AH22 cells expressing *Ysh* $A^{1337}G$ (lane 4, Ysh $D^{446}G$, 40 μg), AH22 cells expressing *Ysh* $G^{1384}A$ (lane 6, Ysh $V^{462}M$, 40 μg) and AH22 cells expressing *Ysh* $A^{1337}G$ and *Ysh* $G^{1384}A$ inserted in pAHH5 in the reverse direction respectively (lanes 5 and 7, 40 μg). The microsomal proteins were electrophoresed and blotted, and the colour was developed as described in the Materials and methods section.

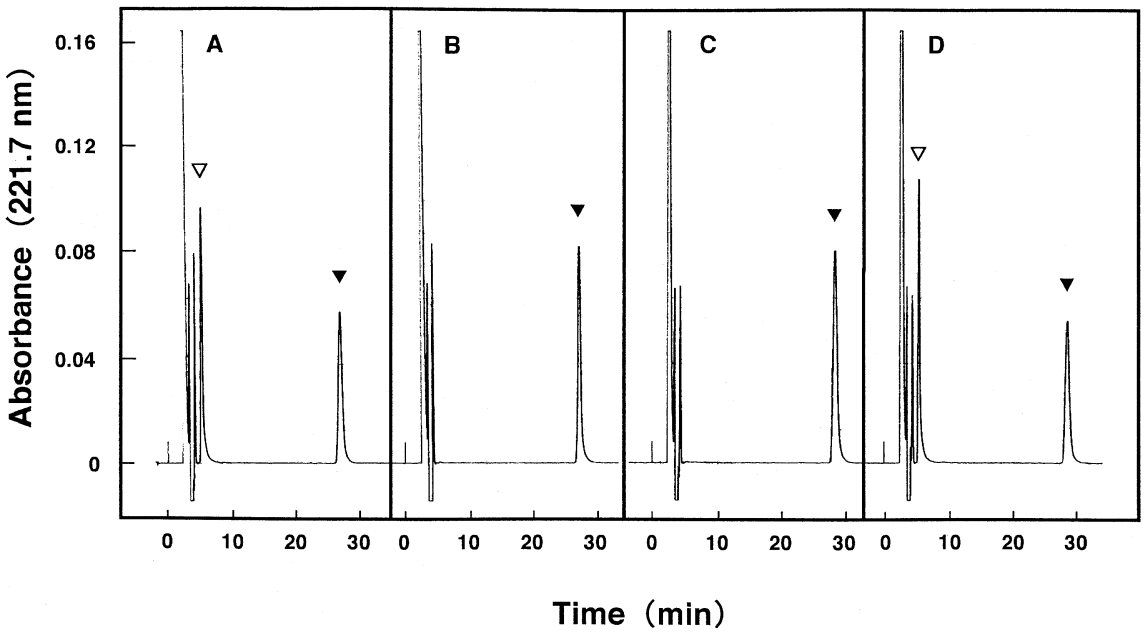

Figure 2 HPLC profiles of reaction products

The products were filtered with a disposable-disc filter (HPLC-DISK™3; Kanto Co.) and analysed on a reverse-phase HPLC system consisting of a Tosoh TSKgel 80TM reversed-phase column (7.8 mm × 30 cm). Samples were injected and eluted with acetonitrile/water/acetic acid (350:650:1, by vol.). Peak 1 (▽), 1-naphthol glucuronide; peak 2 (▼), unconjugated 1-naphthol. Panels **A**, **B**, **C** and **D** show profiles of the products produced by expressed UGT1* (from *Ysh*), Ysh $D^{446}GV^{462}M$, Ysh $D^{446}G$ and Ysh $V^{462}M$ respectively.

Expression of UGT protein in yeast cells

Ysh was inserted into the pAAH5 vector as described in the Materials and methods section. Then *Saccharomyces cerevisiae* AH22 cells were transformed by the expression plasmid. *Ysh* mRNA was detected in the transformed cells by Northern-blotting analysis (results not shown). In order to confirm the expression of the UGT protein, the microsomal proteins were analysed by immunoblotting using antibodies prepared against a rat phenol UGT [12,14], as shown in Figure 1. The microsomal fractions from AH22 cells that expressed *Ysh* had a 54 kDa immunoreactive band (Figure 1, lane 2), although this band did not appear in the AH22 cells transformed with only the pAAH5 vector.

Enzymic activities of the expressed UGTs

The yeast microsomes containing the expressed UGT protein were incubated with 0.05 mM 1-naphthol in 10 mM Tris/HCl buffer, pH 8.0, containing 5 mM $MgCl_2$, and then UDP-glucuronic acid (final concn. 3 mM) was added. The reaction product of Ysh, which had a fluorescent property (excitation peak at 289 nm and emission peak at 336 nm), was identified as 1-naphthol glucuronide by reverse-phase HPLC (Figure 2, panel A). As shown in Table 1, the expressed UGT catalysed the glucuronidation of 1-naphthol, 4-nitrophenol and 4-methylumbelliferone with high activity, and also 5-hydroxytryptamine with low activity. These transferase activities were activated only about 1.2-fold when 0.002% sodium cholate was added in the assay medium (results not shown). This substrate specificity was almost the same as that of the purified UGT from rat liver and kidney (Table 1) [12,13]. The K_m values for 1-naphthol and UDP-glucuronic acid of the expressed Ysh protein in the yeast cells were almost equal to those of native transferase (Table 2) in the presence or absence of detergents. These data described

Table 1 Substrate specificity of expressed UGT (Ysh; wild-type) in yeast strain AH22 cells

The microsomes were prepared from rat liver and the yeast strain AH22 cells by the methods described in the Materials and methods section. Enzyme activity was determined in the rat liver microsomes fully activated by sodium cholate and in the yeast microsomes without sodium cholate. Values represent the means ± S.E.M. for five animals. N.D., not determined.

	Activity (nmol/min per mg)	
Substrate	Rat liver microsomes	AH22 microsomes
1-Naphthol	24.2 ± 0.5	2.53
4-Nitrophenol	25.7 ± 0.6	5.11
4-Methylumbelliferone	35.0 ± 1.3	5.87
5-Hydroxytryptamine	11.4 ± 0.9	0.068
4-Hydroxybiphenyl	26.5 ± 2.0	N.D
Bilirubin	0.61 ± 0.03	N.D.

Table 2 Kinetic parameters of native and expressed UGT in microsomes

Enzyme activity was determined as described in the Materials and methods section and Table 1. The kinetic parameters were evaluated in the presence of various 1-naphthol (1NA) concentrations (0.5–60 μM) for a constant concentration of UDP-glucuronic acid (UDPGA; 5 mM) and by varying the concentration of UDP-glucuronic acid (0.05 mM) at a constant concentration of 50 μM 1-naphthol. Apparent K_m values were determined from a Lineweaver–Burk plot of the data.

Enzyme source	K_{1NA} (μM)	K_{UDPGA} (mM)
Rat liver microsomes	3.4	0.41
Ysh	3.2	0.38
Ysh $V^{462}M$	3.2	0.31

above indicated that Ysh protein expressed in AH22 microsomes was completely functional in transferase activity. *Ysh* $A^{1337}GG^{1384}A$ having two substitutions, $A^{1337}G$ and $G^{1384}A$, that resulted in two amino acid mutations, $D^{446}G$ and $V^{462}M$ (named Ysh $D^{446}GV^{462}M$), was also expressed in AH22 microsomes (Figure 1, lane 3), although the protein was not functional (Figure 2, panel B). Therefore we constructed other mutants, named *Ysh* $A^{1337}G$, having a single substitution, $A^{1337}G$, that resulted in one mutation ($D^{446}G$), and *Ysh* $G^{1384}A$, having a single substitution, $G^{1384}A$, that resulted in one mutation ($V^{462}M$). These mutant cDNAs were also expressed in AH22 microsomes (Figure 1, lanes 4 and 6). The expressed protein (named Ysh $V^{462}M$) from *Ysh* $G^{1384}A$ had transferase activity (Figure 2, panel D) and normal K_m values (Table 1). However, the other mutant protein (Ysh $D^{446}G$) expressed from *Ysh* $A^{1337}G$ had no activity toward 1-naphthol (Figure 2, panel C), 4-nitrophenol and 4-nitrophenol and 4-methylumbelliferone, even at higher concentrations of UDP-glucuronic acid and in the presence of detergents (results not shown).

DISCUSSION

A functional phenol UGT protein could be produced by the yeast expression system using the pAAH5 vector and the yeast strain AH22. The nucleotide sequence of *Ysh* obtained was identical with that of the UGT gene, RATUGT1A1A, recently reported by Emi et al. [5], except for one base substitution resulting in one amino acid change. The expressed transferase had the same substrate specificity (high catalytic activities toward 1-naphthol, 4-nitrophenol and 4-methylumbelliferone) as the purified UGT corresponding to UGT1*06 [3,12], and it also had K_m values for 1-naphthol and UDP-glucuronic acid similar to those of native phenol UGT in rat liver microsomes.

We obtained a mutant cDNA, *Ysh* $A^{1337}GG^{1384}A$, that resulted in two amino acid mutations. The expressed protein, Ysh $D^{446}GV^{462}M$, did not have UGT catalytic activity, indicating that Asp^{446} and/or V^{462} are critical for the exhibition of the enzymic activity. Multiple alignment data of the amino acid sequences of all UGTs reported showed that Asp^{446} was completely conserved in all UGTs (results not shown). We constructed other mutant UGT clones that expressed Ysh $D^{446}G$ and Ysh $V^{462}M$, each having only one amino acid mutation, $D^{446}G$ and $V^{462}M$, respectively. Ysh $V^{462}M$ showed UGT activity, but Ysh $D^{446}G$ had no transferase activity at all. These data suggest that Asp^{446} in all UGTs is an essential amino acid residue. Recently, Schulte and Stoffel [25] reported that the amino acid sequences of rat UDP-galactose:ceramide galactosyltransferase (CGT) showed significant similarity to that of UGT. The amino acid

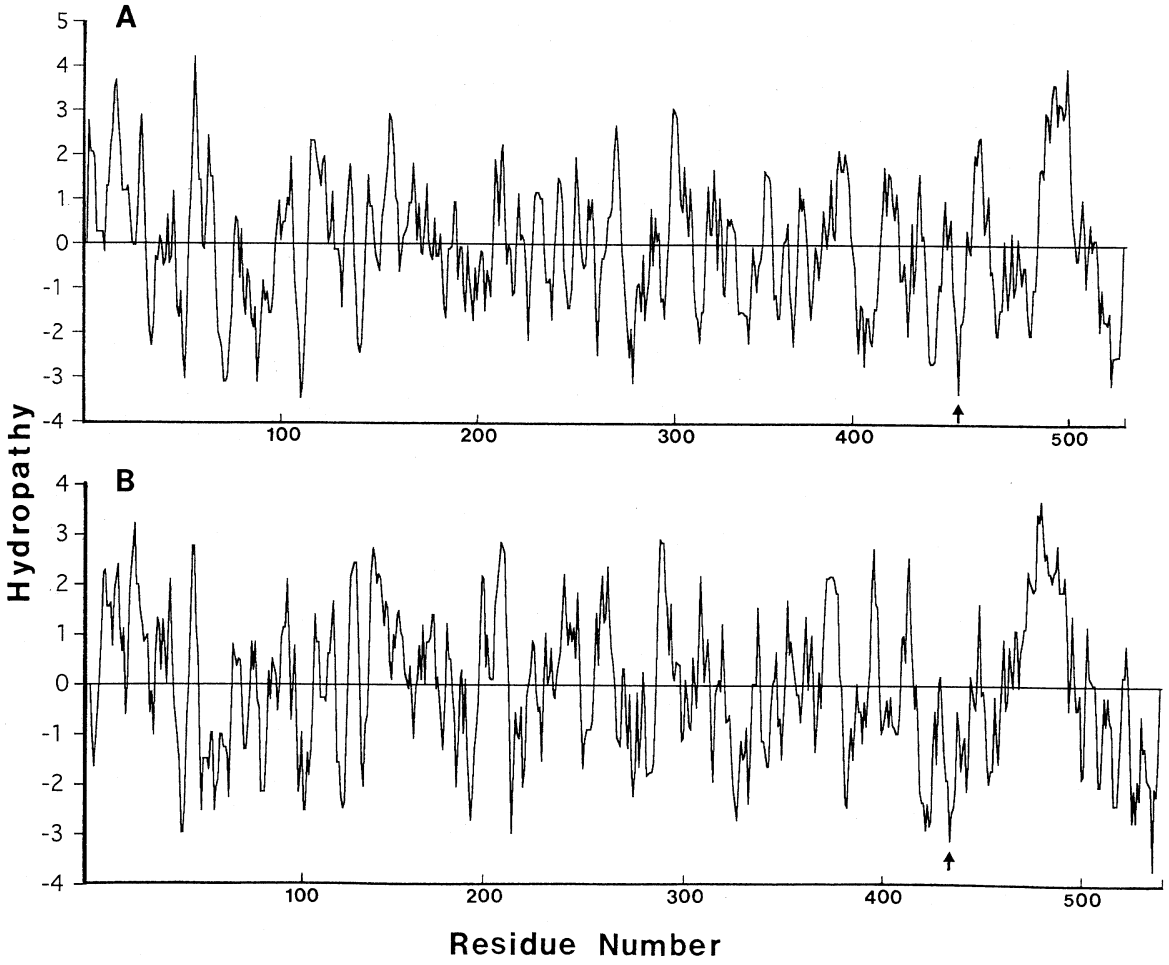

Figure 3 Hydropathy analysis of amino acid sequences of UGT and CGT

The amino acid sequences deduced from *Ysh* (**A**) and rat CGT (**B**) [28] were analysed for hydrophobicity and hydrophilicity as described by Kyte and Doolittle [29] using a window of five residues. The C-terminal highly hydrophilic regions, the Asp^{446} residue in *Ysh* and the Asp^{435} in rat CGT, are indicated by arrows.

sequences of CGTs in rats, mice and humans also have a conserved Asp^{435} residue corresponding to Asp^{446} in UGTs [25–27]. The result of hydropathy analysis of *Ysh* and rat CGT are shown in Figure 3. The Asp residue is located at the hydrophilic peak of the C-terminus in both proteins (Figure 3), as well as in other UGTs and CGTs (results not shown), and the profiles around the Asp residue were highly conserved in all UGTs and CGTs. The two proline residues, Pro^{448} and Pro^{451}, were also conserved in all UGTs and CGTs (Pro^{437} and Pro^{440}), suggesting that the region around the Asp residue forms a fixed structure in both enzymes. Ciotti et al. reported that non-polar residues Gly^{276} and Gly^{277}, which are indispensable for bilirubin UGT activity, possibly contribute to the folding of critical secondary structures such as the β-hairpin loop of birilubin UGT [7], and this Gly–Gly region was also conserved in rat, mouse and human CGTs [24–26]. Pillot et al [28] found that the UDP-binding site was present in the C-terminus (between amino acids 299 and 446) of the human liver UGT2B4. These findings described above suggest that the Asp, a large polar residue, plays an important role, such as in UDP recognition or UDP binding at the surface of UGTs and CGTs.

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