Identification of Aspartic Acid and Histidine Residues Mediating the Reaction Mechanism and the Substrate Specificity of the Human UDP-glucuronosyltransferases 1A*

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The human UDP-glucuronosyltransferase UGT1A6 is the primary phenol-metabolizing UDP-glucuronosyltransferase isoform. It catalyzes the nucleophilic attack of phenolic xenobiotics on UDP-glucuronic acid, leading to the formation of water-soluble glucuronides. The catalytic mechanism proposed for this reaction is an acid-base mechanism that involves an aspartic/glutamic acid and/or histidine residue. Here, we investigated the role of 14 highly conserved aspartic/glutamic acid residues over the entire sequence of human UGT1A6 by site-directed mutagenesis. We showed that except for aspartic residues Asp-150 and Asp-488, the substitution of carboxylic residues by alanine led to active mutants but with decreased enzyme activity and lower affinity for acceptor and/or donor substrate. Further analysis including mutation of the corresponding residue in other UGT1A isoforms suggests that Asp-150 plays a major catalytic role. In this report we also identified a single active site residue important for glucuronidation of phenols and carboxylic acid substrates by UGT1A enzyme family. Replacing Pro-40 of UGT1A4 by histidine expanded the glucuronidation activity of the enzyme to phenolic and carboxylic compounds, therefore, leading to UGT1A3-type isoform in terms of substrate specificity. Conversely, when His-40 residue of UGT1A3 was replaced with proline, the substrate specificity shifted toward that of UGT1A4 with loss of glucuronidation of phenolic substrates. Furthermore, mutation of His-39 residue of UGT1A1 (His-40 in UGT1A4) to proline led to loss of glucuronidation of phenols but not of estrogens. This study provides a step forward to better understand the glucuronidation mechanism and substrate recognition, which is invaluable for a better prediction of drug metabolism and toxicity in human.

UDP-glucuronosyltransferases (UGTs, EC 2.4.1.17) constitute a superfamily of enzymes that are involved in the phase II biotransformation pathway of many drugs and xenobiotics from natural or synthetic origin (1). UGTs are inverting glycosyltransferases belonging to the wide family 1 of NDP-sugar hexosyltransferases (2). They catalyze the transfer of glucuronic acid from the high energy donor substrate UDP-α-D-glucuronic acid on hydroxyl, carboxyl, or amine groups of structurally unrelated acceptor molecules, leading to the formation of β-D-glucuronides. The hydrophilic glucuronides are readily excreted from the body via urine and bile. Endogenous compounds, such as bilirubin and ligands of nuclear receptors (fatty acids, estrogens, retinoic acid) are also substrates of UGTs. Thus, these enzymes expressed in several tissues such as liver, lung, brain, kidney, and gastro-intestinal tract play a major role in both physiological and toxicological processes (3).

The UGT1A6 isoform is encoded by the UGT1 gene locus, which is also responsible for the expression of eight other functional UGT1A proteins (UGT1A1, -1A3, -1A4, -1A5, -1A7, -1A8, -1A9, and -1A10). These isoforms present a distinct but overlapping substrate specificity. The preferred substrates are bilirubin for UGT1A1, carboxyl drugs for UGT1A3, anti-psychotic amines for UGT1A4, 1-hydroxy-[α]-benzopyrene for UGT1A5, planar phenols for UGT1A6, and phenolic and carboxyl substances for UGT1A7, -1A8, and -1A9 (4, 5). Each isoform results from the alternate splicing of different exons 1 encoding the variable N-terminal half of the protein with the four common 2–5 exons encoding the C-terminal half. The N-terminal sequence of different isoforms is, therefore, predicted to interact with the acceptor substrates and is responsible for the substrate specificity, whereas the invariant C-terminal sequence would interact with the common donor substrate, UDP-glucuronic acid (UDP-GlcUA).

UGT1A6 isoform is expressed in hepatic and extrahepatic tissues (6) and is recognized as one of the main enzymes involved in the glucuronidation of phenols (7). Indeed, inhibitory antibodies raised against the C-terminal end of UGT1A6 protein indicated that this isoform contributed up to 60% in the glucuronidation of phenolic substances in human liver (8). In addition, UGT1A6 is involved in glucuronidation of drugs, such as the nonsteroidal anti-inflammatory drug naproxen, the analgesic drug paracetamol (9), the vasorelaxant naftazone (10), and carcinogens (hydroxyl polycyclic aromatic hydrocarbons) (11). Recently, serotonin (5-hydroxytryptamine) and its analogs such as 5-hydroxytryptophol have also been identified as substrates for human UGT1A6 (12, 13).
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Despite the importance of UGTs in the bioavailability of drugs and endogenous compounds, the structural determinants that are critical for the reaction mechanism and the molecular basis of substrate recognition have not been elucidated yet. Recently, the structure of the cofactor binding domain of UGT2B7 has been solved, and several amino acids interacting with the co-substrate (UDP-GlcUA) have been identified (14). However, the lack of information on the three-dimensional structure of the substrate binding domain and of the full-length protein due to difficulties in crystallizing these membrane proteins has impaired a better understanding of the active site organization and of the glucuronidation process.

Among other glycosyltransferases, several members have been crystallized and their three-dimensional structure solved. Interestingly, despite their functional diversity, their structures fall into two different canonical folds only, termed GT-A and GT-B (15). By analogy, the bi-domain organization of the UGTs in which the N- and C-terminal halves appear to be responsible for interaction with the acceptor and donor substrate, respectively, suggests that they would adopt a three-dimensional structure similar to that found in GT-B enzymes.

It has been proposed that the reaction mechanism of GT-B involves a general base (histidine, aspartic, or glutamic acid) initiating catalysis by abstracting a proton from the reactive group of aglycone and allowing the direct nucleophilic attack at the donor sugar C1 carbon center (16). Structural analyses and site-directed mutagenesis studies of GT-B members, MurG (17), GtfA (18), VvGT1 (19), and T4-BGT (20) suggested that histidine and aspartic acid residues His-19, Asp-13, His-20, and Asp-100, respectively, act as a catalytic base (19). Furthermore, His-22 has also been found to be critical for catalysis by the plant UGT71G1, whereas Asp-122 was shown to be a key residue that may assist deprotonation of the acceptor substrate by forming an electron transfer chain with the catalytic base, His-22 (21).

An acid-base mechanism has also been proposed for the glucuronidation reaction catalyzed by human UGT1A6 based on irreversible inactivation of the recombinant UGT1A6 by the histidyl- and carboxyl-directed electrophilic probes, diethylpyrocarbonate and carbodiimides, respectively (22, 23). Using this approach along with site-directed mutagenesis, we showed that histidine residue His-370 plays an important role in the catalytic process (24). Indeed, sequence alignment of GT-B proteins with UGT1A6 revealed that His-370 corresponds to the histidine residue of a common HX-E motif present in GT-B glycosyltransferases, known to play an important role in stabilizing the leaving UDP group of the donor substrate UDP-GlcUA during catalysis (18, 25).

In this paper we have investigated the role of conserved aspartic and glutamic acid residues of the human UGT1A6 in the glucuronidation mechanism. Each of these amino acids was individually mutated, and the mutant enzymes were analyzed in terms of enzyme activity and kinetic properties. Our results suggest that the aspartic residue Asp-150 plays a key role in the glucuronidation reaction and provide evidence that it participates to the catalytic mechanism. Furthermore, analysis of the role of histidine residue His-38 of UGT1A6, which corresponds to a predicted catalytic base His-22 of the plant UGT71G1, and of its counterpart Pro-40 in UGT1A3 and His-40 in UGT1A4 demonstrated that it governs the substrate specificity of these UGT isoforms toward phenolic and carboxylic substrates.

**EXPERIMENTAL PROCEDURES**

Reagents—Aglycone substrates for glucuronidation assays were of the highest purity available and were purchased from Sigma. UDP-[U-14C]glucuronic acid (418 mCi/mmol) was obtained from PerkinElmer Life Sciences. Restriction enzymes were provided by New England Biolabs (Hitchin, UK). The QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA), and Advantage® 2 polymerase mix was from Clontech (Palo Alto, CA). The yeast culture medium was from Difco. The *Pichia pastoris* expression system was from Invitrogen. Alkaline phosphatase-conjugated antibodies for Western blot detection were from Sigma. All other reagents were of the best quality and commercially available.

**Plasmid Construction and Mutagenesis**—cDNA sequences encoding human UGT1A6, UGT1A9, UGT1A4, UGT1A3, and UGT1A1 were cloned from human liver library (Clontech) and inserted into EcoRI-XhoI or PmlI-XhoI sites of the yeast expression vector pPICZB. The recombinant vectors pPICZ-UGT1A6, pPICZ-UGT1A9, pPICZ-UGT1A4, pPICZ-UGT1A3, and pPICZ-UGT1A1 were used for expression of recombinant UGTs in *P. pastoris* as previously described (24). Constructions of amino acid-substituted mutants were performed using the QuikChange site-directed mutagenesis kit according to the recommendations of the manufacturer. The sequence of sense mutation primers is indicated in Table 1. Full-length mutated cDNAs were systematically checked by DNA sequencing.

**Heterologous Expression in the Yeast *P. pastoris*—**Each recombinant pPICZ vector was individually transformed into *P. pastoris* SMD 1168 yeast strain (Invitrogen) using the *P. pastoris* EasyComp™ kit (Invitrogen). Stable transformants were selected on YPD plates (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose) containing 100 μg/ml zeocin. Transformed cells were grown in BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate (pH 6.0), 1.34% (w/v) yeast nitrogen base, and 1% (v/v) glycerol) for 24 h at 30 °C. Expression was induced by methanol in BMMG medium (BMMG with 1% (v/v) glycerol replaced by 2% (v/v) methanol) and carried out for 48 h at 30 °C in a rotary shaker at 215 rpm (26).

**Subcellular Fractionation of Recombinant Yeast Cells—**Yeast cells were harvested by centrifugation at 3000 × g for 10 min and further submitted to subcellular fractionation as previously described (24). Briefly, after harvesting, cells were washed once and suspended in cold breaking buffer (50 mM sodium phosphate (pH 7.4), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 5% (v/v) glycerol). The cells were then broken with glass beads. The resulting homogenate was centrifuged at 5000 × g for 15 min, and the supernatant was further centrifuged at 12,000 × g for 20 min. Membranes were then pelleted from the supernatant by centrifugation for 1 h at 100,000 × g at 4 °C. The membrane fraction was resuspended by Dounce homogenization in 0.25 M sucrose, 5 mM HEPES buffer (pH 7.4).
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### TABLE 1

| Enzyme Activity | Glucuronidation activity toward 4-MU |
|-----------------|-------------------------------------|
| **UGT1A6**      |                                     |
| H38A            | 5′-CGAGGGGACGCCTTGAAGCCTTT-3′       |
| H38D            | 5′-CGAGGGGACGCCTTGAAGCCTTT-3′       |
| H38E            | 5′-CGAGGGGACGCCTTGAAGCCTTT-3′       |
| H38Q            | 5′-CGAGGGGACGCCTTGAAGCCTTT-3′       |
| H38R            | 5′-CGAGGGGACGCCTTGAAGCCTTT-3′       |
| **UGT1A4**      |                                     |
| P40A            | 5′-CGAGGGGACGCCTTGAAGCCTTT-3′       |
| P40H            | 5′-CGAGGGGACGCCTTGAAGCCTTT-3′       |
| **UGT1A9**      |                                     |
| D148A           | 5′-CGAGGGGACGCCTTGAAGCCTTT-3′       |
| D148E           | 5′-CGAGGGGACGCCTTGAAGCCTTT-3′       |
| **UGT1A3**      |                                     |
| H40A            | 5′-CGAGGGGACGCCTTGAAGCCTTT-3′       |
| H40P            | 5′-CGAGGGGACGCCTTGAAGCCTTT-3′       |
| **UGT1A1**      |                                     |
| D151A           | 5′-CGAGGGGACGCCTTGAAGCCTTT-3′       |
| D151E           | 5′-CGAGGGGACGCCTTGAAGCCTTT-3′       |
| H39P            | 5′-CGAGGGGACGCCTTGAAGCCTTT-3′       |
| H39R            | 5′-CGAGGGGACGCCTTGAAGCCTTT-3′       |

**Immunoblot Analysis**—Protein concentration was evaluated by the method of Bradford (27). 30 μg of membrane proteins were analyzed by SDS-PAGE (28) and immunoblotting using anti-C-terminal peptide anti-UGT1A6 antibodies and alkaline phosphatase-conjugated secondary antibodies, as described previously (24).

**Enzyme Activity**—Glucuronidation activity toward 4-MU was measured according to the method of Lilienblum et al. (29). Fluorescence measurement of the glucuronide at excitation and emission wavelengths of 320 and 380 nm, respectively, was carried out on a Hitachi F2000 spectrophotometer (ScienceTec, Les Ulis, France) with 4-MU-β-D-glucuronic acid (0–10 nmol) as a standard.

The activity of recombinant wild-type UGT1A6, UGT1A9, UGT1A4, and UGT1A3 and of corresponding mutants toward selected substrates was determined by thin layer chromatography as described (30). Briefly, incubation in Eppendorf tubes (total volume 40 μl) consisted of 50 μg of microsomal proteins in 100 mM Tris-HCl buffer (pH 7.4), 10 mM MgCl₂, containing 0.1 mM UDP-GlcUA and 0.1 μCi UDP-[U-14C]glucuronic acid. The reaction was started by the addition of substrate (0.5 mM, final concentration) dissolved in 2 μl of dimethyl sulfoxide. A control was performed in which the substrate was omitted and contained dimethyl sulfoxide. After incubation for 1 h at 37 °C, the proteins were precipitated by 40 μl ethanol on ice and removed by centrifugation at 4000 × g for 10 min at 4 °C. The supernatant was loaded onto thin layer chromatography plates (Lk6DF silica gel, 250 μm, Whatman, Clifton, NJ). The plates were developed with n-butanol, acetone, acetic acid, aqueous ammonia (28%), water (70:50:18:1.60, v/v) and with n-butanol, acetone, water, and ammonium hydroxide (35:35:20:10, v/v) in the case of amine substrates. They were dried and sprayed with 1% (v/v) 2-(4-t-butyphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole in toluene. The radioactivity associated to the glucuronide was visualized by autoradiography with X-Omat Kodak film (Sigma) for 3 days or 6 days at −20 °C. The silica gel areas of the glucuronides were scraped off, and the associated radioactivity was quantified on a LKB spectrometer using Fluoran Safe Ultima Gold scintillant mixture (Packard Instrument Co.). The dpm value corresponding to the activity of a given sample was considered significant when it was at least 2-fold that of the control sample.

**Determination of Kinetic Parameters**—Apparent kinetic constants Kₘ and Vₘₐₓ toward 4-MU were determined by incubating yeast microsomes with increasing concentrations of 4-MU (0.01–2.0 mM) in the presence of a fixed concentration of UDP-GlcUA (5.0 mM). The apparent kinetic constants toward UDP-GlcUA were obtained using a constant amount of 4-MU (1 mM) in the presence of increasing concentrations of UDP-GlcUA (0.025 to 10 μM). Kₘ and Vₘₐₓ values were determined using nonlinear least squares analysis of the data fitted to Michaelis-Menten rate equation (v = Vₘₐₓ × [S]/Kₘ + [S]) using the curve-fitter program Sigmaplot 9.0™ (31).

**RESULTS**

**Expression Parameters and Kinetic limits of Wild-type UGT1A6 and Mutants**—In an attempt to identify the carboxylic amino acid potentially involved in UGT catalysis, we have performed a sequence alignment of several mammalian UGT isoforms and identified 14 highly conserved aspartic/glutamic acid residues over the entire sequence of human UGT1A6, i.e. Asp-145, Asp-150, Glu-287, Glu-289, Glu-314, Asp-357, Glu-378, Asp-394, Asp-397, Asp-420, Asp-447, Asp-454, Glu-461, and Asp-488 (Fig. 1). To address the importance of these invariant amino acids in glucuronidation mechanism, each of them was mutated to alanine (non-conservative mutation) and to aspartic or glutamic acids in glucuronidation mechanism, each of them was mutated (24).
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To evaluate the consequences of the mutations on the enzyme function, we analyzed the activity (Fig. 2B) and determined kinetic parameters of recombinant wild-type and mutant enzymes using 4-MU as reference acceptor substrate (Table 2). Interestingly, the results showed that except for D150A and D488A, all the alanine-substituted mutants exhibited enzyme activity, although compared with wild-type it was reduced by 5-, 3.2-, 5.6-, 2-, 29-, and 3-fold for D145A, D357A, D397A, D420A, D447A, and D454A, respectively (Fig. 2B). In the case of D394A mutant, only a slight decrease in activity was observed (about 20%). It is noteworthy that mutation of Asp-447 by alanine (D447A) strongly reduced enzyme activity (29-fold). However, replacement of this residue with glutamic acid (D447E) restored the activity of the enzyme but dramatically increased the $K_m$ value (10 mM) for the donor substrate with no change in the $K_m$ for the acceptor substrate (Table 2), suggesting the importance of Asp-447 residue in the interaction of the enzyme with UDP-GlcUA.

We investigated the role of conserved glutamic acid residues i.e. Glu-287, Glu-289, Glu-297, Glu-314, Glu-378, and Glu-461 in glucuronidation mechanism by engineering the corresponding alanine mutants E287A, E289A, E297A, E314A, E378A, and E461A. Upon expression in P. pastoris, immunoblot analysis showed that all the mutants were produced in similar or higher amounts compared with the wild-type enzyme (Fig. 2C). Analysis of the effect of the mutations of glutamic acid to alanine residues on the enzyme activity showed that, in contrast to aspartic acid to alanine substitutions, none of the alanine mutations abrogated the enzyme activity (Fig. 2D). Indeed, all the mutants exhibited enzyme activity, although compared with wild-type, it was reduced by 1.4-, 3.2-, 2-, and 7-fold for E287A, E289A, E297A, E314A, respectively, and by 150- and 350-fold for E378A and E461A mutants, respectively (Fig. 2D). Further analyses were conducted to determine the importance of Glu-378 and Glu-461 residues. For this purpose we constructed and expressed mutants with Glu-378 and Glu-461 individually replaced by a conservative amino acid, aspartic acid, or with serine or glutamine residues, i.e. E378D, E378S, E461D, E461S, and E461Q. Analysis of the enzyme activity showed that E378D and E378S mutants exhibited similar $V_{max}$ values compared with the alanine-substituted mutant E378A (Table 2). Similarly, E461D and E461S presented $V_{max}$ values close to that of E461A mutant. In contrast, replacement of Glu-461 with glutamine led to E461Q mutant with a $V_{max}$ value 25-fold lower than that of the wild-type enzyme but 20-fold higher than that of E461D and E461S mutants (Table 2). These results showed that both Glu-378 and Glu-461 residues could be replaced to some extent by non-carboxylic amino acids, suggesting that carboxylic amino
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Aspartic acid residues have critical effect on enzyme function, none of them acts catalytically increased (above 10 mM) (Table 2). These results suggested that aspartic acid at position 488 influences the interaction of the enzyme with UDP-GlcUA rather than playing the role of a catalytic base. The topology of UGT1A6 within the endoplasmic reticulum membrane indicates that the Asp-488 residue is the terminal residue of the transmembrane helix and is located at the membrane-luminal side interface. This position is more likely responsible for the positioning of the transmembrane domain of the protein in the endoplasmic reticulum membrane, which is critical for the UGT's function (32).

To investigate the role of aspartic acid Asp-150 in UGT1A6 glucuronidation mechanism, this residue was converted to different non-conservative amino acids including alanine, cysteine, asparagine, glutamine, serine and threonine and to a conservative residue glutamic acid. Upon expression, immunoblot analysis revealed that the mutants were efficiently produced and presented similar or higher levels of expression compared with the wild-type enzyme (Fig. 3).

TABLE 2
Michaelis-Menten parameters for glucuronidation of 4-MU by wild-type UGT1A6 and mutant enzymes

| Enzyme | UDP-GlcA | 4-MU |
|--------|----------|------|
|        | V_max (pmol/min/mg of protein) | K_m (mM) | V_max (pmol/min/mg of protein) | K_m (mM) |
| WT     | 1047.3±23.1 | 0.25±0.01 | 1130.6±19.7 | 0.12±0.01 |
| D145A  | 245.0±4.5  | 1.05±0.07 | 235.0±8.1  | 0.23±0.04 |
| D150A  | ND        | ND      | ND          | ND        |
| D150E  | 3.6±0.2   | 0.24±0.04 | 5.3±0.3    | 0.35±0.10 |
| D287A  | 637.3±13.0 | 0.28±0.02 | 622.6±21.3 | 0.16±0.02 |
| E288A  | 610.1±28.2 | 1.00±0.16 | 516.7±24.4 | 0.21±0.03 |
| E297A  | 776.7±10.5 | 0.83±0.06 | 733.9±24.6 | 0.17±0.02 |
| E314A  | 133.1±8.2  | 0.63±0.15 | 166.0±4.4  | 0.34±0.03 |
| D357A  | 421.3±16.4 | 0.44±0.08 | 462.6±9.0  | 0.14±0.01 |
| E378A  | 30.1±2.0   | >10      | 21.4±1.8   | 0.51±0.11 |
| E378D  | 49.8±6.5  | >10      | 48.1±4.6   | 0.32±0.10 |
| E378S  | 20.6±3.7  | >10      | 23.9±0.6   | 0.58±0.09 |
| D394A  | 2239.5±241.2 | 6.11±1.89 | 2096.0±61.8 | 0.17±0.02 |
| D397A  | 638.6±34.5 | 5.28±1.01 | 549.3±20.3 | 0.23±0.03 |
| D420A  | 1151.2±20.5 | 1.03±0.06 | 1077.0±23.4 | 0.20±0.01 |
| D427A  | 57.0±1.7   | 0.17±0.02 | 46.7±1.3   | 0.16±0.01 |
| D447E  | 1027.0±71.5 | >10        | 838.6±29.8 | 0.11±0.02 |
| D454A  | 522.5±25.9 | 0.82±0.15 | 608.1±44.1 | 0.48±0.09 |
| E461A  | 2.7±0.2   | 0.58±0.14 | 1.8±0.1    | 0.16±0.04 |
| D488A  | ND        | ND      | ND          | ND        |
| D488E  | 129.9±6.9  | >10      | 133.6±7.6  | 0.14±0.03 |

FIGURE 3. Replacement of Asp-488 and Asp-150 by different types of amino acids; effect on UGT1A6 activity. Immunoblot and enzyme activity analyses of UGT1A6 Asp-488 mutants (A and B) and Asp-150 mutants (C and D) are shown. 30 μg of membrane proteins from recombinant yeast cells were loaded in each lane (A and C). Glucuronidation activity (B and D) of wild type (WT) and the mutants toward 4-MU substrate was carried out with 50 μg of protein incubated in the presence of 0.1 mM UDP-GlcUA acid containing 0.1 μCi UDP-[14C]glucuronic acid and 1 mM substrate as indicated under “Experimental Procedures.” The glucuronides were separated by thin layer chromatography, visualized by autoradiography (B and D, inset) (film was exposed for 3 days), and quantitated by liquid scintillation counting. The rate values are the mean of three experiments.
pared with the wild-type recombinant enzyme (Fig. 3C). Analysis of enzyme activity and determination of the kinetic parameters (Table 2) indicated that non-conservative mutations resulted in total loss of enzyme activity, whereas replacement with glutamic acid led to D150E mutant with detectable activity (Fig. 3D). Interestingly, kinetic studies revealed that the D150E mutant presented a very low $V_{\text{max}}$ value compared with that of the wild type, with no change in $K_m$ values (Table 2).

To further determine the importance of the UGT1A6 Asp-150 residue in the glucuronidation reaction, we mutated the corresponding aspartic residue in other UGT1A isoforms UGT1A9, UGT1A4, and UGT1A1, i.e., Asp-148, Asp-152, and Asp-151, respectively, into alanine and glutamic acid and measured the enzyme activity toward reference substrates of each isoform (Fig. 4). Immunoblot analysis showed that all the mutants were expressed at similar levels to that of corresponding wild-type protein (Fig. 4A). Interestingly, enzyme activity analysis showed that for all isoforms, the mutation to alanine resulted in a complete loss of enzyme activity (Fig. 4B). On the other hand, substitution of the aspartic acid residues by glutamic acid produced mutants with low but detectable activity (Fig. 4B). UGT1A9 mutant D148E presented an activity of 3.3 pmol/min/mg of protein toward 4-MU, UGT1A4 mutant D152E exhibited an activity of 0.21 pmol/min/mg of protein toward 4-amino-biphenyl, and UGT1A1 presented an activity of 1 pmol/min/mg of protein toward octylgallate (Fig. 4B). Altogether, these results highlight the importance of aspartic acid residue at position 150 in UGT1A6 as well as its counterpart in other UGT1A isoforms in catalysis.

**Histidine 38 Governs the Substrate Specificity of UGT1A** — According to the three-dimensional structure analysis of two GT-B glycosyltransferase members MurG (17) and VvGT1 (19), histidine residues His-19 and His-20, respectively, were predicted to act as an alternate general base to deprotonate the hydroxyl group of the acceptor substrate instead of carboxylic amino acids, leading us to investigate the role of this residue in the UGT1A family. Sequence alignment indicated that these residues correspond to His-38 in UGT1A6, His-39 in UGT1A1, and His-40 in UGT1A3, whereas in UGT1A4 this position corresponds to proline residue (Pro-40) (Fig. 1). The observation that this residue is not invariant among the members of the UGT family does not favor the hypothesis that it plays the role of a catalytic base.

To clarify the role of His-38 residue of UGT1A6, it was mutated to alanine, aspartic acid, proline, arginine, and tyrosine to generate H38A, H38D, H38P, H38R, and H38Y mutants, respectively. Immunoblot analysis showed that the mutants were expressed at similar or higher levels than that of the wild-type protein (Fig. 5A)

Analysis of the activity and substrate specificity indicated that, except for H38R, these mutants were inactive (data not shown). Nonetheless, the mutant H38R exhibited low enzyme activity (Fig. 5B). Interestingly, H38R mutant presented different substrate specificity compared with wild-type enzyme. Indeed, the mutant was not able to glucuronidate scopolin (Fig. 5, compare B and C), indicating that arginine can, to some extent, replace His-38 residue in terms of substrate specificity. These results suggest that His-38 residue is involved in determining the substrate specificity of UGT1A6 enzyme rather than playing a role of catalyst.

UGT1A4 sequence is 93% identical to that of UGT1A3; however, the two isoforms exhibit major differences in substrate specificity. UGT1A4 forms N-glucuronides from primary, secondary, and tertiary amines and plays a major role in the metabolism of anti-psychotic drugs. In addition to amine substrates, UGT1A3 catalyzes the glucuronidation of phenolic compounds and carboxylic acids that are not glucuronidated by UGT1A4. As indicated above, UGT1A4 contains a proline residue (Pro-40) at the position corresponding to His-38 in UGT1A6 (Fig. 1). Therefore, we investigated the role of this residue by engineering the UGT1A4 mutant P40H, in which proline was replaced by a histidine residue, as found in UGT1A6 and in its homologous UGT1A3 as well as in other UGT isoforms, and by alanine to generate P40A mutant (Fig. 6). Upon expression, UGT1A4 mutants were expressed at similar or higher level than that of the wild-type enzyme (Fig. 6A). Remarkably, analysis of enzyme activity toward a range of compounds revealed that P40H mutant exhibited expanded substrate specificity toward phenolic and carboxylic substrates (Fig. 6C). Indeed, the mutant was able to glucuronidate phenols (4-MU, 1-naphthol, 4-nitrophenol, quercetin, scopolin) and...
carboxylic acids (ibuprofen, ketoprofen). Analysis of the substrate specificity of the mutant toward amine substrates showed that the catalytic activity was restricted to primary amines such as 4-aminobiphenyl and 2-naphthylamine. No activity was detected either for secondary amines such as diphenylamine and N-desmethylclozapine or for ternary amines such as cyproheptadine or doxepin (Fig. 6, compare B and C). These results suggested that histidine mutation alters the organization of the active site cavity to favor the binding of small molecules and to prevent the binding of bulky amines. Interestingly, mutation of Pro-40 residue to alanine did not induce changes in substrate specificity of the enzyme (data not shown).

To confirm the importance of Asp-152 residue in glucuronidation mechanism, we generated a UGT1A4 double mutant P40H-D152A and measured the enzyme activity toward the substrates described above. The results obtained showed that, in contrast to a single mutant P40H, which was able to glucuronidate phenolic, carboxylic, and amine substrates, the P40H-D152A double mutant was totally inactive, thus confirming the essential catalytic role of the Asp-152 residue (data not shown).

As shown above, exchanging Pro-40 residue of UGT1A4 by histidine, as found in UGT1A3, led to a UGT1A4 mutant P40H with substrate specificity similar to that of UGT1A3. We further investigated the role of histidine residue His-40 of UGT1A3 in substrate specificity of the enzyme. For this purpose, we generated and expressed UGT1A3 mutant H40P, in which His-40 residue was exchanged by a proline, as found in UGT1A4, and H40A mutant in which His-40 was replaced with alanine residue (Fig. 7). Upon expression, UGT1A3 mutants were expressed at a similar level as that of the wild-type protein (Fig. 7A). Interestingly, substrate specificity analysis of UGT1A3 mutant H40P revealed that it was shifted toward that of UGT1A4. Indeed, the mutant was active toward amine substrates 4-aminobiphenyl and 2-naphthylamine but was not able to glucuronidate phenolic and carboxylic acid compounds (4-MU, scopoletin, 1-naphthol, 4-nitrophenol, ketoprofen, and ibuprofen) (Fig. 7, compare B and C). Similar results were obtained when His-40 residue was converted to alanine (H40A) (data not shown).

To further investigate the importance of this residue in the glucuronidation of phenols, we analyzed the effects of the...
mutation of His-39 residue in UGT1A1 into proline and alanine. Upon expression, UGT1A1 mutants were expressed at similar or higher levels than that of the wild-type enzyme (Fig. 8A). As expected, analysis of the glucuronidation activity indicated that UGT1A1 catalyzes the glucuronidation of estrogens (β-estradiol, 2-hydroxyestradiol, 2-hydroxyestrone) and phenolic compounds (4-MU, scopoletin, 1-naphthol, eugenol, octylgallate, 4-nitrophenol) (Fig. 8B). Interestingly, replacement of His-39 residue of UGT1A1 by proline led to loss of glucuronidation of phenolic compounds but not of estrogens (Fig. 8, compare B and C). Similar results were obtained when His-39 was replaced by alanine residue (data not shown). Taken together, these data indicate that the amino acid at position 40 in UGT1A3 and UGT1A4 and its counterpart in UGT1A1 (His-39) is an important determinant for the glucuronidation of phenolic and carboxylic acid compounds.

DISCUSSION

The improvement of drug efficacy requires a better understanding of the molecular mechanism of the enzyme activity in charge of their metabolism. In that context, UGTs play a key role in the biotransformation of drugs in human. These proteins also catalyze the glucuronidation of structurally unrelated xenobiotics, some of which are known to be carcinogens, as well as endogenous compounds, such as hormones and other ligands of nuclear receptors. Thus, these enzymes constitute an effective barrier against the entry of potentially toxic substances and play an important role in the physiology of the cells by modulating the concentration of endogenous compounds. Therefore, the mechanism of the glucuronidation reaction as well as the molecular basis of their substrate specificity should be better understood. This work aims at providing more information on these two points.

Identification of amino acids that are essential for catalysis or governing the substrate specificity is a major issue in the understanding of the structure and function of UGTs. Based on kinetic and chemical modification studies as well as glycosylation mechanism supported by inverting glycosyltransferases, it was proposed that the reaction mechanism accounting for glucuronidation would involve a nucleophilic attack of the acceptor substrate on the C1 atom of D-glucuronic acid, leading to the formation and release of UDP (15, 23). Such reaction is believed to proceed according to a S_n2 reaction mechanism with an oxocarbenium ion-like as the transition state.
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state. This type of mechanism would require a general base (histidine and/or aspartate/glutamate) for activation of the nucleophilic hydroxyl group of the acceptor substrate by deprotonation required for its transfer to glucuronic acid together with the departure of UDP.

In previous studies we suggested a carboxylic acid and a histidine residue to play crucial roles in the function of UGT1A6 from its susceptibility to be inactivated by dicyclohexylcarbodiimides and diethylpyrocarbonate, respectively (22, 23). Multiple sequence alignment of several human UGT isoforms with GT-B glycosyltransferases showed that His-370 belongs to the \( \text{HX, E}^{770}\text{HX, E}^{778} \) in UGT1A6) signature motif of this family of enzymes (25, 33). By analogy with the reaction mechanism that has been described for crystallized glycosyltransferases, we postulated that His-370 would stabilize the leaving UDP group during catalysis by neutralizing the negative charge of the pyrophosphate moiety (34). This assumption was recently supported by structure analysis of the C-terminal domain of UGT2B7 (residues 285–451) predicting that His-374 residue (His-370 in UGT1A6) interacts with the \( \beta \)-phosphate of the donor substrate UDP-GlcUA (14).

The identification of a putative crucial aspartic or glutamic acid residue acting as a catalytic amino acid will represent a step forward in understanding the glucuronidation process. In this study search for conserved carboxylic residues revealed the presence of 14 aspartic/glutamic acids present over the entire sequence. These positions are highly conserved among mammalian species, including rats and mice. To identify the catalytic residue, serial point mutations were undertaken, and the activity and kinetic parameters of the mutants were investigated upon expression in \( \text{P. pastoris} \). Except for Asp-150 and Asp-488, the substitution of carboxylic residues by alanine led to active mutants but with decreased enzyme activity and lower affinity for both acceptor and donor substrates, 4-MU and UDP-GlcUA. This observation suggests that these carboxylic acid residues may modify the conformation of the enzyme or may be important in substrates binding. Interestingly, replacement of Asp-394 or Asp-397 by alanine induced a 25- and 22-fold increase in apparent \( K_m \) toward UDP-GlcUA, with no marked change in that of 4-MU, thus suggesting that these mutations specifically affected the binding of the donor substrate. Consistently, Asp-394 and Asp-397 are located in a consensus sequence found in all 110 members of the UGT superfamily (35). This signature sequence is believed to correspond to a series of aspartic acid residues that are known to participate in the binding of the common donor substrate, UDP-GlcUA. Our findings are consistent with Asp-394 and Asp-397 being important for the interaction of UGT1A6 with UDP-GlcUA. This is supported by structural analysis of the C-terminal domain of UGT2B7 that predicted that Asp-398 (Asp-394 in UGT1A6) interacts with GlcUA moiety of the co-substrate (14).

On the other hand, substitution of Asp-447 by alanine severely impaired the enzyme activity. However, our results showed that replacement with glutamic acid, an amino acid with similar polarity but with longer side chain, restored the activity of the enzyme but dramatically increased the \( K_m \) value toward the donor substrate, suggesting that this residue is likely to be important in donor substrate binding and recognition. Furthermore, investigation of the role of Asp-446 of rat UGT1A6 (the counterpart of Asp-447 in human UGT1A6) showed that it was important in the enzyme function but not directly involved in catalysis and can be replaced by threonine or asparagine residues (36). In the case of Glu-461, although alanine replacement produced a severe decrease in UGT1A6 activity, our results indicated that introduction of a glutamine residue (Gln-461) sustained, to some extent, the enzyme activity, suggesting that the carboxyl group was not mandatory at this position.

In contrast, mutation of Asp-150 or Asp-488 by alanine completely abolished the enzyme activity, indicating that they were crucial for the enzyme function. The importance of these residues was further supported by the fact that their substitution by the alternate carboxyl amino acid failed to restore a significant activity. The Asp-488 residue belongs to a strictly conserved, negatively charged stretch of 10 amino acids located in the vicinity of the C-terminal side of the transmembrane segment of UGTs, which is expected to be an important signal in positioning and orientating the transmembrane domain (37, 38). Accordingly, mutation of Asp-488 with negatively charged residue glutamic acid (D488E) led to a mutant with detectable enzyme activity. This is in contrast with deleterious consequences of the mutation of this residue to a noncharged amino acid, alanine (D488A). However, replacement of Asp-488 by glutamic acid strongly increased the \( K_m \) value toward UDP-GlcUA, suggesting its implication in determining the interactions between the enzyme and the donor substrate. Consistent with our results, this residue belongs to a domain that has been shown to be critical for UGT2B13 activity, leading to the suggestion that this part of the protein exhibits rigid structural requirements to maintain UGTs in an active conformation (39).

By analogy with GT-B glycosyltransferases, whose three-dimensional structure has been solved, it was possible to suggest a role for the conserved carboxyl residues Glu-378 and Asp-150 of UGT1A6. Glu-378 belongs to the \( \text{HX, E}^{770}\text{HX, E}^{778} \) signature motif of GT-B glycosyltransferases where glutamic acid was shown to be important for the interaction with the ribose of UDP-GlcUA (25). In agreement, our results showed that exchanging this residue by alanine strongly decreased the \( V_{\text{max}} \) of the reaction and also highly increased the apparent \( K_m \) value (above 10 mM) of the enzyme for the donor substrate. Similarly, it is noteworthy that Asp-150 residue is conserved among UGTs and GT-B members. Mutation of this residue led to inactive UGT, except when glutamic acid was substituted to aspartic acid. However, the mutation strongly affected the \( V_{\text{max}} \), with no marked change in the \( K_m \) values, thus suggesting that Asp-150 is important for catalysis and not for substrate recognition. Additionally, sequence alignment of UGT1A6 with T4-BGT (Fig. 1) indicated that Asp-150 of UGT1A6 corresponds to Asp-100 proposed as a catalytic base of T4-BGT enzyme (20). Consistently, mutations of Asp-148, Asp-152, and Asp-151 in other UGT isoforms UGT1A9, UGT1A4, and UGT1A1, respectively, similarly abrogated enzyme activity, suggesting that aspartic acid at position 150 in UGT1A6 and its counterpart in other UGTs is likely to be a catalytic residue. Analysis of the secondary structure of the
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region encompassing residue Asp-150 in different UGT isoforms indicated that this aspartic residue is located in a loop between two α-helices, a position favoring the catalytic role of this residue.

According to the three-dimensional structure analysis of two GT-B glycosyltransferase members, MurG and VvGT1, histidine residues His-19 and His-20, respectively, were predicted to act as a general base to deprotonate the hydroxyl group of the acceptor (19). Sequence alignment of MurG, VvGT1, and UGT1A6 indicated that His-19 and His-20 of MurG and VvGT1, respectively, correspond to residue His-38 of UGT1A6. Mutation of His-38 by proline completely abolished UGT1A6 enzyme activity. Similar results were obtained when His-38 was replaced by alanine. However, substitution by arginine restored to some extent the enzyme activity and substrate specificity except for scopoletin that is not glucuronidated by the mutant. These results suggest that arginine may establish interactions with phenol substrates via the amine group leading to glucuronidation to some extent of these compounds. Interestingly, sequence alignment of different members of UGTs (Fig. 1) reveals that His-38 residue is highly conserved among UGT1A family, except for UGT1A4, where it is replaced by proline (Pro-40). UGT1A4 enzyme catalyzes the formation of N-glucuronides, and the only substrates accepted are primary, secondary, and tertiary amines among those are important antipsychotic drugs (4). However, UGT1A3, that is highly homologous to UGT1A4, glucuronidates a larger range of substrates. The presence of proline at position 40 (Pro-40) in UGT1A4 isoform was likely to play a pivotal role in the substrate specificity. Indeed, mutation of this residue to histidine (P40H) expanded the glucuronidation activity of the enzyme to phenol and carboxylic compounds, therefore leading to UGT1A3-type isoform in terms of substrate specificity. On the other hand, P40H mutation led to a loss of glucuronidation of secondary and tertiary amines but not of primary amines, suggesting that histidine residue may induce steric hindrance that alters the capacity of the binding site cavity to accommodate bulky amine substrates. In contrast, these changes favor the binding of small molecules such as 1-naphthol and 4-MU probably by establishing interactions between these substrates and histidine residues. Interestingly, mutation of Pro-40 residue to alanine (P40A) did not induce any changes in the substrate specificity of the enzyme, suggesting that alanine replacement did not alter the active site organization. These results indicated that the proline residue at position 40 is critical for glucuronidation of tertiary amines and for the formation of quaternary amine glucuronides by human UGT1A4 enzyme.

Conversely, when the His-40 residue of UGT1A3 was replaced with proline (as found in UGT1A4), the substrate specificity shifted toward that of UGT1A4 as the enzyme was active only toward amine substrates but no longer glucuronidated carboxylic acids and phenols. Replacement by alanine (H40A) did not restore the glucuronidation activity of the enzyme toward phenolic and carboxylic acid compounds. These results reveal the key effect of histidine 40 residue in expanding the glucuronidation activity of UGT1A4 toward phenolic and carboxylic acid compounds possibly by increasing their reactivity via interaction with the imidazole group. The importance of histidine residue at this position in glucuronidation of phenolic compounds was further confirmed by analyzing the effects of its mutation in UGT1A1 isoform. Indeed, replacement of His-39 of UGT1A1 by proline or alanine residues led to UGT1A1 mutant lacking glucuronidation activity toward phenols but still exhibiting detectable activity toward estrogens.

The binding site of UGTs is intrinsically promiscuous in nature, the residue at position 40 in UGT1A3 and UGT1A4 and its counterpart in UGT1A1 obviously play a crucial role in glucuronidation of phenolic and carboxylic substrates. Thus, it will be important to take into account the role of this key residue in further development of predictive models of the specificity of UGT1A4, UGT1A3, and UGT1A1. Similarly, Funhoff et al. (40) showed recently that a histidine residue plays an important role in positioning the substrate of the purple acid phosphatase, and Watts et al. (41) identified a single active site histidine residue governing substrate selection of aromatic lyase family.

In the absence of structure data of the full-length UGT and of the C-terminal domain that binds the acceptor substrate, the structure of the plant flavonoid glucosyltransferase VvGT1 has been used to generate a homology model of UGT2B7 (14). This model revealed that His-35 and Asp-151 of UGT2B7 were analogous to the predicted catalytic residues His-20 and Asp-119 of VvGT1 enzyme. Thus, the authors suggested that His-35 plays a role of the catalytic base to deprotonate the substrate, and the resulting protonated histidine is stabilized by Asp-151 (14). However, a histidine residue at this position is not strictly conserved among all UGT members. Indeed, two UGT isoforms, UGT1A4 and UGT2B10, have proline and leucine, respectively, instead of histidine at this position. Therefore, for these enzymes it is not clear which residue may act as a catalytic base to deprotonate the substrate. In addition, mutation of His-35 residue of UGT2B7 led to a mutant enzyme with residual activity for androsterone (14). Likewise, we showed here that mutation of this histidine residue in different UGT isoforms did not completely inactivate the enzymes, except for phenolic and carboxylic substrates.

Our study indeed suggests that this residue is more likely to confer glucuronidation activity toward phenolic and carboxylic compounds; it cannot be ruled out that it could also act to deprotonate these substrates. The resolution of the three-dimensional structure of the whole protein will surely bring a definitive answer to that question. Our study provides a step forward to better understanding of the glucuronidation mechanism. Such information is invaluable for a rational prediction of drug metabolism and toxicity in human.

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