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ACCELERATED COMMUNICATION

Importance of Histidine Residues for the Function of the Human Liver UDP-Glucuronosyltransferase UGT1A6: Evidence for the Catalytic Role of Histidine 370

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ABSTRACT

The human UDP-glucuronosyltransferase isoform UGT1A6 catalyzes the nucleophilic attack of phenolic xenobiotics on glucuronic acid, leading to the formation of water-soluble glucuronides. Based on the irreversible inhibition of the enzyme activity by the histidyl-selective reagent diethyl pyrocarbonate (DEPC), histidine was suggested to play a key role in the glucuronidation reaction. Therefore, the role of four strictly conserved histidine residues (His38, His361, His370, and His485) in the glucuronidation of 4-methylumbelliferone, as reporter substrate, was examined using site-directed mutagenesis. For this purpose, stable heterologous expression of wild-type and mutant UGT1A6 was achieved in the yeast *Pichia pastoris*. Replacement of histidine residues by alanine or glutamine led to fully inactive H38A, H38Q, and H485A mutants. Substitution of His361 by alanine affected the interaction of the enzyme with the cosubstrate, as indicated by a 4-fold increase in the K_m value toward UDP-glucuronic acid. Interestingly, H370A mutant presented a severely impaired catalytic efficiency (with a V_{max} value approximately 5% that of the wild-type), whereas conservative substitution of His370 by glutamine (H370Q) led to a significant restoration of activity. Whereas H361A was inactivated by DEPC as the wild-type enzyme, this chemical reagent only produced a minor effect on either H370Q or H370A mutant, providing evidence that His370 is probably the reactive histidine residue targeted by DEPC. The dramatic changes in catalytic efficiency on substitution of His370 by alanine and the ability of glutamine to function in place of histidine along with a weak sensitivity of these mutants to DEPC strongly suggest that His370 plays a catalytic role in the glucuronidation reaction.

UDP-glucuronosyltransferases (UGTs, EC 2.4.1.17) are a multigenic family of enzymes actively involved in the detoxication of drugs and other xenobiotics (Mackenzie et al., 1997). They also play a key role in the biotransformation of endogenous compounds, especially those that are ligands of nuclear receptors such as steroid hormones and retinoic acid (Nebert, 1991). These enzymes, which are resident in the endoplasmic reticulum, catalyze the binding of glucuronic acid on the hydroxyl, carboxyl, sulfhydryl, or amine group of these structurally unrelated substances, leading to the formation of water-soluble glucuronides easily excreted into bile or urine (Radominska-Pandya et al., 1999).

In humans, up to 30 UGT isoforms, essentially members of families 1 and 2, have been identified and characterized in terms of substrate specificity on expression of the corresponding cDNA in heterologous cells (Guengerich et al., 1997). They all present distinct but overlapping substrate specificity.

The human liver UGT1A6 is 1 of the 13 isoforms encoded by the complex *UGT1* gene that are generated by alternative splicing of exon 1 to the four common exons (exons 2–5) (Ritter et al., 1992). Exon 1 codes for the variable N-terminal end of the luminal domain of the protein, whereas the common exons code for the identical C-terminal domain includ-

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ABBREVIATIONS: DEPC, diethyl pyrocarbonate; 4-MU, 4-methylumbelliferone; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; UGT, UDP-glucuronosyltransferase.

ing the transmembrane segment and the cytoplasmic tail. Using inhibitory antibodies raised against the N-terminal end of the protein, we previously showed that this isoform contributed up to 60% in the glucuronidation of phenols in human liver (Ouzzine et al., 1994). Structure-activity relationships of the protein expressed in V79 fibroblasts revealed a strict specificity toward planar and short phenols, such as 1-naphthol or 4-methylumbelliferone (4-MU) (Fournel-Gigleux et al., 1991). The enzyme also catalyzes the formation of glucuronides from drugs such as paracetamol and naftazone (Bock et al., 1993; Herber et al., 1995). It is also actively implicated in the metabolism of the potential carcinogens, polycyclic aryl hydrocarbons (Bock, 1991). Recently, serotonin has been shown to be glucuronidated by UGT1A6 (King et al., 1999). UGT1A6 is expressed in the liver and in other organs including kidney (Ouzzine et al., 1994), brain (Martinasevic et al., 1998; Gradinaru et al., 1999), lung (Vainio et al., 1995), and intestine (Strassburg et al., 1998, 1999). By the nature of its substrates, it is believed that UGT1A6 plays an important role as a protective metabolic barrier against the intrusion of xenobiotics.

We have been deeply involved in elucidating the determinants governing the structure and function of UGT1A6 (Ouzzine et al., 1999a,b). In this regard, identification of functionally important amino acids is a major issue toward a better understanding of the molecular basis of the glucuronidation reaction. We previously reported the presence of key histidine, arginine, aspartic, or glutamic residues based on the irreversible inactivation of the recombinant UGT1A6 by the amino acid-modifying reagents diethyl pyrocarbonate (DEPC) (Battaglia et al., 1994a), 2,3-butanedione (Senay et al., 1997), and carbodiimides (Battaglia et al., 1994b), respectively. On the basis of these results, a reaction mechanism through a general acid/base catalysis was postulated whereby a charge relay system between histidyl and aspartate/glutamate residues may facilitate the deprotonation of the phenolic substrates and their transfer to glucuronic acid, leading to the release of UDP. On the other hand, the arginine residue was believed to interact through ionic bonding with the carboxylate of glucuronic acid for the correct positioning of the substrate in the active site (Zakim et al., 1983; Pillot et al., 1993). Attempts to identify these amino acids using site-directed mutagenesis led to the conclusion that the strictly conserved His54 and Arg52 located in the N-terminal end of UGT1A6, although important for the function and structure required for optimal enzyme efficiency, were not catalytic residues (Senay et al., 1997).

Because the three-dimensional structure of the enzyme has not yet been resolved, we systematically investigated, in this work, the potential role of the other strictly conserved histidine residues His38, His361, His370, and His485 using site-directed mutagenesis (Fig. 1). Histidine was replaced by alanine, which presents a radically different side chain, and by glutamine, which is considered a conservative substitution of histidine, because their side chain contains nitrogens with unpaired electrons. For this purpose, a new and powerful expression system was developed using the yeast *Pichia pastoris* as host cell. This system not only provides a convenient source for recombinant wild-type UGT1A6 but also facilitates the functional characterization of mutants of this enzyme.

This study shows that His38 and His485, although important for the structure and function of UGT1A6, were not directly engaged in catalysis. His361 is likely to be involved in the UDP-glucuronic acid binding site. Interestingly, a detailed kinetic analysis of the alanine- and glutamine-substituted His370 mutants along with their weak sensitivity to DEPC inactivation provides evidence for a catalytic role of His370.

Materials and Methods

Chemicals and Reagents. 4-MU (free acid), 4-MU-β-D-glucuronide, 1-naphthol, 4-nitrocatechol, 4-nitrophenol, 2-ethylphenol, 4-ethylphenol, 4-hydroxybiphenyl, α-estradiol, 17α-ethinylestradiol, testosterone, DEPC, and donkey anti-goat alkaline phosphate-conjugated immunoglobulins were purchased from Sigma (L'Isle d'Abeau, St. Quentin Fallavier, France). UDP-glucuronic acid, sodium salt, and UDP-[U-^{14}C]glucuronic acid (285 mCi/mmol) were obtained from Boehringer Mannheim (Mannheim, Germany) and NEN (DuPont, Paris, France), respectively. The yeast culture medium was from Difco (Detroit, MI). The restriction enzymes and *Vent* DNA polymerase were provided by New England Biolabs (Hitchin, UK). T4 DNA ligase, pGEM-3Z, and competent *Escherichia coli* JM109 were purchased from Promega (Charbonières, France). The *P. pastoris* expression system was from Invitrogen (Groningen, The Netherlands). All other reagents were of the best quality available commercially.

Plasmid Construction and Mutagenesis. Human UGT1A6 cDNA was isolated from the mammalian expression vector pcDNA1-UGT1A6 (Ouzzine et al., 1994) and used as a template for polymerase chain reaction (PCR) amplification. For expression of wild-type UGT1A6 in yeast, the UGT1A6 cDNA coding sequence was modified by PCR to contain an *Eco*RI site and a Kozak sequence at the 5′ end using a sense primer (UGT1A6A) and to include an *Xba*I site at the 3′ end using an antisense primer (UGT1A6B), as detailed in Table 1. The PCR fragment was subcloned into the *Sma*I site of pGEM-3Z. The recombinant vector was then digested by *Eco*RI-*Xba*I, and the resulting fragment was subcloned into the *Eco*RI-*Xba*I sites of pPICZB yeast expression vector to generate pPICZ-UGT1A6.

The mutated UGT1A6 cDNAs were obtained by a two-rounds PCR-based method as follows. The first reaction was realized using the sense primer UGT1A6A and an antisense primer introducing the chosen mutation. The second PCR reaction was performed with a sense primer complementary to the antisense primer introducing the

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                         38                                  361      370                           485
hUGT1A6     VGDKLLVVPQDGSHWLSMKDIVEVLSDRGHEIV   ILVKWLPQNDLLGHPMTRAFITHAGSHGVYESICNGV   RPAAHDLTWYQYHSLDVIGFLLAVVL
rUGT1A6     LGDKLLVVPQDGSHWLSMKEIVEHLSERGHDIV   ILVKWLPQNDLLGHPKARAFITHSGSHGIYEGICNGV   RPAAHDLTWYQYHSLDVIGFLLAIVL
hUGT2B17    SCGKVLVWPTEYSHWINMKTILEELVQRGHEVI   RLYKWIPQNDLLGHPKTRAFITHGGANGIYEAIYHGI   RVAAHDLTWFQYHSLDVIGFLLVCVA
hUGT2B15    SCGKVLVWPTEYSHWINMKTILEELVQRGHEVT   RLYKWIPQNDLLGHPKTRAFITHGGANGIYEAIYHGI   RVAAHDLTWFQYHSLDVTGFLLACVA
rUGT2B1     ACGKVLVWPTEYSHWINIKIILNELAQRGHEVT   RLYKWLPQNDLLGHPKTKAFITHGGTNGIYEAIYHGI   RVAAHNLTWIQYHSLDVIAFLLACVA
hUGT2B4     SCGKVLVWPTEFSHWMNIKTILDELVQRGHEVT   RLYKWLPQNDLLGHPKTKAFITHGGTNGIYEAIYHGI   RVAAHNLTWIQYHSLDVIAFLLACVA
hUGT2B7     NCGKVLVWAAEYSHWMNIKTILDELIQRGHEVT   RLYKWIPQNDLLGHPKTKAFVAHGGTNGIYEAIYHGI   RSTLHDLSWFQYHSLDVIGFLLLCVV
rUGT2B6     KCGKVLVWPMEFSHWMNIKTILDELVQRGHEVT   RVYKWLPQNDLLGHPKTKAFVTHGGANGVYEAIYHGI   RPLGHNLPWYQYHSLDVIGFLLACLA
rUGT2B2     HCGKVLVWPMDFSHWMNIKIILDELVQRGHEVT   RVYKWLPQNDLLGHPKTKAFVTHGGANGLYEAIYHGI   RPLGHNLPWYQYHSLDVIGFLLTCFA
               * **     ***   * *   *  ***         ** *********   **  * *  * ** *  *    *   * * * *******  ***
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Fig. 1. Sequences alignment of UGTs. Alignment was performed using programs resident in GCG DNA and Protein Analysis Package (Promega, Madison, WI). Identical amino acid residues are indicated by stars in the bottom row. Conserved histidines are in bold.

mutation and the antisense primer UGT1A6B. After purification from agarose gel, the two PCR fragments were hybridized via the overlapping regions from the sense and antisense primers introducing the mutation and used as a template for the amplification of the full-length UGT1A6 coding region using primers UGT1A6A and UGT1A6B. Details of the primers designed to introduce the mutations are given in Table 1. For each mutation, the resulting PCR fragment was purified from agarose gel and subcloned into the *Sma*I site of pGEM-3Z. The recombinant vector was then digested by *Eco*RI-*Xba*I, and each mutated fragment obtained was subcloned into the *Eco*RI-*Xba*I sites of pPICZB yeast expression vector to generate pPICZ-UGT1A6H38A, pPICZ-UGT1A6H38Q, pPICZ-UGT1A6H361A, pPICZ-UGT1A6H370A, pPICZ-UGT1A6H370Q, and pPICZ-UGT1A6H485A.

Heterologous Expression in the Yeast *P. pastoris*. Each recombinant pPICZ vector was individually transformed into *P. pastoris* SMD1168 yeast strain (Invitrogen) using the *P. pastoris* Easy-Comp 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 BMGM medium [BMGY 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 (Ouzzine et al., 1999).

Subcellular Fractionation, Enzyme Activity, and Protein Analysis of Recombinant Yeast Cells. Cells were harvested after 48 h of induction, washed once, and suspended in 50 mM sodium phosphate (pH 7.4), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 5% (v/v) glycerol. The cells were broken by vortexing with glass beads. The resulting homogenate was centrifuged at 5,000*g* for 15 min, and the supernatant was further centrifuged for 20 min at 12,000*g*. Microsomes were pelleted after centrifugation of the supernatant at 12,000*g* followed by centrifugation for 60 min at 100,000*g* at 4°C. The microsomal fraction was homogenized with a Dounce B homogenizer in 0.25 M sucrose and 5 mM HEPES buffer (pH 7.4) and used for further protein analysis and enzymatic assays.

Protein concentration was measured by the method of Bradford (1976). SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) and immunoblot analysis were performed using anti-C-terminal peptide anti-UGT1A6 antibodies and alkaline phosphatase-conjugated secondary antibodies (Sigma), as described previously (Pillot et al., 1993b; Ouzzine et al., 1994).

Glucuronidation activity was measured with 4-MU as reporter phenolic substrate according to the method of Lilienblum et al. (1982). Fluorescence measurements of the glucuronide, at excitation and emission wavelengths of 320 and 380 nm, were carried out on a Hitachi F2000 spectrofluorometer (ScienceTec, Les Ullis, France) with authentic 4-MU-β-D-glucuronide (0–10 nmol) as the standard.

The activity and substrate specificity of the recombinant wild-type UGT1A6 expressed in the newly introduced *P. pastoris* expression system of different phenolic substances and steroids were determined according to the method of Bansal and Gessner (1980). Briefly, incubation in Eppendorf tubes (total volume, 40 μl) consisted of 30 μg of microsomal protein in 100 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, and 2.5 M UDP-glucuronic acid (0.2 μCi). The reaction was started by the addition of substrate (1 mM final concentration) in 2 μl of dimethyl sulfoxide. A control experiment was performed in which the substrate was omitted. After incubation for 30 min at 37°C, the proteins were precipitated using 40 μl of ethanol in ice and removed by centrifugation for 10 min at 4000*g* (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 (25%), and water (70:50:18:1.5:60 v/v). They were dried and sprayed with 1% (v/v) 2-(4-*t*-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole in toluene. The radioactivity associated with the glucuronide was visualized by autoradiography with X-Omat Kodak films (Sigma) for 3 days at −20°C. The silica gel areas of the glucuronides were scraped off, and the radioactivity associated was quantified on an LKB spectrophotometer using Fluoran Safe Ultima Gold scintillant cocktail (Packard, Rungis, France).

Determination of Kinetic Parameters. Apparent kinetic constants toward 4-MU were determined by incubating microsomes with increasing concentrations of 4-MU (0.01–2.0 mM) in the presence of a fixed concentration of UDP-glucuronic acid (5.0 mM). The apparent kinetic constants toward UDP-glucuronic acid were obtained using a constant amount of 4-MU (1 mM) in the presence of increasing concentrations of UDP-glucuronic acid (0.025–5.0 mM). The kinetic constants were calculated using linear least-squares regression analysis of the double-reciprocal plots of initial activity versus each substrate concentration.

Chemical Modification by DEPC. Inactivation of wild-type UGT1A6 and active mutants by DEPC was carried out at 25°C with microsomal proteins (100 μg) in 50 mM sodium/potassium phosphate buffer (pH 6.0) and 5 mM $MgCl_2$, as described previously (Battaglia et al., 1994a). This pH value was previously shown to increase the specificity of DEPC toward histidine residues (Miles et al., 1993). Increasing amounts of DEPC (concentration range, 0.1- 0.5 mM) in absolute ethanol [2% (v/v) final concentration] were added. Aliquots were removed after 1 min and quenched by a 10-fold dilution in the buffer containing 5 mM imidazole. A control sample containing 2% (v/v) absolute ethanol, representing 100% activity, was run simultaneously. UGT activity was then measured with 4-MU as indicated above.

TABLE 1

List of primers used for the construction of UGT1A6 mutant cDNAs

Mutant amino acid codons are underlined. S, sense primer; AS, antisense primer.

Primer	Sequence (5′–3′)
UGT1A6A	GAATTCACCATGGCCTGCCTCCTTCGGTCATTTC
UGT1A6B	TCTAGATCAATGGGTCTTGGATTTGTGGGCTTTC
H38A(S)	GTCCCTCAGGACGGAAGCGCTTGGCTTAGTATGAAGGA
H38A(AS)	TCCTTCATACTAAGCCAAGCGCTTCCGTCCTGAGGGAC
H38Q(S)	GTCCCTCAGGACGGAAGCCAGTGGCTTAGTATGAAGGA
H38Q(AS)	TCCTTCATACTAAGCCACTGGCTTCCGTCCTGAGGGAC
H361A(S)	CAAAACGATCTGCTTGCTGCTCCGATGACCCGTGCCTT
H361A(AS)	AAGGCACGGGTCATCGGAGCAGCAAGCAGATCGTTTTG
H370A(S)	ACCCGTGCCTTTATCACCGCTGCTGGTTCCCATGGTGT
H370A(AS)	ACACCATGGGAACCAGCAGCGGTGATAAAGGCACGGGT
H370Q(S)	ACCCGTGCCTTTATCACCCAGGCTGGTTCCCATGGTGT
H370Q(AS)	ACACCATGGGAACCAGCCTGGGTGATAAAGGCACGGGT
H485A(S)	CTCACCTGGTACCAGTACGCTTCCTTGGACGTGATTGG
H485A(AS)	CCAATCACGTCCAAGGAAGCGTACTGGTACCAGGTGAG

Results

Expression and Activity of Wild-Type and Mutant UGT1A6 Expressed in *P. pastoris*. To gain insights into the mechanism underlying the glucuronidation of phenolic compounds supported by UGT1A6, we attempted to identify crucial histidine residues targeted by the modifying reagent DEPC. For this purpose, we developed a novel yeast expression system to probe the importance of conserved histidine residues (Fig. 1) using site-directed mutagenesis. Wild-type UGT1A6 cDNA was subcloned in the pPICZ expression vector and transformed into the methyltrophic yeast *P. pastoris*. As illustrated in Fig. 2A, immunoblot analysis of whole extract of recombinant yeast cells showed that the protein was successfully produced on methanol induction (compare lanes a and b). Subfractionation experiments showed that, as expected, UGT1A6 protein was associated with the membrane fraction of recombinant yeast cells, whereas no polypeptide was detected in the cytosolic fraction (Fig. 2A, lanes c and d, respectively). The substrate specificity of the recombinant

enzyme was evaluated toward a range of phenolic compounds and steroids (Fig. 2B). 4-MU, 1-naphthol, 4-nitrophenol, 4-nitrocatechol, and 4-ethylphenol (lanes a through d and lane f) were glucuronidated at similar high rates, whereas 2-ethylphenol glucuronidation occurred very slowly (lane e), probably because of the steric hindrance exerted by the ethyl group at the *ortho* position of the glucuronidation site (Fig. 2B). By contrast, no glucuronide formation could be detected from the bulky phenol 4-hydroxybiphenyl or steroids tested (lanes g through k). This experiment indicated that the activity of the recombinant UGT1A6 expressed in *P. pastoris* is restricted to planar and short phenols. This substrate specificity was similar to that reported for the recombinant enzyme expressed in mammalian cells (Fournel-Gigleux et al., 1991), thus validating the newly developed yeast expression system.

Inactivation of UGT1A6 by the modifying reagent DEPC coupled with multiple sequence alignment of UGT isoforms enabled the rational design of histidine mutants. The strictly conserved histidine residues His38, His361, His370, and His485 (see Fig. 1) were mutated to alanine and/or glutamine. Immunoblot analysis of membrane fraction of recombinant yeast cells expressing wild-type and UGT1A6 mutants is illustrated in Fig. 3A. As the wild-type protein, the mutants were expressed as a membrane-bound 56-kDa polypeptide. Comparison of the level of expression by scanning densitometry indicated that similar amounts of the wild-type and mutants were produced, except for His38 to alanine (H38A) and His370 to glutamine (H370Q) mutants, which were approximately 50% less expressed (see Fig. 3A). Furthermore, the consequences of the mutations on the functional activity of UGT1A6 were investigated, and the results appear in Fig. 3B. Replacement of His38 by alanine or glutamine (H38A, H38Q) and His485 by alanine (H485A) resulted in a complete loss of activity, whereas substitution of His361 by alanine (H361A) reduced the glucuronidation activity by 56%. When the activity of the H361A mutant was normalized by the protein expression level, the actual decrease dropped to 42%. Furthermore, when His370 was replaced by alanine (H370A), the activity of the mutant was dramatically reduced to approximately 95% of that of the

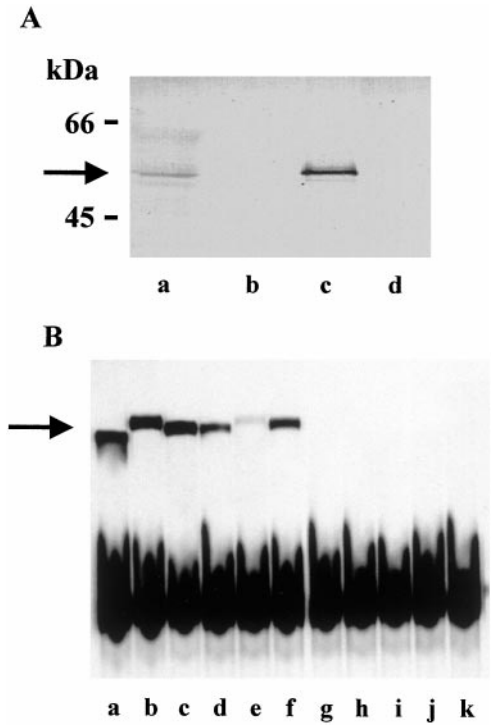

Fig. 2. Heterologous expression of UGT1A6 in *P. pastoris.* A, subcellular localization of wild-type UGT1A6 expressed in *P. pastoris*.: a, whole cell extract from recombinant yeast cells expressing UGT1A6 on methanol induction; b, whole cell extract from recombinant noninduced yeast cells; c, membrane fraction; d, cytosolic fraction from recombinant methanol-induced yeast cells. Protein (20 μg) was loaded on each lane and analyzed using SDS-PAGE and immunoblotting as described under *Materials and Methods*. B, thin-layer chromatographic analysis of the substrate specificity of recombinant UGT1A6 expressed in *P. pastoris*. The catalytic activity of UGT1A6 was analyzed toward various substrates: a, 4-MU; b, 1-naphthol; c, 4-nitrophenol; d, 4-nitrocatechol; e, 2-ethylphenol; f, 4-ethylphenol; g, 4-hydroxybiphenyl; h, α-estradiol; i, 17α-ethinylestradiol; j, testosterone in the presence of radiolabeled UDP-glucuronic acid and separated by thin-layer chromatography as described under *Materials and Methods*. Lane k corresponds to a control experiment performed in the absence of aglycone substrate. The arrow indicates the position of the glucuronides.

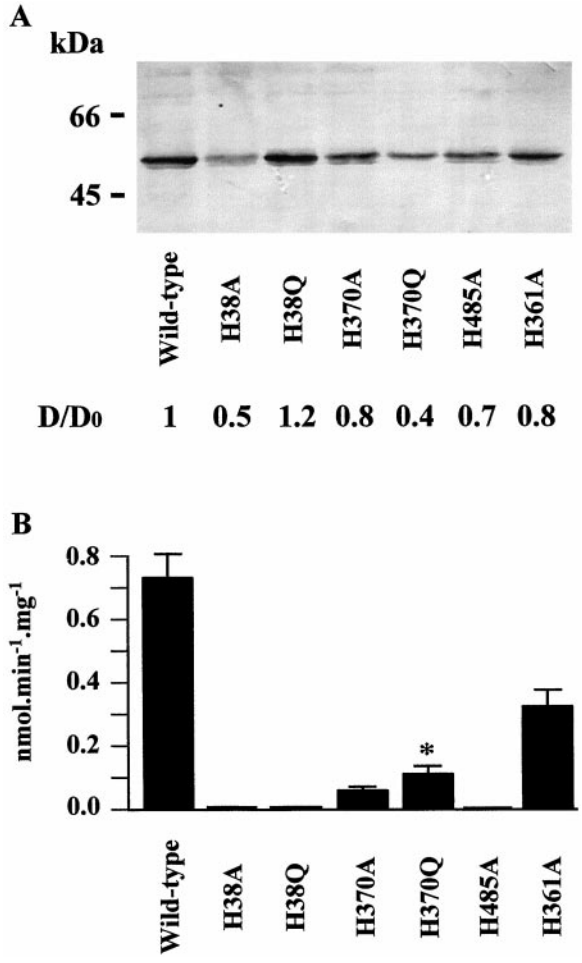

Fig. 3. Expression and activity of wild-type and mutant UGT1A6 expressed in *P. pastoris.* A, SDS-PAGE and immunoblot analysis. Membrane fraction from recombinant yeast cells (20 μg) was loaded on each lane. Evaluation of the relative expression of mutants compared with wild-type protein was performed by scanning densitometry using National Institutes of Health software and given as densitometry ratio mutant/wild-type (D/D0). B, glucuronidation activity toward 4-MU. Activity was determined on yeast microsomes using 1 mM 4-MU, 5 mM UDP-glucuronic acid, and 20 μg of protein in 180 mM Tris-HCl buffer (pH 7.4) and 28 mM $MgCl_2$ for 30 min at 37°C. Values are the mean ± S. D. of three independent determinations. *Significantly different ($P < .05$) from activity value obtained with the H370A mutant.

wild-type enzyme. Interestingly, substitution of His370 residue by glutamine (H370Q) significantly restored the activity compared with H370A, because the activity of this mutant reached approximately 40% of that of the wild-type protein when the activity was normalized by the protein expression level.

Kinetic Analysis and Inactivation of Mutants by DEPC. Because His38 and His485 mutants were inactive, it was not possible to assess the effects of these mutations on kinetic parameters or inhibition by DEPC. In contrast, the active mutants H361A, H370A, and H370Q as well as the wild-type UGT1A6 were characterized kinetically, and their sensitivity to the histidine-specific reagent was investigated. The apparent kinetic constants K_m and V_{max} and the corresponding catalytic efficiency V_{max}/K_m were determined toward each of the two substrates of the enzyme, i.e., UDP-glucuronic acid and 4-MU. Kinetic analysis of the His361 and His370 mutants indicated the importance of these histidine residues on the enzyme function. The H361A mutant exhibited an approximately 4-fold increase in the K_m value for UDP-glucuronic acid, whereas the K_m value for the aglycone substrate 4-MU was slightly modified compared with the wild-type enzyme (Table 2), indicating that the mutation primarily affects the interaction of the enzyme with its cosubstrate. The lower affinity of the mutant toward UDP-glucuronic acid together with a 2-fold reduction in V_{max} value resulted in a strong impairment of the catalytic efficiency. Investigation of the sensitivity of H361A to DEPC revealed that the profile of inhibition of the mutant was similar that of the wild-type enzyme. In both cases, DEPC inhibition was time-dependent (data not shown) and occurred in a dose-dependent manner (Fig. 4) with kinetics typical of an irreversible inhibition. This result strongly suggested that the H361A mutant still contains a reactive histidine residue and that His361 was not the target of DEPC.

In contrast, mutation of His370 had distinct pronounced effects on V_{max}. For H370A, the V_{max} value dramatically dropped up to 20-fold compared with that of the wild-type with no significant change in K_m value for both substrates (Table 2). Interestingly, when His370 was converted to glutamine, a strong increase in V_{max}, compared with the H370A mutant, was observed, reaching approximately 40% of the value determined for the wild-type enzyme when the activity was normalized by protein expression level. This result indicates that glutamine, in contrast to alanine, could sustain the catalytic activity of the enzyme (Table 2). Moreover, DEPC produced only a weak effect on H370A and H370Q mutants compared with the wild-type (Fig. 4), indicating that the critical histidine residue was no longer present to be derivatized. Altogether, these results support a crucial role of His370 in the catalytic mechanism of UGT1A6.

TABLE 2

Kinetic properties of wild-type UGT1A6 and site-directed mutants toward the substrates UDP-glucuronic acid and 4-MU

Apparent kinetic constants of the wild-type and mutant enzymes were determined in membrane fraction of recombinant yeast cells toward UDP-glucuronic acid by varying its concentration (0.025–5.0 mM) while that of 4-MU was kept constant (1.0 mM). Similarly, the apparent kinetic constants toward 4-MU were calculated by varying its concentration (0.01–2.0 mM) for a fixed concentration in UDP-glucuronic acid (5.0 mM). K_m, V_{max}, and the catalytic efficiency V_{max}/K_m were expressed as micromolar concentration, nanomoles per minute per milligram of protein, and liters per minute per milligram of protein, respectively.

Enzyme	UDP-Glucuronic Acid			4-MU		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
Wild-type	110.2	0.76	6.9	168.7	0.81	4.8
H361A	407.8	0.42	1.0	274.9	0.37	1.3
H370A	206.0	0.04	0.2	114.9	0.05	0.4
H370Q	60.0	0.10	1.6	130.0	0.13	1.0

Discussion

The identification of amino acids essential for catalysis or that contribute to the activity through conformational effects is a major issue in the understanding of the structure and function of proteins. In the absence of tridimensional structure, the strategy to detect such residues relies on a combination of site-directed mutagenesis and chemical modification studies. In this work, the functional role of the four invariant histidine residues of UGT1A6 was investigated. Histidine was suspected to play a key role in the structure and function of this isoform from its susceptibility to be inactivated by DEPC (Battaglia et al., 1994a). On the other hand, it has been proposed that the reaction mechanism accounting for the glucuronidation of phenols would involve a nucleophilic attack of the phenol on the C_1 atom of glucuronic acid, leading to β-D-glucuronide formation and the release of UDP, according to an SN_2 mechanism. In that respect, histidine, in combination with aspartate/glutamate, would facilitate this process by increasing the nucleophilicity of the phenolic acceptor substrate. Mutation of strictly conserved histidines of UGT1A6 by nonconservative and conservative substitutions and subsequent determination of the sensitivity of the mutants to DEPC were performed. Using this approach, we previously investigated the potential role of H54, which belongs to a strictly conserved ^{52}RGHE/D^{55} sequence located on a hydrophobic domain of the variable N-

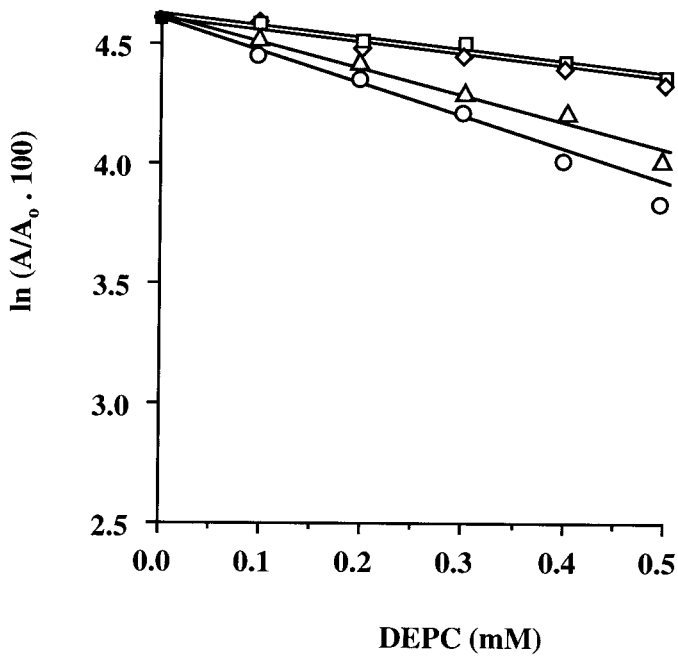

Fig. 4. Inactivation of recombinant wild-type and mutant UGT1A6 by DEPC. Inactivation was carried out by incubating microsomes (100 μg of protein) of recombinant yeast cells with increasing concentrations of DEPC (0.1–0.5 mM) in 50 mM phosphate buffer (pH 6.0) and 5 mM $MgCl_2$ at 25°C. Inactivation was quenched after 1 min by 5 mM imidazole, and the ratio of activity A/A_o was determined. □, H370A; ◇, H370Q; ▲, wild-type; ○, H361A.

terminal portion of the protein. Mutation of H54 into alanine and glutamine revealed that although important, H54 did not play any catalytic role because the mutants were still inhibited by DEPC as the wild-type enzyme (Senay et al., 1997). In this study, further identification of the nucleophilic catalyst highly reactive toward DEPC was performed using site-directed mutagenesis. Each of the four (of five) conserved histidine residues was converted to alanine and, for H38 and H370 to glutamine as well; the mutant proteins were stably expressed in a newly developed *P. pastoris* expression system. Replacement of His38 by alanine or its substitution by a conservative residue glutamine led in either case to inactive enzymes. These results suggest that this residue did not play a catalytic role because the nitrogens with unpaired electrons of glutamine side chain were unable to promote glucuronidation of 4-MU. His38 is located on the N-terminal domain oriented toward the luminal side of the endoplasmic reticulum. This portion is believed to be part of the aglycon binding site (Mackenzie, 1990). Thus, the involvement of His38 in the structural determinants governing the organization of the substrate binding site could account for the deleterious effects of this mutation. It is noteworthy that His38 is localized in a strictly conserved α-helix as predicted by secondary structure analysis, thus emphasizing a possible role in maintaining an active conformation of the enzyme.

On the other hand, His485 was also found critical for enzyme function because mutation of this histidine residue to alanine totally abolished enzyme activity. This residue belongs to a strictly conserved, highly charged stretch of 10 amino acids located in the vicinity of the N-terminal side of the transmembrane segment of UGTs that is expected to be important in positioning the transmembrane domain. Consistent with our results, this motif 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 (Li et al., 1997). Altogether, two lines of evidence argue against a catalytic role for His38 and His485. First, kinetic studies by Battaglia et al. (1994a) with DEPC indicate that inactivation involves only one histidine residue. Second, the resistance exhibited by His370 mutants in which His38 and His485 are not mutated indicates that this position confers most of the DEPC sensitivity to this enzyme. In view of these results, a critical structural role for His38 and His485 seems more likely.

The replacement of His361 by Ala reduced the glucuronidation rate of 4-MU. Kinetic analysis revealed a 4-fold increase in the apparent K_m value toward UDP-glucuronic acid, thus suggesting that the mutation decreased the affinity of the enzyme for the cosubstrate. His361 is located on a consensus sequence found on all 110 members of the UGT superfamily (Mackenzie et al., 1997). This signature sequence is believed to correspond to a series of residues that would interact with UDP-glucuronic acid, the common cosubstrate of all these isoforms. On the other hand, we demonstrated by photoaffinity labeling with azanucleotide analogs of UDP-glucuronic acid ([β-^{32}P]5N_3UDP-glucuronic acid and [β-^{32}P]5N_3UDP-glucose) that a specific UDP-binding site was located between amino acids 299 and 446 of UGT2B4 (Pillot et al., 1993b). Our findings are consistent with His361 being important for the efficient interaction of UGT1A6 with UDP-glucuronic acid.

The histidyl-selective reagent DEPC has been widely used to demonstrate the importance of histidine residues in the structure and function of proteins. In this study, we suggest that H370 is the catalytic residue reacting with DEPC. This conclusion is drawn from three lines of evidence. First, the catalytic efficiency (V_{max}/K_m) of H370A was severely depressed (35-fold) by comparison with that of the wild-type UGT1A6. Second, conservative substitution of H370 by glutamine indicated that glutamine could replace histidine to some extent and lead to a mutant (H370Q) that presented a higher glucuronidation rate by comparison with H370A. Finally, both mutants were much less sensitive to the inhibitory effect of DEPC, even at high concentrations. Furthermore, that His370 is catalytically important is substantiated by the position of this residue in the consensus sequence predicted to interact with UDP-glucuronic acid (Mackenzie et al., 1997) (Radominska-Pandya et al., 1999). Nevertheless, H370A and H370Q mutants exhibited similar apparent affinity constants toward UDP-glucuronic acid compared with the wild-type enzyme, ruling out a direct involvement of His370 in the cosubstrate binding site. The essential role of histidine residues in the chemical mechanism of the reaction catalyzed by several classes of enzymes has been largely emphasized. For example, a histidine residue of D-lactate dehydrogenases acts as an acid/base catalyst donating a proton to the substrate carbonyl or accepting a proton from the substrate hydroxyl in the reverse reaction (Kochlar et al., 2000). For several enzymes, such as proteases (Fersht and Sperling, 1973), esterases (DiPersio et al., 1991), and ribonuclease A (Quirk et al., 1998), the acid/base mechanism is promoted by an interaction between histidine and at least one accessory amino acid residue such as aspartic acid. Because UGT1A6 was sensitive to the carboxyl-directed reagents carbodiimide and *N*-ethyl-5-phenylisoxazolium-3′-sulfonate, we also postulated the implication of an aspartate or a glutamate in the catalytic mechanism of the glucuronidation reaction (Battaglia et al., 1994b). Altogether, the involvement of the prototropic groups histidine and aspartate or glutamate in catalysis is consistent with a general acid/base mechanism via a charge-relay system. The conserved Asp446 of rat UGT1A6 (Asp447 in human) was a potential candidate for acting in conjunction with histidine in the catalysis. However, investigation of the role of this residue using site-directed mutagenesis showed that Asp446, although important for a functional conformation of the enzyme, was not directly involved in catalysis (Iwano et al., 1999). Therefore, the identification of a putative crucial aspartic or glutamic acid residue awaits further investigation.

In conclusion, we have systematically altered the four invariant histidine residues in human UGT1A6 using site-directed mutagenesis. In agreement with the chemical modification data, we demonstrate that His370 is essential for catalysis. Furthermore, our results highlight the crucial role of His38 and His485 in maintaining an active conformation of the enzyme. Finally, we provide evidence that His361 is likely to be involved in the UDP-glucuronic acid binding site.

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