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The chemical modification of human liver UDP-glucuronosyltransferase UGT1*6 reveals the involvement of a carboxyl group in catalysis

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Abstract

The treatment of UDP-glucuronosyltransferase UGT1*6 stably expressed in V79 cells with three carboxyl-specific reagents, dicyclohexylcarbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K), resulted in a fast, dose-dependent decrease of the 4-methylumbelliferone glucuronidation. The inactivation reactions followed pseudo-first order kinetics. The pK_a of the modified residue was close to 5.0. A partial protection against inactivation by Woodward's reagent was observed at pH 7.4 in the presence of UDP-glucuronic acid, UDP, and, to a lesser extent, in the presence of 4-methylumbelliferone. Dicyclohexylcarbodiimide significantly decreased the V_{max}, without affecting the apparent K_m towards UDP-glucuronic acid and 4-methylumbelliferone. The results support the involvement of a carboxyl group in the catalytic process.

Key words: UDP-glucuronosyltransferase; Human liver, Chemical modification; Carboxyl-specific reagent; Essential residue

1. Introduction

UDP-glucuronosyltransferases (EC 2.4.1.17; UGT) represent a superfamily of enzymes which catalyze the conjugation of glucuronic acid from UDP-glucuronic acid (UDP-GlcU) to a variety of structurally unrelated compounds which possess a hydroxyl, carboxyl, sulfhydryl or amino group [1]. Drugs or endogenous compounds that are important for cell differentiation and growth (e.g. steroid and thyroid hormones, fatty acids, retinoic acid) are substrates of these enzymes [2,3]. The glucuronides formed are generally inactive metabolites and are excreted into urine or bile [4]. However, some glucuronides, such as the acylglucuronides formed from carboxylic acid-containing drugs or *N*-hydroxyglucuronides from arylamines are known for their unstability and reactivity, responsible for toxicity reactions in man [5,6]. In order to elucidate the molecular basis of the formation of the glucuronides, we have expressed the human liver UGT1*6[1] isoform in recombinant V79 cells [7]. This enzyme is involved in the glucuronidation of planar and short phenolic substances (such as 4-methylumbelliferone, 4-MU), including the drug paracetamol [8–10]. In an attempt to investigate the key amino acids which play a role in catalysis, we recently demonstrated, by chemical modification of the protein with diethylpyrocarbonate, the involvement of an histidine residue in the glucuronidation process [11]. Moreover, from the pH profile of the V_{max}/K_m data of the enzyme, a possible implication of a carboxylic acid in catalysis was postulated. In the present study, such hypothesis was evaluated by the mean of three carboxyl-specific reagents. Our results clearly support the participation of such a residue in the catalytic process of UGT1*6.

2. Materials and methods

Dicyclohexylcarbodiimide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide were purchased from Aldrich (Saint-Quentin-Fallavier, France); *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K, WK reagent) and UDP were obtained from Sigma (St. Louis, MO). UDP-GlcU was from Boehringer (Mannheim, Germany). All other chemicals were of the best purity commercially availble.

2.1. Cell cultures and preparation of the membrane fractions

Establishment and culture of V79 cells expressing UGT1*6 was performed as previously described [7,11]. Two other V79 cell lines expressing the UGT2B1 and UGT1*02 isoforms were also used. The rat liver UGT2B1 catalyzes the glucuronidation of morphine and carboxylic acids (fatty acids, nonsteroidal anti-inflammatory drugs) [2]; the human liver UGT1*02 glucuronidates bulky phenols [9]. The enriched membrane fractions were obtained from the cell homogenate by differential ultracentrifugation. The homogenate, obtained by a 3 × 10-s sonication (Vibra cell 72446, Bioblock, Illkirch, France) of 100 mg of cells protein resuspended in 60 ml of 0.25 M sucrose, 5 mM HEPES (pH 7.4), was centrifuged at 10,000 × *g* for 10 min. The supernatant was then centrifuged at 100,000 × *g* for 60 min. The resulting pellet was homogenized in 0.25 M sucrose, 5 mM HEPES (pH 7.4) and stored at −20°C. Under these conditions, a fully active UGT was associated to this membrane

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Abbreviations: UGT, UDP-glucuronosyltransferase; UDP-GlcU, UDP- glucuronic acid; 4-MU, 4-methylumbelliferone; DCC, dicyclohexylcarbodiimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; WK reagent, *N*-ethyl-5-phenylisoxazolium-3′-sulfonate; HPLC, high- performance liquid chromatography.

[1] UGT1*6 and UGT1*02 are encoded by two members of family 1 of the UGT gene superfamily [1]. The asterisk indicates that these isoforms are derived by alternative splicing from a primary transcript common to several isoforms encoded by the *UGT1* locus. UGT2B1 is encoded by a gene belonging the subfamily 2B [1].

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fraction. Attempts to further purify this phospholipid-dependent protein [12,13] lead to enzyme inactivation. Protein concentration was measured according to Bradford [14], with bovine serum albumin as standard 40–60% of the total enzymatic activity was recovered in the final membrane fraction. The enrichment in term of specific activity was about 1.3-fold, compared to the starting cell homogenate.

2.2. Inactivation of UGTs by carboxyl-specific reagents

The membrane fractions were adjusted to 1.3 mg protein/ml with 50 mM sodium/potassium phosphate buffer (pH 5.0), 5 mM $MgCl_2$, unless specified. Dilution of carboxyl-specific reagents were prepared just before use. The WK reagent was diluted in 1 mM HCl. The carbodiimide derivatives were prepared in dimethyl sulfoxide. Solvents (2% v/v for experiments with WK reagent and 5% v/v for experiments with carbodiimide derivatives) were shown not to affect the glucuronidation activity. Inactivation experiments, including protection by substrates, were performed at 20°C as follows: inactivation was initiated by addition of the carboxyl-specific reagents at concentrations ranging from 0.1 mM to 5.0 mM (depending on the compound used). A control experiment performed with the solvent alone was run simultaneously and corresponded to 100 percent of activity. The effect of ligands on the inactivation of UGT1*6 by 2 mM WK reagent was performed at concentrations corresponding to 10 apparent K_m (4-MU) (2.5 mM), 10 apparent K_m (UDP-GlcU) (2.1 mM), and 10 apparent K_i (UDP) (5.9 mM), at pH 7.4. The chemical modification was stopped at various periods of time by a 60-fold dilution of the proteins in 180 mM Tris-HCl (pH 7.4), 28 mM $MgCl_2$, 5 mM glutamic acid (buffer 1) and the glucuronidation activity was determined by a fluorimetric assay with 4-MU as substrate; for this purpose, the membrane fractions (3 μg protein) were incubated with 4-MU (1 mM) and UDP-GlcU (5 mM) for 25 min at 37°C [11], and the reaction was stopped by the addition of 800 μl of ice-cold water. Unconjugated 4-MU was then extracted by 4 ml of ethyl acetate. Samples were centrifuged and 200 μl of the aqueous phase was mixed with 1.8 ml of 0.25 M glycine-NaOH buffer (pH 10.3). The fluorescence measurements were performed on a Hitachi F2000 spectrofluorimeter (ScienceTec, Les Ulis, France) at excitation and emission wavelengths of 320 and 380 nm, respectively, with authentic 4-methylumbelliferyl-β-D-glucuronide (Sigma) as standard. No addition of detergent was required for the measurement of the activity since the sonication procedure provided a mecanical activation of the membrane-bound UGT resulting in full activation. The inactivation kinetics of UGT1*6 by carboxyl-specific reagents were fitted to equation I.

$$\log (\%\ \text{residual activity}) = -k'_i \cdot t \quad \text{(I)}$$

where k_i is the pseudo-first order inactivation rate constant and t the time of inactivation for which the residual activity is evaluated. The inactivation order (n) was calculated according to equation II

$$\log k_i = n \cdot \log [\text{inactivator}] + \log k'_i \quad \text{(II)}$$

with k'_i the second-order inactivation constant. The pK_a of the essential residue modified by WK reagent was evaluated following the equation III, while that of the residue modified by DCC was determined according to the equation IV

$$1/k_i = [H^+]/k_m \cdot K_a + 1/k_m \quad \text{(III)}$$

$$1/k_i = K_a/[H^+] \cdot k_m + 1/k_m \quad \text{(IV)}$$

where k_m is the maximal pseudo-first-order inactivation rate constant and K_a the ionization constant of the modified residue. Inactivation experiments with increasing concentrations of the nucleophilic compounds glycine methyl ester and glycine ethyl ester (5–50 mM) were carried out with membrane fractions (1.3 mg proteins/ml) in 50 mM sodium/potassium phosphate (pH 5.0), 5 mM $MgCl_2$. UGT1*6 inactivation was performed with DCC at 1 mM for 1 min following the addition of glycine methyl ester or glycine ethyl ester, and compared to that observed without the presence of both exogenous nucleophiles.

2.3. Treatment of UDP-GlcU with WK reagent

UDP-GlcU (2 mM) was added either to 2 mM WK reagent (2% v/v in 1 mM HCl) or to 1 mM HCl (control) for 3 min in the conditions described for the protection experiments, but without membrane fractions. 50 μl of the incubation medium was injected on a Hibar Lichrosorb RP-18 HPLC column (7 μm) (Merck, Darmstadt, Germany) following a 60-fold dilution in buffer 1. The mobile phase was composed of 50 mM ammonium phosphate/phosphoric acid (pH 3.0) with 2.5% methanol (v/v). The retention time of UDP-GlcU was 16.5 min, upon detection at 260 nm with a constant flow rate of 0.4 ml/min.

3. Results

*3.1. Effect of carboxyl-specific reagents on the UGT1*6 activity*

The glucuronidation of 4-MU catalysed by UGT1*6 was rapidly decreased as a function of time and concen-

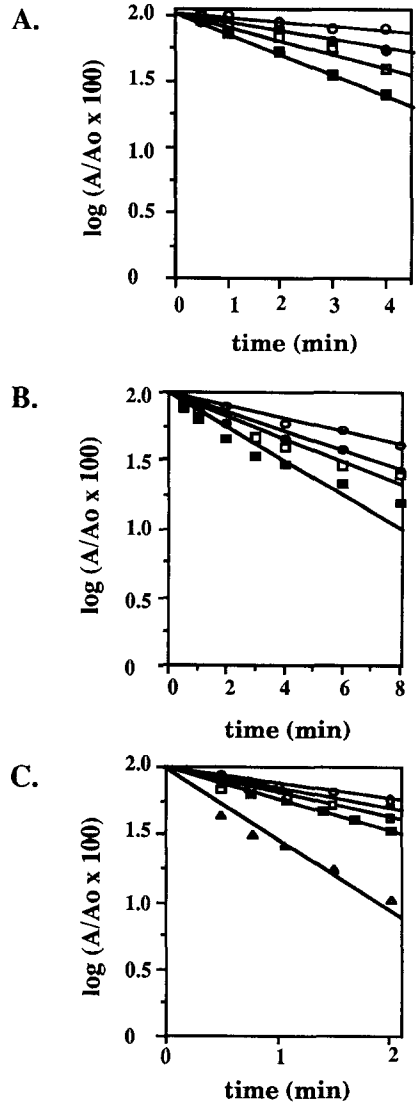

Fig. 1. Inactivation of UGT1*6 enzyme by carboxyl-specific reagents. Membrane fractions (1.3 mg proteins/ml) of the recombinant V79 cells expressing UGT1*6 were incubated at 20°C in 50 mM sodium/potassium phosphate buffer, 5 mM $MgCl_2$ with inactivators at pH 5.0 (DCC and EDC) and pH 6.0 (WK reagent). (A) Inactivation by DCC (○), 0.1 mM; (●), 0.3 mM; (□), 0.4 mM; (■), 0.7 mM. (B) Inactivation by EDC (○), 2 mM; (●), 4 mM; (□), 5 mM; (■), 8 mM. (C) Inactivation by WK reagent (○), 1.0 mM; (●), 1.5 mM; (□)), 2.0 mM; (■), 3.0 mM; (△), 5.0 mM. A/A_o corresponds to the ratio of the activity in the presence of inhibitor, at the time point considered, vs. the initial activity without inhibitor.

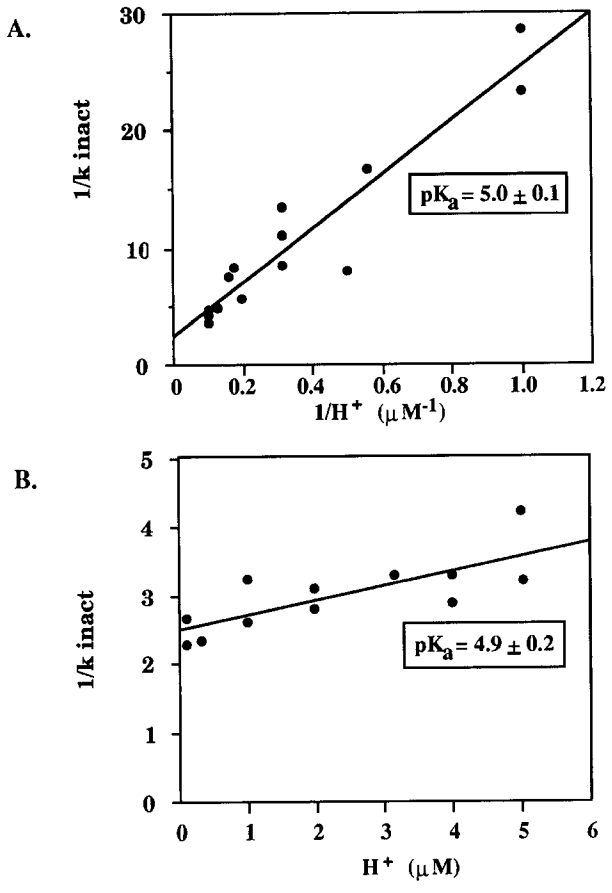

Fig. 2. pH dependence of the inactivation of UGT1*6 by WK reagent and DCC. The membrane fractions were diluted to 1.3 mg proteins/ml in 50 mM sodium/potassium phosphate buffer (pH 5.0–7.0), 5 mM $MgCl_2$. Inactivation was performed with 0.5 mM DCC (A) and 2.5 mM WK reagent (B) at the indicated pHs.

tration of DCC, EDC and WK reagent. Plotting on a semi-logarithmic scale the residual activity as a function of the time of inactivation showed a time and dose dependent inactivation of the enzyme by DCC, EDC and WK reagent (Fig. 1) which indicated that the inactivation of the enzyme by each of the reagents followed a pseudo-first-order kinetic. The reaction order (n) was determined from the plots of logarithm of the apparent inactivation constants versus the logarithm of the concentrations of inactivators (not shown). This allowed the determination of values of n close to 1 whatever the inactivation reagent with respect to 4-MU glucuronidation (Table 1). The data indicated the presence of one residue critical for enzyme activity. The second order inactivation rate constant k'_i was evaluated from the same representation. This constant value for DCC was 1200-fold higher than that for EDC (Table 1). The pK_a of the residue modified by DCC, which reacts with protonated residues [15] or by WK reagent, which reacts with unprotonated residues [16] were both close to 5.0 (Fig. 2). Moreover, the simultaneous addition of the exogenous nucleophile glycine methyl ester or glycine ethyl ester (0–50 mM) with DCC (1.0 mM) did not modify the inhibition of UGT1*6 (not shown). A partial inactivation of UGT1*6 by DCC did not significantly affect the apparent K_m toward 4-MU and UDP-GlcU, whereas the apparent V_{max} was decreased more than 3-fold under these conditions (Table 2).

3.2. Protection against inactivation by the WK reagent

The influence of UDP-GlcU and 4-MU on the inactivation of UGT1*6 was evaluated with the WK reagent only, because it reacts with unprotonated carboxyl group [16] and thus allowed to perform the experiment at the optimal pH (7.4) of 4-MU glucuronidation [11]. We first evaluated the possible reactivity of UDP-GlcU with the WK reagent since the cofactor possesses a carboxyl group which is unprotonated at the optimum pH of the glucuronidation reaction [11,17]. For this purpose we incubated UDP-GlcU with WK reagent in conditions similar to those used for the protection experiment, and then submitted the reaction products to HPLC separation. The retention time, of UDP-GlcU alone or treated by 2 mM WK reagent for 3 min, the relative area of the corresponding peaks and the elution profile were identical (results not shown). Therefore any possible reactivity of the co-substrate with the WK reagent could be excluded in the experimental conditions used. Protection experiments were performed in the presence of UDP-GlcU with the WK reagent at pH 7.4. A partial protection against inactivation was observed in the presence of UDP-GlcU and, to a lesser extent, in the presence of 4-MU for concentrations of UDP-GlcU and 4-MU of about 10-fold the apparent K_m (Fig. 3). Incubation of UDP (5.9 mM, 10-fold the apparent K_i) with the membrane fractions led to a complete protection against inactivation by the WK reagent (Fig. 3).

3.3. Influence of DCC on various recombinant UGTs

Membrane fractions from recombinant V79 cells expressing UGT2B1 [2] and UGT1*02 [9] isoforms were treated with DCC and the inhibitory effect of this compound was compared to that observed with the UGT1*6 enzyme. Both enzymes were inhibited by DCC, although to different extents (Table 3), and the inhibition ranked as follows: UGT1*6 > UGT1*02 >> UGT2B1.

Table 1
Kinetic parameters for the inactivation of UGT1*6 by carboxyl specific reagents

	Carboxyl specific reagents		
	DCC[a]	EDC[a]	WK reagent[b]
n	1.3	0.69	0.97
k'_i ($M^{-1} \cdot min^{-1}$)	2157	1.8	69.3

[a] Inactivation performed at pH 5.0.
[b] Inactivation performed at pH 6.0.

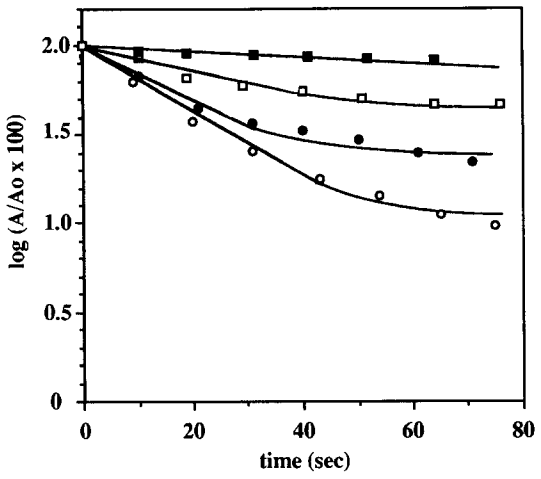

Fig. 3. Protection of UGT1*6 against inactivation by WK reagent. The inactivation was performed in the presence of WK reagent (2 mM) as described in Fig. 1 but at pH 7.4. 4-MU (2.5 mM), UDP-GlcU (2.1 mM) or UDP (5.9 mM) was incubated for 5 min with the membrane fractions before addition of WK reagent. (○), WK reagent; (●), WK reagent plus 4-MU; (□) WK reagent plus UDP-GlcU; (■) WK reagent plus UDP. A/A_0 corresponds to the ratio of the activity in the presence of inhibitor, at the time point considered, vs. the initial activity without inhibitor.

4. Discussion

In this study, three carboxyl-specific reagents (DCC, EDC, WK reagent) have been used to demonstrate the essential role of a carboxyl group in the catalytic function of UGT1*6. Carbodiimide derivatives (DCC, EDC) were chosen for their reaction specificity towards protonated carboxyl groups of proteins [15], and in order to gain insight on the hydrophobicity of the environment of the modified residue within the active site of the enzyme. WK reagent was chosen to modify unprotonated carboxyl group [16], and thus was convenient to perform inactivation experiments in the presence of substrate and cofactor at optimum pH of the glucuronidation reaction (7.4) [11]. This characterization was supported by several conclusive findings. We observed that incubation of membrane fractions of V79 cells expressing UGT1*6 resulted in a time and concentration dependent inhibition of the glucuronidation of 4-MU catalyzed by this isoform of UGT. The inactivation order was close to one for all the reagents and thus suggests that the decrease in activity was dependent on the modification of one single essential residue. The second order inactivation rate constant for the hydrophobic DCC which was by far higher than that measured with the water-soluble reagent EDC, likely reflects the presence of a hydrophobic environment around the essential carboxyl group. UGTs are membrane-bound enzymes [18–20], and this might also be the reason for the lower inactivation rate with EDC, when compared to DCC. We have also evaluated the pK_a of the modified residue, which was close to 5.0, as revealed from inactivation with DCC or WK reagent as a function of pH. This value is in the range of pK_a of carboxyl groups in proteins. This result is also in accordance with the V_{max}/K_m vs. pH plots previously determined [11] which suggests the essential contribution of a second unprotonated residue at optimum (pH 7.4), in combination with the imidazole group of a histidine residue. In order to discriminate between the actual involvement of a carboxyl group in catalysis and a possible internal cross-linking, the influence of an exogenous nucleophile, glycine methyl ester, on the inactivation of UGT1*6 by DCC was evaluated. Pedemonte and Kaplan [21] have described the reaction mechanism of the modification of carboxyl groups of enzymes with a carbodiimide. A protonated carboxyl group is first modified by DCC to give the unstable *O*-acylurea, which leads either to a rearrangement into a stable *N*-acylurea group or to a reaction with a nucleophile (amino group) resulting in the formation of an amide linkage. In this latter case, if the amino group belongs to the enzyme, the amide formation results in a cross-linking within the active site. It is not possible to distinguish between an inhibition due to a modification of the protein conformation upon the formation of this cross-linking, or an inhibition due to the modification of the endogenous nucleophile responsible for the cross-linking. In this work, the lack of effect of increasing concentrations of glycine methyl ester or glycine ethyl ester on the inactivation of UGT1*6 by DCC excluded competition between the exogenous nucleophile and a nucleophile of the active site. This supports a direct role of a carboxyl group in catalysis. A protection by UDP-GlcU and, to a lesser extent, by 4-MU against inactivation of UGT1*6 by WK reagent was observed at the optimum pH of the enzyme (pH 7.4). This result suggests that the essential residue may be located within or near the active site. This is also supported by the protective effect exerted by UDP, a competitive inhibitor towards UDP-GlcUA binding. Moreover, a partial inactivation of the enzyme by DCC led to a decrease of the V_{max} without affecting the apparent K_m toward 4-MU and UDP-GlcU, thus suggesting that the carboxyl group modified by DCC was not in-

Table 2
Kinetic parameters of native and DCC-treated UGT1*6

UGT1*6	App. K_m (mM)		App. V_{max} (nmol/min × mg)
	UDP-GlcU	4-MU	
Native	0.208	0.242	71
DCC-modified	0.195	0.299	19

Membrane fractions (1.3 mg protein/ml) were treated with 0.4 mM DCC at pH 5.0 and 20°C for 3 min. The kinetic parameters were then evaluated in the presence of various UDP-GlcU concentrations (0.05–5 mM) for a constant concentration of 4-MU (1 mM); and by varying the concentration of 4-MU (0.05–1 mM) at a constant concentration of 5 mM UDP-GlcU. Apparent K_m were determined from the Lineweaver–Burk plot of the data.

Table 3
Effect of DCC on various recombinant UGT isoforms stably expressed in V79 cells

DCC (mM)	% inactivation		
	UGT1*6	UGT1*02	UGT2B1
0.6[a]	78.3 ± 0.4	53.8 ± 0.8	4.3 ± 0.3
5.0[a]	81.2 ± 1.0	71.0 ± 5.6	6.8 ± 0.2
20.0[b]	90.3 ± 0.1	82.2 ± 2.8	28.6 ± 1.7

Membrane fractions of recombinant cells (1.3 mg protein/ml) were incubated at the indicated concentrations of DCC at pH 5.0 and 20° C. The percentage of inhibition of 4-MU glucuronidation were then evaluated (mean ± S.D.).
The initial values for 4-MU glucuronidation of UGT1*6, UGT1*02 and UGT2B1 were: 63.4 ± 5.0; 5.9 ± 0.2 and 3.9 ± 0.4 (nmol/min × mg protein), respectively.
[a] Inactivation performed for 5 min.
[a] Inactivation performed for 10 min.

volved in the binding of these ligands but in the catalytic process [22]. Finally, DCC could inhibit three recombinant UGTs, although to a different extent, indicating that the reactive carboxyl residue is likely conserved among the UGT superfamily. The sensitivity of the glucuronidation activity toward DCC varied depending on the isoform considered; UGT1*6 and UGT1*02, which belong to the UGT1 family [1] and are from human origin were more inhibited by DCC than the rat liver UGT2B1 of the UGT2 family. This could reflect a change in the reactivity or in the accessibility of the carboxyl group, because of the low sequence homology between the two groups of proteins.

The involvement of prototropic groups histidine [11] and aspartate or glutamate supports a general acid/base catalysis. These residues are unprotonated at optimum pH (7.4) of the glucuronidation reaction. The base catalyst, which is likely to be involved in the deprotonation of aglycones in the catalytic process could be either the histidine or the aspartate or glutamate residue identified in this study. A neutral imidazole group (histidine) could however be an acid catalyst although the imidazole/imidazolate pair is rather unusual in enzymatic mechanisms compared to the imidazolium/imidazole pair [23]. The second and more likely mechanism would involved the histidine and the aspartate or glutamate residues in which one of these residues is the base catalyst while the second is orienting and polarizing the base catalyst. Such a mechanism (charge-relay system) has been proposed for the reaction catalysed by DNA gyrase [24] and chymotrypsin [25].

Work is in progress to further identify this residue which is involved in the glucuronidation process of 4-MU supported by UGT1*6.

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