Activation of Glucuronidation through Reduction of a Disulfide Bond in Rat UDP-glucuronosyltransferase 1A6

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ABSTRACT: UDP-glucuronosyltransferase- (UGT-) dependent glucuronidation is an important detoxification process for many endogenous and exogenous compounds in mammals. Treatment of rat hepatic microsomes with the reducing reagent dithiothreitol (DTT) resulted in a significant increase in *p*-nitrophenol (*p*-NP) glucuronidation in a time- and concentration-dependent manner. The DTT-dependent activation of glucuronidation was specific for planar phenols but not for bilirubin or testosterone without membrane perturbation of the microsomes. *p*-NP glucuronidation in Gunn rat hepatic microsomes lacking UGT1 isozymes was not affected by DTT, indicating that UGT1A6 in the microsomes is mainly involved in the activation. The DTT-dependent activation was inhibited by 1,6-bis(maleimido)hexane (BMH) but not by *N*-ethylmaleimide, indicating that cross-linking between cysteine residues in UGT1A6 is responsible for the activation. Immunoblot analysis of rat hepatic microsomes on nonreducing SDS−PAGE gels revealed that most of the UGT1A6 migrated as a monomer, suggesting that DTT could affect an intramolecular disulfide bond in the UGT1A6 that may be responsible for the activation. To identify which of the ten cysteines in UGT1A6 are involved in the disulfide bond, rat UGT1A6 wild type and a set of mutants, each with a cysteine to serine substitution, were constructed and expressed in COS cells. Treatment of COS microsomes with DTT had no effect on the activity of the wild type but BMH showed significant inhibition, suggesting that UGT1A6 expressed in COS cells may be in the reduced and activated state. Replacement of either Cys 121 or Cys 125 with serine showed insensitivity to the BMH-dependent inhibition. These results demonstrate that both Cys 121 and Cys 125 are responsible for the activation of the activity through the disulfide bond in rat UGT1A6.

UDP-glucuronosyltransferases (UGTs,[1] EC 2.4.1.17) are located mainly in the endoplasmic reticulum (ER) and play a major role in the detoxification of drugs and other xenobiotics in all vertebrates (*1*, *2*). These enzymes catalyze the transfer of glucuronic acid (GlcUA) to the hydroxyl, carboxyl, sulfhydryl, or amine groups of these structurally unrelated compounds, resulting in the formation of water-soluble glucuronides to be excreted into bile or urine. Many of the UGT isozymes have been identified and characterized in terms of their substrate specificity after expression of the corresponding cDNA in heterologous cells (*3*, *4*). On the basis of the substrate specificity, sequence similarities, and gene structures of the isozymes, mammalian UGTs can be divided into two families, *UGT1* and *UGT2*.

Among UGT isozymes, UGT1A6 is a major isozyme catalyzing the glucuronidation of various simple phenolic compounds such as *p*-nitrophenol (*p*-NP) and 4-methylumbelliferone (4-MU) in the liver. This isozyme is likely to be functionally orthologous across several species including human, rat, mouse, rabbit, dog, bovine, and monkey (*5*−*9*). The UGT1A6 originates from a *UGT1* gene complex by differential splicing of a unique 5′ exon (exon 1A6) to common 3′ exons (exons 2−5) (*10*, *11*). The first exon 1A6 encodes the N-terminal domain of the variable region, which is an important determinant of substrate specificity in UGT1 isozymes. The common exons code for the UGT1-identical C-terminal domain, which is involved in UDP-GlcUA recognition. On the basis of previous kinetic and chemical modification studies, a general acid−base mechanism has been proposed for glucuronidation (*4*). A site-directed mutagenesis study of human UGT1A6 showed that the conserved histidine 370 of UGTs plays a catalytic role in the glucuronidation (*12*). Senary et al. suggested that the conserved motif in phenol binding proteins, 71YXXXKXX-PXP81, is involved in substrate binding to human UGT1A6 (*13*). In addition to these amino acid residues, cysteine residues in UGT1A6 have been considered to be involved in catalysis and the tertiary structure of the protein. Recently, the importance of cysteine 126 in human UGT1A6 was suggested by a mutational analysis (*14*). The mature protein of rat UGT1A6 contains seven and three cysteine residues located in the luminal region and cytoplasmic tail, respec-

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[1] Abbreviations: UGT, UDP-glucuronosyltransferase; GlcUA, glucuronic acid; *p*-NP, *p*-nitrophenol; 4MU, 4-methylumbelliferone; BMH, 1,6-bis(maleimido)hexane; NEM, *N*-ethylmaleimide; ER, endoplasmic reticulum; DTT, dithiothreitol; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; GSH, gluthathione (reduced form).

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tively, but which of the cysteines is involved in the glucuronidation process and/or the formation of disulfide bonds has not been fully resolved.

It is well-known that glucuronidating activity is latent in intact microsomes. Sonication, cycles of freezing and thawing, pore-forming agents, and detergents have all been reported to increase the microsomal UGT activity toward various substrates including *p*-NP. The problem of latency has been the subject of some debate, and two models have been proposed. The compartmental model, supported by the popular model of membrane topology of UGT, suggests that UDP-GlcUA transport across the ER membranes is the rate-limiting step for overall glucuronidation. UDP-*N*-acetylglucosamine as a putative physiological effector may be involved in the activation of glucuronidation via trans stimulation of UDP-GlcUA uptake (*15*, *16*). Although the human UDP-sugar transporters in ER membranes that are responsible for transport of UDP-GlcUA and UDP-*N*-acetylgalactosamine have been identified, the involvement of the proteins in glucuronidation of xenobiotics is still unclear (*17*). Alternatively, the conformational model of Zakim et al. (*18*) suggests that the UGTs in membranes are constrained in an inactive conformation that is released by membrane perturbation. Recently, the oligomeric model, a third possible model for latency, suggests that a dimer or higher oligomer of UGT in membranes may function as the UDP-GlcUA transporter (*19*, *20*). Despite these extensive investigations, the molecular basis of the latency in microsomal glucuronidation, which is catalyzed by UGTs in ER membranes, has not been elucidated.

In the present study, we have found that glucuronidation toward *p*-NP in rat hepatic microsomes is significantly activated by the addition of dithiothreitol (DTT) as a reducing agent. In the presence of DTT, the latency in microsomes is released without membrane perturbation or activation of UDP-GlcUA uptake. To elucidate the molecular mechanism of DTT-dependent activation of glucuronidation in rat hepatic microsomes, the role of cysteine residues in the modulation of activity was assessed by site-directed mutagenesis and cross-linking experiments of rat UGT1A6 in COS microsomes. Replacement of each of the ten cysteine residues in the rat UGT1A6 has identified the cysteine residues responsible for disulfide bond formation as Cys 121 and Cys 125. These results demonstrate that cleavage of the intramolecular disulfide bond in rat UGT1A6 could affect the glucuronidating activity, resulting in the change of latent state to activated state in native membranes.

EXPERIMENTAL PROCEDURES

Preparation of Rat Hepatic Microsomes. Adult male Wistar rats and homozygous Gunn rats (5−6 weeks, 150−180 g) were obtained from Clea, Inc. (Kyoto, Japan) and SLC Inc. (Shizuoka, Japan), respectively. Hepatic microsomes were prepared in 0.25 M sucrose as described previously (*21*). The protein concentration of the microsomes was determined with the bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as a standard.

Construction of Expression Vectors for Rat UGT1A6 and UGT1A7. Rat UGT1A6 and UGT1A7 cDNA coding regions (1.6 kb) were amplified by RT-PCR using isozyme-specific and UGT1-specific primers, and the sequences were verified by DNA sequencing. The coding regions of UGT1A6 and UGT1A7 with blunt ends were each subcloned into the *Sma*I site of the pUCD-SRa expression vector (*22*) to generate pUCD-UGT1A6 and pUCD-UGT1A7, respectively.

Site-Directed Mutagenesis of Rat UGT1A6. Cysteine to serine mutations in rat UGT1A6 cDNA were made by the QuickChange site-directed mutagenesis kit (Stratagene) with the following primers and their complements (mutation sites are underlined): C121S, 5′-ATT GAC ATG T<u>C</u>C TTT TTC AGC-3′; C125S, 5′-TTT TTC AGC T<u>C</u>C CAG AGA ATC-3′; C154S, 5′-GCC ATG CCC T<u>C</u>T GGT GTG ATC-3′; C175S, 5′-GGT TTC CCA T<u>C</u>C TCT CTG GAG C-3′; C223S, 5′-CTT TAT CAT T<u>C</u>T CTG TAC TCA-3′; C277S, 5′-GG ACC AAC T<u>C</u>C AAG AAG AAG GG-3′; C380S, 5′-GAA GGA ATA T<u>C</u>C AAT GGG GTT-3′; and C507/511/514S, 5′-AGT T<u>C</u>T GCC TAT GGC T<u>C</u>C CGG AAA T<u>C</u>C TTTG-3′. For mutations in the variable region, pUCD-UGT1A6 wild type was restricted with *Bam*HI, and the resultant fragment containing the 1A6 variable region was ligated with pUC118. The mutational PCR reactions were performed with the pUC vector containing the *Bam*HI fragment of UGT1A6 as the template. Each mutation was verified by DNA sequencing, and the mutated *Bam*HI fragments were then subcloned into the wild-type pUCD-UGT1A6 plasmid. For two mutations of the commonly used region (C380S and triple mutation C507/511/514S), the mutational PCR reactions were performed with pUCD-UGT1A6 wild type as the template. Each mutation was verified by DNA sequencing.

Cell Cultures, Transfections, and Membrane Preparations. For transient expression of UGT protein, COS cells were cultured in 100 mm dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37 °C in 5% CO_2. Plasmids were transfected into COS cells as described by Chen and Okayama (*23*), and after 16 h, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and further incubated for 48 h in DMEM with 10% fetal calf serum.

After harvesting, cells were washed once with PBS, scraped from the dishes with a rubber policeman, placed in PBS, and subjected to centrifugation (50*g*) at 4 °C for 2 min. The cell pellets were suspended in 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM $MgCl_2$, 0.1 mM *p*-amidinophenylmethanesulfonyl fluoride, 2 μg/mL leupeptin, and 2 μg/mL pepstatin. The cells were broken by vigorously passing the suspension 50 times through a 25-gauge needle. The resulting homogenate was subjected to centrifugation for 10 min at 5000*g*. The supernatant was further centrifuged for 5 min at 10000*g*. Microsomal fractions were pelleted after 10000*g* supernatant followed by centrifugation for 12 min at 105000*g* at 4 °C. The microsomal fractions were suspended in 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, and 1 mM $MgCl_2$ and stored at −80 °C until used for further protein analysis and enzymatic assays.

Enzyme Assay. Glucuronidation of *p*-NP and testosterone was analyzed by HPLC using reverse-phase column chromatography as previously described (*18*, *19*). 4-Methylumbelliferone glucuronidation was analyzed by the HPLC method used for *p*-NP, except that detection was at 315 nm. Bilirubin glucuronidation was determined using reverse-phase HPLC analysis as described by Odell et al. (*24*). The

conjugation activity is expressed as the sum of mono- and diglucuronides formed. The data and peak area determinations were made using an automatic system consisting of an autosampler (Model AS-8020, TOSOH Inc., Japan), data processor system (MacIntegrator I, Rainin Inc., Emoryville, CA), and HPLC system (Hitachi L-6200 Intelligent system, Hitachi Co., Japan). The relative activities of *p*-NP glucuronidation in UGT1A6 mutants can be detected at 0.01% compared to the wild type.

To determine the effects of DTT on glucuronidation, incubation of microsomes with 5 mM DTT was carried out at 37 °C with 10 mM Tris-HCl, pH 7.0, containing 0.25 M sucrose and 1 mM $MgCl_2$. Aliquots of the reaction mixture were removed at the indicated times and quenched with 2 mM NEM. Assays of *p*-NP glucuronidation were then performed using the HPLC method described above. For the assay of glucuronidation, alamethicin, a pore-forming peptide, was added to reaction mixtures (0.05 mg of alamethicin/mg of microsomal protein) after the incubation with DTT. To determine the effects of maleimides on the DTT-dependent activation, incubation of microsomes with 2 mM BMH or 4 mM NEM was carried out at 37 °C for 30 min, followed by DTT treatment.

Assays for Intactness and UDP-GlcUA Uptake of Rat Hepatic Microsomes. The intactness of the microsomes was verified by determination of mannose-6-phosphatase latency (*25*). Uptake of UDP-GlcUA into rat hepatic microsomal vesicles was determined using a rapid filtration assay with [^{14}C]UDP-GlcUA as described previously (*19*). To determine the effects of DTT on the intactness and uptake, incubation of microsomes (100 μg) with 5 mM DTT was carried out at 37 °C for 60 min with 10 mM Tris-HCl, pH 7.0, containing 0.25 M sucrose and 1 mM $MgCl_2$ before each assay.

SDS−PAGE and Immunoblotting. SDS−polyacrylamide gel electrophoresis was performed as described by Laemmli (*26*) using a 4% stacking and a 10% separating gel. For nonreducing SDS−PAGE, microsomes were treated with SDS sample buffer in the presence of 10% (v/v) 2-mercaptoethanol. The gels were blotted using a semidry blotting method, and the detection of protein was performed using chemical luminescence (ECL detection kit, Amersham Pharmacia Biotech). Polyclonal anti-peptide antibodies that specifically recognize the UGT1A1 and UGT1A6 isozymes belonging to the UGT1A family, as well as an antibody against a C-terminal peptide common to all UGT1A isozymes, were used in the immumoblot analysis (*21*).

RESULTS

Incubation of Rat Hepatic Microsomes with DTT Leads to a Significant Increase in Glucuronidation of p-NP. Hepatic microsomes were prepared from male Wistar rats and assayed for glucuronidating activity toward *p*-NP using the HPLC assay system. The activity of glucuronidation in intact microsomes is approximately 3−6 nmol of *p*-NP glucuronides min^{-1} (mg of protein)$^{-1}$ under the standard assay conditions. Incubation of the microsomes with reduced dithiothreitol (DTT) was found to significantly increase the activity to a maximum of approximately 90 nmol min^{-1} mg^{-1}. The activation depends on the incubation conditions with respect to time and DTT concentration (Figure 1A). It should be noted that the time course of the activation had a lag phase of about 20 min. Incubations of the microsomes with 5 mM DTT were performed over of a pH range of 6.2−8.4. As shown in Figure 1B, the activation was observed between pH 6.5 and 7.2, even though the reaction rate of DTT-dependent disulfide reduction is enhanced above pH 7.5. The addition of 2-mercaptoethanol, another monothiol reducing reagent, activated the activity, although the effective concentration was greater than 10 mM (data not shown). The physiological reductant reduced gluthathione (GSH) had no effect on *p*-NP glucuronidation in rat hepatic microsomes.

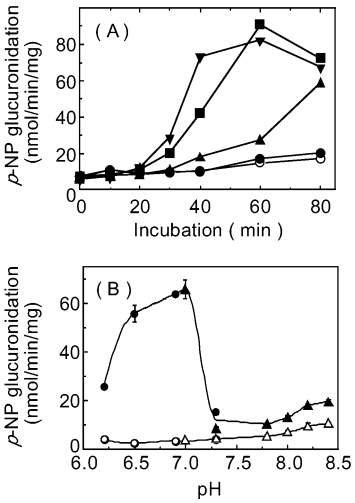

FIGURE 1: DTT-dependent activation of *p*-NP glucuronidation in rat hepatic microsomes. (A) Time courses and concentration dependence of DTT-dependent activation. Wistar rat hepatic microsomes were preincubated without or with various concentrations of DTT at 37 °C for 0−80 min with 10 mM Tris-HCl, pH 7.0, 0.25 M sucrose, and 1 mM $MgCl_2$, followed by the *p*-NP glucuronidating assay. DTT concentrations used were 0 mM (open circles), 1 mM (closed circles), 2 mM (closed triangles), 5 mM (closed squares), and 10 mM (reverse closed triangles). (B) pH profiles of DTT-dependent activation. The microsomes were preincubated without (open symbols) or with 5 mM DTT (closed symbols) at 37 °C for 60 min under various pH conditions. PIPES−NaOH (circles) and Tris-HCl (triangles) buffers were used for pH ranges 6.2−7.3 and 7.0−8.4, respectively.

Table 1 shows the effect of DTT on glucuronidation toward various substrates in rat hepatic microsomes. In the absence of alamethicin, a pore-forming oligopeptide, the DTT-dependent activation is specific for planar phenolic compounds such as *p*-NP and 4-MU but not bilirubin or testosterone. It should be noted that values of the activities in DTT-dependent activation are comparable to alamethicin-induced levels. These results indicate that the DTT affects glucuronidation catalyzed by one or some of the UGT isozymes in the rat hepatic microsomes. No changes in the apparent K_m values of glucuronidation for *p*-NP (0.13 ± 0.02 mM and 0.32 ± 0.03 mM) or UDP-GlcUA (0.13 ± 0.02 mM and 0.12 ± 0.01 mM) were observed in the absence and presence of DTT, respectively. This is indicative of a contribution of DTT to catalysis in the glucuronidation but not binding affinity for substrates.

Glucuronidation is activated by perturbation of microsomal membranes using sonication or detergents (*20*). To examine whether the microsomal membranes are perturbed by the treatment with DTT or not, the intactness of the microsomal vesicles was estimated using the measurement of latency for mannose-6-phosphate phosphatase activity. The values of

Table 1: Effect of DTT on Glucuronidation of Various Substrates[a]

substrates	glucuronidation (nmol min^{-1} mg^{-1})			
	−alamethicin		+alamethicin	
	−DTT	+DTT	−DTT	+DTT
p-nitrophenol	6.2 ± 0.2	89.5 ± 2.8 (14)	120.2 ± 5.1 (19)	89.4 ± 1.7 (14)
4-methylumbelliferone	14.2 ± 0.6	70.4 ± 6.0 (5)	114.8 ± 9.3 (8)	77.8 ± 3.1 (6)
bilirubin	0.4 ± 0.1	0.4 ± 0.1 (1)	2.1 ± 0.1 (5)	2.4 ± 0.2 (6)
testosterone	2.0 ± 0.1	1.4 ± 0.1 (0.7)	11.9 ± 0.6 (6)	14.6 ± 0.5 (7)

[a] The microsomes were preincubated with or without 5 mM DTT at 37 °C for 60 min, followed by glucuronidation assays with various substrates with or without addition of 0.05 mg/mL alamethicin. Values in parentheses represent the ratio compared to each control without DTT and alamethicin.

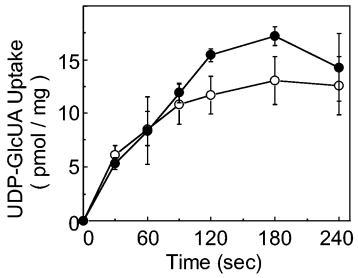

FIGURE 2: Inability of DTT to activate the UDP-GlcUA uptake into microsomes. Wistar rat hepatic microsomes (100 μg) were incubated with (closed circles) or without (open circles) 5 mM DTT at 37 °C for 60 min, followed by the UDP-GlcUA uptake assay as described under Experimental Procedures.

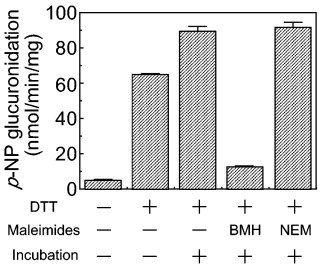

FIGURE 3: DTT-dependent activation is inhibited by BMH but not NEM. Wistar rat hepatic microsomes (20 μg) were incubated in the absence (column 1) or presence (column 2) of 5 mM DTT at 37 °C for 60 min and assayed for *p*-NP glucuronidation. The microsomes that were treated with 5 mM DTT were further incubated in the absence (column 3) or presence of 2 mM BMH (column 4) or 4 mM NEM (column 5) at 37 °C for 30 min and assayed for *p*-NP glucuronidation.

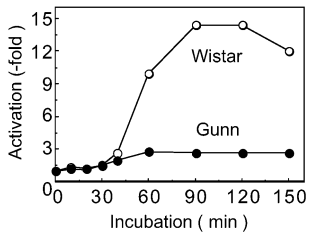

FIGURE 4: Inability of DTT to activate *p*-NP glucuronidation in Gunn rat hepatic microsomes. Wistar (open circles) and Gunn rat hepatic microsomes (closed circles) were preincubated with 5 mM DTT at 37 °C followed by the *p*-NP glucuronidating assay. The activities in the absence of incubation were as follows: 3.0 ± 0.1 and 1.4 ± 0.1 nmol min^{-1} mg^{-1}, respectively.

intactness are 97% and 94% in the absence and presence of DTT, respectively, indicating that incubation of microsomes with DTT had no effect on the integrity of microsomal membranes. The rate of glucuronidation in intact microsomes is also linked to the rate of UDP-GlcUA uptake into the luminal side of microsomes (*14*). As shown in Figure 2, DTT had no effect on the time course of UDP-GlcUA uptake into microsomes. This result demonstrates that the DTT-dependent activation of glucuronidation in microsomes is due to the enhancement of the catalytic step of one or some of the UGT isozymes but not to UDP-GlcUA uptake for availability on the luminal side of the ER. These results are consistent with the substrate-specific activation of glucuronidation by DTT (Table 1).

DTT-Dependent Activation Is Inhibited by BMH but Not by NEM. DTT is known to reduce disulfide bonds in enzymes, resulting in the modulation of enzymatic activity. In this case, the DTT-dependent activation of glucuronidation is probably achieved by cleavage of disulfide bonds in UGT(s). We have examined the reversibility of the effect of DTT on the microsomal glucuronidation using several oxidizing reagents such as oxidized gluthathione, copper phenanthroline, or hydroperoxide. However, the treatment of microsomes with these oxidants resulted in the complete loss of activity in microsomes (data not shown). To mimic the oxidized state of UGTs, 1,6-bis(maleimido)hexane (BMH), a thiol-specific homobifunctional reagent that cross-links the reactive cysteine residues in some enzymes, was used. As shown in Figure 3 (columns 3 and 4), the addition of BMH to the microsomes after incubation with DTT resulted in the inhibition of activity to the basal level. In contrast to BMH, *N*-ethylmaleimide, a monomaleimide, had no effect on the DTT-dependent activation (Figure 3, column 5). BMH with two maleimides reacts proximal to thiol residues in UGT(s) to cross-link, resulting in the BMH-dependent inhibition against the activation. These results suggest that cleavage of inter- or intramolecular disulfide bonds in UGT(s) is responsible for the DTT-dependent activation.

DTT Activates UGT1 but Not UGT2 Isozyme-Dependent Glucuronidation. To identify the isozymes of UGT to be activated by DTT, the effect of DTT on *p*-NP glucuronidation in Gunn rat hepatic microsomes was examined. The Gunn rat is hyperbilirubinemic and lacks all UGT1 isozymes because of a frame-shift mutation in the commonly used exon of the *UGT1* gene complex (*12*, *27*). As shown in Figure 4, in contrast to the Wistar rat, the time course of treatment of the microsomes with DTT showed the inability of DTT to activate *p*-NP glucuronidating activity in Gunn rat microsomes. This result clearly indicates that one or more of the UGT1 isozymes in the hepatic microsomes are sensitive to activation by DTT but UGT2 isozymes are not affected.

UGT1 Isozymes Have Intramolecular Rather Than Intermolecular Disulfide Bonds. The possibility of the formation of intra- or intermolecular disulfides in UGT1 isozymes was examined by immunoblot analysis of microsomes after nonreducing SDS−PAGE. As shown in Figure 5A, immu-

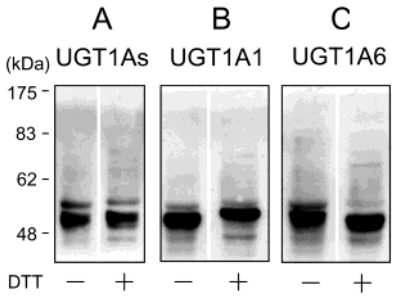

FIGURE 5: UGT1 isozymes have an intramolecular rather than an intermolecular disulfide bond. Rat hepatic microsomes (20 μg) were loaded in each lane and analyzed using SDS−PAGE in nonreducing and reducing conditions. Immunostaining was performed using the anti-peptide antibodies (A) anti-UGT1A, (B) anti-1A1, and (C) anti-1A6.

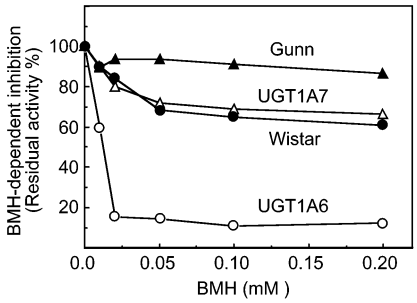

FIGURE 6: BMH-dependent inhibition of glucuronidation in rat hepatic microsomes or COS microsomes containing recombinant rat UGT1A isozymes. Twenty micrograms of Wistar (closed circles) and Gunn (closed triangles) rat hepatic microsomes or 50 μg of COS microsomes containing recombinant UGT1A6 (open circles) and UGT1A7 (open triangles) was incubated with 0.01−0.2 mM BMH at 37 °C for 30 min and assayed for *p*-NP glucuronidation.

noblots using an anti-UGT1 antibody against the C-terminal peptide on nonreducing SDS−PAGE gels showed that the UGT1 isozymes in microsomes run as monomer bands (52−57 kDa). None of the UGT1 isozymes formed disulfide-linked dimers or higher oligomers in microsomes. No change in mobility was observed using isozyme-specific antibodies against UGT1A1 or UGT1A6 (Figure 4B,C). These results revealed that most of the UGT1A6 in hepatic microsomes migrated as a monomer of 53 kDa and disulfide cross-linked products were not formed, suggesting that the treatment of microsomes with DTT may affect intramolecular rather than intermolecular disulfide bonds in rat UGT1A6.

Rat UGT1A6 in COS Cells Is Not Activated by DTT but Is Significantly Inhibited by BMH. To elucidate the molecular mechanism of the DTT-dependent activation in rat hepatic microsomes, cDNAs for rat UGT1A6 or UGT1A7 were expressed in COS cells, and the microsomal fractions were prepared. Unexpectedly, in contrast to rat hepatic microsomes, treatment of the COS microsomes containing UGT1A6 or UGT1A7 with DTT had no effect on *p*-NP glucuronidation activity (only 1.5−2-fold increase). These results suggest that rat UGT1A6 and UGT1A7 may be lacking the cysteine residues responsible for the DTT-dependent activation or that these isozymes may already exist in a reduced state in COS cells. To determine which of these possibilities is correct, the effects of BMH on the activity were examined in those microsomes. Figure 6 shows the BMH concentration dependence of the inhibition of rat hepatic microsomes or COS microsomes containing UGT1A6 or UGT1A7. As shown in Figure 6 (open circles), treatment of UGT1A6 with BMH resulted in a significant inhibition of activity (90% inhibition). In contrast to UGT1A6, BMH had a slight effect on the activity of UGT1A7 (open triangles, 25% inhibition). The insensitivity of UGT1A7 to DTT and BMH indicates that the isozyme is lacking the DTT-sensitive cysteine and does not contribute to the DTT-dependent activation of *p*-NP glucuronidation in rat hepatic microsomes. In the absence of DTT, BMH moderately inhibited the activity in Wistar rat microsomes (30% inhibition) but not in Gunn rat microsomes (10% inhibition). Most of the UGT1A6 in rat hepatic microsomes may be in an oxidized and inactivated state, resulting in the insensitivity to BMH-dependent inhibition. In contrast to rat hepatic microsomes, most of the UGT1A6 expressed in COS cells may already be in a reduced state with BMH-dependent inhibition. The insensitivity of the Gunn rat to DTT and BMH indicates that the UGT2B isozymes have no DTT-sensitive cysteines and could not contribute to the DTT-dependent activation of *p*-NP glucuronidation in the hepatic microsomes. On the basis of these effects of DTT and BMH on the activity of UGT1A6 in rat hepatic microsomes or cDNA-expressed COS microsomes, the enzyme in rat hepatic microsomes is thus likely to be responsible for the DTT-dependent activation of *p*-NP glucuronidation.

The Disulfide Bond between Cys 121 and Cys 125 Is Responsible for the Activity of Rat UGT1A6 Activity. The mature form of rat UGT1A6 contains ten cysteine residues. There are six in the variable region (121, 125, 154, 175, 223, and 277) and four in the commonly used region (380, 507, 511, and 514). To identify which of the cysteine residues in rat UGT1A6 are involved in the DTT-dependent activation of glucuronidation, a set of mutants, each with a cysteine to serine substitution, was constructed in the expression vector pUCD-UGT1A6. Each mutant was expressed in COS cells, and membrane fractions containing the UGT1A6 mutants were prepared. Eight mutants of rat UGT1A6 (C121S, C125S, C154S, C175S, C223S, C277S, C380S, and C517/511/511S as triple mutant) were normally expressed in COS cells as compared to wild type (Figure 7A). The glucuronidating activity of each mutant with *p*-NP was determined in microsomal fractions and expressed relative to the amount of UGT1A6 present, as quantified by immunoblot analysis using the anti-UGT1 peptide antibody (Figure 7B). With the exception of the C125S and C154S mutants, the other cysteine mutants retained catalytic activity (20−95% activity compared to wild type). Cys 125 and Cys 154 are conserved cysteine residues in all known UGT isozymes. The mutation at Cys 154 results in the complete loss of glucuronidation ability (below 0.01% activity compared to wild type). The mutation at Cys 125 significantly decreased activity (0.14% activity compared to wild type), but *p*-NP glucuronide was detected quantitatively using HPLC assay conditions with long incubation times and 0.1 mg of microsomal proteins.

The microsomal fractions containing the UGT1A6 mutants were treated with BMH and assayed for *p*-NP glucuronidation (Figure 8). BMH inhibited the activity of wild type and some mutants (75−98% inhibition). Only two mutations, C121S and C125S, showed significant resistance against BMH-dependent inhibition of glucuronidation (6% and 24% inhibition, respectively). These results clearly reveal that the substitution of cysteine by serine at position 121 or 125 of

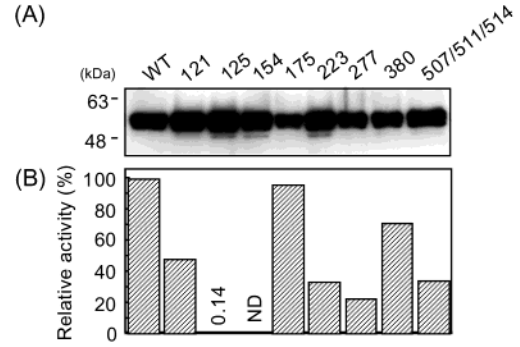

FIGURE 7: Expression and *p*-NP glucuronidation by wild-type and mutant UGT1A6 expressed in COS cells. (A) SDS−PAGE and immunoblot analysis. Microsomal fractions prepared from recombinant COS cells (20 μg) were loaded in each lane and analyzed using SDS−PAGE and immunoblotting as described under Experimental Procedures. Evaluation of the relative expression of the mutants compared with the wild-type protein was performed by scanning densitometry using NIH image software. (B) Glucuronidation activity toward *p*-NP. Activity was determined in COS microsomes using 2 mM *p*-NP, 2 mM UDP-GlcUA, and 20−100 μg of protein in 10 mM Tris-HCl buffer (pH 7.5), 0.25 M sucrose, and 1 mM $MgCl_2$ for 20 min at 37 °C. Values are the means of two independent transfections. ND: glucuronide formation was below 0.01% compared to wild type.

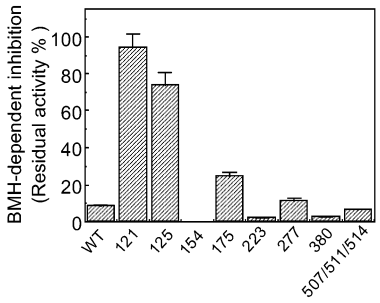

FIGURE 8: Involvement of Cys 121 and Cys 125 in BMH-dependent inhibition of rat UGT1A6. COS microsomes containing wild type or mutants of rat UGT1A6 (20−100 μg) were preincubated in the absence or presence of 2 mM BMH at 37 °C for 20 min and then assayed for *p*-NP glucuronidating activity. The activities were expressed as a percentage of the activity compared to a control without BMH. Values are means ± SD of three to five experiments of two independent transfections.

UGT1A6 interferes with the formation of cross-links by BMH, resulting in the insensitivity against BMH-dependent inhibition of glucuronidation.

Figure 9 shows the sequence alignment of rat UGTs and mammalian UGT1A6s that contain Cys 121 and Cys 125. Cys 125 is a conserved residue in all known UGTs. Among the rat UGTs, only UGT1A6 and UGT2B12 have a cysteine at position 121. In contrast to rat, orthologues in other mammals have a tyrosine or phenylalanine instead of cysteine. Indeed, DTT treatment had no effect on *p*-NP glucuronidation in bovine or mouse hepatic microsomes (data not shown). We have concluded from these results that Cys 121 and Cys 125 in the rat hepatic microsomal UGT1A6 could be responsible for the intramolecular disulfide bond that affects the *p*-NP glucuronidation.

DISCUSSION

In the present study, we have shown that incubation of microsomes with DTT as the reductant significantly activates glucuronidation toward *p*-NP. There are two possibilities for

(A) Rat UGT

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                  121  125
1A6   119DMCFFSCQSLL
1A7   119ELLFSHCRSLF
1A1   123SMLLSGCSHLL
2B1   122DVVENLCKALI
2B2   122YFYLSICKDAV
2B3   122DYYLSLCKDTV
2B6   122DYYLSVCKDAV
2B12  122DYCLTVCKEAV
```

(B) UGT1A6

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                  121  125
Rat       119DMCFFSCQSLL
Human     120GLYFINCQSLL
Monkey    120GMYFINCQSLL
Bovine    117NMYFLNCQSLL
Mouse     120DMFFSNCQSLL
Rabbit    120DMYFINCQSLL
Dog       116DMYFTNCQSLL
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FIGURE 9: Comparison of amino acid sequences of UGTs around positions equivalent to the cysteine residues of rat UGT1A6. (A) Sequence alignment of the region of rat UGT1A6 containing Cys 121 and Cys 125 with the corresponding region of the rat UGT1A and 2B isozymes. (B) Sequence alignment of the region of mammalian UGT1A6 orthologues containing Cys 121 and Cys 125 with the corresponding region of rat UGT1A6.

the mechanism of the DTT-dependent activation: formation of a disulfide between two cysteine residues within a monomer of UGT and disulfide bond formation between the subunits of a dimer. Although UGTs in ER membranes could interact to form homo- or heterodimers (*19*, *28*−*30*), the latter possibility is unlikely because immunoblot analysis under nonreducing conditions provided no evidence for the formation of a disulfide-linked UGT1 dimer (Figure 5). Some Golgi UDP-glycosyltransferases involved in glycolipid and glycoprotein synthesis have been identified as disulfide-linked homodimers (*31*). Unlike Golgi glycosyltransferases, UGT1 isozymes are known to exist as noncovalent dimers or higher oligomers in ER membranes (*19*, *28*). The majority of any disulfide bonds present in UGT1s, including UGT1A6, must thus be intramolecular.

We have used BMH to mimic the reversibility of DTT-dependent activation of glucuronidation in microsomes (Figure 3). BMH is a homobifunctional cross-linker that reacts with sulfhydryl groups. The average distance between the reactive groups is 10.16 Å (*32*). It is possible that BMH could react with proximal free cysteines in UGT, resulting in the inhibition of glucuronidation. We have previously demonstrated that treatment of rat hepatic microsomes with BMH can result in cross-linked homo- or heterodimers of UGTs (*19*). Rat UGT1 isozymes expressed in COS cells were treated with BMH, resulting in the formation of homodimers of UGT1A1 but not UGT1A6.[2] Human UGT1A1 expressed in Gunn rat fibroblasts was found to form BMH-dependent dimers (*29*). These results indicate that the BMH-reactive site of cysteines in UGT1A6 is different from that in UGT1A1. In the case of UGT1A6, BMH reacts with a free sulfhydryl group within a single subunit to form the intramolecular cross-linked product, and this results in the modulation of glucuronidating activity. In an attempt to investigate the effect of DTT and BMH on glucuronidation

[2] S. Ikushiro, Y. Emi, and T. Iyanagi, IXth International Workshop on Glucuronidation and UDP-Glucuronosyltransferase, Brisbane, 1998.

in rat UGT1A6 or UGT1A7, which are responsible for *p*-NP glucuronidation in hepatic microsomes, we have constructed UGT1 cDNA-expressing vectors and expressed them in COS cells (Figure 6). Unexpectedly, the activities of these isozymes were not activated by the addition of DTT. In contrast, incubation with BMH caused a significant inhibition of activity in UGT1A6 but not UGT1A7. The most likely explanations for these data are that UGT1A6 expressed in COS cells is already in the reduced state and that UGT1A7 is an insensitive isozyme because it has no cysteines available for BMH-dependent inhibition.

Mutagenesis of Cys 121 or Cys 125 resulted in rat UGT1A6 that is insensitive to BMH-dependent inhibition, indicating that an intramolecular disulfide bond between the residues is involved in the modulation of activity. As shown in Figure 9, the cysteine residue equivalent to that at position 125 of rat UGT1A6 is conserved in all members of the UGT family. The significant decrease in activity of the C125S mutant clearly shows the primary importance of this cysteine in catalyzing glucuronidation. Substitution of cysteine 125 by serine in rat UGT1A1 also caused a significant loss in bilirubin glucuronidating activity.[3] Recently, Senay et al. reported that the equivalent Cys 126 in human UGT1A6 is an essential residue for the integrity of the substrate binding site (*14*). Mutation of Cys 126 to valine led to a fully inactive mutant, whereas conservative substitution with serine significantly restored the activity. In contrast to Cys 125, Cys 121 in rat UGT1A6 is a unique amino acid at this position among the UGT isozymes except for UGT2B12. Since the C121S mutant has moderate activity compared to wild type, Cys 121 is not likely to be essential for the catalytic process. The rat UGT1A7 lacking the equivalent cysteine showed DTT and BMH independence, confirming the role of this cysteine as one of the cross-linked residues. Both Cys 121 and Cys 125 in UGT1A6 are located within an N-terminal variable region involved in the recognition of a glycone structure and catalysis as shown in human UGT2B7 (33). The disulfide bond formation or BMH-dependent cross-linking between these residues, therefore, may result in some steric hindrance that may affect the catalytic process of glucuronidation. Besides the significance of Cys 121 and Cys 125 in disulfide bond formation, mutational analysis of UGT1A6 revealed that Cys 154 is essential for glucuronidation (C154S mutant in Figure 7). The free sulfhydryl group at this position but not the hydroxyl may be involved in the catalytic process. As shown in Figure 3, *p*-NP glucuronidating activity in hepatic microsomes was not inhibited by NEM, suggesting that Cys 154 in rat UGT1A6 does not react with the maleimide. The exact role of Cys 154 in catalysis needs to be elucidated.

According to the compartmental model, the treatment of microsomes with a detergent could enhance the permeability of ER membranes to UDP-GlcUA, resulting in an activation of glucuronidation activity. In this study, the DTT-dependent activation is comparable to detergent-induced activation without a change in membrane permeability or the rate of UDP-GlcUA uptake into microsomes (Figure 2). An alternate explanation for latency is the conformational model proposed by Zakim et al. (*18*), which proposes that UGTs in the membrane could exist in different functional states with different activities. They suggested that the use of detergents to perturb the lipid environment would alter catalytic activity by modulating the conformation of the protein. In the case of *p*-NP glucuronidation in rat hepatic microsomes, it is possible that UGT1A6 in the latent state may be in the oxidized form, which has an intramolecular disulfide bond between Cys 121 and Cys 125. This would result in a constrained *p*-NP glucuronidation activity in intact membranes. Treatment of Wistar rat hepatic microsomes with BMH had little effect on the activity (Figure 6), confirming most of the UGT1A6 to be oxidized state in intact membranes. Detergent-dependent activation of glucuronidation is also inhibited by BMH but not NEM, suggesting that solubilization of the intact microsomes may cause a conformational change equivalent to the cleavage of the disulfide bond in rat UGT1A6.[4] It is well-known that cDNA-expressed UGTs in membrane fractions showed no latency for glucuronidation because of the low intactness of prepared microsomes in the heterologus expression system (*34*, *35*). Recombinant rat UGT1A6 expressed in COS cells showed insensitivity against the DTT-dependent activation and BMH-dependent inhibition of glucuronidation. In the case of rat UGT1A6, loss of latency in this expression system may be due to the redox state of the UGT in COS cells. In cultured cells, the UGT1A6 is likely to be in the reduced state with high catalytic activity, and this results in an insensitivity to detergents or DTT-dependent activation.

In conclusion, we have demonstrated the DTT-dependent activation of *p*-NP glucuronidation in rat hepatic microsomes. The intramolecular disulfide bond between Cys 121 and Cys 125 in rat UGT1A6 functions to modulate the activity. The oxidized disulfide-linked state of rat UGT1A6 is likely to be equivalent to the latent state in membranes. Our present data can partly support that the latency of *p*-NP glucuronidation in rat hepatic microsomes depends on a conformational change in the rat UGT1A6 molecule. The further elucidation of the molecular mechanism of the DTT-dependent activation of rat UGT1A6 should lead to a better understanding of the structure and function of this enzyme.

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