Evidence for Overlapping Active Sites for 17α-Ethynlestradiol and Bilirubin in the Human Major Bilirubin UDPglucuronosyltransferase

M. Ciotti and Ida S. Owens*

Heritable Disorders Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892-1830

Received March 11, 1996; Revised Manuscript Received June 3, 1996[⊗]

ABSTRACT: The human major bilirubin UDPglucuronosyltransferase (transferase), HUG-Br1, and its mutants were expressed in the COS-1 cells using cDNA-based pSVL expression units to generate isoforms for the comparison of relative activities with 17α-ethynlestradiol (17α-EE) and bilirubin, its natural substrate. In comparison to bilirubin, 17α-EE was a good substrate for HUG-Br1 under typical assay conditions of pH 7.2, confirming published studies [Ebner, T., *et al*. (1993) *Mol. Pharmacol. 43*, 649−654]. It was further shown that the estrogen derivative is 1.2−2-fold more effective as a substrate at pH 6.4 than at pH 7.2. The k_m for 17α-EE was 40 μM under both pH conditions, while the V_{max} values were 400 and 200 pmol per hour per 300 μg of protein at pH 6.4 and 7.2, respectively. The pattern of glucuronidation was similar for both bilirubin and 17α-EE. Previously, a ratio of 2−3-fold more activity for bilirubin glucuronidation at pH 6.4 versus 7.6 was established, and k_m values of 2.5 μM at both pH conditions were determined [Ritter, J. K., *et al*. (1993) *J. Biol. Chem. 268*, 23573−23579]. In this study, the generation of 17α-EE and bilirubin β-glucuronides under both pH conditions was confirmed by the sensitivity of the products to β-glucuronidase treatment. Concurrent glucuronidation reaction mixtures containing equal amounts of wild-type and mutant proteins demonstrated the following. P270G, V273D, and five different G276 mutants nearly or completely inactivated all glucuronidation at both pH levels. V273Q generated 81−94% of the normal activity for 17α-EE and 42% of the normal activity for bilirubin turnover; H173R gave 37−60% of the normal turnover with both substrates, and V275I produced 15−24% of the normal level of glucuronide with both compounds. The most distinguishing amino acid tested was P176G which was approximately 50% normal for 17α-EE at both pH conditions but was totally inactive for bilirubin. A second substitution, P285G, did not affect 17α-EE turnover but was 50% normal for bilirubin. The parallel effects on the metabolism of both substrates by some mutants and the opposite results from two mutants are evidence for a common set of amino acids for their catalysis with the recruitment of additional amino acids to depend upon the substrate to be metabolized. Hence, amino acid substitutions in the protein are not necessarily universally inactivating.

The family of UDPglucuronosyltransferase (transferase) isozymes, located primarily in the liver, is generally considered detoxifying for many different endogenous and exogenous lipophilic or lipophilic-like chemicals. The isozymes convert these compounds to excretable water-soluble derivatives *via* covalent linkage to glucuronic acid donated by UDPglucuronic acid, the common substrate for this family of isozymes. An ever-increasing list of new chemicals undergo glucuronidation by an undetermined number of isozymes utilizing an active center(s) in the proteins which participates in a mechanism that is not yet understood. In most instances, there is considerable overlap in substrate specificity of each isoform that is, no doubt, the underpinning of the extensive glucuronidation capacity of the liver.

Critical aspects of the glucuronidation system have been clarified in recent studies. Defective bilirubin transferase, in particular, has been identified with toxic and lethal effects in the body. The lethal neurotoxicity of bilirubin in Crigler−Najjar type I (Crigler & Najjar, 1952) patients was shown to be due to inheritable mutations in the major bilirubin transferase (Ritter *et al*., 1992). High doses of acetaminophen proved 100 times more toxic to the bilirubin/phenol transferase-deficient Gunn rat than to the control Wistar rat strain (de Morais & Wells, 1989). In an *in vitro* mutagenesis assay, the lack of transferase activity for glucuronidating promutagens demonstrated 12−33-fold more mutagenicity than conditions where the enzyme(s) was active (Owens *et al*., 1979).

Acylglucuronides of certain nonsteroidal anti-inflammatory drugs are reported to cause serious toxic effects that are thought to be due to the covalent binding of the acylglucuronide *via* transacylation or after acyl migration and binding of the sugar to proteins [see review in Spahn-Langguth and Benet (1992)]. In one case, the target protein of the acylglucuronide has been identified in the liver, and the adduct is thought to be responsible for toxicity either directly or as an immunogen (Hargus *et al*., 1995). In addition, insight into the glucuronidation process could have implications for idiosyncratic drug reactions and other toxicities.

Since the glucuronidation process can generate either detoxifying or, in some cases, toxifying consequences, it is essential to understand its aspects such as control of isozyme

* Direct all correspondence to Ida S. Owens, Ph.D., National Institutes of Health, Building 10, Room 9S-242, Bethesda, MD 20892-1830. Telephone: 301-496-6091.

[⊗] Abstract published in *Advance ACS Abstracts,* July 15, 1996.

S0006-2960(96)00584-3

levels involved in the glucuronidation process, the potential to generate reactive glucuronides, the overlapping substrate specificity of isoforms, and the nature of the active site(s) of isoforms. This report focuses on amino acids involved in the active site(s) versus the overlapping substrate specificity of the glucuronidation process. Historically, information on substrate specificity of isoforms has been made on purified preparations and, more recently, on cDNA-based expressed transferases from different species [reviewed in Owens and Ritter (1993), Miners and Mackenzie (1991), and Tephly and Burchell (1990)]. The expression of a single isoform or its mutants in an endogenous transferase-free or -low environment offers a special opportunity to gain information on the active center(s) of an isoform in its native environment.

Because bilirubin glucuronidation is the only mechanism in humans for detoxifying this potentially neurotoxic heme metabolite, we have studied the human major bilirubin isoform, HUG-Br1, with respect to its overlapping substrate specificity in greater detail. We have compared conditions and amino acids in its primary structure that are required for glucuronidating bilirubin and 17α-ethynlestradiol (17α-EE). This study does not, however, distinguish between changes in substrate binding and changes in the catalytic process. This estrogen derivative, known to be a component of female contraceptives, was shown by Ebner *et al*. (1993) to be a substrate for the HUG-Br1 isoform. Also, we would like to know if the HUG-Br1 glucuronidates this alternative substrate at both pH 6.4 and 7.6, similar to that for bilirubin (Ritter *et al*., 1993).

MATERIALS AND METHODS

Materials. The sources of reagents used to carry out recombinant DNA techniques are already described (Ritter *et al*., 1993). The TA vector was obtained from Invitrogen (San Diego, CA). β-Glucuronidase (EC 3.2.1.31) type VII-A from *Escherichia coli* was from Sigma. The sources of materials for the transfection of plasmid DNA into the COS-1 cells and the radiolabeling and immunoprecipitation of the expressed bilirubin transferase protein are already reported (Ritter *et al.*, 1990).

Construction of Mutant pHUG-Br1 Expression Units. All mutants were constructed in the pHUG-Br1 expression unit; its construction was previously described (Ritter *et al*., 1991). Point mutations were introduced at either codon 173, 176, 270, 273, 275, 276, or 285 of the HUG-Br1 protein. The mutated codons are designated as follows: H173R, P176G, P270G, V273D, V273Q, V275I, G276A, G276Q, G276I, G276R, G276E, or P285G. Two independent PCR reactions were carried out for the H173R and P176G mutants using the following primers: H173R, sense (5′-GTATTCTTCT-TGCGTGCACT-3′) and antisense (5′-AGTGCACGCAA-GAAGAATAC-3′); P176G, sense (5′-GCATGCACTGGGT-TGCAGCCT-3′) and antisense (5′-AGGCTGCAACC-CAGTGCATGC-3′); V273D, sense (5′-TGCCCAATATG-GATTTTGTT-3′) and antisense (5′-AACAAAATCCATAT-TGGGCA-3′); and V273Q, sense (5′-TGCCCAATATG-CAGTTTGTT-3′) and antisense (5′-AACAAACTG-CATATTGGGCA-3′). The outside primer set for mutations at positions 173 and 176 was OP170, sense (5′-CAGGGCG-GACGCCCACTTGT-3′), and PXAS6, antisense (5′-TAAA-CACCATGGGAACC-3′). Each sense primer used with the antisense PXAS6 generated a 652 bp fragment, whereas the antisense primer used with the sense primer OP170 generated a 484 bp fragment. The interchanged hybridizing fragment generated by combining the 652 and 484 bp PCR products was used as a primer in a reaction with the primer set, OP170 and PXAS6. The reaction generated a 1136 bp fragment containing the mutation of interest. The fragment was digested with *Apa*I and *Bst*EII; the resulting 1020 bp fragment was ligated into the *ApaI/Bst*EII-digested wild-type expression unit, pHUG-Br1. The replaced fragment contained the two respective mutations. The replaced segment, including the ligation sites, was sequenced to ensure that no other changes occurred in the reading frame. The construction of the other pHUG-Br1 mutants is described in Ciotti *et al*. (1995). The outside primer set for all other mutants was P2S4 (sense) and PXAS6 (antisense); each sense degenerate primer with PXAS6 gave a 341 bp fragment, P2S4 with each antisense degenerate prime gave a 150 bp fragment, and the two fragments together generated a 491 bp fragment which was digested with *Eco*RI/*Bst*EII and cloned into *Eco*RI/*Bst*EII-digested pHUG-Br1.

Expression of Wild-Type and Mutant Expression Units in COS-1 Cells and Immunocomplexing of Protein. COS-1 cells were plated in 100 mm dishes at 10^6 cells and grown to 90% confluency in 24 h in Dulbecco's Modified Eagle's Medium (DMEM) with Hepes buffer and 4% fetal calf serum (FCS). pHUG-Br1 or each of its mutants was transfected into cells using DEAE-dextran as the carrier as described (Ritter *et al.*, 1993; Ciotti *et al*., 1995). The radiolabeling, immunocomplexing, and analysis of expressed wild-type or mutant HUG-Br1 proteins by SDS gel electrophoresis are already described (Ritter *et al*., 1990, 1993).

Assay for 17α-EE and Bilirubin Glucuronidation. Cell homogenate containing expressed wild-type or mutant enzyme was used to determine either 17α-EE or bilirubin glucuronidation as described (Ritter *et al*., 1993) with modifications. The reaction buffers were standardized using between 300 and 600 μg of cell homogenate protein as follows. For the pH 6.4 buffer, 0.1 M sodium phosphate (pH 8.05) was diluted to 20 mM and adjusted to pH 6.4 with 0.65 μL of 1.0 N NaOH/300 μg of protein, and for the pH 7.6 buffer, 0.166 M triethanolamine (pH 8.6) was diluted to 32 mM and adjusted to pH 7.6 with 1.6 μL of 1.0 N NaOH/300 μg of protein. The pH was checked for verification and adjusted, if necessary. The assay conditions and methodology for the glucuronidation of 17α-EE were the same as that for bilirubin except additional NaOH was not required to obtain pH 7.2. Since transfected COS-1 cell homogenates, like hepatocyte microsomes, are latent and require activation/perturbation to allow complete expression of bilirubin transferase activity when analyzed *in vitro*, we used 0.5 mg of 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) per milligram of protein [the zwitterion, CHAPS, can act as an efficient perturbant/solubilizing agent with minimal adverse effects on activity when present at a ratio as high as 1.5 detergent to protein (Mackenzie *et al*., 1984)]. In order to compare the effect of mutations on activity, equal amounts of specific protein were required. [^{35}S]Methionine incorporated into immunocomplexed protein was analyzed on SDS−polyacrylamide gels which were dried and scanned on the Fuji Phosphorimager BAS 2000; the counts were used to establish equivalent wild-type and mutant proteins for the glucuronidation reactions. The amount of 17α-EE and bilirubin glucuronide on TLC

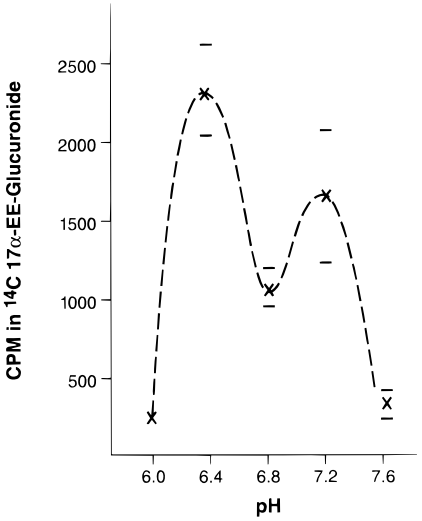

FIGURE 1: pH dependence of 17α-EE glucuronidation using homogenates of transfected COS-1 cells with the pHUG-Br1 expression unit. The glucuronidation of 17α-EE was carried out as described in Materials and Methods using 20 mM sodium phosphate buffer for pH values 6.8 and below or 32 mM triethanolamine for pH 7.2 and above with verification and adjustments, if necessary, before the reactions were initiated. The reaction mixtures contained 1.41 mM [^{14}C]UDPglucuronic acid (1.4 μCi/μmol), 200 μM 17α-EE, and 300 μg of protein of COS-1 cell homogenate; cells were previously transfected with pHUG-Br1 and harvested after 72 h. Glucuronidation incubations were for 16 h at 24 °C. The product was analyzed as described in Materials and Methods.

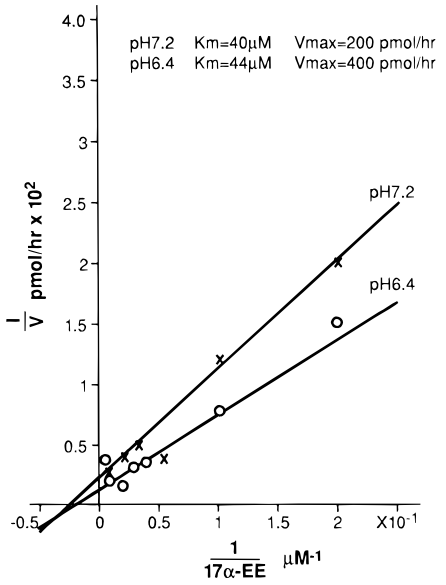

FIGURE 2: Lineweaver−Burk plot of 17α-EE versus reaction velocity of glucuronidation using homogenates of COS-1 cells after transfection with pHUG-Br1. The effect of 17α-EE concentration on velocity of glucuronidation was analyzed using homogenates with a range of 17α-EE concentrations, 1.4 mM [^{14}C]UDPglucuronic acid (1.4 μCi/μmol), and 300 μg of protein with pH adjustments as described in Materials and Methods; incubations were for 2 h at 37 °C.

plates was analyzed on the Ambis Radioanalytical Imaging System Mark II as previously described (Ritter *et al*., 1993). X-ray exposures of the TLC plates were developed for radiograms.

Assay for Sensitivity of Product to β-Glucuronidase Activity. At the end of the glucuronidation incubation, reaction mixtures with either the 17α-EE or bilirubin β-glucuronide formed were untreated or treated with β-glucuronidase at pH 6.8 or 7.0 for 3 h; the samples were then analyzed as usual.

RESULTS

pH Optima for Glucuronidation of 17α-EE by the HUG-Br1 Isoform. Since a main goal of this study was to determine whether the HUG-Br1 protein glucuronidates the exogenous substrate, 17α-EE, under conditions identical to that for its natural substrate, bilirubin, we established the optimal pH conditions for the metabolism of the estrogen derivative. The results in Figure 1 show that *in vitro* glucuronidation of the estrogen derivative has two pH optima of 6.4 and 7.2. These pH values are similar to that established for bilirubin (Ritter *et al*., 1993). Both optima for 17α-EE, like the more acidic one for bilirubin (Ritter *et al*., 1993), are rather sharp, while the higher-pH optimum for bilirubin is generally a plateau between 7.2 and 8.0 (Ritter *et al*., 1993). The level of activity for 17α-EE is approximately 1.3−2.0-fold higher at pH 6.4 than at pH 7.2; for bilirubin, the ratio ranges from 2−3 (Ritter *et al*., 1993). Both substrates are glucuronidated more efficiently under the more acidic condition than under the more alkaline one.

k_m Values for 17α-EE at pH 6.4 and 7.2. Figure 2 shows that the two k_m values for 17α-EE are essentially the same, approximately 40 μM for both pH conditions, but that the

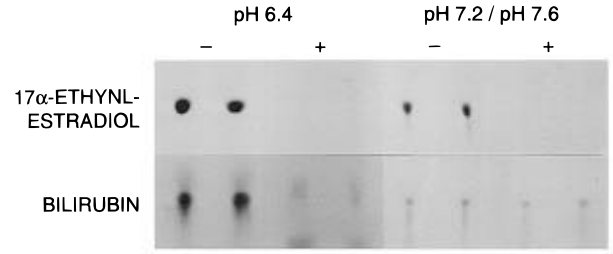

FIGURE 3: Hydrolysis of the 17α-EE and bilirubin β-glucuronides by β-glucuronidase. A homogenate of the pHUG-Br1-transfected COS-1 cells in combination with 1.4 mM [^{14}C]UDPglucuronic acid (1.4 μCi/μmol) and 300 μg of protein with either 200 μM 17α-EE or 100 μM bilirubin was used to generate β-glucuronides of 17α-EE and bilirubin at pH 6.4 and at pH 7.2 (17α-EE) or pH 7.6 (bilirubin) in quadruplets during a 16 h incubation at 24 °C. After the 16 h incubation, half of each set of tubes was untreated (−) or β-glucuronidase-treated (+). *E. coli* β-glucuronidase was added directly to the pH 6.4 and 7.2 reaction mixtures, while the pH 7.6 reaction mixtures were brought to pH 7.0 with 40 mM sodium phosphate (pH 7.0) before adding the enzyme. The 17α-EE and bilirubin reaction mixtures were treated with either 2.5 or 25 units of the enzyme, respectively, for 3 h at 37 °C and then analyzed on TLC plates as previously described (Ciotti *et al*., 1995).

V_{max} values of 400 and 200 pmol per hour per 0.3 mg of cell protein, respectively, are significantly different for pH 6.4 and 7.2. With bilirubin as substrate, the k_m values, approximately 2.5 μM, are the same at both pH values and are 15-fold less than that of this exogenous chemical, 17α-EE.

Sensitivity of Products to β-Glucuronidase. Since glucuronidation reactions are typically carried out at pH 7.2−7.6, it was necessary to demonstrate through their sensitivity to β-glucuronidase that the products generated by HUG-Br1 at pH 6.4 are β-glucuronides. Figure 3 shows that the 17α-EE derivative generated at both pH values and the bilirubin derivative produced at pH 6.4 were completely degraded by the β-glucuronidase; the bilirubin at pH 7.6 was reduced by

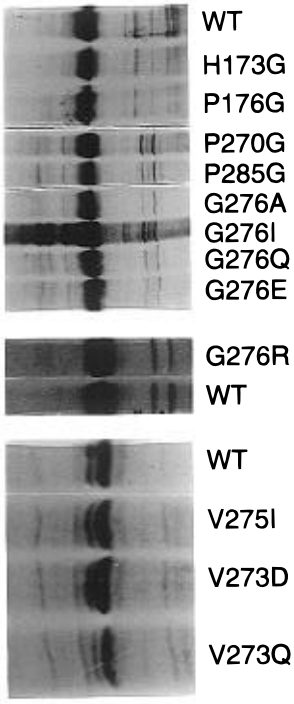

FIGURE 4: Immunocomplexes of the HUG-Br1 isoform or its mutants from COS-1 cells after transfection with pHUG-Br1 or its mutants following [35S]methionine labeling. Cells were transfected with the pHUG-Br1 or with one of its mutants for 72 h, radiolabeled for 4 h with [^{35}S]methionine, solubilized, and immunocomplexed with goat anti-mouse UDPglucuronosyltransferase as described in Materials and Methods. [It has been established that the HUG-Br1 cDNA encodes a 52 kDa protein in COS-1 cells which is immunocomplexed with this antibody (Ritter *et al*., 1993; Ciotti *et al*., 1995).] Washed product was separated on a SDS−polyacrylamide gel during electrophoresis and dried; the specific bands were quantitated by scanning on a phosphorimager as described in Materials and Methods.

26% during the 3 h incubation. Degradation of the bilirubin β-glucuronide required 25 units of this *E. coli* β-glucuronidase, while the 17α-EE product required 2.5 units, signifying greater sensitivity of the steroid glucuronide to this enzyme.

Expression of Wild-Type and Mutant HUG-Br1 Proteins in COS-1 Cells. Since relative amounts of glucuronidation by the wild-type and mutants of HUG-Br1 are to be compared, we first established the relative levels of synthesis for each mutant. Each protein was [^{35}S]methionine-labeled during synthesis as described in Materials and Methods. The demonstration of specific protein for wild-type and each mutant is shown in Figure 4. On the basis of specific counts in the immunoprecipitated protein in each lane, equivalent amounts of wild-type and mutant protein in concurrent cultures/homogenates were established for the glucuronidation assays.

Comparison of Glucuronidation of 17α-EE and Bilirubin by HUG-Br1 Mutants. In order to determine whether the HUG-Br1 isozyme used the identical active center for the glucuronidation of both 17α-EE and bilirubin, we compared the amount of the respective glucuronides formed by mutants of the isozyme. Both glucuronidation assays were carried out concurrently for the same time period on the same cellular preparations with identical amounts of protein and radiolabeled cosubstrate, [^{14}C]UDPglucuronic acid (1.41 mM, 1.4 μCi/μmol). It can be seen in Table 1 that P270G, V273D, and all the G276 mutants were nearly or totally

Table 1: Summary of All the Studies on the Wild-Type HUG-Br1 and Its Mutants Using 1.4 mM [^{14}C]UDPglucuronic Acid (1.4 μCi/μmol) with either 200 μM 17α-EE or 100 μM Bilirubin[a]

HUG-Br1 mutants	% of normal activity pH 6.4 17α-EE	% of normal activity pH 6.4 bilirubin	% of normal activity pH 7.2 17α-EE	% of normal activity pH 7.6 bilirubin
H173R	46	61	37	56
P176G	50	NA	43	NA
P270G	NA	NA	NA	NA
V273D	NA	NA	NA	10
V273Q	94	43	81	42
V275I	17	24	15	15
G276A	NA	NA	NA	NA
G276E	NA	NA	NA	NA
G276Q	NA	NA	NA	NA
G276I	NA	NA	NA	NA
G276R	NA	NA	NA	NA
P285G	98	45	69	65

[a] In each case, the equivalent amount of specific protein, determined by [^{35}S]methionine labeling of parallel cultures, was used in the enzyme studies as described in the legends to Figures 4 and 5. Each analysis was carried out a total of four times using protein from two or more sets of transfections for each expression unit.

inactive for both substrates. V273Q generated 81−94% of normal 17α-EE glucuronide levels, whereas the level dropped to 42% of normal for the bilirubin glucuronide. Additionally, H173R was from 37 to 60% active for both substrates, and the V275I mutant glucuronidated both substrates to a minimal extent, some 15−24% under the two pH conditions. The most distinguishing difference for the production of the two glucuronides occurred with the P176G mutant which showed from 43 to 50% of the normal 17α-EE glucuronidation at the two pH values, but this mutant was totally inactive for glucuronidating bilirubin. A second distinguishing mutant, P285G, showed normal activity for generating 17α-EE glucuronide but was down to 35−74% of normal for the production of the bilirubin derivative. Each experiment in Table 1 was repeated three to four times.

In Figure 5, we show the level of 17α-EE or bilirubin glucuronide generated in typical experiments for some of the HUG-Br1 mutants which were either normal, partially inactivating, or totally inactivating for glucuronidation. It can be seen that P176G was the most distinguishing mutant, showing the total loss of bilirubin turnover with the retention of 50% of the normal turnover of 17α-EE. Further, P285G metabolized 17α-EE to an extent similar to that for the wild-type, but it metabolized bilirubin to only 50% of normal. Generally, the level of product formed for the two substrates was essentially the same despite the 15-fold difference in k_m values.

DISCUSSION

The findings presented here show that, while 17α-EE is an overlapping substrate (Ebner *et al*., 1993) for the major bilirubin transferase, HUG-Br1, under the typical assay condition (pH 7.2), it is also glucuronidated at pH 6.4 and at a rate 1.2−2 times higher than at pH 7.2. This glucuronidation pattern, at least *in vitro*, is similar to that for glucuronidating bilirubin (Ritter *et al*., 1993), its natural substrate. These results with a second substrate confirm that this isoform glucuronidates under the slightly acid condition (pH 6.4), and more efficiently, than at the slightly alkaline one (pH 7.2 or 7.6). Because the HUG-Br1 isoform has a

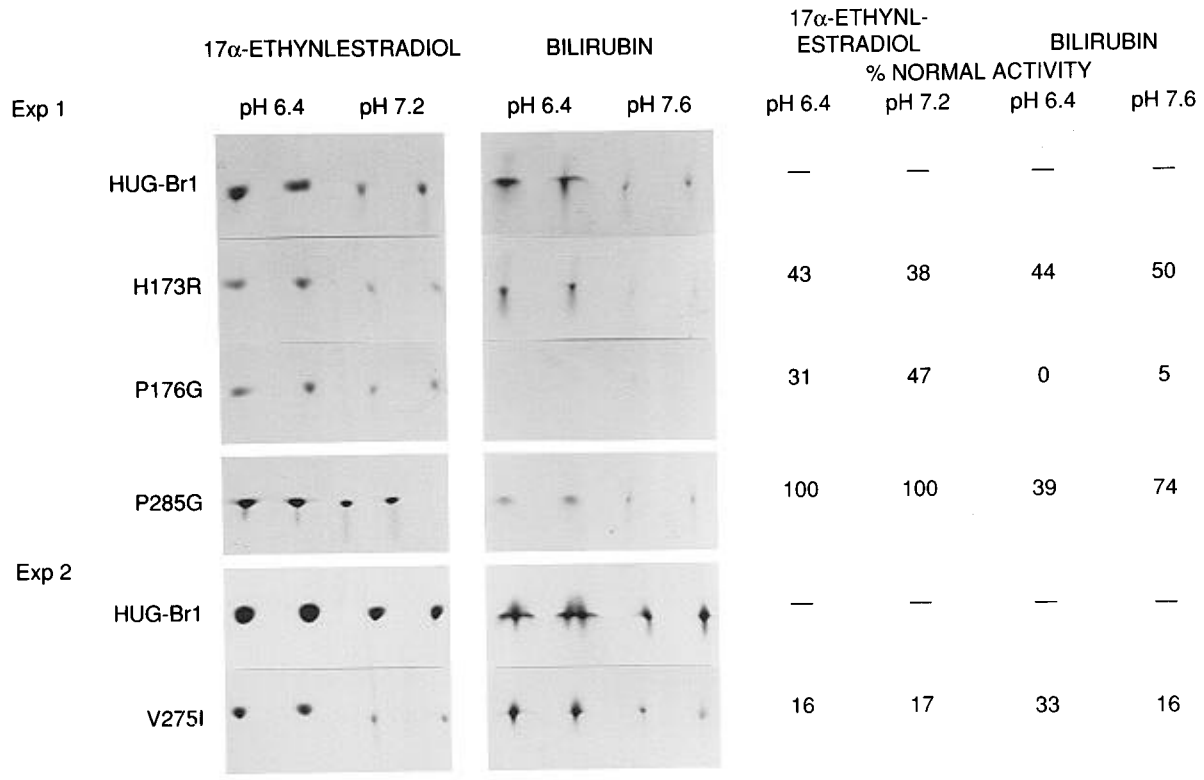

FIGURE 5: Analysis of 17α-EE glucuronide and bilirubin glucuronide generated by the HUG-Br1 isozyme or its mutants. Either the pHUG-Br1 expression unit or its mutant was transfected into COS-1 cells for 72 h. After [^{35}S]methionine labeling, samples were used to quantitate specific protein as described in the legend to Figure 4. Equivalent amounts of the HUG-Br1 protein and its mutants were analyzed for glucuronidation as described in Materials and Methods using 1.4 mM [^{14}C]UDPglucuronic acid (1.4 μCi/μmol) with either 200 μM 17α-EE or 100 μM bilirubin. NA indicates that no activity was detected.

much higher affinity for bilirubin, on the basis of relative k_m values, the isoform will preferentially glucuronidate its natural substrate under competitive conditions. At both pH values per chemical, we obtained a k_m value of 40 μM for 17α-EE versus 2.5 μM for bilirubin (Ritter *et al.*, 1993). Individuals with normal bilirubin activity would necessarily require a significant liver burden of the estrogen derivative to compete effectively with bilirubin (present in serum at ~8.5−12.75 μM) glucuronidation.

Furthermore, we verify through β-glucuronidase sensitivity that the products generated at both pH values are indeed glucuronides. The overall results validate our findings that the HUG-Br1 isoform, at least *in vitro*, has a higher glucuronidating activity at pH 6.4 than at pH 7.6. Presently, the physiological significance of the pH 6.4 activity is not obvious. It is notable that, in two different type I Crigler−Najjar patients who manifested severely reduced bilirubin glucuronidating activity, the expressed mutant cDNAs exhibited significant activity under typical conditions (pH 7.4 or 7.6) (Ritter *et al.*, 1993; Seppen *et al.*, 1995) but expressed none of the major activity (pH 6.4) in the case where it was examined (Ritter *et al.*, 1993). The findings suggest that the pH 6.4 activity plays a significant physiological role. Whether the conditions are obtainable in the endoplasmic reticulum (ER), the site of distribution for this family of isozymes, is debatable. It is known, however, that 5α-reductase (Thigpen *et al.*, 1992) and glucose 6-phosphatase (Arion *et al.*, 1972), integral components of the ER and unrelated to the transferases, have a pH optimum of 5.5 and 6.5, respectively. It is accepted that most UDPglucuronosyltransferases metabolize under the typically more alkaline conditions.

More importantly, the findings in this study address the issue of the nature of the active center(s) for metabolizing two different substrates. For most of the mutants, the nearly parallel level of inactivation of glucuronidation for 17α-EE and bilirubin suggests that a common set of amino acids are involved in the catalysis of the substrates. On the contrary, the total loss of activity for bilirubin by the P176G mutant with the retention of 50% of the normal activity for 17α-EE points to a lack of identity beyond a common group of amino acids responsible for catalyzing the two chemicals. The normal activity for metabolism of the estrogen derivative by the P285G mutant, but only 45−65% of the normal activity for bilirubin, is further evidence in support of variability beyond a common set of amino acids in the HUG-Br1 isozyme with the utilization of additional residues to depend upon the chemical metabolized.

The mutants that inactivate direct attention to critical structural requirements in isoforms. Microregion B (between residues 270 and 288) is conserved in all transferase isoforms (Ciotti *et al.*, 1995), and microregion A (between residues 161 and 180) is contained in at least five bilirubin-like isoforms (Ritter *et al.*, 1993). Within microregion (MR) B, there is a strictly conserved diglycine 276/277 (present in all transferase isoforms). The complete inactivation of both substrate glucuronidations by all the G276 mutants supports the critical role of the diglycine (Ciotti *et al.*, 1995). This diglycine structure, often part of a β-loop in proteins (Thornton *et al.*, 1988; Sibanda *et al.*, 1993), is located near the carboxyl end of the unique and substrate-selecting domain

of transferase isoforms, proteins containing 532 ± 3 amino acids. (The substrate-selecting domain is clearly defined in proteins encoded at the *UGT1* locus which uses alternative exons 1 to define different isoforms.)

In microregions B and A, there are conserved Pro residues at positions 270/285 and at 167/176, respectively. It is realized that all of these conserved Pro residues are not equally critical to activity. That is evident in the metabolism of the two substrates used in this study. On one level, P285G had activity similar to that of the wild-type isozyme for the estradiol derivative and roughly 50% of the normal activity for bilirubin. The P270G mutant, however, was totally inactive. Within the MR A, it is the P176G that generated the greatest distinction at the active site(s); the glucuronidation of 17α-EE was 50% of normal, but that for bilirubin was completely abolished. The Pro residue, creating a ring closure and a restricted angle around the α-carbon of its amide bond in proteins, produces a rigid polypeptide segment with restricted conformational possibilities. Often the residue produces a kink and changes the direction of the peptide chain, and the residue is seldom within an α-helix due to its inability to participate in hydrogen bonding. Although Pro is often classified as an α-helix initiator, it may actually function as a helix breaker (Richardson & Richardson, 1988). On the basis of the location of Pro 176 in a carboxyl position to a clearly hydrophobic MR A (Ritter *et al*., 1993), it is possible that this residue is terminating an α-helix. Pro 167 completely inactivates both 17α-EE and bilirubin glucuronidation (data not shown). The conformation imposed on the active center(s) by the Pro 176 and 285 residues had a different effect on glucuronidation of 17α-EE versus that of bilirubin.

Both chemicals are efficiently metabolized when the slightly basic His residue 173 is replaced with the more basic Arg, the residue that exists at that position in the rat bilirubin isoform (Sato *et al*., 1990). We also point out that position 273 in HUG-Br1, as well as all members of the *UGT1* family, is a hydrophobic Val; in steroid isoforms, it is either the negatively charged Asp or the uncharged polar Gln (Ciotti *et al*., 1995). In this study, the Gln was the most effective for both substrates, while the Asp was essentially totally inactivating.

Although we know very little about the complete amino acid requirements, structure, and conformation at the active center(s) for glucuronidating these two substrates, one can conclude from this study that a single coincident active center for the overlapping substrate activities is not likely for 17α-EE and bilirubin metabolism by the HUG-Br1 isozyme. Rather, it appears that an active center exists which engages additional amino acids depending upon the substrate. This finding argues that the inactivating effects of an amino acid substitution are not necessarily universal; in particular, it appears to depend upon the substrate undergoing metabolism by this isoform.

REFERENCES

Arion, W. J., Wallin, B. K., Carlson, P. W., & Lange, A. J. (1972) *J. Biol. Chem. 247*, 2558−2565.

Ciotti, M., Yeatman, M. T., Sokol, R. J., & Owens, I. S. (1995) *J. Biol. Chem. 270*, 3284−3291.

Crigler, J. F., & Najjar, V. A. (1952) *Pediatrics 10*, 169−180.

de Morais, S. M. F., & Wells, P. G. (1989) *Hepatology 10*, 163−167.

Ebner, T., Remmel, R. P., & Burchell, B. (1993) *Mol. Pharmacol. 43*, 649−654.

Hargus, S. J., Martin, B. M., George, J. W., & Pohl, L. R. (1995) *Chem. Res. Toxicol. 8*, 993−996.

Mackenzie, P. I., Lang, M. A., & Owens, I. S. (1984) *Membr. Biochem. 5*, 193−207.

Miners, J. O., & Mackenzie, P. I. (1991) *Pharmacol. Ther. 51*, 347−369.

Owens, I. S., & Ritter, J. K. (1992) *Pharmacogenetics. 2*, 93−108.

Owens, I. S., Koteen, G. M., & Legraverend, C. (1979) *Biochem. Pharmacol. 28*, 1615−1622.

Richardson, J. S., & Richardson, D. C. (1988) *Science 240*, 1648−1652.

Ritter, J. K., Sheen, Y. Y., & Owens, I. S. (1990) *J. Biol. Chem. 265*, 7900−7906.

Ritter, J. K., Crawford, J. M., & Owens, I. S. (1991) *J. Biol. Chem. 266*, 1043−1047.

Ritter, J. K., Yeatman, M. T., Ferriera, P., & Owens, I. S. (1992) *J. Clin. Invest. 90*, 150−155.

Ritter, J. K., Yeatman, M. T., Kaiser, C., Gridelli, B., & Owens, I. S. (1993) *J. Biol. Chem. 268*, 23573−23579.

Sato, H., Koiwai, O., Tanabe, K., & Kashiwamata, S. (1990) *Biochem. Biophys. Res. Commun. 169*, 260−264.

Seppen, J., Bosma, P. J., Goldhoorn, B. G., Bakker, C. T. M., Roy Chowdhury, J., Roy Chowdhury, N., Jansen, P. L., & Oude Elferink, R. P. J. (1994) *J. Clin. Invest. 94*, 2385−2391.

Sibanda, B. L., & Thornton, J. M. (1993) *J. Mol. Biol. 24*, 229, 428−447.

Spahn Langguth, H., & Benet, L. Z. (1992) *Drug Metab. Rev. 24*, 5−48.

Tephly, T. R., & Burchell, B. (1990) *Trends Pharmacol. Sci. 11*, 276−279.

Thigpen, A. E., Davis, D. L., Milatovich, A., Mendonca, B. B., Imperato-McGinley, J., Griffin, J. E., Francke, U., Wilson, J. D., & Russell, D. W. (1992) *J. Clin. Invest. 90*, 799−809.

Thornton, J. M., Sibanda, B. L., Edwards, M. S., & Barlow, D. J. (1988) *Bioessays* 8, 63−69.

BI960584A