A Functional Role for Histidyl Residues of the UDP-Glucuronic Acid Carrier in Rat Liver Endoplasmic Reticulum Membranes†

Eric Battaglia and Anna Radominska-Pandya*

Department of Internal Medicine, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

Received July 7, 1997; Revised Manuscript Received October 21, 1997⊗

ABSTRACT: Previous studies have documented the presence of protein-mediated transport of UDP-glucuronic acid (UDP-GlcUA) in rat liver endoplasmic reticulum (ER). To determine the crucial amino acids of the membrane transporter and evaluate their function in regulating the glucuronidation reaction, we examined the effect of histidyl-specific irreversible inhibitors on the uptake of radiolabeled UDP-GlcUA in rat liver ER. Inactivation of uptake (initial rate) was more pronounced with hydrophobic reagents [diethyl pyrocarbonate (DEPC), *p*-bromophenacyl bromide] as compared to the more hydrophilic reagent (*p*-nitrobenzenesulfonic acid methyl ester). DEPC was used to further characterize the inhibition because of its greater specificity for protein histidyl residues. While initial [^{14}C]UDP-GlcUA uptake rates were diminished by DEPC treatment of intact microsomes, the accumulation of isotope at equilibrium was not significantly affected, indicating no loss of vesicle integrity. A pK_a of ∼7 for the modified residue(s) of the transporter supported the alkylation of imidazole moieties. Protection against inactivation was observed with UDP-GlcUA as well as other nucleotide-sugars known for their interaction with this transporter. Uptake activity of the transporter (V_{max}) but not UDP-GlcUA binding (K_m) was affected by a limited inactivation. Furthermore, a partial inactivation of the transporter impaired the binding of the photoaffinity label [β-^{32}P]5-azido-UDP-GlcUA to UDP-glucuronosyltransferases (UGTs) in intact, but not in detergent-disrupted, ER vesicles. These results demonstrate the involvement of histidyl residue(s) in the UDP-GlcUA uptake process in rat liver ER, provide additional evidence for the lumenal orientation of the UGT active site, and support the view that translocation of the UGT cosubstrate is a rate-limiting step of the glucuronidation reaction.

Most nucleotide-sugars are synthesized in the cytosol and translocated across the Golgi and endoplasmic reticulum (ER)[1] membranes to the lumenal compartment for multiple glycosylation reactions prior to secretion as processed products *(1)*. These translocations are carried out by different protein carriers in Golgi and ER membranes. Our attention has been focused on the glucuronidation reaction in the ER and transport of UDP-glucuronic acid (UDP-GlcUA), the cosubstrate for the UDP-glucurononsyltransferases (UGTs). A UDP-GlcUA carrier resides in the ER membrane, transferring the sugar-nucleotide, formed from glucose 6-phosphate in the cytosol, to the lumenal face where glucuronidation takes place. Glucuronic acid, through its high-energy form UDP-GlcUA, is incorporated into a variety of compounds, both endogenous [such as bibirubin *(2)*] and exogenous *(3)*, facilitating the excretion of hydrophobic substances from the body. Glucuronic acid conjugation is a detoxification process but is also involved in the biosynthesis of macromolecular structures such as proteoglycans and glycolipids *(4, 5)*. Studies on the regulation of glucuronidation have been performed by focusing on the enzymatic activity of membrane-bound and lumenally oriented UGTs. The transport mechanism of substrates and products of UGTs must also be considered in this regard. Indeed, new inhibitors of protein and lipid glycosylation, targeting the import of sugar-nucleotides in the Golgi-ER network, are emerging as potential therapeutic tools *(6)*.

The UDP-GlcUA transport process in the ER has been phenomenologically characterized: compounds such as UDP-*N*-acetylglucosamine (UDP-GlcNac) *(7, 8)*, UDP-xylose (UDP-Xyl) *(9)*, and 4-nitrophenol- and phenolphthalein-glucuronides *(10)* have been shown to have antiporter characteristics in the UDP-GlcUA uptake process. This antiport could represent a potential driving force for UDP-GlcUA import. We have also shown that uptake data can be fitted to a multicomponent model with two kinetically-distinguishable transport processes *(11)*.

Chemical modification of proteins is well-established as an approach to structure/function studies *(12)*. This method identifies the role of specific amino acid residues in the function of the protein of interest on the basis of the effect

† This work was supported in part by NIH Grant DK-38678. A portion of this work was presented at the 17th International Congress of Biochemistry and Molecular Biology in conjunction with the 1997 Annual Meeting of the American Society for Biochemistry and Molecular Biology, San Francisco, CA, August 24−29, 1997.

* Address correspondence to this author. Telephone: 501-686-5414. Fax: 501-686-6248.

⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1997.

[1] Abbreviations: DEPC, diethyl pyrocarbonate; ER, endoplasmic reticulum; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; UDP-Glc, UDP-glucose; UDP-GlcUA, UDP-glucuronic acid; UDP-GlcNAc, UDP-*N*-acetylglucosamine; UDP-Xyl, UDP-xylose; UGTs, UDP-glucuronosyltransferases; 5N$_3$UDP-GlcUA, 5-azido-UDP-glucuronic acid.

S0006-2960(97)01633-4 CCC: $15.00
Published on Web 01/06/1998

of cross-linking these residues and currently provides the only logical approach for characterization of the UDP-GlcUA transporter protein.

In this work, we have examined the contribution of histidyl residues in the carrier-mediated translocation of UDP-GlcUA across the ER membrane in intact rat liver microsomes. We show that residues modified by histidyl-specific reagents are directly involved in the uptake of UDP-GlcUA. In addition, the results suggest that this transport plays a major role as a rate-limiting factor for the glucuronidation reaction in the ER and clearly demonstrate a lumenal orientation of the UGTs active site.

EXPERIMENTAL PROCEDURES

Materials. Uridine diphosphate glucuronic acid, [*glucuronyl*-^{14}C(U)] (285.2 mCi/mmol) ([^{14}C]UDP-GlcUA), was purchased from American Radiolabeled Chemicals (Saint Louis, MO). Filtron-X and Dimiscint were from National Diagnostics (Manville, NJ), and Soluene 350 was from Packard (Downers Grove, IL). Diethyl pyrocarbonate (DEPC), *p*-bromophenacyl bromide, *p*-nitrobenzenesulfonic acid methyl ester, UDP-GlcUA (triammonium salt), UDP-Glc (disodium salt), UDP-Xyl (sodium salt), and UDP-GlcNAc (sodium salt) were obtained from Sigma (Saint Louis, MO).

Synthesis of [β-^{32}P]UDP-GlcUA and [β-^{32}P]5N$_3$UDP-GlcUA. [β-^{32}P]UDP-GlcUA (specific activity of 5−10 mCi/μmol) was synthesized and purified as previously described *(11)*. The synthesis of [β-^{32}P]5-azido-UDP-glucuronic acid ([β-^{32}P]5N$_3$UDP-GlcUA, specific activity 2−5 mCi/μmol) is described in *(13)*.

Rat Liver Microsomal Preparation. Intact liver microsomes from male rats (Sprague-Dawley, 220−250 g) were prepared, without subfractionation into rough and smooth ER fractions, as previously described *(11)*. Phenylmethanesulfonyl fluoride (PMSF) was omitted from the preparation since we observed an inhibitory effect on the UDP-GlcUA uptake (not shown). Microsomes were rapidly frozen in liquid nitrogen and stored at −80 °C. The protein concentration was determined by the Bradford method *(14)*. The integrity of the microsomal vesicles used for uptake measurement was determined to be greater than 94% by a mannose-6-phosphatase assay as modified by Blair and Burchell *(15, 16)*.

Characterization of the ER membranes with marker enzymes was in accordance with our previous observations *(11)*. No Na^+/K^+ ATPase activity could be detected, suggesting no significant contamination of the preparation with plasma membranes. Galactosyltransferase activities were consistent with minor Golgi contamination, but we have previously determined that the detected UDP-GlcUA uptake activities do not result from this contamination *(11)*. Mannose-6-phosphatase enrichment in the microsomal preparations over the homogenate was ∼4-fold, in accordance with other reports *(11, 17)*.

Inactivation of UDP-GlcUA Uptake. Intact ER vesicles [30 mg of protein/mL in 10 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4, containing 0.25 M sucrose and 1 mM $MgCl_2$] were incubated with DEPC (freshly diluted in ethanol; final concentration 2% v/v), *p*-bromophenacyl bromide, or *p*-nitrobenzenesulfonic acid methyl ester (dissolved in dimethyl sulfoxide, 2% v/v), at concentrations ranging between 1 and 10 mM (as described in the corresponding figure legends), and for 30−60 s. Following inactivation, microsomes were diluted 50 times with ice-cold 10 mM Tris-HCl (pH 7.4) buffer containing 0.25 M sucrose and 1 mM $MgCl_2$ to quench the alkylation, pelleted by centrifugation at 100000*g* at 4 °C for an hour, and resuspended in the same buffer. Assays for UDP-GlcUA uptake were performed by the fast filtration method previously described *(11)*, using either [β-^{32}P]UDP-GlcUA or [^{14}C]UDP-GlcUA (5 μM). As a control, microsomes were subjected to the same procedure in the presence of 2% absolute ethanol (v/v). Uptake at equilibrium was evaluated using [^{14}C]UDP-GlcUA as previously described *(11)*. The pH-dependence of inactivation of uptake by DEPC was studied by incubating the microsomes at 30 mg of protein/mL in 10 mM sodium phosphate buffer containing 0.25 M sucrose and 1 mM $MgCl_2$ at the specific pH values indicated in Figure 4. After rapid mixing of microsomes and buffer, DEPC (5 mM) was added and the mixture was left for 30 s at 25 °C prior to UDP-GlcUA uptake measurement. Since the small volumes used precluded a direct determination of pH, the relative amounts of phosphate buffer needed to reach the desired pH values were estimated by setting up proportionally identical mixtures on larger volumes, allowing direct pH measurement. Control experiments (2% absolute ethanol) were carried out in a similar way to determine total uptake at each pH.

Protection against inactivation by DEPC was studied by preincubating the microsomes (30 mg of protein/mL) with UDP-GlcUA, UDP-Xyl, UDP-GlcNAc, and UDP-Glc for exactly 20 s at 25 °C before incubating with DEPC (5 mM) for 30 s. The external concentration of all UDP-sugars was fixed at 50 μM. In these experiments, the time of preincubation with UDP-sugars and the time of inactivation were minimized to favor *cis*-competition by reducing possible translocation.

Michaelis−Menten parameters were computed by nonlinear regression analysis of the double reciprocal plot of UDP-GlcUA influx versus UDP-GlcUA concentration (1−110 μM) for intact and partially DEPC-inactivated microsomes (5 mM DEPC, 1 min inactivation) using the EnzymeKinetics program (Trinity Software, Campton, NH).

Photoaffinity Labeling with [β-^{32}P]5N$_3$UDP-GlcUA. Intact rat liver microsomes (30 mg of protein/mL) were incubated with DEPC (4 and 8 mM) or ethanol (control, 2% v/v) for 1 min at 25 °C. The inactivation was stopped by a 50-fold dilution in ice-cold 10 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose and 1 mM $MgCl_2$. The diluted microsomes were then centrifuged at 100000*g* for 1 h at 4 °C. Pellets were resuspended in a minimal volume of the same buffer. A portion of the microsomes was treated with Triton X-100 for 10 min on ice at a protein to detergent ratio of 40:1 (w/w), sufficient to disrupt the integrity of the vesicles. Intact and detergent-disrupted ER vesicles were photolabeled as follows: [β-^{32}P]5N$_3$UDP-GlcUA (40 μM) was incubated with microsomal proteins (50 μg) for exactly 20 s at room temperature in 10 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose and 1 mM $MgCl_2$, followed by irradiation for 90 s with a hand-held UV 254 nm lamp (UVP-11, Ultra-violet Products, Inc., Upland, CA). Proteins were processed for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and run on a 10% gel as

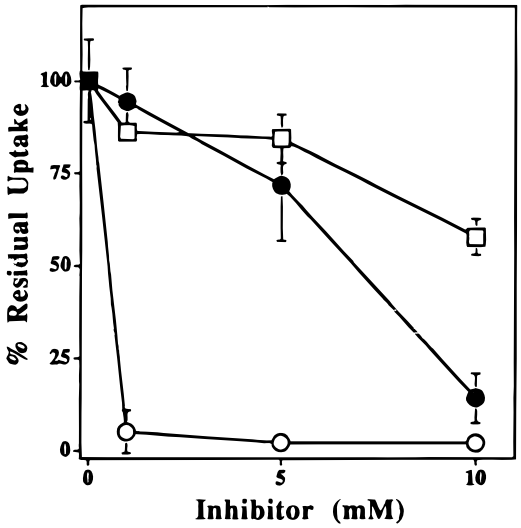

FIGURE 1: Inhibition of microsomal UDP-GlcUA uptake by histidyl-modifying reagents. Rat liver ER membranes (30 mg of protein/mL) were preincubated for 1 min in the presence or absence of *p*-nitrobenzenesulfonic acid methyl ester (□), diethyl pyrocarbonate (●), or *p*-bromophenacyl bromide (○), at the concentrations indicated in the figure. The membranes were then diluted 50 times in ice-cold 10 mM Tris-HCl (pH 7.4) buffer containing 0.25 M sucrose and 1 mM $MgCl_2$, pelleted by centrifugation, and resuspended in the same buffer. UDP-GlcUA transport (15 s) was assayed at 25 °C as described under Experimental Procedures. Values are the mean ± SD for 3 experiments performed on 3 different microsomal preparations.

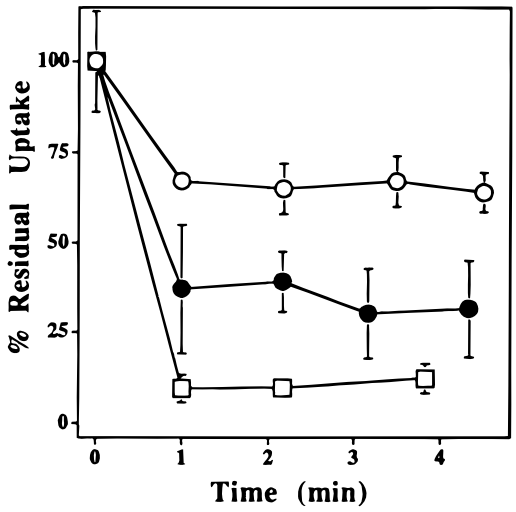

FIGURE 2: Time- and concentration-dependent inhibition by DEPC of UDP-GlcUA transport in ER vesicles. ER membranes (30 mg of protein/mL) were incubated at 25 °C with 2.5 (○), 5 (●), and (□) 10 mM DEPC as indicated in the figure. The membranes were then diluted 50 times in ice-cold 10 mM Tris-HCl (pH 7.4) buffer containing 0.25 M sucrose and 1 mM $MgCl_2$, pelleted by centrifugation, and resuspended in the same buffer. UDP-GlcUA transport (15 s) was assayed as described under Experimental Procedures. Values are the mean ± SD for 3 experiments.

described *(18)*. After electrophoresis, gels were dried and radiolabeled proteins were visualized by autoradiography for 5 days at −80 °C.

RESULTS

Concentration-Dependent Inhibition of UDP-GlcUA Influx by Histidyl-Specific Reagents. In the present work, we employed three imidazole-modifying reagents to delineate the contribution of histidyl residues of the UDP-GlcUA transporter to uptake activity in ER-derived vesicles from rat liver. All three histidyl-selective chemical probes inhibited UDP-GlcUA uptake to various degrees. *p*-Bromophenacyl bromide was the most effective, with almost complete inactivation (95% inhibition) at 1 mM (Figure 1). DEPC inhibited the transport activity in a dose-dependent manner, leading to 86% inhibition at 10 mM. The least potent inhibitor, *p*-nitrobenzenesulfonic acid methyl ester, also inhibited the uptake in a concentration-dependent fashion, but with only 42% inhibition at 10 mM. Inhibition was irreversible as uptake activity was not restored by extensive dilution and washing of microsomes followed by centrifugation and resuspension. DEPC was chosen for further detailed study because it was potent in inhibiting the uptake of UDP-GlcUA, as shown in Figure 1. Furthermore, DEPC is described in the literature as a reagent with relatively high specificity for histidyl residues.

Characterization of the Inactivation of UDP-GlcUA Uptake by DEPC. Previously we described a two-component model for the translocation of UDP-GlcUA in intact ER vesicles *(11)*. The results of this work suggested that multicomponent uptake was probably carried out by a single protein but the presence of multiple carriers could not be ruled out. In order to study the effect of DEPC on the two uptake components, we evaluated dose-dependent inhibition by DEPC of the initial uptake of [^{32}P]UDP-GlcUA at 5 and 75 μM, where the so-called high- and low-affinity components, respectively, would predominate. DEPC inhibition did not discriminate between the two uptake components previously identified (results not shown) which further supports a carrier-mediated process catalyzed by a single protein with two affinities for UDP-GlcUA *(11)*. Consequently, we further characterized the function of histidyl residues of the transporter using a concentration of 5 μM radiolabeled UDP-GlcUA.

Figure 2 shows the time- and concentration-dependent inhibition of UDP-GlcUA uptake by DEPC. Inactivation of UDP-GlcUA uptake by DEPC was rapid, with inactivation taking place within 1 min, and concentration-dependent. Inactivation was not gradual, which precluded the estimation of inactivation rate constants for detailed kinetic analysis.

The effect of DEPC was also evaluated on the uptake at equilibrium (Figure 3). Because of lumenal breakdown of UDP-GlcUA during prolonged incubation and subsequent efflux of [$^{32}P_i$], [^{14}C]UDP-GlcUA (labeled in the glucuronic acid moiety) was used for the determination of accumulated radionuclide. It has been shown that [^{14}C]glucuronic acid resulting from the breakdown of UDP-GlcUA is not transported out of the ER lumen *(17)*. Initial uptake, as well as uptake in the overshoot phase *(7)*, was strongly inhibited by DEPC, while the steady-state reached after 20 min of incubation was unchanged (Figure 3). Therefore, the effect of DEPC was not due to the impairment of membrane integrity but to a direct modification of the protein carrier.

Effect of pH on Inactivation. DEPC reacts much faster with an unprotonated imidazole than with an imidazolium moiety. Modification of the external pH of the microsomes of the transporter prior to inactivation of the uptake by DEPC allows the titration of the essential residues. As shown in Figure 4, the inhibitory potency of DEPC was enhanced as

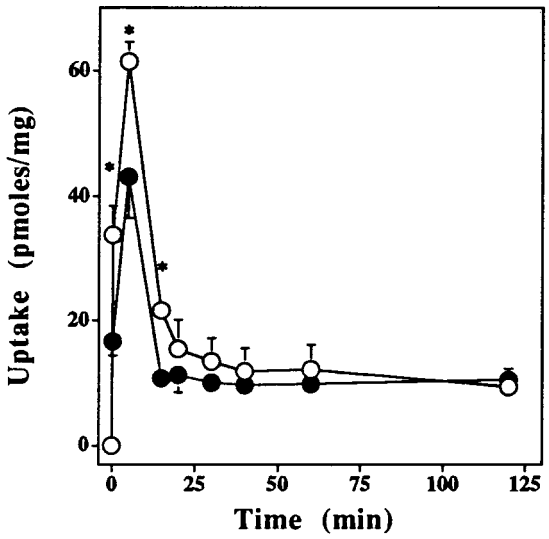

FIGURE 3: Effect of limited inactivation by DEPC on uptake and accumulation of UDP-GlcUA. Microsomes (30 mg of protein/mL) were incubated with (●) or without (○) 5 mM DEPC for 1 min, diluted 50 times in ice-cold 10 mM Tris-HCl (pH 7.4) buffer containing 0.25 M sucrose and 1 mM $MgCl_2$, pelleted, and resuspended in the same buffer. [^{14}C]UDP-GlcUA (5 μM) was used to measure transport activity at 25 °C, and incorporation of radionuclide was determined at the indicated time. Values are the mean ± SD for 3 experiments. Asterisk: Significantly different from control (no DEPC) by Student's *t* test, $P < 0.05$.

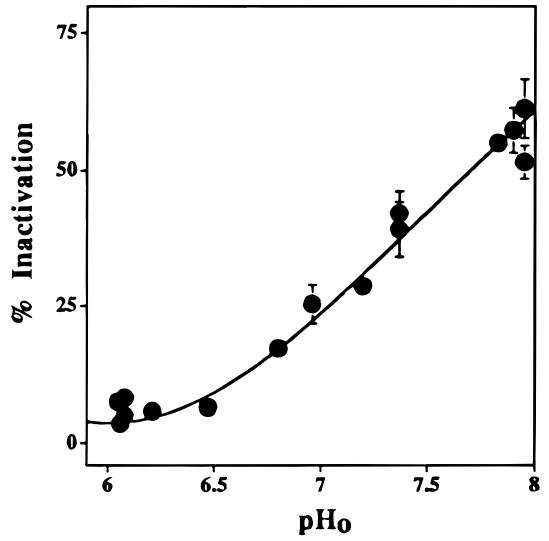

FIGURE 4: pH dependence of the inactivation of UDP-GlcUA uptake by DEPC. Microsomes (30 mg of protein/mL) were mixed with various 10 mM phosphate buffers containing 0.25 M sucrose and 1 mM $MgCl_2$ to obtain an external pH-range (pH_o) between 6.1 and 7.9. DEPC (5 mM) was added, and the initial rate of UDP-GlcUA uptake (15 s) at 25 °C was determined after 30 s of inactivation. Control experiments in which DEPC was omitted were run to evaluate the total uptake activity at the corresponding pH. The data are representative of 1 experiment from a total of 3.

the pH was increased from 6.1 to 7.9, reflecting progressive dissociation of protonated residue(s). The pK_a of the titrated essential residue(s) was ~7.0, which is compatible with carbethoxylation of the imidazole moieties of histidyl residues in proteins.

Effect of Partial Inactivation by DEPC on the Kinetic Parameters of the Transporter. Further understanding of the effect of derivatization of the transporter with DEPC was achieved by evaluating the effect of partial inactivation on the kinetic parameters of the transporter. V_{max} values for both the high- and low-affinity components of UDP-GlcUA uptake were similarly reduced upon partial inactivation, but there was no effect on the apparent K_m for UDP-GlcUA (Table 1). Thus, the histidyl residues identified in this study are not involved in the binding of UDP-GlcUA but in the translocation process itself. V_{max} values are higher than our previously published values for both high- and low-affinity components *(11)*. In the course of this study, we observed that the protease inhibitor PMSF, previously included throughout the microsomal preparation, impaired UDP-GlcUA uptake. This reagent is no longer used. However, it is worth mentioning that inhibition by PMSF did not affect K_m values for either the high- or the low-affinity components [*(11)* and Table 1].

Table 1: Kinetic Parameters of UDP-GlcUA Uptake in Rat Liver Microsomes after Partial Inactivation by DEPC

	high-affinity system		low-affinity system	
	V_{max}[a]	K_m[a]	V_{max}[a]	K_m[a]
control[b]	311.0 ± 19.0	1.5 ± 0.1	557.0 ± 50.0	37.7 ± 6.1
DEPC[c]	112.0 ± 9.0	1.5 ± 0.4	258.0 ± 30.0	38.2 ± 17.0

[a] Parameters described in the table were calculated according to a two-component model (high-affinity and low-affinity components) for uptake of UDP-GlcUA, using EnzymeKinetics. V_{max}, the maximal uptake rate, is expressed, for both components, in pmol min^{-1} (mg of protein)$^{-1}$, and K_m values are in μM. [b] Intact microsomes (2% v/v ethanol). [c] Microsomes modified with 5 mM DEPC for 1 min at room temperature.

UDP-Sugars Protect against Irreversible Inhibition of UDP-GlcUA Influx by DEPC. The effect of *cis*-addition of various UDP-sugars (preincubated with microsomes at a ratio of 10:1 over UDP-GlcUA) on inactivation of uptake by DEPC was studied to determine the location of essential residue(s) identified in this study. Figure 5 shows that UDP-GlcUA, UDP-GlcNAc, and UDP-Xyl reduced the inhibitory effect of DEPC by 75, 53, and 46%, respectively. Conversely, UDP-Glc did not prevent inhibition under the experimental conditions. The protection observed with UDP-sugars is in close agreement with our previous *cis*-inhibition studies of the UDP-GlcUA influx (following this inhibition pattern: UDP-GlcUA > UDP-GlcNAc > UDP-Xyl > UDP-Glc) *(11)*. The protection experiments described support the hypothesis that the locations of essential histidyl residue(s) of the UDP-GlcUA transporter are within or near the UDP-sugar binding site.

Photoaffinity Labeling of UGTs after Treatment with DEPC. Previously, we described a photoaffinity analog of UDP-GlcUA which covalently labeled the active site of UGTs in rat liver microsomes *(13, 18)*. This analog, [β-^{32}P]5N$_3$UDP-GlcUA, was used in the present study as an indicator of UDP-GlcUA transport to investigate the process of UDP-GlcUA translocation as a possible rate-limiting step for the glucuronidation reaction in the lumen of microsomes. Treatment of intact microsomes with DEPC strongly impaired photolabeling of rat liver UGTs (50−54 kDa) (Figure 6). Inhibition of the transporter activity correlated with the decrease of UGT labeling in intact vesicles. When DEPC-treated microsomes were disrupted with Triton X-100 prior to photolabeling, no impairment of photoincorporation of the probe into UGTs was observed (Figure 6). In the latter case, uptake activity could not be detected because of the disrup-

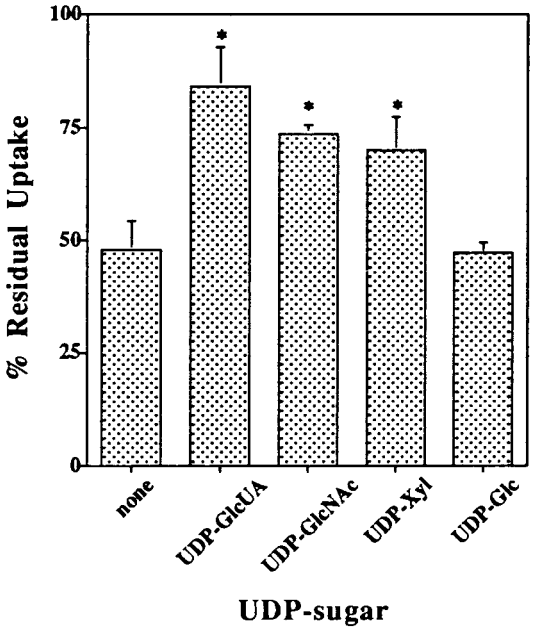

FIGURE 5: Effect of *cis*-addition of UDP-sugars on the modification of UDP-GlcUA transporter by DEPC. Microsomes (30 mg of protein/mL) were preincubated with and without UDP-GlcUA, UDP-GlcNAc, UDP-Xyl, and UDP-Glc for 20 s at 25 °C, followed by incubation with 5 mM DEPC for 30 s. The microsomes were then diluted 50 times in ice-cold 10 mM Tris-HCl (pH 7.4) buffer containing 0.25 M sucrose and 1 mM $MgCl_2$, pelleted by centrifugation, and resuspended in the same buffer. UDP-GlcUA uptake (15 s) at 25 °C was assayed as described under Experimental Procedures. Values are the mean for 3 experiments. Asterisk: Significantly different from control (DEPC, no UDP-sugars) by Student's *t* test, $P < 0.05$.

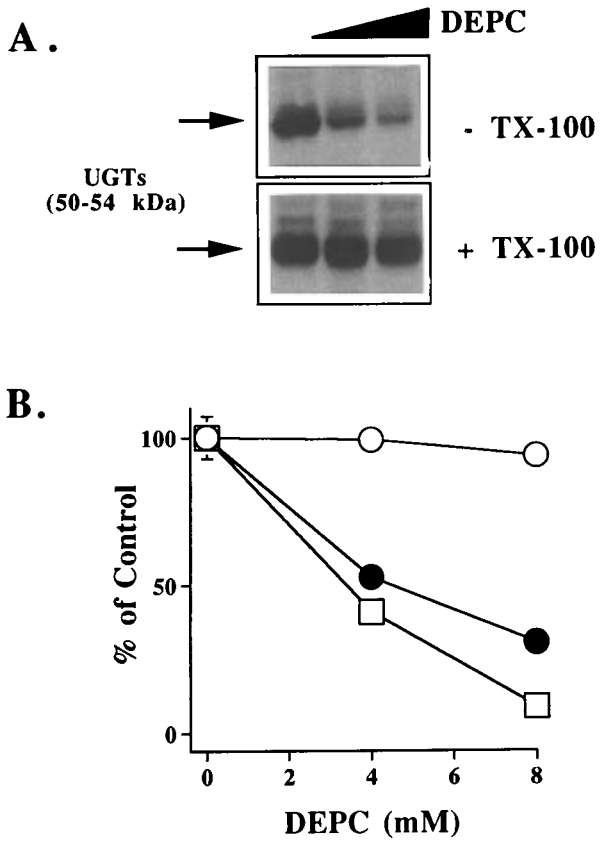

FIGURE 6: Inhibition of UGT photolabeling with [β-^{32}P]5N_3UDP-GlcUA in DEPC-treated ER vesicles. (A) Intact microsomes (30 mg of protein/mL) were incubated with DEPC (4 and 8 mM) or ethanol (control) for 1 min at 25 °C. The inactivation was then quenched by a 50-fold dilution in ice-cold 10 mM Tris-HCl (pH 7.4) buffer containing 0.25 M sucrose and 1 mM $MgCl_2$, and microsomes were pelleted by centrifugation and resuspended in the same buffer. The integrity of a portion of both intact and DEPC-treated microsomes was then disrupted by treatment with Triton X-100 as described under Experimental Procedures. Intact (− TX-100) and detergent-disrupted microsomes (+ TX-100), each containing 50 μg of protein, were photolabeled with 40 μM [β-^{32}P]5N_3UDP-GlcUA for 90 s, and subjected to SDS−PAGE and autoradiography as described under Experimental Procedures. The autoradiograph is one representative experiment from two repeated independently on two different microsomal preparations. (B) UGT photolabeling with [β-^{32}P]5N_3UDP-GlcUA (panel A) in intact (●) and detergent-disrupted microsomes (○) was quantified by densitometric measurement, and the results were expressed as percent of control. Uptake activities (□) at 25 °C were also determined with 5 μM [^{32}P]UDP-GlcUA prior to photolabeling, and results are expressed as percent of control activity.

tion of the vesicle integrity (results not shown). Therefore, protein carbethoxylation by DEPC in ER vesicles induced a concomitant decrease in binding of the photoaffinity analog [β-^{32}P]5N_3UDP-GlcUA to UGTs in intact microsomes, which is due to impairment of the uptake process, and not to a reduced UGT affinity for the photoprobe.

DISCUSSION

In this study, we have addressed the identity of a critical functional group of the UDP-GlcUA transporter in rat liver ER. Our attention was focused on the role of histidyl residues of the transporter. These residues frequently contribute to biochemical processes through substrate binding (H bonds, ionic interaction), cation coordination, or as nucleophilic or acid/base catalysts. We demonstrated the involvement of histidyl residue(s) in the UDP-GlcUA uptake process in rat liver microsomes. Three reagents known to preferably react with histidyl residues, *p*-nitrobenzenesulfonate, *p*-bromophenacyl bromide, and DEPC, were able to inactivate transport of UDP-GlcUA, supporting an inactivation process resulting from modification of one or several imidazole moieties of the transporter. The pH-profile of inactivation of uptake by DEPC also suggested modification of histidyl residue(s) ($pK_a \sim 7.0$). Inhibition by DEPC was due to irreversible modification of the transporter protein itself and was not the result of the spurious side effect of chemical modification, such as impairment of membrane properties. Protection experiments with known substrates and/or *cis*-inhibitors (UDP-GlcUA, UDP-Xyl, UDP-GlcNAc) supported the location of essential residue(s) within or near the UDP-GlcUA binding site. These results also demonstrate that UDP-GlcNAc and UDP-Xyl are likely to bind to a common site with UDP-GlcUA, although a ligand-induced conformational change impairing the inhibition cannot be totally ruled out. The lack of a significant protective effect by UDP-Glc, which did not strongly compete with UDP-GlcUA uptake in *cis*-inhibition experiments *(11)*, further supported the location of an essential residue(s) within the sugar-nucleotide binding site. The residue(s) identified in this work did not appear to be involved in the binding of UDP-GlcUA, but directly in the translocation process as reflected by the effect of limited inactivation on the kinetic parameters.

This work further addressed the possible orientation of the essential residues in the ER membrane. No correlation

between the steric bulk of the reagents and their inhibition potency was apparent from these results (Figure 1). However, the partition coefficient of an inhibitor into the membrane is an important factor when membrane proteins such as transporters are studied. The inhibition potency of histidyl-specific reagents appeared to be stronger with increasing lipophilicity. These results suggested either that the modified histidyl residues were located on the lumenal side of the ER microsomes and modification of the transporter by these reagents was subsequent to membrane partition, or that modified histidyl residues were located in a hydrophobic microenvironment of the transporter. We observed that, first, inhibition did not proceed further upon extensive dilution of inactivated microsomes, suggesting that DEPC exerted its inhibitory effect by interacting with the cytoplasmic side of the transporter. Second, changing the external pH of the vesicles was sufficient to change the reactivity of the residues toward DEPC. Finally, protection against inactivation was observed with *cis*-inhibitors (UDP-sugars) of UDP-GlcUA uptake. The preceding observations support the location of the modified residue(s) on the cytoplasmic side of the transporter. The essential residue(s) could be positioned in a hydrophobic microenvironment of the transporter which may explain the reactivity pattern of the three histidyl-specific reagents used in this study.

Our previous work suggested that the photoaffinity analog of UDP-GlcUA ([β-^{32}P]N_3-UDP-GlcUA) is transported across the ER membrane and subsequently covalently labels UGTs in intact rat liver microsomes *(19)*. Translocation and subsequent binding of photoactive ligands to lumenal proteins have been previously used as evidence of transport in ER vesicles *(19, 20)*. Here, we showed that an intact UDP-GlcUA transporter is a prerequisite for UGT photolabeling by $5N_3$UDP-GlcUA in intact microsomes. Similarly, dose-dependent inhibition of the transporter by *N*-ethylmaleimide resulted in impairment of 4-methylumbelliferone glucuronidation *(21)*, supporting the theory that the transport of UDP-GlcUA is a rate-limiting step for glucuronidation in rat liver microsomes. Therefore, a low rate of UDP-GlcUA uptake is likely to compromise the efficiency of the glucuronidation reaction by depletion of the lumenal UDP-GlcUA pool. Thus, UDP-GlcUA transport is part of the process regulating the UDP-GlcUA level in the ER lumen, thereby contributing to the regulation of glucuronide biosynthesis. Other biochemical pathways could well be affected by the efficiency of UDP-GlcUA translocation since it has been shown that, in the lumen of chondrocytes, UDP-GlcUA can be converted enzymatically to UDP-Xyl, a regulatory step in the elongation of glycosaminoglycan chains of glycoproteins *(4)*. Our photoaffinity labeling experiments in the presence of DEPC also strongly support a lumenal orientation of the UGT active site. The combined application of DEPC inactivation and photoaffinity labeling of microsomal proteins with [β-^{32}P]N_3-UDP-GlcUA is being utilized in our laboratory to identify the protein(s) involved in this carrier-mediated process.

ACKNOWLEDGMENT

We are grateful to Magdalena Mizeracka for her excellent technical assistance. We also thank Dr. Richard R. Drake for synthesizing the [β-^{32}P]N_3-UDP-GlcUA photoprobe, and J. M. Little for critical reading of the manuscript.

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BI9716332