The human $\beta_1,3$-Glucuronosyltransferase I (GlcAT-I) plays a key role in proteoglycan biosynthesis by catalyzing the transfer of glucuronic acid onto the trisaccharide-protein linkage structure Gal$\beta_1,3$Gal$\beta_1,4$Xyl$\beta$-O-Ser, a prerequisite step for polymerization of glycosaminoglycan chains. In this study, we identified His$^{308}$ and Arg$^{277}$ residues as essential determinants for the donor substrate (UDP-glucuronic acid) selectivity of the human GlcAT-I. Analysis of the UDP-glucuronic acid-binding site by computational modeling in conjunction with site-directed mutagenesis indicated that both residues interact with glucuronic acid. Substitution of His$^{308}$ by arginine induced major changes in the donor substrate specificity of GlcAT-I. Interestingly, the H308R mutant was able to efficiently utilize nucleotide sugars UDP-glucose, UDP-mannose, and UDP-N-acetylglucosamine, which are not naturally accepted by the wild-type enzyme, as co-substrate in the transfer reaction. To gain insight into the role of Arg$^{277}$, site-directed mutagenesis in combination with chemical modification was carried out. Substitution of Arg$^{277}$ with alanine abrogated the activity of GlcAT-I. Furthermore, the arginine-directed reagent 2,3-butanedione irreversibly inhibited GlcAT-I, which was effectively protected against inactivation by UDP-glucuronic acid but not by UDP-glucose. It is noteworthy that the activity of the H308R mutant toward UDP-glucose was unaffected by the arginine-directed reagent. Our results are consistent with crucial interactions between the His$^{308}$ and Arg$^{277}$ residues and the glucuronic acid moiety that governs the specificity of GlcAT-I toward the nucleotide sugar donor substrate.

Glycosaminoglycan (GAG)$^1$ side chains of proteoglycans (PGs) are important regulators in a wide range of biological events, such as matrix deposition, cell membrane signal transfer, morphogenesis, cell migration, normal and tumor cell growth, and bacterial and viral infections (1, 2). PGs are abundant polyanionic molecules present in the extracellular matrix and on the cell surface mediating diverse cellular processes through interactions of GAG chains with a variety of protein ligands including growth factors, adhesion molecules, and enzymes. The heparan sulfate (HS) GAG chains attached to PGs located in the plasma membrane are increasingly implicated in the regulation of signal transduction (3, 4). The key role played by HS in the control of the signaling of morphogens such as Hedgehog proteins as well as growth factors during development has been established in Drosophila melanogaster, mouse, and human (for review see Ref. 5). On the other hand, investigation of the role of the chondroitin sulfate (CS) GAG chains of neural PGs such as phosphacan has highlighted their importance in the development of mammalian brain (for review see Ref. 6). The integrity of CS chains of aggrecan, a predominant structural PG of articular cartilage is also essential for maintaining the specific mechanical and functional properties of this tissue (7). Thus, the biological activity of PGs is intimately related to GAG biosynthesis. Indeed, defects in the assembly of GAG chains have severe biological consequences in both vertebrates and invertebrates. Mutations in galactosyltransferase I involved in the initiation of GAG chains are associated with the human inherited Ehlers-Danlos syndrome, which is characterized by many developmental abnormalities (8). The hereditary human multiple exostoses syndrome, which is characterized by skeletal malformations leading in some cases to malignant chondrosarcomas, is caused by defects in EXT genes that encode glycosyltransferases (GT) responsible for the polymerization of HS chains (9).

The assembly of galactosaminoglycan (CS/dermatan sulfate) and glucosaminoglycan (HS/heparin) chains is initiated by the formation of a tetrasaccharide primer, GlcA$\beta_1,3$Gal$\beta_1,3$Gal$\beta_1,4$Xyl$\beta$-Ser, covalently attached to specific serine residues of different core proteins (for review see Ref. 10). This linkage tetrasaccharide serves as a primer for the attachment of either N-acetylgalcosamine (GlcNAc) or N-acetylgalactosamine, thus initiating the formation of HS and CS, respectively. The HS chains are then elongated by the alternate additions of $\beta$-glucuronic acid (GlcA) and $\alpha$-GlcNAc catalyzed by HS polymerase (EXT1/EXT2) (11). Polymerization of the CS chains is performed by the alternate conjugations of $\beta$-GlcA and $\beta$-N-acetylgalactosamine (12).

The final step of the assembly process of the linkage tetrasaccharide sequence is catalyzed by GlcAT-I, a member of the
glucuronyltransferase family catalyzing the formation of GlcAβ1,3Gal linkages on glycoproteins or glycolipids. This enzyme has received much attention because it plays a central role at a branch point common to various GAG chains (13), and it has been suggested to be a regulatory factor in the biosynthesis of GAG in Chinese hamster ovary cells (14). Importantly, we recently demonstrated that the down-regulation of GlcAT-I by interleukin 1 may contribute to the reduced biosynthesis of GAGs observed after treatment of chondrocytes with this proinflammatory cytokine (15). Detailed analysis of the structure-function relationships of human GlcAT-I is thus essential to gain insight into the molecular basis of the assembly of GAG chains in physiological and pathological conditions. Studies involving cDNA cloning and x-ray crystal structure analysis have provided important clues to solve the organization of several GT including GlcAT-I (16–18). Although these proteins have very different primary amino acid sequences, they share several common structural features (19). GlcAT-I is a type II Golgi-resident membrane-bound glycoprotein with a pattern of organization similar to that of other GT members. It consists of a lumen-orientated C terminus containing the catalytic domain followed by an extended region called the “stem region,” a single membrane-spanning region, and a short cytoplasmic N terminus. We have been deeply involved in elucidating the determinants governing the structure and function of human GlcAT-I (15, 20). We recently demonstrated that the enzyme is organized as a homodimer as a result of disulfide bond formation mediated by Cys323 in the stem region (20). However, the molecular basis for the specificity of GlcAT-I toward the nucleotide sugar donor substrate UDP-GlcA has not yet been elucidated. To gain insight into the molecular details of GlcA binding and transfer on the terminal galactose residue of the growing linkage region Galβ1,3Galβ1,4Xylβ-O-Ser, we have engineered GlcAT-I point mutations based on available structural data combined with computational modeling. We examined the consequences of amino acid substitutions on the specificity of GlcAT-I toward UDP-galacturonic acid (UDP-GalA), UDP-glucuronic acid (UDP-GlcA), UDP-glucose (UDP-Glc), UDP-galactose (UDP-Gal), UDP-N-acetylgalactosamine (UDP-GlcNAc), UDP-mannose (UDP-Man), galactosyl-β1,3,4-thiogalactose (Gal-Gal), β-glucuronidase (bovine liver), β-glucosidase (Caldocellulium saccharolyticum), aniribine, anti-rabbit alkaline phosphatase-conjugated immunoglobulins, and methanol were from Sigma. GDP-mannose (GDP-Man) was from CN Biosciences (Nottingham, UK). 2,3-Butanediol was purchased from Fluka (L’Aube, France). UDP-glucuronic acid (UDP-GlcA) was obtained from Roche Molecular Biochemicals. Bacterial and yeast culture media were from Difco (Le Pont de Chaix, France). The protein assay reagent was obtained from Bio-Rad. The restriction enzymes and Vent DNA polymerase were provided by New England Biolabs (Hitchin, UK). T4 DNA ligase, the α XIII DNA ligase, the P. pastoris SMD1168 yeast strain using the Easy-Comp kit (Invitrogen). Transformsants were selected on YPD plates (2% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose) containing 100 μg/ml of Zeocin. The cells were grown in BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) potassium phosphate, pH 6.0, 1.34% (w/v) yeast nitrogen base, and 1% (v/v) glycerol). Expression was induced in BMGY medium with 1% (v/v) glycerol replaced by 2% (v/v) methanol and carried out for 48 h at 30 °C in a rotary shaker (215 rpm). Subcellular Fractionation and Protein Analysis—Yeast cells were harvested by centrifugation at 3,000 × g for 10 min and further submitted to subcellular fractionation as previously described (22). Briefly, after harvest, the cells were washed once and suspended in cold breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 5% (v/v) glycerol). The cells were then broken with glass beads. The resulting homogenate was centrifuged at 5,000 × g for 15 min, and the supernatant was further centrifuged at 12,000 × g for 20 min. The membranes were pelleted from the supernatant for 1 h at 100,000 × g at 4 °C. The membrane fraction was resuspended by Dounce homogenization in 0.25 M sucrose, 5 mM Heps buffer (pH 7.4). The protein concentration was evaluated by the method of Bradford (23) before analysis by SDS-PAGE. For reducing conditions (24). Immunoblotting was performed using a polyclonal anti-GlcAT-I antibody and alkaline phosphatase-conjugated anti-rabbit immunoglobulins as secondary antibodies, as previously described (20). Analysis of GlcAT-I Activity—Activity of the recombinant human GlcAT-I was evaluated using Gal-Gal as an acceptor substrate as previously described (20). Briefly, standard incubations were performed in 100 mM sodium acetate buffer (pH 5.0) with 10 μM GlcAT-I. 10–50 μg of membrane protein, 1 mM Gal-Gal, and 1 mM UDP-GlcA in a total volume of 100 μl. The mixture was incubated at 37 °C for 60 min, and the reaction was terminated by placing the tube on ice and adding 10 μl of 6 N HCl. UDP-GalA, UDP-Glc, UDP-Gal-GlcNAc, UDP-Mann, and UDP-Gal-Gal were similarly tested as nucleotide sugar donor substrates. The proteins were then precipitated by centrifugation, and the enzymatic reaction product was analyzed by high performance liquid chromatography (HPLC) after chromophore labeling by reductive amiation using aniline as previously described (20, 25). The labeled water-soluble product was analyzed by HPLC on a reverse phase C-18 column (4.6 × 150 mm, 4 μm; Waters, Milford, MA) at a detection wavelength of 280 nm according to a method adapted from Pless et al. (26). The mobile phase was composed of 5% (v/v) acetonitrile, 0.015% (v/v) formic acid, and 0.03% (v/v) triethylamine in water (pH 4.0) and used at a flow rate of 0.5 ml/min. Control assays in which either the donor substrate (UDP-GlcA or the other UDP-sugars) or the acceptor substrate Gal-Gal was omitted were simultaneously run under the same conditions. Because the labeled disaccharide and trisaccharide reaction products possess the same extinction coefficient, quantification of the trisaccharide product was performed by comparison with a standard curve established upon injection of increasing amounts of authentic Gal-Gal following chromophore labeling. The response (peak area) was linear from 0.05 to 5 nmol of Gal-Gal injected. The detection limit was 25 pmol of product, under our experimental conditions. Addition of a GlcA residue on the nonreducing end of Gal-Gal was verified by the susceptibility of the products to hydrolysis by β-glucu-
ronidase from bovine liver. /\textit{β}-Glucuronidase (600 units) was dissolved in 200 mM sodium acetate buffer (pH 5.5) and added to 100 μl of the reaction mixture. The incubation was conducted for 4 h at 37 °C. To verify glucose transfer onto the Gal-Gal acceptor, /\textit{β}-glucosidase from \textit{C. saccharolyticum} (10 units) was dissolved in 50 mM sodium phosphate buffer (pH 5.5) and added to 100 μl of the reaction mixture as described above. The reaction was conducted for 6 h at 37 °C and stopped by cooling on ice. The disappearance of the reaction product following /\textit{β}-glucuronidase or /\textit{β}-glucosidase treatment was monitored by HPLC as described above. The apparent kinetic parameters (\( K_m \) and \( V_{\max} \)) were deter-

FIG. 1. Specificity of the human recombinant GlcAT-I toward the UDP-sugar co-substrate. \( A \), activity of the wild-type enzyme evaluated in membranes upon expression in the yeast \textit{P. pastoris}, using UDP-GlcA (column a), UDP-GalA (column b), UDP-Glc (column c), UDP-Gal (column d), UDP-Man (column e), and UDP-GlcNAc (column f) as donor substrate. The results are the means ± S.D. of three determinations. The structure of the corresponding UDP-sugars is represented below the histograms. \( B \), chromatograms of the HPLC resolution of the reaction products after incubation of recombinant wild-type GlcAT-I with UDP-GlcA (line 1) or UDP-GalA (line 2) or without donor substrate (line 3) and labeling with anilin by reductive amination. The incubation conditions and HPLC analysis of the reaction products have been described under “Experimental Procedures.” Peak a, nucleotide sugar; peak b, product of the reaction; peak c, Gal-Gal substrate.
determined using linear least squares regression analysis of double-reciprocal plots of initial velocity versus UDP-sugar (0–2 mM) at a constant concentration of Gal-Gal (1 mM).

**Chemical Modification by 2,3-Butanedione**—Inactivation of wild-type GlcAT-I and of the H308R mutant with the arginine-directed reagent 2,3-butanedione was performed at 25 °C in the dark using 30 μg of protein of membrane fraction in 100 mM sodium acetate buffer (pH 5.0) containing 10 mM MnCl2. Inactivation was started by the addition of 2,3-butanedione (0–15 mM) freshly prepared in ice-cold absolute ethanol. The aliquots were removed at times 0–20 min and quenched by a 10-fold dilution in buffer and activity toward UDP-GlcA or UDP-Glc was determined as described above. For protection experiments, UDP-GlcA or UDP-Glc (10 mM) was preincubated with proteins for 15 min before the addition of 2,3-butanedione. In a second set of experiments, the membrane fractions from recombinant yeast cells were treated with 2,3-butanedione (15 mM for 20 min), pelleted by centrifugation, and washed twice in 0.25 M sucrose, 5 mM HEPES buffer (pH 7.4) before activity measurements as above.

**Molecular Modeling**—The primary model for our calculations was the x-ray structure of the human GlcAT-I bound to UDP and substrate refined at 2.3 Å (17). Modeling and docking of substrates were carried out with SCULPT version 3.0 (Molecular Designs, Ltd., San Leandro, CA) (28), mutagenesis with S-PDB Viewer molecular graphics (29), and protein analysis using RASMOL v.2.7.1.1 (30). The software was run on a PC. Docking experiments were conducted following three steps: (i) binding of substrates by tethering UDP part to UDP template of the crystal structure (Protein Data Bank code 1FGG) and then relaxing the substrate to minimize global free energy (all backbone and side chains were kept frozen); (ii) tethering off and deleting template UDP to substrate to minimize global free energy (all backbone and side chains thawing side chains of residues in contact with the substrate for a last energy minimization run, keeping both Gal residues of the acceptor substrate frozen. This overall process was repeated 20 independent times for each complex studied.

**RESULTS**

**Specificity of Wild-type GlcAT-I Is Restricted to Hexuronic UDP Sugars**—The specificity of the recombinant GlcAT-I toward the UDP sugar donor substrate was analyzed using a series of analogs of UDP-GlcA, UDP-GalA, UDP-Glc, UDP-Gal, UDP-Man, and UDP-GlcNAc (Fig. 1A). Chromatograms of the HPLC resolution of the enzymatic product formed with UDP-GlcA (chromatogram 1, peak b) or UDP-GalA (chromatogram 2, peak b) as co-substrate are illustrated in Fig. 1B. No detectable product was observed when incubation was performed in the absence of donor substrate (Fig. 1B, chromatogram 3) or after treatment by β-glucuronidase (data not shown). As expected, the recombinant wild-type enzyme exhibited the highest activity toward UDP-GlcA reaching about 70 pmol/min/mg–1 protein in the membrane fraction of recombinant yeast cells (Fig. 1A, column a). Interestingly, GlcAT-I was also able to efficiently catalyze the transfer of GaLA from UDP-GalA onto the acceptor digalactose substrate. The activity toward UDP-GalA was about 35 pmol/min/mg–1 protein (Fig. 1A, column b). By contrast, the other nucleotide sugars tested (UDP-Glc, UDP-Gal, UDP-Man, and UDP-GlcNAc) (Fig. 1A, columns c–f, respectively) were not substrates of the recombinant GlcAT-I. The membrane fraction of yeast cells transformed with a nonrecombinant plasmid, used as a control, did not exhibit any detectable activity toward all of the UDP-sugars tested (data not shown).

It thus appears that the presence of a carboxyl group on C-6 of the carbohydrate moiety of the UDP sugar (i.e. UDP-GlcA, UDP-GalA) was a determinant for the donor substrate to accommodate the binding site of GlcAT-I. Interestingly, the fact that UDP-GalA was an alternative donor substrate of GlcAT-I suggested that the active site of the enzyme can accommodate the carbohydrate ring with C-4 hydroxyl in α and β configuration as long as it contains a carbonyl group at C-6 position.

**The Active Site His308 Residue Is a Determinant for the Co-substrate Specificity of the Human GlcAT-I**—The x-ray structure of the catalytic domain of GlcAT-I bound to UDP and acceptor substrate has been recently solved (17). Analysis of the three-dimensional structure revealed that the histidine at position 308 is close to the β-phosphate of UDP and to the O-3 hydroxyl of the terminal galactose, suggesting the possibility that this residue is part of the active site. In addition, His308 is in a remarkably conserved WHRRTRT motif located at the C-terminal end of the GlcAT protein family. We therefore investigated the role of His308 by alanine and arginine substitution to assess the contribution of the size of the side chain of the amino acid and the presence of nitrogen, respectively. Upon expression of the mutants in P. pastoris, similar amounts of wild-type and mutant proteins were produced (data not shown). Replacement of the His308 residue by alanine abrogated the activity of GlcAT-I toward UDP-GlcA, indicating that this histidine residue is crucial for enzyme activity (Fig. 2A, column a). The H308A mutant was not active toward any of the co-substrate analogs UDP-Glc, UDP-Gal, UDP-Man, or UDP-GlcNAc tested (columns b to e, respectively). Interestingly, replacement of the His308 residue by an arginine induced a major change on the GT specificity of the GlcAT-I enzyme. In contrast to the wild-type enzyme, the H308R mutant was able
to efficiently transfer Glc from UDP-Glc onto the acceptor substrate Gal-Gal as evidenced by the HPLC profile presented in Fig. 2B (chromatogram 1, peak b). The structure of the reaction product was further confirmed by digestion with β-glucosidase (not shown). The activity toward UDP-Glc was up to 40 pmol-min$^{-1}$-mgP$^{-1}$ (Fig. 2A, column g). The H308R mutant remained able to catalyze the transfer of GlcA from UDP-GlcA onto the acceptor substrate as evidenced by HPLC analysis (Fig. 2B, chromatogram 2, peak b) but with a 3.5-fold lower activity compared with the wild-type enzyme (Fig. 2A, column f). UDP-GlcNAc was used by the mutant at about the same rate as UDP-Glc (Fig. 2A, column j), whereas UDP-Gal was a weak donor substrate in the transfer reaction (Fig. 2A, column h). Furthermore, UDP-Man was efficiently used as co-substrate (Fig. 2A, column i), whereas no activity could be detected in the membrane fraction of recombinant yeast cells expressing H308R with GDP-Man as a donor substrate.

To provide further information on the functional consequences of the histidine substitution by arginine, we compared the kinetic properties of the H308R mutant with those of the wild-type enzyme. The values obtained by Lineweaver-Burk plots are summarized in Table I. As expected from the data reported above, the efficiency of the H308R mutant toward UDP-GlcA estimated by the $V_{\text{max}}/K_m$ ratio was substantially reduced (2.4-fold) compared with the wild-type enzyme because of a decrease in the apparent $V_{\text{max}}$ value. In addition, the H308R mutant enzyme displayed significant GT activities toward UDP-Glc, UDP-Man, and UDP-GlcNAc, with $V_{\text{max}}$ values in the same range. However, compared with UDP-GlcA, the $K_m$ values were 5- and 2-fold higher in the case of UDP-Glc and UDP-GlcNAc, respectively.

**Investigation of the GlcA-binding Site by Molecular Modeling**—To further investigate His$^{308}$-GlcA interactions, a model of the active site of GlcAT-I in complex with UDP-GlcA was generated based on the crystal structure of the protein bound to UDP and acceptor substrate (Protein Data Bank code 1FGO) (17). Docking experiments were carried out using SCULPT software (28) to determine the orientation and conformation that GlcA moiety of UDP-GlcA may adopt in the GlcAT-I active site (Fig. 3). Analysis of the GlcA interactions by modeling revealed two important sugar-binding site residues, His$^{308}$ and Arg$^{277}$ (Fig. 3). Interestingly, our computational model pointed out the importance of His$^{308}$ as the NE nitrogen of this residue was within hydrogen bond distance to the hydroxyl at the C-2 and C-3 positions of GlcA. Furthermore, this model revealed that O-1 and O-2 of the carboxylate at the C-6 position of GlcA were within hydrogen bond distance to NH$_2$ nitrogen of the Arg$^{277}$ residue.

**Butanedione Irreversibly Inhibited GlcAT-I Activity toward UDP-GlcA**—Incubation of membranes of yeast cells expressing the wild-type enzyme with the arginine-directed reagent 2,3-butanedione resulted in a strong dose- (Fig. 4A) and time-dependent (Fig. 4B) decrease of GlcAT-I activity. Up to 70% of the initial activity was lost upon addition of the 2,3-butanedione and was not recovered after washing the membranes to remove the reagent (data not shown). These results indicated that the 2,3-butanedione produced an irreversible inactivation. However, when UDP-GlcA was preincubated with the membrane fraction prior to the addition of 2,3-butanedione, the enzyme was efficiently protected against inactivation, because 90% of the initial activity was recovered, whereas no protection was observed in the presence of UDP-Glc (Fig. 4B). In the case of H308R, 2,3-butanedione exerted no effect on the activity of this mutant toward UDP-Glc (Fig. 4) but abrogated the activity of H308R toward UDP-GlcA (data not shown). These results support the idea that 2,3-butanedione reacts with an arginine residue involved in UDP-GlcA interactions but not with UDP-Glc. It is thus reasonable to suggest that Arg$^{277}$ may be the target of the dicarbonyl reagent 2,3-butanedione.

**Arg$^{277}$ Is Required for the Glucuronosyltransferase Activity of GlcAT-I**—In an attempt to determine the importance of Arg$^{277}$ in the GlcA-binding site, a mutant form of GlcAT-I was engineered with alanine substitution at position 277. We also constructed and expressed a double H308R/R277A mutant to assess the influence of Arg$^{277}$ on the engineered glucosyltransferase activity of the H308R mutant. Importantly, mutation of Arg$^{277}$ to alanine suppressed GlcAT-I activity toward UDP-GlcA, consistent with a crucial role of this residue in GlcA binding (Fig. 5, compare columns c and a). Similarly, the H308R/R277A mutant did not display any activity when UDP-GlcA was used as a donor substrate (Fig. 5, compare columns g and e). However, this mutant was active toward UDP-Glc as in the case of the single H308R mutant (Fig. 5, compare columns h and f). The $V_{\text{max}}$ and $K_m$ values for the double mutant H308R/R277A toward UDP-Glc (36 nmol-min$^{-1}$-mgP$^{-1}$ protein and 436 μM, respectively) were in the same range with those determined for the single H308R mutant. These results indicated that Arg$^{277}$ was not required for the use of UDP-Glc by the mutant H308R.

**DISCUSSION**

GlcAT-I has recently attracted considerable attention because the enzyme catalyzes the final step of the formation of the glycopeptide sequence priming different GAG chains, a prerequisite reaction for the conversion of core proteins to functional PG. This paper focuses on the analysis of the specificity of the transfer reaction catalyzed by the human GlcAT-I toward the high energy UDP-GlcA donor substrate. Combination of site-directed mutagenesis and computational modeling allowed us to identify crucial residues for GlcA recognition.

X-ray crystallographic data of the catalytic domain of GlcAT-I in complex with UDP and the acceptor substrate indicated that the residue His$^{308}$ is located within the vicinity of UDP and the terminal galactose of the acceptor substrate.
and may be involved in protein-substrate interactions (17). In addition, comparison of the primary sequence of the members of the GlcA-T family together with structure-based alignment of GT showed that the invariant residue His308 is located in a conserved structural domain, suggesting an important role in the function of the protein. This assumption was confirmed in this work by the observation that mutation of His308 to alanine led to a fully inactive enzyme. In addition, analysis of the activity of the H308R mutant revealed a dramatic change in the donor substrate specificity, because UDP-Glc, UDP-Man, or UDP-GlcNAc could be efficiently transferred to the digalactose acceptor substrate, in contrast to the wild-type enzyme, which presented a restricted specificity toward UDP-hexuronic acids.

To provide further information on the crucial role of His308 in the co-substrate specificity, a structural model of GlcAT-I in complex with both the donor and acceptor substrates was constructed to predict the organization of the GlcA-binding site of the human GlcAT-I protein. Analysis of our model indicated that His308 played a major role as a hydrogen bonding partner of the GlcA moiety. Consistently, substitution of His308 by alanine led to a complete loss of catalytic activity resulting from disruption of hydrogen bond interactions. In the same manner, Mirza et al. (31) recently showed the importance of a histidine residue bonded to the O-2 and O-3 of glucose in a
hydrogen bond network governing the interactions of sucrose with the amylomaltase from *Neisseria polysaccharea*. When His<sup>308</sup> was substituted with arginine, the NE nitrogen of this residue was within hydrogen bonding distance to the hydroxyl group on the C-2 of GlcA. In addition, the longer side chain of arginine compared with histidine provided a steric hindrance to the sugar moiety. This effect together with the electronic constraint provided by the hydrogen bond between NE nitrogen of this moiety to the Gal-Gal acceptor substrate, allowing the use of UDP-Glc, UDP-Man, and UDP-GlcNAc as a donor substrate. However, the replacement of His<sup>308</sup> by arginine did not modify the specificity of the enzyme toward the nucleotide part of the co-substrate because UDP-Man was not used as a donor substrate by the mutant.

On the other hand, the restricted specificity of the wild-type GlcAT-I toward UDP-GlcA and UDP-GalA led us to investigate for the presence of active site amino acid residues in the vicinity of the sugar moiety that could interact with the carboxyl group at the C-6 position. Our results provided evidence that inactivation of GlcAT-I by the arginine-directed reagent 2,3-butanedione was efficiently protected by UDP-GlcA but not by UDP-Glc, suggesting that the chemical-targeted residue interacts with the glucuronic part of the co-substrate. In the case of the H308R mutant, the glucosyltransferase activity was insensitive to treatment with 2,3-butanedione. The insensitivity of the glucosyltransferase activity of the mutant H308R toward 2,3-butanedione treatment indicated that this reagent may not affect the Arg<sup>308</sup> residue. This may be due to possible interactions of the guanidinium group with other amino acids, preventing its accessibility to 2,3-butanedione. On the other hand, it cannot be ruled out that chemical modification of the guanidinium group did not affect the interaction of this residue with the sugar part of UDP-Glc. It is noteworthy that according to our model, the amino group of Arg<sup>308</sup> predicted to interact with Glc moiety (i.e. NE nitrogen) is not a target of 2,3-butanedione modification.

We show in this study that a single amino acid change (His<sup>308</sup> to arginine) can dramatically affect the donor substrate specificity. In particular, the H308R mutant described in this work presents a significant flexibility with respect to the UDP sugar donor substrate. In the literature, there are few examples of changing the specificity of glycosyltransferases. Deletion of a five-amino acid loop and insertion of an aromatic residue in the active site of a maltogenic α-amylase converted this enzyme into a cyclodextrine GT (34). Another example of such conversion/alteration was recently reported by Wakaharuk et al. (35), who showed that a single amino acid substitution produced an engineered sialyltransferase able to catalyze the transfer of N-acetylenuraminic acid at the O-6 position in addition to the O-3 position of a galactose of *N. meningitidis* cell surface lipooligosaccharide. Interestingly, we were able to engineer a GlcAT-I form that can use a variety of UDP sugars as a donor substrate. This mutant with higher promiscuity opens up the possibility of generating libraries of carbohydrate epitopes in a combinatorial fashion. Such an approach has been recently illustrated by the production of NDP sugar donor libraries using a genetically engineered nucleotidyltransferase to produce metabolites with potential therapeutic value (36). The engineered GlcAT-I enables enzyme-assisted synthesis of GAG oligosaccharides with different structures. This approach constitutes an important issue in the design of new bioactive molecules.

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