Mutation of Arginine 228 to Lysine Enhances the Glucosyltransferase Activity of Bovine β-1,4-Galactosyltransferase I[†,‡,#]

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Received September 22, 2004; Revised Manuscript Received December 30, 2004

ABSTRACT: β-1,4-Galactosyltransferase I (β4Gal-T1) normally transfers Gal from UDP-Gal to GlcNAc in the presence of Mn^{2+} ion (Gal-T activity) and also transfers Glc from UDP-Glc to GlcNAc (Glc-T activity), albeit at only 0.3% efficiency. In addition, α-lactalbumin (LA) enhances this Glc-T activity more than 25 times. Comparison of the crystal structures of UDP-Gal- and UDP-Glc-bound β4Gal-T1 reveals that the O4 hydroxyl group in both Gal and Glc moieties forms a hydrogen bond with the side chain carboxylate group of Glu317. The orientation of the O4 hydroxyl of glucose causes a steric hindrance to the side chain carboxylate group of Glu317, accounting for the enzyme's low Glc-T activity. In this study, we show that mutation of Arg228, a residue in the vicinity of Glu317, to lysine (R228K-Gal-T1) results in a 15-fold higher Glc-T activity, which is further enhanced by LA to nearly 25% of the Gal-T activity of the wild type. The kinetic parameters indicate that the main effect of the mutation of Arg228 to lysine is on the k_{cat} of Glc-T, which increases 3−4-fold, both in the absence and in the presence of LA; simultaneously, the k_{cat} for the Gal-T reaction is reduced 30-fold. The crystal structure of R228K-Gal-T1 complexed with LA, UDP-Gal, and Mn^{2+} determined at 1.9 Å resolution shows that the Asp318 side chain exhibits a minor alternate conformation, compared to that in the wild type. This alternate conformation now causes a steric hindrance to the O4 hydroxyl group of the Gal moiety of UDP-Gal, probably causing the dissociation of UDP-Gal and the reduced k_{cat} of the Gal-T reaction.

β-1,4-Galactosyltransferase I (β4Gal-T1,[1] EC 2.4.1.38) normally transfers Gal from UDP-Gal to GlcNAc in the presence of Mn^{2+} ion, known as Gal-T activity (*1*). The presence of α-lactalbumin (LA) alters the Gal acceptor specificity from GlcNAc to Glc (*2*). Over the past 25 years, extensive biochemical and biophysical studies have been carried out on β4Gal-T1 in an attempt to understand its mechanism of action and the modulation of the substrate specificity by LA. When the crystal structure of β4Gal-T1 was first determined, the enzyme was found to be in an open conformation (*3*). However, it was found to be in a closed conformation when crystallized in the presence of its substrates and LA or in the presence of UDP-Gal and Mn^{2+} (*4*−*6*). These studies explained the enzyme's kinetic pathway (*7*−*12*).

Apparently, the enzyme β4Gal-T1 does not absolutely require UDP-Gal as the sugar donor. Rather, it exhibits polymorphic donor specificity, transferring glucose (Glc), D-deoxy-Glc, arabinose, GalNAc, and GlcNAc from their UDP derivatives, albeit at low rates (0.3−5%) compared to the rate of Gal transfer (*13*, *14*). Many other glycosyltransferases, such as blood group A and B α-1,3-galactosyltransferase (*15*, *16*), β-1,3-glucouronyltransferase (*17*), glucosylceramide synthase (*18*), and galactosylceramide synthase (*19*), also exhibit such polymorphic donor specificity. As the crystal structures of these enzymes became available, a better understanding of the polymorphic donor properties has emerged. For example, studies on β4Gal-T1, β-1,3-glucouronyltransferase and blood group A and B α-1,3-galactosyltransferase have revealed that a single amino acid in the active site is responsible for the donor sugar specificity (*13*, *15*−*17*). Appropriate mutations of these residues alter or broaden the donor sugar specificity of these enzymes (reviewed in ref *20*). Interestingly, many galactosyltransferase enzymes exhibit a low level of glucosyltransferase (Glc-T) activity where glucose, which differs from galactose only in the orientation of the exocyclic O4 hydroxyl group, is transferred from UDP-Glc to an acceptor molecule. The reverse also seems to be true: glucosylceramide synthase, which normally transfers glucose from UDP-glucose to ceramide, also transfers, at low efficiency, galactose from

[†] This project has been funded in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract N01-C0-12400.

[‡] The content of this publication does not necessarily reflect the view or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

[#] The PDB accession number for R228K-Gal-T1·LA·UDP-Gal·Mn^{2+} is PDB 1YRO.

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[1] Abbreviations: β4Gal-T1, β-1,4-galactosyltransferase; GlcNAc, *N*-acetylglucosamine; UDP-Gal, uridine 5′-diphosphogalactose; UDP-Glc, uridine 5′-diphosphoglucose; UDP, uridine 5′-diphosphate; Gal-T activity, galactosyltransferase activity; Glc-T activity, glucosyltransferase activity; Mn^{2+}, manganese; LA, α-lactalbumin; Mes, 2-morpholinoethanesulfonic acid.

10.1021/bi0479454
Published on Web 02/12/2005

UDP-Gal to the same acceptor ceramide. Knowledge of the these enzyme's three-dimensional structures will help us not only better understand the mechanism of their activities but, more importantly, also enable the design of new glycosyltransferases which can be used to synthesize novel, biologically important carbohydrates.

In attempting to understand the polymorphic sugar donor specificity of β4Gal-T1, we determined crystal structures of the wild-type (*7*−*9*) and mutant forms of this enzyme (*13*). Our investigations on the low activity of *N*-acetylgalactosaminyltransferase (GalNAc-T) of β4Gal-T1 showed that Tyr289 of β4Gal-T1 causes steric hindrance to the binding of UDP-GalNAc. A mutation of the bulky Tyr289 residue to the less bulky leucine, isoleucine, or aspargine results in a mutant enzyme with a GalNAc-T activity as high as its Gal-T activity (*13*). This mutant has been used to develop a rapid and sensitive method for detection of O-GlcNAc modification in proteins (*21*, *22*).

In earlier studies, we provided a structural basis for the 30-fold enhancement by LA of the Glc-T activity of β4Gal-T1 (*14*). In this study comparing the molecular interactions between UDP-Glc and UDP-Gal with β4Gal-T1, we report that the hydrogen bond formed between the O4 hydroxyl group of the sugar moiety of the nucleotide donor and Glu317 appears to be responsible for the low Glc-T activity. Since Glu317 is required for the Gal-T activity (*23*), residues in the vicinity of Glu317 were considered for mutational studies. We found that the mutation of Arg228 to lysine while reducing its Gal-T activity (by nearly 70%) enhances its Glc-T activity, compared to that of wild-type β4Gal-T1.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. Site-directed mutagenesis was performed using the PCR method. Construction of the mutants was carried out using plasmid pEGT-d129 as the template: this plasmid contains a *Bam*HI−*Eco*RI fragment inserted into a pET23a vector, encoding residues 130−402 of bovine β4Gal-T1 (*24*), and has a Cys342 to Thr mutation. Since the intended mutation site was present between the *Bam*HI site and the unique restriction site *Stu*I, these sites were utilized for construction of the mutants. The mutation primer corresponding to the lower DNA strand is as follows: R228K, CA*AGGCCT*CTTTAAAGCCAACATTGAGGAGCTTTGC**TTT**GTTGAACATGGA. The *Stu*I restriction site is shown in italics and the mutation codon in bold letters. Typically, the 5′-terminal fragment of β4Gal-T1 DNA was PCR amplified using the 5′-terminal cloning primer containing the *Bam*HI site and the mutagenesis primer, R228K. Then the PCR fragment carrying the mutation was digested with *Bam*HI and *Stu*I and ligated to the vector fragment obtained from the plasmid pEGT-d129 DNA cut with the same enzymes. Upon transformation of the XL2 supercompetent cells, the mutants were screened for the presence of the full β4Gal-T1 DNA insert and then sequenced to verify the mutation. For protein expression, BL21(DE3)/pLysS competent cells were transformed with the identified cDNA clones according to the manufacturer's protocols. The transformed cells were grown in LB broth containing 50 μg/mL ampicillin to an OD_{600} of ∼0.7, followed by induction with 0.4 mM IPTG. Cultures were harvested after 3−4 h by centrifugation at 2000*g* for 20 min. The inclusion bodies were isolated, solubilized, and refolded as described previously (*13*, *23*).

Enzyme Assays and Measuring the Kinetic Parameters of the Enzyme. The in vitro assay for β4Gal-T1 and R228K-Gal-T1 was carried out as previously described (*16*, *17*). Enzyme activities were measured using UDP-Gal or UDP-Glc as sugar−nucleotide donors, with GlcNAc or Glc as acceptor sugars. The Glc-T reaction was carried out in the presence of saturating amounts of LA. The true K_m of the donor (K_A) and of the acceptor (K_B), the dissociation constant of the donor (K_{ia}), and k_{cat} were obtained by two substrate analyses using the primary plots of five concentrations of donor (UDP-Glc/UDP-Gal), five concentrations of acceptor (GlcNAc), and the corresponding secondary plots of the intercepts and slopes. Conditions were chosen such that the initial rates were linear with respect to time, and the ranges of donor and acceptor substrate concentrations allowed an accurate Michaelis−Menten plot to be derived. The data were also analyzed for a general two-substrate system using eqs 1 and 2 (*23*, *25*) with EnzFitter, a non-linear curve-fitting program for Windows from Biosoft.

$$v = \frac{V_m[A][B]}{K_{ia}K_B + K_B[A] + K_A[B] + [A][B]} \quad (1)$$

$$v = \frac{V_m[A][B]}{K_B[A] + K_A[B] + [A][B]} \quad (2)$$

Equation 1 is the rate equation for a sequential symmetrical initial velocity pattern associated with an ordered or random equilibrium sequential mechanism, whereas eq 2 is the rate equation for an asymmetric initial velocity pattern associated with a sequential mechanism in which substrate A does not dissociate from the ECS complex. Using eqs 1 and 2, the kinetic parameters K_A, K_B, K_{ia}, and V_{max} were obtained from the fitted curves.

Crystallization, Data Collection, and Refinement. The catalytic domain of bovine R228K-Gal-T1 and mouse recombinant LA were cocrystallized in the presence of UDP-galactose and $MnCl_2$. Crystals were grown at room temperature by the "hanging drop" method, using 20 mg/mL R228K-Gal-T1, 10 mg/mL LA, 17 mM $MnCl_2$, and 17 mM UDP-Gal, and using the precipitant containing 100 mM NaCl, 100 mM Mes buffer (pH 6.0), and 12.5% PEG 4000. The crystals of the protein complex could be obtained only in the presence of its substrates. Complete three-dimensional X-ray diffraction data were collected at beam line X9B (National Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY), using a Quantum-4 ccd detector with a wavelength of 0.986 Å. The frames were processed using HKL2000 (*26*). The data collection statistics are listed in Table 1.

Using AMORE (*27*), the crystal structure was determined by molecular replacement methods. The crystal structure of lactose synthase (*7*) with the R228A mutation and without any substrate was used as a model for molecular replacement. After the initial refinement, the difference electron density maps revealed the presence of a lysine residue at position 228 and a UDP-Gal and Mn^{2+} bound to the LS molecule. These were included for further refinement. All the refinements were carried out using CNS1.0 (*28*). The coordination

Table 1: Statistics for Data Collection and Refinement

wavelength used during data collection (Å)	0.986
cell dimensions	$a = 57.3$ Å, $b = 94.1$ Å, $c = 99.7$ Å, $\beta = 101.6°$
resolution range (Å)	40−1.9
space group	$P2_1$
no. of observations	257131
no. of unique reflections	82383
R_{symm} (%) (outermost shell)[a]	5.3 (47)
completeness (%) (outermost shell)[a]	97 (86)
$I/\sigma(I)$ (outermost shell)[a]	18.2 (2.0)
R_{cryst} for $I > 0$ (%)	19
R_{free}[b] for $I > 0$ (%)	23
deviations from ideality	
bond lengths (Å)	0.015
bond angles (deg)	1.8
Ramachandran plot	
core region (%)	88.4
allowed region (%)	11.4
generously allowed region (%)	0.1

[a] The shell being 1.95−1.88 Å. [b] R_{free} is equal to R_{cryst} for a randomly selected 10% of the reflections, not used in refinement.

distances for the Mn^{2+} and Ca^{2+} ions were not restrained. The final refinement statistics are listed in Table 1. All the figures were drawn using MOLSCRIPT (*29*) and BOBSCRIPT (*30*).

RESULTS AND DISCUSSION

Previous crystal structure analyses revealed that the binding of UDP-Glc to β4Gal-T1 is essentially similar to that of UDP-Gal (Figure 1a,b) (*8*, *9*, *14*). However, the difference in the orientation of the O4 hydroxyl group of Glc and Gal results in a significant major difference in the interaction of the sugar moiety with Glu317 of β4Gal-T1. It was postulated that the most likely cause for UDP-Glc being a poor donor substrate (*14*) was the steric hindrance caused by the equatorial orientation of the O4 hydroxyl group of Glc with respect to Glu317, which may be responsible for the minor distortion of the orientation of the O4 hydroxyl group of Glc (*9*). In another study, we had found that Tyr289 caused a steric hindrance to the binding of UDP-GalNAc, a less preferred donor substrate. When Tyr289 was replaced with the less bulky residue Leu, the mutant (Tyr289Leu) exhibited GalNAc-T activity that was as good as the Gal-T activity (*13*). A similar approach to mutating Glu317 to obtain Glc-T activity was not feasible because Glu317 had been established as an essential residue for the Gal-T activity (*23*). Hence, to create the needed space, we explored the mutation of other residues in the vicinity of Glu317. The crystal structure of the wild-type protein reveals that Arg228 lies at the base of the catalytic pocket; its positively charged guanidine group is placed between the side chain carboxylate groups of Asp252 and Glu317. The guanidine group of Arg228 also forms a hydrogen bond with the side chain carboxylate group of Glu317. Arg228 was, therefore, chosen for mutational studies. To preserve the positive charge between these two negatively charged side chain carboxylate groups and to retain the hydrogen bond with the side chain of Glu317, Arg228 was replaced with lysine.

Enzymatic Activity of the R228K-Gal-T1 Mutant and Its Kinetic Parameters. R228K-Gal-T1 is a stable enzyme and folds as efficiently as the wild type. However, it had only

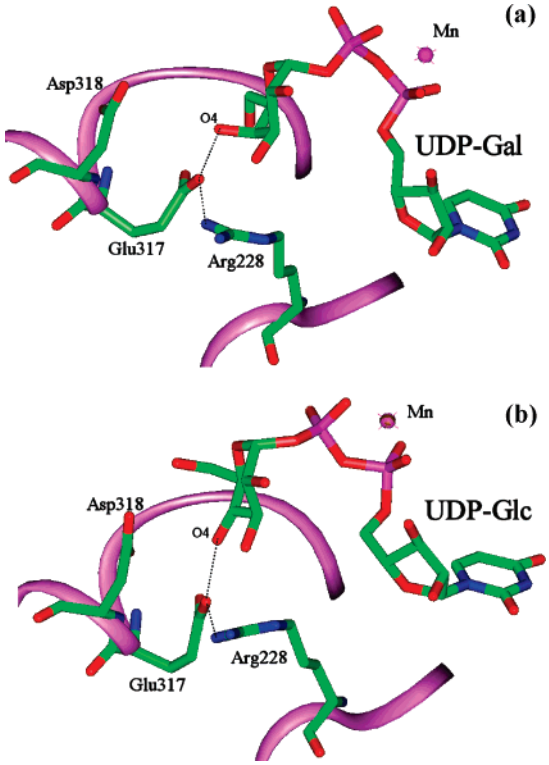

FIGURE 1: Molecular interactions of the O4 hydroxyl group of the sugar moiety in UDP-Gal (a) and UDP-Glc (b) with the β4Gal-T1 molecule in the crystal structures (coordinates taken from PDB entries 1O0R and 1O23, respectively). Although the hydrogen bonding interactions of the O4 hydroxyl group of the sugar moieties are similar, its equatorial orientation in UDP-Glc seems to cause steric hindrance with the side chain carboxylate group of Glu317.

16% of the Gal-T activity of wild-type β4Gal-T1 (Figure 2a and Table 2a). Even in the absence of LA, its Glc-T activity was nearly 15-fold higher than that of the wild type (Figure 2b and Table 2a). LA enhanced this activity even further, to nearly 25% of the Gal-T activity (Table 2a). Clearly, R228K-Gal-T1 in the presence of LA exhibited a Glc-T activity equal to, or higher than, its Gal-T activity. Like the wild-type enzyme, the mutant exhibits Glc-T activity toward various acceptor substrates (Table 2b). As expected, its activity toward disaccharides or β-benzyl-GlcNAc was inhibited by the presence of LA, as the latter binds to the extended oligosaccharide binding site created by the enzyme upon sugar−nucleotide binding.

The steady-state kinetic analyses of the enzymatic reactions by R228K-Gal-T1 were carried out in the presence and absence of LA. In the Glc-T reaction, in the absence of LA, the kinetic data fit best to eq 1, with a calculated dissociation constant, K_{ia}, of 168 μM for UDP-Glc (Table 3a and Figure 3a), 8 times higher (Table 3a) than that observed for the wild-type enzyme (*14*). The K_m values for both UDP-Glc and GlcNAc were lower than those found for the wild-type enzyme (Table 3a). In the presence of LA, both the mutant (Figure 3b) and the wild-type data (not shown) fit best to eq 2, in which the dissociation constant, K_{ia}, for UDP-Glc was much less than its K_m. This is consistent with the earlier finding that in the presence of LA, the Glc-T activity of the wild-type enzyme exhibits a negligible dissociation constant, K_{ia}, for UDP-Glc (*14*). However, the K_m values for UDP-Glc in the presence of LA are quite similar to those of the wild type, even though the k_{cat} is nearly 4-fold higher in the

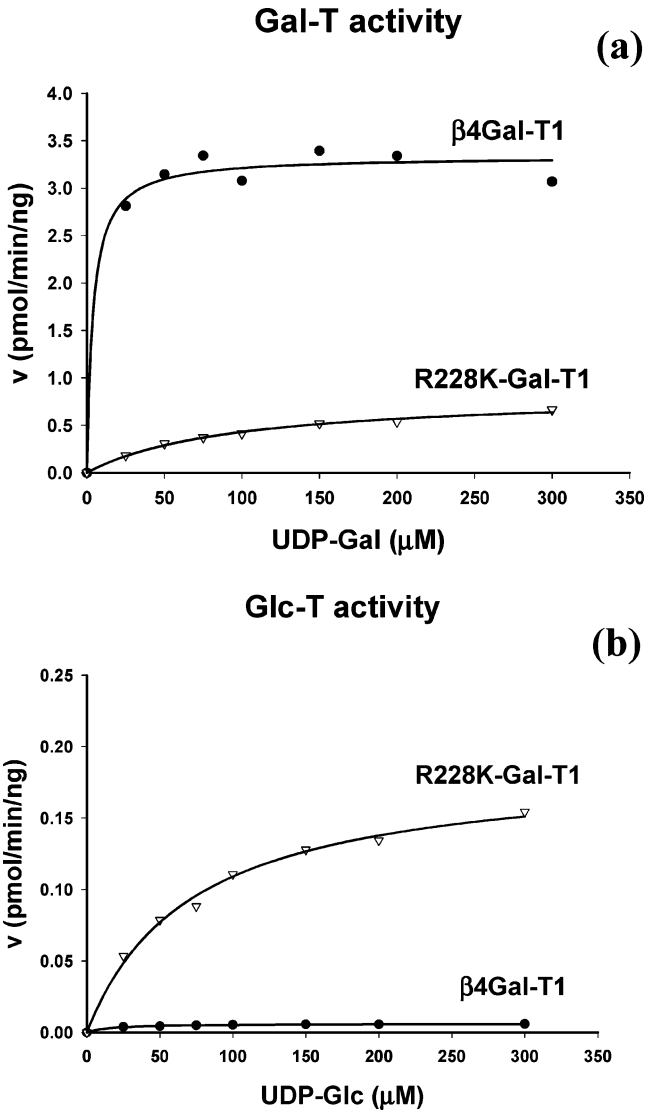

FIGURE 2: Comparison of the catalytic activity of the R228K-Gal-T1 mutant with the wild-type β4Gal-T1 enzyme. (a) In the Gal-T activity, where the enzyme transfers Gal from UDP-Gal to GlcNAc, the mutant exhibits reduced activity, compared to the wild type. However, in the Glc-T activity, where it transfers Glc from UDP-Glc to GlcNAc, the mutant exhibits enhanced catalytic activity, compared to the wild type. Furthermore, LA enhances this high Glc-T activity of the mutant (Tables 2a and 3a).

presence of LA and 3-fold higher in the absence of LA (Table 3a). Compared to that of the wild type, the catalytic efficiency of the mutant with respect to donor and acceptor substrates was increased less in the absence of LA than in the presence of LA. The increase in the k_{cat} of the mutant, in the presence of LA without a change in the K_m of the substrates, reflects a weaker effect on ground-state binding of UDP-Glc to the mutant, while having a greater effect on the transition-state binding of the substrates.

In the Gal-T activity, the kinetic data of the R228K-Gal-T (Figure 3c) fit best to eq 1, which showed a dissociation constant, K_{ia}, of 158 μM for UDP-Gal (Table 3b). In the absence of LA, the catalytic efficiency with respect to donor and acceptor substrates, compared to that of the wild type, decreased 30-fold, and the turnover number, the k_{cat}, also decreased 27-fold (Table 3b). This is in contrast to the case with the wild-type enzyme, whose data fit to eq 2 (*14*, *23*, *31*, *32*), which does not have the dissociation constant term, K_{ia}, for UDP-Gal. The K_m for the substrates, in the absence of LA for the mutant, is comparable to that of the wild-type enzyme (Table 3b). When LA is used in the Gal-T reaction, the kinetic data (Figure 3d) fit to eq 2, and the kinetic constants were much lower than those in the absence of LA (Table 3b). The K_m for UDP-Gal and GlcNAc was reduced approximately 8- and 16-fold, respectively, and the K_{ia} for UDP-Gal approached zero. In the presence of LA, the catalytic efficiency of the donor and the acceptor substrate for the mutant was reduced 5- and 3-fold, respectively. In the absence of LA, the larger effect on k_{cat}/K_m than on K_m indicates that the mutation of Arg228 to lysine does not affect the ground-state binding of the substrates as much as it does the transition-state binding. In contrast, the effect of LA on the ground-state binding by the mutant is much more pronounced than by the wild type, as indicated by the lowered K_m values.

Table 2

(a) Specific Activities of the Wild-Type β4Gal-T1 and the Mutant R228K-Gal-T1 in the Gal-T and Glc-T Reactions

	Gal-T activity[a] UDP-Gal → GlcNAc	Glc-T activity[a] UDP-Glc → GlcNAc	
enzyme	−LA	−LA	+LA
wild type	3.07 (100%)	0.01 (0.3%)	0.16 (5%)
R228K-Gal-T1	0.50 (16%)	0.15 (5%)	0.78 (25%)

(b) Glc-T Activity of the R228K-Gal-T1 Mutant Using Various Acceptors

acceptor	−LA	+LA	*x*-fold increase (+LA/−LA)
GlcNAc	0.09	0.59	6.3
N-propionyl-GlcN	0.09	0.84	8.8
N-butanoyl-GlcN	0.10	0.74	7.7
Glc	0	0.17	NA
β-benzyl-GlcNAc	0.16	0.03	NA
β-GlcNAc-1,4-GlcNAc	0.11	0.04	NA

[a] The specific activities are expressed in units of picomoles per minute per nanogram of protein. Gal-T activity of both proteins is inhibited by LA and therefore not listed.

Crystal Structure of the R228K-Gal-T1·LA·UDP-Gal·Mn^{2+} Complex. The mutation of Arg228 of β4Gal-T1 to lysine enhances the Glc-T activity and reduces its Gal-T activity. The presence of LA is yet required for the mutant to exhibit a maximum Glc-T activity, suggesting that steric hindrance of the O4 hydroxyl group of the Glc moiety of UDP-Glc with Glu317 was not completely eliminated. This was also evident from the kinetic studies which showed that the mutant still exhibits a dissociation constant, K_{ia}, for UDP-Glc. Further, it also exhibits a dissociation constant for UDP-Gal in the Gal-T reaction, indicating that the mutation also causes a steric hindrance for the binding of UDP-Gal. To explore the effect of the mutation on the binding of UDP-Gal and UDP-Glc, we undertook a crystal structure determination of the mutant in the presence of these two donor substrates. The mutant could be crystallized in the presence of UDP-Gal, or UDP-Glc, in the complex with mouse LA and $MnCl_2$. Surprisingly, in the crystal structure of the UDP-Glc complex, only UDP, not the glucose moiety, was observed. Analysis of the data collected on several crystals of the mutant R228K-Gal-T1 in complexes with UDP-Glc, Mn^{2+}, and LA failed to identify the intact UDP-Glc molecule. Apparently, the Glc moiety was hydrolyzed. However, in

Table 3[a]

ligand	parameter	R228K-Gal-T1 −LA	R228K-Gal-T1 +LA	β4Gal-T1 −LA	β4Gal-T1 +LA
	(a) Kinetic Constants for Glc-T Activity				
UDP-Glc	K_a (μM)	91 ± 5	44 ± 4	148 ± 4	31 ± 2
	K_{ia} (μM)	168 ± 10	0	20 ± 6	0
	k_{cat}/K_a (s^{-1} μM^{-1})	0.0018	0.02	0.0004	0.008
GlcNAc	K_B (mM)	9 ± 1	1.3 ± 0.12	74 ± 3	2.5 ± 1
	V_{max} (pmol min^{-1} ng^{-1})	0.30 ± 0.01	1.7 ± 0.09	0.11 ± 0.01	0.45 ± 0.1
	k_{cat} (s^{-1})	0.17	0.96	0.06	0.25
	k_{cat}/K_b (s^{-1} mM^{-1})	0.0188	0.73	0.0008	0.1
	(b) Kinetic Constants for Gal-T Activity[b]				
UDP-Gal	K_a (μM)	87 ± 10	10.5 ± 0.9	93 ± 6	ND
	K_{ia} (μM)	158 ± 10	0	0	ND
	k_{cat}/K_a (s^{-1} μM^{-1})	0.005	0.03	0.15	ND
GlcNAc	K_b (mM)	11 ± 2	0.7 ± 0.02	11 ± 1	ND
	V_{max} (pmol min^{-1} ng^{-1})	0.92 ± 0.3	0.59 ± 0.01	10 ± 2	ND
	k_{cat} (s^{-1})	0.52	0.33	14	ND
	k_{cat}/K_b (s^{-1} mM^{-1})	0.04	0.47	1.27	ND

[a] The wild-type kinetic parameters were taken from ref *14*. [b] Due to strong inhibition of the Gal-T activity in the presence of LA by the wild type, the corresponding kinetics parameters were not determined.

crystals of the R228K-Gal-T1·LA·UDP-Gal·Mn^{2+} complex, the Gal moiety was clearly present (Figure 4a).

The crystal structure of the mutant R228K-Gal-T1 in complex with UDP-Gal and LA was very similar to that of wild-type β4Gal-T1 in complex with LA and UDP (*7*, *9*). The mean rms deviation of the Cα atoms in the mutant, compared to the wild-type β4Gal-T1 structure, is 0.5 Å, indicating that mutation of Arg228 to lysine has not perturbed the overall crystal structure of β4Gal-T1. There were two protein R228K-Gal-T1·LA complexes in the asymmetric unit, where each bound with one UDP-Gal molecule and a Mn^{2+} ion. In the crystal structure, the R228K-Gal-T1 molecules were in a closed conformation and interacted with the LA molecules. The molecular interactions between LA and R228K-Gal-T1 molecules were quite similar to those found in the previous crystal structures (*7*, *9*).

In both R228K-Gal-T1 molecules, Lys228 side chain atom Nζ forms hydrogen bonds with the Glu317 side chain carboxylate oxygen atom and the O3 hydroxyl group of the Gal moiety of UDP-Gal (Figure 4a). In the wild-type enzyme, the side chain guanidinium nitrogen atoms of Arg228 form hydrogen bonds with the side chain carboxylate oxygen atoms of Glu317 and Asp252 (Figure 4b). An Arg-to-Lys mutation created an additional space, utilized by Asp318, around the Glu317 side chain. This is evident from a weak residual positive electron density observed around the Asp318 side chain that corresponds to the minor alternate conformation for this side chain (Figure 5). The major conformation corresponds to the highly conserved conformation found in the wild-type crystal structure, in which Asp318 is positioned to bind to the acceptor substrate and points away from the O4 hydroxyl group of the Gal moiety of UDP-Gal (Figure 5). In the alternate conformation, the side chain carboxylate group of Asp318 fills the space created by the mutant and interacts with the Lys228 side chain Nζ atom. Asp318 also causes a steric hindrance to UDP-Gal binding, due to its proximity to the O4 hydroxyl group of the Gal moiety of the UDP-Gal (Figure 5). During catalysis, the side chain carboxylate group of Asp318 plays an important role by binding to the O4 hydroxyl group of the acceptor substrate (*33*). Mutation of Asp318 has been shown to reduce the specific activity of the enzyme by nearly 100-fold (*23*), and the Asp318Asn mutant exhibits a dissociation constant for UDP-Gal in the Gal-T reaction (*30*). It appears that the alternate conformation observed for the Asp318 chain could also be responsible for the reduced, specific activity of R288K-Gal-T1.

The binding of UDP-Gal and its interactions with the R228K-Gal-T1 molecule are mostly similar to those found in the β4Gal-T1·UDP-Gal·Mn^{2+} complex (Figure 4). Surprisingly, Asp318 in the R228K-Gal-T1·LA·UDP-Gal·Mn^{2+} crystal structure had acquired a minor alternate conformation that is different from that in the wild-type UDP-Gal complex, resulting in a steric hindrance from the Gal moiety of UDP-Gal. This in turn could result in the dissociation of UDP-Gal from the complex that is reflected in the dissociation constant, K_{ia}, found for UDP-Gal in the Gal-T reaction of the mutant (Table 3b). The presence of an additional hydrogen bond observed between the O3 hydroxyl group of Gal and the Nζ atom of Lys228 (Figure 5) could restrict the flexibility of the Gal moiety required for the formation of the transition-state complex. This would account for the reduced k_{cat} of the enzyme. Because of the equatorial orientation of the O4 hydroxyl group of the Glc moiety, UDP-Glc, unlike UDP-Gal, could be better accommodated in the carved space in the mutant, resulting in enhanced Glc-T activity. Additional mutations of other nearby residues either abolish the enzyme activity altogether or decrease the stability of the protein without a further increase in the Glc-T activity (unpublished data).

A structure-based explanation for the role of LA in the Glc-T reaction of the wild-type enzyme is found by comparing the previously determined crystal structures of Gal-T1·UDP-Gal (Figure 4b) and LA·Gal-T1·UDP-Glc complexes (*8*, *9*, *14*). When modeling the Glc moiety to replace the Gal moiety in the crystal structure of the Gal-T1·UDP-Gal complex lacking LA, we observe that the O4 hydroxyl group of the Glc moiety causes a severe steric hindrance with the Glu317 side chain carboxylate group. Therefore, the UDP-Glc molecule cannot form a stable complex with Gal-T1. By interacting with Gal-T1 in the closed conformation, LA forms a stable complex with Gal-T1·UDP-Glc and helps to

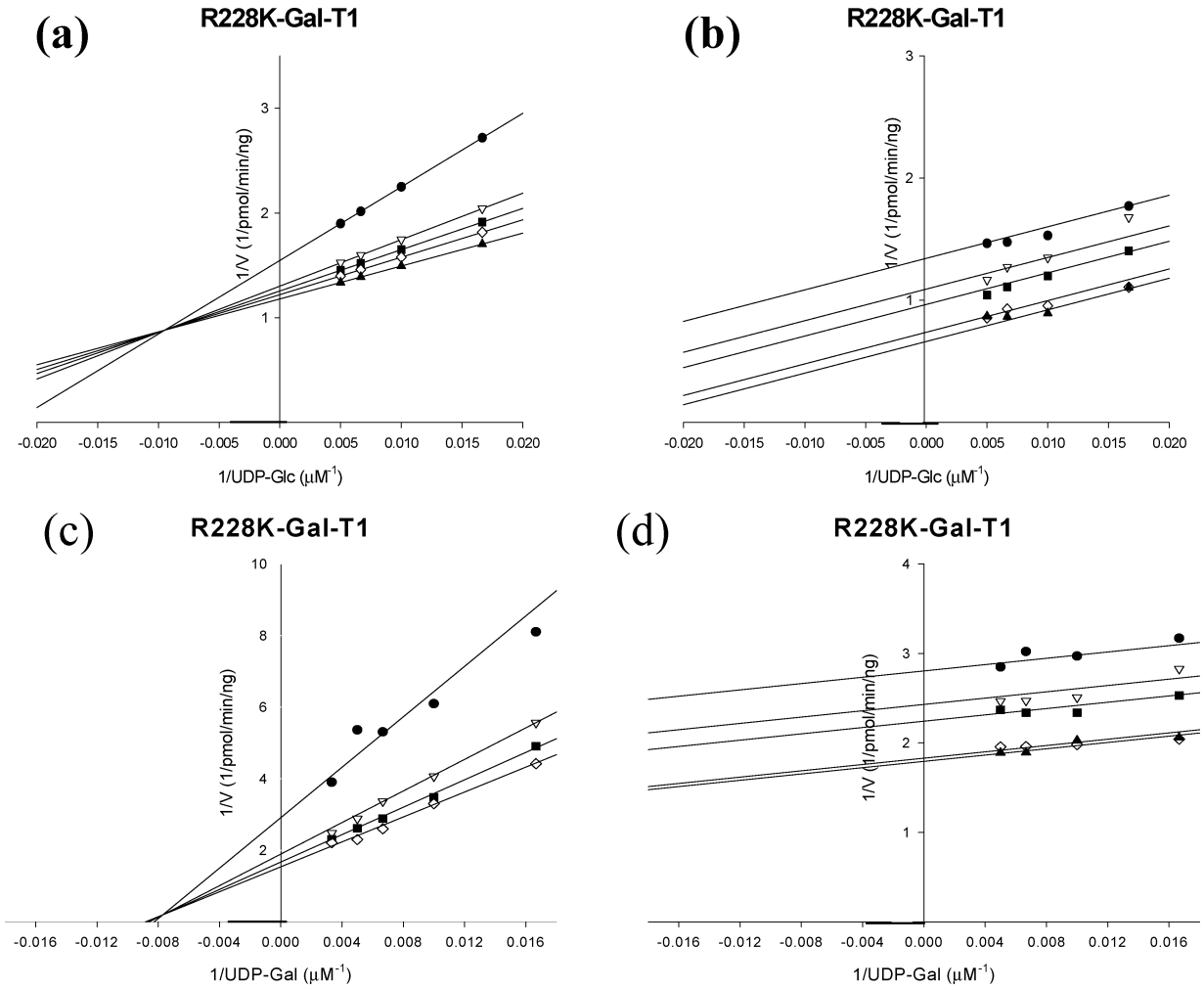

FIGURE 3: Steady-state kinetic properties of the mutant R228K-Gal-T1. Double-reciprocal plots showing the variation of Glc-T (a and b) and Gal-T (c and d) activities at a fixed concentration of GlcNAc and various concentrations of UDP-Glc and UDP-Gal, respectively. (a) Glc-T activity in the absence of LA at a fixed GlcNAc concentration of 5 (●), 10 (▽), 15 (■), 20 (◇), and 25 mM (▲). (b) Glc-T activity in the presence of 7 μg of LA at a fixed GlcNAc concentration of 1 (●), 1.5 (▽), 2 (■), 5 (◇), and 10 mM (▲). (c) Gal-T activity in the absence of LA at a GlcNAc concentration of 7 (●), 15 (▽), 20 (■), and 25 mM (◇). (d) Gal-T activity in the presence of 7 μg of LA at a fixed GlcNAc concentration of 1 (●), 1.5 (▽), 2 (■), 7.5 (◇), and 10 mM (▲). Assays were performed for 15 min at 30 °C. The parallel lines represent an ordered sequential or "ping-pong" mechanism, with a dissociation constant (K_{ia}) for UDP-sugar approaching zero. The nonparallel lines represent a sequential ordered mechanism, with a non-zero K_{ia} value.

position the Glc moiety of UDP-Glc in a way similar to that of the Gal moiety of UDP-Gal in the wild-type complex. This facilitates the transfer of the Glc moiety to an acceptor. In the crystal structure of the LA·Gal-T1·UDP-Glc complex (*14*), we found that although the binding of the Glc moiety is similar to that of Gal, the orientation of the O4 hydroxyl group and its interactions with the Glu317 side chain are different. From this finding, we infer that in the Glc-T reaction, LA (a) stabilizes the closed conformation of the enzyme so that the dissociation constant for UDP-Glc, K_{ia}, approaches zero and (b) places the Glc moiety in the binding pocket in a position similar to that of the Gal moiety. This facilitates the successful transfer of glucose to the acceptor with an enhanced k_{cat} value.

A similar explanation is possible for the observed Gal-T and Glc-T activity of the mutant R228K-Gal-T. It is interesting to note that in the Gal-T reaction of the mutant, the presence of LA hinders the dissociation of UDP-Gal from the complex while it has no significant effect on the k_{cat} of the reaction (Table 3b). On the other hand, in the Glc-T reaction, by both the wild-type and mutant enzymes, the presence of LA not only abolishes the dissociation constant for the binding of UDP-Glc but also enhances the k_{cat} (Table 3a). Thus, it seems that in the Glc-T reaction, the binding of LA positions UDP-Glc in such a way that formation of the transition-state complex is promoted. Since only the crystal structure of the LA·R228K-Gal-T1 form in complex with only UDP-Gal, and not with UDP-Glc, is available (Figure 4a), we modeled the Glc moiety in place of the Gal moiety in this structure. The steric hindrance between the O4 hydroxyl group of the Glc moiety and the side chain carboxylate group was still present. LA appears to be required, thus, not only to stabilize the R228K-Gal-T and UDP-Glc complex but also to position properly the Glc moiety in the binding pocket of the R228K-Gal-T1 mutant. The steric hindrance between the Gal moiety of UDP-Gal and the alternate conformation of the Asp318 residue will prevent the formation of a stable complex between UPD-Gal and the R228K-Gal-T1 mutant (Figure 5). Thus, LA is needed to stabilize the R228K-Gal-T1·UDP-Gal complex by interacting with the flexible loop of β4Gal-T1 in the closed conformation. On the other hand, UDP-Gal being the natural

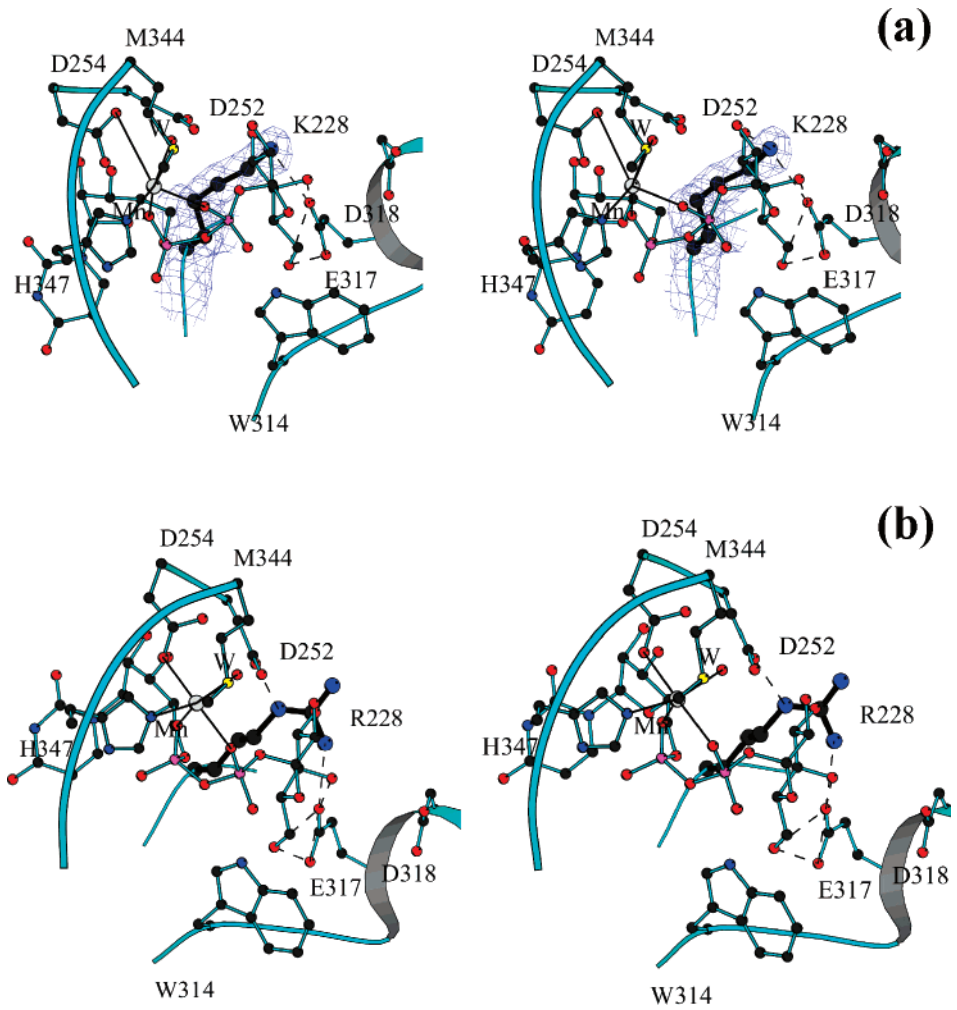

FIGURE 4: (a) Stereoview of the binding of UDP-Gal to the R228K-Gal-T1 mutant in the crystal structure of the R228K-Gal-T1·LA·UDP-Gal·Mn^{2+} complex. The final electron density maps contoured at 1σ around the residue K228 are colored blue lines. (b) Similar view shown for the binding of the UDP-Gal to the wild-type enzyme (PDB entry 1O0R), where an Arg residue is found at position 228. As can be seen, the overall binding of UDP-Gal to the R228K-Gal-T1 mutant is similar to that of the wild type. The Nζ atom of the Lys228 side chain forms a hydrogen bond with the Glu317 residue.

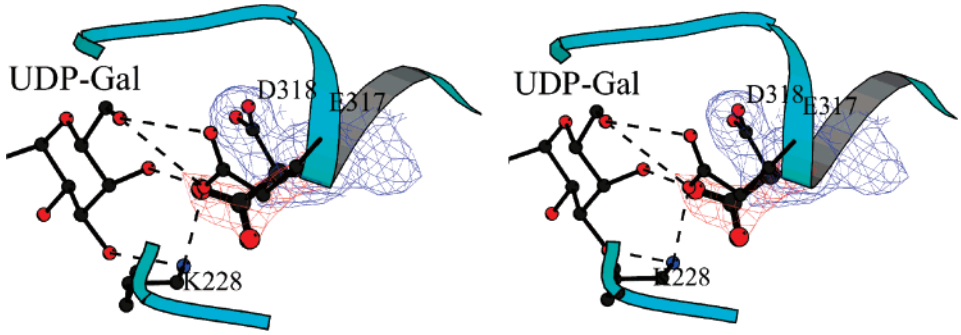

FIGURE 5: Stereoview of the electron density maps around the Asp318 residue and its interactions with the Gal moiety of UDP-Gal in the R228K-Gal-T1·LA·UDP-Gal·Mn^{2+} crystal structure. The final electron density map around the major Asp318 side chain conformation is colored blue (contoured at 1σ), while the residual difference Fourier electron density maps (shown in red) around Asp318 are contoured at 2.5σ. Although an alternate conformation for the Asp318 side chain carboxylate group can be fitted into this electron density (shown as a larger ball-and-stick diagram), its carboxylate oxygen atom is found to have steric hindrance with the O4 hydroxyl group of UDP-Gal, with a distance of only 1.8 Å. Due to the short contact, this conformation is not included in the refinement.

donor substrate, LA is not needed to assist in positioning the Gal moiety properly in the binding pocket of the R228K-Gal mutant.

In a previous study, the *N*-acetylgalactosaminyltransferase (GalNAc-T) activity of β4Gal-T1 was enhanced to equal that of its Gal-T activity when the steric hindrance between the *N*-acetyl moiety of UDP-GalNAc and the side chain of Tyr289 was relieved by mutating the Tyr289 residue to Leu. In fact, mutation of both Arg228 and Tyr289 to create the R288K/Y289L-Gal-T1 mutant yields an enzyme exhibiting a high GlcNAc-T activity (unpublished data). This study and a previous structure-based design study (*13*) demonstrate that it is possible to significantly alter the sugar donor specificity of β-1,4-galactosyltransferase by means of a few selective

point mutations in its active site amino acid residues. In this connection, it is of interest to note that β-1,4-*N*-acetylglucosaminlytransferase (β4GlcNAc-T) from *Lymnaea stagnalis* exhibits a high degree of sequence homology with β-1,4-galactosyltransferase and also has the same substitutions, such as Lys for Arg228 and Leu for Tyr289 (R228K and Y289L) (*35*). Further, a recently cloned β-1,4-*N*-acetylgalactosylaminyltransferase (β4GalNAc-T) from *Caenorhabditis elegans* with a high degree of sequence homology with β-1,4-galactosyltransferase also carries a substitution of Ile for Tyr289. Such a mutation has been previously demonstrated to convert the β4Gal-T1 to a β4GalNAc transferase (*36*).

ACKNOWLEDGMENT

We thank Dr. Alexander Wlodawer and Prof. Soma Kumar for the critical reading of the manuscript. We thank Dr. Zbigniew Dauter, NCI, Brookhaven National Laboratory, for his help in data collection.

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BI0479454