

# $\alpha$ -Lactalbumin (LA) Stimulates Milk $\beta$ -1,4-Galactosyltransferase I ( $\beta$ 4Gal-T1) to Transfer Glucose from UDP-glucose to *N*-Acetylglucosamine

CRYSTAL STRUCTURE OF  $\beta$ 4Gal-T1·LA COMPLEX WITH UDP-Glc\*

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**$\beta$ -1,4-Galactosyltransferase I (Gal-T1) transfers galactose (Gal) from UDP-Gal to *N*-acetylglucosamine (GlcNAc), which constitutes its normal galactosyltransferase (Gal-T) activity. In the presence of  $\alpha$ -lactalbumin (LA), it transfers Gal to Glc, which is its lactose synthase (LS) activity. It also transfers glucose (Glc) from UDP-Glc to GlcNAc, constituting the glucosyltransferase (Glc-T) activity, albeit at an efficiency of only 0.3–0.4% of Gal-T activity. In the present study, we show that LA increases this activity almost 30-fold. It also enhances the Glc-T activity toward various *N*-acyl substituted glucosamine acceptors. Steady state kinetic studies of Glc-T reaction show that the  $K_m$  for the donor and acceptor substrates are high in the absence of LA. In the presence of LA, the  $K_m$  for the acceptor substrate is reduced 30-fold, whereas for UDP-Glc it is reduced only 5-fold. In order to understand this property, we have determined the crystal structures of the Gal-T1·LA complex with UDP-Glc·Mn<sup>2+</sup> and with *N*-butanoyl-glucosamine (*N*-butanoyl-GlcN), a preferred sugar acceptor in the Glc-T activity. The crystal structures reveal that although the binding of UDP-Glc is quite similar to UDP-Gal, there are few significant differences observed in the hydrogen bonding interactions between UDP-Glc and Gal-T1. Based on the present kinetic and crystal structural studies, a possible explanation for the role of LA in the Glc-T activity has been proposed.**

Glycosyltransferases constitute a superfamily of an increasing number of enzymes that are involved in the synthesis of complex carbohydrates present on glycoproteins and glycolipids. They transfer the glycosyl moiety of nucleotide derivatives to various sugar acceptors.  $\beta$ -1,4-Galactosyltransferase ( $\beta$ 4Gal-T)<sup>1</sup> (EC 2.4.1.38), is one of the subfamilies of glycosyltrans-

ferase superfamily, which comprises at least seven members, Gal-T1 to Gal-T7 (1–9). They catalyze the transfer of galactose (Gal) from UDP-Gal to different sugar acceptors. For example, Gal-T1, the milk galactosyltransferase enzyme transfers Gal to the non-reducing terminal *N*-acetylglucosamine (GlcNAc) residue of glycans to form  $\beta$ -1,4-linked galactosylated glycan and to free GlcNAc to form *N*-acetylglucosamine (1), whereas Gal-T6 transfers Gal to Glc in glucosylceramide synthesizing lactosylceramide (10). Gal-T1 has been reported to be able to use, at a lower efficiency, other *N*-acetyl/*N*-acyl substituted sugars such as *N*-acetyl-D-mannosamine, *N*-acetylmuramic acids as sugar acceptors (11). It also does not show an absolute requirement for UDP-Gal as a sugar donor since it can transfer glucose (Glc), 2-deoxy-Glc, arabinose, and GalNAc from their UDP derivatives, albeit at very low efficiencies (12–14). The transfer of Glc occurs at a rate of only 0.3–0.5% of that of Gal, and the product Glc $\beta$ 1–4GlcNAc has been well characterized (13, 14). In recent years, many other transferases have been shown to transfer related sugars from their UDP-derivatives, albeit with a lesser efficiency. For example, the glucosylceramide synthase, which transfers Glc from UDP-Glc to ceramide to synthesize glucosylceramide, can also transfer Gal from UDP-Gal to ceramide to synthesize galactosylceramide with ~10% efficiency (15).

The normal galactosyl acceptor specificity of some  $\beta$ 4Gal-T family members, in particular of Gal-T1, is altered from GlcNAc to Glc by  $\alpha$ -lactalbumin (LA), a mammary gland-specific Ca<sup>2+</sup> ion-binding protein that has sequence and structural similarity to c-type lysozyme (16). The transfer of galactose to glucose to synthesize lactose by Gal-T1 in the presence of LA constitutes the lactose synthase (LS) activity of Gal-T1. In the present study we show that, in the presence of LA, Gal-T1 can transfer Glc from UDP-Glc to GlcNAc, at an ~30-fold higher efficiency than in the absence of LA. This constitutes its glucosyltransferase (Glc-T) activity, which compares to an efficiency of ~10% of Gal transfer from UDP-Gal to GlcNAc. In order to better understand the broad donor specificity of Gal-T1, we have in this report attempted to investigate the Glc transfer activity of Gal-T1 in greater detail.

In our continued efforts to understand the structure-function relationship between Gal-T1 and its nucleotide-sugar donor and acceptor substrates, we have previously determined several crystal structures of the complex of Gal-T1 with LA in the presence of various substrates (17). These studies, together

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The atomic coordinates and structure factors (code 1JN8, 1JNA, and 1JNC) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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<sup>1</sup> The abbreviations used are:  $\beta$ 4Gal-T,  $\beta$ -1,4-galactosyltransferase;

LA,  $\alpha$ -lactalbumin; Gal-T, galactosyltransferase; Glc-T, glucosyltransferase; LS, lactose synthase; GdnHCl, guanidine HCl; r.m.s., root mean square; PCR, polymerase chain reaction.

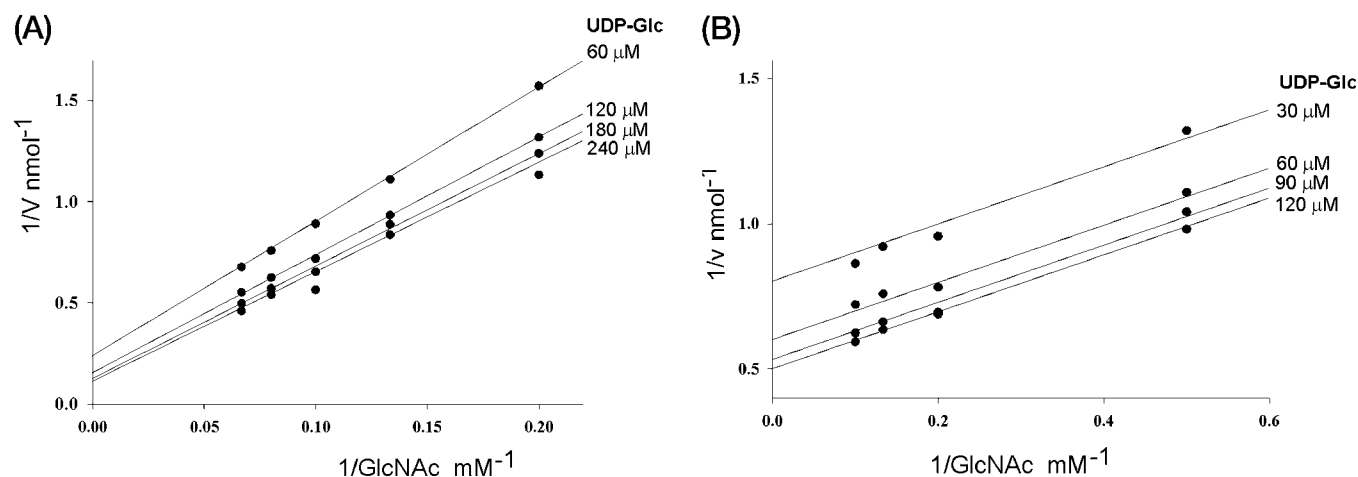


FIG. 1. **Representative double-reciprocal plots of glucosyltransferase reaction exhibited by the recombinant C342T-Gal-T1.** Steady-state velocities were determined in the absence of (A) and presence of (B) LA. The curves were fitted by the EnzFitter program, using appropriate equations (see "Experimental Procedures"). Plots were generated by SigmaPlot.

with the previously determined crystal structure of Gal-T1 by Gastinel *et al.* (18), have led to an understanding of the enzyme mechanism of Gal-T1 and the modulation of the acceptor specificity by LA. In order to understand the observed Glc-T activity by Gal-T1 and the role of LA, we have determined the crystal structure of Gal-T1 complex with UDP-Glc in the presence of LA. Our attempts to grow the crystals of this complex in the absence of LA have not yet been successful. We did succeed, however, in obtaining the crystals of the complex of Gal-T1 with LA in the presence of UDP-Glc-Mn<sup>2+</sup> and also in the presence of *N*-butanoylglucosamine (*N*-butanoyl-GlcN), a preferred sugar acceptor in Glc-T activity. Here we present these crystal structures in view of the newly observed Glc-T activity. We also found that the mutation of Cys-342 to Thr in Gal-T1 enhances the *in vitro* folding of the protein from the inclusion bodies and increases the stability of Gal-T1 without perturbing the crystal structure of the enzyme.

#### EXPERIMENTAL PROCEDURES

**Expression, Folding, and Purification of Bovine Gal-T1**—Bacterial growth and plasmid transformations were performed using standard procedures (19). The plasmid pEGT-d129, which encodes the catalytic domain (residues 130–402) of bovine Gal-T1, was used for mutation (20). Site-directed mutagenesis was performed using a CLONTECH site-directed mutagenesis transformer kit. The transformation mixture contained the template pEGT-d129, a selection primer (pNotApa), and the mutagenic primer 5'-GTGATCGGGAAGACCCGGATGCGCCA-3'. Mutants were screened for the incorporated mutations by looking for changes in restriction enzyme digestion patterns and confirmed by DNA sequencing. The positive clones were transformed into B834(DE3)pLysS cells. The expression and purification of the inclusion bodies were carried out as described previously (19). The inclusion bodies were *S*-sulfonated by dissolving in 5 M GdnHCl, 0.3 M sodium sulfite, and the addition of di-sodium 2-nitro-5-thiosulfobenzoate to a final concentration of 5 mM (21). The sulfonated protein was precipitated by dilution with water, and the precipitate was washed thoroughly. It was re-dissolved in 5 M GdnHCl to a protein concentration of 1 mg/ml (1.9–2.0 optical density at 275 nm). The protein solution was diluted 10-fold, in 10 portions, in a folding solution to give a final concentration of 100  $\mu$ g/ml Gal-T1, 0.5 M GdnHCl, 50 mM Tris-HCl, 5 mM EDTA, 4 mM cysteamine, and 2 mM cystamine, pH 8.0 at 4°C. It was left at 4°C for 48 h to allow the protein to fold and then dialyzed against 3  $\times$  4 liters of water containing 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 4 mM cysteamine, and 2 mM cystamine at 4°C to remove GdnHCl. Any protein that precipitated during dialysis was removed by centrifugation, and the supernatant was concentrated. Typically, when 100 mg of sulfonated protein was folded in a 1-liter folding solution, it yields 2–5 and 10–12 mg of active, soluble, and pure wild-type d129-Gal-T1 and C342T-Gal-T1, respectively. The folded proteins were purified further on the LA-agarose column (Sigma). The final, purified protein had a specific activity that was slightly higher than that of purified milk Gal-T1.

**Construction, Expression, and Folding of Mouse LA**—The mouse LA gene was PCR-amplified from the clone p35MLA that contains mouse LA cDNA cloned into pBR322 vector.<sup>2</sup> The oligonucleotides with a built-in restriction site for *Nde*I and *Bam*HI (shown in bold type) were used as the 5' end and 3' end primers and were used for the PCR amplification, respectively: 5'-GGAATTCATATGACAGAGGCTTACA-AAATGCAAGGTGACCC-3' and 5'-CGCGGATCCTCAGGGCTTCTCAACGCCA-3'.

The PCR-amplified products were cloned into the *Nde*I/*Bam*HI restriction sites of the modified pET17 expression vector. The bacterial growth, the inclusion body purification, and sulfonation methods were quite similar to those used for Gal-T1. However, unlike Gal-T1, the *S*-sulfonated LA remained soluble and was dialyzed against water thoroughly. The concentration of the dialyzed sulfonated LA solution was adjusted to ~1 mg/ml by monitoring its absorption at 275 nm (2.4–2.6 optical density). It has been shown previously that CaCl<sub>2</sub> is required for the folding of the recombinant LA (22). An equal volume of a 2 $\times$  folding solution containing 100 mM sodium borate buffer (pH 8.5), 5 mM calcium chloride, 8 mM reduced glutathione, and 4 mM oxidized glutathione was added slowly to the protein solution. The resulting solution was left at room temperature for 48 h to fold and then dialyzed against 1 mM calcium chloride to remove the buffer and the oxidizing agents. The dialyzed protein solution was centrifuged at 15,000  $\times$  *g* to remove any precipitate formed and then concentrated. The active protein was isolated from the partially folded inactive protein by fractional precipitation at 40–70% ammonium sulfate concentration (23). It was dissolved in water and dialyzed extensively against 1 mM calcium chloride, and the resulting protein solution was directly used for crystallization or other studies. Using this method, 60–70% of the starting *S*-sulfonated LA material was obtained in an active form.

**Gal-T1 Enzyme Assays and Measuring the Kinetic Parameters of the Enzyme**—The *in vitro* assay for Gal-T1 was carried out as described previously (20, 23). The activities of the enzymes were measured using UDP-Gal or UDP-Glc as sugar nucleotide donors, and GlcNAc or Glc as the 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) and five concentrations of acceptor, 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 donor and acceptor concentrations were in a range that allowed for an accurate Michaelis-Menten plot to be derived (Fig. 1). The data were also analyzed for a general two-substrate system using Equations 1 and 2 (24) with the EnzFitter program, a non-linear curve fitting program for Windows from Biosoft.

$$v = \frac{V_{max}[A][B]}{K_{iA}K_B + K_B[A] + K_A[B] + [A][B]} \quad (\text{Eq. 1})$$

$$v = \frac{V_{max}[A][B]}{K_B[A] + K_A[B] + [A][B]} \quad (\text{Eq. 2})$$

<sup>2</sup> P. V. Balaji and P. K. Qasba, unpublished observation.

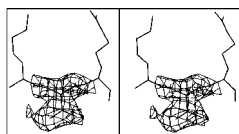


FIG. 2. Stereo diagram showing the  $2F_o - F_c$  electron density for the C342T mutation. The maps were contoured at  $1\sigma$  level.

Equation 1 is the rate equation for sequential symmetrical initial velocity pattern associated with an ordered or random equilibrium sequential mechanism, whereas Equation 2 is the rate equation for asymmetric initial velocity pattern associated with a sequential mechanism in which substrate A does not dissociate well from the *E*-S complex. The kinetic parameters  $K_A$ ,  $K_B$ ,  $K_{ia}$ , and  $V_{max}$  were obtained from the fitted curves using the above rate equations. The graphical method and the EnzFitter program gave very similar kinetic parameters.

**Crystallization, Data Collection, and Refinement**—The catalytic domain of recombinant bovine Gal-T1, residues 130–402 (d129-Gal-T1) (mass ~33 kDa), and mouse recombinant LA (mass ~14 kDa) were co-crystallized either in the presence of *N*-butanoyl-GlcN or UDP-glucose. Crystals were grown at room temperature by hanging drop methods, using 20 mg ml<sup>-1</sup> Gal-T1 and 10 mg ml<sup>-1</sup> LA in the presence of a substrate with the precipitant containing 100 mM NaCl, 100 mM sodium citrate buffer (pH 5.6), and 12.5% polyethylene glycol 4000. The crystals of the complex could only be obtained in the presence of substrates. The concentration of substrates used during the crystallization were 10 mM *N*-butanoyl-GlcN and 17 mM UDP-Glc and MnCl<sub>2</sub>. 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 0.98 Å. The frames were processed using DENZO (25). The data collection statistics are listed in Table I.

The crystal structures were solved by molecular replacement methods using AMORE (26). The crystal structure of lactose synthase (17) without any substrate was used as a model for molecular replacement. After initial refinement, the difference electron density maps (Fig. 2) revealed the substrate bound to the LS molecule and were included for further refinement. The two LS molecules in the asymmetric unit are related by a pseudo 2-fold symmetry. During refinement, the two LS molecules are restrained by this pseudo 2-fold symmetry. All the refinements were carried out initially by XPLOR 3.85, followed by CNS1.0 (27). The coordination distances for a Mn<sup>2+</sup> ion were not restrained, whereas those for Ca<sup>2+</sup> in LA were restrained. Only reflections with  $F > 2\sigma(F)$  were used for the refinement. The final refinement statistics are listed in Table I. All the figures were drawn using MOLSCRIPT (28). The Protein Data Bank access numbers for the coordinates of C342T-Gal-T1·LA·GlcNac, LS·*N*-butanoyl-GlcN, and LS·UDP-Glc·Mn complexes are 1JN8, 1JNA, and 1JNC, respectively.

## RESULTS

**Mutation of Cys-342 to Thr of Gal-T1 Imparts Stability and Increases Folding Yields**—In a separate study on the role of cysteine residues in Gal-T1, it was observed that the mutation of Cys-342 to Thr in the catalytic domain of the wild-type protein resulted in a 2–3-fold higher yield of the folded protein. The mutant C342T-Gal-T1 was significantly more stable at room temperatures and showed ~18% higher activity compared with the wild type (Table II). In the crystal structure of wild-type Gal-T1, Cys-342 residue exists in the reduced form, whereas the remaining four Cys residues form two disulfide bonds. Recent mass spectroscopic studies have shown that, during denaturation of milk Gal-T1, this free Cys-342 residue is responsible for the formation of mixed disulfide bonds (29). Therefore, the increased *in vitro* folding efficiency of the mutant C342T-Gal-T1 may be attributed to the diminished mixed disulfide bond formation during folding.

**Specific Activities for the Three Gal-T1-catalyzed Reactions**—Gal-T1 catalyzed the transfer of Gal from UDP-Gal to GlcNac, generating a  $\beta$ 1–4-linked disaccharide (Gal-T activity) (Table II). The modifier protein, LA, changed the sugar acceptor specificity of Gal-T1 to Glc, the LS activity of the enzyme complex. The Gal-T1, however, could also transfer Glc from UDP-Glc to GlcNac, exhibiting a Glc-T activity (Table II), although the

efficiency of this reaction was only 0.3% as compared with Gal-T activity (13). We have compared these three activities of the recombinant wild type enzyme, d129-Gal-T1, and of C342T, in the presence and absence of LA (Table II). In the absence of LA, Glc-T activity toward GlcNac acceptor was only ~0.3% of that of Gal-T activity. In the presence of LA, however, this activity increased ~30-fold, equivalent to ~10% of the Gal-T activity. The mutant protein C342T-Gal-T1 also showed 10–15% higher Glc-T activity and ~40% more LS activity, compared with wild type.

**Glucosyltransferase Activity of C342T-Gal-T1 toward Substrates Containing Different N2-acyl Derivatives of Glucosamine**—In the presence of LA, the transfer of Glc from UDP-Glc to an acceptor molecule (the Glc-T activity) was enhanced by ~50–75-fold, depending upon the N2-acyl substituent on the glucosamine (GlcN) molecule (Table III, part A). The transfer to unsubstituted Glc was approximately one-twentieth as that to GlcNac, indicating the importance of the *N*-acyl substitution at the 2-amino group of GlcN. The acceptor binding site can accommodate *N*-acetyl, *N*-propionyl, and even the bulkier *N*-butanoyl group. In the absence of LA, the presence of a  $\beta$ -benzyl group at the reducing end of GlcNac showed an activity higher than with GlcNac. However, LA did not increase this activity any further as it did with GlcNac (Table III, part A). *N,N'*-Diacylchitobiose (GlcNac $\beta$ 1–4GlcNac) was a less efficient acceptor, and LA inhibited this weak activity even further. In the LS reaction, the 2-hydroxyl group in Glc was necessary for maximum enzymatic reaction since 2-deoxyglucose or 2-fluoro-glucose were less efficient acceptors (Table III, part B).

**The Kinetic Parameters for the Glc-T Reaction**—The steady state kinetic analyses of C342T were carried out in the presence and absence of LA, in which acceptor and donor substrate concentrations were varied (Fig. 1). The initial velocity data obtained in the absence of LA with varying concentrations of UDP-Glc at fixed concentrations of GlcNac gave nonparallel lines in the double-reciprocal plot, and the data fitted best to Equation 1. Under these conditions the true  $K_m$  values for UDP-Glc and GlcNac were much higher than the values found for UDP-Gal and GlcNac in the Gal-T reaction. In the presence of LA, the data of double-reciprocal plots showed essentially parallel lines and the data fitted best to Equation 2, where  $K_{ia}$ , the dissociation constant for UDP-Glc, was 0 (Table IV); the  $K_m$  values for both substrates were considerably reduced to ~5-fold for UDP-Glc and 30-fold for GlcNac, whereas the  $k_{cat}$  increased by 30-fold (Table IV).

**Crystal Structure of C342T-Gal-T1·LA Complex with GlcNac**—The crystal structure of the mutant C342T-Gal-T1 was quite similar to the wild-type Gal-T1 in complex with LA and GlcNac. The mean r.m.s. deviation of the C $\alpha$  atoms in the mutant compared with the wild-type Gal-T1 structure was 0.4 Å. Although there was an additional methyl group present in the Thr side chain compared with the Cys residue, it was well accommodated and showed no perturbation to the local structure. The conformation and the binding of LA to C342T-Gal-T1 were quite similar to that observed with the wild type Gal-T1·LA complex with GlcNac (17). The molecular interactions between GlcNac and the C342T-Gal-T1·LA complex were quite similar to those found in the Gal-T1·LA·GlcNac (LS·GlcNac) complex (17). The *N*-acetyl group of GlcNac interacted with a hydrophobic pocket on Gal-T1 formed by the residues Arg-359, Phe-360, and Ile-363. A hydrogen bonding interaction was observed between Arg-359 and the carbonyl oxygen atom of the *N*-acetyl group of GlcNac (Fig. 4A).

**The Crystal Structures of the Complexes of Gal-T1 and LA with Either *N*-Butanoyl-GlcN or UDP-Glc·Mn<sup>2+</sup>**—The individ-

TABLE I  
Crystal structure refinement statistics

The crystals belong to a monoclinic system, with space group P2<sub>1</sub>.  $\beta$ 4Gal-T1-LA is a 1:1 complex between bovine  $\beta$ 4Gal-T1, residues 130–402, and mouse LA.

	Crystals grown in the presence of		
	(C342T- $\beta$ 4Gal-T1)-LA-GlcNAc	$\beta$ 4Gal-T1-LA-N-butanoyl-GlcN	$\beta$ 4Gal-T1-LA-UDPGlc-Mn <sup>2+</sup>
Unit cell parameters			
<i>a</i> (Å)	57.1	57.2	55.1
<i>b</i> (Å)	95.7	97.1	98.9
<i>c</i> (Å)	100.3	99.7	102.0
$\beta$ (°)	101.4	101.1	103.9
Resolution (Å)	2.0	2.3	2.3
Reflections collected	265,383	84,832	72,447
Unique reflections	71,569	38,898 (84%)	39,806 (85%)
<i>R</i> <sub>sym</sub>	0.079	0.062	0.117
Reflections used	69,391	38,816	37,326
Final <i>R</i> factor/ <i>R</i> <sub>free</sub>	0.25/0.30	0.26/0.32	0.25/0.30
r.m.s. deviations on			
Bonds (Å)	0.008	0.007	0.006
Angles (°)	1.5	1.4	1.5

TABLE II  
Relative specific activities of the wild type catalytic domain of Gal-T1 (d129-Gal-T1, residues 130–402) and C342T-Gal-T1, in the Gal-T, LS, and Glc-T reactions and comparison of the influence of  $\alpha$ -LA on these reactions

All reactions were performed under saturating conditions of all substrates and at 7  $\mu$ M  $\alpha$ -lactalbumin concentration (see text for assay conditions). 100% activity is defined as 51 pmol min<sup>-1</sup> ng<sup>-1</sup> of Gal transfer to GlcNAc in the Gal-T reaction. The LS and Glc-T activities are expressed as a percentage of Gal-T activity, which is set as 100%.

Enzyme	- $\alpha$ -Lactalbumin		+ $\alpha$ -Lactalbumin	
	UDP-galactose $\rightarrow$ GlcNAc (Gal-T activity)	UDP-glucose $\rightarrow$ GlcNAc (Glc-T activity)	UDP-galactose $\rightarrow$ Glc (LS activity)	UDP-glucose $\rightarrow$ GlcNAc (Glc-T activity)
	%	%	%	%
Bovine milk Gal-T1	100	0.3	110	10
d129-Gal-T1	100	0.3	129	7.8
C342T-Gal-T1	118	0.3	180	11.8

TABLE III  
Catalytic activity of C342T mutant using various acceptors in glucosyltransferase (part A) and lactose synthase (part B) reactions

A. Glucosyltransferase activity (Gal-T1 + UDP-Glc)	Activity		Increase (+LA/-LA)
	-LA	+LA	
	$\times 10^{-3}$ pmol/min/ng		-fold
GlcNAc	28	1486	53
N-Propionyl-GlcN	20	1466	73
N-Butanoyl-GlcN	18	1031	57
Glc	0	63	
$\beta$ -Benzyl-GlcNAc	168	162 <sup>a</sup>	
$\beta$ -GlcNAc-1,4-GlcNAc	17 <sup>b</sup>	9 <sup>c</sup>	
B. Acceptor specificities of lactose synthase (Gal-T1 + UDP-Gal + LA)			Activity
Acceptor			pmol/min/ng
Glc			19.17
2-Deoxy-Glc			0.47
2-Fluro-Glc			0.11
Glucosamine (GlcN)			0.0
Glucosamine 2-sulfate			0.0

<sup>a</sup> Activity was measured at 15 mM acceptor concentration.

<sup>b</sup> Activity was measured at 5 mM acceptor concentration.

<sup>c</sup> Activity was measured at 3 mM acceptor concentration.

ual structures of Gal-T1 and LA in both the crystal structures, Gal-T1-LA complex with *N*-butanoyl-GlcN and Gal-T1-LA complex with UDP-Glc-Mn<sup>2+</sup>, were quite similar (Fig. 3, A and B). The r.m.s. deviation for their C $\alpha$  atoms was only 0.6 Å. These structures were also similar to those of Gal-T1-LA-GlcNAc complex and the Gal-T1-LA-UDP-Mn<sup>2+</sup> complex (17). The binding of *N*-butanoyl-GlcN was quite similar to the GlcNAc binding in the C342T-Gal-T1-LA-GlcNAc complex (Fig. 4, A and B). *N*-Butanoyl group was very well accommodated in the *N*-acetyl binding pocket formed by residues Arg-359, Phe-360, and Ile-

TABLE IV  
Kinetic parameters for glucose transfer to GlcNAc by C342T-Gal-T1 in the presence and absence of LA

Substrate	Parameter	-LA	+LA
UDP-Glc	<i>k</i> <sub>cat</sub> (s <sup>-1</sup> )	0.058 (5)	0.247 (5)
	<i>K</i> <sub>A</sub> ( $\mu$ M)	148 (4)	31 (2)
	<i>K</i> <sub>ia</sub> ( $\mu$ M)	19.5 (6)	0
GlcNAc	<i>K</i> <sub>B</sub> (mM)	73.8 (3)	2.5 (1)

363 (Fig. 4B). The molecular interactions between the *N*-butanoyl group and Gal-T1 were also similar to those found with *N*-acetyl group (Fig. 4, A and B). In addition, the extended aliphatic chain of the *N*-butanoyl group is in the close vicinity to the side chain of Met-110 of the LA molecule. Longer aliphatic chain in the *N*-acyl group would cause steric hindrance with Met-110 residue of LA and expected to affect LA binding to Gal-T1, which is in agreement with the previous results (30).

**Comparison of UDP-Glc-Mn<sup>2+</sup> Binding with UDP-Gal-Mn<sup>2+</sup>**—Just as in the crystal structure of the Gal-T1-LA-UDP-Mn<sup>2+</sup> complex (17), Mn<sup>2+</sup> ion in the crystal structure of the Gal-T1-LA-UDP-Glc-Mn<sup>2+</sup> complex has five coordinations: two with the pyrophosphate oxygen atoms, one with Asp-254 carboxyl oxygen atom, one with S $\delta$  atom of Met-344, and one with the N $\epsilon$ 1 nitrogen atom of His-347. All the hydroxyl groups of the Glc moiety of UDP-Glc were involved in hydrogen bonding interactions with the Gal-T1 residues, Asp-252, Asp-318, and Glu-317 (Fig. 5, A and B). In the crystal structure, a strong hydrogen bond with a distance of 2.7 Å was observed between the O4 hydroxyl group of Glc of UDP-Glc and the carboxylate oxygen atom of the Glu-317 side chain (Fig. 5, A and B). A similar but weaker hydrogen bond (3.3 Å) was observed for the Gal of UDP-Gal in the crystal structure of

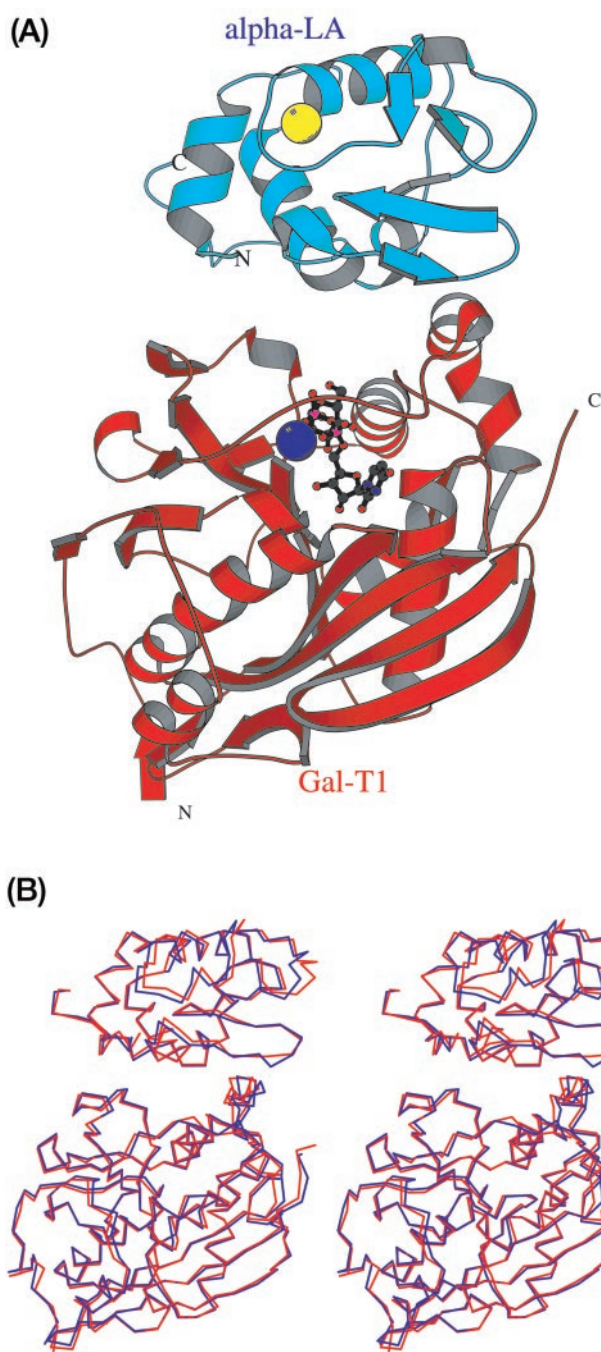


FIG. 3. *A*, molecular diagram of Gal-T1-LA-UDP-Glc-Mn<sup>2+</sup> complex. The molecular interactions between Gal-T1 and LA molecules are quite similar to those observed in the Gal-T1-LA-N-butanoyl-GlcN complex. However, in the protein complex, LA molecule does not interact with the donor UDP-Glc molecule. The Ca<sup>2+</sup> and Mn<sup>2+</sup> ions are shown as yellow and blue spheres, respectively. *B*, superposition of the C $\alpha$  atoms of the LS molecules from the crystal structure of the complex Gal-T1-LA-UDP-Glc-Mn<sup>2+</sup> (blue) and Gal-T1-LA-N-butanoyl-GlcN (red). The r.m.s. deviation between these structures is only 0.8 Å, indicating that these two protein structures are quite similar. Although there are few residues in the loop region, residues 60–65 on LA molecules that exhibit differences are not involved in any interactions either with the Gal-T1 or with substrate molecules.

Gal-T1-UDP-Gal-Mn<sup>2+</sup> (solved at 2.8-Å resolution).<sup>3</sup> The O4 hydroxyl group was equatorial in Glc, whereas it was axial in Gal. Due to this difference, the O4 hydroxyl oxygen of the Gal

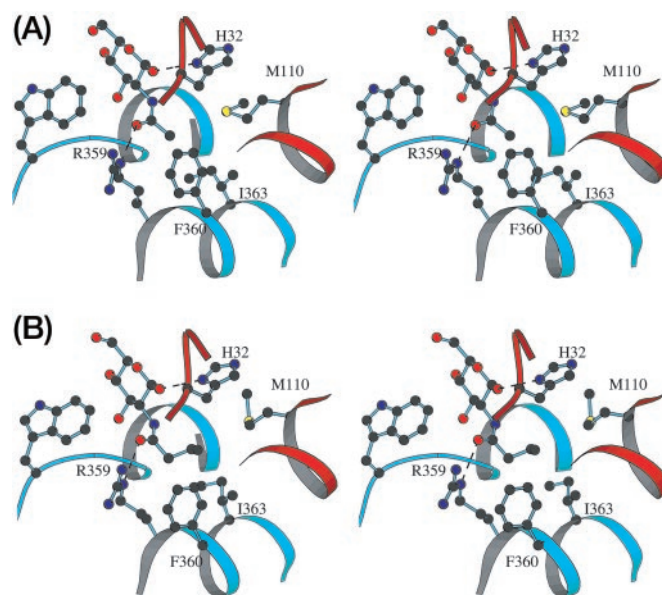
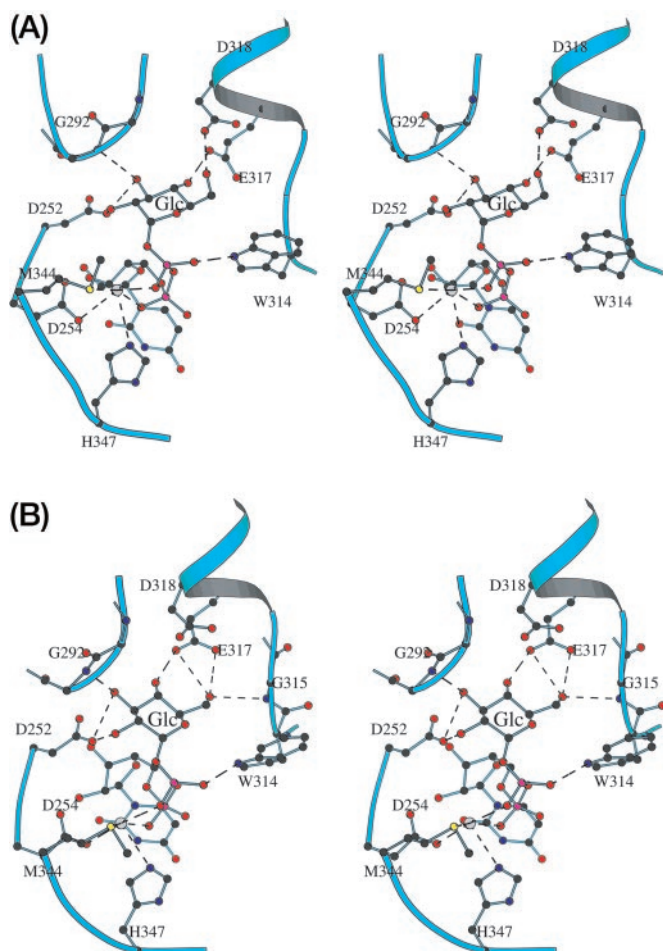


FIG. 4. Stereo diagram showing the binding of GlcNAc (*A*) and *N*-butanoyl-GlcN (*B*) to Gal-T1-LA complex. The LA and Gal-T1 secondary structures are shown in red and blue, respectively. The O1 hydroxyl group of the acceptor is hydrogen-bonded with the His32 residue of the LA molecule (dotted line). In these, interactions of both the acceptor substrates with the Gal-T1 and LA molecules are quite similar. *A*, the *N*-acetyl moiety of GlcNAc binds to a hydrophobic pocket on Gal-T1 formed by the residues Arg-359, Phe-360, and Ile-363. The carbonyl oxygen atom of the *N*-acetyl group forms a hydrogen bond with the Arg-359 side chain, and the methyl group is surrounded by the hydrophobic residues Phe-360 and Ile-363. *B*, like the *N*-acetyl group, the *N*-butanoyl group also binds to the same hydrophobic pocket of Gal-T1. In addition, the extended aliphatic chain of the *N*-butanoyl group interacts with the side chain of Met-110 of LA.

in the Gal-T1-UDP-Gal-Mn<sup>2+</sup> complex was pointed toward the acceptor binding site and formed a weak hydrogen bond with the Asp-318 side chain carboxylate oxygen atom. In the present crystal structure, although the O4 hydroxyl group of Glc was hydrogen-bonded to Glu-317, it pointed away from the acceptor binding site and Asp-318 (distance, 3.8 Å).

There were two Gal-T1-LA complexes present in the asymmetric unit, in which each complex had one bound UDP-Glc molecule and one Mn<sup>2+</sup> ion. Although the interactions of the donor substrate UDP-Glc-Mn<sup>2+</sup> with Gal-T1 were quite similar in both complexes in the asymmetric unit, the hydrogen bonding interactions observed for the O6 hydroxyl group of Glc with Gal-T1 were quite different in these two molecules (Fig. 5, *A* and *B*). In one molecule, the O6 hydroxyl group interacted with Asp-318, whereas, in the other, it interacted with the Gly-315 and Glu-317 in a manner similar to those observed for UDP-Gal binding in the Gal-T1-UDP-Gal-Mn<sup>2+</sup> complex.<sup>2</sup> The differences in the O6 hydroxyl orientation could have been a result of the orientation of O4 hydroxyl group in the Glc moiety. When UDP-Glc was bound, the O4 hydroxyl group (equatorial orientation) was pointing away from Asp-318, resulting in a space between the C4 atom of Glc and the Asp-318 side chain, which was filled by the O6 hydroxyl group (Fig. 5*A*). If the O4 hydroxyl group were in axial orientation as in Gal, it would be pointing toward the Asp-318 side chain, leaving not enough space between O4 atom of Gal and Asp-318. The O6 hydroxyl group must orient away from Asp-318, similar to what is observed in Fig. 5*B*. The hydrogen bonding interactions between O6 and Gal-T1 seem to be important for the binding of nucleotide-sugar since UDP-Glc, but not UDP-xylose, is a competitive inhibitor of Gal-T1 reaction (31, 32).

<sup>3</sup> B. Ramakrishnan and P. K. Qasba, unpublished observation.



**FIG. 5. Binding of UDP-Glc and  $Mn^{2+}$  ion to the Gal-T1-LA complex.** There are two Gal-T1-LA complexes in the asymmetric unit (A and B), each with one bound UDP-Glc- $Mn^{2+}$ . The  $Mn^{2+}$  ion coordination is the same in both the molecules. Although the molecular interactions between UDP-Glc and Gal-T1 are mostly the same in both the molecules of the asymmetric unit, the interactions of the O6 hydroxyl group with Gal-T1 are different in the two molecules. In one, the O6 hydroxyl group is hydrogen-bonded to the side chain carboxylate group of Asp-318 (A), whereas, in the other, it is hydrogen-bonded to the side chain carboxylate group of Glu-317 and the backbone nitrogen atom of Gly-315 residue (B). All the H-bonds and the  $Mn^{2+}$  coordination bonds are shown by dotted lines.

#### DISCUSSION

The present crystallographic study shows that the interactions of UDP-Glc and Gal-T1 exhibit few significant differences from those of UDP-Gal and Gal-T1. In the presence of LA, these differences are reflected in the  $K_m$  values for the substrates as well in the catalytic efficiencies between Gal-T reaction and Glc-T reaction (Tables II and IV). The property of Gal-T1 utilizing alternate nucleotide-sugar donor is also shared by other glycosyltransferases, albeit with lesser efficiency. For example, glucosylceramide synthase, which transfers Glc from UDP-Glc to ceramide, still exhibits 10% activity when UDP-Gal is used as the sugar donor (15). However, the difference between glucosylceramide synthase and Gal-T1 is that Gal-T1 exhibits ~10% glucosyltransferase (Glc-T) activity only in the presence of LA; in contrast, glucosylceramide synthase exhibits 10% activity with the alternate donor substrate UDP-Gal, even in the absence of any enhancer protein. It is possible that this may be an intrinsic property of the galactosyltransferases to exhibit glucosyltransferase activity or *vice versa*, albeit with lesser efficiency.

In order to understand the  $K_m$  values for the substrates in

the Glc-T reaction in the absence of LA, the crystal structure of Gal-T1-UDP-Glc complex in the absence of LA is essential. Since we could not obtain such crystals, it remains to be seen why the substrates have high  $K_m$  values. LA does not interact with either UDP-Glc or UDP-Gal in any of the crystal structures of Gal-T1-LA complex with various substrates determined so far. Thus, it appears that the role of LA in stimulating the Glc-T activity of Gal-T1 would only be at the enzyme kinetic level and not at the level of molecular interactions between UDP-Glc and Gal-T1. It has been suggested previously (31) that, during Gal-T reaction, Gal-T1- $Mn^{2+}$  complex is formed first, to which UDP-Gal and sugar acceptor bind sequentially. In the presence of LA during LS reaction, LA and Glc molecules bind synergistically (33). After catalysis the product disaccharide and LA dissociate, followed by UDP and  $Mn^{2+}$ . The Glc-T reaction is also expected to follow the same ordered sequential kinetic mechanism, and LA participates in the reaction in a way similar to LS reaction as described below.

Previously, we provided a structural basis for the Gal-T and LS kinetic mechanism based on the crystal structures of Gal-T1 (17). Briefly, Gal-T1 exists in two different conformations, I and II, which differ in the conformation between residues 345 and 365 and in the side chain orientation of Trp-314 (17, 18). In conformation I, these residues do not interact with UDP-Gal as they are away from the UDP-Gal binding site. In conformation II, on the other hand, they are placed in such a way that many of these residues not only bind to UDP-Gal but also bury it. In the crystal structure of substrate free Gal-T1 (18), Gal-T1 is found in conformation I, whereas it is found in conformation II in its complex with UDP-Gal- $Mn^{2+}$ ,<sup>2</sup> or as in the UDP-Glc- $Mn^{2+}$ -LA complex (Fig. 3A). It has been proposed that Gal-T1 exists in conformation I to which a free UDP-Gal molecule can bind (17). Upon binding, a conformational change takes place, involving residues 345–365 and Trp-314, which gives rise to conformation II of Gal-T1. This conformational change is important for the catalytic reaction to proceed forward, since it creates not only the sugar acceptor binding pocket but also the LA binding site so that the sugar acceptor GlcNAc can bind to the Gal-T1-UDP-Gal- $Mn^{2+}$  complex in conformation II. After catalysis, the product disaccharide leaves first followed by the reversal of conformation II to I, which releases the UDP molecule. The UDP-Gal-dependent conformational change is supported by earlier spectroscopic and photoinactivation studies; these studies had suggested that, upon UDP-Gal binding, Gal-T1 undergoes a conformational change protecting one Trp residue from photo-inactivation (30, 34). It was inferred that the sugar moiety of UDP-sugar is important for this conformational change, as UDP alone did not induce the conformational change to the same extent as UDP-Gal (30). The inability of UDP to stabilize the conformation II is crucial for its dissociation from the binding site.

On the basis of the Glc-T kinetic data obtained in the present study and taking into consideration the results of the earlier spectroscopic studies (30) and the structural data (17, 18), we propose the following role for LA in its Glc-T reaction. In the absence of LA, the kinetic data suggest that UDP-Glc dissociates from the enzyme-UDP-Glc complex ( $K_{ia} = 20 \mu M$ ), whereas in Gal-T reaction, UDP-Gal does not dissociate from the enzyme-UDP-Gal complex (31). The free UDP-Glc molecule can bind to Gal-T1 only when Gal-T1 is in conformation I, and, once Gal-T1 changes its conformation to II, the bound UDP-Glc gets buried in this conformation and cannot dissociate from enzyme (conformation II)-UDP-Glc complex. For UDP-Glc to dissociate from the enzyme-UDP-Glc complex, Gal-T1 must exist in conformation I. Therefore, it appears that, similar to UDP, UDP-Glc also does not induce the conformational change from I to II

as efficiently as UDP-Gal, thus hindering the catalytic reaction from proceeding.

In the presence of LA, according to the kinetic data, UDP-Glc does not dissociate from the enzyme-UDP-Glc complex ( $K_{ia} = 0$ ); therefore, the presence of LA seems to stabilize the enzyme-UDP-Glc complex in conformation II (Table IV). LA binds to Gal-T1 only if Gal-T1 is in conformation II (17), and, for steric reasons, upon its binding, Gal-T1 cannot reverse its conformation from II to I (17). Therefore, by selectively binding to enzyme-UDP-Glc complex in conformation II, LA might shift the equilibrium toward conformation II of enzyme-UDP-Glc complex. Further, it is possible that LA may not dissociate unless the product *N*-acetylcellobiosamine is formed, thereby enhancing the catalytic reaction to proceed forward. By stabilizing the conformation II and through direct interactions with the acceptor substrate, LA may enhance the binding of the acceptor GlcNAc in the Glc-T reaction. In all the three catalytic reactions (Gal-T, Glc-T, and LS), the conformational change from I to II is important not only for the LA and acceptor binding but also for the donor binding. In conformation II only, the sugar acceptor and LA binding sites are exposed for the binding and the His-347 residue can participate in  $Mn^{2+}$  ion coordination (17). Clearly, the ability to introduce conformational change in Gal-T1 by the UDP-sugar seems to be important for the enzyme catalysis even though the specific role of the sugar moiety in UDP-sugars in this process remains to be determined.

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