UDP-galactose:β-galactosyl-α,1,3-galactosyltransferase (α3GT) catalyzes the synthesis of galactosyl-α,1,3-β-galactosyl structures in mammalian glycoconjugates. In humans the gene for α3GT is inactivated, and its product, the α-Gal epitope, is the target of a large fraction of natural antibodies. α3GT is a member of a family of metal-dependent-retaining glycosyltransferases that includes the histo blood group A and B enzymes. Mn$^{2+}$ activates the catalytic domain of α3GT (α3GTcd), but the affinity reported for this ion is very low relative to physiological levels. Enzyme activity over a wide range of metal ion concentrations indicates a dependence on Mn$^{2+}$ binding to two sites. At physiological metal ion concentrations, Zn$^{2+}$ gives higher levels of activity and may be the natural cofactor. To determine the role of the cation, metal activation was perturbed by substituting Co$^{2+}$ and Zn$^{2+}$ for Mn$^{2+}$ and by mutagenesis of a conserved D$^{334}$YD$^{335}$ sequence motif that is considered to act in cation binding in many glycosyltransferases. The aspartates of this motif were found to be essential for activity, and the kinetic properties of a Val$^{150}$ to Ala mutant with reduced activity were determined. The results indicate that the cofactor is involved in binding UDP-galactose and has a crucial influence on catalytic efficiency for galactose transfer and for the low endogenous UDP-galactose hydrolase activity. It may therefore interact with one or more phosphates of UDP-galactose in the Michaelis complex and in the transition state for cleavage of the UDP to galactose bond. The DXD motif conserved in many glycosyltransferases appears to have a key role in metal-mediated donor substrate binding and phosphate-sugar bond cleavage.

UDP-Gal:β-galactosyl-α,1,3-galactosyltransferase (α3GT), a Golgi membrane-bound enzyme, catalyzes the synthesis of galactosyl-α,1-3-galactosyl β-OR structures in glycoconjugates (Refs. 1 and 2; see Fig. 1). α3GTcd and the products of its action are found in most mammals but not in humans and their closest relatives, the old world monkeys and apes (3, 4). In these species the gene for α3GT is mutationally inactivated (4, 5), and the absence of active enzyme allows the production of antibodies against the product of α3GT action, the α-Gal epitope (Fig. 1). Primates lacking this enzyme have natural antibodies (1–3% of circulating IgG, designated anti-Gal) that bind α-Gal (5) and facilitate immune defenses against pathogens but also present a barrier to the xenotransplantation of organs from mammalian species that produce active α3GT (6).

Like other glycoprotein glycosyltransferases, α3GTs are type-2 membrane proteins with a short N-terminal cytosolic domain, a transmembrane helix, a stem, and a C-terminal catalytic domain (7–10). Only a few subgroups of glycosyltransferases that function in the processing of glycoproteins and glycolipids show global homology in primary structure. At present, a 2.4-Å structure of the catalytic domain of β-1,4-galactosyltransferase I is the only reported three-dimensional structure of an enzyme of this type (11). α1,3-GT is homologous to histo blood group glycosyltransferases A and B and to Forssman glycolipid synthase but is not significantly similar in overall sequence to β-1,4-galactosyltransferase I (12–13). Nevertheless, these and other divalent cation-dependent glycosyltransferases share a DXD sequence motif that is thought to represent at least part of a cation binding site (14–16), suggesting that they may share a metal binding domain or substructure.

Recombinant forms of bovine and other α3GTs have been previously expressed (18, 19); the cytosolic and transmembrane domains together with a 67-residue stem can be deleted to produce a fully active soluble enzyme (18). We have described the use of a bacterially expressed soluble form of the catalytic domain of bovine α3GT (residues 80–367; α3GTcd) for the enzymatic production of substantial amounts of α-Gal-containing oligosaccharides (20). Here we have investigated the role of the metal cofactor in the catalytic mechanism using the recombinant enzyme. Like many other glycoprotein glycosyltransferases, α3GTcd requires a divalent cation for activity that has been thought to be Mn$^{2+}$ (1, 2). However, the reported affinity of the enzyme for this ion ($K_d$ of 6 mM) exceeds the physiological concentration of Mn$^{2+}$ by about 3 orders of magnitude, raising questions about the cation dependence in vivo. Studies of metal activation over a wide concentration range reveal a high affinity binding site for Mn$^{2+}$ and other metals, including Zn$^{2+}$, which is present at higher levels in biological systems.

Glycosyltransferases that, like α3GT, catalyze “retaining” reactions are expected to utilize double displacement mechanisms in which the UDP to galactose bond is cleaved, with formation of an intermediate before transfer of galactose to an acceptor. The mechanism of the reaction catalyzed by α3GTcd is sequential, UDP not being released before the completion of catalysis. However, a low level of UDP-galactose hydrolytic activity indicates that the UDP to galactose bond can be cleaved in the absence of a carbohydrate acceptor, possibly via formation of an oxycyanobium intermediate. The properties of α3GT activated by different metal ions and a mutant with a...
substitution in a metal binding sequence motif indicate a role for the metal ion in UDP-galactose binding and catalysis.

**EXPERIMENTAL PROCEDURES**

**Construction of pET15b E80rGT**—The expression plasmid was generated from a cDNA of bovine α1,3-GT in pSVSPORT vector provided by Dr. L. Inverardi, Diabetes Research, Cell Transplant Center, University of Miami School of Medicine. This clone had a deletion corresponding to Tyr-64 to Phe-95 that included a 15-residue sequence previously reported to be essential for enzyme activity. The region required for activity was restored by polymerase chain reaction using the primers designated ”extension” and “BGT-C.” The product was gel-purified and used as a template in a second amplification with primers BE80GT-N and BGT-C. Extension (coding), GAAAAGCTTAAAGCTATGCGACTGTTGTCACACCCCATTTAAAGGC; BE80GT-N (coding), CGAATATCATATGCAACAGTCTAGGCTTACGTCATCG; BGT-C (complementary), CGCGGATCCAAAAAGTCAGATTTCTAACCAC.

The product from the second polymerase chain reaction was directly used for TA cloning. Positive colonies were isolated and screened by restriction mapping with Ndel and BamHI. A clone with the appropriate insert was selected and digested with the same restriction enzymes. The insert was purified by agarose gel electrophoresis and cloned into a preparation of pET15b vector that had been previously cleaved with Ndel and BamHI. The product was transformed into *Escherichia coli* DH5α competent cells, and plasmids were prepared and characterized by restriction mapping and DNA sequencing.

**Bacterial Expression and Purification**—Cultures of *E. coli* BL21(DE3) transformed with pET15b-α3GT were grown in LB medium containing 100 μg/ml ampicillin with rapid shaking (250 rpm) at 37 °C. When the A600 max of the culture reached 0.8–1.0, isopropyl-1-thio-β-D-galactopyranoside was added to a concentration of 0.4 mM to induce expression of T7 RNA polymerase and recombinant α3GTcd. The cultures were then incubated at 27 °C for 20 h with slow shaking (200 rpm) and harvested by centrifugation at 3,000 rpm for 20 min. Bacteria were washed with washing buffer (20% sucrose, 20 mM Tris-HCl, pH 8.0), suspended in 50 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 0.1 M NaCl, and lysed using a French pressure cell. The soluble fraction was collected by centrifugation at 12,000 rpm for 30 min.

The supernatant was applied to a Ni2+-NTA column at 4 °C, and the proteins were expressed as described for the wild-type enzyme.

**Activity Measurements**—α3GT assays typically included 2–4 μg/ml enzyme, 50 mM MES buffer, pH 6.0, 10 mM lactose, 0.3 mM UDP-[3H]galactose (specific activity, 500 cpm/nmol), 0.1% bovine serum albumin, and metal cation in a total volume of 100 μl and were incubated at 37 °C for 5–15 min. In steady-state kinetic studies, the concentrations of metal ion, lactose, and UDP-galactose were varied with other substrates and cofactors at fixed concentrations. Blanks were reactions from which the acceptor substrate is omitted. Studies at high enzyme concentrations indicated that the enzyme has a low level of UDP-galactose hydrolase activity, which contributes to the backgrounds measured in this way; however, because hydrolase activity is only 0.25% of the transferase activity, the effects of the hydrolase activity on the calculated transferase activities is insignificant. Assays were terminated by adding ice-cold 0.1 M EDTA (100 μl). The reaction mixture was then applied to a 2-ml AG1-X8 (Bio-Rad) column, and the radioactive product was eluted with 0.5 ml followed by 1 ml of water. The eluate was collected in a plastic vial, mixed with 10 ml of Econolume (ICN Biomedicals, Costa Mesa, CA), and counted in a liquid scintillation counter (LKB). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the transfer of 1 pmol of galactose from UDP-galactose to lactose/min at 37 °C. Conditions were chosen so that <25% of the radioactivity from the UDP-[3H]galactose was incorporated into product in most experiments. Kinetic data were analyzed by fitting to appropriate rate equations using the curvefitter program of SigmaPlot™. For metal activation studies, data were fitted to equations describing activation through binding to a single metal binding site (Equation 1) or activation to V by binding to site 1 with affinity K1 and to V2 by additional binding to site 2 with a K2 of K1 (Equation 2).

\[
v = V \times [M]/(K_d + [M])
\]

(Eq. 1)

\[
v = (V_1 \times K_2 \times [M] + V_2 \times [M]^2)/(K_a \times K_b + K_a \times [M] + [M]^2)
\]

(Eq. 2)

Data from studies in which the two substrates are varied were fitted to equations for a symmetrical sequential initial velocity pattern associated (3) and an asymmetrical initial velocity pattern (4).

\[
v = V_{o} [A] [B]/(K_a \times K_b + K_a \times [A] + [A] [B])
\]

(Eq. 3)

\[
v = V_{o} [A] [B]/(K_a \times [B] + K_a \times [A] + [A] [B])
\]

(Eq. 4)

**CD Spectroscopy**—Near and far UV CD spectra of recombinant proteins were determined with a Jasco J-710/720 spectropolarimeter. Twenty spectra were scanned for each sample at a speed of 100 nm/min, averaged, and smoothed. Near UV CD spectra (250–320 nm) were determined using a cell with a path length of 1 cm, and far UV spectra (200–250 nm) were determined using a cell with a path length of 0.1 cm. Proteins were dissolved in 10 mM MES, pH 6.0, containing 50% glycerol at concentrations between 0.15 and 0.5 mg/ml. Far UV CD data were analyzed to estimate secondary structure composition using the k2d neural network program (24).
RESULTS

Preparation and Properties of Bovine Recombinant α3GTcd—α3GTcd (residues 80–367 of bovine α1,3GT) was expressed cytoplasmically in E. coli as a soluble active enzyme and was purified, reproducibly, in yields of more than 7 mg/liter bacterial culture. Active enzyme is also produced by expression at 37 °C, but the yield is lower by about 20% than at 27 °C. The enzyme migrates as a single component on SDS gel electrophoresis with an apparent molecular weight of 36,000 (data not shown). After precipitation between 10 and 80% saturation with ammonium sulfate, α3GTcd is soluble at concentrations of up to 7.5 mg/ml in 20 mM MES, pH 6.0, containing 50% (v/v) glycerol. A form of α3GTcd with an additional N-terminal truncation of six residues was also expressed. In this case, when the expression was at 27 °C, a similar yield was obtained, but at 37 °C, essentially no active enzyme was produced. Steady state kinetic studies with the purified smaller enzyme, in which the concentrations of UDP-galactose or lactose were varied at a fixed concentration of the second substrate at 10 mM Mn²⁺, showed that the apparent $K_m$ and $V_m$ values were closely similar to those of the larger enzyme (data not shown).

Fig. 2 shows the near and far UV CD spectra of α3GTcd. CD spectra have not been previously reported for either natural or recombinant forms of this enzyme. The near UV spectrum has a similar magnitude to those of other proteins and can be expected to be that of the correctly folded catalytic domain. The far UV CD spectrum was analyzed using the k2d neural network program to give estimates of 28% α-helix and 38% β sheet; the predicted spectrum from the analysis is a reasonable fit to the experimental data (Fig. 2). The secondary structure predicted from the sequence with a consensus of the PREDATOR (25, 26), PHD (27), and Quadratic Logistic (28) methods is 27% α-helix and 18% β sheet. Differences in the β sheet content obtained from these analyses may reflect the inaccuracy of β-structure predictions from far UV CD spectra resulting from the relatively weak ellipticity of β sheets compared with α helices as well as the inherent limitations of sequence-based secondary structure predictions. The predictions are similar to the secondary structure composition of the β-1,4-GT catalytic domain: 25% helix, 20% β sheet (11). The form of α3GTcd with the additional six-residue truncation had CD spectra closely similar to those of the larger enzyme.

α3GTcd Has High and Low Affinity Binding Sites for Mn²⁺ and Other Metals—As previously reported for natural α3GT, the bacterially expressed enzyme is inactive in the absence of metal ions and is strongly activated by Mn²⁺. Activity measurements in the presence of other metal ions at concentrations of 10 mM and in the absence of Mn²⁺ indicated that Co²⁺ and Fe²⁺ also activate the enzyme but Zn²⁺, Cu²⁺, and Mg²⁺ do not.

Detailed studies with Fe²⁺ were not performed because of its tendency to oxidize in the assay system, but the kinetic properties of the Co²⁺-activated enzyme were characterized in detail (see below and Table I). Enzyme activities at Mn²⁺ concentrations from 10 μM to 15 mM at fixed concentrations of lactose (10 mM) and UDP-galactose (0.3 mM) did not fit well to a rate equation describing the dependence of activity on metal binding to a single site, giving residuals that vary systematically with log([Mn²⁺]). However, the rate equation for a two-site model of metal activation gave a better fit (Fig. 3B). The results of this analysis indicate that α3GTcd is activated by binding of Mn²⁺ to a high affinity site with an apparent $K_a$ value of 80 ± 20 μM; additional binding to a second site with an apparent $K_d$ of 2.3 ± 0.5 mM gives a 6-fold higher maximum activity at the substrate concentrations used in these assays.

This result led us to re-investigate the effects of other metal ions over a broader range of concentrations (10–10,000 μM). Although no activity was observed with Cu²⁺ or Mg²⁺ at any concentration, Zn²⁺ was found to activate up to a concentration of 500 μM and to progressively inhibit at higher concentrations. In the lower concentration range, Zn²⁺ produces a similar level of activity to Mn²⁺ and is more effective than Co²⁺ (Fig. 3). The data obtained with Zn²⁺ do not fit well to an equation describing activation by binding to a high affinity site and inhibition resulting from binding to a second lower affinity site, and the line through these data in Fig. 3A was generated by a nonlinear spline method. Inhibition by Zn²⁺ may result from multisite binding and/or denaturation at higher concentrations. The activity profile with Co²⁺ fits well to the equation for single-site activation with a $K_a$ of 4.8 ± 0.5 mM and a lower maximum activity than for Mn²⁺. It is possible that this cation also binds to two sites, but that the enzyme form with a single metal ion bound to the high affinity site is inactive or has extremely low activity. The apparent binding constants for activation by different cations derived from these studies with fixed substrate concentrations are summarized in Table II.

To determine whether α3GT, when partially activated by Mn²⁺ binding to the high affinity site, can be further activated by a different metal binding to a second lower affinity site, different metal ions (Ca²⁺, Mg²⁺, Zn²⁺, Ni²⁺, Cu²⁺, Te³⁺, and Eu³⁺) were added at concentrations of 10 mM in the presence of 80 μM Mn²⁺. None enhanced the activity. In the enzyme activated by 10 mM Mn²⁺, Cu²⁺ or Eu³⁺ were found to strongly inhibit activity at micromolar concentrations, indicating that they bind to a high affinity cation binding site(s). Because Eu³⁺ is a very potent inhibitor, it was further characterized and found to be a mixed inhibitor with respect to Mn²⁺ (0.5–6 mM) with a small intercept effect and large slope effect in a double reciprocal plot (Fig. 4). In contrast, Eu³⁺ is a competitive in-
hbitors against Co$^{2+}$ (2.2 to 15 mM; data not shown). These results are consistent with the presence of two metal binding sites for Mn$^{2+}$ and a single site for Eu$^{3+}$; it appears also that a binding of Co$^{2+}$ to a single site is required for catalysis. An apparent $K_i$ of 0.5 μM was calculated from the inhibitory activity against Co$^{2+}$ and $K_i$ and $K_{ii}$ values for inhibition against Mn$^{2+}$ were 11.3 ± 1.7 and 3.3 ± 0.7 μM, respectively.

**Steady State Kinetic Studies with Different β-Galactoside Acceptors Show That α3GTcd Catalyzes Galactose Transfer by a Sequential Mechanism—**Bisubstrate reactions can utilize ping-pong (double displacement) or sequential mechanisms; these can be distinguished by steady state kinetic experiments in which acceptor and donor substrate concentrations are varied. Galactose transfer to lactose, lactosamine, and β-azidolectose was measured at UDP-galactose concentrations from 0.03 to 0.6 mM and different acceptor concentrations at a fixed 10 mM concentration of UDP-galactose (0.3 mM) and lactose (10 mM). $K_{ii}$ and $K_{ia}$ calculated from these data are 11.3 ± 1.7 and 3.3 ± 0.7 μM.

**lactose against Co$^{2+}$**

Galactose transfer to lactose, lactosamine, and these can be distinguished by steady state kinetic experiments ping-pong (double displacement) or sequential mechanisms; a Sequential Mechanism—

Bisubstrate reactions can utilize

| Metal Parameter | Wild type | Wild type | Wild type | V150A |
|-----------------|-----------|-----------|-----------|-------|
| Metal activator | Mn$^{2+}$ | Co$^{2+}$ | Zn$^{2+}$ | Mn$^{2+}$ |
| $k_{cat}$ (s$^{-1}$) | 6.4 ± 0.7 | 3.8 ± 0.5 | 0.72 ± 0.06 | 3.0 ± 0.3 |
| $K_i$ (μM) | 0.43 ± 0.07 | 0.83 ± 0.31 | 0.80 ± 0.20 | 2.6 ± 0.5 |
| $K_{ii}$ (μM) | 0.14 ± 0.03 | 1.5 ± 0.5 | 1.4 ± 0.6 | 1.0 ± 0.45 |
| $k_{cat}/K_i \times 10^{-6}$ (s$^{-1} \cdot$ M$^{-2}$) | 19.9 ± 3.4 | 12.1 ± 3.5 | 4.8 ± 1.0 | 30.5 ± 6.5 |
| $K_{ii}$ (μM) | 6.5 ± 1.8 | 21.9 ± 12.6 | 4.5 ± 4.5 | 11.7 ± 6.2 |
| $k_{cat}/K_{ii}$ (s$^{-1} \cdot$ M$^{-1}$) | 2.3 ± 0.7 | 0.21 ± 0.11 | 0.11 ± 0.05 | 0.10 ± 0.05 |

Hydrolase activity

| Metal Parameter | Wild type | Wild type | Wild type | V150A |
|-----------------|-----------|-----------|-----------|-------|
| Metal activator | Mn$^{2+}$ | Co$^{2+}$ | Zn$^{2+}$ | Mn$^{2+}$ |
| $k_{cat}$ (s$^{-1}$) | 16 ± 1.4 | 12.2 ± 0.4 | 7.9 ± 0.1 | 4.8 ± 0.7 |
| $K_i$ (μM) | 0.103 ± 0.001 | 0.51 ± 0.04 | 1.0 ± 0.2 | 1.0 ± 0.3 |
| $k_{cat}/K_i$ (s$^{-1} \cdot$ M$^{-1}$) | 155 ± 13 | 23.9 ± 2.7 | 7.9 ± 1.6 | 4.8 ± 1.6 |

* Lactose was used as acceptor substrate in these experiments.

**Metal ion activation of wild-type α3GTcd and the V150A mutant**

The values for the parameters for Zn$^{2+}$ were obtained by fitting the data for the lower activating concentration range (≤300 μM).

| Metal Parameter | Units | Wild type | V150A |
|-----------------|------|-----------|-------|
| Mn$^{2+}$ | μM | 80 ± 20 | ND$^b$ |
| $K_{i1}$ | mM | 2.3 ± 0.5 | 4.7 ± 0.5 |
| $V_{max}$ | μmol/mg · min | 0.91 ± 0.16 | ND |
| Zn$^{2+}$ | μM | 58 ± 5 | 267 ± 37 |
| Co$^{2+}$ | μM | 0.75 ± 0.02 | 0.32 ± 0.02 |
| μmol/mg · min | 4.8 ± 0.5 | 9.4 ± 4.2 |
| Mn$^{2+}$ | μmol/mg · min | 1.3 ± 0.1 | 0.04 ± 0.008 |

$^a$ Apparent (app) values determined at fixed concentrations of donor and acceptor substrates.  
$^b$ ND, not detected; i.e., enzyme activity showed dependence on metal binding to a single site.

**Mixed inhibition by Eu$^{3+}$ of the Mn$^{2+}$-stimulated activity of α3GTcd**

The double-reciprocal plots show enzyme activity at a fixed concentrations of Eu$^{3+}$ (0, 2, 4, and 8 μM) and varying concentrations of Mn$^{2+}$, at fixed concentrations of UDP-galactose (0.3 mM) and lactose (10 mM). $K_{ii}$ and $K_{ia}$ calculated from these data are 11.3 ± 1.7 and 3.3 ± 0.7 μM.

![Fig. 3. Activation of α3GTcd by different metal ions. A. activation profiles for Zn$^{2+}$ (●), Mn$^{2+}$ (○), Co$^{2+}$ (▲). B. residuals from fitting Mn$^{2+}$ activation data to equations describing single-site binding (open circles) and two-site binding (closed circles).](image)

![Fig. 4. Mixed inhibition by Eu$^{3+}$ of the Mn$^{2+}$-stimulated activity of α3GTcd. The double-reciprocal plots show enzyme activity at a fixed concentrations of Eu$^{3+}$ (0, 2, 4, and 8 μM) and varying concentrations of Mn$^{2+}$, at fixed concentrations of UDP-galactose (0.3 mM) and lactose (10 mM).](image)
a3GTcd Catalyzes a Low Rate of UDP-galactose Hydrolysis, Indicating That It Does Not Bind Acceptor before Donor Substrate in an Obligatory Order—UDP-galactose hydrolyase activity was initially noticed in the form of high backgrounds (1H release from UDP-galactose in the absence of acceptor into a product that does not bind to the anion exchange resin) at higher enzyme concentrations. The activity increases linearly with enzyme concentration and time, up to 15 min. Hydrolyase activity was characterized using a 10–20-fold higher concentration of enzyme than in standard assays; it is Mn2⁺-dependent (data not shown) and also displays saturation kinetics with varying concentrations of UDP-galactose (Fig. 6). The $k_{\text{cat}}$ for hydrolysis is 0.25% of the corresponding transferase activity with lactose as acceptor.

The hydrolytic activity of a3GTcd indicates that the enzyme binds the donor substrate and catalyzes cleavage of the bond between UDP and galactose in the absence of a carbohydrate acceptor. Although the enzyme complexes for the hydrolysis reaction may differ structurally from those for the transfer reaction, the hydrolyase activity is inconsistent with a sequential mechanism with obligatory ordered binding of acceptor before donor substrate. The remaining alternative mechanisms are random sequential or ordered sequential binding of donor and acceptor.

The kinetic parameters listed in Table III were calculated by fitting to Equation 3 designating UDP-galactose as substrate A and lactose as substrate B. The same rate equation applies to an ordered sequential mechanism and random equilibrium mechanism; the values for $K_{ib} = K_{ia} \times K_{a} / K_{b}$ given in Tables I and III represent the $K_{b}$ of the enzyme-acceptor complex in the case of a random equilibrium mechanism.

**Table III**

| Acceptor              | Lactose | β-Azido lactose | Lactosamine |
|-----------------------|---------|-----------------|-------------|
| $k_{\text{cat}}$ (s⁻¹) | 6.4 ± 0.7 | 4.9 ± 0.3       | 3.8 ± 0.5   |
| $K_{a}$ (mM)          | 0.43 ± 0.07 | 0.28 ± 0.03     | 0.52 ± 0.12 |
| $K_{b}$ (mM)          | 0.14 ± 0.03 | 0.25 ± 0.05     | 0.09 ± 0.03 |
| $K_{ib} / K_{ia}$ (s⁻¹ m⁻²) | 2.3 ± 0.7 $\times 10^6$ | 8.5 $\pm 2.0 \times 10^6$ | 2.2 $\pm 0.9 \times 10^6$ |

**Fig. 5.** Double-reciprocal plots for galactose transfer to lactose catalyzed by a3GTcd in the presence of different metal ions. Steady state velocities were determined in the presence of 10 mM Mn²⁺ (A) and 10 mM Co²⁺ (B). Lactose is plotted as the variable substrate at the following fixed concentrations of UDP-galactose: A, 0.6, 0.15, 0.075, 0.045, 0.03 mM; B, 3, 1.5, 0.9, 0.6, 0.3 mM (from bottom to top). The lines are generated by curve fitting using the values given in Table I.

**Fig. 6.** Effects of varying substrate concentration on the rate of UDP-galactose hydrolysis catalyzed by a3GTcd. Activities were determined in the presence of 10 mM Mn²⁺ (○), Zn²⁺ (■), or Co²⁺ (▲). The lines are generated by curve fitting using the values given in Table I.

Metal Cofactor Substitution Affects UDP-galactose Binding and, More Strongly, Catalytic Efficiency—Because Mn²⁺, Co²⁺, and Zn²⁺ are active as cofactors for a3GTcd, the role of the metal ion can be investigated by determining the effects of substituting different metals on different kinetic parameters. The three metal ions were used at concentrations of 10 mM, 10 mM, and 300 μM, respectively, levels that gave optimal activities in assays conducted at fixed substrate concentrations. Table I shows that the metal substitution has a relatively small but significant effect on $k_{\text{cat}}$ and on the $K_{m}$ for acceptor (lactose), but $K_{m}$, the $K_{d}$ of UDP-galactose from the enzyme-UDP-galactose complex in an ordered or random equilibrium sequential mechanism, was increased 10-fold, and $k_{\text{cat}} / K_{m}$ a parameter reflecting catalytic efficiency in a bisubstrate reaction (30), was reduced 11–38-fold.

These three metal ions are also effective cofactors for the low hydrolyase activity of a3GTcd. Hydrolyase activity was measured at a range of UDP-galactose concentrations with the same fixed concentrations of these metals (Fig. 6). As also shown in Table I, metal substitution has similar effects on this activity, perturbing $k_{\text{cat}}$ and the $K_{m}$ for UDP-galactose, which results in a larger reduction in catalytic efficiency ($k_{\text{cat}} / K_{m}$), as observed with the transferase activity.

The Effects of Mutations in the Conserved DVD Sequence Indicates a Crucial Role in Functional Properties Associated with Metal Binding—A sequence motif, DXD, with some less
The activity of α3GTcd is modulated by high and low affinity binding sites for metal cations ($K_a$ of $>10^3$ and $10^5–10^6$ M$^{-1}$) as previously observed with β-1,4GT (31). Inhibition by Eu$^{3+}$ and Cu$^{2+}$ is associated with $K_i$ values in the low micromolar range. The involvement of two metal ions in catalysis has been observed in several metalloenzymes that catalyze phosphoryl transfer reactions including 3’-5’ DNA exonuclease (32), alkaline and acid phosphatases (33, 34), purple acid phosphatase (35, 36), and phosphoprotein Ser/Thr phosphatase, PP-1 (37). In these enzymes, the metal ions form a bimetal center that in purple acid phosphatase and PP-1 has been found to act as a ligand for the phosphate, facilitating its stabilization and correct orientation in the active site and also in generating a hydroxide nucleophile involved in catalysis (35–37). An analogous role for the metal as a ligand for phosphate(s) in the donor substrate in α3GT is supported by the present results.

In vivo only the high affinity site may be relevant since the cellular concentrations of the ions that can activate α3GTcd are in the micromolar range. In this concentration range, Zn$^{2+}$ is as effective as Mn$^{2+}$ (Fig. 3A). The concentrations of different metals in the trans-Golgi lumen, the cellular site of action of α3GT, are unknown. However, body fluids that originate in part from this compartment such as milk have Zn$^{2+}$ concentrations (50–200 μM) that exceed those of Mn$^{2+}$ by about 2 orders of magnitude (38). Co$^{2+}$ is irrelevant as an activator in vivo because of its low abundance relative to the level required for activation. It is therefore possible that, as with methionine aminopeptidase I (39), the biologically active cofactor has been misidentified, and Zn$^{2+}$ is the relevant cofactor for α3GT. However, previous studies with β-1,4GT-1 show that the enzyme in Golgi vesicles is optimally activated by lower concentrations of Mn$^{2+}$ and that an endogenous high molecular weight molecule participates in the activation of this glycosyltransferase in vivo (40). Although the present studies indicate that Zn$^{2+}$ is the likely natural cofactor(s) of α3GT, the possibility of the involvement of an endogenous macromolecule cannot be discounted.

There is limited information presently available on the mechanisms of glycoprotein glycosyltransferases that catalyze retaining reactions. The steady state kinetic properties of α3GTcd indicate, as for β-1,4GT (41), a sequential mechanism in which metal cofactor, donor, and acceptor bind enzyme before catalysis, with no release of UDP before the transfer of galactose to an acceptor. Thus, a double displacement mechanism in which UDP is released with formation of an interme-


**α-1,3-Galactosyltransferase, Cation Specificity, and Role**

The aspartates of the D149VD151 motif of α3GTcd are essential for catalysis as found for analogous residues in yeast MNN1 α-1,3-mannosyltransferase (15) and clostridial cytotoxins (16) and for the aspartate and histidine of an analogous DXH sequence in polyprotein GalNAc transferase (17). The Asp151 → Asn mutation changes the near and far UV CD spectra, indicating that this substitution perturbs the local structure. Difference spectra for the mutant show a peak in the far UV CD spectrum around 230 nm and a broad peak in the near UV CD spectrum centered around 265 nm (Fig. 7); both of these peaks are consistent with an effect of the mutation on the environment of a buried tryptophan side chain (47). Since residues of this sequence motif are essential for function in highly divergent cation-dependent glycoprotein glycosyltransferases, which are otherwise not significantly similar in sequence, it appears that these enzymes may be distantly related or share a homologous domain (14). Sequence comparisons suggest that the catalytic domains of glycoprotein-processing glycosyltransferases have modular structures and are composed of domains of different origins (17). The elucidation of high resolution crystallographic structures for multiple representatives is needed to clarify this issue. At present, the only known structure is that of the truncated catalytic domain of β-1,4GT-1 (11); the structure of α3GTcd is presently unknown, but a model for a putative sugar nucleotide binding domain of α3GTcd has been described based on the use of a T4 phage DNA-modifying glycosyltransferase (48) as a template. However, the phage enzyme appears to be structurally unrelated to β-1,4 GT (11), whereas secondary structure topology predicted for α3GTcd from multiple alignment methods and the secondary structure content indicated by the far UV CD spectrum are consistent with an α/β fold similar to that found in β1,4GT-1. Also, the D289VD294 sequence in β1,4GT-1 has a role in binding the UDP-galactose substrate (11), in agreement with the role for D149VD151 in α3GTcd indicated by the present study which shows that the Val150 to Ala substitution reduces the affinity for UDP-galactose and more strongly lowers the catalytic efficiency for transferase and hydrolase activities. Although there is insignificant global similarity between the sequences of the catalytic domains of α3GTcd and β1,4GT family, this apparent similarity between the two enzymes in structure-function relationships suggests that part of their structures may have a common origin. Unfortunately, the level of resolution of the structure of β1,4GT-1 does not provide any information about bound metal ions. The properties of the Val150 to Ala mutant of α3GTcd suggest that the mutation disrupts properties of the enzyme that are associated with cation activation, supporting the view that this region has a role in binding the metal ion and UDP-galactose.

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Primary kinetic parameters show that the metal strongly affects the affinity for UDP-galactose (Km) but has a lesser effect on affinity for acceptor substrate (Km and Kcat); this is borne out by the kinetic parameters for UDP-galactose hydrolysis with different cofactors (Table I). Although there are large standard errors in the values for the catalytic efficiency for galactose transfer, kcat/Km, these are compounded from the errors in three experimentally determined parameters, it is clear that metal substitution has its largest effect on this parameter. For UDP-galactose hydrolysis, the substitution of Co2+ or Zn2+ produces increases in the Km similar to those for Km for the transferase reaction and greater reductions in catalytic efficiency (kcat/Km); the Km for UDP-galactose will closely approximate the Km of the E/S complex for hydrolysis because of the low kcat. As discussed above, the transition state for the α3GTcd-catalyzed reaction may be similar to those in other retaining glycosyltransferases such as glycosgen phosphorylase and its close relative, maltodextrin phosphorylase. In these enzymes, where the reaction is analogous to the reverse of the α3GTcd-catalyzed reaction, the monosaccharide in the enzyme-bound oligosaccharide substrate that is transferred is rotated relative to its preferred structure in solution, and C1-O bond cleavage results in formation of an oxyacarbonium intermediate (43). A similar process may occur with the UDP-galactose substrate of α3GT, the formation of such an intermediate independent of acceptor substrate binding is consistent with the low level of hydrolytic activity. Data supporting cleavage of the nucleoside diphosphate-monosaccharide bond before transfer have been previously reported for two inverting transferases, α-1,3-fucosyltransferase V (43, 44) and β-1,4GT-1 (45, 46), suggesting that this may be a common intermediate in retaining and inverting transferases. A plausible role for the metal would involve an interaction with the phosphates of the UDP-sugar and of the UDP formed by cleavage of the UDP to galactose bond.
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