Changing the Donor Cofactor of Bovine α1,3-Galactosyltransferase by Fusion with UDP-galactose 4-Epimerase

MORE EFFICIENT BIOCATALYSIS FOR SYNTHESIS OF α-Gal EPITOPES

Two fusion enzymes consisting of uridine diphospho-galactose 4-epimerase (UDP-galactose 4-epimerase, EC 5.1.3.2) and α1,3-galactosyltransferase (EC 2.4.1.151) with an N-terminal His6 tag and an intervening three-glycine linker were constructed by in-frame fusion of the Escherichia coli galE gene either to the 3′ terminus (f1) or to the 5′ terminus (f2) of a truncated bovine α1,3-galactosyltransferase gene, respectively. Both fusion proteins were expressed in cell lysate as active, soluble forms as well as in inclusion bodies as improperly folded proteins. Both f1 and f2 were determined to be homodimers, based on a single band observed at about 67 kDa in SDS-polyacrylamide gel electrophoresis and on a single peak with a molecular mass around 140 kDa determined by gel filtration chromatography for each of the enzymes. Without altering the acceptor specificity of the transferase, the fusion with the epimerase changed the donor requirement of α1,3-galactosyltransferase from UDP-galactose to UDP-glucose and decreased the cost for the synthesis of biomedically important Galα1,3Gal-terminated oligosaccharides by more than 40-fold.

For enzymatic synthesis of Galα1,3Galβ1,4Glc a from UDP-glucose and lactose, the genetically fused enzymes f1 and f2 exhibited kinetic advantages with overall reaction rates that were 300 and 50%, respectively, higher than that of the system containing equal amounts of epimerase and galactosyltransferase. These results indicated that the active sites of the epimerase and the transferase in fusion enzymes were in proximity. The kinetic parameters suggested a random mechanism for the substrate binding of the α1,3-galactosyltransferase. This work demonstrated a general approach that fusion of a glycosyltransferase with an epimerase can change the required but expensive sugar nucleotide to a less expensive one.

Oligosaccharides are attractive targets for the development of new pharmaceuticals because of their important roles in cell recognition, cell signaling, and other biological processes (1, 2). α-Gal1 epitopes (Galα1,3Gal-terminated oligosaccharide sequences including di-, tri-, and pentasaccharides) have drawn increasing attention since it was discovered that the interaction of preexisting natural antibodies in human serum with this specific xenobiotic oligosaccharide sequence on animal cells is the main cause of hyperacute rejection in xenotransplantation (3). α-Gal epitopes exist as glycolipids or glycoproteins on the cell surface of mammals other than humans, apes, and Old World Monkeys (4, 5). The unique enzyme responsible for the formation of the terminal glycoside bond in nature is UDP-Gal:Galβ1,4GlcNHAc α1,3-galactosyltransferase (α1,3GalT), a protein that is absent in humans due to mutational inactivation of the gene (6, 7). In contrast, humans produce a large amount of anti-Gal antibodies including IgG, IgM, and IgA isotypes (8).

The discovery of the interaction of anti-Gal and α-Gal epitopes has led to experimental attempts to overcome hyperacute rejection by either depleting the recipient’s anti-Gal through α-Gal-immobilized affinity columns or antagonizing anti-Gal by infusing the recipient’s body with soluble synthetic α-Gal oligosaccharides (9, 10). However, such procedures require access to a substantial amount of α-Gal oligosaccharides as well as synthetically derived α-Gal analogs and mimetics. Due to the high cost associated with multiple protection and deprotection steps with a tedious separation procedure at each step in traditional chemical synthesis of oligosaccharides (11–13), the most practical production of α-Gal oligosaccharides is by glycosyltransferase-catalyzed enzymatic synthesis (14). Nevertheless, since sugar nucleotides required by glycosyltransferases are exceptionally expensive, much work has been focused on in situ sugar nucleotide regeneration through multiple-enzyme systems (15–18) and on enzymatic transformation to inexpensive sugar derivatives. We have demonstrated that a number of α-Gal oligosaccharides can be synthesized by glycosyltransferase-catalyzed reactions with regeneration of UDP-Gal through a five-enzyme system (19). Such a multiple-enzyme system undoubtedly increased the complexity of the reaction. We also showed that a simpler alternative was a two-enzyme system in which UDP-galactose 4-epimerase (GaE) converted relatively inexpensive sugar nucleotide UDP-Glc to UDP-Gal, and α1,3-galactosyltransferase carried out the subsequent glycosylation reaction (19). In order to further reduce the cost of α-Gal synthesis as well as to avoid multiple fermentation for enzyme preparations, in this work two bifunctional fusion proteins containing both GaE and α1,3GalT were constructed. GaE carries out the interconversion of UDP-Glc to UDP-Gal, and α1,3GalT catalyzes the transfer of galactose from UDP-Gal to N-acetyllactosamine or its derivatives (Fig. 1A). The overall function of the fusion proteins is the transfer of galactose from UDP-Glc to the acceptor (Fig. 1B). These fusion enzymes may therefore be expected to replace the cost of sugar nucleotides with a cheaper source of galactose (20). In addition, the fusion of the two enzymes may provide access to 3Gal-terminated oligosaccharides by more than 40-fold.

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enzymes change the donor requirement of the α1,3GalT from UDP-Gal to UDP-Glc. Furthermore, the fused enzyme system that catalyzes a sequential reaction may also have a kinetic advantage over the mixture of two separated enzymes, since the product of one enzyme travels a shorter distance to be captured by the next enzyme (20, 21).

Glycosyltransferases can be classified mechanistically into two major families, inverting glycosyltransferases and retaining glycosyltransferases, depending on the anomic configuration at the reaction center. α1,3GalT belongs to the retaining family, since it transfers a galactose residue from an α-linked nucleotide diphospho sugar (UDP-galactose) to the acceptor, forming an α-linked product via the retention of the anomeric configuration. The reaction mechanism was proposed to consist of two nucleophilic substitutions at the sugar anomeric carbon including the transient formation of a glycosyl enzyme intermediate (22–24). However, the details of the catalytic mechanism of α1,3GalT remain unknown due to the unavailability of the three-dimensional structure of any retaining glycosyltransferase. The comparison of the kinetic parameters of two fusion proteins with reverse sequence as well as of the native α1,3GalT could provide insights into the mechanism of the α1,3GalT-catalyzed reaction.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—Plasmid vector pET15b was purchased from Novagen Inc., Madison, WI. N<sup>α</sup>-NTA-agarose, polymerase chain reaction purification kit, QIAEX II gel extraction kit, QIAMP tissue kit, and DNA miniprep spin kit were from Qiagen (Santa Clarita, CA). All restriction enzymes, Taq DNA polymerase, 1-kb DNA ladder, and T4 DNA ligase were obtained from Promega (Madison, WI). β-Lactose (4-O-β-D-galactopyranosyl-β-D-glucose), D<sup>+</sup>galactosamine, melibiose (6-O-α-D-galactopyranosyl-N-glucose), UDP-Gal, UDP-Glc, UDP-α-[6-<sup>3</sup>H]Gluco, ampicillin, ammonium sulfate, 2-mercaptoethanol, Polia and Coleculture’s phenol reagent, and DOWEX 1 × 8 anion exchange resin were obtained from Sigma. N-Ribose and 2-hydroxyethyl disulfide were from Aldrich. Guanidine hydrochloride, sodium chloride, and ScintiVerse BD were from Fisher. Low range protein standards was from Bio-Rad. High and low molecular weight gel filtration calibration kits and UDP-α-[6-<sup>3</sup>H]Galactose were from Amersham Pharmacia Biotech. All other chemicals were obtained in reagent grade from commercially available sources.

**Bacterial Strains and Plasmids**—Escherichia coli strain K-12 (sub-strain MG1655) was from ATCC (ATCC catalog no. 47076). Plasmid vector pET15b and *E. coli* competent cell BL21(DE3) [F ompT hsdSB (r<sup>−</sup> m<sup>−</sup> b<sup>−</sup>) gal dcm (DE3)] were from Novagen Inc. (Madison, WI). Plasmid pET15b-α1,3GalT was constructed as described previously (19). *E. coli* competent cell DH5α (lacZΔM15 hsdR recA) was from Life Technologies, Inc.

**Construction of Plasmids pET15b-f1 and pET15b-f2**— Chromosomal DNA of *E. coli* strain K-12 was purified using the QiAamp tissue kit. All of the glyceraldehyde 3-phosphate dehydrogenase (GAP) gene containing 5′ flanking region of 0.2 μm, different concentrations of each of two corresponding primers, MgCl<sub>2</sub> (2.5 mM), 5 μl of 10× buffer B (100 mM Tris-HCl, pH 8.3, at 25 °C, 500 mM KCl), 1 mM dNTPs, and 2.5 units of Taq polymerase. The reaction mixture was covered with 50 μl of mineral oil and subjected to 30 cycles of amplification with an annealing temperature of 55 °C in a Thermolyne AmpliTaq thermal cycler (Barnstead Thermolyne Corp., Dubuque, IA). Restriction digests and DNA ligations were performed as directed by the enzyme manufacturers. The resulting plasmids were transformed into *E. coli* cloning strain DH5α and then expression strain BL21 (DE3). Selected clones were characterized by restriction mapping and DNA sequencing. The strategy for constructing plasmids for fusion enzymes is described in “Results.”

**Overexpression and Purification of the Fusion Enzymes**—The expression and purification of the fusion enzymes from the cell lysate were as described before (19). Briefly, the overexpression of the enzymes was induced by 0.4 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C in a C25 incubator shaker (New Brunswick Scientific Co., Inc., Edison, NJ). The cell lysate and inclusion bodies were separated by centrifugation at 12,000 rpm for 20 min.

From cell lysate, the active enzymes were purified using a Ni<sup>2+</sup>-NTA-agarose affinity column. After elution, the fractions containing the purified enzyme (detected by UV-visible spectrometry) were combined, and ammonium sulfate was slowly added with stirring to 80% saturation. The suspension was kept at 4 °C for an additional 30 min and then centrifuged. The pellet was dissolved in 20 μl Tris-HCl, pH 7.9, and applied to a Superdex 200 preparation FPLC column presaturated with Tris-HCl buffer (50 mM, pH 8.0) containing 2-mercaptoethanol (10 mM), glycerol (10%), and NaCl (0.25 mM). The column was then eluted with the same buffer, and fractions containing fusion protein activity were pooled and concentrated in a Centricon 3 concentrator (Millipore Corp., Bedford, MA).

To obtain the active fusion proteins from the inclusion bodies, the purification and refolding procedures were carried out as described in Ref. 25 except that a calculated E<sub>280</sub> value of 1.6 (26) was used for the fusion enzymes for the determination of enzyme concentration.

**SDS-PAGE**—SDS-PAGE was performed in a 10% gel in a Mini Protein III cell gel electrophoresis unit (Bio-Rad) at DC 200 V. High range (40–212 kDa) SDS-PAGE standards (Promega) were used as molecular weight standards, and the gel was stained with Coomassie Blue.

**Assay for Fusion Enzymes**—Enzyme assays for fusion proteins were performed at 37 °C for 30 min in a final volume of 100 μl containing Tris-HCl (10 mM, pH 7.0), MnCl<sub>2</sub> (10 mM), bovine serum albumin (0.1%), UDP-n-[6-<sup>3</sup>H]galactose (0.3 mM) or UDP-n-[6-<sup>3</sup>H]glucose (final specific activity of 1000 cpm/nmol), fusion enzyme (0.3 pmol), and acceptor (50 mM). Lactose was used as acceptor. Acceptor was omitted for blank. The reaction was stopped by adding 100 μl of ice-cold EDTA (0.1 mM). Dowex 1 × 8–200 chloride anion exchange resin was then added in a water suspension (0.8 ml, 1:1 (v/v)). After centrifugation, supernatant (0.5 ml) was collected in a 20-ml plastic vial, and ScintiVerse BD (5 ml) was added. The vial was vortexed thoroughly before the radioactivity of the mixture was counted in a liquid scintillation counter (Beckmann LS-3801 counter). One unit of fusion enzyme activity is defined as the amount of enzyme that catalyzes the transfer of 1 μmol of galactose from UDP-Glc to lactose/min at 37 °C.

**Determination of Kinetic Parameters**—Kinetic parameters for the coupled reaction (UDP-Glc → Galα1,3Lac) were gained by assays with UDP-6<sup>-</sup>H<sup>3</sup>Gal (0.3 mM) and varying concentrations of lactose (7, 8, 10, 12.5, 17, 25, and 50 mM) at 37 °C for 30 min, in which the formation of the product 6<sup>-</sup>H<sup>3</sup>Galα1,3Lac was measured by scintillation counting. Kinetic parameters for the α1,3-galactosyltransferase moiety (UDP-Gal → Galα1,3Lac reaction) in f1 or f2 were obtained by a modification of the standard assay for the transferase (19), with UDP-6<sup>-</sup>H<sup>3</sup>Gal (0.3 mM) and varying concentrations of lactose (7, 8, 10, 12.5, 17, 25, and 50 mM). Enzymatic activity of the epimerase moiety (UDP-Gal → UDP-6<sup>-</sup>H<sup>3</sup>Glc) in the fusion enzyme was obtained with the following concentrations of UDP-6<sup>-</sup>H<sup>3</sup>Glc (1, 1.14, 1.4, 1.6, 2, 2.6, and 4 mM) at 24 °C for 5 min. After deactivating the enzyme by heating at 100 °C for 10 min, the amount of UDP-6<sup>-</sup>H<sup>3</sup>Glc formed by the epimerase was quantitatively determined by using an excess amount of α1,3GalT to transfer all of the UDP-6<sup>-</sup>H<sup>3</sup>Gal to 6<sup>-</sup>H<sup>3</sup>Galα1,3Lac. This previously established α1,3GalT-coupled radioactivity assay was proven to be a convenient
FIG. 2. Construction of the plasmids for fusion enzymes pET15b-f1 (A) and pET15b-f2 (B). The galE gene for UDP-Gal 4-epimerase was cloned directly from the genome of E. coli K-12 and constructed into pET15b vector. The gene of α1,3-galactosyltransferase was amplified from a previously constructed plasmid and ligated into the same pET15b vector.

### RESULTS

#### Construction of pET15b-f1 and pET15b-f2—The bifunctional enzyme f1 was prepared by in-frame linking of the galE gene downstream to the truncated α1,3GalT gene, whose translational stop signal has been removed, through an in-frame linker (GGTGGAGGC) coding for three glycine residues. To obtain plasmid pET15b-f1, the plasmid pET15b-galE (f1) was digested with NdeI and BamHI and ligated into the same pET15b vector.

#### Expression and Purification of Fusion Enzymes—Both fusion proteins f1 and f2 were efficiently expressed in E. coli expression host BL21 (DE3). Selected colonies were confirmed by restriction mapping and DNA sequencing. When pET15b-f1 was digested with NdeI and BamHI, two fragments with sizes of 5.7 kb (pET15b vector) and 2 kb (α1,3GalT gene plus galE) were observed in agarose gel electrophoresis. When pET15b-f1 was digested with SpeI and SalI, two fragments with sizes of 6.6 kb (pET15b vector plus α1,3GalT gene) and 1100 bp (galE) were observed.

### Table I

| Enzyme        | Expression Host | Refolding | Activity |
|---------------|-----------------|-----------|----------|
| f1            | BL21 (DE3)      | Yes       | 20 units |
| f2            | BL21 (DE3)      | Yes       | 10 units |

#### Expression and Purification of Fusion Enzymes—The expressions of both f1 and f2 were under the control of the T7 lac promoter and induced by the addition of isopropyl-1-thio-β-D-galactopyranoside.

The plasmid pET15b-f1 or pET15b-f2 was transformed into E. coli cloning host DH5α, and positive recombinants were transformed into E. coli expression host BL21 (DE3). Selected colonies were confirmed by restriction mapping and DNA sequencing. When pET15b-f1 was digested with NdeI and BamHI, two fragments with sizes of 5.7 kb (pET15b vector) and 2 kb (α1,3GalT gene plus galE) were observed in agarose gel electrophoresis. When pET15b-f1 was digested with SpeI and SalI, two fragments with sizes of 6.6 kb (pET15b vector plus α1,3GalT gene) and 1100 bp (galE) were observed (Fig. 3A). Similarly, when pET15b-f2 was digested with NdeI and BamHI, two fragments with sizes of 5.7 and 2 kb were shown. If f2 was digested with SalI and BamHI, two fragments with sizes of 6.8 and 900 bp (α1,3GalT gene) were shown (Fig. 3B).

#### Expression and Purification of Fusion Enzymes—Both fusion proteins f1 and f2 were efficiently expressed in E. coli expression host BL21 (DE3) and appeared as predominant bands corresponding to a molecular mass of 67 kDa, which were absent in the BL21 (DE3) transformed with the pET15b vector.

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failed. The successful refolding was achieved in the same buffer except that the dithiothreitol was substituted by a reduct system containing 10 mM 2-mercaptoethanol and 1 mM 2-hydroxyethyl disulfide. The necessity of a reduct system for generating active enzymes indicated the importance of disulfide bond formation during refolding of the fusion proteins.

A better expression level was achieved by lowering the temperature to 30 °C and elongating the incubation time to 20 h after the addition of isopropyl-1-thio-β-D-galactopyranoside. However, as the case under the normal expression conditions (37 °C, 3 h), the amount of the proteins obtained from 1 liter of bacteria culture for f1 remained similar to that for f2, as determined by SDS-PAGE and the activity assay. These results indicated that the relative location of the transferase and the epimerase in the fusion enzymes was not critical for the expression level in the current overexpression system.

Purification of the fusion proteins was achieved by a nickel affinity chromatography and a subsequent FPLC HL Superdex 200 (16/60) gel filtration chromatography. After the affinity chromatography, the fusion proteins were purified to about 90% purity. Further purification was achieved for both f1 and f2 by FPLC gel filtration chromatography, which gave a single peak containing fusion protein activity in FPLC elution and a clean single band of about 67 kDa in SDS-PAGE (Fig. 4).

**Molecular Mass Determination**—The molecular weights of the fusion enzymes were estimated by gel filtration chromatography on a Superdex 200 column. Both hybrid enzymes eluted as single peaks corresponding to a molecular mass of 140 kDa, as determined by comparison with the protein standards performed under the same conditions. The molecular mass for either f1 or f2 determined by SDS-PAGE was estimated to 67 kDa (Fig. 4). The monomers of both fusion proteins have theoretical calculated molecular masses of 74 kDa. These results suggest that both f1 and f2 are homodimers with total molecular mass of about 140 kDa under native conditions.

**Apparent Kinetic Parameters for the Fusion Enzymes**—The measured kinetic parameters in Table II reveal some properties of the fusion enzymes. First, the $k_{cat}$ values of the α1,3GalT moieties (UDP-Gal → Galα1,3Lac reaction) in f1 and f2 are similar to that of the native α1,3GalT. However, the $K_m$ value of f1 for lactose (8.5 mM) is decreased to about half of that of the native α1,3GalT (15 mM). Therefore, the catalytic efficiency of the α1,3GalT moiety in f1 is higher ($k_{cat}/K_m = 0.053 \text{ s}^{-1} \text{ mM}^{-1}$) compared with that of f2 or of the native α1,3GalT. It is important to point out that the epimerase part of the fusion enzymes participated in the conversion of UDP-Gal to UDP-Glc, which decreased UDP-Gal concentration and slowed down the reaction, during the measurement of the α1,3GalT activity for f1 and f2. Therefore, the kinetic parameters shown in Table II are only approximate determinations. Second, it is found that the catalytic efficiencies of the epimerase moieties (UDP-Gal → UDP-Glc reaction) in f1 and f2 are greatly reduced (∼25-fold) in comparison with that of the native GalE. Dissecting to individual parameters, the $k_{cat}$ value of f1 is similar to that of the native GalE, and the efficiency loss of the GalE in f2 mainly comes from the poorer binding to UDP-Glc. For f2, the decrease of the efficiency comes from both a lower $k_{cat}$ and a higher $K_m$. Third, when considering the overall fusion enzyme activity in the coupled reaction (UDP-Gal → Galα1,3Lac reaction), the catalytic efficiency of f1 is about 8 times higher than that of f2. This is mainly due to the fact that f1 has a lower $K_m$.

**Proximity Effect**—UDP-Gal 4-epimerase catalyzes the interconversion of UDP-Glc to UDP-Gal (Reaction 1). α1,3-galactosyltransferase catalyzes the addition of a galactose from UDP-Gal to its acceptor lactose (Reaction 2). The fusion enzymes contain both epimerase and transferase activities and will catalyze the transformation of galactose from UDP-Glc (instead of

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**Table I**

**List of oligonucleotide primers used in the construction of plasmids pET15b-f1 and pET15b-f2 for the fusion proteins**

| Primer name | Primer sequence  |
|-------------|------------------|
| α1,3GalT-F-f1 | 5’-CGAATATCATATGGAAAGCAGCTTTAAGCTATGC-3’ |
| GalE-R-f1 | 5’-GGCATATGACCTATGTAAGAGGTCTGTGTCTTAC-3’ |
| GalE-F-f2 | 5’-GGGTCACATATGGACCTTACTATATGC-3’ |
| GalE-R-f2 | 5’-GGGATATGACTAGTATGAGAGTTCTGGTACC-3’ |
| α1,3GalT-R-f2 | 5’-GGGGATCCCTGGACCTGCACGTCGCCCGGATATCCCTGTAAGCTATTAAC-3’ |

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**Fig. 4**. SDS-PAGE of fusion enzymes f1 and f2. Performed in a 10% acrylamide at 200-V direct current, Coomassie Blue-stained. Lanes 1 and 3, high range molecular standard (from top to bottom: 212 kDa, 166 kDa, 97 kDa, 66.2 kDa, 57.5 kDa, and 40 kDa); lane 2, f1 purified by Ni$_2^+$-NTA affinity column and Superdex gel filtration FPLC chromatography; lane 3, f2 purified by Ni$_2^+$-NTA affinity column and Superdex gel filtration FPLC chromatography; lane 4, whole cell extract of BL21(DE3) transformed with pET15b vector plasmid. 

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**Fig. 3**. Restriction mapping of the plasmid for f1 (A) and f2 (B). Restriction mapping was performed in a 1% agarose gel in 1× TAE buffer (Tris acetate buffer containing 0.04 M Tris acetate and 0.001 M EDTA, pH 8.0) under 110-V direct current. Lanes 1, 6, 7, and 12, 1-kb DNA ladder (from top to bottom: 10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3.5 kb, 3 kb, 2.5 kb, 2 kb, 1.5 kb, 750 bp, 500 bp, and 250 bp); lane 2, pET15b-f1 digested by NdeI and BamHI; lane 3, pET15b-f1 digested by SpeI and SalI; lane 4, pET15b-galE (f1) digested by NdeI and BamHI; lane 5, pET15b-α1,3GalT (f1) digested by NdeI and BamHI; lane 6, pET15b-f1 digested by NdeI and BamHI; lane 7, pET15b-f2 digested by SalI and BamHI; lane 8, pET15b-f2 digested by NdeI and BamHI; lane 9, pET15b-f2 digested by SalI and BamHI; lane 10, pET15b-α1,3GalT (f2) digested by NdeI and BamHI; lane 11, pET15b-galE (f2) digested with NdeI and BamHI.
from UDP-Gal) to lactose (Reaction 3). The net result is the change of donor requirement of α3GalT from UDP-Gal to UDP-Glc, which decreases the cost for the synthesis of α-Gal epitope and its derivatives more than 40-fold.

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\text{UDP-Glc} \leftrightarrow \text{UDP-Gal} \\
\text{UDP-Gal} + \text{lactose} \leftrightarrow \alpha-\text{Gal epitope} + \text{UDP} \\
\text{UDP-Glc} + \text{lactose} \leftrightarrow \alpha-\text{Gal epitope} + \text{UDP}
\]

REACTIONS 1–3

Due to their proximity effects, the fusion proteins may also have kinetic advantages over the system containing two separate enzymes in terms of catalytic efficiency. To test this hypothesis, the rates of the coupled reaction catalyzed by the same amount of f1, f2, or a combination of the two native enzymes were measured and compared under same conditions (Fig. 5).

**Acceptor Specificity**—The natural acceptor for native α3-galactosyltransferase is N-acetyllactosamine. The recombinant α3,1-galactosyltransferase was shown to be able to accept a wide range of substrates including lactose and galactose derivatives but not glucose derivatives (29). In order to determine whether the acceptor specificity of the α3,1-galactosyltransferase had been changed after the fusion with epimerase, acceptor specificities were measured for fusion protein f2. Selected monosaccharides and disaccharides were used as the acceptors, and either UDP-Gal or UDP-Glc was used as the sugar nucleotide donor for the hybrid protein f2 and the α3,1-galactosyltransferase moiety in f2 with the activities corresponding to the substitution of aglycones as N3 > OH > SPh. The presence of axial configuration of hydroxyl group at the C-4 position of the Gal residue is important. Lactose and galactose derivatives are acceptors; however, glucose derivatives are not accepted by the enzyme. As in the case of the recombinant α3GalT, α- or β-linkage of the Gal terminus at the nonreducing end was confirmed to be an important but not crucial factor for f2. All of the Gal β-linked compounds (entries

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**TABLE II**

| Enzyme       | Activity | \(K_{\text{m, UDP-Glc}}\) | \(K_{\text{m, lactose}}\) | \(k_{\text{cat}}\) | \(k_{\text{cat}}/K_{\text{m}} \times 10^{2}\) |
|--------------|----------|---------------------------|--------------------------|-------------------|----------------------------------|
| α3GalT       | UDP-Gal  →Galα1,3Lac | 1.2                       | 15                       | 0.37              | 2.5                              |
| GalE         | UDP-Glc  → UDP-Gal  | 7.3                       | 6.3                      | 5.3               | 62                               |
| f1           | UDP-Glc  → Galα1,3Lac| 25                       | 15                       | 60                |                                   |
| α3GalTa      | UDP-Gal  → Galα1,3Lac| 24                       | 15                       | 1.0               |                                   |
| GalE         | UDP-Glc  → UDP-Gal  | 7.3                       | 4.5                      | 62                |                                   |

*Kinetic parameters for the coupled reaction (UDP-Glc → Galα1,3Lac) were gained by assays with UDP-[6-3H]Glc (0.3 mM) and varying concentrations of lactose (7, 8, 10, 12.5, 17, 25, and 50 mM) at 37 °C for 30 min, in which the formation of the product [6-3H]Galα1,3Lac was measured by scintillation counting. Kinetic parameters for the α3,1-galactosyltransferase moiety (UDP-Gal → Galα1,3Lac reaction, underlined) in f1 or f2 were obtained by a modification of the standard assay for the transferase, with UDP-[6-3H]Gal (0.3 mM) and varying concentrations of lactose (7, 8, 10, 12.5, 17, 25, and 50 mM). Enzymatic activity of the epimerase moiety (UDP-Glc → UDP-Gal reaction, italic type) in the fusion enzymes was obtained with different concentrations of UDP-[8-3H]Gal (1, 1.14, 1.4, 1.6, 2, 2.6, and 4 mM) at 24 °C for 5 min using an α3-galactosyltransferase-coupled radioactive assay according to Ref. 27.

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**TABLE III**

| Entry | Donor candidates | α3GalT Activity | f2 Activity |
|-------|-----------------|-----------------|------------|
| 1     | Galβ1,4Glc(NHAc)| 100             | 100        |
| 2     | Galβ1,4Glcβ1-N3 | 121             | 108        |
| 3     | Galβ1,4Glc     | 91              | 90         |
| 4     | Galβ1,4Glcβ1-SPh| 46              | 43         |
| 5     | Galα1,6Glc     | 4.7             | 8.9        |
| 6     | Galβ1-1(OCH2CH2)3Cl| 7.1           | 9.3        |
| 7     | Galβ1-1Me      | 6.1             | 4.5        |
| 8     | Galα1-OMe      | 2.1             | 2.3        |
| 9     | Glcβ1,4Glc     | <0.01           | <0.01      |
| 10    | Glc             | <0.01           | <0.01      |

*Assay using UDP-Gal as the donor, the acceptor specificity of the α3GalT moiety in f2.

*Assay using UDP-Glc as the donor, the acceptor specificity of the overall fusion enzyme f2.
1–4, 6, 7) are much better acceptors than the Gal α-linked compounds (entries 5, 8).

**DISCUSSION**

Full-length bovine α1,3-galactosyltransferase is a type II membrane protein with a short N-terminal cytosolic domain, a transmembrane domain, a stem region, and a C-terminal catalytic domain (30). The native α1,3-galactosyltransferase is a monomer with a molecular mass of 43 kDa (31). We reported previously a high yield expression of a truncated (the first 79 N-terminal amino acid residues were deleted) recombinant bovine α1,3-galactosyltransferase (19). FPLC gel filtration analysis and SDS-polyacrylamide gel electrophoresis data indicate that the recombinant α1,3-galactosyltransferase is a monomer.

UDP-Gal 4-epimerase is one of the key enzymes of the Leloir pathway for galactose metabolism (32). The epimerase from *E. coli* is a homodimer with an overall molecular mass of 79 kDa (33). The epimerase was cloned from *E. coli* K-12 into pET15b expression vector (27). FPLC gel filtration and SDS-PAGE data indicate that the recombinant epimerase is a homodimer. It is interesting to know that both fusion proteins f1 and f2 have dimeric configurations as that for the native epimerase. The relative position of the transferase and the epimerase does not alter this property. Also, the His$_6$ tag at the N terminus of protein does not prevent dimerization. Since the recombinant α1,3GalT is a monomer, the recombinant epimerase is a homodimer, it is reasonable to assume that, the interactions between the two subunits of the fusion proteins result from the interactions of the epimerase moieties in the fusion enzymes (Fig. 6). The same conclusion was made by Bulow and Mosbach (34) that a dimeric hybrid polypeptide α-ββ-α was usually obtained by the fusion of a dimeric protein (ββ) with a monomeric one (α).

A previous report about a fusion protein composed of UDP-Gal 4-epimerase and galactose-1-phosphate uridylyltransferase (GalT) with an intervening Ala$_4$ linker was shown to exist in three forms: a monomer, dimer, or tetramer (20). An interesting fact is the presence of four or six subunits, as well as other forms (21). Unlike these reports, due to the monomeric nature of the α1,3GalT, both fusion proteins f1 and f2 in the present study exist as homodimers.

It was reported that each subunit of the dimeric epimerase contained one irreversibly, noncovalently bound NAD$^+$ (33), and no additional NAD$^+$ was required for the epimerase-catalyzed reaction (32). This is also the case for the fusion enzymes. No external NAD$^+$ is necessary for either the coupled reaction or the activity of the epimerase moiety in the fusion enzymes. This indicates that NAD$^+$ is also tightly bound to the epimerase during the expression of fusion proteins in *E. coli.*

Our kinetic studies indicate that the fusion proteins have kinetic advantages over the system of individual enzymes for the overall coupled reaction. Significantly, the reaction rates in producing GaLa1,3Lac from UDP-Glc and lactose are increased 4- and 1.5-fold by f1 and f2, respectively, in comparison with the native enzymes. This result can be explained by the substrate channeling effect (21), indicating that the UDP-Gal produced by the epimerase is captured faster by the transferase moiety in either f1 or f2 than that in the system of native enzymes. Furthermore, the higher activity of f1 over f2 suggests that the active sites for the epimerase and α1,3-galactosyltransferase part are in a more optimal configuration in f1.

It is noticed that the overall fusion enzyme activities (UDP-Glc → GaLa1,3Lac reaction) are higher than the activity of the corresponding transferase moieties (UDP-Gal → GaLa1,3Lac reaction) in both f1 and f2. This provided us with an insight into the mechanism of α1,3-galactosyltransferase. The steady state kinetic properties of α1,3-galactosyltransferase indicate a sequential mechanism in which metal cofactor, donor, and acceptor bind enzyme prior to catalysis. The low level of UDP-Gal hydrolase activity found in α1,3-galactosyltransferase excluded the possibility of an ordered sequential mechanism with binding of acceptor prior to donor. Previous studies were not able to determine whether an ordered mechanism with the binding of donor prior to acceptor or a random mechanism applies for α1,3-galactosyltransferase. Our results on the kinetic parameters of coupled reaction and the native transferase reaction in both fusion enzymes suggest that a random mechanism should be a more plausible explanation. For instance, if α1,3-galactosyltransferase obeys an ordered mechanism with the binding of donor prior to acceptor, then the absence of UDP-Gal (as in the coupled reaction), UDP-Glc has to be captured by the epimerase and be converted to UDP-Gal prior to the function of transferase. Opposite to the results we obtained, the initial rate in the coupled reaction should be lower than that in the activity assay for the α1,3-galactosyltransferase moieties in fusion enzymes, in which UDP-Gal is present and able to be captured and utilized by the transferase right away.

Beside N-acetyllactosamine, lactose and lactose derivatives are found to be good acceptors for the recombinant α1,3-galactosyltransferase. Our results indicate that the in-frame fusing with epimerase does not change the acceptor specificity of the transferase. This suggests that without changing many important properties of individual native enzymes, fusing two enzymes together has the advantages of simplicity, economy, and efficiency. In our laboratory, the fusion enzymes f1 and f2 have been successfully applied in the synthesis of α-Gal epitope and its derivatives (35, 36). This novel approach reduces the cost for the synthesis of oligosaccharide by over 40-fold and provides an easy and economic access to a wide spectrum of α-galactosyl epitopes and their derivatives to support the continuous studies on xenotransplantation as well as other pharmaceutical research (37, 38). The methodology can also be applied for other galactosyltransferases that require UDP-Gal as the donor, such as α1,4-galactosyltransferase, α1,6-galactosyltransferase, β1,4-galactosyltransferase, β1,3-galactosyltransferase, human blood type B galactosyltransferase, etc. From a broader point of view, depending on which sugar nucleotide is the most economical source, the fusion of a glycosyltransferase and an epimerase can change a required, but more expensive, sugar nucleotide to a less expensive one. For instance, broader application of such an approach may include the combination of a variety of glycosyltransferases with different epimerases, such as N-acetylgalactosaminyltransferase and UDP-N-acetylgalactosamine 4-epimerase, galactosaminyltransferase and UDP-N-glucosamine 4-epimerase, or galacturonyltransferase and UDP-glucuronate 4-epimerase.

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