Glycosyl Fluorides Can Function as Substrates for Nucleotide Phosphosugar-dependent Glycosyltransferases*

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α-Galactosyl fluoride is shown to function as a substrate, in place of uridine-5′-diphosphogalactose, for the α-galactosyltransferase from Neisseria meningitidis. The reaction only occurs in the presence of catalytic quantities of uridine 5′-diphosphate. In the presence of galactosyl acceptors, the expected oligosaccharide product is formed in essentially quantitative yields, reaction having been performed on multi-milligram scales. In the absence of a suitable acceptor, the enzyme synthesizes uridine-5′-diphosphogalactose, as demonstrated through a coupled assay in which uridine-5′-diphosphogalactose is converted to uridine-5′-diphosphoglucuronic acid with conversion of NAD to NADH. These glycosyl fluoride substrates therefore offer the potential of an inexpensive alternative donor substrate in the synthesis of oligosaccharides as well as generating steady state concentrations of nucleotide diphosphate sugars for in situ use by other enzymes. Further, they should prove valuable as mechanistic probes.

Oligosaccharides, primarily in the form of various glycoconjugates, are involved in a wide variety of biological functions (1–3) and consequently show enormous potential as therapeutic agents for a number of conditions ranging from infectious diseases to cancer therapies (4–7). Their development as drugs, however, has been slow, in part because of difficulties with the large scale synthesis of oligosaccharides in an economic manner. Despite significant advances, chemical syntheses are generally impractical on the large scale since multiple protection and deprotection steps are typically required to control regiochemistry and because control of stereochemistry remains a challenge (8–10). Enzymatic synthesis provides an attractive alternative. The use of glycosidases run “backward” has a long history, but one which has been troubled by poor yields (11, 12), although the recent development of mutant glycosidases (glycosynthases) shows promise (13).

The alternative enzymatic approach involves the use of glycosyltransferases, nature’s own anabolic enzymes, to form the very specific glycosyl linkages required (14, 15). Two problems have plagued this approach, one being the availability and stability of the enzymes, and the other being the cost of the nucleotide diphosphate sugar substrates. The former problem is being solved through the cloning and high level expression of the soluble catalytic domains, particularly of bacterial enzymes rather than their mammalian counterparts. The latter problem has been tackled through the development of complex, but effective recycling schemes to enzymatically regenerate the nucleotide diphosphate sugars (15). Although prices of nucleotide diphosphosugars are decreasing as recombinant strains producing them are developed, a problem still exists with end product (UDP) inhibition. A need therefore exists for detailed mechanistic information on these important enzymes, and for the development of alternative substrates that could prove more amenable to kinetic study and also less expensive for large scale synthesis. Such a system is described herein.

NDP-α-sugar glycosyltransferases have been assigned to families on the basis of sequence similarities (16). They can also be classified mechanistically as either inverting or retaining, depending on the relative anomeric stereochemistries of their substrate and product, exactly as has been done with the well studied glycosidases (17, 18). This has led to the tacit assumption that similar mechanisms are followed by the two classes of enzyme. Mechanistic studies to date have been largely limited to kinetic analyses that reveal no common mechanism. Studies on the retaining glucosyltransferase glycan synthase pointed to a rapid equilibrium random bi bi mechanism (19–21), while those on the inverting galactosyl (22) and fucosyl (23) transferases suggested rapid equilibrium random and ordered bi bi schemes, respectively. Other studies have included measurement of positional isotope exchange and secondary deuterium kinetic isotope effects, as well as a few affinity labeling and photoaffinity labeling studies (see Ref. 17, and references therein). The only three-dimensional structures available to date are those of the phage T4 β-glucosyltransferase, which unfortunately appears to be unrelated in amino acid sequence to any other enzyme (24) and very recently published structures of a bovine galactosyltransferase (25) and a presumed N-acetylgalcosaminyltransferase from Bacillus subtilis (26).

Considerable effort has been expended in the probing of substrate specificities using modified donor and acceptor sugars (27–29). However, no functional substrate analogs have yet been reported in which the nucleotide diphosphate moiety is substantially altered or removed. Glycosyl fluorides have proven to be valuable substrates for glycosidases, both for use in synthesis and in probing mechanisms (30–35). The small size and high reactivity of the fluoride leaving group tends to ensure reasonable reaction rates with glycosidases, and the reaction can be easily monitored by use of a fluoride ion-selective electrode. It therefore seemed to be of value to test whether glycosyl fluorides could in fact function as alternative glycosyl

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The abbreviations used are: NDP, nucleotide diphosphate; FCHASE, 6-5-fluorescein (carboxamido)-hexanoic acid succinimidyld; FITC, fluorescein isothiocyanate; lgtC–19, lipopolysaccharide galactosyltransferase C from N. meningitidis missing the 19 C-terminal amino acid residues; DTT, dithiothreitol; HPLC, high performance liquid chromatography.
MATERIALS AND METHODS

**General Procedures**—Recombinant α-galactosyltransferase from *Neisseria meningitidis* lacking the 19 C-terminal amino acid residues was expressed in, and purified from, *Escherichia coli* as described previously (36). Concentrations of lgtC−19 solutions were determined based on ε254 = 1.74 ml mg⁻¹ cm⁻¹ (36). Type II rabbit muscle pyruvate kinase and lactate dehydrogenase were obtained from Sigma as suspensions in 3.2 M ammonium sulfate, pH 6.0. UDP-galactose-4-epimerase from *E. coli* and UDP-glucose dehydrogenase from group A *Streptococcus*, were gifts from Dr. H. M. Holden (University of Wisconsin) and Dr. M. Tanner (University of British Columbia), respectively. α-Galactosyl fluoride was synthesized by reaction of penta-O-acetylgalactose with HF/pyridine, followed by deprotection using sodium methoxide in methanol as described previously (37, 38). Thin-layer chromatographic (TLC) separations were performed using Merck Kieselgel 60 F254 analytical plates. Compounds were detected visually based on e280.

**Enzymatic Synthesis**—Analytical runs were performed by adding 5 µl of 6 mg/ml lgtC−19 (0.9 nmol) to 3 µl of 115 mM α-galactosyl fluoride (0.35 µmol), 5 µl of 100 mM UDP (0.3 µmol), 5 µl of 5 mM acceptor (0.025 µmol), and 15 µl of 500 mM HEPES buffer (pH 7.5) containing 3.0 mM MnCl2, 0.5 mM UDP, 5 mM DTT, and 2 mM UTP plus appropriate concentrations of lactose and UDP-glucose dehydrogenase, and 1.03 mg/ml UDP-glucose dehydrogenase (39). Derivation and manipulation of Equations 1 and 2 was performed by direct fit of the data using the software program Grafit (39). Kinetic parameters were determined by direct fit of the data using the software program Mathematical Treatment of Data—All kinetic parameters were determined by direct fit of the data using the software program Math.

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The continuous coupled assay monitoring UDP-Gal production was carried out in assay cells containing 100 mM HEPES (pH 7.5), 15 mM MnCl2, 0.5 mM UDP, 5 mM DTT, 0.5 mM NAD+, 4.3 mg/ml UDP-galactose 4-epimerase, and 1.03 mg/ml UDP-glucose dehydrogenase plus α-galactosyl fluoride (0–200 mM) in a total volume of 190 µl. Reactions were initiated by the addition of lgtC−19 (3.07 nmol), bringing the final volume to 200 µl, and monitored through the change in absorbance at a wavelength of 340 nm (ε340 = 6.22 mM⁻¹ cm⁻¹). Control experiments were performed to confirm that UDP-glucuronic acid did not inhibit the coupling system significantly at the concentrations formed during initial rate analysis. Similarly, control experiments in which lgtC−19 was omitted confirmed that α-galactosyl fluoride plus UDP could not function as substrates for UDP-galactose-4-epimerase and UDP-glucose dehydrogenase. Subsequent investigations revealed that the substrate specificity of the dehydrogenase is sufficiently broad that the epimerase is not needed.

**Competitive rate reduction upon addition of lactose was measured by incubating cells as above, but containing a fixed concentration (50 mM) of α-galactosyl fluoride and varying the concentration of lactose over a range of 0–120 mM.**

**Mathematical Treatment of Data**—All kinetic parameters were determined by direct fit of the data using the software program Math (39). Derivation and manipulation of Equations 1 and 2 was performed using the method of King-Altman (40) and the software program Math.
RESULTS AND DISCUSSION

α-Galactosyl fluoride was first tested as a donor substrate for the recombinant lipopolysaccharide galactosyltransferase C (lgtC-19) from N. meningitidis (36) in the presence of several readily-detected fluorescent galactosides as acceptors. When reaction mixtures containing α-galactosyl fluoride plus, individually, FITC lac, FCHASE gal, and FCHASE lac were incubated with lgtC-19 in buffer containing Mn²⁺ and DTT, no reaction product could be detected after 1 h by thin-layer chromatography or HPLC analysis, or by use of a fluoride electrode. However, when uridine 5′-diphosphate (UDP) was also added, complete conversion of the fluorescent galactoside to a disaccharide and of the fluorescent lactosides to the corresponding trisaccharides was observed within that same time frame. Neither UMP nor UTP functioned equivalently. The products observed were chromatographically identical to that formed using UDP-Gal as glycosyl donor. Further, mass spectrometric analysis of the Sep-Pak-purified products confirmed the expected masses of 905.4 for FCHASE-gal-gal, 1067.6 for FCHASE-lac-gal, and 985.2 for FITC-lac-gal. Repeat of the reaction on a larger scale but using lactose as the acceptor allowed the production of galactose from α-galactosyl fluoride, a linear dependence of rate upon α-galactosyl fluoride concentration was observed (Fig. 1a), allowing the determination of the second order rate constant to be $k_{cat}/K_m = 1 \times 10^{-4} \text{ mM}^{-1} \text{ s}^{-1}$. However, as the concentration of α-galactosyl fluoride was increased, no saturation behavior was observed. Instead, a distinct upward curvature in the plot of rate as a function of α-galactosyl fluoride concentration was seen (Fig. 1b). This result suggests that, at sufficiently high concentrations, a second molecule of α-galactosyl fluoride can become involved in the reaction leading to an increase in the observed rates. Since the acceptor for this enzyme is a galactosyl moiety, it is likely that this second molecule of α-galactosyl fluoride is functioning as an alternative acceptor, giving α-Gal-1,4-α-GalF as the product. The formation of this product has indeed been confirmed by TLC and HPLC analysis of a reaction mixture containing UDP, α-galactosyl fluoride, and enzyme. In addition, the enzyme was also shown to be capable of reaction when assayed with only α-galactosyl fluoride and UDP, in the absence of other glycosyl acceptors (Fig. 2). The second order rate constant for this transfer of galactose from α-galactosyl fluoride to a second molecule of α-galactosyl fluoride was determined to be $2 \times 10^{-4} \text{ mM}^{-1} \text{ s}^{-1}$.

The dual role of α-galactosyl fluoride in the presence of another glycosyl acceptor such as lactose can be described kinetically by three possible models, depending on whether substrate binding proceeds via a ping pong, ordered, or random mechanism (Scheme 1, a–c, respectively). The simplified initial velocity equation for each of these mechanisms is given below. The individual rate constants comprising each constant term in these equations are summarized in Table I.

$$v = \frac{V[B][A]}{(K_1 + [B])[A] + K_1[A]}$$

(Eq. 1)

$$v = \frac{V[B][A]}{(K_1 + [B])+ [K_2]+ [A]}$$

(Eq. 2)

$$v = \frac{(V[B][A]) + (V_2 + V[B][A]) + V[A]}{(K_1 + [B]) + (K_2 + [B]) + [A] + (K_3 + [B]) + [A] + (K_4 + [A])}$$

(Eq. 3)

At a fixed concentration of lactose, the rate equations in terms of α-galactosyl fluoride concentration have the same general form for both ping pong (Equation 1) and ordered (Equation 2) mechanisms. On the other hand, the rate equation describing the random mechanism (Equation 3) is quite different. Nonetheless, the fact that the data in Fig. 1b can be fitted equally well by each equation demonstrates that it is possible for α-galactosyl fluoride to behave as both donor and alternate...
acceptor substrates regardless of the kinetic mechanism employed for substrate binding. Differentiation between these mechanisms will require measurements of rates at a series of concentrations of α-galactosyl fluoride, UDP, and lactose, as well as product inhibition studies.

The complicated nature of α-galactosyl fluoride functioning in the capacity of both donor and acceptor substrate has made it difficult to determine individual values of $k_{\text{cat}}$ and $K_m$ for α-galactosyl fluoride and lactose in the galactosylation reaction. An estimate of the enzyme’s affinity for UDP was obtained by measuring rates at fixed concentrations of α-galactosyl fluoride (100 mM) and lactose (100 mM) while varying UDP concentration. Here, saturation kinetics were observed (Fig. 3) and an apparent $K_m$ value of 0.08 mM was obtained.

The absolute requirement for UDP is of interest, but not entirely unexpected, since UDP is probably required to optimally orient residues for catalysis. The small size of the fluorine substituent on the galactose moiety must allow for both donor and acceptor substrate to bind coincidentally in the donor site. By analogy with retaining glycosidases, an intermediate of some kind is presumably formed, which then reacts with a glycosyl acceptor (Scheme 2, upper pathway). This intermediate is shown here as a covalent glycosyl-enzyme, but may well take any form. It is conceivable that such an intermediate could also react with UDP to form UDP-Gal (Scheme 2, lower pathway). However, the detection of UDP-Gal formation is a challenging endeavor given that UDP-Gal binds tightly to the enzyme ($K_m = 30 \mu M$) and will cause severe product inhibition. As a consequence, no significant accumulation of UDP-Gal will occur. In addition, any UDP-Gal formed can be turned over by the enzyme in the galactosylation of α-galactosyl fluoride, which can function as an acceptor substrate. To circumvent this problem, a coupled assay was developed to partition off any UDP-Gal formed, thereby allowing for its detection and quantitation (Scheme 2). In this assay, the enzyme UDP-Gal 4-epimerase was used to convert any UDP-Gal formed by the transferase to UDP-Glc. The latter was then oxidized by a second enzyme UDP-Glc dehydrogenase to UDP-glucuronic acid with concomitant reduction of NAD$^+$ to NADH.

**Table I**

| Ping pong | | Ordered | | Random |
|---|---|---|---|
| $V_i = \frac{k_i[k_i]}{k_i + k_2} V_s = \frac{k_i[k_i]}{k_i + k_2} \frac{k_i[k_i]}{k_i + k_2 + k_4} K_i = \frac{k_i[k_i]}{k_i + k_3} \frac{k_i[k_i]}{k_i + k_3 + k_4} K_s = \frac{k_i[k_i]}{k_i + k_4} \frac{k_i[k_i]}{k_i + k_3 + k_4 + k_5} K_o = \frac{k_i[k_i]}{k_i + k_6} \frac{k_i[k_i]}{k_i + k_3 + k_4 + k_6} K_n = \frac{k_i[k_i]}{k_i + k_3 + k_4 + k_6} | $V_i = \frac{k_i[k_i]}{k_i + k_2} V_s = \frac{k_i[k_i]}{k_i + k_2} \frac{k_i[k_i]}{k_i + k_2 + k_4} K_i = \frac{k_i[k_i]}{k_i + k_3} \frac{k_i[k_i]}{k_i + k_3 + k_4} K_s = \frac{k_i[k_i]}{k_i + k_4} \frac{k_i[k_i]}{k_i + k_3 + k_4 + k_5} K_o = \frac{k_i[k_i]}{k_i + k_6} \frac{k_i[k_i]}{k_i + k_3 + k_4 + k_6} K_n = \frac{k_i[k_i]}{k_i + k_3 + k_4 + k_6} |
| $V_i = \frac{k_i[k_i]}{k_i + k_2} V_s = \frac{k_i[k_i]}{k_i + k_2} \frac{k_i[k_i]}{k_i + k_2 + k_4} K_i = \frac{k_i[k_i]}{k_i + k_3} \frac{k_i[k_i]}{k_i + k_3 + k_4} K_s = \frac{k_i[k_i]}{k_i + k_4} \frac{k_i[k_i]}{k_i + k_3 + k_4 + k_5} K_o = \frac{k_i[k_i]}{k_i + k_6} \frac{k_i[k_i]}{k_i + k_3 + k_4 + k_6} K_n = \frac{k_i[k_i]}{k_i + k_3 + k_4 + k_6} | $V_i = \frac{k_i[k_i]}{k_i + k_2} V_s = \frac{k_i[k_i]}{k_i + k_2} \frac{k_i[k_i]}{k_i + k_2 + k_4} K_i = \frac{k_i[k_i]}{k_i + k_3} \frac{k_i[k_i]}{k_i + k_3 + k_4} K_s = \frac{k_i[k_i]}{k_i + k_4} \frac{k_i[k_i]}{k_i + k_3 + k_4 + k_5} K_o = \frac{k_i[k_i]}{k_i + k_6} \frac{k_i[k_i]}{k_i + k_3 + k_4 + k_6} K_n = \frac{k_i[k_i]}{k_i + k_3 + k_4 + k_6} |

![Fig. 3. A plot demonstrating the saturating behavior of UDP at high concentrations.](image-url)

As shown in Fig. 4a, a rate that was dependent upon the concentration of α-galactosyl fluoride was indeed observed. Once again, the absence of any saturation is consistent with a low affinity of the transferase for α-galactosyl fluoride. From the linear dependence of the data, the $k_{\text{cat}}/K_m$ value was determined to be $7 \times 10^{-7} \text{mm}^{-1} \text{s}^{-1}$. The reasonable agreement of this value with that obtained for the galactosylation of α-galactosyl fluoride in the absence of another glycosyl acceptor ($2 \times 10^{-5} \text{mm}^{-1} \text{s}^{-1}$) is consistent with the notion that the same step, formation of the intermediate, is rate-limiting in both cases. Furthermore, the introduction of lactose also resulted in the suppression of UDP-Gal production, as can be seen in the decreased rates at increasing concentrations of this additional acceptor (Fig. 4b). This is not surprising, as the presence of a second glycosyl acceptor would cause a greater partitioning of the intermediate toward the synthesis of oligosaccharides instead of UDP-Gal. The saturable dependence of this rate decrease as a function of lactose concentration allowed the determination of an apparent $K_m$ value of 7 mM for lactose under these conditions (50 mM α-galactosyl fluoride, 0.5 mM UDP).

The ability of glycosyl fluorides to act as substrates for NDP-
Sugar-dependent glycosyltransferases carries two major implications. One is that these retaining transferases are likely mechanistically analogous to retaining glycosidases and proceed through some kind of glycosyl-enzyme intermediate. They may therefore be amenable to study using many of the mechanistic tools developed for glycosidases. The other is that such glycosyl fluorides could serve as inexpensive alternatives to NDP-sugars in the synthesis of oligosaccharides, the nucleotide diphosphate being needed only in catalytic quantities. The system could also serve as a means of generating UDP-sugars in situ for use by other enzymes. The rather low $k_{cat}/K_m$ value is not as serious a problem as might appear to be the case, being due mostly to a high $K_m$ value for $\alpha$-galactosyl fluoride, which can be overcome through the use of high substrate concentrations. As shown in the preparative synthesis, the use of high concentrations of both the donor and acceptor resulted in good product yields with no significant quantities of side products.

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