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UDP-(5F)-GlcNAc Acts as a Slow-Binding Inhibitor of MshA, a Retaining Glycosyltransferase

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Glycosyltransferase (GT) enzymes, which catalyze the transfer of a sugar moiety to an acceptor molecule, are involved in numerous cellular pathways, including bacterial cell wall biosynthesis,[1] post-translational modification of proteins,[2] and signal transduction.[3] These enzymes can be classified as either retaining or inverting on the basis of the stereochemical outcome of the anomeric carbon center of the donor sugar.[4] Despite their participation in many therapeutically relevant pathways, there is a paucity of information on how to effectively inhibit this class of enzymes. The lack of information can be traced to the observations that only a small percentage of GT enzymes have been characterized and that the affinity of the substrates for characterized enzymes tends to be low. We recently reported the three-dimensional structure and basic kinetic characterization of the retaining glycosyltransferase MshA from *Corynebacterium glutamicum* (CgMshA).[5] MshA catalyzes the transfer of *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to 1-L-*myo*-inositol-1-phosphate (L-I1P) in the first committed step in the biosynthesis of the small-molecule reductant mycothiol (Scheme 1).

It is generally accepted that the chemical mechanism of glycosyltransferase enzymes involves the development of substantial oxocarbenium character in the transition state, similar to glycosidase enzymes.[6] In support of this hypothesis, sugar nucleotides with electron-withdrawing substituents are inhibitors of glycosidases. Recently, the synthesis of UDP-(5F)-GlcNAc (Scheme 2) provided the ability to probe the mechanism of glycosyltransferase enzymes as well.[7,8]

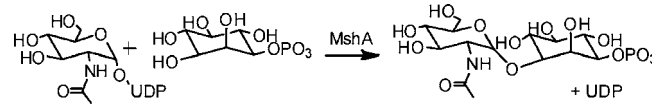
On the basis of steady-state kinetic data, UDP-(5F)-GlcNAc acts as a competitive inhibitor of CgMshA versus UDP-GlcNAc with a K_{is} value of 1.4 ± 0.2 μM, which is ~150-fold lower than the Michaelis constant determined for UDP-GlcNAc (210 μM) (Figure S1 in the Supporting Information).[5] The 5-fluoro compound did not act as a viable donor substrate for the MshA-catalyzed reaction at concentrations up to 200 μM under conditions similar to those in the inhibition assays. These results are similar to those from previous studies demonstrating that the 5-fluoro compound does not act as a donor substrate but acts as a functional acceptor substrate in glycosyltransferase reactions.[8] The results shown here provide further support for the formation of substantial charge development on the donor sugar in glycosyltransferase reactions and demonstrate the power of 5-F sugars as mechanistic probes.

Unlike previous studies, however, the time courses of CgMshA activity obtained in the presence of UDP-(5F)-GlcNAc displayed nonlinear kinetics described by a rapid initial rate followed by a slower steady-state rate (Figure 1A). The time-courses were fit using eq 1:

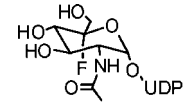
$$\frac{[P]_t}{[E]} = v_{ss}t + \frac{v_i - v_{ss}}{k_{obs}}(1 - e^{-k_{obs}t}) + C \qquad (1)$$

where $[P]_t$ is the concentration of product formed, [E] is the total enzyme concentration, v_i is the initial velocity, v_{ss} is the steady-state velocity, t is time, k_{obs} is the exponential rate constant for equilibration, and C is a finite intercept.[9] The inhibition was demonstrated to be reversible by incubating the enzyme with the inhibitor prior to initiation of the reaction with UDP-GlcNAc; this resulted in a progress curve that consisted of a lag phase followed by reactivation of the enzyme comparable to levels seen in the absence of the inhibitor (Figure 1B).

Scheme 1



Scheme 2



UDP-(5F)-GlcNAc

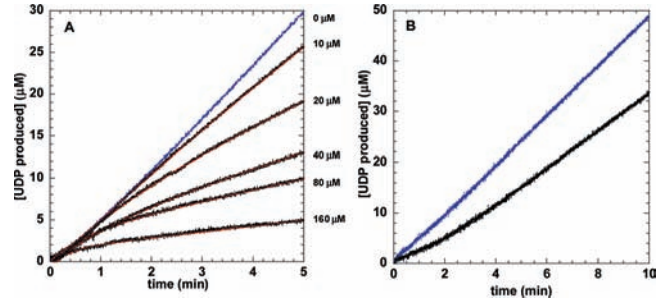


Figure 1. (A) Kinetics of time-dependent inhibition of CgMshA by UDP-(5F)-GlcNAc. Progress curves for MshA in the absence (blue) and presence (black) of UDP-(5F)-GlcNAc are shown. The red lines are fits to eq 1. Saturating substrate concentrations of 2 mM UDP-GlcNAc and 2 mM L-I1P were used in this experiment. (B) Effect of preincubation with UDP-(5F)-GlcNAc on CgMshA activity initiated with UDP-GlcNAc. A solution of 8 nM enzyme, 2 mM L-I1P, and 5 μM UDP-(5F)-GlcNAc was incubated at 25 °C for 10 min prior to the addition of 1 mM UDP-GlcNAc (black line). The volume of UDP-GlcNAc added was 1% of the reaction volume. The blue line represents the same experiment in the absence of UDP-(5F)-GlcNAc. See the Supporting Information for full experimental details.

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Scheme 3

Two possible kinetic mechanisms that can explain the nonlinear kinetics are shown in Scheme 3.[9] Mechanism A depicts a two-step mechanism involving the formation of an initial inhibitory complex followed by slow equilibration to a more tightly bound complex (EI*). Mechanism B depicts a single-step mechanism in which the formation of the initial complex is the slow step relative to catalysis. A plot of k_{obs} versus inhibitor concentration (Figure 2) displays a linear dependence, consistent with the single-step mechanism depicted in mechanism B.

The dependence of k_{obs} on [UDP-(5F)-GlcNAc] can be fit to eq 2:

$$k_{obs} = k_2 + \frac{k_1[I]}{1 + \frac{[S]}{K_S}} \quad (2)$$

where k_1 and k_2 are the rate constants shown in mechanism B, [I] is the concentration of the inhibitor, [S] is the concentration of UDP-GlcNAc, and K_S is the Michaelis constant for UDP-GlcNAc. From the results of the fit, values of $0.14 \pm 0.01\ \mu M^{-1}\ min^{-1}$ and $0.26 \pm 0.06\ min^{-1}$ can be calculated for k_1 and k_2, respectively. Using these values derived from k_{obs} gives a calculated K_i value of $1.8 \pm 0.4\ \mu M$, which is in excellent agreement with the K_{is} value determined from the steady-state inhibition experiments ($1.4 \pm 0.2\ \mu M$).

Structural studies have demonstrated that CgMshA and other GT-B fold glycosyltransferases can adopt both open and closed forms. It has been proposed that a large conformational change takes place after the sugar nucleotide binds to the enzyme, creating the closed form of the enzyme and a binding site for the second substrate.[5,10−13] One might expect the inhibitor to bind in a similar fashion, consistent with mechanism A. However, the kinetic data indicate that UDP-(5F)-GlcNAc inhibits the enzyme in a single step that occurs slowly relative to catalysis.

On the basis of the steady-state kinetic data, CgMshA follows an ordered bi−bi sequential mechanism with binding of UDP-GlcNAc first and then L-I1P.[5] As UDP-(5F)-GlcNAc is a competitive inhibitor versus UDP-GlcNAc, it is expected that both compounds bind to the apo-form of the enzyme. Attempts to cocrystallize CgMshA with the 5-fluoro compound have been unsuccessful. One possible explanation for the slow-onset phenomenon is that UDP-(5F)-GlcNAc binds slowly to the open form of the enzyme. However, in view of the modest structural change versus UDP-GlcNAc, this seems unlikely. Instead, if UDP-(5F)-GlcNAc binds to a closed form of the apo enzyme, a possible physical mechanism for the slow binding may arise from a small loop (residues 16−22) that becomes ordered in the closed conformation. This loop is proposed to sequester the uridine moiety of UDP-GlcNAc from solvent. It is possible that the movement of this loop limits the rate at which UDP-(5F)-GlcNAc can bind to the apo enzyme, resulting in the time-dependent inhibition.

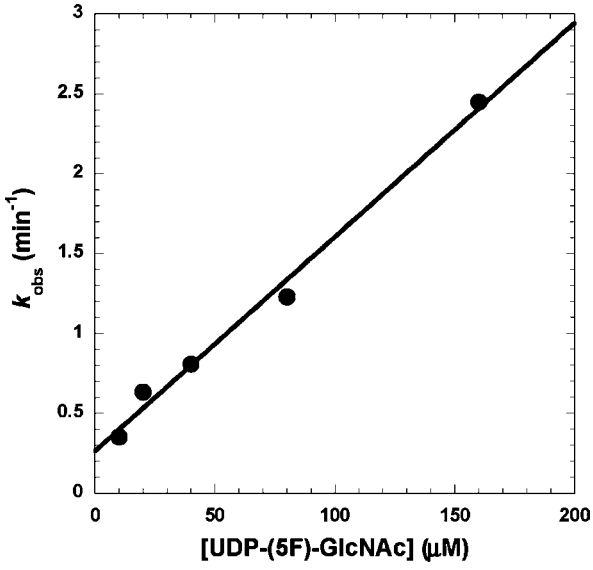

Figure 2. Plot of k_{obs} vs [UDP-(5F)-GlcNAc]. The values for k_{obs} were determined from fits of the progress curves to eq 1. The solid black line is a fit of the data to eq 2.

While a number of time-dependent inhibitors of glycosidase enzymes have been reported,[14] we believe that this is the first reported glycosyltransferase inhibitor to act in a slow-onset manner. The potent inhibition of CgMshA by the fluoro-substituted compound is consistent with the oxocarbenium transition-state structure proposed for retaining glycosyltransferase enzymes on the basis of kinetic isotope effects.[15,16] While structural studies indicate a large conformational change upon sugar nucleotide binding, the kinetic results suggest that more subtle structural changes are involved in the time-dependent inhibition of CgMshA by UDP-(5F)-GlcNAc. Taken together, these conclusions provide data on the electronic structure of the transition state of this retaining glycosyltransferase and the possibility of alternate conformations of the enzyme. This information will aid in the design of future inhibitors for the large family of GT-B glycosyltransferase enzymes.

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Supporting Information Available: Experimental procedures and steady-state inhibition plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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