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# Strategies for the Chemoenzymatic Synthesis of Deoxysugar Nucleotides: Substrate Binding versus Catalysis

Kwang-Seuk Ko, Corbin J. Zea, and Nicola L. Pohl*

*Department of Chemistry and the Plant Sciences Institute, Gilman Hall, Iowa State University, Ames, Iowa 50011-3111*

*npohl@iastate.edu*

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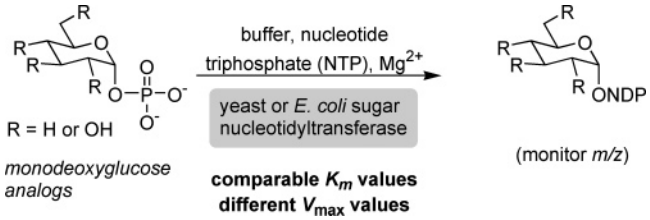


Sugar nucleotidyltransferases, also known as sugar pyrophosphorylases, catalyze the formation of a phosphate linkage to produce sugars activated for use by Leloir pathway glycosyltransferases and are subjects of protein engineering for chemoenzymatic synthesis strategies. Herein we present evidence that differences in substrate binding affinity do not primarily account for substantial contrasts in deoxysugar nucleotide product yields with this class of enzymes. Prokaryotic and eukaryotic glucose-1-phosphate uridylyltransferases (EC 2.7.7.9) can exercise kinetic discrimination in choosing carbohydrates of comparable binding affinity for catalytic turnover. These findings have implications for the in vivo and in vitro function and use of these enzymes.

Numerous studies have begun to delineate the binding determinants of sugars to receptors such as lectins and antibodies,[1] but relatively little is understood about how binding relates to catalysis by the many enzymes involved in carbohydrate biosynthesis. These enzymes, particularly sugar nucleotidyltransferases and glycosyltransferases, are used extensively for the chemoenzymatic synthesis and in vivo biosynthesis of carbohydrate natural products and their analogues.[2] The desire to expand the substrate range of these enzymes has also sparked protein engineering efforts.[3] However, syntheses using nonnatural substrates, even those with conservative modifications, often fail for reasons that are not clearly understood. Unfortunately, the usual percent conversion studies do not shed light on whether the fault lies in low substrate affinity for the enzyme, low turnover rates, or both, and therefore, reaction optimization must be empirical. Herein we present evidence from the first kinetics analyses of sugar nucleotidyltransferases with deoxysugar substrates that these enzymes can exercise kinetic discrimination in choosing carbohydrates of comparable binding affinity for catalytic turnover. The consequences of this finding for using this class of enzymes for the synthesis of sugar nucleotides, as well as for understanding of their biological roles, will be discussed.

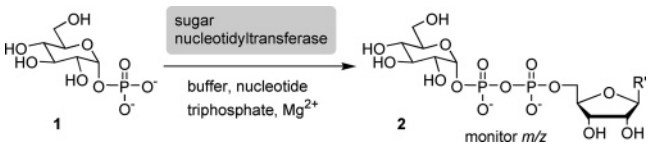


**FIGURE 1.** Sugar nucleotidyltransferases catalyze the synthesis of activated sugar nucleotides, such as uridinediphosphoglucose (**2**) when R′ = uridine, from sugar-1-phosphates, such as glucose-1-phosphate (**1**) and nucleotide triphosphates. Analysis of product formation by ESI-MS allows the facile kinetic analysis of the reaction independent of the structure of the carbohydrate portion of the sugar-1-phosphate.

Sugar nucleotidyltransferases catalyze the formation of a phosphate linkage to produce sugars activated for use by Leloir pathway glycosyltransferases (Figure 1). The reaction is known to proceed via nucleophilic attack of the sugar-1-phosphate on the α-phosphate of the nucleotide triphosphate;[4] hence, the carbohydrate portion is somewhat removed from the reactive site during catalysis. Although the structures of only a few sugar nucleotidyltransferases are presently known[5,6] and no apparent sequence homology between enzymes of similar function from eukaryotes and prokaryotes is evident, the process is crucial for carbohydrate biosynthesis. Sugar nucleotidyltransferases have been proposed as potential antibiotic targets,[5] but a more profound knowledge of differences in carbohydrate substrate recognition between bacteria and humans is needed to design compounds with the necessary selectivity. Greater knowledge of these enzymes also will aid in their use for the synthesis of nonnatural sugar nucleotides as glycobiology tools and precursors for in vitro glycosylation strategies.

A major impediment to studying sugar nucleotidyltransferases is the lack of an assay that can quickly determine kinetic parameters and inhibition constants

* To whom correspondence should be addressed. Phone: 515-294-2339 Fax: 515-294-0105.

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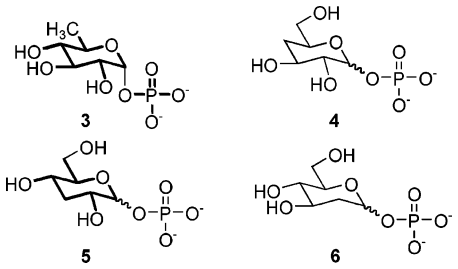

**FIGURE 2.** Synthetic substrates 6dGlc-1-phosphate (**3**), 4dGlc-1-phosphate (**4**), 3dGlc-1-phosphate (**5**), and 2dGlc-1-phosphate (**6**) used to test the substrate tolerance of UDP-glucose pyrophosphorylases from yeast and *E. coli*.

for a range of compounds. The use of coupled assays[7] or radioactive assays[8] limits the use of nonnatural substrates. HPLC-based assays[9,] require separation protocols for every new substrate and are so relatively time-consuming to probably have inhibited kinetics studies that require analysis of multiple reaction points in triplicate. Our recent development of an electrospray ionization mass spectrometry (ESI-MS)-based assay[10] enables the rapid determination of kinetic parameters for both natural and nonnatural substrates of this class of enzymes and prompted our investigation into the origins of substrate discrimination by sugar nucleotidyltransferases to optimize their use in synthesis.

A sugar nucleotidyltransferase from yeast that activates the most common naturally occurring sugar, glucose, is commercially available, and therefore, we commenced our studies with probing the tolerance of this enzyme to conservative substrate modifications. The systematic removal of hydroxyl moieties should provide substrates that still fit into the enzyme active site; however, previous work with a bacterial enzyme has shown that even such minor changes can significantly impact product yields.[9b] We therefore first undertook the synthesis of the required 6-, 4-, 3-, and 2-deoxyglucose-1-phosphates[9b] (dGlc-1-phosphate) (Figure 2) to probe the origin of these differences in product yields.

With the four required deoxyglucose-1-phosphates in hand, we could test the ability of the commercially available yeast enzyme, a representative eukaryotic enzyme, to accept these nonnatural substraes. The acceptance of the natural substrate (**1**) and various deoxyglucose-1-phosphates (**3**−**6**) by the yeast UDP-glucose pyrophosphorylase enzyme was tested in the presence of 0.02 U of enzyme, 10 mM uridine 5′-triphosphate (UTP) or 2′-deoxythymidine 5′-triphosphate (dTTP), and 10 mM $Mg^{2+}$. Inorganic pyrophosphorylase (IPP) was added to all reactions to degrade the inhibitory pyrophosphate generated in the course of the reaction. The relative acceptance rate is defined as the ratio of nonnatural substrate (**3**−**6**) to natural substrate (**1**) converted into the corresponding nucleotide diphosphate sugar. As shown in Figure 3A, the yeast UDP-glucose pyrophosphorylase enzyme only accepted the 6-deoxysubstrate **3** in the presence of UTP. No product formation was observed in the absence of the enzyme, $Mg^{2+}$, the nucleotide triphosphate, or the α-D-hexopyranosyl-1-phosphate. Unfortunately, the commercial enzyme was rather selective and therefore did not show promise for chemoenzymatic synthesis of activated deoxyglucose analogues. Therefore, a recombinant bacterial enzyme was investigated next. Fortunately, the relative acceptance of the UDP-glucose pyrophosphorylase from *Escherichia coli*[10] of the nonnatural substrates in the presence of UTP and dTTP was much better than the yeast enzyme (Figure 3B). Only the 2-deoxysubstrate **6** failed to generate product in the presence of UTP. However, the percent conversions were significantly lower for the deoxysubstrates than the natural substrate, despite no obvious negative steric factors.

To ascertain whether the differences in product yields were the result of decreased substrate binding or decreased turnover efficiencies, a kinetic analysis of the reactions was necessary. We therefore determined the kinetic parameters of the substrate−enzyme systems in which turnover was noted in the relative acceptance study. The kinetic parameters were measured by varying the concentration of the modified substrates **3**−**5** (2−100 $\mu$M) at a constant UTP or dTTP concentration (300 $\mu$M) in the presence of 10 mM $Mg^{2+}$ and 0.02 U of UDP-glucose pyrophosphorylase from *E. coli* or yeast. The reactions were quenched after 5 min, and the initial reaction rate was determined by the change in concentration of UDP or dTDP-dGlc-1-phosphate measured via ESI-MS. The kinetic values $K_m$ and $V_{max}$, Table 1, were determined from nonlinear regression of the Michealis-Menten plots (See Supporting Information).

Surprisingly, all of the substrates **1**, **3**, **4**, and **5** had essentially identical $K_m$ values for the *E. coli* enzyme. However, $V_{max}$ values decreased from the natural substrate (**1**) to the nonnatural substrates (**3**−**5**) thereby explaining the difference in product yields that were seen in the relative acceptance study. The yeast enzyme exhibited a slightly lower affinity for **2**, and the $V_{max}$ value dropped 2-fold replicating what was noted for the *E. coli* enzyme.

Clearly, differences in substrate affinity do not explain the differences in product yields for these two UDP-glucose pyrophosphorylases. It has been proposed that a glycosyltransferase involved in blood group determinant biosynthesis can distinguish between UDP-galactose and UDP-*N*-acetylgalactosamine based on kinetics rather than binding affinity;[11] however, this reaction involves both bond-making and bond-breaking directly on the sugar ring. That sugar nucleotidyltransferases can use this same strategy to select among carbohydrate substrates even though the sugar is three atoms from the site of bond formation is a surprising observation. However, the consequence is that use of higher enzyme concentrations rather than higher substrate concentrations can provide optimal yields of sugar nucleotides.

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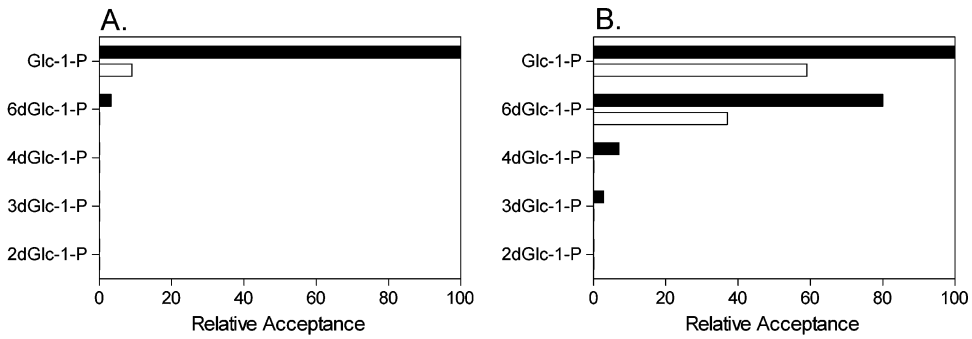


**FIGURE 3.** Relative substrate acceptance of UDP-glucose pyrophosphorylase from yeast (A) and *E. coli* (B) in the presence of UTP (solid bar) and dTTP (open bar) with the α-anomer of various glucose-1-phosphate analogues after 30 min.

**TABLE 1. Kinetic Analysis of Glucose-1-phosphate Uridylyltransferases**

| source | substrates | $K_M$ ($\mu$M) | $V_{max}$ ($\mu$M/min) | $k_{cat}/K_M$ ($s^{-1}$ $\mu M^{-1}$) |
|---|---|---|---|---|
| *E. coli* | **1** + UTP[a] | 12 ± 2 | 1.15 ± 0.06 | 1.45 |
| | **1** + dTTP[a] | 13 ± 2 | 0.90 ± 1.05 | 1.05 |
| | **3** + UTP | 15 ± 3 | 0.57 ± 0.03 | 0.58 |
| | **3** + dTTP | 17 ± 3 | 0.39 ± 0.03 | 0.35 |
| | **4**[b]+ UTP | 17 ± 3 | 0.47 ± 0.03 | 0.42 |
| | **5**[b]+ UTP | 18 ± 3 | 0.27 ± 0.01 | 0.23 |
| yeast | **1** + UTP[a] | 7 ± 1 | 3.8 ± 0.1 | 10.9 |
| | **1** + dTTP | 11 ± 2 | 1.84 ± 0.09 | 3.4 |

[a] Reference 10. [b] Adjusted for the anomeric mixture of compounds as only the α-anomer reacts.

Indeed, addition of higher *E. coli* enzyme concentrations to the reactions with the 3-, 4-, and 6-deoxysubstrates allows complete conversion of the substrates to their respective deoxysugar nucleotides.

The kinetic analysis reported herein is the first evidence that sugar nucleotidyltransferases can make use of not only differences in substrate binding affinity but also kinetic differences in discriminating among carbohydrate substrates. This finding has several implications. Sugar nucleotidyltransferase substrates, such as **3**, that are turned over preferentially by prokaryotic enzymes relative to eukaryotic enzymes could serve as prodrugs if the resulting products block glycosyltransferase activity. Protein engineering efforts cannot expect mutations that allow steric access of the sugar to the binding pocket to necessarily result in efficient turnover of the modified substrate. Chemoenzymatic reactions may benefit from an increase in enzyme concentration rather than substrate concentration to increase product yields as is the case with the reactions reported here. The same might be true for the turnover of nonnatural substrates in vivo. Upregulation of sugar nucleotidyltransferases also might result in the undesired turnover of alternate substrates that could lead to negative phenotypes. Finally, since the level of active enzyme is hard to quantify until that activity is known, newly isolated proteins believed to have sugar nucleotidyltransferase activity should be screened against a library of possible substrates to accurately determine the biological function of the enzyme.

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**Supporting Information Available:** Details of the mass spectrometry analysis including calibration curves, general methods, and Michaelis−Menten plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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