Enhancing the Latent Nucleotide Triphosphate Flexibility of the Glucose-1-phosphate Thymidylyltransferase RmlA

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Nucleotidyltransferases are central to nearly all glycosylation-dependent processes and have been used extensively for the chemoenzymatic synthesis of sugar nucleotides. The determination of the NTP specificity of the model thymidylyltransferase RmlA revealed RmlA to utilize all eight naturally occurring NTPs with varying levels of catalytic efficiency, even in the presence of non-native sugar-1-phosphates. Guided by structural models, active site engineering of RmlA led to alterations of the inherent pyrimidine/purine bias by up to three orders of magnitude. This study sets the stage for engineering single universal nucleotidyltransferases and also provides new catalysts for the synthesis of novel nucleotide diphosphosugars.

Carbohydrates are vital in nature, not only for energy metabolism, but also as structural scaffolds, recognition motifs, solubility aids, and functional modulators (1, 2). Yet, despite the vast structural and functional diversity of natural glycoconjugates, they are constructed via a common biosynthetic theme. Specifically, sugars are attached to most proteins, lipids, carbohydrates, and small molecules by glycosyltransferases, which with few exceptions use sugar nucleotides as the monosaccharide building blocks, and functional modulators (1, 2). Yet, despite the vast structural and functional diversity of natural glycoconjugates, they are constructed via a common biosynthetic theme. Specifically, sugars are attached to most proteins, lipids, carbohydrates, and small molecules by glycosyltransferases, which with few exceptions use sugar nucleotides as the monosaccharide building blocks, and functional modulators (1, 2). Yet, despite the vast structural and functional diversity of natural glycoconjugates, they are constructed via a common biosynthetic theme. Specifically, sugars are attached to most proteins, lipids, carbohydrates, and small molecules by glycosyltransferases, which with few exceptions use sugar nucleotides as the monosaccharide building blocks, and functional modulators (1, 2). Yet, despite the vast structural and functional diversity of natural glycoconjugates, they are constructed via a common biosynthetic theme. Specifically, sugars are attached to most proteins, lipids, carbohydrates, and small molecules by glycosyltransferases, which with few exceptions use sugar nucleotides as the monosaccharide building blocks, and functional modulators (1, 2). Yet, despite the...
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**FIGURE 1.** A, the wt RmlA reaction. B, an overview of glycorandomization. The inherent substrate flexibility of most natural product glycosyltransferases subsequently allow the ability to use unique sets of sugar nucleotides to generate libraries of differentially glycosylated natural products. The sphere represents any given natural product, and a key to this process is the ability of all participating enzymes to accommodate substrate diversity.

**TABLE 1**

| Mutation | Primer Sequence (5’ → 3’) |
|----------|---------------------------|
| Q83E     | AATGAGTCAGAGACCATTTAGGGG |
| Q83A     | AATGAGTCAGAGACCATTTAGGGG |
| Q83S     | AATGAGTCAGAGACCATTTAGGGG |
| Q83D     | AATGAGTCAGAGACCATTTAGGGG |
| Q83N     | AATGAGTCAGAGACCATTTAGGGG |

**EXPERIMENTAL PROCEDURES**

**General Materials and Methods**—Glucose-1-phosphate, mannose-1-phosphate, glucosamine-1-phosphate, N-acetylglucosamine-1-phosphate, galactose-1-phosphate, and NTPs were obtained from commercial sources (Sigma or Invitrogen) and used without further purification. The synthesis of 3-O-glucosamine-1-phosphate, galactose-1-phosphate, and NTPs were expressed as LT2 wt RmlA and all engineered RmlA mutants were obtained from commercial sources (Sigma or Invitrogen) or a Micromass LCT mass spectrometer (Agilent, Palo Alto, CA). Standard mass spectrometry utilized electrospray ionization and was performed using an Agilent 1100 HPLC-MSD mass spectrometer (Agilent, Palo Alto, CA). HPLC was performed using a Varian ProStar HPLC system (Varian Inc., Palo Alto, CA). Standard mass spectrometry utilized electrospray ionization and was performed using either an Agilent 1100 HPLC-MSD mass spectrometer (Agilent Technologies, Palo Alto, CA) or a Micromass LCT mass spectrometer (Waters, Milford, MA).

**Protein Expression and Purification**—Salmonella enterica typhimurium LT2 wt RmlA and all engineered RmlA mutants were expressed as N-His6 fusion proteins from pET28a-based expression plasmids (Novagen, Madison, WI) in Escherichia coli BL21(DE3) in a manner similar to previous methods (17–21). Specifically, an overnight starter culture of LB medium containing 50 μg/ml kanamycin was directly inoculated from a glycerol stock of the desired expression strain. After growth overnight (37 °C, 250 revolutions/min), this culture was diluted 1:100 with 2× YT medium (42) containing 50 μg/ml kanamycin typically to a total volume of 1 l. The large scale culture was subsequently grown (37 °C, 250 revolutions/min) to mid-log phase (A600 ~ 0.6), at which point isopropyl-β-D-thiogalactopyranoside was added to a 1 mM final concentration. Growth was continued for an additional 2–4 h, and the cells were collected by centrifugation (15 min, 5000 × g) and resuspended in 100 ml of 50 mM sodium phosphate, pH 8.0, containing 300 mM NaCl and 20 mM imidazole on ice. The cells were lysed via incubation with 1 mg/ml lysozyme (~50,000 units/mg; Sigma) for 30 min on ice followed by sonication (VirSonic 475, Virtis, Gardiner, NY; 100 W, 4 × 30 s pulses, ~1 min between pulses) on ice. Protein was purified with nickel-nitrotriacetic acid-agarose resin or spin columns (Qiagen, Hilden, Germany) using manufacturer’s protocols. As RmlA is not stable in the elution buffer (50 mM sodium phosphate, pH 8.0, containing 300 mM NaCl and 250 mM imidazole), the buffer was exchanged with 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 200 mM NaCl, and 10% (v/v) glycerol via PD-10 gel filtration columns (GE Healthcare, Uppsala, Sweden). Purified RmlA was subsequently concentrated to ~10 mg/ml, flash frozen in liquid nitrogen, and stored at −80 °C. Protein concentrations were determined by Bradford assay (Bio-Rad) using bovine serum albumin as a standard and a molecular mass of 34.6 kDa for RmlA (43). Spectrophotometric determination of protein concentration (calculated ε280 = 33,350 cm−1 M−1) (44) were consistent with the Bradford assay.

**RmlA Mutagenesis**—RmlA mutants were generated with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) using the wt RmlA-pET28a parent expression plasmid as the template and the primers indicated in Table 1. Individual progeny plasmids were confirmed by DNA sequencing to carry the desired mutations. Driven by these mutant plasmids, the corresponding RmlA variants were subsequently expressed and purified as described above.

**Enzymatic Reactions**—The enzyme assay was accomplished via slight modification of previously reported methods (10–13). In a typical reaction, sugar-1-phosphate (1 mM final) and enzyme (0.01–50 μM) were mixed with NTP (0.01–40 mM) in the presence of MgCl2 ([NTP] + 5 mM) and 10 units/ml inorganic pyrophosphatase (Sigma) in 100 mM Tris HCl, pH 8.0 (20 μl final volume). Typical reactions were analyzed after 10 min.
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at 37 °C, and reaction times were extended for some slow reacting sugar/NTP combinations. Reactions were stopped by the addition of 80 μl of prewarmed HPLC buffer and heat inactivation (95 °C, 5 min). The amount of enzyme used and the incubation time were adjusted so that the reactions never proceeded to >10% turnover for kinetics or 30% for activity assays. Equivalent reactions with heat-denatured enzyme displayed no product formation as determined by HPLC.

**Product Characterization**—Reactions were analyzed via analytical HPLC (Supelcosil LC18-T, Supelco, Bellefonte, PA; 5 μm, 250 × 3 mm, 40 mM phosphoric acid, adjusted to pH 6.5 with triethylamine, with a gradient of 0–10% MeOH over 20 min, 0.8 ml/min, A223). Due to earlier elution, a 5-min 0% MeOH isocratic hold was performed prior to starting the gradient for CTP-containing reactions. For nucleotide sugar products with commercially available standards, HPLC peak identity was confirmed by co-elution. The retention times for sugar-nucleotide product peaks under the above HPLC program are listed in supplemental Table S1. The identities of representative new reaction products were confirmed by mass spectrometry (supplemental Table S2).

**Kinetic Measurements**—Pseudo-first order kinetics were obtained by fixing glucose-1-phosphate at a saturating concentration of 10 mM (9) and titrating NTPs. At least eight different concentrations in the range of 1/4 to 4 × K_m (0.09–20 mM for dTTP, 0.2–40 mM for others) were assayed in triplicate. Reaction rates were confirmed to be linear over twice the incubation time. Activities were corrected for time and enzyme concentrations, and the kinetic curves were fit to the Michaelis-Menten equation using SigmaPlot with the Enzyme Kinetics module (SPSS, Chicago, IL).

**Structural Models of Active Site Mutants**—Atomic coordinates of enzyme structures were obtained from the RSCB Protein Data Bank (for RmlA with dTTP, 1IIM (9); RmlA with UDP-Glc, 1IIN (9); and MobA with GTP, 1FRW (45)). Point mutations were simulated by the mutation function of Swiss-PdbViewer software (46). Models of non-crystallographic ligands were created by manual overlay of nucleotide bases so that they were in the same plane as that of the crystallographic base. Mutant side chain positions were selected from the rotamer set provided, and no additional energy minimization was performed on the structures. Figures were generated with VMD (47) and rendered with POV-Ray software.

**RESULTS AND DISCUSSION**

**Protein Expression and Purification**—The wild-type and mutant enzymes were expressed in soluble form at high levels (10–100 mg/liter of culture) and were purified to >95% purity, as estimated by Coomassie-stained SDS-PAGE (supplemental Fig. S1). No significant differences in yield were noticed between the expression of wt RmlA and any of the RmlA mutants described herein.

**NTP Specificity of Wild-type RmlA**—Previous RmlA studies indicate a preference for the pyrimidine nucleoside triphosphates dTTP and, to a lesser extent, UTP using standard assay conditions (9–13, 49). Recent work on thermophilic uridylyl and thymidylyltransferases (Thermus caldophilus UDP-sugar pyrophosphorylase, or Usp, and Sulfolobus tokodaii ST0452, respectively) revealed the conversion of alternative nucleotides, including both ribo and deoxyribo variants of one or more purine nucleotides (33, 34). Specifically, Usp was found to turn over glucose-1-phosphate with UTP, ATP, GTP, and CTP, and dTTP (33). Alternatively, whereas ST0452 could not utilize ATP, CTP, or GTP, the enzyme showed good activity with UTP and the four deoxynucleoside triphosphates dATP, dTTP, dGTP, and dCTP (34). Given the high degree of amino acid similarity between Usp or ST0452 to RmlA (44 and 36%, respectively), these pioneering studies prompted a re-evaluation of the RmlA purine nucleotide specificity.

**Pseudo-first order Michaelis–Menten kinetic analysis** revealed RmlA to turn over all eight “natural” NTPs in the presence of glucose-1-phosphate (Table 2), although with appreciably reduced activity (~15–500-fold reduction in the apparent k_cat value) in comparison to dTTP. The apparent K_m for variant NTPs, including UTP, was also notably higher (~13–50-fold) than that for dTTP. This cumulative analysis translates to a drastic reduction in the apparent RmlA specificity constants (k_cat/K_m) for variant NTPs ranging from ~12-fold (UTP) to ~15,000-fold (ATP) and is noteworthy for a number of reasons. First, although RmlA has been used for the efficient synthesis of a variety of “unnatural” UDP-sugars (9–13), this study reveals the dTTP bias of RmlA to be dictated by K_m. Second, this study highlights the apparent K_m values of all alternative natural NTPs to increase ~10-fold, whereas the large differences in the apparent k_cat values bias RmlA toward dTTP and UTP. Third, a comparison between the determined kinetic parameters for deoxyribose-containing nucleotides (dTTP, dATP, dCTP, and dGTP) and ribose-containing nucleotides (UTP, ATP, CTP, and GTP, respectively) suggests the ribose 2'-hydroxyl to contribute ~10-fold to the overall RmlA apparent nucleotide specificity (k_cat/K_m). This is consistent with the previously reported influences of 2'-hydroxylating upon nucleotidyltransferase activity (50–52). Finally, it should be noted that, because of the high apparent K_m values for alternative nucleotides, preliminary assays were unsuccessful until the level of Mg²⁺ was adjusted according to Morrison (53), lending support to the theory that the true nucleotidyltransferase substrate is a Mg²⁺-NTP complex (54).

**TABLE 2**

Pseudo first order kinetic constants for RmlA

| Mutant         | Substrate | k_cat/K_m app | K_m app | k_cat/K_m app |
|----------------|-----------|---------------|---------|---------------|
| Wild-type      | dTTP      | 1850 ± 40     | 0.29 ± 0.04 | 3630 ± 700   |
|                | UTP       | 2460 ± 130    | 4.6 ± 0.7 | 530 ± 60      |
|                | dATP      | 113 ± 5       | 7.8 ± 0.9 | 14.5 ± 1.1    |
|                | dCTP      | 73 ± 3        | 10.5 ± 1.1 | 7.0 ± 0.4    |
|                | dGTP      | 127 ± 5       | 3.7 ± 0.5 | 34 ± 4        |
|                | ATP       | 3.32 ± 0.09   | 9.0 ± 0.6 | 0.39 ± 0.02   |
|                | CTP       | 5.9 ± 0.2     | 13.7 ± 1.1 | 0.43 ± 0.02  |
|                | GTP       | 8.4 ± 0.2     | 8.5 ± 0.6 | 0.99 ± 0.04   |
| Q83D           | dTTP      | 217 ± 14      | 6.2 ± 1.2 | 35 ± 5        |
|                | dGTP      | 300 ± 20      | 11.5 ± 1.8 | 26 ± 2        |
|                | GTP       | 12.8 ± 0.9    | 1.3 ± 0.4 | 10 ± 3        |
| Q83S           | dTTP      | 380 ± 30      | 2.4 ± 0.6 | 160 ± 30      |
|                | dATP      | 320 ± 20      | 1.1 ± 0.3 | 280 ± 50      |
|                | ATP       | 28.8 ± 1.4    | 3.4 ± 0.6 | 8.3 ± 1.1    |
Sugar-1-phosphate Specificity with Variant NTPs—In light of the newly discovered ability of RmlA to employ variant NTPs, the specific activity of RmlA in the presence of a variety of unnatural sugar phosphate/NTP combinations was assessed. Turnover was observed with nearly all sugar phosphate/NTP combinations examined, although with reductions in overall efficiencies up to 10^7-fold (Fig. 2). Consistent with previous studies (10, 13), wt RmlA was found to be least tolerant of sugar C3/C4 substitutions in the presence of alternative NTPs, and alteration of both the NTP and sugar-1-phosphate typically exceeded an additive effect, previously noted as “adversely cooperative” (10). Yet, despite the adverse cooperativity and large reductions in catalytic efficiency, the clean production of this new set of unnatural sugar nucleotides could be accomplished by simply increasing enzyme concentration and incubation time (supplemental Fig. S2).

Engineering RmlA NTP Specificity—Examination of RmlA structural homologs emphasized that guanylyltransferases, such as *E. coli* MobA (which catalyzes the phosphate-phosphate coupling reaction highlighted in Fig. 3A (55)) employ an aspartate to bind the GTP base-pairing face (45). In RmlA, the structural equivalent of this moderately conserved MobA Asp-71 (Fig. 3B (45)) is RmlA Gln-83 (Fig. 3C), the side chain of which hydrogen bonds with the dTTP/UTP base-pairing face. We postulated this interaction and the steric bulk of Gln-83 (Fig. 3D) to contribute to the pyrimidine bias and mutation of RmlA Gln-83 might open the door

Picture 2: Specific activity of the wild-type RmlA toward variant sugar/nucleotide combinations at 5 mM NTP and 10 mM sugar-1-phosphate. Substrates were reacted with 0.05–50 μM enzyme in 100 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, and 10 units/ml inorganic pyrophosphatase at 37 °C for 10–1000 min, and substrates/products were resolved via C18 reverse phase HPLC in a triethylamine phosphate buffer/methanol system. Activities are plotted on a log scale to accommodate the large range of values.

**Figure 2.** Specific activity of the wild-type RmlA toward variant sugar/nucleotide combinations at 5 mM NTP and 10 mM sugar-1-phosphate. Substrates were reacted with 0.05–50 μM enzyme in 100 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, and 10 units/ml inorganic pyrophosphatase at 37 °C for 10–1000 min, and substrates/products were resolved via C18 reverse phase HPLC in a triethylamine phosphate buffer/methanol system. Activities are plotted on a log scale to accommodate the large range of values.

**Figure 3.** The structural basis for engineering RmlA nucleotide specificity. A, reaction catalyzed by the MobA guanylyltransferase (55). B, crystal structure of *E. coli* MobA (45) with hydrogen bonding contacts between the nucleotide base and aspartate 71 highlighted. C, crystal structure of wt RmlA bound to UTP (9). The uracil base makes hydrogen bonding contacts with the glutamine 83 side chain of the enzyme. D, model of wild-type RmlA bound to GTP, showing the steric clash of the nucleotide base and Gln-83. E, model of the RmlA Q83D mutant bound to GTP. In this model, steric infringements have been relieved, and the side chain oxygens are appropriately positioned to the hydrogen bond with the N1 nitrogen and the N2 exocyclic nitrogen of guanine. F, model of the RmlA Q83S mutant bound to ATP. In this model, the purine/glutamine steric clash has been relieved, and the serine hydroxyl may be able to form a hydrogen bond to the N1 of adenine.
to engineering a universal nucleotidyltransferase. Thus, Q83D (Fig. 3E) and the isosteric Q83N mutations were pursued. In addition, mutants that incorporated smaller non-polar (Q83A) or polar (Q83E) at this position were also studied as well as larger iso-electronic substitutions (Q83E) at this position were also studied.

The activities of this mutant series, in the presence of alternative NTPs, was compared (Table 3), and the salient kinetic parameters for uniquely active mutants were subsequently determined (Table 2). Consistent with our model, changing glutamine to aspartic acid (Q83D) increased the guanine/thymidine discrimination (Table 2). In a similar manner, substituting glutamine for a smaller amino acid (Q83S) favored the adenine/thymidine bias (as measured by the ratio of specificity constants) by approximately three orders of magnitude, wherein this altered specificity derives in large part from a 6.5-fold improvement in apparent $K_m$ for GTP and a corresponding 21-fold increase in the dTTP $K_m$ (Table 2). In a similar manner, substituting glutamine for a smaller amino acid (Q83S) favored the adenine/thymidine bias by approximately three orders of magnitude, wherein dATP improvements were predominately apparent $K_m$-derived, whereas ATP improvements were primarily apparent $k_{cat}$-dictated (Table 2). In this latter case, the effect of mutations on apparent $k_{cat}$ value was not entirely expected, as reduction in steric overlap and the alteration of hydrogen bonds were anticipated to mainly influence substrate and product binding. Surprisingly, no significant increase in (d)CTP turnover was observed in any of the mutants tested (Table 3). Given thymidine and cytidine are isosteric and the backbone nitrogen of glycine 88) may contribute to thymidine/cytosine discrimination.

**CONCLUSIONS**

The determination of the RmlA NTP specificity revealed this catalyst to utilize all eight naturally occurring NTPs with varying levels of catalytic efficiency, even in the presence of non-native sugar-1-phosphates. The uniquely broad synthetic utility of RmlA was further "generalized" by structure-based engineering. The ability to modulate the *in vitro* specificity of RmlA is consistent with the theory that enzymes have evolved to be perceived as specialists in the context of a discrete *in vivo* environment (56) but are perhaps not far-removed from more promiscuous progenitors (48, 57, 58).

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