

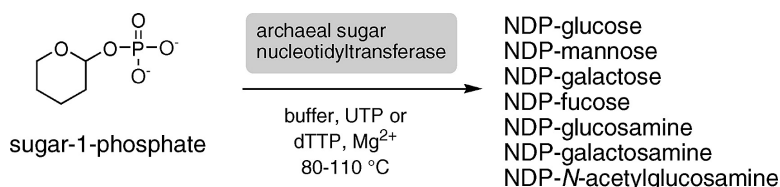
Article

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Unusually Broad Substrate Tolerance of a Heat-Stable Archaeal Sugar Nucleotidyltransferase for the Synthesis of Sugar Nucleotides

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Abstract: Herein, we report the first cloning, recombinant expression, and synthetic utility of a sugar nucleotidyltransferase from any archaeal source and demonstrate by an electrospray ionization mass spectrometry (ESI-MS)-based assay its unusual tolerance of heat, pH, and sugar substrates. The metal-ion-dependent enzyme from *Pyrococcus furiosus* DSM 3638 showed a relatively high degree of acceptance of glucose-1-phosphate (Glc1P), mannose-1-phosphate (Man1P), galactose-1-phosphate (Gal1P), fucose-1-phosphate, glucosamine-1-phosphate, galactosamine-1-phosphate, and *N*-acetylglucosamine-1-phosphate with uridine and deoxythymidine triphosphate (UTP and dTTP, respectively). The apparent Michaelis constants for Glc1P, Man1P, and Gal1P are 13.0 ± 0.7 , 15 ± 1 , and $22 \pm 2 \mu\text{M}$, respectively, with corresponding turnover numbers of 2.08, 1.65, and 1.32 s^{-1} , respectively. An initial velocity study indicated an ordered bi-bi catalytic mechanism for this enzyme. The temperature stability and inherently broad substrate tolerance of this archaeal enzyme promise an effective reagent for the rapid chemoenzymatic synthesis of a range of natural and unnatural sugar nucleotides for *in vitro* glycosylation studies and highlight the potential of archaea as a source of new enzymes for synthesis.

Introduction

The enzymes responsible for creating the incredible diversity of carbohydrate structures that mediate many biological processes are the glycosyltransferases.¹ Most glycosyltransferases that incorporate a wide range of sugars into both primary and secondary metabolites rely on uridine- and thymidinediphospho nucleotide sugars that are produced by sugar nucleotidyltransferases, also known as sugar pyrophosphorylases.² Several glycosyltransferases involved in secondary metabolism recently have been shown to be very promiscuous with respect to their nucleotidediphospho (NDP) sugar donor.² A limiting factor in exploiting this promiscuity to produce new compounds is the lack of the required unusual NDP sugars.³ Since these activated sugar donors are difficult to synthesize chemically, current efforts to generate these structures center on using an array of enzymes or extremely high enzyme concentrations to circumvent poor acceptance of non-natural substrates.^{2a} This prior work has focused entirely on enzymes from eukaryotic and bacterial sources. Our recent studies of a glycosyltransferase involved in primary metabolism from the third branch of life, archaea, have hinted that these untapped genomes could be a source of enzymes with even greater carbohydrate substrate tolerances

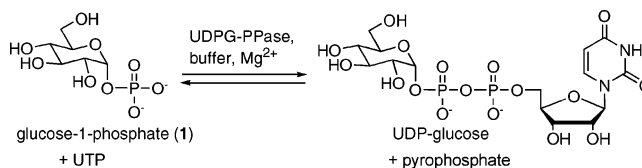


Figure 1. Reaction scheme for the sugar nucleotidyltransferase UDPG-PPase that couples glucose-1-phosphate and uridine triphosphate (UTP) to form activated UDP-glucose as a Leloir pathway glycosyl donor.

than seen to date with enzymes cloned from larger genomes.⁴ In addition, archaea contain many extremophiles and therefore can be a source of thermoactive and heat-stable enzymes for biotechnology applications if the necessary substrate tolerances can be found.⁵ As part of our program to understand the origins of fidelity in carbohydrate biosynthesis, we report the first study of a sugar nucleotidyltransferase from any archaeal source, the hyperthermophile *Pyrococcus furiosus* DSM 3638, and show that its inherent substrate tolerance allows its use for the synthesis of commercially unavailable non-natural NDP sugars, such as uridinediphospho (UDP) mannose, with over 300-fold fewer units of enzyme than previously reported.^{2a}

Most sugar nucleotidyltransferases catalyze the transfer of a sugar-1-phosphate to nucleotidetriphosphate (NTP) with the concomitant release of pyrophosphate (Figure 1). Chemoenzymatic synthesis of a number of natural and unusual UDP and deoxythymidine (dTDP) sugars has been carried out using wild-

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type and engineered variants of α -D-glucopyranosyl phosphate thymidyltransferase (also known as E_p) from the bacteria *Salmonella enterica*, LT 2.^{2a,3} The wild-type enzyme and a number of its mutants have been used for the synthesis of NDP sugar libraries with high enzyme concentrations (3.5 units; a unit is defined as the amount of enzyme needed to produce one micromole of nucleotidediphospho sugar per minute).^{2a} Although no kinetic studies have been carried out with this *Salmonella* enzyme with any non-natural substrates, our results with a related enzyme from *Escherichia coli*⁶ suggest that substrate binding affinity differences are not the major problem, and high enzyme concentrations are necessary to override the limited turnover of non-natural substrates. Expansion and diversification of both natural and non-natural NDP sugar libraries still await the discovery of enzymes with wide glycosyl phosphate acceptability, ideally without the introduction of mutations or active site engineering and with a tolerance of robust working conditions. The study of sugar nucleotidyltransferase has also attracted interest for their potential as antibiotic targets.⁷ For example, UDP-glucose, synthesized by UDP- α -D-glucose pyrophosphorylases (UDPG-PPases), has a number of vital cellular functions, including the synthesis of glycogen,⁸ the synthesis of the carbohydrate moiety of glycolipids,⁹ glycoproteins,¹⁰ and proteoglycans,¹¹ the entry of galactose into glycolysis,¹² the synthesis of UDP-glucuronic acid,¹³ and the pathogenesis of a number of bacteria.¹⁴

Results and Discussion

Preparation of an Archaeal Sugar Nucleotidyltransferase.

To date, UDPG-PPase has been isolated and characterized from both eukaryotic¹⁵ and prokaryotic¹⁶ sources. On the basis of sequence similarities, UDPG-PPases have been classified into two groups despite their similar catalytic properties: the prokaryotic enzymes with about 300 amino acids residues and the eukaryotic enzymes with about 500 amino acids residues.¹⁷ Surprisingly, no study has been conducted so far on sugar nucleotidyltransferases from the third class of living beings,

archaea. The amino acid sequence of a putative archaea UDP- α -D-glucose pyrophosphorylase (UTP: α -D-glucose-1-phosphate uridylyltransferase, EC 2.7.7.9) from *P. furiosus* DSM 3638 was compared to related bacterial and eukaryotic sequences using BLAST (www.ncbi.nih.gov) to identify conserved residues. *P. furiosus* was chosen as a representative archaea because a large structural proteomics effort centered on this extremophile promises protein data to match biochemical studies.¹⁸ Pairwise alignment of the enzyme showed a high degree of residue identity between archaea and bacteria ranging from 40 to 51%. Sixty-two residues are strictly conserved among UDPG-PPases from a variety of evolutionary diverse organisms. Among the conserved residues, glycine is the most abundant, followed by glutamate, lysine, and proline. However, among the amino acids of *P. furiosus* UDPG-PPase, glutamate is the most prominent, followed by leucine, isoleucine, lysine, and glycine. The percentage of Glu and Lys increased in the hyperthermophilic proteomes, and the percent of Gln and His decreased, so that (Glu + Lys)/(Gln + His) is greater than 4.5.¹⁹ This ratio for the *P. furiosus* UDPG-PPase is 4.8, which would predict thermostability. A single motif can be clearly identified at alignment position 5-30 (Figure S4 of the Supporting Information), which is in the N-terminal position of the enzyme. All other conserved residues are distributed throughout the sequence. Amino acid sequence alignments among the UDPG-PPases from *Saccharomyces cerevisiae*, *E. coli*, and *P. furiosus* were also done using BLAST. As shown in Figure 2, 38% overall sequence identity was found between the *E. coli* and *P. furiosus* enzymes (Figure 2). In contrast, the archaeal enzyme showed no sequence similarity with the yeast enzyme. Therefore, the archaea enzyme could be expected to have significantly different properties than the related bacterial and eukaryotic enzymes and thereby could be a good target for exploring its potential for chemoenzymatic synthetic strategies.

To assess the potential of archaeal proteins in the synthesis of sugar nucleotides, this open reading frame predicted to encode the *P. furiosus* UDPG-PPase (852 base pairs) was amplified by PCR and cloned into a pET21a vector for expression of the protein in *E. coli* cells with a hexahistidine tag at the C-terminus. To purify the enzyme, the cells were lysed by sonication, heat-treated to denature native *E. coli* proteins, and centrifuged. The resulting cell-free extract was passed through a Ni-affinity column and eluted with imidazole buffer. SDS-PAGE analysis of the purified enzyme showed a protein with an apparent molecular mass of 36 kDa (see the Supporting Information), which was in good agreement with the calculated molecular mass of the gene product (31.2 kDa). The enzyme was used directly for determination of its activity and substrate specificity or stored at -20 °C as a glycerol stock solution for future use with little loss of activity after 90 days.

Enzymatic Analysis. The difficulty of assays, such as those based on HPLC,²⁰ needed to measure sugar nucleotidyltransferase activity with non-natural substrates has been a significant deterrent to kinetic studies. Our recent development of an electrospray ionization mass spectrometry assay has significantly shortened the analysis time, and since it is not substrate-specific,

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Pfu:2	KVRKAVIPAAAGLGRMLPLTKAQPKEMLPVVRKPTIQYVLEEAYEAGIREVLIITGKHKR	61
Eco:7	KVKKAVIPVAGLGRMLPATKAIKPEMLPLVDKPLIQYVVNECIAAGITEIVLVTHSSKN	66
Pfu:62	AIEDHFD -----RYEHEVKNPHXXXXXXXX-XXXXNIYYARQVQRGLGDAIKYAEAFV	113
Eco:67	SIENHFDT SFELEAMLEKRVKRQLLDEVQSI CPPHVTIMQVRQGLAKGLGHAVLCAHPVV	126
Pfu:114	GDEPFALLLGD TI-----TLPSCTAGIIESYEELKAPVIAVEEVQEEKISLYGVV---	163
Eco:127	GDEPVA VILPDVILDEYESDLSQDNLAEMIRRFDETGHSSQIMVEPVAD--VTAYGVVDCK	184
Pfu:164	GIGRYINERIFEINKLVEKPEIHEAPSNLAILGRYILTPEIFEYLEEVKPKDKKGEIQLTD	223
Eco:185	GVELAPGESVPMVG-VVEKPKADVAPSNLAI VGRYVLSADIWPELLAKTPPGAGDEIQLTD	243
Pfu:224	ALELMVQNGKKIYGYVFKGRRYDIGNIFD WLRANIELGLEDEELSEKLRRELIKSLVG	280
Eco:244	AIDMLIEK-ETVEAYHMKGKSHDCGNK LGYMQAFVEYGI RHNTLGTEFKAWLEEEMG	299

Figure 2. Amino acid sequence alignment of *P. furiosus* UDPG-PPase with an *E. coli* homolog. Pfu: *P. furiosus* (accession no. NC003413.1). Eco: *E. coli* (accession no. AP002556.1). The identical amino acids are indicated in bold. The yeast enzyme used in this study showed no sequence similarity with the *P. furiosus* enzyme.

it can be used for any sugar nucleotidyltransferase.⁶ With this assay, the activity of both crude protein extracts and purified enzyme was determined at 90 °C using glucose-1-phosphate (1, Glc1P) and uridinetriphosphate (UTP) as initial trial substrates. Formation of UDP-glucose at these elevated temperatures in both the crude extract and the purified protein confirmed the presence of an active UDPG-PPase, thereby verifying the tentatively assigned function of this gene. Because uridinemonophosphate transfer to Glc1P releases pyrophosphate, which can serve as an inhibitor, a commercially available thermostable inorganic pyrophosphatase (IPP) was added to the reaction mixture. Although heat treatment should have denatured all native proteins, an additional control experiment was run with extracts of *E. coli* carrying a pET21a vector without an inserted gene. No additional protein band corresponding to 36 kDa was detected by SDS-PAGE analysis of the crude extract after passing through the Ni-affinity column, and no UDPG-PPase activity was detected at 90 °C. No product formation was observed in the absence of UDPG-PPase, sugar phosphates, Mg²⁺, or NTP.

The temperature, pH ranges, and divalent cation requirements of the sugar nucleotidyltransferase were ascertained next. This first purified archaeal UDPG-PPase showed maximum activity at 99 °C (Figure S1 of the Supporting Information) with little loss of activity at 110 °C in phosphate buffer with glycerol. The enzyme exhibited activity between pH 5.5 and 9.5, with a maximum around pH 7.5–8.0 in phosphate buffer (Figure S2 of the Supporting Information). The shape and maxima of the pH velocity curves, however, were found to vary somewhat with the nature of the buffer ion present. The enzyme showed an absolute requirement of divalent cations for activity, as established for other UDP-glucose pyrophosphorylases.^{15,16} Magnesium, manganese, cobalt, and calcium were found to be effective in decreasing order (Table 1). In the direction of UDP-glucose synthesis, a magnesium concentration of 3 mM elicited the maximum reaction rate (Figure S3 of the Supporting Information); enzyme activity did not decrease extensively, however, even at higher concentrations of magnesium. Although molecular and structural data are not available for any UDPG-PPase, it has been suggested that the major function of magnesium in enzyme catalysis is in the formation of MgUTP²⁻ complexes.²¹ However, recent structural data of the *Salmonella* sugar nucleotidyltransferase, E_p,²² suggest that Mg²⁺ plays a structural role in organizing the substrate binding region of the E_p around itself to fix the nucleotidetriphosphate at an optimal position for catalysis.

Table 1. Effects of Divalent Cations on Enzymatic Activity of the Sugar Nucleotidyltransferase from *P. furiosus*

divalent cation ^a	relative activity (%)
Mg ²⁺	100
Mn ²⁺	72
Cu ²⁺	44
Co ²⁺	14
Ca ²⁺	9
Zn ²⁺	9
None	0

^a The purified enzyme (0.007 U) was incubated with standard assay mixtures at pH 7.5 with the addition of various metal ions at 3 mM (chloride form) and incubated for 5 min at 90 °C. The UDP-glucose that formed was analyzed by ESI-MS, and the relative activity is expressed as the percentage of the activity measured in the presence of MgCl₂. "None" indicates that the reaction was carried out in the absence of any divalent cations.

Substrate Specificity Studies. We are particularly interested in developing enzymatic routes to natural and non-natural UDP and dTDP sugars, ideally without the use of multiple enzymes and without the need for extremely high enzyme concentrations. In addition, the discovery of enzymes that demonstrate intrinsic catalytic competence with a range of substrates provides clues to how biosynthetic machinery has evolved to maintain fidelity in carbohydrate biosynthesis. Although the *P. furiosus* enzyme catalyzed the formation of UDP-glucose, as predicted from gene sequence analysis, experiments with the glycogen synthase⁴ from this organism demonstrated the danger of testing only one substrate pair to assign the chemical function of a gene. Therefore, a range of substrates (Figure 3) were assayed with the enzyme. Mixtures containing the reaction components were initiated by addition of 0.01 units of the enzyme and incubated for 1 h at 90 °C. The percent conversion of sugar-1-phosphate with UTP and dTTP was then measured by mass spectrometry. In addition to UTP, dTTP was found to serve as a nucleoside monophosphate donor (Table 2), similar to data reported from enzymes from *E. coli* and *Salmonella typhimurium*.^{22,23} No

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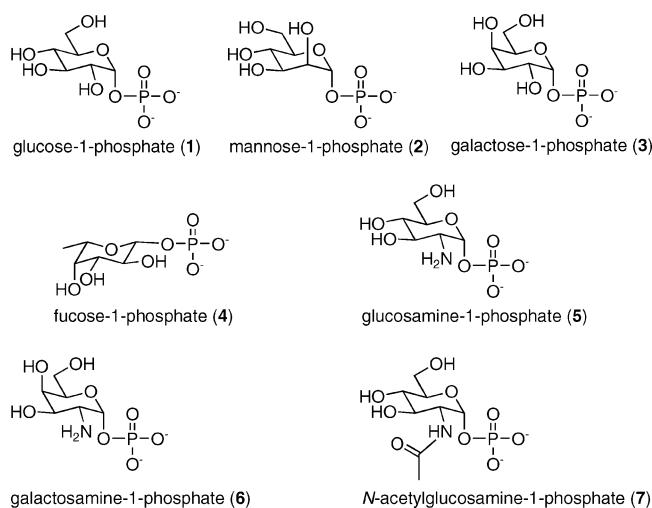
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Table 2. Comparison of Kinetic Parameters of UDPG-PPase from *S. cerevisiae*, *E. coli*, and *P. furiosus*

substrate	<i>S. cerevisiae</i> ^a			<i>E. coli</i> ^b			<i>P. furiosus</i> ^b		
	K_M (μM)	V_{max} ($\mu\text{M}/\text{min}$)	k_{cat}/K_M ($\mu\text{M}^{-1}\text{s}^{-1}$)	K_M (μM)	V_{max} ($\mu\text{M}/\text{min}$)	k_{cat}/K_M ($\mu\text{M}^{-1}\text{s}^{-1}$)	K_M (μM)	V_{max} ($\mu\text{M}/\text{min}$)	k_{cat}/K_M ($\mu\text{M}^{-1}\text{s}^{-1}$)
UTP–Glc1P	7 ± 1	3.8 ± 0.1	10.9	12 ± 2	1.2 ± 0.1	1.45	13 ± 1	2.8 ± 0.1	0.16
dTTP–Glc1P	not accepted			13 ± 2	0.9 ± 0.1	1.05	19 ± 2	2.6 ± 0.1	0.09
ATP–Glc1P	7 ± 1	1.7 ± 0.1	4.8	not accepted			not accepted ^b		
UTP–Gal1P	not accepted ^b			not accepted ^b			22 ± 2	2.00 ± 0.07	0.06
UTP–Man1P	not accepted ^b			not accepted ^b			15 ± 1	2.10 ± 0.04	0.11

^a Previously reported (ref 6). ^b From this study. Kinetic parameters were determined in 25 mM phosphate buffer (pH 7.5) at 90 °C using 2.5×10^{-4} U of UDPG-PPase. Reported error bars represent the standard deviation, which was obtained from three independent experiments. UDPG-PPases from *E. coli* and *S. cerevisiae* were run in triplicate at varying concentrations of Man/Gal1P and at fixed concentrations of UTP using the same enzyme unit concentration as was used for the *P. furiosus* enzyme.

**Figure 3.** Sugar-1-phosphates used in this study.

activity was recorded against all other nucleotide triphosphates in those cases. Although several enzymes from *P. furiosus* exhibit an unusual usage of nucleotides,^{4,24} our archaeal sugar nucleotidyltransferase also showed this discrimination against other NTPs. However, unlike other reported UDPG-PPases and dTDPG-PPases, a substantial amount of activity against mannose-1-phosphate (2, Man1P) and galactose-1-phosphate (3, Gal1P) with UTP and dTTP was found when the reaction mixture was incubated for 30 min with only 0.007 units of the enzyme. This finding is in stark contrast to results obtained with the related *Salmonella* enzyme E_p in which 500 times more units of enzyme were used to get only 18% conversion of Man1P into the non-natural UDP-mannose donor.^{2a} In addition, fucose-1-phosphate (Fuc1P), glucosamine-1-phosphate (GlcN1P), galactosamine-1-phosphate (GalN1P), and N-acetylglucosamine-1-phosphate (GlcNAc1P) also demonstrated significant product formation of their respective UDP and dTDP sugars when the reaction mixture was incubated with 0.01 units of the enzyme (Table 3). For comparison, very low rates of activity (<1% compared to those of Glc1P) were evident against Gal1P and Man1P with UTP of the enzyme purified from human erythrocyte²⁵ and <5% activity was reported against Gal1P and Man1P with the enzyme purified from *Thermus caldophilus*.²⁶

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Table 3. Conversion of Sugar-1-Phosphates by UDPG-PPase from *P. furiosus*

sugar-1-phosphate ^a	UTP conversion (%) ^b	dTTP conversion (%) ^b
glucose-1-phosphate (1)	99	91
mannose-1-phosphate (2)	92	36
galactose-1-phosphate (3)	96	15
fucose-1-phosphate (4)	82	10
glucosamine-1-phosphate (5)	66	43
galactosamine-1-phosphate (6)	28	5
N-acetylglucosamine-1-phosphate (7)	84	56

^a The purified enzyme (0.01 U) was incubated in a 50 μL reaction volume with 3 mM Mg^{2+} , 5 mM UTP or dTTP, and 5 mM sugar-1-phosphate, including all other reaction components. The reaction was incubated at 90 °C for 60 min. NDP-sugars that formed were analyzed by ESI-MS. ^b Percent conversion is defined as 100 times the ratio of the amount of sugar-1-phosphate remaining in the reaction mixture over the amount of initial sugar-1-phosphate. No substrate hydrolysis was seen under these conditions.

Control experiments were run to ensure that the archaeal protein was solely responsible for this unusually broad substrate turnover. It has been reported that small amounts of UDP-mannose/galactose/xylose were formed in the presence of UDPG-PPase purified from calf liver.²⁷ However, coelution of other contaminating sugar nucleotidyltransferases in the purified liver enzyme preparation has not been ruled out. Control experiments using heat treatment of the crude extract, as well as a plasmid without insert, eliminate the possibility of contaminating *E. coli* proteins as a source of alternate sugar nucleotidyltransferase activities in the case of the archaeal protein. In addition, we have tested the recombinant *E. coli* UDPG-PPase⁶ activity at 90 °C, and no activity was observed at this elevated temperature. The presence of only *P. furiosus* UDPG-PPase can account for the conversion of the various sugars to activated sugar donors.

Enzymatic Mechanisms. Relative substrate specificity studies are useful for ascertaining the potential of an enzyme for chemoenzymatic synthesis; unfortunately, such data provide no clues as to the reasons why one substrate is preferred over another. Therefore, the mechanism of this thermostable archaeal enzyme was probed next. Previous functional investigations of a related thymidyltransferase have provided alternate hypotheses on the catalytic mechanism.²⁸ On one hand, the bimolecular group transfer reaction has been described by a ping-pong bi–bi mechanism, based on the analysis of double reciprocal plots of initial velocity as a function of different

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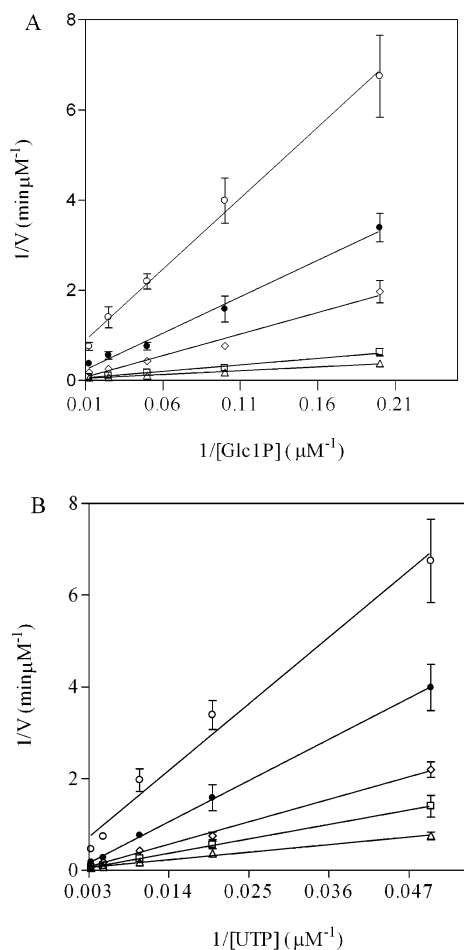


Figure 4. Steady-state kinetic parameters of UDPG-PPase from *P. furiosus*. (A) The double reciprocal plots are from assays with varying glucose-1-phosphate (1, Glc1P) concentrations as a function of UTP (μM): 20 (\circ), 50 (\bullet), 100 (\diamond), 200 (\square), and 300 (\triangle). (B) The double reciprocal plots are from assays with varying UTP concentrations as a function of Glc1P concentration (μM): 5 (\circ), 10 (\bullet), 20 (\diamond), 40 (\square) and 80 (\triangle). The enzymatic reaction mixtures also contained phosphate buffer (25 mM, pH 7.5), inorganic pyrophosphorylase (0.2 U), MgCl_2 (3 mM), and the sugar nucleotidyltransferase (0.00025 U), with a final volume of 50 μL . Reactions were carried out at 90 $^\circ\text{C}$ for 5 min. Error bars represent the standard deviation of three independent averaged data points.

concentrations of the two substrates.^{28a} Achievement of a ping-pong-type catalytic mechanism would require the availability of reactive (nucleophilic) residues within the structural surroundings of the dTTP α -phosphate group to yield the first covalent enzyme–dTTP intermediate. Alternatively, the achievement of a sequential bi–bi mechanism requires proper noncovalent binding of dTTP and Glc1P within the active site (yielding an enzyme substrate ternary complex) for the onset of catalysis.^{28b} In both cases, activation of the substrate reactive phosphate groups (i.e., α -phosphate on dTTP and phosphate-1 on Glc1P) for the group transfer reaction through local interactions with active site residues is required. To determine the kinetic mechanism of the archaeal enzyme, double reciprocal plots of the initial velocity were obtained by varying the concentration of one substrate at fixed concentrations of the other. The initial kinetic patterns of velocity with the Glc1P as the variable substrate are shown in Figure 4A, and those with UTP as the variable substrate are shown in Figure 4B. The clear intersecting patterns left of the $1/V$ axis is indicative of a sequential bi–bi mechanism in which the nucleotide is the first

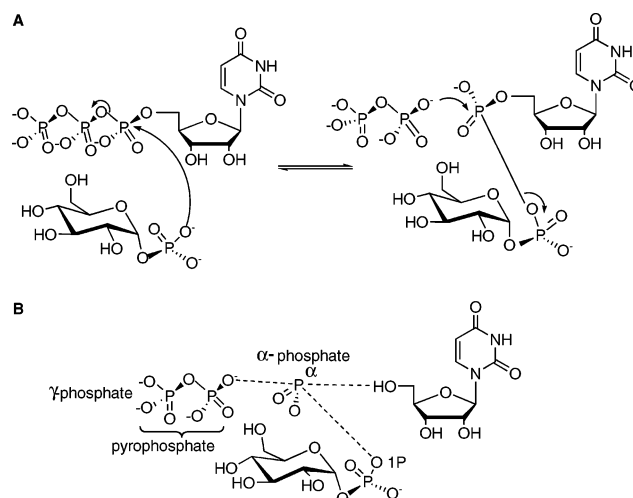


Figure 5. (A) Mechanism of the reaction catalyzed by UDPG-PPase. (B) Distinct chemical groups that form a ternary complex with the protein.

substrate to bind to the enzyme and is also the last to be released. Therefore, the archaeal enzyme follows the same mechanism previously demonstrated by bacterial and eukaryotic sugar nucleotidyltransferases.^{7,22,25} This type of reaction has been suggested as an $\text{S}_{\text{N}}2$ type, with dTTP binding to the protein, followed by Glc1P, which acts as the α -phosphate of dTTP.²⁰ The β - and γ -phosphates of dTTP are displaced and leave as pyrophosphate. In $\text{S}_{\text{N}}2$ -type reactions, the leaving group and the attacking nucleophile (the oxygen atom of the α -phosphate) reside at opposite faces of the central atom. On the basis of the above evidence and argument, the catalytic mechanism of UDPG-PPase is diagrammed in Figure 5.

Given this sequential bi–bi mechanism, we next wanted to determine if the different relative conversions of sugar substrates were a function of differences in substrate binding to the enzyme active site or a difference in turnover rates of bound substrates. Therefore, the kinetic properties of the enzyme with a number of glycosyl phosphates and UTP and dTTP were determined (Table 2). The concentrations of Glc1P (2–80 μM) or Man1P/Gal1P (5–200 μM) were varied with a fixed concentration of UTP/dTTP (400 μM). A Michaelis–Menten plot of the velocity versus sugar-1-phosphate ion concentration was determined using ESI-MS for the *P. furiosus* UDPG-PPase in the presence of UTP for all three sugar-1-phosphates and dTTP for Glc1P (Supporting Information). Nonlinear regression analysis of the data yields K_{M} and V_{max} values of $13.0 \pm 1 \mu\text{M}$ and $2.8 \pm 0.1 \mu\text{M min}^{-1}$, respectively, for the enzyme with Glc1P and UTP. These values are similar to values for the related enzymes from yeast and *E. coli* (Table 2).⁶ Surprisingly, the K_{M} and $k_{\text{cat}}/K_{\text{M}}$ values for glucose-, galactose-, and mannose-1-phosphate were all comparable. This finding indicates that the archaeal sugar nucleotidyltransferase truly is unusual in its ability to bind and turnover a range of substrates.

Conclusion

This initial study of any sugar nucleotidyltransferase from an archaeal source has uncovered a valuable heat-stable enzyme for the chemoenzymatic synthesis of a range of activated sugar nucleotides. The catalytic and mechanistic properties of the *P. furiosus* enzyme are almost the same as those of the prokaryotic and eukaryotic enzymes based on our findings. However, unlike

bacterial, plant, and animal enzymes, *P. furiosus* UDPG-PPase has an unusual tolerance of high temperatures. More surprisingly, this archaeal sugar nucleotidyltransferase can accept an unusually broad range of sugar phosphates with reasonably efficient turnover rates, unlike previously reported enzymes that carry out this reaction. The archaeal UDPG-PPase demonstrated a unique ability to activate a range of sugar substrates and, therefore, should serve as an ideal biocatalytic tool in order to simplify the synthesis of useful nucleotide sugars. In the context of significant promiscuity displayed by a number of glycosyltransferases and the availability of appropriate aglycones, the UDPG-PPase-catalyzed production of activated sugar donor libraries is a particularly promising approach toward the generation of a diverse library of glycosylated structures. With a *Salmonella* sugar nucleotidyltransferase, a number of engineered variants had to be obtained in order to have a wider acceptance of substrates.²³ At the same time, approximately 350–25 000-fold higher enzyme units, in comparison to those of the *P. furiosus* UDPG-PPase, were used to get a reasonable turnover and thereby an increased product yield.^{1,30} A slight increase of inorganic pyrophosphorylase and UDPG-PPase should allow >99% conversion of a wide range of natural and non-natural substrates by the *P. furiosus* enzyme. Future work with the archaeal enzyme will assess its tolerance for substrates that include, specifically, reactive groups for chemoselective ligation reactions or other tags to allow for the downstream

diversification or labeling of natural products and glycosylated proteins using sugar nucleotide reactions.^{29,30} The catalytic efficiency and thermostability of this enzyme also make it potentially attractive for industrial applications in the biochemical synthesis of various sugar nucleotides.

Ambiguous enzyme activities may well reside in the genomes of organisms as a result of either the partial retention of an earlier optimized catalytic function or the redundancy of molecular structures in the many modern metabolic pathways.³¹ Whether the broader substrate acceptance of the archaeal sugar nucleotidyltransferase reported here is a general characteristic of this class of life, just characteristic of enzymes from smaller genomes, or a special case requires further investigation. However, the first substrate specificity studies of an archaeal enzyme that activates carbohydrate substrates show the value of more extensive probing of archaeal genomes to expand the range of chemoenzymatic synthesis strategies.

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Supporting Information Available: Full experimental details and additional data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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