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A thermostable promiscuous glucose-1-phosphate uridyltransferase from *Helicobacter pylori* for the synthesis of nucleotide sugars

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Abstract

UDP-α-D-glucose pyrophosphorylase (α-D-glucose-1-phosphate uridyltransferase) (UGPase) catalyzes the formation of activated UDP-glucose from glucose-1-phosphate and UTP. Herein we report the cloning and characterization of a thermostable UGPase from a mesophilic, Gram-negative pathogen *Helicobacter pylori*. An electrospray ionization mass spectrometry (ESI-MS)-based assay demonstrates that the recombinant enzyme absolutely requires divalent cation for its activity, reaching a maximum in the presence of 3 mM Mg^{2+}. The optimum activity of the enzyme was around pH 8–9.5 in Tris–HCl buffer and at 37–70 °C; maximum activity was observed at pH 8.5 and 60 °C. Apart from its natural substrate, glucose-1-phosphate, the enzyme also accepts mannose-, galactose-, and glucosamine-1-phosphates. The apparent Michaelis constants of the purified enzyme for glucose-1-phosphate with UTP and dTTP and for mannose-1-phosphate with UTP are 15 ± 2, 32 ± 4 and 77 ± 9 μM, respectively, with the corresponding turnover numbers 5.4, 5.4 and 1.2 min^{-1}, respectively. An initial velocity study of the forward reaction of the enzyme indicates an ordered bi–bi catalytic mechanism. Analysis of the genomes of other organisms that grow at 37 °C predicts many more such thermostable biocatalysts.

Keywords: *Helicobacter pylori*; Nucleotidyltransferase; Thermostability; Mesophile; Charged amino acids

1. Introduction

Sugar nucleotidyltransferases catalyze the synthesis of activated sugar donors for transfer by a range of glycosyltransferases. One ubiquitous form of this class of enzymes, UDP-α-D-glucose pyrophosphorylase (UTP-α-D-glucose-1-phosphate uridyltransferase, EC 2.7.7.9) (UGPase), is central to primary metabolism and is widely distributed in all forms of life. UGPase catalyzes the condensation of uridine triphosphate (UTP) and α-D-glucose-1-phosphate (Glc1P) to form UDP-glucose with the concomitant release of pyrophosphate (Fig. 1). UDP-glucose synthesized by UGPase has a number of vital cellular functions, including the synthesis of glycogen, the synthesis of the carbohydrate moiety of glycolipids, glycoproteins, and proteoglycans, the entry of galactose into glycolysis and the synthesis of UDP-glucuronic acid [1–6]. UGPase is an essential protein for adhesion and virulence in various Gram-negative bacteria as it is required for the biosynthesis of capsular polysaccharides and lipopolysaccharide core biosynthesis [7]. The GalU gene, encoding UGPase, of *Pseudomonas aeruginosa* is required for corneal infection and efficient systemic spread following pneumonia [8]. Hence, UGPase has also been considered as an appropriate target for the development of new antibiotics [9]. Recently, a crystal of a putative UGPase from *Helicobacter pylori* has been reported [10]. *H. pylori*, a micro-aerophilic, Gram-negative, slow growing, spiral-shaped and flagellated organism, is probably the most common chronic bacterial infection of humans, present in almost half of the world population [11]. Although the UGPase of the bacterium could be a potential drug target, surprisingly no biochemical and kinetic data is available in which to interpret any data that might arise from structural analysis of protein crystals.

There have been a number of studies of UGPases from different organisms [12–16], but little biochemical data has been reported on mesophile protein stability and activity versus temperature. Thermostable enzymes could be advantageous over their mesophilic counterparts in industrial applications as these biocatalyst are stable at harsh environmental con-

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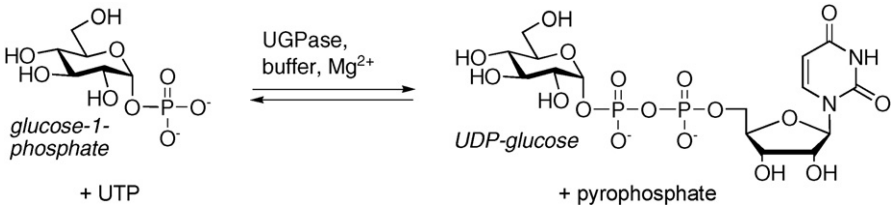

Fig. 1. Reaction scheme for the sugar nucleotidyltransferase (UGPase) that couples glucose-1-phosphate (Glc1P) and uridine triphosphate (UTP) to form activated UDP-glucose as a Leloir pathway glycosyl donor.

ditions and limit unwanted microbial growth in the reaction vessels [17]. Thermostable proteins can most likely be found in those unusual organisms whose optimal growth temperatures are over 60 °C. Unfortunately, these proteins are rarely very active at the lower temperatures required for many biomedical and synthetic purposes or applications requiring additional enzymes [18–20]. Thermostable enzymes potentially appealing synthetic tools as we have shown in our previous studies that the hyperthermostable sugar nucleotidyltransferases from *Pyrococcus furiosus* are promiscuous in substrate recognition [21,22]. We also reported a moderately thermostable sialic acid activating enzyme from *Clostridium thermocellum* that showed significant substrate ambiguity [23]. Thermostable enzymes also active at moderate temperatures could circumvent the problems associated with standard mesophilic or hyperthermophilic enzymes.

The recent comparative analysis of codon usage from 28 complete genomes suggested that the ratio of lysine (K) and glutamic acid (E) to histidine (H) and glutamine (Q) serves as a marker of the optimal growth temperature of an organism [24,25]. The ratio for mesophiles was less than 2.5 whereas for hyperthermophiles the ratio was above 4.5. For instance, the hyperthermophile *P. furiosus* shows a significant bias toward use of lysine and glutamic acid. Two sugar nucleotidyltransferase from this hyperthermophile have recently been studied and found to have optimal enzyme activities over 80 °C [21,22]. During this study, we analyzed the (E + K)/(H + Q) of *H. pylori* UGPase and found that the ratio of this protein is 3.2, which implies thermostability, although the ratio of the whole proteome of this organism is only 2.7 (Table 4) and the optimal growth temperature is 37 °C. We envisioned that this ratio might be a marker to identify thermostable proteins from mesophilic organisms. In order to test the biochemical and kinetic properties of a potential therapeutic target gene product and to test the predictive power of the (E + K)/(Q + H) ratio in our search for a thermostable nucleotidyltransferase for the biosynthesis of activated sugars for subsequent coupling with glycosyltransferases, we have chosen to produce the *GalU* gene (locus tag HP0646) from *H. pylori*.

2. Materials and methods

2.1. Materials

Enzymes and reagents used for the molecular biology procedures, DNA ladders, deoxynucleotide triphosphates (dNTPs), were purchased from Promega (Madison, WI) or New England Biolabs (Beverly, MA). Oligonucleotides for DNA amplification were synthesized by SIGMA Genosys (Woodland, TX). Thermostable inorganic pyrophosphatase (IPP) from *Thermococcus litoralis* (EC 3.6.1.1, M0296S) was purchased from New England Biolabs as a 2000 U/mL 50% glycerol solution in Tris–HCl buffer (pH 8.0). Isopropylthiogalactoside (IPTG) was obtained from Labscientific, Livingston, NJ. Protein molecular weight standards were obtained from BioRad (Hercules, CA). QIAquick gel extraction kit was obtained from Qiagen (Valencia, CA) and ZeroBlunt PCR cloning kit was purchased from Invitrogen (Carlsbad, CA). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise stated.

2.2. Bacterial strains and growth conditions

Genomic DNA of *H. pylori* (ATCC number 700392D-5), obtained from the American Type Culture Collection (Manassas, VA), was used as the source for the cloning experiments described herein. Oneshot Top10 competent cells (Invitrogen, Carlsbad, CA) and *E. coli* XL-10Blue (Stratagene, La Jolla, CA), and PCR-Blunt vector (Invitrogen, Carlsbad, CA) were used for direct cloning of PCR products. *E. coli* strain BL21 (DE3) (Stratagene, La Jolla, CA) was used in combination with the T7 expression system (pET21a vector; Novagen, Madison, WI) for expression of UGPase gene. *E. coli* cells were grown on Luria–Bertani (LB, Sigma, St. Louis, MO) medium at 37 °C on an incubator shaker at 225 rpm. When required, antibiotics were added at the following concentrations to make the selective media: carbenicillin 50 μg/mL, kanamycin 50 μg/mL, chloramphenicol 25 μg/mL.

2.3. General methods

DNA techniques standard procedures, including plasmid DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, DNA ligation and transformation of *E. coli*, were performed by conventional methods [26]. The PCR was carried out in an Eppendorf Mastercycler gradient thermocycler (Eppendorf Scientific Inc., Westbury, NY). Protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Tris–HCl 10–20% gradients, Bio-Rad Laboratories, Hercules, CA). The gels were stained with Coomassie brilliant blue. Protein concentrations were determined with the Bio-Rad protein assay kit according to the method of Bradford [27], using bovine serum albumin as the standard.

2.4. PCR amplification, cloning, expression, and purification of enzyme

Genomic DNA of *H. pylori* (ATCC number 700392D-5) was amplified by PCR synthesis using two oligonucleotide primers. The primers were designed in order to construct the UGPase expression plasmid. The forward primer, 5′-AAAC**CATATG**ATTAAAAAATGCCTTTTTCCTGC-3′, contains an NdeI restriction site (in bold) and the reverse primer, 5′-AAA**CTCGAG**TTATAAGCGTTTTTTATAATAAGCG-3′, contains an XhoI restriction site (in bold) were synthesized from the putative UGPase gene of *H. pylori*. The amplification reaction mixture contained standard *Pfu* DNA polymerase buffer, 375 μM of dNTPs, 3 ng of each primer, 4 ng of total genomic DNA and 2.5 units of *Pfu* DNA polymerase. The cycling parameters of 94 °C for 2 min 40 s followed by 30 cycles of 94 °C for 30 s, 56 °C for 45 s and 72 °C for 2 min 15 s, with a final elongation step of 72 °C for 15 min. The amplified DNA, after agarose gel electrophoresis (1%) was purified using QIAquick Gel Extraction kit and subcloned into a ZeroBlunt vector using ZeroBlunt PCR cloning kit, and was subsequently transformed into Oneshot Top10 and *E. coli* XL10 competent cells to check the correct insert. The resulting construct was then digested with NdeI and XhoI and was ligated to a pET21a vector containing a C-terminal polyhistidine tag sequence (Novagen, Madison, WI) and previously digested with the same restriction enzymes. Aliquots of the ligation mixture were transformed into competent *E. coli* BL21 (DE3) cells. Transformants were selected at 37 °C grown on LB medium supplemented with carbenicillin. The freshly transformed cells containing the desired plasmid were grown in LB until the optical density at 600 nm of the cell culture reached 0.6–0.8. Enzyme production was initiated by the addition of IPTG (1 mM) and the culture was incubated at 37 °C for the additional 4 h. Cells were harvested by centrifugation at 3500 × *g* for 10 min at 25 °C. The enzyme was purified essentially at 4 °C unless otherwise stated. The cells were disrupted by sonication (Fisher model 100 Sonic Dismembranator, Fisher Scientific, Pittsburgh, PA), after which unbroken cells and debris were removed by centrifugation (30 min at 10,000 × *g*). The cleared lysate was then purified by metal chelate chromatography by following the recommended procedures provided by Novagen. The purified protein was concentrated and dialyzed into the Tris–HCl buffer (50 mM, pH 8.5) using a Microcon Centrifugal Filter Device, MWCO 10 kDa (Millipore, Billerica, MA). The protein, obtained at 8 mg/L of culture, was analyzed by SDS-PAGE analysis.

2.5. Mass spectrometry-based enzyme assay

A Shimadzu LCMS 2010 quadruple mass spectrometer (Shimadzu Scientific Instruments, Columbia, MD) equipped with an electrospray ionization (ESI) source was used in this study. The enzyme activity essentially was monitored by our previously developed electrospray ionization mass spectrometry (ESI-MS)-based assay [28]. The enzymatic reaction was initiated by the addition of Glc1P (5 mM) to a reaction mixture of 50 μL containing Tris–HCl buffer (25 mM, pH 7.5), inorganic pyrophosphatase (IPP, 0.2 U), purified enzyme solution (10 μL), $MgCl_2$ (3 mM), and UTP (5 mM). Before adding Glc1P the reaction components were incubated at 37 °C for 5 min. Reactions were carried out at 37 °C for 10 min and 15 μL of the reaction mixture was quenched by the addition of 30 μL of 70% methanol/water containing AMP (3 mM) as an internal standard. The quenched solutions were centrifuged 10 min at 10,000 × *g* to precipitate the protein. Aliquots of the reaction mixtures were diluted with 135 μL of acetonitrile (acetonitrile/water/triethylamine (35/65/0.2). These samples (5 μL) were subjected to analysis via ESI-MS to determine the amount of UDP-glucose formed which was compared to a blank containing no enzyme. A control reaction was run in a separate tube containing all the reaction components except for the enzyme. One unit of the enzyme is defined as the amount of enzyme needed to produce 1 μmol of UDP-glucose per minute at the specified temperature and conditions.

2.6. Optimal activity determination of the enzyme

The optimal activity for the *H. pylori* UGPase was measured at 37 °C between pH 4.0 and 9.6 using 25 mM acetate, phosphate, and Tris–HCl buffer. The optimal temperature was measured at pH 8.5 between 0 and 90 °C. Relative acceptance by the enzyme of D-glucose-1-phosphate (Glc1P), D-galactose-1-phosphate (Gal1P), D-mannose-1-phosphate (Man1P), L-fucose-1-phosphate (Fuc1P), D-glucosamine-1-phosphate (GlcN1P), D-galactosamine-1-phosphate (GalN1P), and *N*-acetyl-D-glucosamine-1-phosphate (GlcNAc1P) and NTPs (UTP, dTTP, GTP, CTP and ATP), effects of divalent cations on catalytic conversion and the effects Mg^{2+} ion concentrations were determined in Tris–HCl buffer (25 mM, pH 8.5) and at 37 °C. Percent conversion was calculated as the amount of sugar phosphate remaining in the reaction after incubation at specified time divided by the amount of substrate used in the reaction multiplied by 100. Each reaction was run in duplicate and the mean percent conversion was used. In order to check non-enzymatic conversion of sugar-1-phosphate to nucleoside diphospho-sugars (NDP-sugars) the reaction mixture containing all the components except UGPase was also used as a control reaction for each set of reaction.

2.7. Kinetic analysis

The values for K_m and V_{max} were derived from enzymatic reactions run in triplicate and determined from the initial rates of NDP-sugar formation using ESI-MS. The enzymatic reaction was initiated by the addition of Glc1P (2–80 μM) in a reaction mixture containing Tris–HCl buffer (25 mM, pH 8.5), IPP (0.2 U), UGPase (2.5×10^{-3} U), and UTP/dTTP (400 μM) with a final volume of 50 μL. In order to obtain kinetic values for Man1P (5–200 μL) the enzyme concentration used was 5×10^{-3} U. Reactions were carried out at 37 °C for 5 min and 30 μL of reaction mixture was quenched by addition of 30 μL of 70% methanol/water containing AMP (6 μM) as an internal standard. ESI-MS-based analysis of the kinetic data was

obtained following the method described elsewhere [28]. In order to determine the kinetic mechanism of UGPase, the experiments were designed to provide data by varying the concentration of Glc1P at several fixed concentrations of UTP and also varying the concentration of UTP at several fixed concentration of Glc1P according to the approach described by Cleland [29].

2.8. Multiple sequence alignment

A multiple sequence alignment was performed using BLAST (www.ncbi.nih.gov). Amino acid sequences and ratio of (E + K)/(H + Q) were analyzed using the DNA strider version 1.2.

3. Results and discussion

3.1. Cloning and purification of UGPase

The open reading frame predicted to encode the *H. pylori* UGPase (822 base pairs) was PCR amplified and cloned into a ZeroBlunt vector. In order to identify the activity of the cloned gene, it was expressed in a pET21a vector, which was then transformed into the expression host *E. coli* BL21 (DE3). Cell free extracts of *E. coli* BL21 (DE3) harboring the desired plasmid were purified to apparent homogeneity according to the methods described above. The activity of both the crude extract and purified enzyme was determined at 37 °C using Glc1P and UTP as substrates with inorganic pyrophosphatase added to degrade the released pyrophosphate, thereby making the reaction unidirectional. Formation of UDP-glucose was monitored using an ESI-MS-based assay [28] and confirmed the putative chemical function of the *H. pylori* protein. SDS-PAGE analysis of the purified enzyme showed an apparent molecular weight of 35,500 (Fig. 2) that is higher than the calculated molecular mass of 32,000. In a previous study, it has been reported that the amino acid composition of a protein, particularly higher numbers of acidic amino acids, might cause inefficient binding with SDS, thereby lowering the mobility through SDS-PAGE [30]. The *H. pylori* UGPase contains 38 acidic amino acids—2 amino acids more than of positively charged residues—that might alter the protein's reptation through the gel.

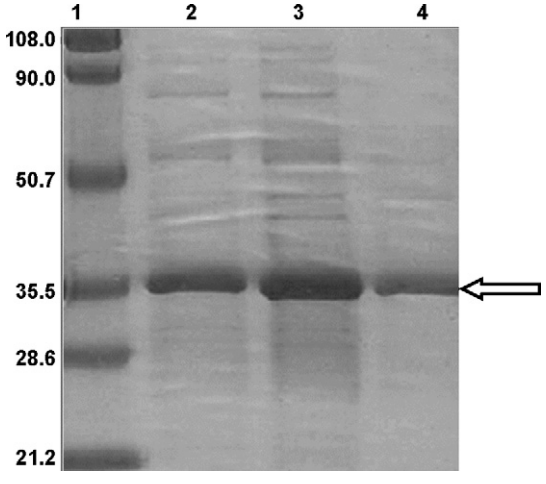

Fig. 2. SDS-PAGE after Coomassie staining of the purification process for the 6x-*His* tagged *H. pylori* UGPase expressed in *E. coli* BL21 (DE3) using a Ni-NTA spin column. Lane 1, molecular mass standard proteins in kDa (Biorad, Hercules, CA): phosphorylase *b* (108), bovine serum albumin (90), ovalbumin (50.7), carbonic anhydrase (35.5), soybean trypsin inhibitor (28.6), lysozyme (21.2); lane 2, cell pellet; lane 3, soluble extract; lane 4, elution. An arrow indicates purified UGPase.

3.2. Reaction characteristics

The enzyme exhibited activity at a broad pH range of 4–9.5 with a maximum around pH 8–9.5 in Tris–HCl buffer (Fig. 3a). 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 cation for activity as established for other nucleotidyltransferases [12–16,21,22]. Magnesium, zinc, copper, calcium and manganese 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; enzyme activity did not decrease extensively even at higher concentrations (data not shown). Due to the lack of structural data of any UGPase, the specific role played by Mg^{2+} in catalysis is still unclear. A recent report of a nucleotidyltransferase suggested that Mg^{2+} plays a structural role in organizing the substrate binding region of the specific enzyme around itself to fix the nucleotide triphosphate at an optimal position for catalysis [31].

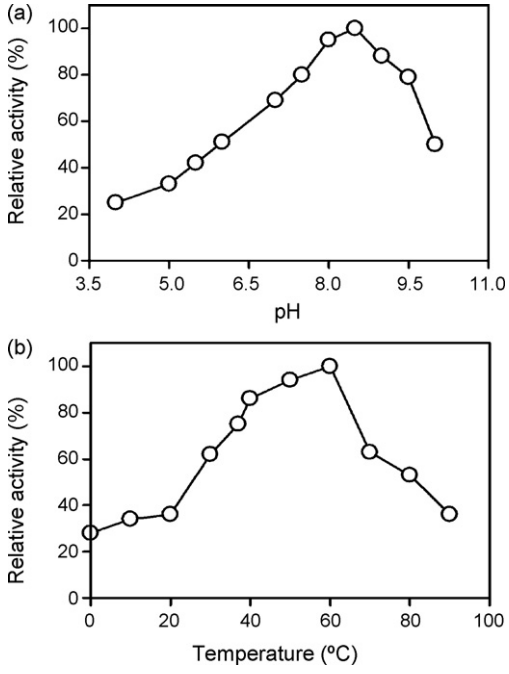

Fig. 3. Effects of pH (a), and temperature (b) on the activity of *H. pylori* UGPase. Activity of the purified enzyme (0.05 U) was checked in different buffers with a range of pHs at 37 °C for 10 min containing all of the reaction components. To check activity at various temperatures, the enzyme (0. 05 U) was incubated with standard assay components for 10 min. The UDP-glucose formed was analyzed by an ESI-MS-based assay and expressed as the percentage of maximum activity.

Table 1
Effects of divalent cations on enzymatic activity of the UGPase from *H. pylori*

Divalent cation[a]	Relative activity (%)
Mg^{2+}	100
Ca^{2+}	78
Co^{2+}	64
Cu^{2+}	85
Mn^{2+}	74
Zn^{2+}	92
None	0

[a] The purified enzyme (0.05 U) was incubated with standard assay mixtures at pH 8.5 with the addition of various metal ions at 3 mM (chloride form) and incubated for 10 min at 37 °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 $MgCl_2$. "None" indicates that the reaction was carried out in the absence of any divalent cations. Each value was from the mean of two independent data points.

Next, the activity of the enzyme was tested in the presence of the reaction components at various temperatures. As shown in Fig. 3b, unlike the reported activities of homologous UGPases from its mesophilic counterparts, the *H. pylori* enzyme shows an unusual activity profile at higher temperature. To the best of our knowledge, this *H. pylori* UGPase is the first sugar nucleotidyltransferase from any mesophilic organism shown to have maximum activity above 50 °C with maintenance of activity up to 90 °C. The origins of thermostability in proteins are unclear. Functionally, identical proteins from hyperthermophiles that grow above 80 °C and proteins from mesophiles share the same catalytic mechanism and overall structures [32–35]. Stability can be the result of other protectant molecules or require special chaperones for proper folding. In the protein itself, the exclusion of thermolabile amino acids such as asparagine and glutamine, and an increased number of charged amino acids that contribute surface ionic interactions can confer increased temperature stability [36–42]. The simple amino acid ratio we used to select this protein as potentially thermostable points to the last method as the most likely source of the thermostability of this *H. pylori* enzyme.

3.3. Substrate specificity

Along with UTP, dTTP was found to serve as a nucleoside diphosphate donor in the presence of Glc1P. Like other bacterial and archaeal UGPases, the *H. pylori* enzyme shows discrimination against other nucleotide triphosphates. However, unlike other reported UGPases from bacterial sources, the enzyme displayed significant conversions of Man1P and even some conversion of GlcN1P and Gal1P (Table 2). Control experiments without UGPase showed no turnover of any of the sugar substrates tested. The purified fraction of the *E. coli* harboring the plasmid pET21a without the UGPase insert also showed no activity. The enzyme is relatively less specific to its sugar-1-phosphates than those of most other bacterial and eukaryotic enzymes. For comparison, low activity (less than 1% in compared to Glc1P) was observed against Gal1P and Man1P with UTP of the enzyme purified from human erythrocyte [43] and less than 5% activity was reported against Man1P and Gal1P

Table 2
Relative acceptance of sugar-1-phosphates by UGPase from *H. pylori*

Sugar-1-phosphate[a]	UTP[b] acceptance (%)	dTTP[b] acceptance (%)
Glucose-1-phosphate	100	77
Mannose-1-phosphate	63	37
Galactose-1-phosphate	26	7
Glucosamine-1-phosphate	32	9

[a] The purified enzyme (0.05 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 37 °C for 10 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.

of the enzyme purified from *Thermus caldophilus* [44]. UGPase from *E. coli* showed some tolerance of Man1P and Gal1P, but no activity was recorded for Man1P with the yeast enzyme [28]. Control experiments were run to ensure that the *H. pylori* protein was solely responsible for this substrate turnover. This substrate selectivity is particularly important in terms of the production of NDP-sugar analogs by an enzyme which is active at 37 °C and can maintain activity at higher temperature. This intrinsic catalytic competence of the enzyme with a range of sugar-1-phosphates might be due to its inherent stability at higher temperature. Our recent investigation of two hyperthermophilic sugar nucleotidyltransferases demonstrated that these enzymes are also unusually tolerant to their sugar substrates [21,22]. Interestingly, most recently several different bacterial thymidyl- rather than uridyltransferases—namely Cps2L, RmlA and RmlA3—were shown to have variable but promiscuous substrate specificity; however, no temperature profiles of protein activity have been reported yet to determine the thermostability of these proteins [45].

3.4. Kinetic properties

To determine the kinetic parameters and mechanism of the *H. pylori* UGPase reactions, the concentrations of Glc1P (2–80 μM) and Man1P (5–200 μM) were varied with a fixed concentration of UTP/dTTP (400 μM). The values for K_m and V_{max} were derived from enzymatic reactions run in triplicate and determined from the initial rates of NDP-sugar formation using ESI-MS. The standard enzymatic analysis, a Michaelis–Menten plot of the velocity versus Glc1P ion concentration, was determined using ESI-MS to yield values for the enzyme with Glc1P and UTP of $K_m = 15 \pm 2$ μM and $V_{max} = 3.0 \pm 0.2$ μM/min, which are comparable to values for the enzymes from *P. furiosus*, and *E. coli* (Table 3) [21,28]. To determine the mechanism of the 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 and those with UTP as the variable substrate are indicative of a sequential bi–bi mechanism in which nucleotide binds first as a substrate to the enzyme and is the last to be released (data not shown). Initial velocity study of the enzyme as a function of

Table 3
Comparison of kinetic parameters of UGPases from *H. pylori*, *E. coli* and *P. furiosus*

Substrates	*H. pylori*[a]		*E. coli*[b]		*P. furiosus*[c]	
	K_m (μM)	V_{max} (μM/min)	K_m (μM)	V_{max} (μM/min)	K_m (μM)	V_{max} (μM/min)
UTP-Glc1P	15 ± 2	3.0 ± 0.2	12 ± 2	1.2 ± 0.1	13 ± 1	2.8 ± 0.1
dTTP-Glc1P	32 ± 4	3.0 ± 0.2	13 ± 2	0.9 ± 0.1	19 ± 2	2.6 ± 0.1
UTP-Man1P	77 ± 9	1.00 ± 0.05	Not accepted[b]		15 ± 1	2.10 ± 0.04

[a] This study. Kinetic parameters were determined in 25 mM Tris buffer (pH 8.5) at 37 °C using 2.5×10^{-3} U of UGPase. Reported error bars represent the standard deviation, which was obtained from three independent experiments.
[b] Previous report, Ref. [28].
[c] Previous report, Ref. [21].

both substrates gives clear intersecting lines which is indicative of a mechanism with formation of a ternary enzyme–substrate complex before the release of either product. This type of reaction has been suggested as an S_N2 type as was reported in the structural analysis of a sugar nucleotidyltransferase [31] and observed in the homologous enzyme from *P. furiosus* [21].

3.5. Amino acid sequence alignment

Whole proteome analysis of the five mesophiles *H. pylori*, *P. aeruginosa*, *Vibrio cholerae*, *Neisseria meningitidis*, and *E. coli* found the ratio of the charged amino acid pairs to the uncharged pairs were all below three as expected for organisms that grow optimally around 37 °C (Table 4). On the other hand, the hyperthermophile *P. furiosus* showed a significant bias toward use of lysine and glutamic acid. As expected, amino acid analysis of the UGPases from the mesophiles mostly predicted proteins stable around 37 °C. The one exception was the protein from *H. pylori*; the (E + K)/(Q + H) ratio for the *H. pylori* UGPase is 3.2, which would predict thermostability for the enzyme. Pairwise alignment of the enzyme from *H. pylori* showed a high degree of residue identity among the other pathogenic organisms. Amino acid alignment shows 63, 59, 62, 60, 52, and 39% identity between *P. aeruginosa*, *V. cholerae*, *N. meningitidis* and *E. coli*, respectively. Amino acid alignment of the *H. pylori* enzyme with its hyperthermophilic counter part from *P. furiosus* showed a significant 43% pairwise identity (Fig. 4). The comparative analysis of codon usage from 28 complete genomes suggested that the ratio of lysine (K) and glutamic acid (E) to histidine (H) and glutamine (Q) serves as a marker of the optimal growth temperature of an organism [24,25]. More recently, the differences in amino acids of psychrophilic Archaea and hyperthermophiles [46] and a study of proteins related to transcription and replication in thermophiles [47] support the concept of higher proportions of charged amino acids in thermophiles as compared to mesophiles and hence the thermostability of such proteins. With the possibility of thermostable enzymes lurking in mesophile genomes and an interest in finding reagents for

Table 4
Relationship of the (E + K)/(Q + H) ratio for whole proteomes and for enzymes that form UDP-glucose with the optimum growth temperature of organisms

Organism	Optimum growth temperature (°C)	(E + K)/(Q + H) ratio for the whole proteome	(E + K)/(Q + H) ratio for the UGPase
H. pylori	37	2.7	3.2
N. meningitidis	35–37	2.1	2.9
P. aeruginosa	37	1.2	2.9
V. cholerae	37	1.7	2.9
E. coli	37	1.5	2.6
P. furiosus	100	4.8	4.8

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Hpy: 2   IKKCLFPAAGYGTRFLPITKTIPKEMLPIVDKPLIQYAVEEAMEAGCEVMAIVTGRNKRS 61
Pfu: 3   VRKAVIPAAGLGTRMLPLTKAQPKEMLPVVRKPTIQYVLEEAYEAGIREVLIITGKHKRA 62
Hpy: 62  LEDYFDTSYEIEHQIQGTNKENALKSIRNIIEKCCFSYVRQKQMKGLGHAILTGEALIGN 121
Pfu: 63  IEDHFD---RYEHEV----KNPHLDKLDKILDDINIYYARQRVQRGLGDAIKYAEAFVGD 115
Hpy: 122 EPFAVILADDLCISHDHPSVLKQMTSLYQKYQCSXXXXXXXXXXXXSKYGVIR-GEWLEE 180
Pfu: 116 EPFALLLGDTITL----PSCTAGIIESYEELKAPVIAVEEVQEEKISLYGVVGIGRYINE 171
Hpy: 181 GVYEIKDMVEKPNQEDAPSNLAVIGRYILTPDIFEILSETKPGKNNEIQITDALRTQAKR 240
Pfu: 172 RIFEINKLVEKPEIHEAPSNLAILGRYILTPEIFEYLEEVKPDKKGEIQLTDALELMVQN 231
Hpy: 241 -KRIIAYQFKGKRYDCGSVEGYIEAS 265
Pfu: 232 GKKIYGYVFKGRRYDIGNIFDWLRAN 257
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Fig. 4. Amino acid sequence alignment of *H. pylori* UGPase with the hyperthermostable *P. furiosu*s homologs. (Hpy) *H. pylori*; (Pfu) *P. furiosus*. The conserved residues are indicated as bold letters.

glycobiology, we checked genomes to see how many glycosyltransferases and glycosidases are predicted to be thermostable. Of the 20 putative glycosyltransferases [48,49] found in each of the genomes of three different organisms that grow optimally between 30 and 45 °C, 25% of the *H. pylori* sequences, 20% of the *Bacillus cereus* sequences, and a surprising 75% of the *Campylobacter jejuni* sequences have amino acid ratios above 3.0 indicative of thermostability. Of the glycosidases, 2 of 5 *B. cereus* and 2 of 3 *C. jejuni* sequences are predicted to code thermostable proteins.

4. Conclusion

Biochemical studies of a UGPase reported in this study have revealed a thermostable protein as predicted from its amino acid content that is also substrate tolerant. Despite the lack of prior biochemical characterization, the protein has been crystallized [10]; with luck a structure that could shed clues on the protein's thermostability should be forthcoming. The unusual thermostability and the tolerance of a number of sugar-1-phosphates indicate that the enzyme could be an important tool for the practical biosyntheses of nucleotide sugars. The relative increase in specific charged residues could serve as a predictor of thermostability for mesophilic proteins and points to the need for such temperature/activity studies even when proteins from mesophilic sources are reported. In fact, the amino acid sequence analysis of glycosidases and glycosyltransferases from different mesophilic organisms indicates that there might be many more thermostable enzymes than is appreciated from organisms' growth temperatures alone.

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