Phosphomannose isomerase/GDP-mannose pyrophosphorylase from *Pyrococcus furiosus*: a thermostable biocatalyst for the synthesis of guanidinediphosphate-activated and mannose-containing sugar nucleotides†

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Herein we present an analysis of the chemical function of a recombinant bifunctional phosphomannose isomerase/GDP-mannose pyrophosphorylase (manC) from *Pyrococcus furiosus* DSM 3638 and its use in the synthesis of guanidinediphospho-hexoses and a range of nucleotidediphospho-mannoses. This enzyme is unusually promiscuous in both its nucleotide triphosphate (NTP) and sugar-1-phosphate acceptance. It accepts all five naturally occurring NTPs (ATP, CTP, GTP, dTTP and UTP) and a range of sugar-1-phosphates (glucose-, mannose-, galactose-, glucosamine-, *N*-acetylglucosamine- and fucose-1-phosphate). A truncated GDP-mannose pyrophosphorylase domain of the whole length enzyme showed almost 100-fold less sugar nucleotidyltransferase activity with only GTP and mannose 1-phosphate as substrates. The temperature stability and inherently broad substrate tolerance of this archaeal enzyme make it an effective reagent for the rapid chemoenzymatic synthesis of a range of natural and unnatural sugar nucleotides that are challenging to make by chemical means alone.

Introduction

Most monosaccharides are activated as sugar nucleotides before their incorporation into glycoconjugates and cell wall polysaccharides by glycosyltransferases. Many of these glycosyltransferases employ uridine- or deoxythymidine-diphosphosugars, and therefore most biosynthetic efforts have focused on accessing these two classes of sugar nucleotide.[1] However, there are many mannose-transferring enzymes that form cell wall components such as lipoarabinomannan and other key oligosaccharides utilizing guanidinediphosphate (GDP)-activated sugar nucleotides.[2] The enzymes that make these GDP-mannose building blocks are necessary for the survival of eukaryotes.[3] Although not vital for pathogens such as *Leishmania*, these enzymes are required for their virulence and are therefore a proposed target for therapeutics.[4] Detailed studies of these pathways will require both activated mannoses and analogous GDP-sugars. For example, recently chemical synthesis has afforded fluorescent and azido-substituted GDP-mannose analogs for probing mannosyltransferase activities.[5,9m]

A key enzyme for the synthesis of GDP-mannose is GDP-mannose pyrophosphorylase (GMP), which catalyzes the synthesis of GDP-mannose from mannose-1-phosphate and GTP with the release of pyrophosphate (Scheme 1). The activated mannose is the precursor for mannosylation of *N*-linked glycoproteins and glycosylphosphoinositol anchors[6] and is an essential metabolic intermediate for the biosynthesis of other GDP-sugars such as GDP-fucose, GDP-colitose, GDP-talose, GDP-perosamine and GDP-D-rhamnose.[7] In addition to GDP-mannose, NDP-mannoses, although rarely found in nature, have also been used as substrates for structural studies of glycosyltransferases.[8]

Several mesophilic bacterial and eukaryotic GDP-mannose pyrophosphorylases have been reported.[6,9] A recombinant version of the bacterial enzyme from *Salmonella enterica* has been shown to be effective in the enzymatic synthesis of natural GDP-mannose[9j–l] as well as azido-substituted versions of the sugar nucleotide.[9m] One crenarchaeal GDP-mannose synthesizing gene from the hyperthermophilic *Sulfolobus solfataricus* has been identified, but the resulting protein produced only GDP-mannose and not GDP-glucose.[10] This inflexible crenarchaeal protein is actually more closely related in sequence to the corresponding protein from *Arabidopsis thaliana* than to that of the euryarchaea *Pyrococcus.*

Only a few of the type II phosphomannose isomerases (PMIs), bifunctional prokaryotic proteins with both PMI (E.C. 5.3.1.8) and GMP (E.C.2.7.7.22) activities, however, have been reported. Enzymes from several bacterial species that include *Burkholderia cenocepacia* $BceA_J$,[9h] *Pseudomonas aeruginosa* AlgA[9g], *Xanthomans campestris* XanB,[9i] *Gluconobacter xylinum* AceF,[9f] and *Helicobacter pylori* HP0043[9b] have been studied so far. The PMI motif of the bifunctional PMI-GMP also has been proposed as a target for the development of inhibitors.[9k] The PMI motif of PMI-GMP catalyses the reversible conversion of D-fructose-6-phosphate into D-mannose-6-phosphate, which is then isomerized to mannose-1-phosphate (Man1P) by a phosphomannomutase; the GMP motif then converts the Man1P into GDP-mannose in the presence of GTP and a divalent cation (Scheme 1).

In contrast to bacterial enzymes, surprisingly no functional PMI-GMP activity has been reported from archaea, the third branch of life. A few bacterial and archaeal nucleotidyl transferases have been shown to be highly flexible with regard to their nucleoside triphosphate substrates.[9m,11] For instance, ST0452 of the *Sulfolobus tokodaii* accepts all four major deoxyribonucleoside

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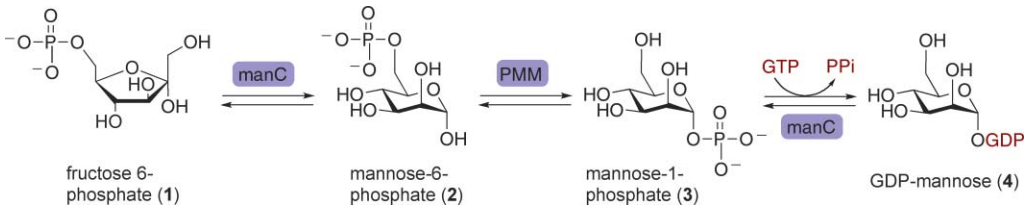


Scheme 1 Synthesis of GDP-mannose from fructose-6-phosphate by a bifunctional phosphomannose isomerase/GDP-mannose pyrophosphorylase (manC) and phosphomannomutase (PMM).

triphosphates (dTTP, dATP, dCTP and dGTP) and UTP as the only ribonucleoside triphosphate in the presence of glucose-1-phosphate (Glc1P). In addition to Glc1P, ST0452 also accepts *N*-acetylglucosamine-1-phosphate (GlcNAc1P) in the presence of UTP and dTTP.[11a] Substrate flexibility studies of three bacterial thymidyltransferases—Cps2L from *Streptococcus pneumoniae* R6, RmlA from *S. mutans* and RmlA3 from *Aneurinibacillus thermoaerophilus* DSM 10155—show them to accept NTPs (ATP, CTP, GTP, dTTP and UTP) in the presence of G1c1P to thus produce NDP-glucoses.[11b] An engineered version of Cps2L from *S. pneumoniae*, designated as the Q24S mutant, was shown to be active in the synthesis of various NDP-sugars including NDP-mannoses except CDP-mannose.[11c] The GMP (manC) from *E. coli* was reported to activate M1P to form GDP-, ADP-, CDP- and UDP-mannose to varying degrees but does not accept dTTP. In addition, this enzyme can synthesize GDP-glucose and GDP-2-deoxyglucose.[9a] However, in addition to their thermo-sensitivity, none of the enzymes were reported to be highly effective in the synthesis of a range of GDP-sugars and NDP-mannoses.

The thermal vent archaea *P. furiosus* DSM 3638 has been the source of several surprisingly flexible carbohydrate-active enzymes that are stable at elevated temperatures.[1b,1e,12] This genome contains a gene (PF0589) annotated as a bifunctional phosphomannose isomerase/GDP-mannose pyrophosphorylase (manC) that catalyzes the first and third step of GDP-mannose synthesis starting from fructose-6-phosphate (Scheme 1), but its biochemical function has not yet been verified. Interestingly, the recent biochemical characterization of the phosphomannomutase from *P. horikoshii OT3* uncovered its ability to act on not only mannose-6-phosphate (**2**), but also glucose-6-phosphate.[13] This study further stimulated our curiosity as to the biochemical function and potential utility of the manC gene. We report herein the identification of a bifunctional PMI-GMP from the hyperthermophile *P. furiosus* and its utility in the syntheses of a number of activated mannoses and GDP-sugars.

Results and discussion

A putative 463 amino acid residue of *P. furiosus* gene product (PF0589) was annotated as mannose-6-phosphate isomerase/mannose-1-phosphate guanyltransferase (manC) enzyme. BLAST search revealed that manC has a nucleotidyltransferase motif at the *N*-terminal site and a mannose-6-phosphate isomerase (phosphomannose isomerase) motif at the *C*-terminal site starting from residues 2 to 275 and 291 to 456, respectively. The C-terminal domain has similarity with a type II phosphomannose isomerase which is generally a bifunctional enzyme. The putative enzyme shares 44 and 47% identity with the *E. coli* and *Pseudomonas aeruginosa* enzymes respectively (see ESI†). In our previous studies we have reported that the hyperthermostable archaeal enzymes possess broad substrate specificities with the potential of expanding the synthesis of various natural and non-natural sugar nucleotides for biotechnological applications.[1e] However, those enzymes are not suitable for the synthesis of GDP-activated sugars. To explore the value of the euryarchaea *P. furiosus* genome as a reagent source for GDP-sugar syntheses, the 1286 base pair manC gene was cloned and expressed in *Escherichia coli* BL21 Codon Plus® (DE3)-RIPL strain. An approximately 53 kDa protein, consistent with the molecular mass calculated from the nucleotide sequence, was purified by simple Ni-affinity column and was analyzed by SDS-PAGE (see ESI†). The resulting purified protein did indeed have phosphomannose isomerase activity as confirmed by a coupled assay that measures the formation of NADPH at 340 nm.[9b] In addition to isomerase activity, the protein could also produce GDP-mannose from mannose-1-phosphate and GTP in the presence of inorganic pyrophosphatase (Scheme 1) as monitored by an electrospray ionization mass spectrometry (ESI-MS) assay,[14] indicating that the manC is a bifunctional enzyme. Although heat treatment should have denatured all native proteins, in order to verify that the Ni-affinity purified protein does not contain enzyme activity deriving from the *E. coli* itself, 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 53 kDa was detected by SDS-PAGE analysis of the crude extract after passing through the Ni-affinity column and no GDP-mannose pyrophosphorylase activity was detected at 80 °C. No product formation was observed in the absence of the enzyme, sugar phosphates, Mg^{2+} or NTP.

Since we hoped to find an effective, hyperthermostable version of a GDP-sugar synthesizing biocatalyst, we focussed further studies on the sugar nucleotidyltransferase activity of the enzyme. Biochemical and kinetic properties of the enzyme were determined by monitoring the formation of GDP-mannose from mannose-1-phosphate and GTP. Because the recombinant protein was originally cloned from a hyperthermophilic archaeon, *P. furiosus*, which grows optimally at 100 °C, the temperature dependence of the enzymatic activity was analyzed between 0 and 100 °C. Maximum activity of the purified manC was observed at 80 °C; however, little loss of activity was detected at 99 °C even after 24 h of incubation at this elevated temperature (Fig. 1A). The enzyme remains stable for over 300 min without losing its activity when stored at 80 °C. The recombinant protein exhibited relatively high activity around pH 6.0 and 9.5, with a maximum in the range of pH 7.0–8.0 in phosphate buffer (Fig. 1B).

It is well established that sugar nucleotidyl transferases require divalent cations for their activity. To check the dependency of the

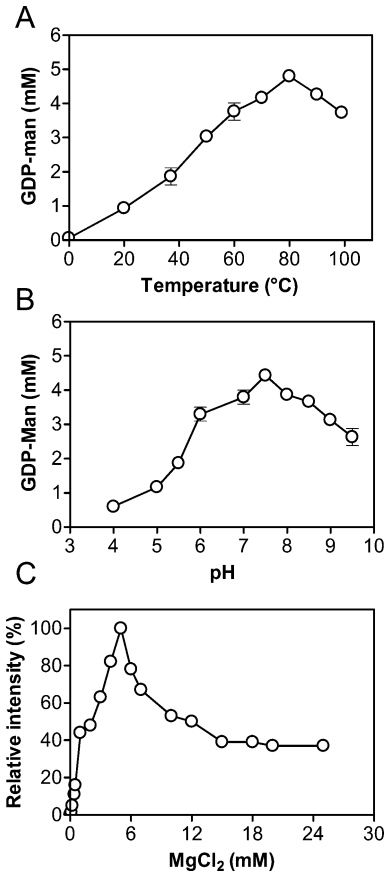


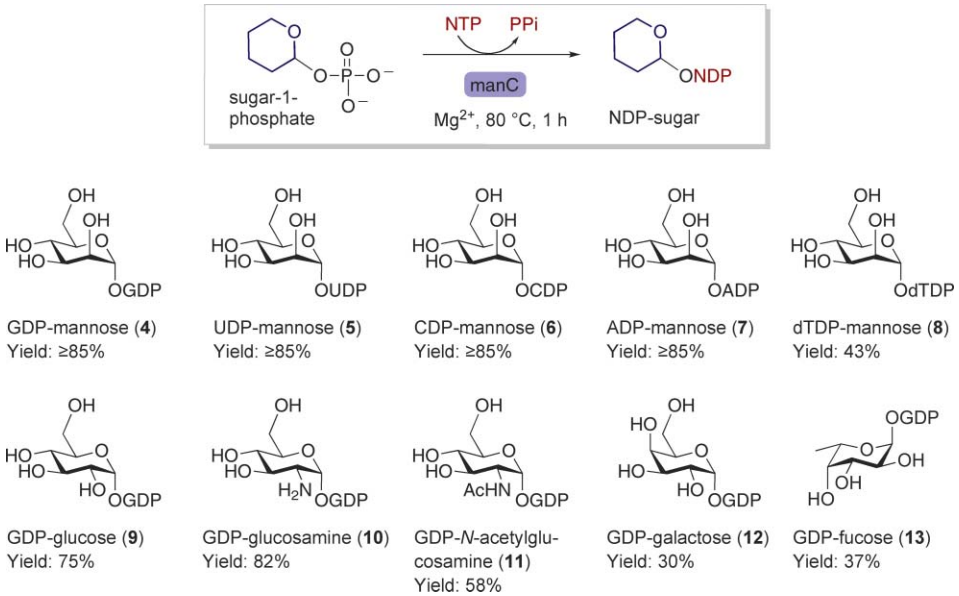
Fig. 1 Effect of temperature (A), pH (B) and Mg^{2+} ion concentration (C) on the activity of the bifunctional manC. The enzyme (0.02 U) was incubated with standard assay components at various temperatures for 10 min to determine the best temperature for activity. To check the pH profile the enzyme (0.02 U) was incubated with standard assay components at 80 °C for 10 min. The GDP-mannose formed was analyzed by an ESI-MS based assay and expressed as the amount of GDP-mannose formed after the reaction time. The optimum Mg^{2+} ion concentration was found by monitoring the formation of GDP-mannose in the presence of various concentrations of the divalent cation and the results expressed as percentages of maximum activity.

enzyme on cations, various concentrations of divalent cations were investigated in the forward reaction. Like other sugar nucleotidyltransferases the GDP-mannose pyrophosphorylase activity of the bifunctional enzyme is absolutely dependent on divalent cations with Mg^{2+} at 5 mM serving the best; however, the enzyme activity remains high between 2–15 mM concentrations (Fig. 1C). The order of effectiveness of the metal ions on the enzyme was shown to be $Mg^{2+} > Cu^{2+} > Zn^{2+} > Co^{2+} > Ca^{2+}$ and Mn^{2+}.

Substrate promiscuity of a number of previously reported *P. furiosus* enzymes from our group prompted us to verify the specificity of manC with different substrates. Consequently the substrate specificity of the enzyme was tested in the presence of mannose-1-phosphate and various NTPs. The enzyme not only accepts its natural substrate GTP, but also accepts ATP, CTP, UTP and dTTP, which was confirmed by monitoring the formation of ADP-man, CDP-man, UDP-man and dTDP-man using ESI-MS. With the exception of dTDP-mannose, greater than 80% yield was recorded in the syntheses of the four NDP-mannoses. These results indicate that the sugar nucleotidyl transferase activity of the bifunctional manC is not specific to GTP; rather it accepts all five major nucleoside triphosphates (Scheme 2). Next, the tolerance of the enzyme to various sugar-1-phosphates with GTP was tested. Relatively high activity was detected against glucose-, galactose-, glucosamine-, *N*-acetylglucosamine- and fucose-1-phosphate with the overall yield 30–85% (Scheme 2). In addition to the GDP-sugars and NDP-mannoses, the enzyme is also effective in the synthesis of NDP-glucoses and ADP-, CDP-, UDP-GlcN (see ESI†). Overall, the enzyme can be used for the synthesis of seventeen different nucleotide sugars from the commercially available sugar 1-phosphates and NTPs. Based on HPLC calculations, 85, 80, 70, and 45% yields were obtained when the reactions were carried out on a 50 mg scale with GTP and Man1P, Glc1P, GlcN1P and GlcNAc1P, respectively. Interestingly, the enzyme did not have to be affinity purified in order to convert the above mentioned sugar-1-phosphates to their corresponding nucleotide sugars; the supernatant after heat treatment of the recombinant *E. coli* extract worked as well as the purified biocatalyst. This euryarchaeal manC clearly can turn over a much greater range of substrates than the previously studied crenarchaeal enzyme or enzymes (wild type or recombinant) from mesophilic sources. For example, ATP, CTP and UTP were poor substrates for the *E. coli* manC and only glucose-1-phosphate and 2-deoxyglucose-1-phosphate could serve as alternate sugar-1-phosphate substrates in low yields.[9a] Heat treatment of the crude extract not only simplifies protein purification, but also eliminates the possibility of contaminating *E. coli* proteins being a source of alternate sugar nucleotidyltransferase activity. This finding indicates that the archaeal sugar nucleotidyltransferase is unusual in its ability to bind and turn over a range of nucleotide triphosphate substrates.

Since the enzyme was shown to accept all 5 NTPs tested, we were particularly interested in determining the effect of substrate concentration on the enzymatic activity. The reaction was performed with varying and fixed concentrations of Man1P and NTP respectively as described above. Michaelis-Menten plots of the velocity *versus* sugar-1-phosphate concentrations were determined for the *P. furiosus* manC. Nonlinear regression analysis of reactions run in triplicate using Man1P as the variable substrate in the presence of fixed concentrations of ATP, CTP, GTP, dTTP and UTP resulted in K_M values of 120 ± 8, 160 ± 20, 72 ± 7, 190 ± 20, and 150 ± 10 μM respectively (Table 1). As shown in Table 1, the K_M value obtained with Glc1P as the variable concentration in the presence of fixed concentration of GTP was comparable to that of Man1P. The results indicated that although the enzyme accepts all NTPs, GTP and Man1P are the most preferred substrates of the enzyme.

Because the enzyme has been shown to be highly effective in the synthesis of a number of GDP-sugars and NDP-sugars we decided to examine the role of the C-terminal domain in the overall activity and substrate specificity of the N-terminal nucleotidyltransferase domain. We created a truncated mutation of the GMP by PCR amplification of the nucleotidyltransferase domain gene and cloned and expressed it in *E. coli.* Substrate specificity study of the truncated GMP showed that the mutant accepts GTP and Man1P with about 100 fold lower GDP-man pyrophosphorylase activity when the reaction was carried out in the presence of Man1P and GTP (Table 1). However, unlike the full length enzyme the truncated enzyme did not accept any



Scheme 2 Synthesis of various sugar nucleotides. The conversion determined by ESI-MS was calculated as the difference between the amount of sugar phosphate remaining in the reaction and the initial amount, then divided by the initial amount of substrate used in the reaction.[1e]

Table 1 Kinetic parameters of *P. furiosus* manC assayed with varying concentrations of sugar-1-phosphate and fixed concentrations of NTPs

Substrate	K_M (μM)	V_{max} (μM/min/mg)	k_{cat}/K_M (μM^{-1} min^{-1})[a]
Man1P(**3**) (ATP)	120 ± 8	11 ± 1	11
3 (CTP)	160 ± 20	11 ± 1	8
3 (GTP)	72 ± 7	50 ± 2	90
	62 ± 9[b]	0.63 ± 0.03[b]	0.81[b]
3 (TTP)	190 ± 20	6 ± 1	4
3 (UTP)	150 ± 10	14 ± 1	11
Glc1P (GTP)	94 ± 7	14 ± 1	18

[a] k_{cat} was calculated based on the concentration of the purified enzyme. [b] indicates the kinetic parameters of the truncated enzyme.

other sugar-1P or NTPs. The temperature stability of the enzyme remains the same as that of the full length enzyme. Likewise, the mutant enzyme activity is absolutely dependent on divalent cations with Mg^{2+} at 5 mM being the best. The pH profile of the truncated enzyme remained the same as that of the wild type version. These results indicate that the *C*-terminal domain is crucial for the substrate promiscuity of the enzyme. The deletion of the *C*-terminal domain may have changed the tertiary structure of the enzyme; also, some key residues required for substrate promiscuity might be missing. A related study found a similar phenomenon; a thermostable truncated sugar-1-phosphate nucleotidyltransferase (RmlA) from *S. tokadaii* strain 7 was shown to have 23 times lower activity than that of the full length enzyme.[11a] This truncated mutant also was shown to be inactivated at temperatures higher than 60 °C.[11a]

Conclusions

In summary, we have identified the first hyperthermostable, bifunctional PMI-GMP from archaea which could be utilized for the one-pot syntheses of a range of GDP-activated sugars and NDP-mannoses. Although the general biochemical properties of this euryarchaeal enzyme are similar to those of previously identified prokaryotic and eukaryotic enzymes, the manC from *P. furiosus* has an unusual tolerance of high temperatures thereby facilitating purification and storage of the recombinant protein. More surprisingly, unlike previously reported enzymes that synthesize GDP-mannose, this euryarchaeal sugar nucleotidyltransferase can accept an unusually broad range of sugar phosphates and sugar nucleotide triphosphates with reasonably efficient turnover rates. This recombinant enzyme can provide various GDP-sugars and NDP-mannoses without any costly and laborious protein engineering approaches. The discovery of this stable, promiscuous enzyme's ability to provide analogs of GDP-mannose finally enables detailed structure/function studies and possibly inhibition of the pathways that build many of the oligosaccharide structures necessary for pathogen virulence.

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