Bifunctional phosphomannose isomerase/GDP-D-mannose pyrophosphorylase is the point of control for GDP-D-mannose biosynthesis in *Helicobacter pylori*

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Abstract In this report a recombinant bifunctional phosphomannose isomerase/GDP-D-mannose pyrophosphorylase from *Helicobacter pylori* has been studied. The enzyme catalyzes the first and third steps of GDP-D-mannose biosynthesis from D-fructose-6-phosphate. The first step, isomerization from D-fructose-6-phosphate to D-mannose-6-phosphate, is found to be rate-limiting in GDP-D-mannose biosynthesis due to feedback inhibition. The inhibition is of non-competitive (mixed) type. As the enzyme is found only in bacteria probably participating in capsular polysaccharide biosynthesis, it could be a specific therapeutic target against bacterial infection. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Phosphomannose isomerase/GDP-D-mannose pyrophosphorylase; GDP-D-mannose; Feedback inhibition; *Helicobacter pylori*

1. Introduction

Phosphomannose isomerase (PMI) catalyzes the interconversion of D-fructose-6-phosphate (F6P) and D-mannose-6-phosphate (M6P). It plays a critical role in the metabolism of D-mannose and supply of GDP-D-mannose, which is necessary for mannosylation of various structures such as lipopolysaccharides (LPS) and glycoproteins. In addition, GDP-D-mannose is a precursor for other activated sugar nucleotides including GDP-L-fucose, GDP-colitose, GDP-perosamine and GDP-D-rhamnose, which are also involved in the synthesis of various glycoconjugates. Proudfoot et al. [1] defined three types of PMI. Type I enzymes include all eukaryotic PMIs identified so far and the enzymes from *Salmonella typhimurium* [2] and *Escherichia coli* (ManA) [3]. The type II enzymes are bifunctional phosphomannose isomerase/GDP-D-mannose pyrophosphorylases (PMI/GMPs) found in bacteria such as *Pseudomonas aeruginosa* (AlgA) [4], *Xanthomonas campestris* (XanB) [5], and *S. typhimurium* [2,6]. No overall homology is found between type I and type II enzymes except a very short PMI motif [7]. The type III group has only one protein from *Rhizobium meliloti* [8].

The type I PMIs have been well studied including those from *X. campestris* [9], *Candida albicans* [10] and fungal and mammalian sources [1,11]. The crystal structure of *C. albicans* PMI has been resolved at 1.7 Å resolution. The protein is a metalloenzyme requiring one atom of zinc that is bound to a pocket within the catalytic center [12]. The impact of type I PMIs on pathogenicity has been reported for *Cryptococcus neoformans* [13] and *Leishmania mexicana* [14]. Since the *pmi* gene in *Saccharomyces cerevisiae* [15] and *C. albicans* [10] is essential for the biosynthesis of the cell wall, PMI from the latter is currently being investigated as a target to combat fungal infections [12,16]. In contrast, the study of type II bifunctional PMI/GMPs has been quite limited. PMI/GMP has been detected in several bacteria including *P. aeruginosa* (AlgA) [4], *S. typhimurium* [2,6], *X. campestris* (XanB) [5] and *Acetobacter xylinum* (AceF) [17]. But up to now, the only PMI/GMP that has been carefully studied is the enzyme from *P. aeruginosa* (AlgA), which has been demonstrated to be composed of two separate domains, an N-terminal GMP domain and a C-terminal PMI domain [4,18]. It is essential for the synthesis and accumulation of alginate, an extracellular glycoconjugate entrapping the bacteria, which enables the pathogen to resist most antibiotics and evade host immunity. PMI/GMP catalyzes the first and third steps in the production of GDP-D-mannose from F6P, a glycolytic intermediate (Fig. 1). Since the enzyme stands at a branch point from the essential central pathway towards glycoconjugate synthesis, it had been expected that the enzyme is well regulated for metabolic flow control. Unexpectedly, the study on AlgA showed no sign of such regulation [4]. Here we will report for the first time how *Helicobacter pylori* PMI/GMP regulates GDP-D-mannose synthesis through feedback inhibition.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli DH5α [*lacZΔM15 hsdR recA*] and BL21(DE3) [F^- *ompT* $hsdS_B(r_B^-m_B^-)$ *gal dcm* (DE3)] were from Gibco-BRL and Novagen, respectively. *H. pylori* 26695 chromosomal DNA was purchased from ATCC (ATCC700392D) and plasmid DNA pET15b was bought from Novagen.

2.2. Chemicals and reagents

Restriction enzymes and T_4 DNA ligase were obtained from Promega. Vent DNA polymerase was purchased from New England Biolabs. GTP, GDP-D-mannose, GDP-L-fucose, D-fructose-6-phosphate

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Abbreviations: DTT, dithiothreitol; F6P, D-fructose-6-phosphate; GMP, GDP-D-mannose pyrophosphorylase; IPTG, isopropyl-D-thiogalactopyranoside; M1P, D-mannose-1-phosphate; M6P, D-mannose-6-phosphate; $NADP^+$, nicotinamide adenine dinucleotide phosphate; PMI, phosphomannose isomerase; PMI/GMP, bifunctional phosphomannose isomerase/GDP-D-mannose pyrophosphorylase; PMM, phosphomannomutase; Ppi, inorganic pyrophosphate

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(F6P), D-mannose-1-phosphate (M1P), D-mannose-6-phosphate (M6P), D-glucose-1,6-bisphosphate, nicotinamide adenine dinucleotide phosphate ($NADP^+$), inorganic pyrophosphate (Ppi) and pyrophosphatase, phosphomannose isomerase and D-glucose-6-phosphate dehydrogenase were products of Sigma. D-Glucose-6-phosphate isomerase was purchased from ICN. Other chemicals were of the highest grade from commercially available sources.

2.3. Overexpression and purification of H. pylori PMI/GMP and phosphomannomutase

The primers used for the amplification of the PMI/GMP gene were P1: 5′-GGGAATTCCATATGAAAATTAAAAATATC (*Nde*I), P2: 5′-GGTACGGATCCTTAGGCGTTTTGATTTTGTC (*Xho*I) and the primers for the phosphomannomutase (PMM) gene were P1: 5′-GGGAATTCCATATGGACATTAGCATTTTTAG (*Nde*I), P2: 5′-GGTACGGATCCTTAAAGTTTTTCAATAAATTG (*Bam*HI). PCR was performed in a total of 50 μl consisting of 20 mM Tris–HCl, pH 8.8, 10 mM KCl, 2 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 50 ng template DNA, 200 nM P1 and P2 each, 0.2 mM dNTPs each, and 0.5 U DNA polymerase. After heating at 96°C for 2 min, 25 cycles were carried out including denaturation at 96°C for 20 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min followed by a final elongation of 7 min. The DNA fragment obtained was inserted downstream of T_7 promoter in pET15b. The *E. coli* BL21 (DE3) harboring the recombinant plasmid was grown in LB medium until OD_{590} reached 0.8. The expression of the protein was induced by 0.1 mM IPTG at 30°C for 17 h. The bacteria were harvested and disrupted by sonication on ice. The lysate was cleared by centrifugation and loaded onto a Ni-NTA column (Qiagen). Further purification was carried out by FPLC using a HiLoad 16/60 Superdex 200 column (Pharmacia), equilibrated and eluted with 50 mM Tris–HCl buffer (pH 7.0) containing 0.2 mM dithiothreitol (DTT) and 100 mM NaCl. Protein concentration was analyzed by the Lowry method [19].

2.4. Enzyme activity analysis

Detection of PMI activity was performed at 25°C in 1 ml of 50 mM Tris–HCl buffer (pH 7.0) containing 2 mM Co^{2+}, 1 mM M6P, 1 mM $NADP^+$, 5 U phosphoglucose isomerase and 5 U phosphoglucose dehydrogenase. The reduction of $NADP^+$ was followed by monitoring UV absorption at 340 nm. The kinetic study of PMI was performed at varied concentrations of M6P in the presence or absence of GDP-D-mannose. The kinetic parameters were calculated by plotting $1/v$ versus 1/[S] (Lineweaver–Burk). The K'_i and K_i were obtained by plotting the reciprocals of apparent maximal velocities and K_m/V_{max} against the inhibitor concentration, respectively.

Analysis of GMP forward reaction was performed at 37°C for 1 min in 50 mM Tris–HCl buffer (pH 7.0) containing 0.2 mM DTT, 2 mM Mg^{2+}, 1 mM GTP and 1 mM M1P. Analysis of the reverse reaction was carried out at 37°C for 1 min in 50 mM Tris–HCl buffer (pH 8.0) containing 0.2 mM DTT, 2 mM Mg^{2+}, 1 mM GDP-D-mannose and 1 mM Ppi. To determine the kinetic parameters of GMP, varying concentrations of either M1P or GDP-D-mannose were used while the concentration of GTP or Ppi was kept at 1 mM, respectively.

PMM activity was analyzed at 25°C in 1 ml of 50 mM Tris–HCl buffer (pH 7.0) containing 1 mM Zn^{2+}, 10 μM glucose-1,6-diphosphate, 1 mM M1P, 1 mM $NADP^+$, 5 U phosphomannose isomerase, 5 U phosphoglucose isomerase and 5 U phosphoglucose dehydrogenase. Again $NADP^+$ reduction was followed by monitoring $OD_{340\,nm}$.

2.5. Determination of the rate-limiting step in GDP-D-mannose biosynthesis

M1P, M6P and F6P were used to synthesize GDP-D-mannose. The reaction was performed at 37°C in a reaction mixture containing 50 mM Tris–HCl (pH 7.0), 5 mM $MgCl_2$, 0.2 mM DTT, 10 μM glucose-1,6-diphosphate, varied concentrations of each precursor and GTP, 0.1 U/ml inorganic pyrophosphatase, suitable amounts of PMM and PMI/GMP.

2.6. Analysis of GDP-D-mannose by capillary electrophoresis

Capillary electrophoresis was performed using an ISCO 3850 capillary electropherograph equipped with a UV detector. The capillary was of bare silica (75 μm × 50 cm) with the UV detector mounted at 40 cm of the capillary. The analysis was run in 25 mM sodium tetraborate (pH 9.42) at 22 kV/72 μA, and the sugar nucleotide was detected at 254 nm. Peak integration was performed by a HP 3395 integrator.

2.7. Sequence alignment and database search

Sequence alignment was performed by ClustalW. Motif detection and database search by position-specific scoring matrices were carried out according to Bailey et al. [20].

3. Results

3.1. Expression and characterization of bifunctional PMI/GMP from H. pylori

L-Fucose has been found in the LPS O-antigen of *H. pylori*, which has been implicated in its pathogenesis and persistence [21–23]. In our search for the enzymes involved in the fucosylation of the bacterial surface [24], an open reading frame named HP0043 was found to be highly homologous to PMI/GMPs from *P. aeruginosa* (AlgA) (40% amino acid identity) [4] and *X. campestris* (XanB) (39% amino acid identity) [5]. Therefore the gene was cloned and overexpressed in *E. coli* with an N-terminal His tag. The recombinant protein was purified to apparently homogeneity as judged by SDS–PAGE (Fig. 2). The denatured protein migrated as a single band at the position corresponding to about 55 kDa, close to the calculated value (55 303 Da) from the deduced protein sequence. The purified protein was subjected to analysis for both PMI and GMP activities.

M6P was used as the substrate for PMI analysis. The formation of F6P can be detected by monitoring $OD_{340\,nm}$ after being coupled to $NADP^+$ reduction through phosphoglucose isomerase and phosphoglucose dehydrogenase. Divalent metal ions are necessary for PMI activity, with an order of activation being $Co^{2+} > Mg^{2+} > Mn^{2+} > Zn^{2+}$ when they are present at 2 mM.

M1P and GDP-D-mannose were used for activity analysis of GMP forward and reverse reactions, respectively. Mg^{2+} or Mn^{2+} is necessary for both directions although Mn^{2+} is less effective (30% of the efficiency with Mg^{2+}). It is very interesting to find that pH dependence differs for forward and reverse directions (data not shown). The forward reaction, with a pH optimum of 7, is very sensitive to pH fluctuation, while the reverse reaction rate does not change much in the pH range of 7–9. This might suggest that the enzyme requires proper ionization of either M1P and/or GTP for binding and catalysis.

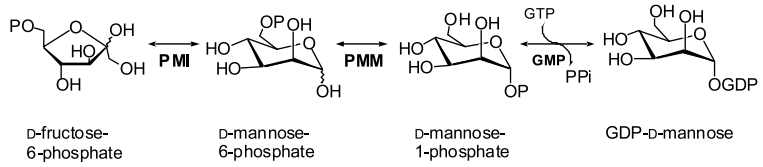

Fig. 1. The GDP-D-mannose biosynthetic pathway. The enzyme catalyzing each step is shown below the arrow. PMI, phosphomannose isomerase; PMM, phosphomannomutase; GMP, GDP-D-mannose pyrophosphorylase.

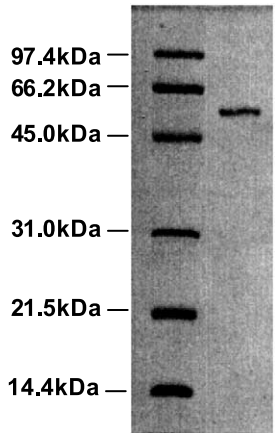

Fig. 2. SDS–PAGE analysis of purified PMI/GMP. SDS–PAGE was run in 12% polyacrylamide gel. Lane 1, molecular weight marker; lane 2, purified PMI/GMP.

3.2. Identification of the rate-limiting step in GDP-D-mannose biosynthesis in vitro

M1P, M6P and F6P were used as precursors to synthesize GDP-D-mannose (Fig. 3). When M1P or M6P was used in equal molar ratio to GTP, the reaction completed very quickly, with approximately 90% of the substrate transformed into GDP-D-mannose. But if the synthesis started from F6P, only 9% of the substrate was converted to GDP-D-mannose under the same condition. The reaction was followed in more detail and a time profile was constructed (Fig. 4). The production of GDP-D-mannose was significantly improved by increasing the F6P/GTP molar ratio.

3.3. PMI activity of PMI/GMP is inhibited by GDP-D-mannose

PMI activity was analyzed in the presence or absence of various compounds involved in the GDP-D-mannose biosynthetic pathway (Fig. 5A). It was found that PMI activity was severely inhibited by GDP-D-mannose, the end product of the pathway, suggesting the existence of feedback inhibition. The inhibition type was demonstrated to be non-competitive (mixed) as both apparent K_m and V_{max} were changed in the presence of GDP-D-mannose (Fig. 5B). The K_i and K'_i were 25.50 ± 0.67 μM and 96.86 ± 0.98 μM, respectively.

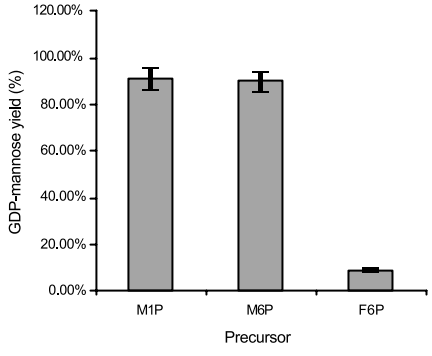

Fig. 3. Synthesis of GDP-D-mannose using intermediates from different stages of the pathway. M1P, M6P and F6P at 5 mM were used to react with 5 mM GTP. The reaction was carried out at 37°C for 2 h. GDP-D-mannose yield (%) was based on GTP (mol/mol).

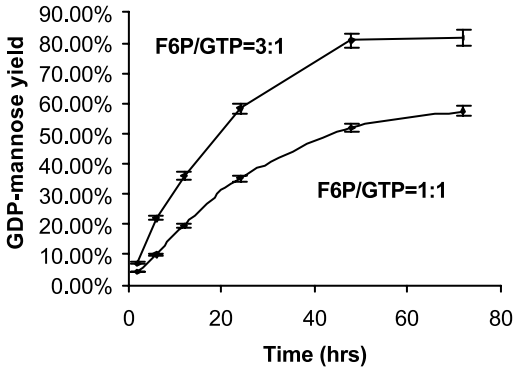

Fig. 4. Profile of GDP-D-mannose synthesis with varied F6P/GTP molar ratios. F6P at 5 mM and 15 mM was used to react with GTP (5 mM). The reaction was carried out at 37°C. GDP-D-mannose yield (%) was based on GTP (mol/mol). See Section 2 for details.

3.4. PMI/GMPs are present only in bacteria based on sequence alignment and database search

Amino acid sequence alignment of HP0043 with AlgA and XanB (Fig. 6), two other confirmed bifunctional PMI/GMP enzymes, revealed that all of them have at their N-terminal a highly conserved region containing a pyrophosphorylase sig-

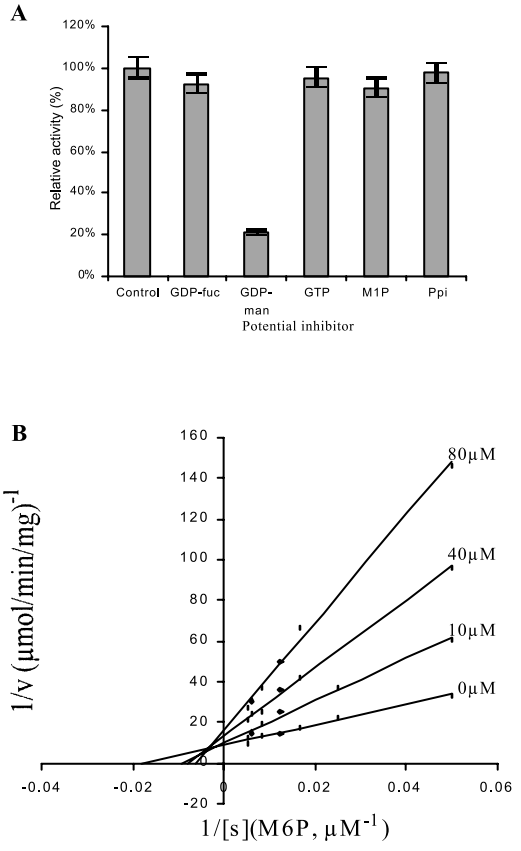

Fig. 5. A: Inhibition of PMI activity by GDP-D-mannose. PMI activity was analyzed in the presence of 0.5 mM of GDP-L-fucose (GDP-fuc), GDP-D-mannose (GDP-man), GTP, M1P and Ppi. Relative activity (%) was calculated against control (100%), the PMI activity measured without the presence of the above-mentioned compounds. B: Double reciprocal plot at various inhibitor concentrations. The inhibitor concentrations are shown above the plot.

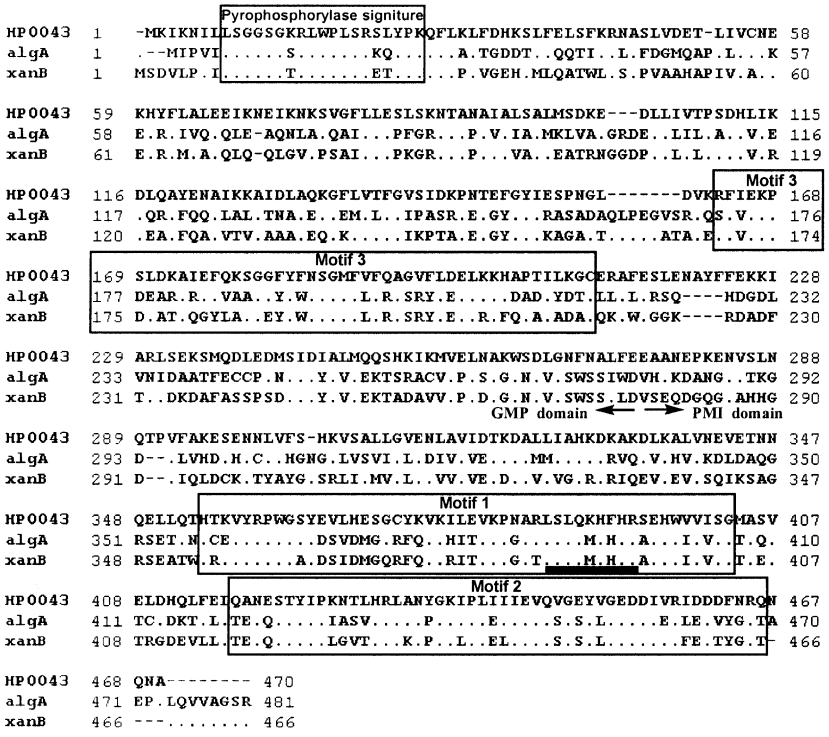

Fig. 6. Amino acid sequence alignment and motif analysis of bifunctional PMI/GMPs. HP0043 (AAD07111) from *H. pylori* was aligned with PMI/GMPs from *P. aeruginosa* (AlgA) (P07874) and *X. campestris* (XanB) (P29956). Identical amino acids are represented by dots. GMP domain and PMI domain are marked by arrows. Pyrophosphorylase signature sequences and three motifs are boxed. The underlined region within motif 1 is the consensus sequence shared by type I and type II PMIs.

nature sequence, GXGXR(L)-PK. In addition, three separate motifs were detected by position-specific scoring matrices. Motif 3 was found in the GMP domain while motifs 1 and 2, closely linked, were found in the PMI domain. The position-dependent scoring matrices corresponding to the three motifs were used by MAST [20] programs to perform database search and 69 protein sequences containing the three motifs were retrieved with *E* values ranging from 3.2e-156 to 3.9e-52. The *E* value of a sequence is the expected number of sequences in a random database of the same size that would match the motifs as well as the sequence does [20]. Obviously, all these sequences are very specific as judged from their extremely low *E* values and therefore are most likely type II bifunctional enzymes. Most importantly it was also found that all these proteins exist only in bacteria.

4. Discussion

In this study we have identified and characterized *H. pylori* HP0043 as a bifunctional PMI/GMP. Our results show a nearly absolute requirement for divalent ions by the enzyme. While GMP activity depends solely on Mg^{2+} or Mn^{2+}, PMI activity can use a variety of metal ions with Co^{2+} as the most effective activator and Zn^{2+} as the least among the divalent ions tested. This suggests that the combination between the metal ion and the PMI domain of PMI/GMP is loose and less specific, which is in sharp contrast to type I PMI, a metalloenzyme containing one atom of zinc per protein molecule [12,25]. But sequence alignment and domain analysis (Fig. 6) reveal that the PMI domain of the bifunctional enzymes still conserves the zinc binding pattern, e.g. QXH (X represents any amino acid) as seen in zinc metalloenzymes [26] including type I PMIs. The reason why the PMI domain of PMI/GMPs does not specifically bind Zn^{2+} is unknown. The apparent K_m of recombinant *H. pylori* PMI/GMP for M6P is significantly lower than that of AlgA (55.56 μM against 3.03 mM). The high K_m of AlgA for M6P has been proposed to be in favor of M6P formation, and therefore to be well suited for alginate production but not for D-mannose utilization by *P. aeruginosa* [4]. If this is true for *P. aeruginosa*, then *H. pylori* might have a well-balanced bi-directional pathway as suggested by the close values of K_{cat}/K_m for M6P, M1P and GDP-D-mannose (Table 1). *H. pylori* differs from *P. aeruginosa* in that it does not need to accumulate huge amounts of a single metabolite like alginate from the pathway. This might also help to explain why AlgA does not exhibit feedback inhibition [4], which would make it possible to produce alginate continuously.

Table 1
Kinetic parameters of recombinant *H. pylori* PMI/GMP

	M6P	M1P	GDP-D-mannose
K_m (μM)	55.56 ± 0.96	22.08 ± 0.94	101 ± 0.93
K_{cat} (s^{-1})	6.14 ± 0.32	10.27 ± 0.42	43.38 ± 0.40
K_{cat}/K_m (μM^{-1} s^{-1})	0.11	0.47	0.43

See Section 2 for detailed description of activity analysis.

As shown in Fig. 1, the major pathway for GDP-D-mannose biosynthesis starts from F6P, consisting of three steps with two intermediates, M6P and M1P. Combined use of recombinant PMI/GMP and PMM allows us to synthesize GDP-D-mannose from intermediates of different stages, which leads to the finding that the first step, isomerization of F6P to M6P, is rate-limiting (Fig. 3). Different concentrations of substrates and enzymes have been tested to remove any artifacts caused by selection of reaction conditions. Another possibility is that F6P could block one step of the pathway, but it is less likely since the production of GDP-D-mannose is greatly improved by increasing F6P/GTP molar ratio (Fig. 4). As PMI/GMP catalyzes the first and third steps of the pathway (Fig. 1), the enzyme is actually interacting with all the intermediates involved in GDP-D-mannose biosynthesis and it is reasonable to believe that some of them might participate in the regulation of the enzyme. PMI activity analysis in the presence of various intermediates from the pathway shows that GDP-D-mannose, the end product of the pathway, severely inhibits PMI activity (Fig. 5A). Therefore the bottleneck is caused by feedback inhibition of PMI activity via GDP-D-mannose. As there is no indication that GDP-D-mannose could bind to the PMI domain, this suggests that although separate domains specify PMI and GMP activities, binding of GDP-D-mannose to the GMP domain can greatly influence the substrate binding as well as the catalytic activity of the PMI domain. On the other hand, the difference between K_i and K'_i (25.50 μM versus 96.86 μM) indicates that binding of M6P with the PMI domain can decrease the affinity between GMP domain and GDP-D-mannose. These results are consistent with the observation that Ser^{12} and Val^{321} mutations in AlgA severely deteriorate both PMI and GMP activities by disrupting an important structure required for both PMI and GMP activities [18].

The feedback control in the biosynthesis of sugar nucleotides has been well studied for the inhibition of D-glucose-1-phosphate thymidyltransferase by dTDP-L-rhamnose via the competitive and non-competitive manner [27,28]. The competitive inhibition is caused by binding of dTDP-L-rhamnose to the same sites in roughly the same manner as the substrates in both forward and reverse directions while the mechanism for non-competitive inhibition is less clear [27]. It has also been reported that CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac) inhibits the UDP-GlcNAc 2-epimerase activity of bifunctional UDP-GlcNAc 2-epimerase/ManNAc kinase in rat liver, catalyzing the first and second reactions of CMP-Neu5Ac biosynthesis from UDP-GlcNAc [29]. But CMP-Neu5Ac is not an immediate substrate or product of the bifunctional enzyme. The PMI/GMP reported here obviously presents a new control mechanism for sugar nucleotide biosynthesis. It is also different from the most extensively studied bifunctional enzyme, chorismate mutase-prephenate dehydratase (P-protein), which plays a critical role in the biosynthesis of aromatic acids. The P-protein, like PMI/GMP, has two distinct catalytic domains for chorismate mutase and prephenate dehydratase, respectively. But it also has an extra regulatory domain (R-domain) for binding and feedback inhibition by phenylalanine [30,31]. Another bifunctional enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, affects glucose metabolism through its ability to both synthesize and degrade fructose-2,6-bisphosphate. The kinase activity of the bifunctional protein is feedback-inhibited by specific binding of succinate, a citric acid cycle intermediate, to the kinase domain [32]. A similar mechanism has also been reported for feedback inhibition by proline of the γ-glutamyl kinase activity of bifunctional γ-glutamyl kinase/γ-glutamyl phosphate reductase, catalyzing the first two steps of proline biosynthesis from glutamate in plant [33].

Sequence alignment and database search reveals that all PMI/GMP bifunctional proteins, either experimentally confirmed or putative based on sequence analysis, are highly homologous, possessing two separate domains containing three conserved motifs (Fig. 6). The GMP domain also shows overall homology to monofunctional GMP enzymes (data not shown) and it appears that motif 3 is highly conserved among them, containing Lys^{175} that has been shown to participate in M1P binding as well as in catalysis [18,27]. On the other hand, the PMI domain of type II bifunctional enzymes shares no homology with type I PMI proteins except a very short consensus sequence of nine amino acids within motif 1, containing the completely conserved residues like Gln^{389} and His^{391} that are found at the active site of *C. albicans* PMI [7,12]. Motif 2 is located close to the C-terminus of the bifunctional proteins. The requirement of motif 2 for PMI activity can be inferred from the observation that the 52-kDa chymotryptic fragment of AlgA (deletion of C-terminus by about 1 kDa) retained only 8% of the PMI activity of the undigested protein but retained 81% of the GMP activity of the intact protein [18]. It is also supported by the finding that the insertion at the corresponding site of XanB abolishes PMI activity but maintains 40% of the wild-type GMP activity [5].

As the impact of type I PMIs on pathogenicity has been implicated [13,14], PMI of pathogenic *C. albicans* has become a potential target to combat fungal infections [12,16]. However, due to the high level of sequence identity ($>40\%$) among type I PMI enzymes from bacteria to human beings [11], it might be very difficult to achieve species-specific inhibition of PMI. On the other hand, database searching using the three motifs found in PMI/GMPs shows that proteins containing all three motifs (confirmed or putative type II PMIs) are only found in bacteria. It has been suggested that type I enzymes participate in mannose metabolism while type II enzymes function in capsular polysaccharide biosynthesis [1]. Therefore type II PMIs can be a specific target to fight bacterial infection. Further work including site-directed mutagenesis and crystallization will be needed to clarify the catalytic and regulatory mechanism of PMI/GMPs, which will lead to rational design of highly efficient and specific inhibitors.

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