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Research paper

Molecular characterization of a novel thermostable mannose-6-phosphate isomerase from *Thermus thermophilus*

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ABSTRACT

Mannose-6-phosphate isomerase catalyzes the interconversion of mannose-6-phosphate and fructose-6-phosphate. The gene encoding a putative mannose-6-phosphate isomerase from *Thermus thermophilus* was cloned and expressed in *Escherichia coli*. The native enzyme was a 29 kDa monomer with activity maxima for mannose 6-phosphate at pH 7.0 and 80 °C in the presence of 0.5 mM Zn^{2+} that was present at one molecule per monomer. The half-lives of the enzyme at 65, 70, 75, 80, and 85 °C were 13, 6.5, 3.7, 1.8, and 0.2 h, respectively. The 15 putative active-site residues within 4.5 Å of the substrate mannose 6-phosphate in the homology model were individually replaced with other amino acids. The sequence alignments, activities, and kinetic analyses of the wild-type and mutant enzymes with amino acid changes at His50, Glu67, His122, and Glu132 as well as homology modeling suggested that these four residues are metal-binding residues and may be indirectly involved in catalysis. In the model, Arg11, Lys37, Gln48, Lys65 and Arg142 were located within 3 Å of the bound mannose 6-phosphate. Alanine substitutions of Gln48 as well as Arg142 resulted in increase of K_m and dramatic decrease of k_{cat}, and alanine substitutions of Arg11, Lys37, and Lys65 affected enzyme activity. These results suggest that these 5 residues are substrate-binding residues. Although Trp13 was located more than 3 Å from the substrate and may not interact directly with substrate or metal, the ring of Trp13 was essential for enzyme activity.

1. Introduction

Mannose-6-phosphate isomerase (EC 5.3.1.8) catalyzes the interconversion of D-fructose 6-phosphate and D-mannose 6-phosphate via an aldose–ketose isomerization reaction. In prokaryotes, this enzyme plays a critical role in the supply of guanosine diphosphate-D-mannose (GDP-D-mannose) that is the activated mannose donor for the biosynthesis of the extracellular polysaccharide [1,2] and the precursor for the synthesis of GDP-D-rhamnose and GDP-L-fucose (Fig. 1). These nucleotide sugars are also involved in the synthesis of various glycoconjugates, including the antitumor agent bleomycin and the antibiotics hygromycin A, nystatin, amphotericin, pimaricin, and candicidin [3]. In humans, a deficiency of mannose-6-phosphate isomerase activity leads to carbohydrate-deficient glycoprotein syndrome type Ib, a severe metabolic disorder with a hepatic-intestinal presentation [4,5]. However, the syndrome is successfully treated with oral mannose [6].

Mannose-6-phosphate isomerases have been identified in a wide range of organisms, and several of these have been cloned and characterized [2,7]. The enzymes have been classified into three types [8]. The type I enzymes include all eukaryotic and prokaryotic mannose-6-phosphate isomerases that are homologous monofunctional enzymes, catalyzing the single isomerization reaction [9,10]. The type II enzymes are bifunctional phosphomannose isomerases/GDP-D-mannose pyrophosphorylases found in certain bacteria [2,10–13] and exhibit no extensive homology with the type I enzymes except a very short motif [7]. The type III enzyme consists of only one protein from *Rhizobium meliloti* [14] and is able to catalyze reversible isomerization but shares little or no sequence identity with the type I and II enzymes.

The physicochemical and kinetic characterization of the type I mannose-6-phosphate isomerases from various sources [7,15–17] and the X-ray crystal structures of the type I mannose-6-phosphate isomerases from *Candida albicans* [18] (PDB code 1PMI), *Salmonella typhimurium* [19] (2WFP), *Archaeoglobus fulgidus* (1ZX5) [20], and *Bacillus subtilis* (1QWR) have been reported to

Abbreviations: GDP, guanosine diphosphate; LB, Luria-Bertani; IPTG, isopropyl-β-D-thiogalactopyranoside; FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; EPPS, N-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid); MD, molecular dynamics; ICP-MS, inductively coupled plasma mass spectrometry.

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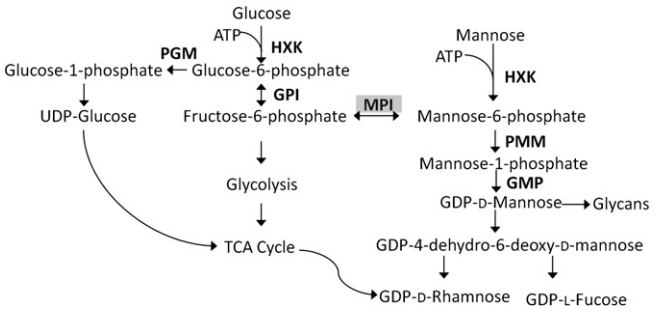

Fig. 1. Schematic representation of the central role of mannose-6-phosphate isomerase (MPI) in glycolysis, glycosylation, and nucleoside sugars. MPI catalyzes the interconversion of fructose 6-phosphate and mannose 6-phosphate. PGM, phosphoglucomutase; HXK, hexokinase; GPI, glucose-6-phosphate isomerase; PMM, phosphomannomutase; and GMP, GDP-mannose pyrophosphorylase.

date. However, the type I mannose-6-phosphate isomerases from thermophiles for the isomerization of mannose 6-phosphate have not been characterized. Although only one structure [19] and a few theoretical models of complexes between type I mannose-6-phosphate isomerases and substrate or inhibitor [21–23] have been reported, their exact active-site residues have never been identified.

In this study, the gene encoding a putative mannose-6-phosphate isomerase from the hyperthermophile *Thermus thermophilus* was cloned and expressed in *Escherichia coli*. The biochemical properties of the mannose-6-phosphate isomerase, such as the effects of metal ions, pH, temperature, and substrate specificity, were evaluated, and the metal-binding and substrate-binding residues were identified by conducting sequence alignments, site-directed mutagenesis, and kinetic analyses for the predicted active-site residues based on the homology model.

2. Materials and methods

2.1. Materials

The kits for PCR product purification, gel extraction, and plasmid preparation as well as the DNA-modifying enzymes were purchased from Promega (Fitchburg, WI, USA). The phosphate sugar standards mannose 6-phosphate, fructose 6-phosphate, glucose 6-phosphate, ribose 5-phosphate, ribulose 5-phosphate, and arabinose 5-phosphate were purchased from Sigma (St Louis, MO, USA).

2.2. Bacterial strains, plasmids, and culture conditions

The genomic DNA of *T. thermophilus* KCCM 40879, *E. coli* ER2566, and plasmid pET-28a (+) (Novagen, Germany) were used as a source of mannose-6-phosphate isomerase gene, host cells, and expression vector, respectively. *T. thermophilus* was grown aerobically at 75 °C on a growth medium containing 0.4% yeast extract, 0.2% polypeptone, and 0.2% sodium chloride. The recombinant *E. coli* cells for protein expression were cultivated in Luria-Bertani (LB) medium (1.0% tryptone, 0.5% yeast extract, and 1.0% sodium chloride) in a 2000 mL flask containing 20 μg/mL kanamycin at 37 °C with shaking at 250 rpm. When the optical density of bacteria reached 0.6 at 600 nm, isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture medium at a final concentration of 0.1 mM, and then the culture broth was incubated with shaking at 150 rpm at 16 °C for 16 h to express mannose-6-phosphate isomerase.

2.3. Gene cloning and site-directed mutagenesis of mannose-6-phosphate isomerase

The gene encoding a putative mannose-6-phosphate isomerase was amplified by PCR using the genomic DNA isolated from *T. thermophilus* KCCM 40879 as a template. Oligonucleotide primer sequences used for gene cloning were designed using the published DNA sequence of mannose-6-phosphate isomerase from *T. thermophilus* HB8 (GenBank accession number AP008226). Forward (5- TTTCATATGAGGCGGTTGGAGCCCAA) and reverse primers (5-TTTGAATTCACTCACGCCCCCTCCTT) were designed for introduction of the underlined *Nde*I and *Eco*RI restriction sites, respectively. The amplified DNA fragment was purified using PCR purification kit (Promega) and was ligated into the *Nde*I and *Eco*RI sites of pET-28a(+). The resultant plasmid was used to transform *E. coli* ER2566. The recombinant *E. coli* cells containing the plasmid were grown on LB medium. The expression of the gene encoding mannose-6-phosphate isomerase was determined by both sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% gels) and the assay of enzyme activity. Site-directed mutagenesis was performed using the Quick-Change kit and protocol (Stratagene Beverly, MA, USA).

2.4. Purification of mannose-6-phosphate isomerase

The washed recombinant cells were resuspended in 50 mM phosphate buffer containing 300 mM NaCl, 10 mM imidazole, and 0.1 mM PMSF as a protease inhibitor. The resuspended cells were disrupted by ultrasonication (Fisher Scientific, Pittsburgh, PA, USA) on ice. The cell debris was removed by centrifugation at 13,000 × *g* for 20 min at 4 °C, and the supernatant was filtered through a 0.45 μm pore-size filter. The filtrate was applied to a HisTrap HP chromatography column (GE Healthcare, Uppsala, Sweden) equilibrated with 50 mM phosphate buffer. The column was washed extensively with the same buffer, and the bound protein was eluted with a linear gradient from 10 to 250 mM imidazole at a flow rate of 1 mL/min. The active fraction was dialyzed at 4 °C for 24 h against 50 mM Tris–HCl buffer (pH 7.5). After dialysis, the resultant solution was used as the purified enzyme. The purification steps using the column were carried out by a fast protein liquid chromatography (FPLC) system (Bio-Rad Laboratories Hercules, CA, USA) in a cold room. The protein concentrations were quantified by the method of Bradford. The purified proteins were confirmed by SDS-PAGE.

2.5. Determination of molecular mass

The subunit molecular mass of mannose-6-phosphate isomerase was examined by SDS-PAGE under denaturing conditions, using the proteins of a pre-stained ladder (MBI Fermentas, Glen Burnie, MD, USA) as reference proteins. All protein bands were stained with Coomassie Blue for visualisation. The molecular mass of the native enzyme was determined by gel-filtration chromatography on a Sephacryl S-300 preparative-grade column HR 16/60 (GE Healthcare). The purified enzyme was applied to the column and eluted with 50 mM Tris–HCl (pH 7.5) buffer containing 150 mM NaCl at a flow rate of 1 mL/min. The column was calibrated with aldolase (158 kDa), bovine serum albumin (67 kDa), chymotrypsin (29 kDa), and RNaseA (13.7 kDa) as reference proteins (GE Healthcare), and the native enzyme was calculated by comparing with the migration length of reference proteins.

2.6. Measurement of specific activities and kinetic parameters of the wild-type and mutant enzymes

One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of fructose 6-phosphate per