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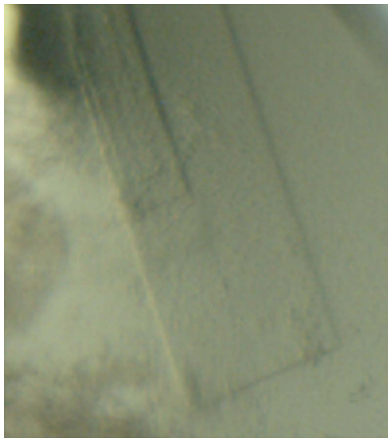
Cloning, expression, purification, crystallization and preliminary X-ray crystallographic analysis of the mannose 6-phosphate isomerase from *Salmonella typhimurium*

Mannose 6-phosphate isomerase (MPI; EC 5.3.1.8) catalyzes the reversible isomerization of D-mannose 6-phosphate (M6P) and D-fructose 6-phosphate (F6P). In the eukaryotes and prokaryotes investigated to date, the enzyme has been reported to play a crucial role in D-mannose metabolism and supply of the activated mannose donor guanosine diphosphate D-mannose (GDP-D-mannose). In the present study, MPI was cloned from *Salmonella typhimurium*, overexpressed in *Escherichia coli* and purified using Ni–NTA affinity column chromatography. Purified MPI crystallized in space group $P2_12_12_1$, with unit-cell parameters a = 36.03, b = 92.2, c = 111.01 Å. A data set extending to 1.66 Å resolution was collected with 98.8% completeness using an image-plate detector system mounted on a rotating-anode X-ray generator. The asymmetric unit of the crystal cell was compatible with the presence of a monomer of MPI. A preliminary structure solution of the enzyme has been obtained by molecular replacement using *Candida albicans* MPI as the phasing model and the program *Phaser*. Further refinement and model building are in progress.

1. Introduction

Mannose 6-phosphate isomerase catalyzes the reversible isomerization of fructose 6-phosphate (F6P) and mannose 6-phosphate (M6P) (Fig. 1). MPI is essential for bacterial growth using mannose as the sole carbon source. It is also necessary for the formation of GDP-L-fucose, the precursor of L-fucose. In all prokaryotes and eukaryotes, the reaction catalyzed by MPI is the initial committed step for the supply of guanosine diphosphate D-mannose (GDP-D-mannose), the activated mannose donor for the biosynthesis of many mannosylated structures, including glycoproteins, glycolipids, exopolysaccharides and cell-wall components in microorganisms. In the biosynthesis of GDP-D-mannose, F6P is first isomerized by MPI to M6P, which is further converted to mannose 1-phosphate (M1P) by phosphomannomutase. GDP-mannose pyrophosphorylase then catalyzes the



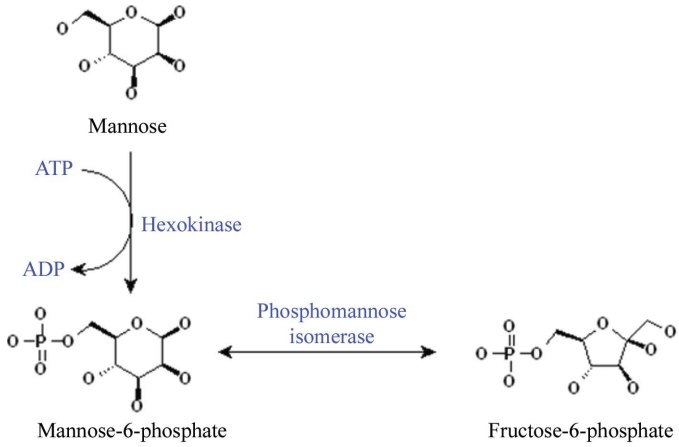


Figure 1
The reaction catalyzed by MPI.

incorporation of GTP onto M1P to produce the final product GDP-D-mannose.

Since the activity of MPI is essential for the survival and pathogenesis of several microbes, it is considered to be a likely target for drugs against various microorganisms. Absence of MPI activity in yeast causes cell lysis (Smith *et al.*, 1995). Deficiency of MPI activity leads to the human disease congenital disorder of glycosylation 1b. The disorder results from the hypoglycosylation of serum and other glycoproteins (Jaeken *et al.*, 1998; de Koning *et al.*, 1998). Ablation of mouse MPI causes M6P accumulation, toxicity and embryonic lethality (DeRossi *et al.*, 2006). MPI is needed for the development of the mucoid strain of *Pseudomonas aeruginosa* that causes life-threatening lung infections in cystic fibrosis patients (Shinabarger *et al.*, 1991). It is also important for the virulence of the protozoan parasite *Leishmania mexicana* (Awadalla *et al.*, 1987).

Based on the domain organization, MPIs have been classified into two types (types I and II; Jensen & Reeves, 1998). Type I, which includes proteins from *Aspergillus nidulans*, *Candida albicans*, *Escherichia coli*, *Homo sapiens*, *Salmonella enterica* and *Cryptococcus neoformans*, are homologous monofunctional enzymes that catalyze a single isomerization reaction. The type I MPI isolated from *Saccharomyces cerevisiae* (Gracy & Noltmann, 1968) has been shown to be a zinc-dependent metalloenzyme with one metal atom per monomer. Type II MPIs are bifunctional enzymes that possess both MPI and guanosine diphospho-D-mannose pyrophosphorylase activities in separate catalytic domains. However, in the cyanobacteria the occurrence of a gene homologous to the C-terminal half of type II MPI and hence probably coding only for a single catalytic domain with MPI activity has been reported (Shinabarger *et al.*, 1991). Types I and II have a common conserved MPI catalytic motif.

Crystal structures of MPI are known from *C. albicans* (Cleasby *et al.*, 1996; PDB code 1pmi), *Bacillus subtilis* (PDB code 1qwr), *Archaeoglobus fulgidus* (PDB code 1zx5) and *Pyrobaculum aerophilum* (Swan *et al.*, 2004; PDB codes 1tzb and 1tzc). The amino-acid sequence of *Salmonella typhimurium* MPI is most similar to that of *C. albicans*. In comparison, its similarity to other bacterial or archaeal MPIs of known structure is significantly lower.

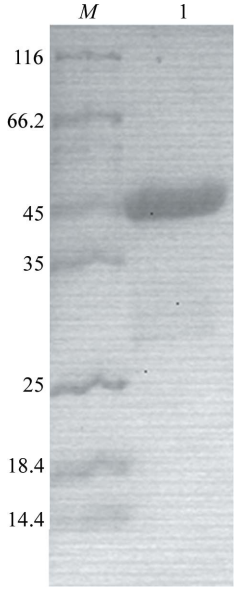


Figure 2
SDS–PAGE of MPI. The protein was analyzed on 12% SDS–PAGE and stained with Coomassie blue. Lane 1, purified His-tagged MPI; lane *M*, molecular-weight markers (kDa; Fermentas 431 molecular-weight marker kit).

In the present study, we initiated structural studies on MPI from the pathogenic *S. typhimurium*. The three-dimensional structure of this enzyme will help in understanding the mechanism of the isomerization reaction and the role of metal atoms in catalysis. In turn, it will help in the rational design of inhibitors for this therapeutically important enzyme. MPI has been cloned from *S. typhimurium*, overexpressed in *E. coli* and purified using Ni–NTA affinity column chromatography. Crystals obtained using purified His-tagged MPI diffracted X-rays to 1.66 Å resolution.

2. Materials and methods

2.1. Cloning

The gene coding for MPI was PCR-amplified from *S. typhimurium* genomic DNA using XT-polymerase (Genei, India) and specific sense (GGC**GCTAGC**ATGCAAAAACTCATTAACTCAGT) and antisense (GGC**GGATCC**CTACAGCTTGTTATAAACACG) primers. The sequences in bold correspond to *Nhe*I and *Bam*HI sites in the sense and antisense primers, respectively. The PCR-amplified fragment was digested with *Nhe*I and *Bam*HI. It was then ligated with pRSET-C (Invitrogen) vector previously digested with the same restriction enzymes. The genomic DNA and plasmid DNA isolation for cloning were carried out according to the method of Sambrook & Russell (2001). The clone thus obtained was confirmed by DNA sequencing. Such a cloning strategy resulted in the expression of *S. typhimurium* MPI with 14 additional amino acids (MRGSHHHHHHGMAS) from the vector, including six histidines at the N-terminus which facilitated purification by Ni–NTA affinity chromatography.

2.2. Overexpression and purification

The recombinant MPI clones were transformed into BL21(DE3)-pLysS cells and plated on LB with ampicillin. The pre-inoculum prepared from a single colony was transferred to a 500 ml culture of terrific broth containing 50 µg ml^{-1} ampicillin and incubated at 310 K until the OD at 600 nm reached 0.6. Expression of the enzyme was induced with 0.3 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG)

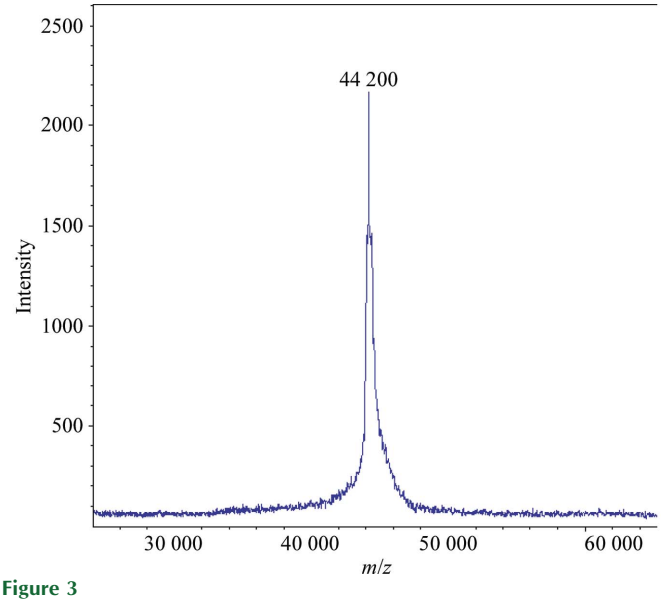


Figure 3
MALDI–TOF mass spectrum. The peak representing MPI corresponds to 44.2 kDa.

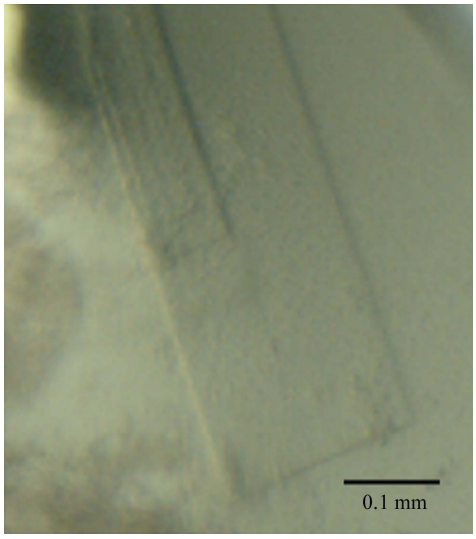


Figure 4
Crystals of mannose 6-phosphate isomerase.

and the cells were allowed to grow for a further period of 5–6 h at 303 K. The cells were pelleted and resuspended in 50 m*M* Tris–HCl pH 8.0 with 300 m*M* NaCl, 10% glycerol and 1% Triton X-100. The cells were sonicated until the solution became clear. Soluble and insoluble fractions were separated by centrifugation. Fractions were run on 12% SDS–PAGE (Laemmli, 1970) along with marker proteins in order to monitor the expression. MPI was purified from the soluble fraction using Ni–NTA affinity column chromatography following the manufacturer's protocol. Eluted fractions containing MPI were pooled and dialyzed against buffer containing 20 m*M* Tris–HCl pH 8.0 with 100 m*M* NaCl. The protein was used in the dialysis buffer for the crystallization experiments. The molecular weight and purity of the enzyme were checked on 12% SDS–PAGE (Fig. 2) and using a MALDI–TOF (Fig. 3) mass spectrometer. The enzyme was concentrated to the concentration required for crystallization by several cycles of low-speed centrifugation using a 10 kDa molecular-weight cutoff Centricon (Amicon). The protein concentration was estimated by the Bradford method (Bradford, 1976).

Table 1
Data-collection statistics.

Values in parentheses are for the last resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters	
a (Å)	35.90
b (Å)	92.47
c (Å)	111.87
Resolution range (Å)	30–1.66 (1.72–1.66)
Total no. of reflections	1552287
No. of unique reflections	45058
Completeness (%)	98.9 (89.6)
$R_{\rm merge}$† (%)	6.2 (55.6)
$R_{\rm p.i.m.}$‡ (%)	1.5 (12.1)
$\langle I/\sigma(I)\rangle$	48.5 (5.2)
Multiplicity	19.4 (16.0)
Matthews coefficient (Å^3 Da^{-1})	2.08
No. of molecules per ASU	1

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \overline{I(hkl)}|/\sum_{hkl}\sum_i I_i(hkl)$. ‡ $R_{\rm p.i.m.} = \sum_{hkl}[1/(N-1)]^{1/2}\sum_i |I_i(hkl) - \overline{I(hkl)}|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of $I(hkl)$, $\overline{I(hkl)}$ is its mean intensity and N is the number of times a given reflection was measured.

2.3. Crystallization, data collection and processing

Initial crystallization experiments were carried out at room temperature (293 K) with protein containing the hexahistidine tag using a crystallization robot (Cartesian dispensing systems). Crystallization attempts were made with Hampton Research Crystal Screen kits including Crystal Screen, Crystal Screen 2, Index Screen, PEG/Ion Screen and SaltRx Screen. Each crystallization droplet contained 100 nl screen solution and 100 nl protein solution. Small crystals were obtained in three conditions. These were further refined by the hanging-drop vapour-diffusion and microbatch methods. Crystallization drops were prepared by mixing 3 µl protein solution (8 mg ml^{-1}) with 3 µl reservoir solution. In the hanging-drop setups, the crystallization drops were suspended over 500 µl reservoir solu-

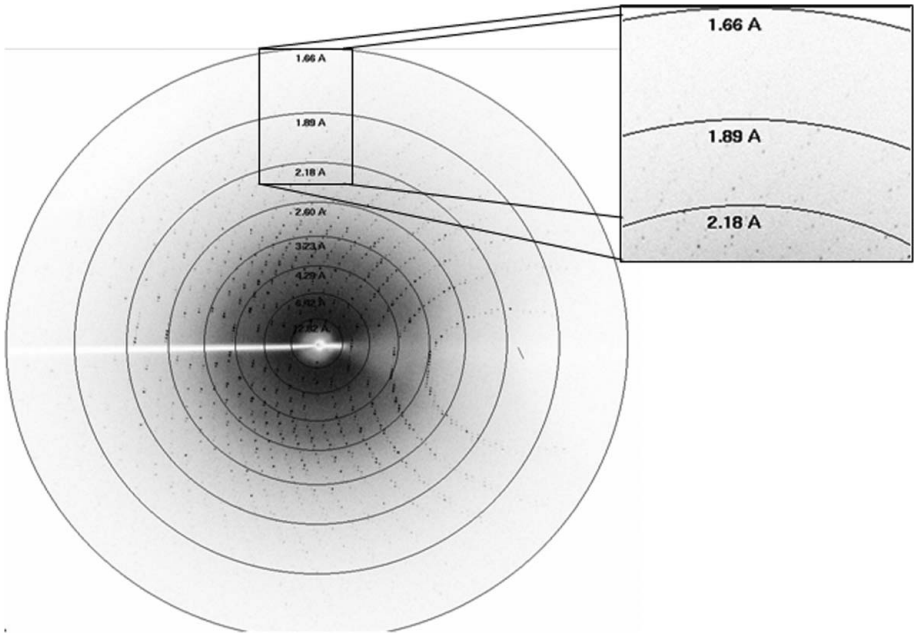


Figure 5
Diffraction pattern obtained from a crystal of MPI.

tion. In the microbatch method, the protein solution and the crystallization reagent were pipetted into a layer of paraffin oil and silicon oil (Hampton Research) in a 1:1 ratio. Diffraction-quality crystals were obtained at 293 K using the following conditions: (i) 0.2 *M* magnesium acetate, 0.1 *M* sodium cacodylate pH 6.5, 20% PEG 8000, (ii) 0.2 *M* calcium acetate, 0.1 *M* sodium cacodylate pH 6.5, 20% PEG 8000 and (iii) 0.2 *M* sodium acetate, 0.1 *M* Tris–HCl pH 8.5, 30% PEG 8000. Further optimization of condition (i) gave well diffracting crystals (Fig. 4) using the microbatch method and a crystal thus obtained was used for data collection.

X-ray diffraction data were collected at 100 K on a Rigaku RU200 rotating-anode X-ray generator equipped with a MAR Research image-plate detector. The crystal-to-detector distance was set to 120 mm. All frames were collected at 100 K with a 1.0° oscillation angle and an exposure time of 600 s per frame. The crystallization condition containing 20%(*v*/*v*) ethylene glycol was used as the cryoprotectant. The crystal was soaked for 18 h in the cryoprotectant at 297 K. The data were indexed, integrated and scaled using *DENZO* and *SCALEPACK* from the *HKL*-2000 suite (Otwinowski & Minor, 1997). The data revealed significant diffraction spots to 1.66 Å resolution (Fig. 5).

3. Results and discussion

Crystals of MPI suitable for collecting X-ray diffraction data were obtained using 10 mg ml^{-1} protein and optimized condition (i), which consisted of 0.1 *M* magnesium acetate, 0.2 *M* sodium cacodylate pH 6.5, 20% PEG 8000 and 5% dioxane. The data collected from the MPI crystals extended to 1.66 Å resolution (Fig. 5). Systematic absences showed that the crystal belongs to space group $P2_12_12_1$, with a monomer in the asymmetric unit (Matthews coefficient of 2.08 Å^3 Da^{-1}; Matthews, 1968). Unit-cell parameters and data-collection statistics are shown in Table 1. Preliminary structure-solution trials were performed by the molecular-replacement method using phosphomannose isomerase (PDB code 1pmi) from *C. albicans* (Cleasby *et al.*, 1996) as the search model (29.7% sequence identity). A tentative structure solution was obtained using the program *Phaser* (McCoy *et al.*, 2007). Further refinement and model building are in progress.

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