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Crystallization and initial crystallographic analysis of phosphomannomutase/phosphoglucomutase from *Pseudomonas aeruginosa*

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The enzyme phosphomannomutase/phosphoglucomutase (PMM/PGM) catalyzes the conversion of mannose 6-phosphate to mannose 1-phosphate in the second step of the alginate biosynthetic pathway of *Pseudomonas aeruginosa*. PMM/PGM has been crystallized by hanging-drop vapor diffusion in space group $P2_12_12_1$. Crystals diffract to 1.75 Å resolution on a synchrotron X-ray source under cryo-cooling conditions. PMM/PGM substituted with selenomethionine has been purified and crystallizes isomorphously with the native enzyme. Structure determination by MAD phasing is under way. Because of its role in alginate biosynthesis, PMM/PGM is a potential target for therapeutic inhibitors to combat *P. aeruginosa* infections.

1. Introduction

The infectious bacterium *P. aeruginosa* secretes a protective exopolysaccharide called alginate that forms a viscous capsule around the organism. This alginate coating is believed to contribute to the bacteria's widespread resistance to antibiotics and its ability to evade the host's immune response (Govan & Deretic, 1996). Chronic *P. aeruginosa* infections are the leading cause of morbidity and mortality in cystic fibrosis patients (Govan & Deretic, 1996) and are also common in cancer, burn and immunocompromised patients. Because of alginate's role in the pathogenicity of *P. aeruginosa*, enzymes in the alginate biosynthetic pathway are attractive candidates for clinical inhibitors.

Pseudomonad alginate is a linear polymer of (1–4)-linked β-D-mannuronate and α-L-guluronate residues. Many of the mannuronate residues are O-acetylated at the 2- or 3-position or both. The alginate biosynthetic pathway as it is currently understood is shown in Fig. 1. PMM/PGM is encoded by the *algC* gene and catalyzes the conversion of mannose 6-phosphate to mannose 1-phosphate in the second step of the alginate biosynthetic pathway. It is also required for A-band lipopolysaccharide biosynthesis, where it utilizes glucose 6-phosphate as a substrate. These dual biosynthetic roles for PMM/PGM were confirmed by *algC* mutants that lacked the alginate phenotype and O-side chain and core lipopolysaccharides (Ye *et al.*, 1994). PMM/PGM has a molecular weight of 50 200 Da and is reported to be a monomer in solution (Ye *et al.*, 1994). It requires Mg^{2+} for maximal activity and is activated by glucose-1,6-bisphosphate. The reaction is believed to proceed through a phosphoenzyme intermediate at Ser108 (Shankar *et al.*, 1995).

P. aeruginosa PMM/PGM shows only moderate sequence identity (30–50%) with other bacterial phosphomannomutases and phosphoglucomutases and very little homology with the rabbit muscle PGM which has been structurally characterized by Ray *et al.* (1993). *P. aeruginosa* PMM/PGM also differs from rabbit muscle PGM in a number of its functional characteristics, including its substrate specificity and metal-ion preference (Ye *et al.*, 1994).

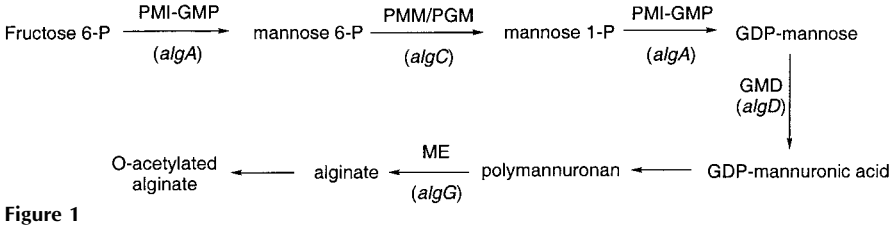

Figure 1
Biosynthetic pathway of alginate in *P. aeruginosa*. Several key enzymes are indicated: PMI-GMP, phosphomannose isomerase-GDP pyrophosphorylase; PMM/PGM, phosphomannomutase/phosphoglucomutase; GMD, GDP-mannose dehydrogenase; ME, mannuronan epimerase. Genes encoding each enzyme are indicated in italics.

2. Materials and methods

The *algC* gene was amplified from *P. aeruginosa* strain PAO1 by the polymerase chain reaction using primers based on the published sequence. The forward primer incorporated an *Nde*I site and the reverse primer incorporated a *Bam*HI site, so the amplicon could be cloned directly into the pET3-a (Novagen) vector. The resulting construct was used to transform BL21(DE3) cells. Freshly transformed cells were grown to an optical density at 600 nm of ~0.6 and expression of the *algC* gene was induced by the addition of IPTG to a final

Table 1
Statistics for PMM/PGM native data set obtained at beamline X8C.

Numbers in parentheses represent statistics for the outer resolution shell.

Resolution range (Å)	20.0–1.75
R_{sym}† (%)	4.6 (31.8)
$\langle I\rangle/\langle\sigma\rangle$‡	27.9 (4.9)
Completeness (%)	94.9 (97.1)
Reflections $I > 3\sigma(I)$ (%)	84.3 (55.4)
Multiplicity	4.8 (4.8)
No. of unique reflections	46700

† $R_{sym} = 100(\sum_h |I_h - \langle I\rangle|)/\sum I_h$, where $\langle I\rangle$ is the mean intensity of all symmetry-related reflections I_h. ‡ $\langle I\rangle$ = average intensity of reflections, $\langle\sigma\rangle$ = average σ or error of $\langle I\rangle$.

concentration of 0.4 m*M*. Cells were harvested 4–5 h after induction and stored at 195 K until use. PMM/PGM was purified to homogeneity following the published procedure (Ye *et al.*, 1994). Approximately 200 mg of purified protein was obtained from 17 g of frozen cell paste. MALDI mass-spectrometric analysis (Mass Consortium, La Jolla, CA, USA) of the purified PMM/PGM indicates two predominant species differing by approximately 77 Da, consistent with isolation of both phospho and dephospho forms of the enzyme (a phosphoryl group is 79 Da).

Initial crystallization screens employed conditions found in the Hampton Research Crystal Screen kits 1 and 2. The PMM/PGM solution was at 12–15 mg ml^{-1} in 10 m*M* MOPS buffer pH 7.0. Crystals grow by hanging-drop vapor diffusion from 1.4 *M* sodium/potassium tartrate and 100 m*M* Na HEPES pH 7.5 from drops containing 2 µl of protein and 2 µl of well buffer. Crystals grow from heavy precipitate in drops; *de novo* growth time varies widely from two weeks to four months. Use of micro- and macro-seeding techniques has reliably produced crystals within several weeks. A solution of 40% glycerol and 1.4 *M* sodium/potassium tartrate was used to cryoprotect the PMM/PGM crystals for low-temperature data collection. Crystals were transferred gradually into solutions of increasing glycerol concentration and were flash-frozen in a nitrogen stream for data collection.

To facilitate structure solution, a selenomethionine (SeMet) substituted PMM/PGM (eight methionine residues) was produced in *Escherichia coli* by growing bacteria in minimal media and in the presence of SeMet and lysine, threonine, phenylalanine, leucine, isoleucine and valine in order to inhibit methionine biosynthesis (Van Duyne *et al.*, 1993). The SeMet-substituted enzyme was active and was purified by the same protocol as the wild-type enzyme. Approximately 50 mg of purified protein was obtained from a 2 l culture broth. Incorporation of selenomethionine was verified by MALDI mass spectrometry (data not shown). The SeMet protein did not crystallize *de novo*, but native PMM/PGM crystals were successfully used to seed into SeMet PMM/PGM drops containing the crystallization buffer described above plus 1.0 m*M* β-mercaptoethanol. SeMet protein concentration was 6 mg ml^{-1} for seeding. Small *de novo* SeMet crystals produced from seeding with native crystals were used as seeds to grow larger SeMet PMM/PGM crystals for data collection.

The SeMet PMM/PGM crystallizes isomorphously with the native enzyme. Although the SeMet PMM/PGM crystals are quite sensitive to radiation damage at room temperature (lifetime on rotating anode <1 h), the cryoprotectant used for the native crystals is also suitable for the SeMet crystals and extends crystal lifetime in the beam indefinitely. Presumably because of their smaller size, the SeMet PMM/PGM crystals diffract to a nominal resolution of 2.2 Å on a synchrotron X-ray source.

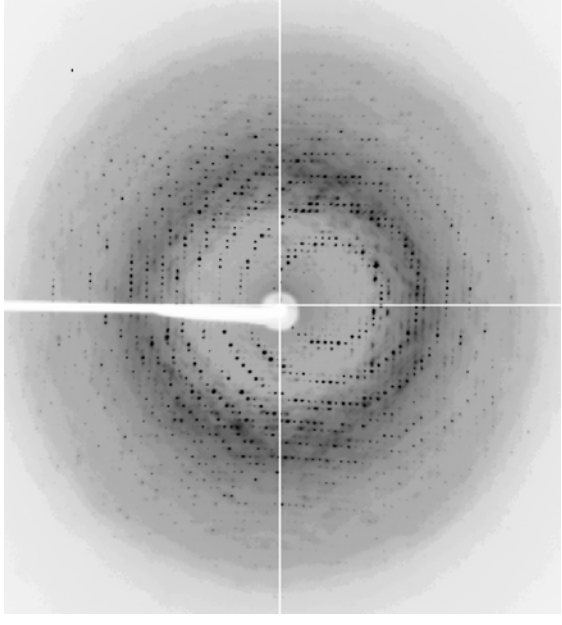

Figure 2
Diffraction pattern of native PMM/PGM crystals at beamline X8C at the National Synchrotron Light Source.

3. Results and discussion

A native data set to 1.75 Å for PMM/PGM was collected from a single crystal on beamline X8C at Brookhaven National Laboratory (Table 1; Fig. 2). Data were collected at approximately 100 K using the cryoprotectant described above. The X-ray wavelength was 0.98 Å, the oscillation angle was 0.75° and the crystal-to-detector distance was 130 mm. Crystals belong to space group $P2_12_12_1$ and have unit-cell parameters $a = 70.89$, $b = 73.02$, $c = 92.57$ Å, $\alpha = \beta = \gamma = 90°$. Data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Packing density calculations for PMM/PGM indicate $V_m = 2.43$ Å^3 Da^{-1}, assuming one molecule of PMM/PGM per asymmetric unit. This corresponds to a solvent fraction of about 49%, a typical value for protein crystals (Matthews, 1968).

The protein of known structure most closely related to PMM/PGM is PGM from rabbit. Several models derived from rabbit PGM structure were used for molecular-replacement attempts. However, no solution was found, presumably owing to the low sequence identity between the two proteins (~21%). Structure-determination efforts will proceed *via* the SeMet PMM/PGM crystals that were recently used in MAD experiments at beamline X8C at Brookhaven National Laboratory. Refinement of PMM/PGM with 1.75 Å native data should provide an excellent starting point for studies of enzyme–inhibitor complexes.

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