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Purification, crystallization and preliminary X-ray analysis of the *Escherichia coli* glucose-1-phosphatase

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Encoded by the *agp* gene, *Escherichia coli* glucose-1-phosphatase hydrolyzes glucose-1-phosphate in the periplasmic space of the bacterium. It is a potential drug-design target because inositol phosphatases have been identified as important virulence determinants in several human and animal pathogens. The enzyme was isolated and purified to homogeneity from a strain of *E. coli* CU1867 (an *appA*-deficient mutant). Crystals were obtained overnight by the equilibrium vapour-diffusion method from a solution containing 10 mg ml^{-1} enzyme, 1.2 *M* ammonium sulfate and 25% polyethylene glycol monomethyl ether 5000 in 0.1 *M* MES at pH 6.5. The crystals belong to space group *R*3, with unit-cell parameters $a = b = 156.0$, $c = 92.2$ Å. The diffraction limit was 2.6 Å at a rotating-anode X-ray source; a 2.7 Å resolution data set has been collected using light mineral oil as a cryoprotectant. The data set was 95.2% complete, with an $R_{\rm sym}$ of 0.058. There were two monomers of glucose-1-phosphatase in the asymmetric unit, which correspond to a $V_{\rm M}$ of 2.36 Å Da^{-1} and 47.5% solvent content. Self-rotation analysis unambiguously shows a twofold non-crystallographic symmetry.

1. Introduction

The enteric bacterium *E. coli* produces periplasmic phosphatases capable of hydrolyzing the phosphate moiety from a variety of phosphorylated compounds at acidic pH. Among these phosphatases is the acid glucose phosphatase (*agp*) encoded glucose-1-phosphatase, a homodimeric enzyme with a subunit molecular mass of 45 kDa (Pradel & Boquet, 1988, 1989). A documented role of *E. coli* glucose-1-phosphatase is to scavenge glucose from glucose-1-phosphate, a conclusion drawn from the lack of regulation of glucose-1-phosphatase activity by phosphate concentration and the unique ability of *agp*$^+$ strains to grow in a high-phosphate media with glucose-1-phosphate as the sole carbon source (Dassa *et al.*, 1990).

In addition to the variety of phosphorylated compounds previously characterized as substrates, glucose-1-phosphatase has recently been shown to possess myo-inositol hexaphosphatase activity (Golovan *et al.*, 2000). This inositol phosphatase activity is intriguing for two reasons. It has recently been shown that inositol phosphatases of the related *Enterobacteriaceae* family members *Salmonella dublin*, *S. typhimurium* and *Shigella* spp. are translocated directly into the eukaryotic host cell *via* type III secretion systems where they interfere with a variety of host cell-signalling pathways (De Vinney *et al.*, 2000). Inositol phosphatases are therefore important virulence determinants and may prove to be useful targets for new antimicrobial agents, the development of which could be accelerated with structural data from a number of different enzymes with similar activities. Secondly, an interesting structural comparison is possible between *E. coli* glucose-1-phosphatase and phytase. Both enzymes are members of the histidine acid phosphatase family and share 31% amino-acid identity (Dassa *et al.*, 1990). These enzymes employ a two-step catalytic mechanism: nucleophilic attack of the scissile phosphate by a conserved histidine residue followed by hydrolysis of the phosphohistidine intermediate (Van Etten, 1982). Comparing the crystal structures of glucose-1-phosphatase and phytase is expected to help determine the structural features that influence substrate recognition and catalysis in these and other inositol phosphatases (Lim *et al.*, 2000; Golovan *et al.*, 2000).

2. Enzyme purification

The *E. coli* glucose-1-phosphatase was purified to homogeneity using a method similar to that established for *E. coli* phytase (Jia *et al.*, 1998). An overnight culture of *E. coli* CU1867 was used to inoculate 8 l of LB broth containing 100 µg ml^{-1} ampicillin and 20 µg ml^{-1} kanamycin. *E. coli* CU1867 is an *appA*-deficient mutant, as the gene encodes a bifunctional enzyme that exhibits both phytase and acid phosphatase activity (Golovan *et al.*, 2000).

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Table 1
Statistics of X-ray diffraction data.

Values given in parentheses refer to reflections in the outer resolution shell, 2.8–2.7 Å.

Wavelength (Å)	1.54
Temperature† (K)	100
No. of measured reflections	299912
No. of independent reflections	21735
Resolution (Å)	2.7
R_{sym}‡ (%)	5.8 (58.6)
Completeness (%)	95.2 (56.0)
Average $I/\sigma(I)$	11.4

† Light mineral oil was used as the cryoprotectant. ‡ Defined as $R_{sym} = \sum|I(k) - \langle I\rangle|/\sum I(k)$, where $I(k)$ and $\langle I\rangle$ represent the diffraction intensity values of the individual measurements and the corresponding mean values, respectively. The summation is over all measurements.

The culture was shaken at 200 rev min^{-1} in a reciprocating shaker for 24 h at 305 K. Cells were collected by centrifugation and suspended in 1 l of 50 m*M* Tris–HCl, 20%(*w*/*v*) sucrose and 1 m*M* EDTA. Lysozyme was added to a concentration of 1 mg ml^{-1} and the solution was incubated with shaking for 20 min (Ostanin *et al.*, 1992). This solution was concentrated by ultrafiltration through a PM10 membrane (Amicon) and then applied to DEAE-Sepharose Cl-6B. The column was washed and proteins were eluted with a linear gradient of 0–300 m*M* NaCl. Fractions were pooled on the basis of glucose-1-phosphatase activity and purity by SDS–PAGE. Pooled fractions were concentrated again by ultrafiltration through a PM10 membrane (Amicon) and dialyzed against 25 m*M* imidazole–HCl pH 7.0. The dialyzed sample was applied to a column packed with PBE 94 polybuffer exchanger (Pharmacia) equilibrated in 25 m*M* imidazole–HCl and eluted with a 1:8 dilution of polybuffer 94 (pH 4.0). Fractions were again monitored for glucose-1-phosphatase activity and those free from contaminating proteins were pooled. Polybuffer 94 was removed by gel filtration on Sephadex G-75 and fractions containing only the desired protein were concentrated by ultrafiltration and dialyzed with 10 m*M* sodium acetate buffer (pH 4.5).

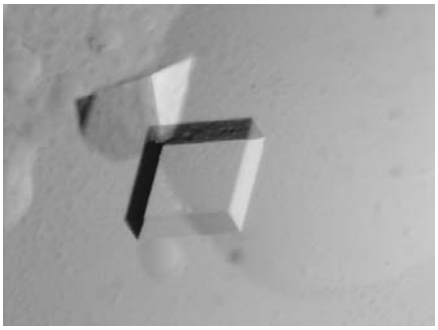

Figure 1
Typical crystals of *E. coli* glucose-1-phosphatase, with dimensions ~0.7 × 0.5 × 0.2 mm.

3. Enzyme assays

Glucose-1-phosphatase assays were performed in 200 µl of 0.1 *M* sodium acetate buffer pH 4.5 using 5 m*M* of glucose-1-phosphate as the substrate at 310 K for 10 min. The reaction was stopped by adding 133 µl of a mixture of ammonium molybdate, ammonium vanadate and nitric acid (Engelen *et al.*, 1994). The concentration of inorganic phosphate released was determined by comparison with a K_2HPO_4 standard measured at 405 nm.

4. Crystallization

The protein was concentrated to 10 mg ml^{-1} in 10 m*M* sodium acetate pH 4.5 for crystallization trials. Initial crystal screening by equilibrium vapour diffusion (hanging drop) was carried out using a sparse-matrix screen (Jancarik & Kim, 1991) with Crystal Screen kits (Hampton Research, California, USA) at room temperature (293 K). Crystals were first obtained within one week with 1.8 *M* ammonium sulfate and 10 m*M* cobalt chloride in 0.1 *M* MES at pH 6.5. However, it was later found that cobalt chloride was not necessary for crystallization and that reproducible crystals require an ammonium sulfate concentration of 2.4 *M*, as the first crystals observed from 1.8 *M* ammonium sulfate were the result of incomplete sealing.

Another crystallization condition consisting of ammonium sulfate and polyethylene glycol monomethyl ether 5000 produced crystals with higher quality and efficiency. After optimization, crystals were obtained overnight with 1.2 *M* ammonium sulfate and 25% PEG–MME 5000 in 0.05 *M* MES buffer at pH 6.5. Rhombohedral crystals grew to up to ~0.7 mm in the longest dimension (Fig. 1). Similar crystals could be obtained over the temperature range 277–310 K. Large crystals in the sealed chamber had a tendency to dissolve owing to temperature fluctuation resulting from the microscope light source. This problem was solved by placing the crystallization plate on an ice bed when the microscope was used.

5. X-ray diffraction

As crystals suffer from high radiation damage at room temperature, data collection was carried out at 100 K using a cryocooling apparatus (Oxford Cryosystems, Oxford, UK). Three cryoconditions were established: (i) 10% glycerol in crystallizing buffer, 3 min soaking, (ii) light mineral oil (Sigma) and (iii) 45% PEG 400 for 10 s.

The data were collected on a 30 cm MAR Research imaging plate equipped with a Rigaku rotating-anode X-ray generator operated at 50 kV, 100 mA and were processed using *DENZO* and *SCALEPACK* (Otwinowski, 1993). The crystals of *E. coli* glucose-1-phosphatase diffracted to at least 2.6 Å resolution and data were collected to 2.7 Å resolution. One of the most successful data sets so far was obtained using light mineral oil as cryoprotectant and the statistics are summarized in Table 1. The space group is *R*3, with unit-cell parameters $a = b = 156.0$, $c = 92.2$ Å. The data set is 95.2% complete, with $R_{sym} = 0.058$. Diffraction to 2.2 Å was observed using synchrotron radiation (CHESS) with a wavelength of 0.94 Å.

Since the two major components of the crystallizing buffer (1.2 *M* ammonium sulfate and 25% PEG–MME 5000) are immiscible, we often obtained small immiscible aqueous droplets attached to the concave crystal surface, giving rise to an icing problem during data collection. Neither a cryoprotectant of 10% glycerol in the crystallization buffer nor light mineral oil could completely solve this problem. However, 45% PEG 400 was found to dissolve these droplets rapidly and did not damage the crystals in a typical soaking time of 10 s.

Given that the molecular weight of *E. coli* glucose-1-phosphatase is 45 683 Da (413 amino acids), the calculated solvent content is 47.5% with $V_M = 2.36$ Å^3 Da^{-1}, assuming two enzyme molecules in the asymmetric unit. This result supports the previous observation that *E. coli* glucose-1-phosphatase exists as dimers in solution (Pradel & Boquet, 1988). In order to determine the relationship of the two monomers in the asymmetric unit, a self-rotation function calculation was preformed using the program *POLARRFN* from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994). Strong and unambiguous peaks were found at ($\psi = 90$, $\varphi = 220$, $\kappa = 180°$) with 92.7% magnitude of the origin peaks, in comparison with the next highest peaks of 36.9%. This result clearly indicates a non-crystallographic twofold axis relating two monomers of *E. coli* glucose-1-phosphatase in the asymmetric unit.

Using structural models of a number of proteins in the histidine acid phosphatase family (*E. coli* phytase, *A. niger* phytase and rat prostatic acid phosphatase) as probes, molecular replacement has not been successful. The same difficulty was experi-

enced in the structural determination of *E. coli* phytase (Lim *et al.*, 2000). Even though the various structures in histidine acid phosphatase family have a similar overall fold, the difference in structure is still large. As a result, a search for heavy-atom derivatives such as tungsten, silver, platinum, mercury and gold are in progress by both soaking and co-crystallization.

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