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Crystallization and preliminary X-ray characterization of a glycerol dehydrogenase from the human pathogen *Salmonella enterica* serovar Typhimurium

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Glycerol dehydrogenase (GldA) encoded by the STM4108 gene (*gldA*) has been related to the synthesis of HilA, a major transcriptional regulator that is responsible for the expression of invasion genes in the human pathogen *Salmonella enterica* serovar Typhimurium. Single colourless crystals were obtained from a recombinant preparation of GldA overexpressed in *Escherichia coli*. They belonged to space group $P222_1$, with unit-cell parameters a = 127.0, b = 160.1, c = 665.2 Å. The crystals contained a very large number of molecules in the asymmetric unit, probably 30–35. Diffraction data were collected to 3.5 Å resolution using synchrotron radiation at the European Synchrotron Radiation Facility.

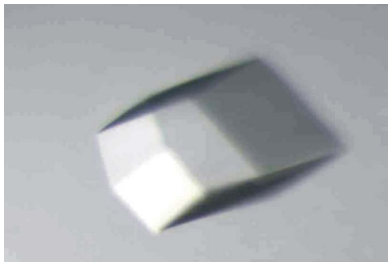
1. Introduction

Salmonella enterica serovar Typhimurium (*S. typhimurium*) is a Gram-negative rod-shaped bacteria that belongs to the *Enterobacteriaceae* family and is one of the most frequent causes of human foodborne infections (Lavigne & Blanc-Potard, 2008). The complete genome of *S. enterica* serovar Typhimurium LT2 has been sequenced (McClelland *et al.*, 2001) and contains at least two putative glycerol dehydrogenases genes: *gld*A and *glh*A.

Glycerol dehydrogenases have been related to several metabolic processes within the *Enterobacteriaceae* family, such as the oxidation of glycerol for energy purposes (Forage & Lin, 1982) and protection against toxic agents (Subedi *et al.*, 2008). In *S. typhimurium*, glycerol dehydrogenases have been described as being involved in the regulation of genes required for invasion of the host cell (Nakayama & Watanabe, 2006), which is the first and a necessary step for *Salmonella* pathogenesis.

Most of the genes required for the invasion of host epithelial cells are localized in a chromosome region termed *Salmonella* pathogenicity island 1 (SPI-1; Darwin & Miller, 1999). This region encodes effectors that are essential invasion mediators and components of the type III secretion machinery. HilA, a transcriptional regulator of the OmpR/ToxR family (Bajaj *et al.*, 1996), is believed to be the first global activator for the expression of SPI-1 genes (Eichelberg & Galan, 1999). Expression of HilA is regulated by a number of environmental and genetic factors (Lucas & Lee, 2000). Two putative NAD-dependent glycerol dehydrogenases genes, *gld*A and *glh*A, have been related to HilA regulation (Nakayama & Watanabe, 2006). These enzymes can use 1,2-propanediol (1,2-PD) as a substrate, which is a repressor of HilA expression (Nakayama & Watanabe, 2006).

In this study, we describe the purification, overexpression, crystallization and preliminary X-ray analysis of GldA from *S. typhimurium*, a member of the family III metal-dependent polyol dehydrogenases (Ruzheinikov *et al.*, 2001), which includes enzymes isolated from bacteria (Scopes, 1983) and yeast (Williamson & Paquin, 1987). Some members of this family require the presence of Fe^{2+} for catalysis (*e.g. Escherichia coli* propanediol dehydrogenase; Montella *et al.*, 2005), while others are Zn^{2+}-dependent (*e.g. Bacillus stearothermophilus* glycerol dehydrogenase; Ruzheinikov *et al.*, 2001).



The members of this family may also require cofactors such as NAD or NADP (Bouvet *et al.*, 1995). The three-dimensional structures of type III alcohol dehydrogenases roughly resemble the so-called 'medium-chain' alcohol dehydrogenases, generally showing a conserved secondary-structure pattern called the Rossmann fold, which is composed of six parallel β-strands surrounded by α-helices (Ruzheinikov *et al.*, 2001). Type III polyol dehydrogenases show a nucleotide-binding motif comprised of six parallel strands with a three-dimensional structure that is reminiscent of the classic Rossmann fold, but the connectivity between the secondary-structure elements is radically different (Montella *et al.*, 2005).

GldA is a polypeptide of 367 amino acids with a theoretical isoelectric point of 4.7 and a calculated molecular weight of 38 742 Da. The most closely related structures deposited in the Protein Data Bank, based on sequence similarity (Altschul *et al.*, 1997), are glycerol dehydrogenases from *Schizosaccharomyces pombe* (43% sequence identity; PDB code 1ta9), *Thermotoga maritima* (53% sequence identity; PDB code 1kq3) and *B. stearothermophilus* (50% sequence identity; PDB code 1jqa). All of them are members of the type III polyol dehydrogenase family.

2. Experimental

2.1. Protein expression and purification

The STM4108 (*gld*A) gene was amplified from *S. typhimurium* genomic DNA by a polymerase chain reaction (PCR) using complementary gene-specific primers (forward primer, ATGGATCGCATTATTCAGTCACC; reverse primer, TTATTCCCATTCCTGCAAGAAGC) and *Pfu* Turbo high-fidelity DNA polymerase (Stratagene). An initial DNA-denaturation step was performed at 368 K for 5 min, followed by 35 cycles of 1 min at 368 K, 1 min at 321 K and 1 min at 345 K and a final 15 min extension at 345 K. The generated blunt-end amplicons were purified using a PCR Clean-Up Kit (Promega). The obtained fragment was cloned into the pET151/D-TOPO vector using the TOPO cloning kit (Invitrogen). The vector adds an N-terminal His_6 tag, a V5 epitope and a tobacco etch virus (TEV) protease cleavage site to the expressed recombinant protein. Recombinant clones were selected through ampicillin resistance and colony PCR analysis, using T7 promoter and gene-specific oligonucleotide primers to confirm the presence of a correctly sized insert. The integrity of the cloned *gld*A gene was determined by DNA sequencing.

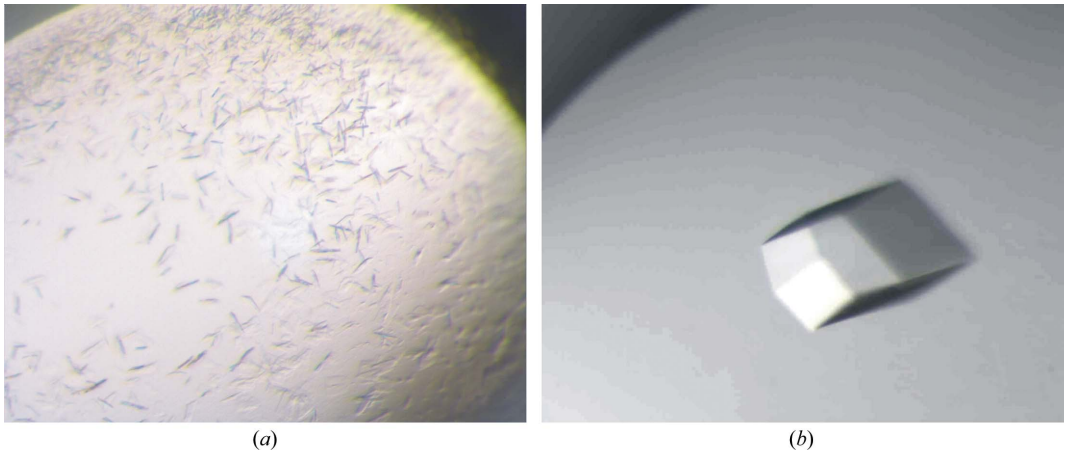
The expression construct was transformed into chemically competent *E. coli* BL21 STAR (DE3) cells (Invitrogen) using a heat-shock technique.

A small overnight culture grown at 310 K in Luria–Bertani broth containing 100 µg ml^{-1} ampicillin was used to inoculate a 1.4 l culture. Cells were grown to an OD_{600} of 0.7 in the same broth. Expression of the recombinant protein was then induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.75 m*M*. Cells were allowed to grow for 4 h after induction and were harvested by centrifugation at 7000*g* for 30 min at 277 K. They were frozen at 193 K, thawed and resuspended in 50 m*M* HEPES buffer pH 7.5 containing 1 *M* NaCl (buffer *A*). Lysis was performed using a French Press at 131 MPa and the cell-free supernatant was collected by centrifugation at 30 000*g* for 60 min at 277 K.

A 5 ml Hi-Trap chelating column (Amersham Biosciences) previously loaded with nickel ion and equilibrated with buffer *A* containing 20 m*M* imidazole was attached to an ÄKTA Explorer system (GE Healthcare). The soluble fraction was applied onto the column and washed with 30 ml buffer *A* containing 20 m*M* imidazole followed by 50 ml buffer *A* containing 60 m*M* imidazole. The target protein was eluted with a linear gradient of imidazole in buffer *A* from 60 to 500 m*M* in five column volumes. GldA fractions were identified by SDS–PAGE analysis and applied onto a Superdex S-75 gel-filtration column (Amersham Pharmacia) pre-equilibrated with 50 m*M* HEPES pH 7.4 containing 150 m*M* NaCl for size-exclusion chromatography. Fractions containing GldA were identified by SDS–PAGE, pooled and concentrated to 21 mg ml^{-1} in the same buffer using a Vivaspin concentrator (30 kDa molecular-weight cutoff). The protein concentration was estimated by absorption spectroscopy at 280 nm using a calculated extinction coefficient of 31 900 M^{-1} cm^{-1} (Gasteiger *et al.*, 2005). The estimated sample purity from SDS–PAGE was around 95%.

2.2. Crystallization

Initial crystallization screening was carried out at 295 K by the sitting-drop vapour-diffusion method in 96-well plates (Greiner) using a Cartesian Minibee nanolitre robot (Genomics Solutions). Crystallization screens from Qiagen (Classics, Classics Lite, PEGs, pH Clear and Cryos Suite) and Molecular Dimensions (Pact Premier) were used. Each drop had a final volume of 300 nl (1:1 ratio of protein and reservoir solution) and was equilibrated against 150 µl reservoir solution.



(*a*) (*b*)

Figure 1
Glycerol dehydrogenase (GldA) crystals grown at 293 K in 0.1 *M* Tris–HCl pH 8.5 with 25%(*w*/*v*) PEG 3000. (*a*) Crystals obtained with the automated crystallization method; (*b*) GldA crystal (0.25 × 0.4 × 0.3 mm in size) obtained in 5 d in a manually set-up drop in a 24-well plate using the sitting-drop diffusion method.

Table 1
Diffraction data-processing statistics.

Values in parentheses are for the highest resolution shell.

Source	ESRF ID23-1
Detector	MAR Mosaic 225 CCD
Space group	$P222_1$
Unit-cell parameters (Å)	a = 127.09, b = 160.11, c = 665.21
Wavelength (Å)	1.0673
No. of unique intensities	173742
Redundancy	3.5
Resolution (Å)	100–3.5 (3.63–3.5)
Completeness (%)	98.5 (95.2)
$R_{\rm merge}$ (%)	11.5 (33.2)
$R_{\rm p.i.m.}$ (%)	6.0 (18.0)
$I/\sigma(I)$	9.7 (2.0)

Crystals of GldA were obtained within 5–10 d in seven conditions from three distinct screens (Pact Premier, PEG I and Cryos Suite). Initially, all of them were manually reproduced on a microlitre scale (1:1 ratio of protein and reservoir solution equilibrated against 500 µl reservoir solution) in 24-well sitting-drop plates at 293 K. The crystals obtained were used for the first X-ray diffraction trials. Three crystallization conditions yielded higher diffracting crystals (conditions with crystals diffracting to 8 Å resolution or lower were excluded) and were subsequently modified by varying the pH and the temperature and using a screen of cationic additives. However, the best diffraction-quality crystals were found with an unmodified screen solution composed of 0.1 *M* Tris–HCl pH 8.5 and 25%(*w*/*v*) PEG 3000. Clusters of very thin needle-shaped crystals obtained using the automated crystallization method (Fig. 1*a*) were replaced by well ordered single crystals (Fig. 1*b*) in manually produced drops using exactly the same crystallization conditions and the sitting-drop vapour-diffusion method.

2.3. Cryoprotection

Crystals mounted in capillary tubes diffracted to approximately 4 Å resolution at room temperature using a rotating-anode generator (Bruker–Nonius). After cryocooling, most of the crystals diffracted only to low resolution (7 Å or less) and various post-crystallization treatments for improving the crystal diffraction were attempted. Sugars, low-molecular-weight PEGs and MPD were tested as cryoprotectants, without success.

The most successful post-crystallization treatment was the gradual equilibration of crystals against a cryoprotective agent. Crystals were grown in sitting drops by the vapour-diffusion method as already described, using 2 µl drops containing a 1:1 ratio of protein to reservoir solution. Cryoprotection was performed by adding mother liquor supplemented with a cryoprotective agent slowly to the drop (in steps of 0.2–0.4 µl) and allowing short equilibration periods of 5 min between each step. Two solutions were used in a total of 33 steps. The first 15 steps were performed using mother liquor supplemented with 5% ethylene glycol. In a second stage, a solution supplemented with 25% ethylene glycol was used. The drop volume was always maintained below 10 µl by removing two aliquots of 4 µl during the cryoprotection process at the end of each stage. This method yielded the best diffracting crystals.

2.4. Data collection

Several data sets were collected using synchrotron radiation at the ESRF, Grenoble (Table 1). The highest resolution data to date were obtained from a crystal submitted to a stepwise addition of cryobuffer, as described above, with dimensions of 0.25 × 0.7 × 0.25 mm vitrified in liquid nitrogen and measured on the ID23-1 beamline of the ESRF. Diffraction data were collected at 100 K using an Oxford Cryostream. To accommodate the problems of a large unit cell and poor spot separation, the crystals were mounted in carefully bent loops in order to avoid alignment of the longer axis with the direction of the beam. A multi-axis crystal-alignment apparatus (mini-kappa goniometer from Maatel) was used in some of the experiments. A perfect alignment of the crystallographic *c* axis with the rotation axis of the goniometer was never achieved and data sets with few overlaps of reflections were collected from crystals mounted close to this ideal orientation. Diffraction images were integrated with *MOSFLM* (Leslie, 2006) and the experimental intensities were scaled using *SCALA* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The *gldA* gene from *S. typhimurium* was successfully cloned in *E. coli* and the encoded hexahistidine-tagged GldA was purified to homogeneity by metal-chelating affinity chromatography. Good-sized and good-shaped crystals were obtained in drops set up manually with 25%(*w*/*v*) PEG 3000 as a precipitant agent and typically appeared in 5–10 d. The crystals suffered greatly upon freezing and a number of cryoprotection strategies were attempted. The most successful was a protocol designed in-house to slowly equilibrate the crystal against the cryoprotective agent, which is a modification of a protocol described in Heras & Martin (2005). Data collection was troublesome owing to difficulty with spot separation when the crystal was orientated close to alignment with the longer cell axis. This was coped with by mounting the crystals in carefully bent loops in order to avoid the longer axis orientation and in some cases using a multi-axis crystal-alignment setup. Structure solution by molecular replacement (MR) is currently under way using several homologous structures present in the PDB database.

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