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Crystallization and preliminary crystallographic analysis of bifunctional γ-glutamylcysteine synthetase–glutathione synthetase from *Streptococcus agalactiae*

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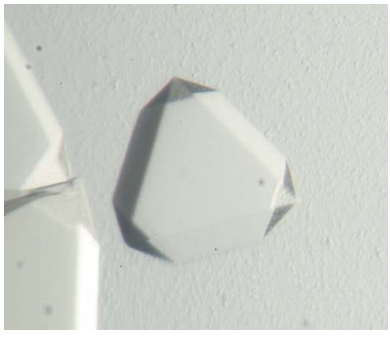
γ-Glutamylcysteine synthetase–glutathione synthetase (γGCS-GS) is a bifunctional enzyme that catalyzes two consecutive steps of ATP-dependent peptide formation in glutathione biosynthesis. *Streptococcus agalactiae* γGCS-GS is a target for the development of potential therapeutic agents. γGCS-GS was crystallized using the sitting-drop vapour-diffusion method. The crystals grew to dimensions of 0.3 × 0.2 × 0.2 mm under reducing conditions with 5 m*M* TCEP. X-ray data were collected to 2.8 Å resolution from a tetragonal crystal that belonged to space group $I4_1$.

1. Introduction

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH) is the main low-molecular-weight thiol in virtually all eukaryotes (Fahey *et al.*, 1984), many Gram-negative bacteria (Fahey *et al.*, 1978; Newton *et al.*, 1996) and some Gram-positive bacteria (Fahey *et al.*, 1978; Sherrill & Fahey, 1998). γ-Glutamylcysteine synthetase–glutathione synthetase (γGCS-GS) was first found in a Gram-positive bacterium, *Streptococcus agalactiae*, as a bifunctional enzyme (Janowiak & Griffith, 2005) that is a dimer with identical subunits (molecular mass 2 × 85 600; Janowiak *et al.*, 2006; Vergauwen *et al.*, 2006). This bifunctional enzyme catalyzes two consecutive steps of ATP-dependent peptide formation in GSH biosynthesis, the ATP-dependent ligation of glutamate and cysteine to form γ-glutamylcysteine (reaction 1) and the ATP-dependent ligation of glycine to that intermediate (reaction 2), which are catalyzed by different enzymes, γGCS and GS, respectively, in most organisms other than some Gram-positive bacteria. The N-terminal amino-acid sequence of the *S. agalactiae* γGCS-GS subunit (amino acids 1–520) has significant homology to *Escherichia coli* γGCS (32% identity, 43% similarity), but the C-terminal sequence (amino acids 360–750) contains an ATPase grasp domain that is homologous to *E. coli* D-Ala:D-Ala ligase (24% identity, 38% similarity). The N- and C-terminal constructs of γGCS-GS have been independently expressed and folded and showed significant γGCS and GS activity, respectively (Janowiak *et al.*, 2006). These results suggested that the original γGCS and D-Ala:D-Ala ligase genes became overlapped and acquired the activity of continuous GSH biosynthesis as a consequence of their evolutional adaptation. Since the plausible evolutional precusor of the GS domain has a dimer structure as a biological unit (Zawadzke *et al.*, 1991) and *E. coli* γGCS is a monomer (Gushima *et al.*, 1983), γGCS-GS was suggested to be dimerized through the GS domain. The functional roles of the dimerization and the domain–domain linkage still largely remain unknown, although the half-of-the-sites activity of γGCS-GS from Gram-negative *Pasteurella multocida* has been indicated, possibly as a consequence of intimate domain–domain interaction (Vergauwen *et al.*, 2006).

γGCS-GS has been identified as being responsible for GSH accumulation in some species of Gram-positive bacteria (Janowiak & Griffith, 2005; Gopal *et al.*, 2005) and Gram-negative bacteria (Ver-



gauwen *et al.*, 2006). Reaction 1 is generally a rate-limiting step in GSH biosynthesis and the cellular level of GSH is controlled through feedback inhibition of γGCS by competitive binding of GSH (Huang *et al.*, 1988; Richman & Meister, 1975). Strangely, neither the γGCS nor the GS activity of *S. agalactiae* γGCS-GS was inhibited by GSH at concentrations of up to 100 m*M*. This result provides us with a possible explanation for the observation that *S. agalactiae* maintains a much higher intracellular GSH concentration than *E. coli* in spite of its lower γGCS activity. Interestingly, most Gram-positive bacteria bearing γGCS-GS genes, such as *Streptococcus*, *Listeria* and *Clostridium*, are human pathogens that produce various infectious diseases. Although the physiological function of GSH in these pathogens has remained elusive, γGCS-GS has been indicated to be essential for the aerobic growth and virulence of the pathogens based on the observation that a glutathione-deficient strain of *L. monocytogenes* in which γGCS-GS was truncated at residue 466 was found to be markedly more sensitive to oxidative stress (Gopal *et al.*, 2005). These pieces of evidence led us to become interested in the structure and mechanism of the enzyme in order to design and develop inhibitors as antibiotic chemotherapeutic agents. Here, we describe the crystallization and preliminary crystallographic investigation of the bifunctional γGCS–GS.

2. Materials and methods

2.1. Protein expression and purification

The γGCS-GS protein was overexpressed according to the method of Janowiak & Griffith (2005) with slight modification. Chemically competent cells of *E. coli* strain SG13009[pREP4] (Qiagen) were transformed with the expression vector pQE30 bearing the *gshAB* gene (SAG1821) for full-length γGCS-GS with N-terminal His-tag residues (MRGSHHHHHHGS). The transformant cells were grown at 310 K in M9 culture medium containing 2%(*w*/*v*) casamino acids, 100 mg ml^{-1} ampicillin and 34 mg ml^{-1} chloramphenicol with constant shaking. When the absorbance of the culture at 660 nm reached 0.6, isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 0.1 m*M* and the culture was grown for an additional 15 h at 298 K.

The bacterial cells (∼10 g) were harvested by centrifugation and the cell pellet was resuspended in 20 m*M* sodium phosphate buffer pH 7.4 containing 1 m*M* EDTA and 5 m*M* 2-mercaptoethanol and then disrupted using a Vibra-Cell VCX500 ultrasonic processor (Sonics) at 277 K. The cell lysate was subjected to centrifugation at 20 000*g* for 20 min to remove the cell debris. Ammonium sulfate powder was slowly added to the cell-free extract to bring the salt concentration of the extract to 2.0 *M* and the precipitated protein was removed by centrifugation. The soluble supernatant was applied onto a 300 ml butyl-Toyopearl 650M column (Tosoh) equilibrated with 20 m*M* sodium phosphate buffer pH 7.4, 1 *M* ammonium sulfate and 10 m*M* NaCl and the bound protein was eluted using a linear gradient of 1.0–0.0 *M* ammonium sulfate. The fractions containing active γGCS-GS were pooled and dialyzed against 20 m*M* sodium phosphate buffer pH 7.4, 40 m*M* imidazole, 500 m*M* NaCl and 5 m*M* 2-mercaptoethanol, which was also used to equilibrate an Ni-affinity chromatography column (HiTrap HP column, GE Healthcare Bioscience). The applied enzyme was eluted by stepwise elution using 500 m*M* imidazole. Fractions containing active γGCS-GS were pooled and dialyzed against 50 m*M* Tris–HCl buffer pH 7.4, 5 m*M* $MgCl_2$, 5 m*M* 2-mercaptoethanol and 10%(*w*/*v*) glycerol. Final purification was achieved by chromatography on a CIM QA-8 tube monolithic column (BIA Separations) equilibrated with 50 m*M* Tris–HCl pH 7.4, 5 m*M* $MgCl_2$ and 10%(*w*/*v*) glycerol. The bound enzyme was eluted using a linear gradient of 0.0–1.0 *M* NaCl. The eluted enzyme was concentrated by centrifugal ultrafiltration (Amicon Ultra-15 10 kDa cutoff; Millipore) and dialyzed against 20 m*M* HEPES–NaOH buffer pH 7.8 containing 1 m*M* EDTA and 25%(*w*/*v*) glycerol; finally, TCEP was added to 10 m*M*. The purified enzyme was analyzed by SDS–PAGE, enzyme-activity assay and dynamic light-scattering (DLS) measurements. The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard. The γGCS activity and GS activity were assayed according to the method described by Janowiak & Griffith (2005). The DLS measurements were carried out using a DynaPro Titan DLS instrument (Wyatt). The purified enzyme solution was used for crystallization experiments in 20 m*M* HEPES–NaOH buffer pH 7.8 containing 1 m*M* EDTA, 10 m*M* TCEP and 25%(*w*/*v*) glycerol and was stored at 193 K until use.

2.2. Crystallization and X-ray data collection

For crystallization, the purified protein was concentrated to 20 mg ml^{-1} by centrifugal ultrafiltration (Amicon Ultra-500, 10 kDa cutoff; Millipore). Crystals of γGCS-GS were grown by the sitting-drop vapour-diffusion technique using a Cryschem crystallization plate (Hampton Research). 2 µl protein solution was mixed with an equal volume of reservoir solution containing 1.8 *M* ammonium sulfate, 0.2 *M* potassium sodium (+)-tartrate tetrahydrate, 0.1 *M* trisodium citrate dihydrate pH 5.6 and 0–5 m*M* tris(2-carboxyethyl)-phosphine (TCEP). The mixture was equilibrated against 1 ml reservoir solution at a constant temperature of 293 K.

X-ray diffraction data were collected from the native γGCS-GS crystal using an ADSC Quantum 4R CCD on synchrotron beamline BL-6A at the Photon Factory, KEK, Japan. The crystal was soaked momentarily in a cryoprotectant solution containing 10%(*w*/*v*) glycerol and the concentration of the cryoprotectant was increased to 25% by repetitively pipetting aliquots of 15–25% cryoprotectant solution onto the drop. The crystal obtained was flash-cooled in a 100 K dry nitrogen stream and then exposed to X-rays at 100 K. Individual frames consisted of a 0.25° oscillation angle measured for 10 s at a crystal-to-detector distance of 236 mm. Intensity data were processed, merged and scaled with *MOSFLM* and the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

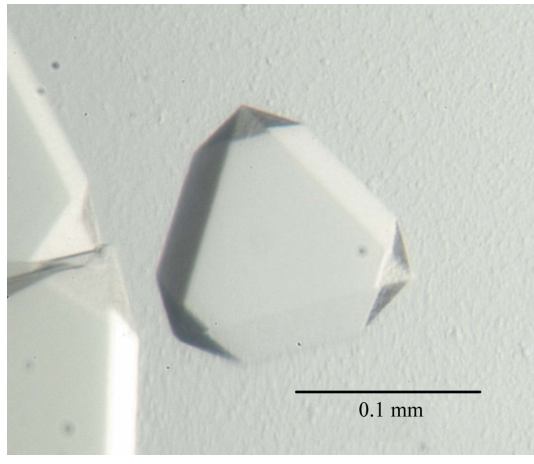


Figure 1
Crystals of γGCS-GS from *S. agalactiae* obtained using the sitting-drop method.

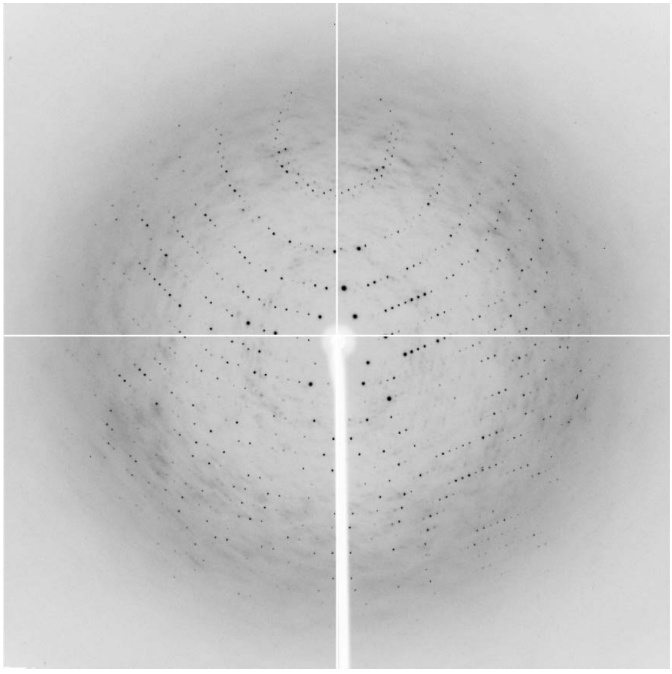


Figure 2
Diffraction image obtained on Photon Factory beamline BL-6A.

3. Results and discussion

γGCS-GS was overexpressed and purified, with a yield of 60 mg protein from 1 l culture. The purity of the obtained enzyme was judged to be >95% from SDS–PAGE analysis; dynamic light-scattering (DLS) analysis gave a monodisperse size distribution (data not shown). Crystallization of the enzyme was achieved using the sitting-drop method at 298 K. Initial crystallization conditions were found using the Crystal Screen HT kit (Hampton Research). Small prism-shaped crystals of less than 20 µm were obtained using condition No. 62 (0.1 *M* sodium citrate tribasic dihydrate pH 5.6, 2.0 *M* ammonium sulfate, 0.2 *M* potassium sodium tartrate tetrahydrate). Native PAGE and DLS analysis of the enzyme after the crystallization indicated its heterogeneous size distribution. Reduction of the enzyme molecules using dithiothreitol as a reducing agent significantly improved the size distribution after storage at 293 K for 12 h and the addition of TCEP provided the best result for crystal growth of the three reducing agents investigated (TCEP, 2-mercaptoethanol and dithiothreitol). As a result of further trials optimizing the crystallization conditions, crystals grew from a 20 mg ml^{-1} protein solution in 1.8 *M* ammonium sulfate, 0.2 *M* potassium sodium (+)-tartrate tetrahydrate, 0.1 *M* trisodium citrate dihydrate pH 5.6, 5 m*M* TCEP (Fig. 1). Crystals suitable for X-ray diffraction experiment grew to maximum dimensions of 0.2 × 0.2 × 0.1 mm within a month. These crystals were used to collect a native data set.

Diffraction data were collected from cryocooled (100 K) crystals using an ADSC CCD on beamline BL-6A at the Photon Factory (Fig. 2). Data were collected using 1° oscillations with the crystal-to-detector distance set to 236 mm. Analysis of merging statistics and systematic absences indicated that the crystals belonged to space group $I4_1$, with unit-cell parameters $a = b = 141.5$, $c = 208.1$ Å. Estimation of the content of the asymmetric unit based on a single γGCS-GS subunit gave a Matthews coefficient V_M of 3.05 Å^3 Da^{-1} (Matthews, 1968), corresponding to 59.4% solvent content. Data-collection and processing statistics are shown in Table 1.

Table 1
Data-collection statistics.

Values in parentheses are for the outer shell.

Space group	$I4_1$
Unit-cell parameters (Å, °)	$a = b = 141.45$, $c = 208.15$
No. of monomers per ASU	2
Wavelength (Å)	0.9726
Resolution (Å)	52.1–2.80 (2.95–2.80)
Total No. of reflections	380543
No. of unique reflections	50180
Completeness (%)	100.0 (100.0)
$I > 3\sigma(I)$ (%)	88.5
$\langle I/\sigma(I)\rangle$	30.6 (8.8)
Redundancy	7.6 (7.6)
$R_{\rm merge}$†	0.049 (0.211)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations i of reflection hkl.

Structure determination was attempted using the molecular-replacement method as implemented in the program *Phaser* (McCoy *et al.*, 2007). In spite of the sequence identity (∼30%) between γGCS-GS and the search models of other members of the γGCS or GS family, no solution could be found that correctly placed the template molecules in the crystal unit cells. The failure of the molecular replacement is probably a result of significant structure differences in the fusion enzyme. A search for heavy-atom derivatives for use in the multi-wavelength anomalous diffraction method is now under way.

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