Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Escherichia coli B γ-glutamylcysteine synthetase: modification, purification, crystallization and preliminary crystallographic analysis

Takao Hibi,[a]* Hiromoto Hisada,[b]† Toru Nakatsu,[c] Hiroaki Kato[c] and Jun'ichi Oda[a]

[a]Department of Bioscience, Fukui Prefectural University, Fukui 910-1195, Japan, [b]Institute for Chemical Research, Kyoto University, Uji 611-0011, Japan, and [c]Membrane Dynamics Research Group, RIKEN, Harima Institute at SPring-8, 1-1-1 Kouto, Mikazuki-cho, Sayo, Hyogo 679-5148, Japan

† Current address: Research Institute, Gekkeikan Sake Co. Ltd, 24 Shimotobakoyanagi-cho, Fushimi-ku, Kyoto 612-8385, Japan.

Correspondence e-mail: hibi@fpu.ac.jp

Received 14 September 2001
Accepted 19 November 2001

Escherichia coli B γ-glutamylcysteine synthetase (γGCS) catalyzes the ATP-dependent coupling of L-Glu and L-Cys to form the glutathione precursor γ-L-Glu-Cys and is a target for development of potential therapeutic agents. By introducing four point mutations of surface-exposed cysteine residues to serine, the γGCS was purified to homogeneity; single crystals have been obtained using the hanging-drop vapour-diffusion method with sodium formate. The γGCS crystal diffracted to 2.8 Å and belongs to space group $R3$, with unit-cell parameters $a = b = 326.7$, $c = 103.9$ Å.

1. Introduction

γ-Glutamylcysteine synthetase (γGCS) catalyzes the ATP-dependent coupling of L-Glu and L-Cys to form the glutathione precursor γ-L-Glu-Cys (Meister, 1974). This reaction is a rate-limiting step in glutathione biosynthesis and the cellular level of glutathione is controlled through its feedback inhibition by glutathione (Richman & Meister, 1975; Huang *et al.*, 1988). Glutathione plays a pivotal role in maintaining cellular redox balance as a non-protein sulfhydryl and also constitutes a front line of detoxification of reactive oxygen and other xenobiotics such as heavy metals, organic peroxides and electrophilic alkylating agents (Meister, 1974; Soltaninassab *et al.*, 2000). γGCS is a target for development of potential therapeutic agents such as parasiticides or drugs that can suppress multi-drug resistance of cancer cells (Bailey, 1998; Griffith, 1999). Several approaches aimed at controlling the level of glutathione have been investigated by the design and synthesis of potent inhibitors (Arrick *et al.*, 1981; Moncada *et al.*, 1989; Lautier *et al.*, 1992; Koizumi *et al.*, 1993; Yokomizo *et al.*, 1995; Anderson *et al.*, 1996; Tokutake *et al.*, 1998).

The γGCS used in this study was originally isolated from *E. coli* B mutant strain RC912 (Watanabe *et al.*, 1986), but has the same amino-acid sequence as the *E. coli* K12 enzyme. This prokaryotic enzyme is a single polypeptide chain with a molecular weight of 58 300 Da, unlike the eukaryotic enzymes which are composed of two non-identical subunits. However, *E. coli* and rat kidney γGCS not only have similar apparent K_m values, turnover numbers and substrate specificity, but are also both inhibited by glutathione (Huang *et al.*, 1988). Recently, significant homology was detected among all known γGCSs using analysis of conserved motifs and secondary-structure predictions; two highly conserved regions were predicted to be metal- and substrate-binding sites of γGCS (Abbott *et al.*, 2002). Interestingly, GCS were also homologous to the C-terminal domain of glutamine synthetase, which makes two hexamer rings. The crystallographic analysis of the *E. coli* enzyme will elucidate the mechanism of catalysis and the basis for feedback inhibition and will provide a framework for structure-assisted drug design for any species of γGCS.

Initial crystallization trials of this enzyme failed and this seemed to be because of its heterogeneous size distribution, as indicated by native PAGE and dynamic light-scattering (DLS) analysis. Reduction of the enzyme molecules with dithiothreitol provided an improvement in the distribution size, but no crystals were obtained. Our strategy to improve the chances of crystallization success was to identify surface-exposed cysteine residues by modification with a sulfhydryl fluorescence reagent and to mutate them to serine residues. Here, we report the first crystallization and preliminary X-ray diffraction analysis of γGCS.

2. Materials and methods

2.1. Chemical modification experiment

Fresh γGCS (0.93 mM) was incubated with 100 mM *N*-[*p*-(2-benzimidazoyl)-phenyl]-maleimide (BIPM, Wako Chemicals) at 298 K for 50 min in 25 mM Tris–HCl pH 7.5 containing 5 mM $MgSO_4$ and 1 mM EDTA. The BIPM-labelled enzyme was dialyzed against 50 mM Tris–HCl pH 9.0 containing 2 M urea overnight and then digested at 310 K for

24 h with lysyl endopeptidase at a protease-to-substrate ratio of 1:200(*w*/*w*). The resulting peptide fragments were separated by reverse-phase HPLC with a Capcell Pak C_{18} (Shiseido) column with the absorbance of peptides being continuously monitored at 215 nm and the label was detected by the fluorescence emission at 365 nm with excitation at 313 nm. The four labelled peptides were obtained in the first HPLC of the modified γGCS and were further purified to homogeneity by repeated reverse-phase HPLC. N-terminal amino-acid sequencing was performed with a gas-phase sequencer (Applied Biosystems Model 477 A) linked to a phenylthiohydantoin analyzer (Applied Biosystems Model 120 A).

2.2. Site-directed mutagenesis, expression and purification

Site-specific mutagenesis was performed by the method of Kunkel (1985). Mutations were checked using a Dye Terminator Cycle Sequencing FS Ready Reaction kit and an ABI PRISM 377 sequencer (Perkin–Elmer).

The C106S, C164S, C205S, C223S quadruple mutant of γGCS (γGCS4CS) was overexpressed according to Gushima *et al.* (1983) with slight modification. The expression vector pKGC20 bearing the *gsh*I gene of γGCS4CS was transformed into competent cells of *E. coli* strain JM109. The transformant cells were grown at 310 K in Terrific broth containing 50 mg ml^{-1} ampicillin with constant shaking. When absorbance of the culture reached 0.7 at 600 nm, isopropyl-β-D-galactoside was added to a final concentration of 1 m*M* and the culture was grown for an additional 14 h at 310 K.

The bacterial cell pellet was extracted in a buffer containing 50 m*M* Tris–HCl pH 7.5 and 5 m*M* $MgSO_4$ (buffer *A*) and after sonication the cell lysate was subjected to centrifugation at 20 000*g* for 20 min to remove the cell debris. The resulting cell-free extract was brought to 40% saturation by addition of solid ammonium sulfate and the precipitated protein was removed by centrifugation. The soluble supernatant was applied to 300 ml of a hydrophobic column (butyl-Toyopearl 650M, Tosoh), equilibrated with buffer *A* containing 40% saturation of ammonium sulfate and the bound protein was eluted using a linear gradient of 40–0% saturation of ammonium sulfate. Fractions containing active γGCS were pooled and dialyzed against 50 m*M* MOPS–NaOH pH 6.5, 5 m*M* $MnCl_2$ and 5 m*M* L-glutamic acid (buffer *B*). Further purification was achieved by affinity chromatography on an N-6 ATP agarose (Sigma) column equilibrated with buffer *B*. The enzyme was eluted from the column using a linear gradient of NaCl (0–0.25 *M*). Final purification was achieved by chromatography on an anion-exchange column (DEAE-Toyopearl 650M, Tosoh) equilibrated with 50 m*M* Tris–HCl pH 7.5, 5 m*M* $MgCl_2$ (buffer *C*). The enzyme was eluted from the column using a linear gradient of NaCl (0–0.15 *M*). The eluted enzyme was dialyzed against 5 m*M* sodium phosphate buffer pH 7.5 and concentrated using an Amicon ultrafiltration device (Stirred Cell 8400, 30 kDa cutoff). The purified enzyme was analyzed by SDS–PAGE, enzyme activity assay and DLS. The protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard. The enzyme activity was assayed according to the method described by Huang *et al.* (1988). The DLS measurement was carried out using a Dyna PRO-801 DLS instrument (Protein Solutions).

2.3. Crystallization

For crystallization, the purified protein was concentrated to 22.6 mg ml^{-1} by ultrafiltration (Ultrafree-MC; 30 kDa cutoff; Millipore). Crystals of γGCS were grown by the hanging-drop vapour-diffusion technique. 5 µl of protein solution was mixed with an equal volume of reservoir solution containing 3.9 *M* sodium formate pH 7.4 and the mixture was equilibrated against 1 ml of reservoir solution at 293 K.

2.4. Data collection

X-ray diffraction data were collected using a Rigaku R-AXIS IV++ imaging-plate detector and synchrotron radiation on beamline BL40B2, SPring-8, Harima, Japan. Native γGCS crystals were exposed to X-rays at 100 K after soaking the crystal momentarily in a cryoprotectant solution containing 4.0 *M* sodium formate and 20% glucose. A total of 90° of data were measured using the oscillation method. Individual frames consisted of a 1° oscillation measured for 5 min at a crystal-to-detector distance of 300 mm. Intensity data were processed, merged and scaled with *MOSFLM* and the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The sulfhydryl fluorescence reagent BIPM was used for identification of the cysteine residues on the solvent-accessible surface of wild-type γGCS. There was no effect on the enzymatic activity, although the incorporation of BIPM yielded 3.5 BIPM residues per enzyme molecule after saturation. The extent of incorporation did not change in the presence of the substrate, MgATP, L-Glu or L-Cys. To identify the BIPM-labelled sites, the modified enzyme was digested with lysyl endopeptidase and the digest was separated on a reverse-phase column. Four major peaks of fluorescence appeared in the chromatogram; each eluent with fluorescence was then subjected to N-terminal amino-acid sequence analysis. Consequently, the residues labelled with BIPM were identified as Cys106, Cys164, Cys205 and Cys223.

The four BIPM-labelled cysteine residues of γGCS were replaced with serine residues. The C106S, C164S, C205S, C223S quadruple mutant of γGCS (γGCS4CS) has been overexpressed in *E. coli* JM109 and purified. The purified protein migrated on SDS–PAGE as a single band of about 58 000 Da, which was in agreement with the predicted value of 58 117 Da. The quadruple mutation did not significantly affect the enzymatic activity, but γGCS4CS yielded a single band on native PAGE (Fig. 1) and had a stable monodisperse size distribution when analyzed by DLS. Its activity and monodisperse state did not change during two weeks at room temperature.

Crystallization of γGCS4CS was achieved by the hanging-drop method with Linbro tissue-culture plates. Crystallization conditions were found using the Crystal Screen I kit (Hampton Research). Small rhombic prismatic crystals of less than 20 mm in size

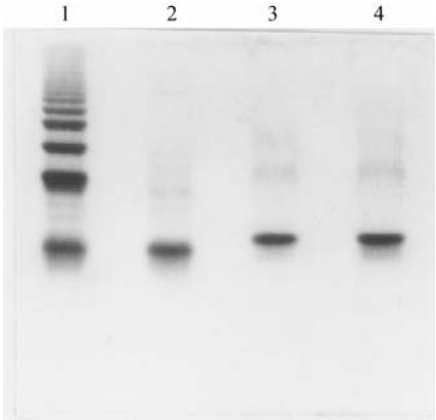

Figure 1
Native PAGE showing purified wild-type γGCS and mutant γGCS4CS samples. Each protein was subjected to electrophoresis on 9% polyacrylamide gel. Lane 1, wild-type γGCS kept under non-reducing conditions at 293 K for two weeks; lane 2, γGCS reduced with 20 m*M* cysteine; lane 3, mutant γGCS4CS kept under non-reducing conditions at 293 K for two weeks; lane 4, γGCS4CS reduced with 20 m*M* cysteine.

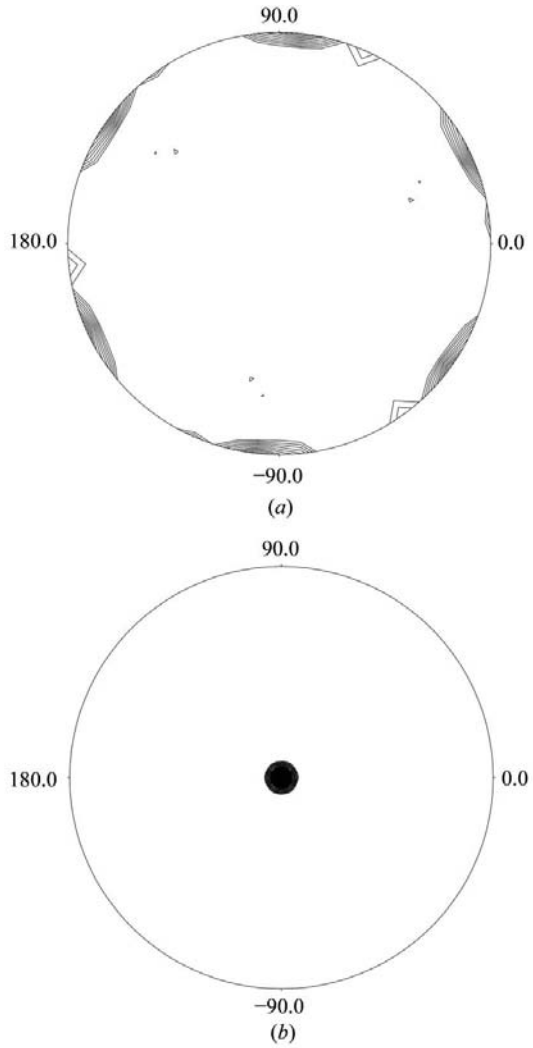

Figure 2
The self-rotation function of γGCS at κ angles of (*a*) 180° and (*b*) 120°. The data are in the resolution range 10.0–3.0 Å and the integration radius is 30.0 Å.

were obtained from condition No. 33 (4.0 *M* sodium formate pH 7.5). Further trials optimizing the concentration of precipitant yielded crystals suitable for X-ray diffraction using 3.9 *M* sodium formate. Crystals grew to maximum dimensions of 0.2 × 0.2 × 0.1 mm within a month. These crystals were used to collect native data.

Diffraction data were collected from cryocooled (100 K) crystals on an R-AXIS IV++ detector (Rigaku Co.) on beamline BL40B2 at SPring-8. Data were collected in 1° oscillations with the crystal-to-detector distance set to 300 mm. From auto-indexing in *MOSFLM*, the crystal was found to index in a rhombic lattice corresponding to space group *R*3 or *R*32. Merging the data in *R*32 gave poor statistics. The crystals were therefore scaled and merged in *R*3, with unit-cell parameters $a = b = 326.74$, $c = 103.96$ Å. There was no twofold symmetry in a pseudo-precession plot of the X-ray data. Using the rotation-function program *POLARRFN* (Collaborative Computational Project, Number 4, 1994), a general self-rotation function was computed with the reflections from 10 to 3 Å and a 30 Å radius of integration. Three twofold axes occur on the $\kappa = 180°$ section at $\omega = 90°$ and every 60° on φ (Fig. 2*a*), but no peaks appear on the $\kappa = 120°$ section except for the origin peak (Fig. 2*b*). These results suggest the non-crystallographic threefold axis is parallel or very close to the *c* axis and the three non-crystallographic twofold axes are perpendicular to the crystallographic threefold axis. Therefore, assuming six γGCS monomers per asymmetric unit, the Matthews coefficient is calculated to be 3.05 Å^3 Da^{-1} (Matthews, 1968), corresponding to a solvent content of 59.4%. The data are 99.8% complete to 2.8 Å resolution, consisting of a total number of 39 187 reflections containing 14 846 unique reflections. The completeness in the 2.95–2.80 Å shell is 99.5% and 40.9% of the data in this shell have $I > 3\sigma(I)$. $R_{\rm merge}$ for the entire data set collected on a single crystal is 6.9%, the overall value for $I/\sigma(I)$ is 9.5 and the averaged redundancy is 2.8 [for the outer shell, $R_{\rm merge} = 26.1\%$, $I/\sigma(I) = 2.8$ and the redundancy is 2.6]. The search for heavy-atom derivatives is now under way.

The synchrotron-radiation experiments were performed at SPring-8 with the approval of the Japan Synchrotron Radiation Research Institutes (JASRI; Proposal No. 2000-A0144-NL-np). The authors would like to thank Dr H. Moriyama and Dr K. Miura for excellent technical assistance. This work was in part supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture. TH was supported by a Grant-in-Aid for Scientific Research from Fukui-ken University Science Foundation.

References

Abbott, J. J., Pei, J., Ford, J. L., Qi, Y., Grishn, V. N., Pitcher, L. A., Phillips, M. A. & Grishin, N. K. (2002). In the press.
Anderson, J. K., Mo, J. Q., Hom, D. G., Lee, F. Y., Harnish, P., Hamill, R. W. & McNeil, T. H. (1996). *J. Neurochem.* **67**, 2164–2171.
Arrick, B. A., Griffith, O. W. & Cerami, A. (1981). *J. Exp. Med.* **153**, 720–725.
Bailey, H. H. (1998). *Chem.-Biol. Interact.* **111–112**, 239–254.
Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Griffith, O. W. (1999). *Free Rad. Biol. Med.* **27**, 922–935.
Gushima, H., Miya, T., Murata, K. & Kimura, A. (1983). *J. Appl. Biochem.* **5**, 43–52.
Huang, C.-S., Moore, W. R. & Meister, A. (1988). *Proc. Natl Acad. Sci. USA*, **85**, 2464–2468.
Koizumi, T., Negishi, M. & Ichikawa, A. (1993). *Biochem. Pharmacol.* **45**, 2457–2464.
Kunkel, T. A. (1985). *Proc. Natl Acad. Sci. USA*, **82**, 488.
Lautier, D., Luscher, P. & Tyrell, R. M. (1992). *Carcinogenesis*, **13**, 227–232.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Meister, A. (1974). *The Enzymes*, vol. 10, edited by P. D. Boyer, pp. 671–697. New York: Academic Press.
Moncada, C., Repetto, Y., Aldunate, J., Letelier, M. E. & Morello, A. (1989). *Comput. Biochem. Physiol. Res.* **94**, 87–91.
Richman, P. & Meister, A. (1975). *J. Biol. Chem.* **250**, 1422–1426.
Soltaninassab, S. R., Sekhar, K. R., Meredith, M. J. & Freeman, M. L. (2000). *J. Cell. Physiol.* **182**, 163–170.
Tokutake, N., Hiratake, J., Katoh, M., Irie, T., Kato, H. & Oda, J. (1998). *Bioorg. Med. Chem.* **6**, 1935–1953.
Watanabe, K., Yamano, Y., Murata, K. & Kimura, A. (1986). *Nucleic Acids Res.* **14**, 4393–4400.
Yokomizo, A., Kohno, K., Wada, M., Ono, M., Morrow, C. S., Cowan, K. H. & Kuwano, M. J. (1995). *J. Biol. Chem.* **270**, 19451–19457.