Cloning, Biochemical and Phylogenetic Characterizations of \(\gamma\)-Glutamylcysteine Synthetase from \textit{Anabaena} sp. PCC 7120

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\(\gamma\)-Glutamylcysteine synthetase (EC 6.3.2.2, \(\gamma\)-GCS) catalyzes the first step of glutathione synthesis: L-Glu + L-Cys + ATP = \(\gamma\)-L-glutamyl-L-cysteine (\(\gamma\)-GC) + ADP + Pi. We have cloned the gene \textsc{alr3351} of \textit{Anabaena} sp. PCC 7120, expressed the recombinant enzyme in \textit{Escherichia coli}, and characterized its product as \(\gamma\)-GCS by analyzing \(\gamma\)-GC production, ADP formation and Pi release. Apparent \(K_m\) values for L-Glu, ATP and L-Cys were estimated to be 0.82, 0.23 and 0.14 mM, respectively. Glutathione and L-buthionine sulfoximine were inhibitors with \(K_i\) values of 6.5 and 29.3 mM, respectively. The molecular mass of \textit{Anabaena} \(\gamma\)-GCS was estimated to be 43.4 kDa by SDS–PAGE and matrix-assisted laser desorption/ionization time of flight mass spectrometry. The important sequence for the activity of plant \(\gamma\)-GCS was found in \(\alpha\)-proteobacterial \(\gamma\)-GCSs but not in cyanobacterial enzymes, suggesting that the cyanobacterial \(\gamma\)-GCS gene is not the primary progenitor for the plant genes.

Keywords: \textit{Anabaena} — Cyanobacteria — \(\gamma\)-Glutamylcysteine synthetase — Glutathione — Molecular evolution

Abbreviations: ABA, L-\(\alpha\)-aminobutyric acid; BSO, L-buthionine sulfoximine; \(\gamma\)-GC, \(\gamma\)-glutamylcysteine; \(\gamma\)-GCS, \(\gamma\)-glutamylcysteine synthetase (EC 6.3.2.2); GSH-II, glutathione synthetase (EC 6.3.2.3); GSH, reduced form of glutathione; IPTG, isopropyl-\(\beta\)-D-thiogalactopyranoside; ORF, open reading frame; PCC, Pasteur Culture Collection.

Introduction

Glutathione, a tripeptide (L-\(\gamma\)-glutamyl-L-cysteinyl-glycine), is ubiquitous primarily in eukaryotes and Gram-negative bacteria, but is found in some strains of low-GC Gram-positive bacteria (Copley and Dhillon 2002). The reduced form of glutathione (GSH) participates in many biological processes: (i) the metabolism of sulfur-containing amino acids; (ii) the biosynthesis of leukotrienes and DNA; (iii) the defense systems involved in the detoxification of toxic xenobiotics and reactive oxygen species by scavenging free radicals and their intermediates or by working as an electron donor for glutathione peroxidase to reduce hydrogen peroxide; and (iv) the regulation of cellular redox states by reducing the disulfide linkage of proteins and other cellular thiol-containing molecules (Meister and Anderson 1983, Griffith and Mulcahy 1999). Another role for GSH has been reported: (v) the initiation and maintenance of cell division during post-embryonic root development (Vernoux et al. 2000). Recently, Yanagida et al. (2004) showed that (vi) GSH induces vernalization-induced bolting in rosette plants.

Cellular GSH may be largely maintained by reduction of the oxidized form of glutathione via glutathione reductase (EC 1.6.4.2), and through GSH de novo synthesis in two sequential ATP-dependent steps from its constituent amino acids. In the first step, catalyzed by \(\gamma\)-glutamylcysteine synthetase (\(\gamma\)-GCS), the dipeptide \(\gamma\)-L-glutamyl-L-cysteine (\(\gamma\)-GC) is produced from L-Glu and L-Cys: L-Glu + L-Cys + ATP = \(\gamma\)-GC + ADP + Pi. In the second step catalyzed by glutathione synthetase (GSH-II), Gly is added to the C-terminal site of \(\gamma\)-GC to form GSH: \(\gamma\)-GC + Gly + ATP = GSH + ADP + Pi. The first reaction of glutathione synthesis is thought to be the rate-limiting step because \(\gamma\)-GCS is inhibited by GSH. L-Buthionine sulfoximine (BSO) is also an inhibitor for \(\gamma\)-GCS (Griffith and Mulcahy 1999).

Although these catalytic properties and responses to inhibitors appear to be common to all \(\gamma\)-GCSs, the subunit structures of the enzymes in different species diverge from one other. Human and rat \(\gamma\)-GCSs are heterodimers, composed of catalytic (73 kDa) and regulatory (about 30.5 kDa) subunits (Huang et al. 1993). The regulatory subunit is also noted in \textit{Mus muscaris} and \textit{Rattus norvegicus} enzymes (Griffith and Mulcahy 1999). However, the oligomeric structures of these enzymes are not conserved, because yeast (78.3 kDa; Ohtaake and Yabuuchi 1991) and prokaryotic \(\gamma\)-GCSs (\textit{Escherichia coli}, 58.2 kDa; \textit{Thiobacillus ferroxoxidans}, 49 kDa) are monomeric enzymes (Griffith and Mulcahy 1999, Powles et al. 1996). In contrast, the genetic and molecular properties of GSH-II from prokaryotes to eukaryotes appear to share common molecular characteristics (Griffith and Mulcahy 1999).

Cyanobacterial cells contain a high concentration of intracellular glutathione (2–5 mM), mainly in the reduced form (Tel-Or et al. 1985, Mittler and Tel-Or 1991). Moreover, cyanobacterial cells can accumulate glutathione by feeding precursor amino acids, especially Cys (Suginaka et al. 1998), as in the...
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The occurrence of glutathione reductase in the cyanobacterium, Anabaena sp. PCC 7120 has been confirmed by cloning, sequencing and activity (Jiang et al. 1995). In the CyanoBase (http://www.kazusa.or.jp/cyanobase), 12 genes in the genome of Anabaena sp. PCC 7120 are ascribed to encode enzymes using glutathione as the substrate, such as glutathione reductase, glutathione \( S \)-transferases and glutathione peroxidase (Kaneko et al. 2001). Moreover, the open reading frame (ORF) of GSH-II has been confirmed in cyanobacterial genes, alr3859 of Anabaena sp. PCC 7120 and slr1238 of Synechocystis sp. PCC 6803 (Okumura et al. 1997). These results and backgrounds strongly suggest de novo glutathione synthesis in cyanobacterial cells, but no information on the genetic, enzymatic and molecular properties of cyanobacterial \( \gamma \)-GCS is available to date. We found that partial amino acid sequences of alr3351 from Anabaena sp. PCC 7120 are identical or similar to the sequences of plant \( \gamma \)-GCSs. Then, we cloned alr3351, expressed in E. coli, characterized the enzymatic properties of the purified protein, and concluded that the gene product was \( \gamma \)-GCS. A molecular phylogenetic tree showed that the plant \( \gamma \)-GCS is more closely related to \( \alpha \)-proteobacterial enzymes than cyanobacterial \( \gamma \)-GCS, leading us to conclude the molecular independency of cyanobacterial \( \gamma \)-GCS from that of plants.

**Results**

**Molecular cloning of Anabaena alr3351**

The ORF-designated alr3351, encoding a hypothetical protein consisting of 379 amino acid residues with a calculated \( M_r \) of 42,870 Da, was cloned and its coding product was expressed as a fusion protein, including a His tag-fused portion (2,540 Da). The fusion protein, designated hereafter as ALR3351, was purified by affinity chromatography. The \( M_r \) of ALR3351 was estimated to be about 45,000 Da by SDS–PAGE (Fig. 1A). The mass of the ALR3351 was confirmed further to be 45,382 Da by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (data not shown). These values were about 2 kDa higher than the \( M_r \) of the deduced amino acid sequence due to the His tag portion. The results in Fig. 1B show one protein band on the native PAGE (lane 1) of purified ALR3351. Activity staining of the enzyme by detecting the released Pi from ATP in the standard reaction mixture as the precipitate of Ca-phosphate (Manchenko 1994) revealed a band in the purified preparation (lane 2), which confirmed ALR3351 as a \( \gamma \)-GCS.

**\( \gamma \)-GCS activity of ALR3351**

To confirm the \( \gamma \)-GCS activity of the ALR3351, the NADH oxidation (Fig. 2) coupled with the ADP formation (May et al. 1998, Abbott et al. 2001) was examined. The reaction was dependent on the addition of L-Cys (2 mM; line 1) or L-\( \alpha \)-aminobutyric acid (ABA) (line 2) to the mixture containing L-Glu, ATP and ALR3351. GSH (5 mM) and BSO (20 mM)
Anabaena \(\gamma\)-glutamylcysteine synthetase added to the reaction mixture inhibited the ABA-dependent reaction by 30 and 15.5%, respectively.

Another reaction product, \(\gamma\)-GC, was identified by high-performance liquid chromatography (HPLC) analysis as described by Parmentier et al. (1998). The reaction containing ALR3351, ATP, L-Glu and L-Cys produced a compound having an elution time which coincided with that of authentic \(\gamma\)-GC, and this was undetectable in the absence of the enzyme (data not shown). These results obtained by different assay methods clearly indicated that ALR3351 was a functional \(\gamma\)-GCS.

The optimum pH of ALR3351 activity was 8.4 in Tris–HCl and bicarbonate–carbonate buffers. The \(k_{\text{cat}}\) value for ALR3351 was estimated to be 6.72 s\(^{-1}\) mol\(^{-1}\). The apparent \(K_m\) values of ALR3351 for L-Glu, L-Cys, ABA and ATP were 0.824, 0.14, 0.84 and 0.23 mM, respectively, and apparent \(K_i\) values for GSH and L-BSO were estimated to be 6.5 and 29.3 mM, respectively. These values do not differ greatly from those of \(\gamma\)-GCSs from bacteria to mammals (Griffith and Mulcahy 1999).

**Discussion**

The ORF of the filamentous cyanobacterium *Anabaena* sp. PCC 7120, alr3351, was confirmed to code \(\gamma\)-GCS, by cloning and characterization of the activity of the recombinant ALR3351. Most of the kinetic values as well as the responses to inhibitors of ALR3351 reported here were similar to those reported with rat kidney \(\gamma\)-GCS (Griffith and Mulcahy 1999). The estimated molecular mass of 43.4 kDa for *Anabaena* \(\gamma\)-GCS coincided with the calculated molecular mass of the deduced amino acid sequence. Cyanobacterial \(\gamma\)-GCS has a lower molecular mass than those of mammalian catalytic subunits, and those of monomeric forms from many organisms.

GSH has many important biological functions, and most of the cells maintain a concentration of above several mM, especially 2–5 mM in cyanobacteria (Tel-Or et al. 1985, Mittler and Tel-Or 1991). Cyanobacterial \(\gamma\)-GCS may be active under physiological GSH concentration (3.2 mM) (Suginaka et al. 1998), because the estimated \(K_i\) value of *Anabaena* \(\gamma\)-GCS for GSH was 6.5 mM.

According to molecular phylogenetic approaches, the hypothetical and ancestor \(\gamma\)-GCS would have evolved in three lines as shown in Fig. 3. Many investigators have pointed out that the ability to synthesize GSH probably originated from...
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cyanobacteria or photosynthetic purple bacteria and then
migrated to eukaryotes during an end-symbiotic process, result-
ing in the chloroplasts of modern plants (Fahey and Sundquist
1991, Griffith and Mulcahy 1999, Copley and Dhillon 2002). It
is thought that the enzymes involved in GSH biosynthesis of
cyanobacteria and chloroplast of plants descended from a com-
mon ancestor, therefore, knowledge of the genetic and molecu-
lar properties of cyanobacterial
\( \gamma \)-GCS is necessary to evaluate
the evolutionary process of plant
\( \gamma \)-GCSs. We compared amino
acid sequences of cyanobacterial
\( \gamma \)-GCS with those of chloro-
plast and cytosolic enzymes. In this study, amino acid se-
quences of representative
\( \gamma \)-GCSs from plants, cyanobacteria
and α-proteobacteria were compared (Fig. 4) as these proteo-
bacterial \( \gamma \)-GCSs are close to those from plants in the phylo-
genetic tree (Fig. 3). Of the two gene products of plants (top
three lines in Fig. 4), the protein coded by O23736 of
Brassica
juncea
(Chloroplast), P46309 for Arabidopsis
thaliana, Q9ZNX6 for Medicago trancac-
tula, Q8PPF8 for Xanthomonas axonop-
oides, Q89V83 for Bradyrhizobium
japonicum, SLR0990 for Synechocystis
sp. PCC 6803 and ALR3351 for Ana-
baena sp. PCC 7120. The numbers in
parentheses indicate the total amino acid residues of each γ-GCS.

![Fig. 4](https://academic.oup.com/pcp/article-abstract/46/4/557/1872932)

Thirty amino acid residues (colored purple) were con-
served among the sequences of \( \gamma \)-GCSs from chloro-
plasts (top two lines in Fig. 4), two α-proteobacteria, Q8PPF8 of
Xanthomonas axonopodis and Q89V83 of Bradyrhizobium
japonicum (middle two lines), and two cyanobacterial species
(bottom two lines), suggesting that these conserved amino acid
residues are related to the three-dimensional structure for the
same or similar enzymatic characteristics. Only 30 amino acid
residues (indicated by purple) in addition to the 10 green
colored amino acid residues were common between cyano-
bacteria and plant enzymes. On the other hand, >35% of iden-
tical amino acid residues (colored red) in Q8PPF8 (454 amino
acids) and Q87V83 (456 amino acids) were present in the cor-
responding sequences of plants. It may be important that the
Cd-sensitive Arabidopsis mutant lacks activity due to the 6 bp
deletion of the \( \gamma \)-GCS gene resulting in the deletion of two
amino acid residues, P and K at 241–242 (indicated by an
asterisk) shown in Fig. 4, and the replacement of one, V to L at
243, in the sequence of 240–263 (MPKVGTLGLDMML-
RTCTVQVNLDF, boxed by the red line) (Vernoux et al. 2000).
Another Arabidopsis mutant, designated rml1, in which
Asp262 was replaced by Asn, was reported to have extreme
sensitivity to Cd due to the undetectable activity and the very
low level of glutathione (Vernoux et al. 2000). Accordingly, the
boxed portion of the amino acid sequences is very important
for the functionality of \( \gamma \)-GCS in
Arabidopsis
cells. This
sequence is conserved, with the highest homology in enzymes
of α-proteobacteria, but not in the boxed region or in other
parts of the overall sequence of cyanobacterial \( \gamma \)-GCSs. These
results led us to hypothesize that plant \( \gamma \)-GCSs evolved from an
α-proteobacterial gene after horizontal transfer (Aoki and
Syono 1999) rather than from a cyanobacterial gene via endo-
symbiosis (Abdallah et al. 2000, Martin et al. 2002).

A similarity search has proved that 24,990 Arabidopsis
proteins have the greatest similarities with their yeast homo-
logs; the second largest fraction of genes are cyanobacterial
acquisitions, and a surprisingly large fraction of proteins are branched with homologs from Gram-positive bacteria (Martin et al. 2002). Martin et al. interpreted this by suggesting that the Arabidopsis lineage acquired genes from a Gram-positive donor subsequent to its divergence from the yeast lineage. Because plant γ-GCSs are far from yeast enzymes (Fig. 3), the gene for γ-GCS of α-proteobacteria was believed to have migrated to the nucleus of a host cell and replaced that of yeast or a eukaryotic host. During an endo-symbiotic event with a cyanobacteria-like organism, chloroplasts lost many cyanobacterial genes (Kaneko et al. 1996), thereby losing their autonomy (Abdallah et al. 2000). The gene for γ-GCS (454–456 amino acids) of α-proteobacteria incorporated into the nuclear genome of a host cell might have to be modified to attain the transit peptide portion for the chloroplast-localizing γ-GCS (514–522 amino acids). About 12% of nuclear-encoded Arabidopsis proteins are assumed to be localized in chloroplasts, because they possess a predicted transit peptide (Abdallah et al. 2000).

Materials and Methods

Materials

All chemicals were from Wako Pure Chemical Industries (Japan). Restriction enzymes and oligonucleotides were purchased from TaKaRa Biochemicals (Japan) and Greiner bio-one (Japan), respectively. E. coli cells and plasmids for gene manipulation were obtained from Novagen, Madison, WI, U.S.A. Anabaena sp. PCC7120 cells were grown photoautotrophically at 25°C in BG-11 medium (Rippka 1998) under illumination provided by fluorescent lamps at 100 µmol photons m⁻² s⁻¹. The culture was agitated on a reciprocal shaker (BR-300L, TAITEC, Japan) at 100 strokes min⁻¹. The cells were grown photoautotrophically at 25°C in 2-YT medium supplemented with 50 µg ml⁻¹ ampicillin. After 2 h, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.4 mM and incubation was continued for an additional 18 h at 30°C. The E. coli cells were harvested and then homogenized with BugBuster protein extraction buffer (Novagen). After centrifugation at 15,000 g for 15 min, the fusion protein was purified with His-Bind-Resin (Novagen, Madison, WI, USA) according to the manufacturer’s instruction. The concentration of protein was determined using a BioRad protein assay kit with bovine serum albumin (BSA) as the standard. SDS–PAGE was performed on a 10–15% (w/v) gradient polyacrylamide gel. Proteins on the gels were stained with Coomassie brilliant blue R-250.

Overexpression of the product of ORF alr3351 in E. coli

Chromosomal DNA from the Anabaena sp. PCC7120 cells was prepared by the method of Tandeau de Marsac et al. (1982) and used as a template for polymerase chain reaction (PCR). The 1.1 kb fragment of DNA that contained ORF alr3351 was amplified by PCR with the following primers: 5′-CATATGAGGTTCTATGGAAAGGC-3′ and 5′-GGATCTTACCCTTTAGCTG-3′. The amplified fragment was purified and cloned into a pT7 Blue-T vector. The resultant plasmid was used for transformation of E. coli JM109. Both strands of the cloned fragment were sequenced with an automated DNA sequencer (ABI 310, Perkin Elmer, Foster City, CA, USA.) to confirm the identity of the product of PCR. The plasmid was digested with Ndel and BamHI, and the resultant 1.1 kb DNA fragment was cloned into the vector pET16b for the subsequent expression of a His-tagged fusion protein. The product was designated pET3351 and used for the transformation of E. coli BL21 (DE3)-pLysS. Transformed cells were grown at 37°C in 2-YT medium supplemented with 50 µg ml⁻¹ ampicillin. After 2 h, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.4 mM and incubation was continued for an additional 18 h at 30°C. The E. coli cells were harvested and then homogenized with BugBuster protein extraction buffer (Novagen). After centrifugation at 15,000 × g for 15 min, the fusion protein was purified with His-Bind-Resin (Novagen, Madison, WI, USA) according to the manufacturer’s instruction. The concentration of protein was determined using a BioRad protein assay kit with bovine serum albumin (BSA) as the standard. SDS–PAGE was performed on a 10–15% (w/v) gradient polyacrylamide gel. Proteins on the gels were stained with Coomassie brilliant blue R-250.

Assays of γ-GCS activity

The enzymatic activity of γ-GCS was assayed by three methods detecting the reaction products, γ-GC, ADP or Pi. For γ-GC formation, the substrate Cys and the product γ-GC were assayed by HPLC with o-phthalaldehyde post-column derivatization methods (Parmentier et al. 1998). The ADP formation in the reaction was followed by a pyruvate kinase–lactate dehydrogenase coupled assay (May et al. 1998, Abbott et al. 2001). Oxidation of NADH was monitored at 340 nm (ε = 6.2 mM⁻¹ cm⁻¹) and was assumed to equal the rate of ADP formation. Steady-state kinetic parameters were determined by the formation of ADP by systematically varying the concentrations of substrate (1–Glu, 0.2–0.4 mM; l-Cys, 0.0625–0.5 mM; ATP, 0.08–0.4 mM, and ABA, 0.4–2.0 mM). The incubation was performed in 50 mM Tris–HCl, pH 8.4 at 25°C. The kinetic constants were calculated from double reciprocal plots of the initial rates of NADH oxidation. Maximal activity (Vₘₐₓ) was expressed as the turnover number (s⁻¹) with an estimated Mₘₐₓ of 45,400 Da. For inhibition studies, BSO (10 and 20 mM) or GSH (2.5, 5 and 7.5 mM) were added to the γ-GCS reaction mixture for the assay of ADP formation, and Glu was varied over its dynamic concentration range while ATP and ABA were kept constant at saturation-level concentrations. For the detection of Pi released during the γ-GCS reaction, crude extract from E. coli BL21(DE3)-pLysS with pET3351 and purified enzyme were separated by electrohoresis on a 12.5% native polyacrylamide gel. The gel was rinsed with 50 mM Tris–HCl, pH 8.4, and incubated at 25°C in the staining medium containing 0.14 M Tris–HCl, pH 8.4, 0.14 M MgCl₂, 14 mM l-Glu, 14 mM ATP, 38 mM CaCl₂ and 14 mM ABA, producing Ca-phosphate as a white precipitate (Mancherek 1994).

Construction of the phylogenetic tree

Inferences concerning the evolution of γ-GCSs were constructed using the PHYLIP 3.5c. software (Felsenstein 1996).

Acknowledgments

This work was supported in part by the Ministry of Education, Sports, Science and Culture of Japan (Grant-in-Aid for Scientific Research, No. 15658033).

References


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