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Redox-dependent stability of the γ-glutamylcysteine synthetase enzyme of *Escherichia coli*: a novel means of redox regulation

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Glutathione is a thiol-containing tripeptide that plays important roles in redox-related processes. The first step in glutathione biosynthesis is catalysed by γ-GCS (γ-glutamylcysteine synthetase). The crystal structure of *Escherichia coli* γ-GCS has revealed the presence of a disulfide bond. As the disulfide-bonding cysteine residues Cys^{372} and Cys^{395} are not well conserved among γ-GCS enzymes in this lineage, we have initiated a biochemical genetic strategy to investigate the functional importance of these and other cysteine residues. In a cysteine-free γ-GCS that was non-functional, suppressor analysis yielded combinations of cysteine and aromatic residues at the position of the disulfide bond, and one mutant that lacked any cysteine residues. Kinetic analysis of the wild-type and mutant enzymes revealed that the disulfide bond was not involved in determining the affinity of the enzyme towards its substrate, but had an important role in determining the stability of the protein, and its catalytic efficiency. We show that *in vivo* the γ-GCS enzyme can also exist in a reduced form and that the mutants lacking the disulfide bond show a decreased half-life. These results demonstrate a novel means of regulation of γ-GCS by the redox environment that works by an alteration in its stability.

Key words: degradation, γ-glutamylcysteine synthetase (γ-GCS), glutathione, redox regulation, stability.

INTRODUCTION

Glutathione, the most abundant small molecular mass thiol-containing compound present in living cells, carries out several important functions that include a role as the cellular redox buffer, as an antioxidant, and in the cellular response to different stress conditions [1].

In prokaryotes, glutathione is not as universally present as in eukaryotes. Furthermore, unlike eukaryotes, where glutathione has been found to be an essential metabolite, in prokaryotes glutathione appears to be dispensable under normal growth conditions, although it is required in bacterial responses to different kinds of osmotic, oxidative and metal stresses [2–4].

The biosynthesis of GSH occurs by two sequential ATP-dependent enzymatic steps. The first enzyme γ-GCS (γ-glutamylcysteine synthetase, *gshA*) ligates glutamate and cysteine to yield γ-glutamylcysteine. GS (glutathione synthetase, *gshB*), the second enzyme, then catalyses the addition of glycine to yield glutathione. In eukaryotes these activities are encoded by two distinct enzymes as indicated above. In some prokaryotes, such as *Escherichia coli* and *Vibrio cholerae*, separate enzymes exist for these two reactions. However, in some prokaryotes, such as *Streptococcus agalactiae* [5], *Pasteurella multicoda* [6] and *Listeria monocytogenes* [7], both of these activities are encoded by a single bifunctional enzyme (*gshF*).

The analysis of the γ-GCS enzyme from different organisms has revealed that the evolution of γ-GCS has occurred by convergent evolution in three different lineages with no significant sequence similarities between the lineages. The *E. coli* γ-GCS enzyme belongs to lineage I and includes other γ-proteobacteria. It also includes the γ-GCS domain of the bifunctional *gshF* enzymes. Lineage I enzymes are distinct from those of non-plant eukaryotes (animal/fungal) (lineage II) and from the plant γ-GCS lineage III that includes *Brassica juncea*, *Arabidopsis thaliana* and some plant-associated bacteria [8].

γ-GCS is also the rate-limiting and regulated step of GSH biosynthesis, being both transcriptionally and post-translationally regulated. A post-translational regulation of the γ-GCS enzyme by the redox environment was first shown for the mammalian [9] and *Drosophila* [10] enzymes (lineage II) through biochemical studies. The γ-GCS enzymes from these organisms have been shown to exist as heterodimers with catalytic and regulatory subunits. Conserved cysteine residues in the catalytic and regulatory subunit have been found to be involved in the disulfide bond formation between the two subunits. The disulfide bond that is formed under oxidizing conditions increases the substrate affinity and decreases the feedback inhibition by glutathione and thus the enzyme also functions as a redox sensor [10,11].

A study of the regulation by the cellular redox environment was carried out with the plant γ-GCS enzyme (lineage III), which also includes some plant-associated bacteria. The crystal structure of the γ-GCS enzyme from *B. juncea* [12] and studies with both the *B. juncea* and *A. thaliana* enzymes [12,13] have revealed the presence of two intramolecular disulfide bonds, CC1 and CC2. CC1 had limited influence on enzyme activity, but disruption (or reduction) of the CC2 disulfide bond severely affected the enzyme activities, and was responsible for the enzyme's redox sensitivity. Both studies have clearly demonstrated that this disulfide bond

Abbreviations used: AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; CF, cysteine-free; DTT, dithiothreitol; DTT_{Ox}, *trans*-4,5-dihydroxy-1,2,dithane; γ-GCS, γ-glutamylcysteine synthetase; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria–Bertani; LDH, lactate dehydrogenase; MM(PEG), methyl-[poly(ethylene glycol)] maleimide; Ni-NTA, Ni^{2+}-nitrilotriacetate; PK, pyruvate kinase; SD, synthetic define; SMG, *S*-methyl glutathione; TCA, trichloroacetic acid.

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acts as a redox sensor to modulate the activity of γ-GCS [12,13]. Interestingly, the plant-associated bacteria, that are also part of the plant lineage and include *Xanthomonas campestris* and *Agrobacterium tumefaciens*, contained the equivalent of the CC2 disulfide. However, despite the presence of this disulfide bond, these bacteria were, surprisingly, not found to display similar redox-sensitive activities on reduction/oxidation of the disulfide bond similar to that observed in plants [14].

The *E. coli* γ-GCS that belongs to lineage I has been shown to exist as a monomeric enzyme. The crystal structure of the *E. coli* γ-GCS enzyme has revealed one disulfide bond between Cys^{372} and Cys^{395} [15], but the role of the disulfide bond, if any, has not been investigated. On the one hand, the possibility of the disulfide bond being involved as a redox sensor has seemed unlikely given the findings with the plant lineage bacteria. However, on the other hand, the very existence of a disulfide bond in the structure of *E. coli* γ-GCS, a cytosolic enzyme, when most cytosolic enzymes rarely have disulfide bonds, has suggested that it might still play an important role. The present study addresses the need, if any, of the disulfide-bonded cysteine residues Cys^{372} and Cys^{395}, as well as other cysteine residues in *E. coli* γ-GCS using a genetic and biochemical approach. Our findings reveal a novel means to redox regulation that is mediated through stability of the reduced form of γ-GCS, a key enzyme involved in glutathione homoeostasis.

MATERIALS AND METHODS

Chemicals and reagents

All of the chemicals used in the present study were either of analytical or molecular biology grade and were obtained from commercial sources. Media components were purchased from Difco. Oligonucleotides were purchased from Sigma. Restriction enzymes, Vent DNA polymerase and other DNA-modifying enzymes were obtained from New England Biolabs. The enzymes LDH (lactate dehydrogenase) and PK (pyruvate kinase) were procured from Roche. The DNA sequencing kit (ABI PRISM 310 XL with dye termination cycle sequencing ready reaction kit) was obtained from PerkinElmer. Gel extraction kits, plasmid miniprep columns and the Ni-NTA (Ni^{2+}-nitrilotriacetate) agarose resin were obtained from QIAGEN. His-tagged mouse monoclonal antibody was bought from Cell Signaling Technology and ECL (enhanced chemiluminescence) Plus Western Blotting detection reagents were purchased from Amersham Biosciences. The radiolabelled ^{35}S-Eligmix (70 % L-[^{35}S]methionine and 30 % L-[^{35}S]cysteine) was procured from BRIT. Other reagents were from Sigma–Aldrich, HiMedia, Merck or USB Corporation. The monobromobimane AMS (4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid) and MM(PEG) {methyl-[poly(ethylene glycol)] maleimide} were from Invitrogen BioServices India.

Strains, media and growth conditions

The *E. coli* strain DH5α was used as a cloning host. For protein expression, either *E. coli* BL21 (DE3) or *E. coli* Origami was used. The *Saccharomyces cerevisiae* strain ABC1195 (*MAT*α *his3*Δ1 *leu2*Δ-0 *lys2*Δ-0 *ura3*Δ-0 *gsh*1Δ:: *LEU2*) was used for the genetic screens and for complementation assays. The *S. cerevisiae* strain was maintained on YPD [1 % (w/v) yeast extract/2 % (w/v) peptone/2 % (w/v) glucose] medium. SD (synthetic define) minimal medium contained yeast nitrogen base, ammonium sulfate and dextrose, and was supplemented with histidine, leucine and lysine at concentrations of 80 mg/l. Glutathione was added as required. Yeast transformation was carried out by the lithium acetate method [16].

Creation of CF (cysteine-free) *E. coli gshA* mutants

The *E. coli gshA* gene was amplified by PCR from genomic DNA and cloned downstream of the single-copy yeast expression vector pTEF416 using the restriction enzyme sites BamHI and XhoI. The *E. coli gshA* open reading frame with surface cysteine residues at positions 106, 164, 205 and 223 mutated to serine cloned into pKGC20 vector was a gift from Dr Takao Hibi (Fukui Prefectural University, Fukui, Japan). The fragment containing 4CF to serine mutations (EcoRI–SnaBI) was excised from pKGC20 and subcloned into *E. coli gshA* in pTEF416.

Site-directed mutagenesis was used to mutate the remaining native cysteine residues at positions 357, 372, 395, 433 and 439 sequentially to serine by Splice Overlap Extension PCR by using the 4CF *E. coli gshA* gene as a template and pairs of complementary oligonucleotides (Supplementary Table S1 at http://www.biochemj.org/bj/449/bj4490783add.htm). Oligonucleotides were designed to introduce a single nucleotide change resulting in an amino acid substitution from cysteine to serine. Amplified plasmid DNA of the resulting mutants was isolated and sequenced to confirm the presence of the desired nucleotide changes and to rule out any undesired mutations introduced during the mutagenic procedure. In the process of generating a 9CF *gshA*, a series of CF mutants bearing different numbers and combinations of cysteine-to-serine mutations were generated (Table 1).

Hydroxylamine mutagenesis

The *in vitro* mutagenesis of the 9CF *gshA* clone was performed using hydroxylamine as described previously [17] with some modifications [18].

Cloning, expression and purification of *E. coli* wild-type and mutant *gshA*

For protein expression studies, the *E. coli* and mutant *gshA* genes were His-tagged and expressed in the pET23a vector. For the His-tagging of wild-type and mutants, 1.52 kb of *E. coli gshA* gene was PCR amplified with SmaIFw and EcGshA_CHis _BamHIRev primers. The amplified 1.52 kb gene was digested with SmaI and BamHI restriction enzymes and cloned into the NdeI (that was blunted) and BamHI sites in the pET23a vector. The in-frame fusions were confirmed by sequencing.

All proteins were expressed as C-terminal His_6-tagged fusion proteins in *E. coli* BL21 or *E. coli* Origami strains in LB (Luria–Bertani) broth with 100 μg/ml ampicillin and at a temperature of 37 °C. At a D_{600} of $\sim$0.6, the cultures were induced with 1 mM IPTG (isopropyl β-D-thiogalactopyranoside). Cells were harvested after 5 h by centrifugation at 2500 ***g*** for 5 min at 4 °C following which they were lysed by sonication in the presence of 50 mM Tris/HCl, pH 8.0, 300 mM NaCl and 1 mM PMSF. The lysate was centrifuged at 15000 ***g*** for 30 min at 4 °C to remove the insoluble debris. Cleared lysate was then loaded on to an equilibrated Ni-NTA column and subsequently washed with wash buffer (50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 1mM PMSF and 20 mM imidazole). Elution was performed in the presence of elution buffer carrying 250 mM imidazole and the samples were analysed on SDS/PAGE (10 % gel). Protein quantification was performed by measuring absorbance at A_{280}.

Table 1 ***In vivo*** **and** ***in vitro*** **functional status of different mutants**

+++, yes; −, no; N.D., not determined; SDM, obtained by site-directed mutagenesis.

Strain	*In vivo* complementation	*In vitro* activity	Derived from
Wild-type GshA	+++	+++	
2CF (C433S,C439S)	+++	N.D.	SDM
4CF (C106S,C164S,C205S,C223S)	+++	N.D.	SDM
6CF (C106S,C164S,C205S,C223S,C433S,C439S)	+++	N.D.	SDM
7CF (C106S,C164S,C205S,C223S,C357S,C433S,C439S)	+++	+++	SDM
8CF (C106S,C164S,C205S,C223S,C357S,C372S,C433S,C439S)	−	N.D.	SDM
9CF (C106S,C164S,C205S,C223S,C357S,C372S,C395S,C433S,C439S)	−	−	SDM
9CF+S372F+S395C	+++	+++	9CF suppressor
9CF+S372F+C395S	−	N.D.	SDM
9CF+S372C+S395W	+++	+++	9CF suppressor
9CF+C372S+S395W	−	N.D.	SDM
9CF+S372C+S395Y	+++	+++	9CF suppressor
9CF+C372S+S395Y	−	N.D.	SDM
9CF+R374Q+V375F	+++	+++	9CF suppressor
9CF+V375F	+++	N.D.	Subcloning of R374QV375F
9CF+R374Q	−	N.D.	Subcloning of R347QV375F
9CF+S372W+S395C	+++	N.D.	SDM
F357 P358 C372 W395	+++	N.D.	SDM
F357 P358 C372 W395S	−	N.D.	SDM
F357 P358 C372S W395S	−	N.D.	SDM

Multiple sequence analysis and phylogenetic analysis

The *E. coli* γ-GCS protein sequence was used as the query sequence to retrieve the γ-GCS homologues of different organisms of lineage I (Supplementary Table S2 at http://www.biochemj.org/bj/449/bj4490783add.htm). The multiple sequence alignment of the protein sequences was generated using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) using default parameters and the MEGA 3.1 software was used to construct the phylogenetic tree using the neighbour-joining method.

γ-GCS activity assay

The activity of *E. coli* γ-GCS was assayed by measuring the rate of ADP formation using a coupled assay that involved PK and LDH [19]. A standard reaction mixture of 800 μl contained 150 mM Tris/HCl, pH 8.0, 150 mM KCl, 40 mM $MgCl_2$, 2 mM sodium phosphoenol pyruvate, 4 mM sodium ATP, 20 mM sodium L-glutamate, 15 mM L-cysteine, 1.6 units of PK (rabbit muscle), 4 units of LDH (rabbit muscle type II) and 0.24 mM NADH. The reaction was initiated by adding 1 μg of protein. The rate of decrease in NADH concentration was monitored at 340 nm using a spectrophotometer (UV-1800, Shimadzu, UV spectrophotometer) for 5 min. The reaction was carried out at 25 °C.

For the determination of the K_m value for L-glutamate, L-cysteine was added at 15 mM and ATP at 4 mM. For determining the K_m value for cysteine, L-glutamate was added at 20 mM and ATP at 4 mM whereas the K_m determination for ATP was carried out at 20 mM L-glutamate and 15 mM L-cysteine. The kinetic parameters were calculated using GraphPad Prism software.

Functionality assay by *in vivo* complementation in *S. cerevisiae*

The yeast strain ABC 1195 (Sc*gsh*1Δ) was transformed with wild-type or mutant *gshA* gene downstream of the TEF promoter in pTEF416. Transformants were grown in minimal media containing 0.2 mM glutathione and other amino acid supplements. The overnight culture was used to reinoculate the secondary culture and incubated until $D_{600} = 0.6$. Equal numbers of cells were harvested, washed with water and suspended in sterile water to a $D_{600} = 0.2$. Serial dilutions were prepared and 10 μl was spotted on to SD medium with or without glutathione.

CD spectroscopy

Thermal denaturation studies were performed by monitoring the change in signal at 222 nm using CD on a Jasco 810 spectropolarimeter. Purified protein at a concentration of 0.15 mg/ml, in 50 mM Tris/HCl, pH 8.0, 20 mM NaCl and 0.5 M guanidinium chloride was used for these investigations, with a temperature ranging from 25 °C to 90 °C. Data obtained were plotted as a function of mean residue ellipticity against temperature. Spectra were also collected for the wild-type and mutant proteins with a wavelength range 205–255 nm in the presence of buffer (50 mM Tris/HCl, pH 8.0, 20 mM NaCl and 0.5 M guanidinium chloride) to estimate the secondary structural content of the samples. The graph was plotted using Origin software and the data was smoothed by point 5 using the Savitzky–Golay algorithm.

Identification of the reduced form of γ-GCS protein *in vivo*

The wild-type (or mutant) *gshA* plasmid was transformed into *E. coli* BL21, the secondary cultures were induced at $D_{600} = 0.4$ with 1 mM IPTG and cells were harvested 90 min post-induction. Prior to harvesting, the culture was treated with 100 % TCA (trichloroacetic acid) to a final concentration of 20 % to trap the *in vivo* thiols in their original status and to prevent aerial oxidation.

Protein extraction was performed following the glass bead lysis method and the supernatants were centrifuged at 15000 ***g*** for 10 min at 4 °C. The pellets obtained were washed three times with chilled acetone and dried in the speedvac concentrator.

The dried pellet was dissolved in Hepes (50 mM Hepes, pH 7.0, and 2.5 % SDS) buffer and 5 mM MM(PEG) (alkylation agent) was added. For the AMS treatment, the pellet was dissolved in TES (100 mM Tris/HCl, pH 8.0, 10 mM EDTA and 1 % SDS) buffer and 30 mM AMS (alkylation agent) was added. The MM(PEG)-treated samples were incubated in the dark at 25 °C whereas AMS-treated samples were incubated at 37 °C for 2 h with 1400 rev./min agitation in a thermo mixer. Purified protein was precipitated by adding an equal volume of 20 %

TCA. Precipitated protein was washed twice with ice-cold acetone and air-dried. The protein sample was then treated with 5 mM MM(PEG) as described above. After 2 h the samples were centrifuged at 15000 ***g*** for 15 min at room temperature (25 °C) and the supernatant was collected. The protein concentration was determined by the Bradford method and 1 μg of protein was mixed with non-reducing loading dye and boiled for 5 min. SDS/PAGE (14 % gel) was run followed by Western blotting [20].

Pulse–chase experiments

The *E. coli* BL21 strains were transformed with plasmids carrying either wild-type or 9CF + R374Q + V375F *gshA* gene. The primary culture was set up in M9 minimal medium supplemented with all 20 amino acids except methionine and cysteine and grown at 37 °C overnight. The primary culture was used to seed the secondary culture at $D_{600} = 0.1$ in M9 minimal medium and allowed to grow until $D_{600} = 0.5$, when protein expression was induced with 1 mM IPTG. The culture was induced for 2 h at 37 °C followed by pulse-labelling by addition of 40 μCi/ml ^{35}S-Eligmix (70 % L-[^{35}S]methionine and 30 % L-[^{35}S]cysteine) and the culture was incubated at 37 °C. After 5 min of pulse-labelling, the medium was supplemented with 5 mg/ml of non-labelled L-methionine and L-cysteine mixture (7:3 ratio). A 0.2 ml aliquot of the culture was taken at 0, 2, 4 and 7 h intervals and mixed with 50 μl of the protease inhibitor PMSF (1 mM). Cells were lysed in 90 μl of 1× SDS-reducing dye and 15 μl of lysates were electrophoresed on reducing SDS/PAGE (10 % gel). The gel was exposed on a phosphoimager screen and autoradiographed and the band corresponding to 58.2 kDa was observed in induced cells. The band intensity was analysed by using scion software. This protocol was modified from Parsell and Sauer [21].

Midpoint redox potential of *E. coli* γ-GCS

The midpoint redox potential of *E. coli* γ-GCS was achieved by titrating the protein with defined ratios of DTT_{Ox} (*trans*-4,5-dihydroxy-1,2,dithane) and DTT (dithiothreitol). The protein (50 μg) was equilibrated in buffer containing 100 mM Hepes (pH 7.0) and different ratios of DTT (80–0.01 DTT_{Ox}/DTT) with a total concentration of 10 mM for 3 h at 25 °C to achieve the equilibrium. The experiment was carried out and midpoint potential calculations were made as described previously [22,23].

RESULTS

The conservation patterns and functional importance of cysteine residues in *E. coli* γ-GCS

Conservation patterns and co-evolution of pairs of cysteine residues often reveals important insights on the functionality of cysteine residues in proteins. To examine the extent to which the nine cysteine residues present in *E. coli* γ-GCS are conserved, we carried out multiple sequence alignments with the γ-GCS enzymes of lineage I to which the *E. coli* γ-GCS belongs. This also included the γ-GCS domain of the bifunctional enzymes (g*shF*) observed in several bacteria [6]. To ensure that these putative orthologues from other organisms might indeed be functioning as γ-GCSs, we first examined whether the active site residues identified from the crystal structures were conserved in these proteins. All of the active site residues were conserved in the selected proteins, suggesting that they might be true orthologues (Supplementary Figure S1 at http://www.biochemj.org/bj/449/bj4490783add.htm). Even the bifunctional *gshF* proteins contained these conserved residues. We then examined the conservation pattern of these cysteine residues. We observed that, among the nine cysteine residues seen in *E. coli* γ-GCS, three of the residues, Cys^{106}, Cys^{205} and Cys^{357} were conserved in the majority of the orthologues, barring the bifunctional *gshF* proteins that fell into a completely distinct cluster (Figure 1). However, surprisingly, the two cysteine residues involved in making the disulfide bond, Cys^{372} and Cys^{395}, were conserved (along with Cys^{433}) in only a small subset that primarily included the enterobacteria (Supplementary Figure S2 at http://www.biochemj.org/bj/449/bj4490783add.htm). The least-conserved cysteine residues were Cys^{164}, Cys^{233} and Cys^{439}. In the analogous enzymes of the other lineages, conservation of the cysteine residues correlated with a functional role in disulfide-bonding participation. The relative lack of conservation seen in the disulfide-bonded cysteine residues in the *E. coli* γ-GCS lineages was surprising.

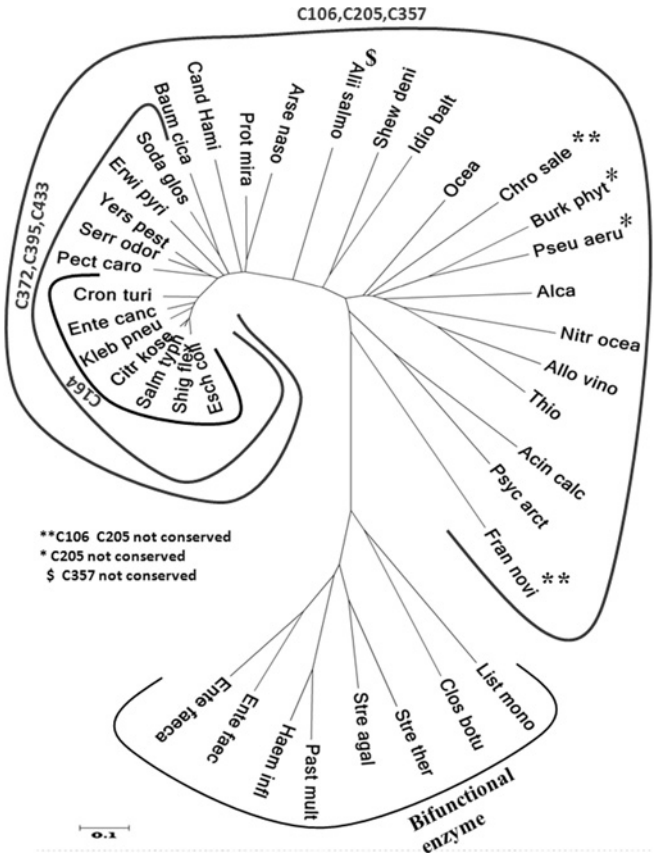

Figure 1 Phylogenetic tree analysis of lineage I

The *E. coli* γ-GCS protein sequences were used to retrieve the γ-GCS homologues of different organisms of lineage I. The MEGA 3.1 software was used to construct the phylogenetic tree. The conservation patterns of cysteine residues are shown. The representative members used in multiple sequence alignment are listed in Supplementary Table S2 (at http://www.biochemj.org/bj/449/bj4490783add.htm).

To understand these aspects and also evaluate the role of the different cysteine residues, we created a series of cysteine-to-serine mutants, where eventually all of the cysteine residues were mutated to serine. Functionality was assessed by complementation of the *S. cerevisaie gsh1Δ* phenotype in medium lacking glutathione. Unlike *E. coli gshA* deletions which are viable even in the absence of glutathione, *S. cerevisiae gsh1Δ* deletions are strict glutathione auxotrophs. As *E. coli* γ-GCS can complement *S. cerevisiae gsh1Δ*, it enabled a simple complementation-based growth assay for the mutant γ-GCS

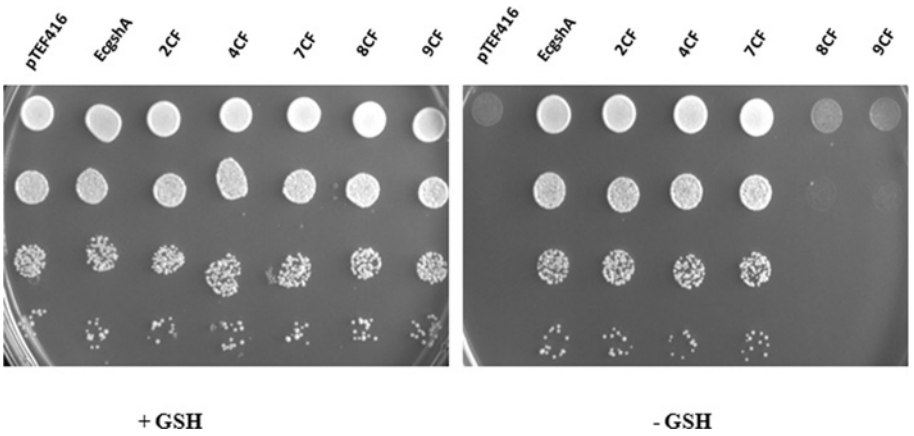

Figure 2 Complementation analysis of wild-type and CF mutants of *E. coli* γ-GCS

S. cerevisiae strain ABC1195 (*MATα his3*Δ1 *leu2*Δ-0 *lys2*Δ-0 *ura3*Δ-0 *gsh1*Δ:: *LEU2)* was transformed with plasmids bearing genes for the wild-type *gshA* or the different CF *gshA* mutants cloned under the TEF promoter. The transformants were serially diluted and spotted on to SD medium containing/lacking GSH as the sole source of organic sulfur. The vector pTEF416 was used as a control.

clones on plates lacking glutathione. We observed that the 2CF, 4CF, 6CF and even the 7CF mutants were all functional in this assay (Table 1 and Figure 2). However, on introduction of further cysteine-to-serine mutations by mutating the Cys^{372} and then the Cys^{395} residues to yield the 8CF and 9CF mutants, activity was immediately lost. The 8CF and 9CF mutants lack the disulfide bond, whereas the 7CF mutant retains the cysteine residues that form the disulfide bond. Thus, in spite of the fact that Cys^{372} and Cys^{395} were not the most conserved, they still appeared to be functionally very important on the basis of this analysis.

Isolation of intragenic functional suppressors of a non-functional CF *E. coli* γ-GCS

The complete loss in activity seen in the 8CF and 9CF mutants suggested that the disulfide bond was likely to be critical for activity. We took an independent approach to confirm if these cysteine residues were essential for activity, or if we might identify a functional molecule devoid of cysteine residues. This approach exploited a genetic selection strategy in *S. cerevisiae gsh1*Δ where functional suppressors could be selected.

We carried out *in vitro* hydroxylamine mutagenesis of the CF *E. coli gshA* (9CF) gene expressed from a TEF promoter, transformed this into an *S. cerevisiae gsh1*Δ strain, and selected for functional clones on minimal medium lacking glutathione.

A total of 53 suppressors were isolated from two independent mutagenic events. Among these mutants, 22 transformants showing different levels of complementation were selected for further analysis. Among the 22 selected mutated transformants, only 14 behaved true on secondary screening and were chosen for further investigation. The plasmids were isolated from those yeasts, transformed in *E. coli* and, after purification through *E. coli*, reconfirmed by transformation in the *S. cerevisiae gsh1*Δ strain. All 14 clones were able to functionally complement the *S. cerevisiae gsh1*Δ and were subjected to sequencing. To eliminate the possibility of mutations in the promoter being responsible for the phenotypes, the coding regions of the functional mutants were also excised and subcloned under a TEF promoter of a non-mutated plasmid. In cases where more than one mutational change was detected, we further subjected these to subcloning to determine the minimal residues that were permitting the molecule to regain activity. These data are summarized in Table 1. The majority of the mutants carried double mutants in which both the Ser^{372} and Ser^{395} residues (originally cysteine) had reverted back to cysteine. As these residues have been shown to form a disulfide bond, the simultaneous appearance of both these cysteine residues in the majority of suppressors (nine suppressors among 14) strongly suggested that the disulfide bond is a very important determinant for functionality. However, in addition to these mutants, we interestingly also obtained the double mutants 9CF + S372C + S395W, 9CF + S372F + S395C and 9CF + S372C + S395Y. These mutants had a pattern where only one of either Cys^{372} or Cys^{395} was restored as cysteine, whereas the other change, although it also involved the corresponding 372 or 395 residues, contained an aromatic residue at the position (Figure 3A). Although some of the mutants contained mutations in addition to these mutations (Table 1), subcloning experiments that were done to separate the mutations revealed that they were not required for functionality (results not shown). With only a single cysteine residue in the mutants, they lacked the ability to form an intramolecular disulfide bond observed in the *E. coli* γ-GCS structure, clearly indicating that an intramolecular disulfide mutant was not essential for activity.

In addition to these mutants that contained either a single or double cysteine residue at one or other of these positions, we were also able to isolate a single mutant that lacked any cysteine residues. This mutant contained two changes, R374Q and V375F, both changes proximal to the disulfide bond (Supplementary Figure S3 at http://www.biochemj.org/bj/449/bj4490783add.htm). To determine whether both changes were needed for functionality, we separately reconstructed these mutants, and observed that it was only the V375F mutation that was responsible for the restoration of functionality (Figure 3B).

To investigate the cysteine-aromatic suppressors, and how important the relative positioning of the aromatic and cysteine residues were in this mutant, we created a mutant in which the 9CF + S372C + S395W mutation position was swapped. The 9CF + S372W + S395C mutation so created was found to be functionally similar to 9CF + S372C + S395W, suggesting that although an aromatic and cysteine residue were important in these positions, swapping the positioning was also acceptable for function. This suggested that the nature of the interaction rather than the precise positioning might be important (Supplementary Figure S4A at http://www.biochemj.org/bj/449/bj4490783add.htm). However, mutations at both of these positions were necessary for the activity since a serine-aromatic

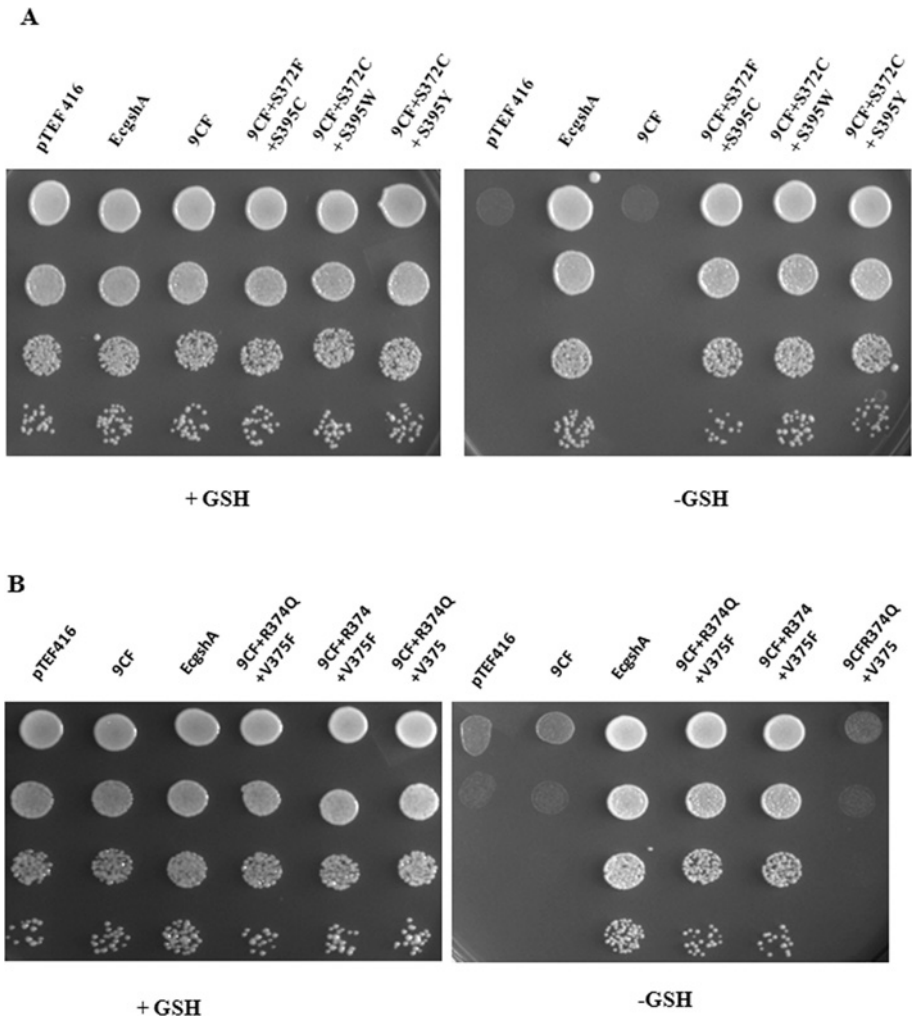

Figure 3 Functional complementation displayed by suppressor of 9CF γ-GCS

E. coli 9CF γ-GCS suppressors were transformed in *S. cerevisiae* strain ABC1195 (*MATα his*3Δ1 *leu2*Δ-0 *lys2*Δ-0 *ura*3Δ-0 *gsh1*Δ:: *LEU2)* and spotted on to SD medium containing/lacking glutathione as the sole source of organic sulfur. The vector pTEF416 was used as a control. (**A**) Functional complementation of cysteine aromatic suppressor mutants. (**B**) The complementation of *Scgsh1Δ* by CF suppressors 9CF + R374Q + V375F, 9CF + R374Q + V375 and 9CF + R374 + V375F.

combination that we created was not functional (Supplementary Figure S4B).

Wild-type and 7CF *E. coli* γ-GCS capable of forming a disulfide bond show reduced activity in the presence of DTT/GSH, but the mutants 9CF + S372C + S395W and 9CF + R374Q + V375F are unaffected

As the *in vivo* complementation assay in *S. cerevisiae* was a qualitative assay and did not give an indication of other properties of the protein that might be affected by the mutations, we resorted to *in vitro* purification and characterization of these mutant proteins from *E. coli*. The wild-type, 7CF (Cys^{372} and Cys^{395}), 9CF + S372C + S395W, 9CF + S372C + S395Y and 9CF + R374Q + V375F mutants were His-tagged at their C-terminal, and purified from *E. coli* using Ni-NTA affinity chromatography. The proteins were purified to >90 % homogeneity (Supplementary Figure S5 at http://www.biochemj.org/bj/449/bj4490783add.htm).

Previous studies with *E. coli gshA* have revealed an inhibition by GSH [24]. We reinvestigated this inhibition by GSH with the wild-type and mutant proteins and observed that at 2 mM GSH the activity of wild-type and 7CF was reduced by 25 %, whereas at 10 mM GSH a 45 % reduction in enzyme activity was observed. However, the mutants 9CF + S372C + S395W and 9CF + R374 + V375F that lacked disulfide-bonding ability, did not show any inhibition with glutathione (Figure 4A). As the inhibitory effect of glutathione could be either a consequence of its reductant property or as a consequence of its ability to cause product inhibition, we investigated the effect of DTT on the activity of these mutants. Interestingly, we observed inhibition by DTT that paralleled the inhibitory effect of GSH in the wild-type and 7CF mutant but caused no inhibition in activity in the 9CF + S372C + S395W and 9CF + R374 + V375F mutants (Figure 4B). This suggested that the inhibition by glutathione was not by virtue of its being a final product of the reaction or of allosteric regulation, but as a consequence of the reductant property of glutathione. To further confirm this aspect we used the glutathione analogue SMG (*S*-methyl glutathione). In this analogue, the free thiol group is blocked by the methyl group. The properties of the compound as a product analogue are largely unaffected owing to the presence of only a very small methyl group, but as a consequence of a blockage of the thiol group it lacks the reductant property of glutathione. The wild-type

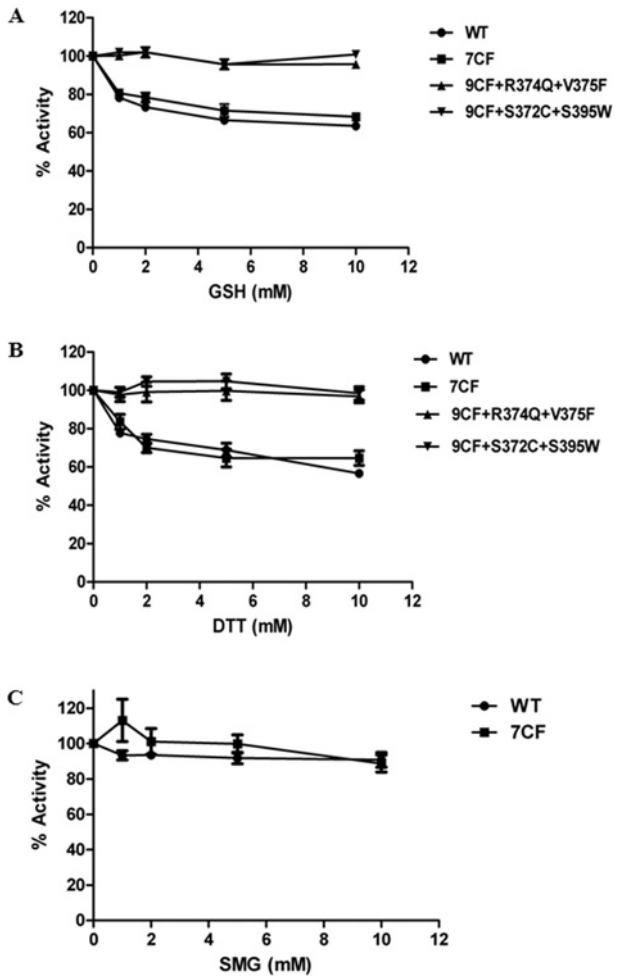

Figure 4 Inhibition study of *E. coli* γ-GCS wild-type and mutant enzymes by GSH, DTT and SMG

The purified wild-type and mutant proteins were treated with different concentrations of GSH (**A**), DTT (**B**) and SMG (**C**) and incubated for 30 min at 4 °C. The enzyme activity of the treated protein was assayed as described in the Materials and methods section. The saturated substrate concentration was used. The enzyme activity of wild-type (WT, ●), 7CF (■), 9CF + R374Q + V375F (▲) and 9CF + S372C + S395W (▼) were considered as 100 % at 0 mM GSH, DTT and SMG respectively and the activity at other concentrations was calculated relative to that. The experiment was carried out in triplicate and means ± S.D. are shown.

γ-GCS enzyme was incubated with different concentrations of SMG (0–10 mM) for 30 min and assayed for enzyme activity (Figure 4C). We observed that SMG was unable to inhibit the enzyme activity even at 10 mM concentrations, further confirming that the glutathione inhibition of *E. coli gshA* was a consequence of its ability to reduce the disulfide bond.

Kinetic studies of wild-type and mutant γ-GCS enzymes reveal no role of the disulfide bond in determining the affinity of the enzyme towards its substrates

In the human and plant γ-GCS enzymes, the reduction of the disulfide bond led to an altered affinity of the enzyme to its substrates. To determine whether the presence or absence of the disulfide bond in *E. coli* γ-GCS also influenced the kinetic parameters towards the substrates, we evaluated the mutants for their kinetic parameters in respect to all three substrates, cysteine, glutamate and ATP. Cysteine, although added at a concentration of 15 mM, was not expected to affect the redox status of the protein since no prior pre-incubation was involved and the kinetics were determined based on initial velocities. The wild-type and 7CF mutant were found to have an almost similar K_m value for the different substrates. Surprisingly, the mutants 9CF + S372C + S395Y, 9CF + S372C + S395W and 9CF + R374Q + V375F also had very similar K_m values for glutamate, cysteine and ATP. These results indicate that the presence or absence of a disulfide bond in *E. coli* γ-GCS does not determine the affinity of the enzyme for any of its substrates to a significant extent (Table 2). However, we observed a lower V_{max} of the mutants. Comparisons of k_{cat} and k_{cat}/K_m (i.e. the turnover number and catalytic efficiency) of wild-type and mutant revealed that some mutants have a reduced turnover number and catalytic efficiency. The catalytic efficiency of 9CF + S374C + S375Y is reduced by 2-fold for cysteine and ATP. To confirm that this is the result of the absence of a disulfide bond, we incubated the wild-type and 7CF protein with 2 mM DTT for 30 min at 4 °C followed by the assay of these enzymes for their kinetic parameters for all three substrates. We observed that reduction of the disulfide bond in the wild-type and 7CF γ-GCS protein had mild effects on the K_m value of the enzyme for all substrates, but significantly affected the catalytic efficiency (k_{cat}/K_m) (Table 3). We observed that the reduced wild-type and 7CF (DTT-treated) had a lower k_{cat}/K_m value than the untreated enzyme. These observations on the wild-type and mutant enzymes demonstrate that, although the absence of the disulfide bond in the mutant does not affect the substrate affinities of enzyme, the mutant enzymes displayed reduced turnover number and catalytic efficiency. More importantly, the kinetic behaviour of the reduced protein was found to be very similar to the pattern shown by the 9CF + R374Q + V375F and 9CF + S372C + S395Y mutants.

Decreased *in vitro* stability in the mutants lacking the capability to form a disulfide bond

During the initial characterization of the different wild-type and mutant purified proteins, we observed a differential thermolability of the different proteins. This prompted us to carry out more detailed investigations on the stability assays of representative mutants from each class. In this assay we pre-incubated the wild-type and mutant proteins at 37 °C, 25 °C and 4 °C for 1 h followed by an activity assay at room temperature. The wild-type had almost similar activity at all three different pre-incubated temperatures, whereas the 9CF + S372C + S395W and 9CF + R374Q + V375F mutants had significantly lower activity. 9CF + S372C + S395W lost approximately 30 % activity by pre-incubation at 37 °C, whereas the 9CF + R374Q + V375F mutant lost 75 % of its activity by pre-incubation at 37 °C compared with its activity when pre-incubated at 4 °C (Figure 5). In contrast, the 7CF mutant, which retained the ability to form a disulfide bond, showed almost similar thermal stabilities as the wild-type. To investigate whether reduction of the disulfide bond would reduce the stability of the wild-type and 7CF proteins, we incubated the wild-type and 7CF γ-GCS protein with 5 mM DTT and GSH for 1 h at 37 °C, 25 °C and 4 °C followed by measurement of enzyme activity at room temperature (25 °C). The stability pattern of these DTT-treated proteins was very similar to that obtained with mutants lacking the ability to form a disulfide bond. The wild-type and 7CF proteins that were pre-incubated at 4 °C showed maximum activity, whereas activity decreased with the increase in pre-incubation temperature (Figure 6). The activity of wild-type and 7CF in DTT or GSH showed no significant change in activity when pre-incubated at 25 °C, but a drastic loss in activity

Table 2 Kinetic parameters of wild-type and mutants

All reactions were performed as described in the Materials and methods section. The values are presented as means ± S.D. for three independent experiments. The statistical significance of the differences was with respect to the wild-type protein, $*P \leqslant 0.05$.

Substrate	Parameters	Wild-type	7CF	9CF + R374Q + V375F	9CF + S372C + S395Y	9CF + S372C + S395W
Glutamic acid	K_m (mM)	3.9 ± 0.4	4.0 ± 1.6	4.0 ± 0.5	3.2 ± 0.4	4.1 ± 1.8
	V_{max} (nmol/min per μg)	32.3 ± 2.6	34.9 ± 11.6	30.4 ± 5.7	22.7 ± 9.7	31.2 ± 2.9
	k_{cat} (s^{-1})	31.4 ± 2.5	34.0 ± 11.3	29.6 ± 5.5	22.1 ± 9.4	30.4 ± 2.8
	k_{cat}/K_m (s^{-1}·mM^{-1})	8.1	8.5	7.4	6.9	7.4
Cysteine	K_m (mM)	0.10 ± 0.01	0.11 ± 0.04	0.14 ± 0.02*	0.11 ± 0.05	0.16 ± 0.08
	V_{max} (nmol/min per μg)	47.9 ± 15.7	40.8 ± 20.9	30.0 ± 10.0	22.5 ± 5.8*	32.7 ± 12.2
	k_{cat} (s^{-1})	46.6 ± 15.3	39.7 ± 20.3	29.2 ± 9.8	21.9 ± 5.7	31.8 ± 11.8
	k_{cat}/K_m (s^{-1}·mM^{-1})	466.0	396.9	208.6	199.1	198.8
ATP	K_m (mM)	0.14 ± 0.04	0.10 ± 0.04	0.19 ± 0.03	0.53 ± 0.15*	0.24 ± 0.23
	V_{max} (nmol/min per μg)	48.2 ± 12.9	40.8 ± 6.5	27.9 ± 5.7	25.4 ± 4.1*	31.3 ± 1.2
	k_{cat} (s^{-1})	46.9 ± 12.6	39.7 ± 6.3	27.2 ± 5.5	24.7 ± 4.0*	30.5 ± 1.2
	k_{cat}/K_m (s^{-1}·mM^{-1})	335.0	397.0	142.9	46.6*	127.1

Table 3 Kinetic parameters of wild-type and 7CF in the presence of DTT

All reactions were performed as described in the Materials and methods section. The values are presented as means ± S.D. for three independent experiments. The statistical significance of the differences was with respect to the unreduced wild-type protein, $*P \leqslant 0.05$.

Substrate	Parameters	Wild-type (reduced)	7CF (reduced)
Glutamic acid	K_m (mM)	5.1 ± 0.7*	5.3 ± 2.0
	V_{max} (nmol/min per μg)	22.8 ± 4.9*	24.4 ± 7.7
	k_{cat} (s^{-1})	22.1 ± 4.8*	23.8 ± 7.5
	k_{cat}/K_m (s^{-1}·mM^{-1})	4.3*	4.5*
Cysteine	K_m (mM)	0.14 ± 0.01*	0.10 ± 0.02
	V_{max} (nmol/min per μg)	24.9 ± 8.9	22.8 ± 7.4
	k_{cat} (s^{-1})	24.2 ± 8.7	22.2 ± 7.2
	k_{cat}/K_m (s^{-1} mM^{-1})	172.9*	222.0*
ATP	K_m (mM)	0.46 ± 0.48	0.25 ± 0.01*
	V_{max} (nmol/min per μg)	29.5 ± 9.0	30.9 ± 5.7
	k_{cat} (s^{-1})	28.7 ± 8.8	30.1 ± 5.6
	k_{cat}/K_m (s^{-1}·mM^{-1})	62.4	120.4*

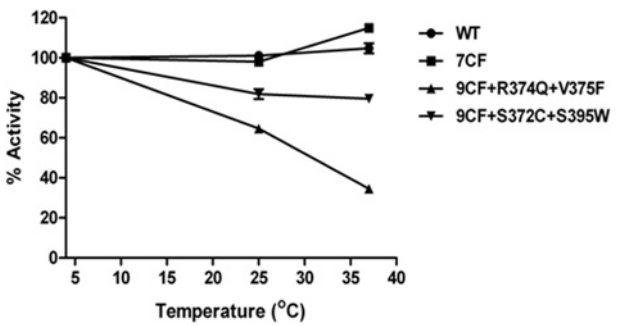

Figure 5 Effect of temperature on wild-type and mutant activity

The effect of temperature was studied with wild-type (WT) and mutant proteins. The wild-type and mutant proteins were pre-incubated at 37 °C, 25 °C and 4 °C for 1 h. The enzyme activity of the wild-type and mutants were assayed at saturated substrate concentration at room temperature (25 °C) as described in the Materials and methods section. The activity was expressed as a percentage of the highest activity at the pre-incubated temperature of 4 °C. The experiment was carried out in triplicate and means ± S.D. are shown.

when pre-incubated at 37 °C. This finding further suggests that the disulfide bond is very important for stability.

To investigate the thermal instability of the mutant proteins, we performed CD spectroscopy [25] of wild-type and mutant proteins. The results revealed that all mutants maintained

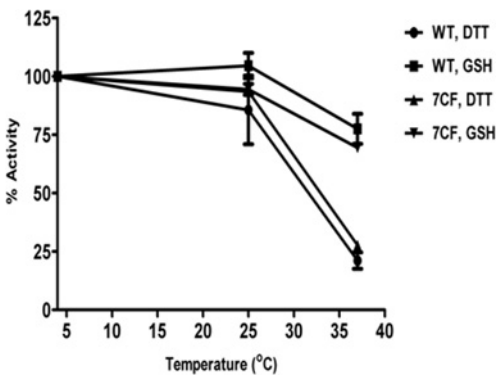

Figure 6 Stability assay of wild-type and 7CF γ-GCS in the presence of DTT and GSH

The purified wild-type (WT) and 7CF γ-GCS protein was treated with 5 mM DTT and GSH and pre-incubated at 37 °C, 25 °C and 4 °C for 1 h followed by assay of activity at room temperature (25 °C). The experiment was carried out in triplicate and means ± S.D. are shown.

their secondary α-helix structure compared with the wild-type (Supplementary Figure S6A at http://www.biochemj.org/bj/449/bj4490783add.htm). The melting temperature scan of mutants along with wild-type, however, revealed that although the wild-type had a T_m at 47.5 °C, the 9CF + S372C + S395W mutant showed lesser thermal stability with a T_m at 44 °C, whereas the mutant 9CF + R374Q + V375F had a T_m at 42.5 °C. This was in contrast with the 7CF mutant that had a T_m similar to wild-type at 47 °C (Supplementary Figure S6B). The similar T_m values of wild-type and 7CF mutant strongly implicates these cysteine residues in disulfide bond formation that provided greater thermostability and higher T_m of unfolding transitions. To confirm this fact, we carried out T_m scans of the wild-type protein that was reduced with DTT and found a decreased T_m ($T_m = 43$ °C) (Supplementary Figure S6C).

Reduced form of *E. coli* γ-GCS can be detected *in vivo*

The crystal structure of *E. coli* γ-GCS has shown the presence of a disulfide bond between Cys^{372} and Cys^{395} [15]. The biochemical data described above have suggested that if this disulfide bond is reduced under reducing physiological conditions it might have an altered stability and consequent physiological implications. It was therefore important to establish whether *E. coli* γ-GCS

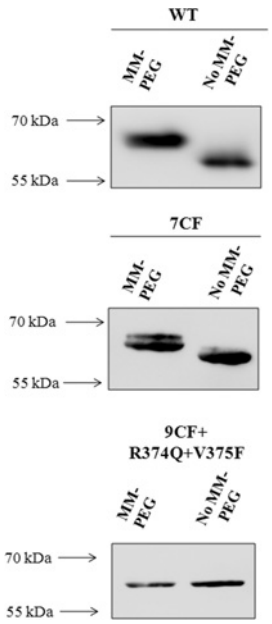

Figure 7 Identification of a reduced form of γ-GCS *in vivo* using MM(PEG)

The wild-type (WT), 7CF and 9CF + R374Q + V375F plasmid harbouring the *E. coli* strain was grown in LB broth supplemented with 100 μg/ml ampicillin. The expression was induced at $D_{600} = 0.4$ with 1 mM IPTG. At 90 min post-induction, the growing culture was treated with 100 % TCA to a final concentration of 20 %. The extracted protein was treated with 5 mM MM(PEG), an alkylation agent, as described in the Materials and methods section. The reduced and oxidized proteins were separated by SDS/PAGE (14 % gels) under non-reducing conditions. The detection of protein was carried out by Western blotting using an anti-His antibody. Molecular masses in kDa are shown to the left-hand side.

was found only as an oxidized form *in vivo* as seen in the structure, or if an equilibrium might exist between the reduced and oxidized forms *in vivo*. To demonstrate whether the disulfide bond can form under physiological conditions, we determined the midpoint redox potential of the disulfide bond by redox titration. We used the 7CF mutant that contains only two disulfide-bonding cysteine residues for this analysis. We obtained an approximate midpoint redox potential of $E_m = -330$ mV for the *E. coli* γ-GCS (Supplementary Figure S7 at http://www.biochemj.org/bj/449/bj4490783add.htm). The redox potential of the *E. coli* cytosol has been estimated in the range -260 to -280 mV [26]. This suggests that the *E. coli* γ-GCS enzyme must largely exist in the oxidized form *in vivo* and the detection of the disulfide bond in the crystal structure is in agreement with this observation.

To investigate whether a reduced form of γ-GCS could nevertheless be detected *in vivo*, we carried out experiments using MM(PEG) and AMS alkylation to determine whether the disulfide bond of γ-GCS might also exist as a reduced form *in vivo*. MM(PEG) is an alkylating agent that can bind to a free thiol group of cysteine and increase the molecular mass by 0.71 kDa, whereas AMS increased the molecular mass by 0.5 kDa. The procedure involves the rapid acidification of *E. coli* cells by TCA that rapidly trap the thiols in the native *in vivo* form and helps protein precipitation. To validate the method, the wild-type *E. coli* γ-GCS, which has nine cysteine residues, seven of which in reduced form, was examined in an MM(PEG) and AMS experiment and as expected a significant shift in mobility of approximately 5–6.5 kDa and 3.5–4.5 kDa respectively was observed on MM(PEG) and AMS treatment corresponding to the expected number of free cysteine residues (Figure 7 and Supplementary Figure S8A at http://www.biochemj.org/bj/449/bj4490783add.htm). We then subjected the 7CF mutant to MM(PEG) and AMS treatment. The presence of a shift in mobility of the protein band on MM(PEG) treatment or AMS treatment would reflect the presence of a reduced cysteine in the 7CF molecule. We observed that the MM(PEG)-treated samples shifted compared with the un-treated controls. Surprisingly though, we observed that in the MM(PEG)-treated samples there were two shifted bands (this was seen reproducibly in all of the experiments carried out). The presence of two bands, both of them shifted up, probably reflects binding to either one or two of the cysteine residues. Normally one expects both cysteine residues to be conjugated to the alkylating reagent. However, it is possible that steric hindrance owing to the proximity of the two cysteine residues might be hindering the rate of reaction, thereby enabling us to detect the singly conjugated and dually conjugated samples. We also observed a discernable shift on AMS treatment (Supplementary Figure S8A). We carried out the MM(PEG) modification experiments with purified wild-type and mutant proteins (with either zero, one or two cysteine residues) and observed similar shifts, validating the interpretations with the crude extracts. One difference seen in the purified samples of 7CF was the absence of a double shift that may be complete oxidation of both cysteine residues in the purified samples (Supplementary Figure S8B). These results suggested that the reduced form of *E. coli* γ-GCS exists *in vivo* and can be detected under *in vivo* physiological conditions. The presence of only the reduced form might be a consequence of the overexpression of the γ-GCS enzyme and might explain the detection of the reduced protein despite the low midpoint redox potential. As controls we have included the 9CF + R374Q + V375F mutant with and without MM(PEG) or AMS treatment and, as expected, no differences in mobility were observed in this mutant (either in the extracts or in the purified controls).

The mutant enzyme lacking cysteine residues shows a decreased *in vivo* half-life

The *in vitro* studies with wild-type and mutants had clearly shown the effects of disulfide on the stability of protein. We hypothesized that the absence of a disulfide bond could possibly affect the *in vivo* half-life of the protein. As it was difficult to achieve completely reduced or oxidized forms of the protein *in vivo*, we exploited the mutants to evaluate this possibility. The *in vivo* protein degradation of wild-type and mutant 9CF + R374Q + V375F was studied by pulse–chase experiments. The wild-type and 9CF + R374Q + V375F clones were grown in M9 minimal medium supplemented with all amino acids except methionine and cysteine. Expression was induced for 2 h with 1 mM IPTG followed by a pulse with ^{35}S-Eligmix. This was followed by a chase with excess non-labelled methionine and cysteine mixture at 37 °C. Aliquots were taken out at different time intervals. The wild-type protein appeared to be very stable even after 7 h of chase. We observed that the mutant which lacked the disulfide bond was significantly more susceptible to degradation, with a half-life of approximately 4 h in contrast with the wild-type which was relatively stable during this period (Figure 8). The result demonstrated that the disulfide bond was important for the *in vivo* stability of *E. coli* γ-GCS.

DISCUSSION

The present study provides new insights into how a key redox enzyme, γ-GCS of *E. coli*, which largely determines the levels of the abundant and key redox metabolite glutathione in the cell, is

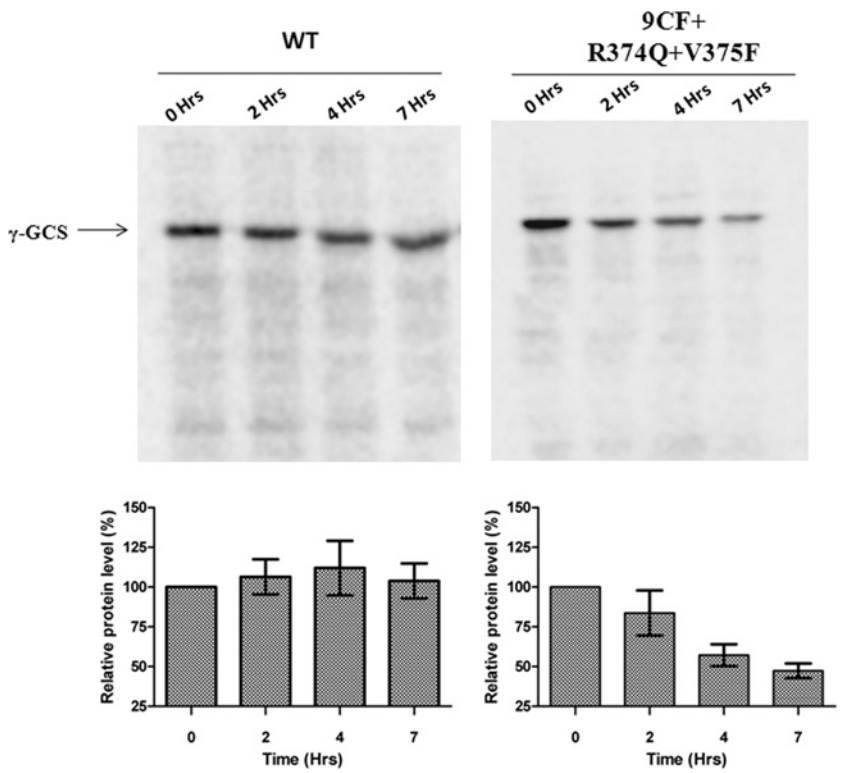

Figure 8 Pulse–chase analysis for the degradation of wild-type and mutant 9CF + R374Q + V375F

The wild-type (WT) and mutant 9CF + R374Q + V375 *gshA* plasmid was transformed in *E. coli* BL21 strain and grown in M9 minimal medium. The exponential phase cells were induced with 1 mM IPTG and after 2 h of post-induction the culture was pulse-labelled with 40 μCi/ml ^{35}S-Eligmix. The sample was collected at different time intervals after chase with a non-labelled methionine and cysteine mixture and electrophoresis was carried out as described in the Materials and methods section. The SDS/PAGE represents a single experiment with the means ± S.D. for three experiments provided in the bottom panel.

itself regulated by the redox status of the cell. In the analogous enzymes of plants and humans a disulfide bond also exists either between two subunits (humans) or within a subunit (plants) and is a key regulator of the efficiency of this enzyme. In these cases, the reduction of the disulfide bond led to an altered affinity for the substrates and altered feedback sensitivity leading to decreased synthesis of GSH and this has a direct impact on the redox state of the cell. In contrast with this situation, in *E. coli* we demonstrate that, although a reduction in the disulfide bond of γ-GCS does not lead to an alteration of the affinity towards the substrates, it did lead to an alteration of the stability and increased degradation of the protein. The consequences are decreased catalytic efficiency, reduced synthesis of glutathione and a consequent effect on the cellular redox status.

The importance of the disulfide bond in proteins in imparting stability to folded proteins is well known. The importance of folding and stability in conferring resistance to intracellular proteolytic digestion of proteins is also well known. However, the fact that these two well-known features of proteins may be conjoined to yield a mechanism by which the redox status of the protein would lead to a differential degradation thereby enabling the cells to alter the GSH level and therefore the redox state, had not been considered so far, at least to our knowledge. The findings of the present study thus demonstrate a new means by which redox homoeostasis can be maintained.

The inhibition of *E. coli* γ-GCS activity in the presence of increasing concentrations of GSH was observed many years ago [24]. However the mode of inhibition was never investigated at that time, and it was largely assumed that the inhibitory mechanism might parallel what was observed with the human enzyme where non-allosteric substrate inhibition was observed. However, the *E. coli* enzyme appears to be quite distinct from the mammalian enzyme in several aspects. It is insensitive to cystamine, and weakly sensitive to iodacetamide and *N*-methylmaleimide. It is also insensitive to 4-methylene glutamate, *S*-sulfocysteine and *S*-sulfohomocysteine, to which the mammalian enzymes are very sensitive [24,27–29]. Although the mammalian enzyme was also relatively insensitive to SMG, it was also insensitive to DTT and 2-mercaptoethanol [27]. The present study, with the *E. coli* γ-GCS wild-type and mutants, and use of DTT and the glutathione analogue SMG, however, clearly demonstrated that the inhibition in *E. coli* γ-GCS enzyme activity was a consequence of the reduction of the disulfide bond leading to both altered stability and activity.

In the bifunctional enzyme *gshF* of *S. agalactiae*, it is interesting to note that no allosteric inhibition of the enzyme by GSH was observed, although inhibition at high concentrations of GSH was observed with the *P. multocida* enzyme. However, as the structure of the *gshF* enzyme does not reveal the presence of a disulfide bond even in the dimer [30], the inhibitory activity of GSH in *P. multocida* is unlikely to be functioning through its action on a disulfide bond as observed in the present study.

In our investigations with the *E. coli* γ-GCS enzyme, we have taken a biochemical genetic strategy to investigate the role of the cysteine residues and the disulfide bond. This was largely because the cysteine residues of *E. coli* showed an unusual conservation pattern, which, along with the lack of redox dependence of the *Xanthomonas* and *Agrobacterium* enzymes of the plant lineage,

initially questioned the functional importance of the disulfide bond in the enzyme. However, the suppressor strategy turned out to be extremely rewarding as it not only yielded insights into the disulfide bond, but also yielded a functional γ-GCS variant that lacked any cysteine residues. This has proved very useful in our analysis and has greatly facilitated our reaching the above conclusions. Furthermore, the use of the 7CF mutant which only contained the two cysteine residues involved in disulfide bond formation, but lacking other cysteine residues, ensured that no other non-physiological disulfides might be forming to stabilize the structure.

The appearance of the combination of cysteine and aromatic amino acids at positions 372 and 395 in many of the suppressors in place of the disulfide-bonding cysteine residues was initially puzzling. However, it has been reported that cysteine–aromatic amino acid interactions are stabilized by π-type hydrogen bonding between the aromatic ring and thiol group of cysteine and the interaction between the aromatic ring and aliphatic group ($-CH_2$) of cysteine [31]. This interaction might have been sufficient to stabilize the folding conformation of the catalytic domain. It is interesting to note that the putative γ-GCS of *Erwinia pyrifoliae* contains a Cys^{372}/Tyr^{395} combination, whereas the putative γ-GCS of *Candidatus hamiltonella* and *Baumanni cicadellinicola* carry a Tyr^{372}/Thr^{395} and Tyr^{372}/Gly^{395} combination respectively, and it would be interesting to see the properties of these enzymes in the light of the observations made in the present study. In the *E. coli* CF γ-GCS enzyme, the conversion of valine to phenylalanine at position 375 even in the absence of any cysteine residues leading to the appearance of a functional CF molecule is also intriguing. Once again, the change occurred at a residue near the disulfide-bond-forming residues, suggesting that a similar interaction might be stabilizing the active site structure, but this awaits more detailed structural insights.

Although the findings and postulated mechanism of redox regulation have been demonstrated for the *E. coli* γ-GCS enzyme, it is likely that the mechanism may also, in part, function in the case of the plant and human enzymes as well, but this aspect of these enzymes has never been investigated, and actual demonstration would await further experiments on these enzymes.

AUTHOR CONTRIBUTION

Shailesh Kumar isolated most of the genetic suppressors, carried out the biochemical, biophysical and kinetic studies, analysed the results, and helped in writing the paper. Neha Kasturia isolated and evaluated the functionality of a few of the suppressors. Amit Sharma constructed the different CF mutants of *E. coli* γ-GCS. Manish Datt helped in building a three-dimensional model of γ-GCS with the wild-type and the CF suppressor. Anand Bachhawat supervised the project, analysed the data and contributed towards writing the paper.

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online data

SUPPLEMENTARY ONLINE DATA

Redox-dependent stability of the γ-glutamylcysteine synthetase enzyme of *Escherichia coli*: a novel means of redox regulation

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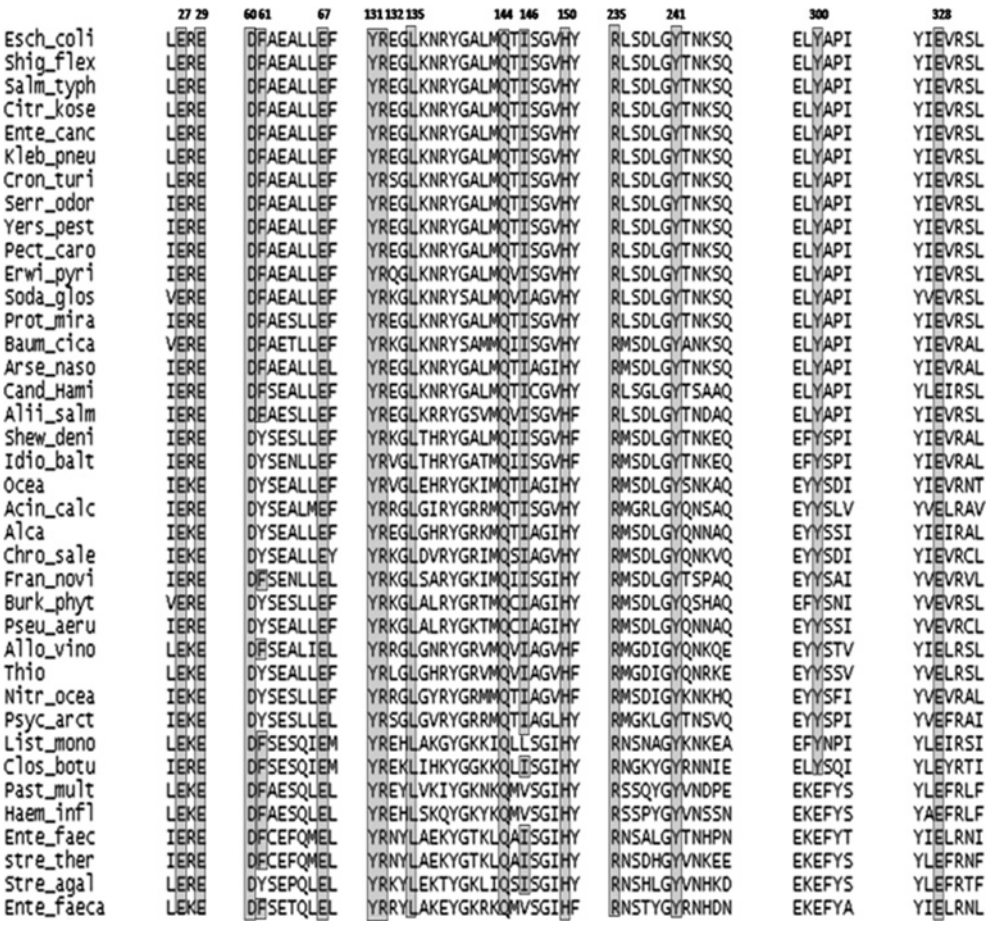

Figure S1 Conservation patterns of active site residues in γ-proteobacterium

The *E. coli* γ-GCS protein sequences were used to retrieve the γ-GCS homologues of different organisms of lineage I. The multiple sequence alignment of the protein sequences were generated using ClustalW using default parameters. The conserved active site residues are highlighted.

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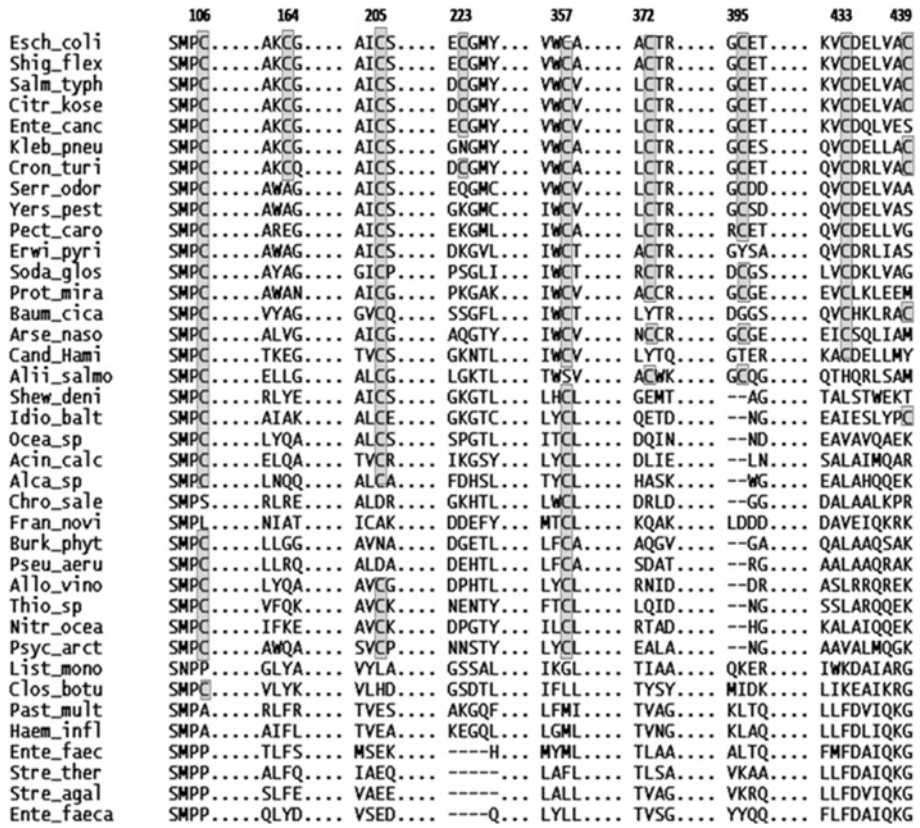

Figure S2 Multiple sequence alignment of *E. coli* γ-GCS with a member of lineage I

The *E. coli* γ-GCS sequences were used to retrieve the γ-GCS homologues of different organisms of lineage I. The multiple sequence alignment of the protein sequences was generated using ClustalW program using default parameters. The conserved cysteine residues are highlighted.

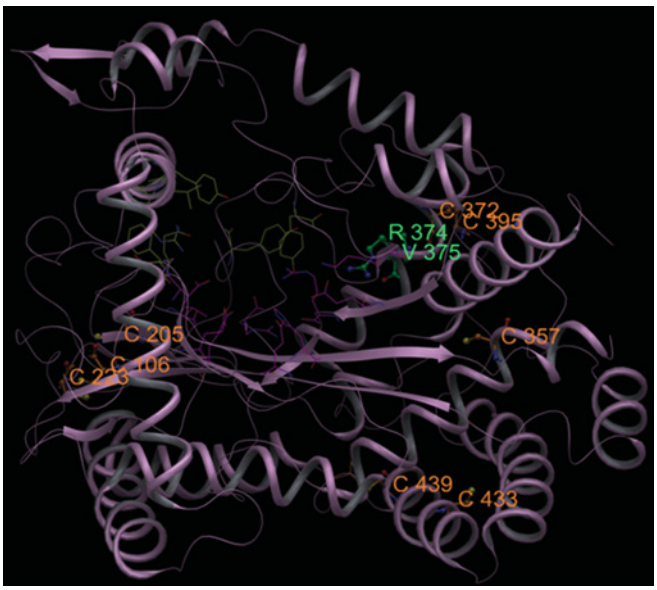

Figure S3 Cartoon representation of the crystal structure of *E. coli* γ-GCS (PDB code 1V4G)

The position of the cysteine residues are labelled and shown as ball and stick. Four of these cysteine residues (Cys^{106}, Cys^{164}, Cys^{205} and Cys^{223}) were mutated to serine to facilitate crystallization; those rotamers were reverted to cysteine *in silico*. The co-ordinates of Cys^{164} are missing in the crystal structure and hence not shown here. Two sites of mutations identified in our experiments are shown as ball and stick and labelled in green. Amino acid residues lining the glutamate- and cysteine-binding sites are shown as wires in magenta and lime respectively.

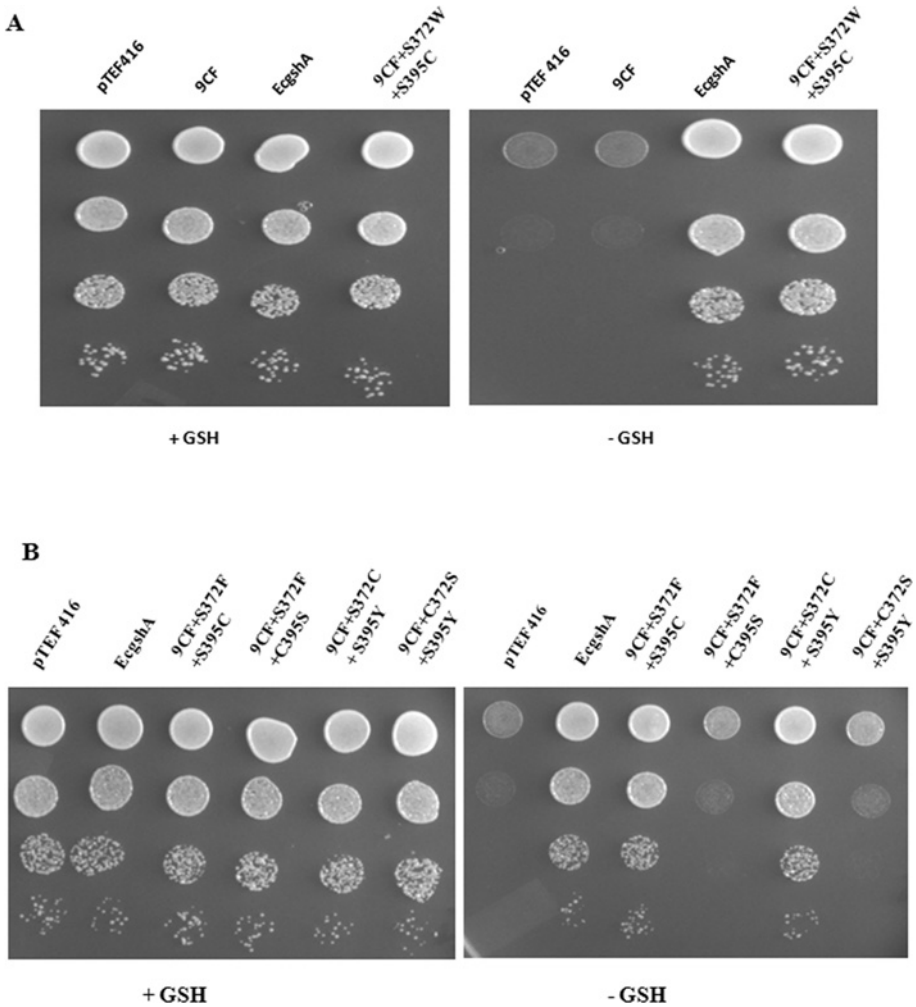

Figure S4 Functional complementation displayed by suppressor of 9CF γ-GCS

E. coli 9CF γ-GCS suppressors were transformed in *S. cerevisiae* strain ABC1195 (*MAT*α *his*3Δ1 *leu2*Δ-0 *lys*2Δ-0 *ura*3Δ-0 *gsh1*Δ:: *LEU2*) and spotted on to SD medium containing/lacking glutathione as the sole source of organic sulfur. The vector pTEF416 was used as a control. (**A**) Swapping of the aromatic cysteine position from 9CF + S372C + S395W to 9CF + S372W + S395C. (**B**) Functional complementation of serine aromatic mutants at position 372/395.

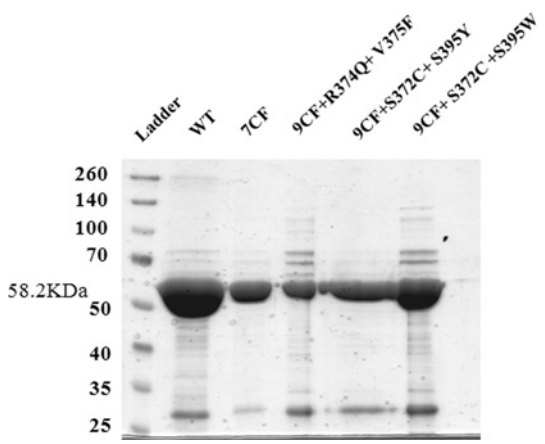

Figure S5 Coomassie Blue gel showing the purity of purified protein

Eluted fractions (20 μl) of the wild-type (WT) and mutant proteins from the Ni-NTA columns were run on SDS/PAGE (10 % gel) and stained with Coomassie Blue dye. Molecular masses are indicated in kDa to the left-hand side.

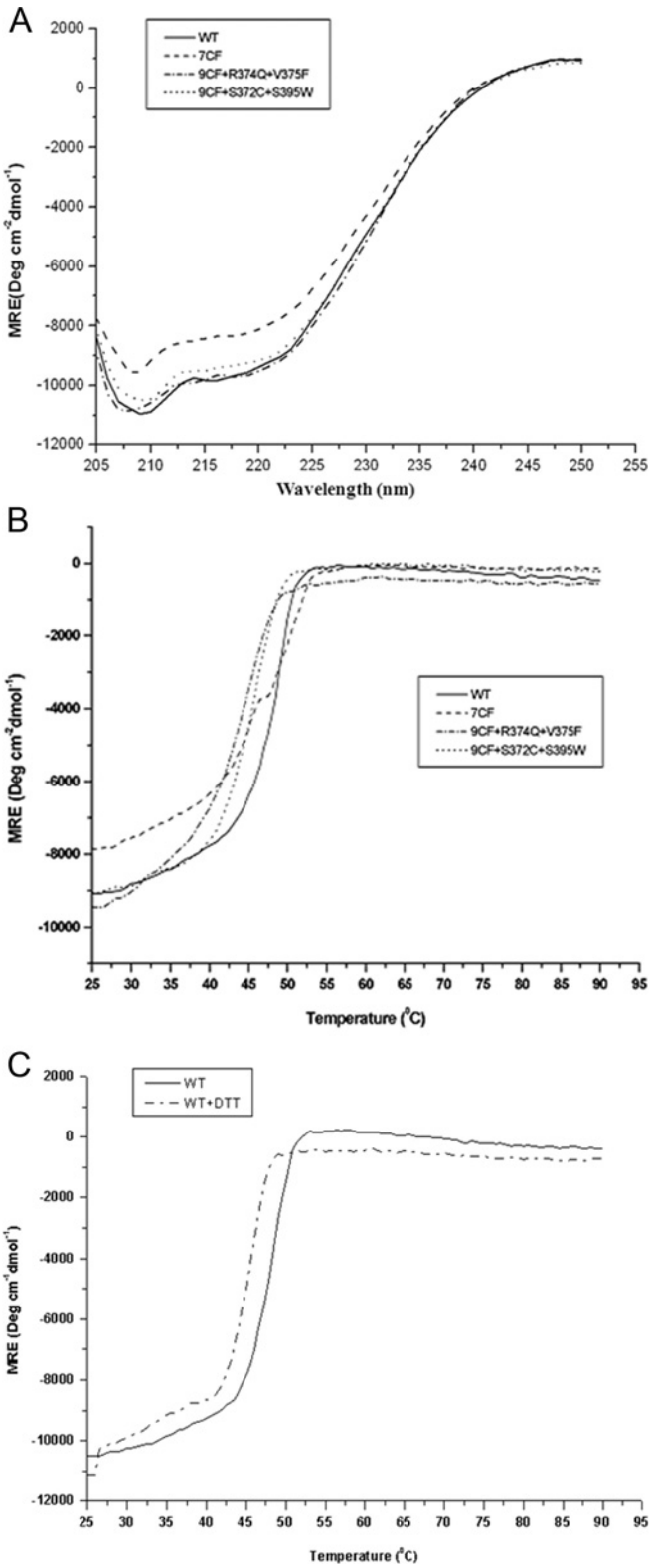

Figure S6 CD spectra and melting temperature scan of wild-type and mutant γ-GCS

The sample preparation for CD spectra of purified wild-type (WT) and mutant protein was carried out in 50 mM Tris/HCl, pH 8.0, 20 mM NaCl and 0.5 M guanidinium chloride. A protein concentration of 150 μg/ml was used. (**A**) CD spectra of wild-type and mutant γ-GCS. The CD spectra of wild-type, 7CF, 9CF + R374Q + V375F and 9CF + S372C + S395W were acquired at a wavelength range 205–255 nm. (**B**) A CD spectroscopy temperature scan of *E. coli* γ-GCS wild-type and mutant protein. The T_m scan of wild-type and mutants were carried out in the temperature range 25–90 °C. The spectra of wild-type, 7CF, 9CF + R374Q + V375F and 9CF + S372C + S395W was acquired at 222 nm. (**C**) CD spectroscopy temperature scan of *E. coli* γ-GCS in the presence of DTT. The wild-type γ-GCS sample was prepared in 50 mM Tris/HCl, pH 8.0, 20 mM NaCl, 0.5 M guanidinium chloride and 2 mM DTT. Protein concentration was 150 μg/ml. The temperature scan with DTT and without DTT was carried out at 222 nm. MRE, mean residue ellipticity.

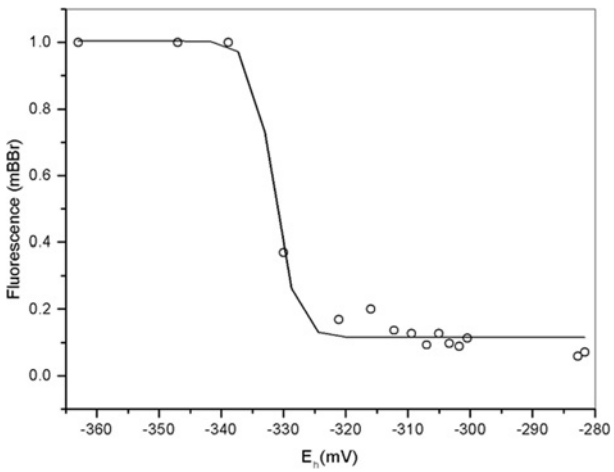

Figure S7 Redox midpoint potential measurement of 7CF γ-GCS protein

The oxidation–reduction titration for 7CF γ-GCS protein was carried out in 100 mM Hepes buffer, pH 7.0, that contained DTT at a total concentration of 10 mM. Proteins (50 μg) were incubated for 3 h to achieve redox equilibrium. The sample was then treated with monobomobimane (mBBr) and the E_h value was calculated from the Nernst equation as described previously [1,2].

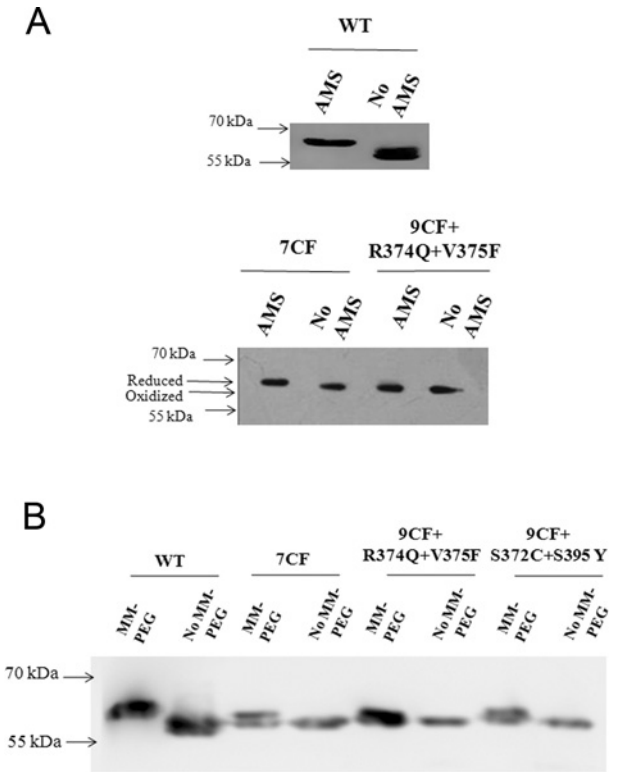

Figure S8 Identification of the reduced form of γ-GCS *in vivo* using AMS and *in vitro* using MM(PEG)

(**A**) The wild-type (WT), 7CF and 9CF + R374Q + V375F plasmids harbouring the *E. coli* strain were grown in LB medium supplemented with 100 μg/ml ampicillin. Expression was induced at $D_{600} = 0.4$ with 1 mM IPTG. At 90 min post-induction, the growing culture was treated with 100 % TCA to a final concentration of 20 %. The extracted protein was treated with 30 mM AMS as described in the Materials and methods section of the main text. The extracted protein was separated by SDS/PAGE (14 % gel) under non-reducing conditions. The detection of protein was carried out by Western blotting using an anti-His antibody. (**B**) The wild-type, 7CF, 9CF + R374Q + V375F and 9CF + S372C + S395Y proteins were purified from *E. coli*. Purified protein (400–500 μg) was precipitated with 20 % TCA by adding an equal volume. Precipitated protein was washed with ice-cold acetone twice and air-dried. The protein sample was treated with 5 mM MM(PEG) as described in the Materials and methods section of the main text. Protein detection was carried out by Western blotting using an anti-His antibody. Molecular masses are indicated to the left-hand side in kDa.

Table S1 List of oligonucleotides and their sequences used in the present study

EC, *E. coli*; Fw, forward; Rev, reverse.

Oligomer name	Sequence (5′→3′)
EcGshAC357S Rev	CTGCTCATTTCCGGTGCATCAGCCAGCGCAGACCAGACCATAAACAGGTCGAGG
EcGshAC372S Rev	GGTTCCAGTTAACGCGTGTAGAGGCAAGTTCGCTACTGCTCATTTCCGGTCATC
EcGshAC395S Rev	GAACAGATCTTTACCCACCTGCGGTAACGGGAACTGTGCGGTTTCGGAGCCGATACCCAGCGTCGTCAGAC
EcGshAC433C439S Fw	GCGAAGCGTATCAGAAAGTGTCTGATGAACTGGTTGCCTCCTTCGATAATCCCGATCTGA
EcGshAC433C439S Rev	GTCAGATCGGGATTATCGAAGGAGGCAACCAGTTCATCAGACACTTTCTGTACGCTTCGC
EcGshA400Rev_seq	CAGCCCTTCACGATACAGCG
EcGshA400Fw_seq	CGCTGTATCGTGAAGGGCTG
EcGshABamHI Fw	CTGCAGGGATCCATGATCCCGGACGTATCACAG
EcGshAXhoI Rev	CAGCTGCTCGAGTCAGGCGTGTTTTTCCAGCC
EcGshA S372W _Rev	GTTCCAGTTAACGCGTGTCCAGGCAAGTTCGCTACTGCTCATTTC
EcGshA S395F _ Fw	GTCTGACGCTGGGTATCGGCTTCGAAACCGCACAGTTCCCG
EcGshA S395F _ Rev	CGGGAACTGTGCGGTTTCGAAGCCGATACCCAGCGTCAGAC
EcGshA CHis BamH1 _Rev	ACCTAGGGATCCTTAATGATGGTGATGGTGATGGGCGTGTTTTTCCAGCCACACC
EcGshA_Sma1 Fw	GATATCCCCGGGATGATCCCGGACGTATCACAGG
R374Q F375V Rev	CCGGTTCCAGTTAACTTGTGTAGAGGC
Q374R V375F Fw	GCGAACTTGCCTCTACACGCTTTAACTGGAACCGGG
Q374R V375F Rev	CCCGGTTCCAGTTAAAGCGTGTAGAGGCAAGTTCGC
EcGshA C372A Rev	GTACCAGTTAACGCGTGTAGCGGCAAGTTCGCTACTGCTCATTTCC
EcGshA C395A Rev	GTCAGTAGATCTTTACCCACCTGCGGTAACGGGAACTGTGCGGTTTCGGCGCCGATACCCAGCGTC

Table S2 List of the representative members of lineage I used for sequence alignment to construct the phylogenetic tree in Figure 1 of the main text

Abbreviation used	NCBI accession number	Organism name
Esch_coli	NP_417173.1	*Escherichia coli* str. K-12 substr. MG1655
Shig_flex	NP_838224.1	*Shigella flexneri* 2a str. 2457T
Salm_typh	NP_461744.1	*Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2
Citr_kose	YP_001455545.1	*Citrobacter koseri* ATCC BAA-895
Ente_canc	ZP_05969993.1	*Enterobacter cancerogenus* ATCC 35316
Kleb_pneu	YP_001336655.1	*Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578
Cron_turi	YP_003211651.1	*Cronobacter turicensis* z3032
Serr_odor	ZP_06192317.1	*Serratia odorifera* 4Rx13
Yers_pest	NP_668220.1	*Yersinia pestis* KIM 10
Pect_caro	YP_003018766.1	*Pectobacterium carotovorum* subsp. *carotovorum* PC1
Erwi_pyri	YP_002649770.1	*Erwinia pyrifoliae* Ep1/96
Soda_glos	YP_454221.1	*Sodalis glossinidius* str. *'morsitans'*
Prot_mira	YP_002150150.1	*Proteus mirabilis* HI4320
Baum_cica	YP_588658.1	*Baumannia cicadellinicola* str. Hc
Arse_naso	CBA73578.1	*Arsenophonus nasoniae*
Cand_hami	YP_002925016.1	*Candidatus Hamiltonella defensa* 5AT (*Acyrthosiphon pisum*)
Alii_salmo	YP_002262162.1	*Aliivibrio salmonicida* LFI1238
Shew_deni	YP_562228.1	*Shewanella denitrificans* OS217
Idio_balt	ZP_01043504.1	*Idiomarina baltica* OS145
Ocea	ZP_01166769.1	*Oceanospirillum* sp. MED92
Acin_calc	ZP_06058310.1	*Acinetobacter calcoaceticus* RUH2202
Alca	ZP_05043145.1	*Alcanivorax* sp. DG881
Chro_sale	YP_574700.1	*Chromohalobacter salexigens* DSM 3043
Fran_novi	ZP_04989211.1	*Francisella novicida* GA99-3548
Burk_phyt	YP_001893776.1	*Burkholderia phytofirmans* PsJN
Pseu_aeru	YP_001351264.1	*Pseudomonas aeruginosa* PA7
Allo_vino	YP_003442779.1	*Allochromatium vinosum* DSM 180
Thio	YP_002512775.1	*Thioalkalivibrio* sp. HL-EbGR7
Nitr_ocea	YP_343611.1	*Nitrosococcus oceani* ATCC 19707
Psyc_arct	YP_263343.1	*Psychrobacter arcticus* 273-4
List_mono	ZP_05300878.1	*Listeria monocytogenes* LO28
Clos_botu	YP_001920910.1	*Clostridium botulinum* E3 str. Alaska E43
Past_mult	NP_245985.1	*Pasteurella multocida* subsp. *multocida* str. Pm70
Haem_infl	ZP_01786436.1	*Haemophilus influenzae* R3021
Ente_faec	ZP_05667624.1	*Enterococcus faecium* 1,141,733
Stre_ther	ADH82115.1	*Streptococcus thermophilus*
Stre_agal	EGS26792.1	*Streptococcus agalactiae* FSL S3-026
Ente_faeca	ZP_05594212.1	*Enterococcus faecalis* AR01/DG

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