Structural Basis for the Redox Control of Plant Glutamate Cysteine Ligase*§

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Glutathione (GSH) plays a crucial role in plant metabolism and stress response. The rate-limiting step in the biosynthesis of GSH is catalyzed by glutamate cysteine ligase (GCL) the activity of which is tightly regulated. The regulation of plant GCLs is poorly understood. The crystal structure of substrate-bound GCL from Brassica juncea at 2.1 Å resolution reveals a plant-unique regulatory mechanism based on two intramolecular disulfide bonds. Reduction of one disulfide bond allows a β-hairpin motif to shield the active site of B. juncea GCL, thereby preventing the access of substrates. Reduction of the second disulfide bond reversibly controls dimer to monomer transition of B. juncea GCL that is associated with a significant inactivation of the enzyme. These regulatory events provide a molecular link between high GSH levels in the plant cell and associated down-regulation of its biosynthesis. Furthermore, known mutations in the Arabidopsis GCL gene affect residues in the close proximity of the active site and thus explain the decreased GSH levels in mutant plants. In particular, the mutation in rax1-1 plants causes impaired binding of cysteine.

Glutathione (GSH) is the predominant non-protein thiol compound in eukaryotic and prokaryotic cells (1). Through reversible oxidation to glutathione disulfide, it acts as a major cellular redox buffer. In the plant chloroplast GSH can reach millimolar concentrations depending on diverse external stress stimuli and on the day-night transition. Light/dark-induced changes in stromal pH further modulate the redox potential of GSH (2).

In plants, GSH detoxifies reactive oxygen species via the ascorbic acid-GSH-cycle (3) and glutathione peroxidases (4). It is involved in the detoxification of xenobiotics via glutathione S-transferases (5), represents the precursor for the heavy metal ions sequestering phytochelatins (6), and serves as a major defense component against a wide range of abiotic and biotic stress factors (7). GSH can post-translationally modify proteins via glutathionylation (8, 9) and may act as a signaling molecule (10, 11). Notably, GSH represents a major storage and transport form of reduced sulfur in plants (12).

The biosynthesis of GSH occurs in two sequential ATP-dependent steps. Glutamate cysteine ligase (GCL, EC 6.3.2.2.) produces the reaction intermediate γ-glutamylcysteine (Scheme 1). Glutathione synthetase (EC 6.3.2.3.) then catalyzes the addition of a glycine residue.

\[
\text{L-glutamate + L-cysteine + ATP} \rightarrow \text{L-γ-glutamyl-L-cysteine + ADP + Pi}
\]

SCHEME 1

Both enzymes are encoded by single genes in Arabidopsis, belonging to the Brassicaceae family (13–15). In this plant family GCL is localized exclusively in the plastids, whereas glutathione synthetase is found both in plastids and in the cytosol (16). Under most conditions, GCL activity is rate-limiting in plant GSH biosynthesis (17). This is highlighted by significantly reduced glutathione levels in AtGCL mutants (18, 19).

Plant GCL protein sequences diverge from those of non-plant eukaryotic organisms (mammals, Drosophila). Although some bacteria share low sequence homology with BjGCL (15% identity with the Escherichia coli enzyme) other species such as Agrobacterium tumefaciens show up to 70% sequence identity. The molecular architecture of GCL in non-plant eukaryotes and bacteria is remarkably distinct. Whereas mammalian and Drosophila GCL consist of a catalytic and a regulatory subunit that reversibly dissociate (20, 21), the E. coli enzyme is a functional monomer (22).

Catalytic properties of plant GCL have been studied in vitro (23), but the regulation of the plant enzyme is poorly understood. Currently, it is known that GCL mRNA levels are increased in response to stress (24). However, a stress-induced increase of glutamate cysteine ligase activity has been moni-

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The atomic coordinates and structure factors (code 2GWC and 2GWD) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ [http://www.rcsb.org/].

[1] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

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3 The abbreviations used are: GCL, glutamate cysteine ligase (alternate name: γ-glutamylcysteine synthetase); BjGCL, B. juncea glutamate cysteine ligase; AtGCL, A. thaliana glutamate cysteine ligase; BS0, L-buthionine-(S,R)-sulfoximine; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; CC1, disulfide bridge 1 (Cys178-Cys356); CC2, disulfide bridge 2 (Cys178-Cys359); r.m.s.d., root mean square deviation; Tricine, N[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
Plant Glutamate Cysteine Ligase Regulation

tored in cell suspension cultures while no transcriptional up-regulation of AtGCL was observed (25).

These findings point toward a post-translational regulation of plant GCL that was suggested by Hell and Bergmann (26). Addition of reducing agents led to severe inhibition of plant GCL directly purified from tobacco cell suspensions. Similar findings could recently be quantified by monitoring the enzymatic activity of recombinant AtGCL in the presence and absence of reducing agents (23). The authors suggested the presence of an intramolecular disulfide bridge that, upon reduction, would trigger the inactivation of the enzyme.

In this study we have used X-ray crystallography to address the question of whether such a redox-sensitive mechanism exists in plant GCL and how it is implemented at the structural level. The first crystal structure of a plant GCL reveals the presence of two intramolecular disulfide bridges. Using site-directed mutants in biochemical experiments, we have analyzed their role in the regulation of the enzyme.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—BjGCL (acc AJ563921) was PCR-amplified using sense primer 5'-ACTGCCAT-GGGGCGGCGAGTCCTCCCAC-3' and antisense primer 5'-TAAATCGACTAGTTAAAGCAGTTCCTGGAA-3', thereby omitting the predicted plastidic transit peptide (residues 1–65). Subsequently, the NcoI/SalI-excised fragment was ligated into vector pETM20 (27). The thioredoxin fusion protein was expressed in E. coli Rosetta gami DE3 (Novagen, Madison, WI). Cells grown to an A600 of 1.5 were induced with 1 mM isopropyl-β-D-thiogalactopyranoside in Terrific Broth at 28 °C for 18 h. Pelleted cells were resuspended in lysis buffer (50 mM NaPi (pH 8.0), 300 mM NaCl, 10 mM imidazole, 0.2 M Mg(CH3COO)2·4H2O, 0.1 M Tricine, pH 8.0) and flash-frozen in liquid nitrogen. A hexagonal crystal form developed from unlabeled protein at a slightly increased polyethylene glycol concentration (18% (w/v) polyethylene glycol 3,350) and in the presence of 0.1 M ATP. These crystals were used in micro-seeding protocols to grow larger hexagonal crystals in the presence of L-glutamate as the only additive. Multiple wavelength anomalous dispersion data were collected from a monocrystal containing selenomethionine-labeled protein at beam line ID14-4 at the European Synchrotron Radiation Facility, Grenoble, France. In the case of the hexagonal crystal form, a dataset at 2.1-Å resolution has been recorded at beam line PX01 at Swiss Light Source, Villigen, Switzerland, using a large area detector. Data processing and scaling were carried out with XDS (version: May 2005) (28).

Structure Solution and Refinement—For structure solution of BjGCL, multiple wavelength anomalous dispersion data were used to locate 142 (out of the final 168) selenium sites with the programs SHELXD (29) and PHENIX.HYSS (30). Consistent sites as determined with SITCOM (31) were input in the program SHARP (32) for site refinement and phasing. NCS averaging and density modification were carried out with RESOLVE (33). Using the phases from RESOLVE, about two-thirds of the model could be automatically built using ARP/wARP (34). The structure was completed in alternating cycles of model correction in COOT (35) and restrained TLS-refinement in REFMAC5 (36; see Table 1).

The structure of the hexagonal crystal form has been determined by molecular replacement with the program PHASER (37). Inspection of the refined models with PROCHECK (38) revealed excellent stereochemistry with more than 90% of the residues in the most favored regions of the Ramachandran plot and none in disallowed regions. Structure visualization was done with POVSCRIPT (39) and POVRAY (www.povray.org).

Site-directed Mutagenesis and Preparation of Mutant Proteins—Site-specific mutations (C341S, C356A, C178S, and R220K)4–5 were introduced with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions and subsequently verified by DNA sequencing. Mutant proteins were produced using similar protocols as described for wt-BjGCL and purified to homogeneity by preparative size-exclusion chromatography using a Superdex 200 HR 16/60 column (Amersham Biosciences) equilibrated in 50 mM HEPES (pH 8.0) and 50 mM L-glutamate.

Selenomethionine-labeled BjGCL was expressed in E. coli strain B834(DE3) at 28 °C, after induction for 18 h with 1 mM isopropyl-β-D-thiogalactopyranoside, using Terrific Broth medium supplemented with 40 μg/ml seleno-L-methionine and purified as described above.

Crystallization and Data Collection—Monoclinic crystals of selenomethionine-labeled GCL (form A, see Table 1) were grown at room temperature by vapor diffusion from hanging drops composed of equal volumes (1 + 1 μl) of protein solution and crystallization buffer (15% (w/v) polyethylene glycol 3,350, 0.2 M Mg(CH3COO)2·4H2O, 0.1 M Tricine, pH 8.0) sus-
sham Biosciences) pre-equilibrated in 50 mM HEPES (pH 8.0), 50 mM NaCl, and 50 mM L-glutamate. 50 μl of the sample (10 mg/ml) was loaded onto the column, and elution at 0.8 ml/min was monitored by ultraviolet absorbance at 280 nm. For reduction experiments, half of the C341S or C356A mutant protein from one preparation was loaded on the column equilibrated in 50 mM HEPES (pH 8.0), 50 mM NaCl, and 50 mM L-glutamate, whereas the other half was extensively di-

### TABLE 1
Summary of crystallographic analysis

| PDB-ID | 2GWC | 2GW/D |
|---|---|---|
| Space group; unit cell (Å, °) | P2₁; a = 88.08, b = 199.11, c = 115.11, β = 99.450 | P6₂; a = b = 54.81, c = 518.04 |
| Wavelength (Å) | 0.97966 | 0.978180 |
| Resolution (Å) | 2.18 | 2.09 |
| Highest shell (Å) | 2.27-2.18 | 2.22-2.09 |
| Unique reflections | 385,971 (40,871) | 27,913 (3,283) |
| Multiplicity | 4.1 (2.0) | 11.0 (2.1) |
| 1/σ(I) | 13.6 (2.3) | 27.2 (5.7) |
| R_{int} (%) | 10.4 (45.7) | 6.2 (13.1) |
| Completeness (%) | 95.5 (89.9) | 94.8 (70.5) |
| Phasing | Phasing power | 1.855 |
| Figure of merit | 0.66 |
| Refinement | Resolution range (Å) | 99.50-2.18 | 47.46-2.09 |
| | Highest shell (Å) | 2.24-2.18 | 2.149-2.09 |
| | R_{work} (%) | 21.4 (29.1) | 17.8 (17.9) |
| | R_{free} (%) | 24.2 (32.6) | 22.8 (24.2) |
| Number of atoms | Protein | 28,070 | 3,509 |
| | Solvent | 1,017 | 245 |
| | Mg²⁺ | 8 | 1 |
| | BSO/L-glutamate | 112 | 10 |
| | Acetate | | 20 |
| r.m.s.d. | Bond length (Å) | 0.016 | 0.012 |
| | Angles (°) | 1.48 | 1.31 |

a Numbers in parentheses give respective statistics for the highest resolution shell.
b As defined in XDS (28).
c As defined in SHARP (32).
d As defined in REFMAC5 (36).

FIGURE 1. Plant GCL shows unique structural features. Front and side views of BjGCL shown in ribbon representation. The central β-sheet is depicted in dark blue, the N- and C-terminal helical regions in light blue, and the plant unique arms in dark and light green, respectively. The L-glutamate bound in the active site is represented in bond representation along with the Mg²⁺ ion (in cyan). The two disulfide bridges CC1 and CC2 are highlighted in yellow; the β-hairpin module is shown in red.
FIGURE 2. Substrate binding in plant GCL. A, close-up view of the glutamate binding site with the inhibitor BSO (in yellow; sulfur depicted in magenta) in bond representation and including the final $2F_{o}-F_{c}$ electron density map contoured at 1.5 $\sigma$. Residues reaching from the central $\beta$-sheet (in blue) to coordinate the Mg$^{2+}$ ion (in cyan) are depicted in blue. Residues contributed by the helical arms are shown in light green. B, schematic representation of the inhibitor BSO binding to BjGCL. The LigPlot diagram (50) summarizes key interactions between the BSO ligand and active site residues. Yellow lines, BSO ligand; green lines, BjGCL residues; semicircles with radiating lines; atoms or residues involved in hydrophobic contacts between protein and ligand. C, stereo close-up view of the plant GCL cysteine binding pocket formed by mostly hydrophobic residues (in blue) around the aliphatic side chain of BSO (in light gray). The corresponding secondary structure elements and residues in *E. coli* GCL (PDB-ID: 1VA6) are shown in orange. D, known mutations in the *Arabidopsis* GCL gene are in proximity of the substrate binding sites in plant GCL. BjGCL in ribbon representation is shown with BSO and ADP (modeled) in bond representation (in yellow). Small spheres indicate the positions of residues affected in AtGCL mutant plants (in magenta). Enlarged versions provide models on how the affected residues in *rax1-1* and *rml1* mutants may interact with GCL substrates. The *rax1-1* arginine residue (Arg$^{220}$) is shown in a modeled rotamer configuration bringing its guanidinium group in close proximity to the terminal methyl of BSO that corresponds to the sulfhydryl group of cysteine (in green). Interactions are highlighted by dotted lines (in magenta).
lyzed over night against 50 mM HEPES (pH 8.0), 50 mM NaCl, 50 mM l-glutamate, and 5 mM TCEP or 5 mM dithiothreitol, respectively, and then applied to the column equilibrated in the reducing buffer. For re-oxidation the reduced protein was dialyzed overnight against 50 mM HEPES (pH 8.0), 50 mM NaCl, and 50 mM l-glutamate and then reapplied to the column equilibrated in the oxidizing buffer.

**CD Spectroscopy**—This was performed on a temperature-controlled J-710 spectropolarimeter (Jasco, Easton, MD) at a cell path length of 0.2 cm and at a sensitivity of 50 millidegrees using protein samples at a concentration of 0.25 mg/ml, dialyzed against 5 mM NaPi (pH 8.0), 5 mM NaCl. Spectra were analyzed using the statistical package R (40).

**Enzyme Assays**—Pure samples of recombinant BjGCL were analyzed in a coupled enzymatic assay as described before (41). A standard reaction mixture (0.5 ml) contained 100 mM Tris (pH 8.0), 150 mM KCl, 20 mM MgCl₂, 10 mM l-cysteine, 20 mM l-glutamate, 5 mM ATP, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 5 units of type II rabbit muscle pyruvate kinase, and 10 units of type II rabbit muscle lactate dehydrogenase (chemicals and enzymes were purchased from Sigma). Reactions were initiated by addition of BjGCL (100–400 ng). The resulting decrease of A₃₄₀ nm was followed, and steady-state kinetic parameters were determined by initial velocity experiments. For determining the Kₘ values for cysteine and glutamate, their concentrations were varied from 0.2 to 20 mM (cysteine) or 0.5 to 25 mM (glutamate) while keeping the other component concentrations constant. Kinetic parameters were calculated to fit data to ν = [S]/(Kₘ + [S]) using the plotting method described by Hanes (42).

**Atomic Coordinates**—and structure factors have been submitted to the Protein Data Bank (www.rcsb.org) with code 2GWG (crystal form A) and 2GWD (crystal form B), respectively.

**RESULTS AND DISCUSSION**

**An Unexpected β-Hairpin in Plant GCL**—Plant GCL from *Brassica juncea* (residues 66–514) has been produced in a bacterial expression system allowing for the formation of disulfide bonds (see "Experimental Procedures"). BjGCL was crystallized under oxidizing conditions, and its structure was solved and refined in two crystal forms at resolutions of 2.18 Å (form A, Rfree 0.24) and 2.1 Å (form B, Rfree 0.23), respectively (Table 1). The molecules in the asymmetric units of both crystal forms are very similar and comprise residues 79–514. Form A contains the inhibitor BSO along with Mg²⁺ in the active site. Form B shows the enzyme bound to l-glutamate.

In contrast to previous predictions (13), plant GCL shares significant structural similarity with the *E. coli* enzyme (43). The primary sequence folds into a six-stranded anti-parallel β-sheet forming a bowl-like structure flanked by helical regions as shown in Fig. 1. This central part of BjGCL superimposes well with its bacterial counterpart (root mean square deviation (r.m.s.d.) between 223 corresponding Ca atoms <2.2 Å; see supplemental Fig. S1).

A plant-unique feature in BjGCL is a β-hairpin motif (residues 326–346; shown in red in Fig. 1) that is stabilized by a disulfide bridge (Cys³⁴¹–Cys³⁵⁶, named ‘CC1’ hereafter; see Fig. 1). The active site is located at the bottom of a solvent-accessible cavity that is formed by two arm-like structures flanking the central β-sheet (shown in light and dark green in Fig. 1). Two helices from the N and C termini are linked by a second disulfide bridge (Cys¹⁷⁸–Cys³⁹⁸, named “CC2” hereafter; see Fig. 1). The presence of two disulfide bonds in BjGCL has important implications for the regulation of plant GCL activity, which we discuss below.

**Catalytic Residues Are Conserved among Plant and *E. coli* GCL**—The crystal forms of BjGCL in complex with l-glutamate and BSO and Mg₂⁺ allow identifying residues important for substrate binding and catalysis in plant GCL. One Mg²⁺ ion is coordinated by three glutamate residues (Fig. 2A), one of which is replaced by aspartate in *E. coli* (supplemental Fig. S2) (43). BjGCL has been crystallized in the absence of the nucleotide, but the position of its binding pocket can be inferred from the high structural similarity with the *E. coli* enzyme, bound to ADP (see supplemental Fig. S3).

Structural comparison of form A and B crystals (Table 1) shows l-glutamate in the same position as is the glutamate mimicking portion of BSO (Fig. 2A). The substrate is coordinated by the highly conserved Arg²⁹² along with Thr²⁴² (Ile⁴⁴⁶ in *E. coli*, Fig. 2A and B) and supplemental Fig. S2). In BjGCL, additional contacts are made by Trp²⁹⁶ that are not present in the *E. coli* protein (Fig. 2A and supplemental Fig. S2). These variations in the substrate-binding residues might account for the differences in affinity for l-glutamate in bacterial and plant GCL (23, 44).

The aliphatic side chain of the inhibitor BSO is bound in the cysteine binding site. This portion of the binding pocket is formed by mainly hydrophobic residues reaching the alkyl chain of BSO from four directions (Fig. 2C), very similar to *E. coli* GCL (shown in orange in Fig. 2C) (43). Notably, the positions of Arg²²⁰ and Tyr²²¹ in BjGCL are reversed with respect to the *E. coli* enzyme (Fig. 2C and supplemental Fig. S2).

Depressed GSH Levels in the Arabidopsis rax1-1 Mutant Are Caused by Impaired Cysteine Binding—Recently, the mutant regulator of ascorbate peroxidase 2 (rax1-1) has been identified as a missense mutation in the *Arabidopsis* GCL gene, resulting in decreased glutathione levels (19). In the protein, the mutation substitutes Arg²²⁹ (Arg²²⁰ in *B. juncea*) for lysine. The arginine is located at the proximal side of the cysteine binding pocket (Fig. 2C), and its guanidinium group may be important for recognition of the sulfhydryl group of cysteine in plant GCL (Fig. 2D). We have engineered the mutation and analyzed its consequences on the affinity of BjGCL for the three substrates with respect to the wild-type protein. As shown in Table 2, the Kₘ value for cysteine is ~5-fold increased, whereas the affini-

**TABLE 2**

|       | Cysteine | Glutamate | ATP |
|-------|----------|-----------|-----|
| Vₘₐₓ |          |           |     |
| Kₘ   |          |           |     |

|       | wt-BjGCL | rax1-1 (R220K) |
|-------|----------|----------------|
| %     | 100      | 48             |
| Vₘₐₓ | 0.12 ± 0.01 | 0.58 ± 0.06  |
| Kₘ   | 8.5 ± 0.4 | 9.7 ± 0.5     |

Where 100% corresponds to 3.336 ± 114 nmol of γ-glutamylcysteine min⁻¹ mg⁻¹.

*V. A. Tropin, G. B. E. Blumberg, and A. Scherthan, J. Biol. Chem. 275, 27561 (2000).*
ties for either glutamate or ATP are unaffected. The decrease in binding affinity highlights the importance of Arg^{220} in the recognition of cysteine. Because the \( K_m \) for cysteine of wild-type BjGCL is comparable to the cellular concentration of cysteine (45), even the moderate \( K_m \) increase for cysteine in \( rax1-1 \) can account for significantly reduced glutathione levels observed in the mutant plants.

A cadmium-sensitive Arabidopsis mutant (cad2-1) deficient in AtGCL activity has been mapped to a 6-bp deletion in the GCL gene (18) that affects residues corresponding to the positions 229–231 in BjGCL. These residues are located in a loop region, and their deletion most likely alters the position of residues involved in substrate binding (e.g. Arg^{220} and Thr^{242}; Fig. 2D and supplemental Fig. S2) and may thus explain the altered GCL activity in planta. Furthermore, a missense mutation in the Arabidopsis GCL gene (root meristemless1, rml1) has been identified (11). It substitutes Asp^{259} (Asp^{250} in B. juncea) for asparagine, resulting in loss of GCL activity in vivo. As depicted in Fig. 2D, this residue most likely plays an important role in recognizing the adenine nucleotide (see also supplemental Fig. S3). Taken together, all abovementioned mutations are located in regions critical for substrate binding and catalysis.

Redox Regulation of Plant GCL Activity—It has been previously suggested that a decrease in activity of Arabidopsis GCL in the presence of reducing agents might be caused by a reduction of a disulfide bond that serves as a switch between active and inactive forms of the enzyme (23).

As a unique feature of plant GCL, we find two intramolecular disulfide bridges (CC1 and CC2; Fig. 1) in oxidized BjGCL crystals. Because they both may represent sites of redox regulation, we have dissected their individual contributions to GCL activity.

Redox regulation of BjGCL is implemented via two intramolecular disulfide bonds. A, stereo view of oxidized BjGCL (gray surface) indicates the relative position of a \( \beta \)-hairpin motif (in red). The disulfide bond CC1 (in yellow) may function as a loaded spring that fixates the hairpin over the substrate binding sites. BSO bound in the active site is shown in bond representation (in yellow) along with ADP (in black) whose position has been inferred from the E. coli structure (PDB ID: 1VA6). The side chain of \( rax1-1 \) R220 is shown as a space-filling model. B, redox regulation of BjGCL occurs via dimer to monomer transition. Surface view of the dimer found in crystals grown under non-reducing conditions. At the level of the dimer interface, the surface has been removed showing the contributing helical elements (in blue). The disulfide bond CC2 in both molecules is highlighted in yellow, a molecule of ADP is shown in the nucleotide binding site. C, enlarged view of the dimer interface in the same orientation as seen in B and D, following a 90° rotation. Seven salt bridges (in magenta) with bonding distances smaller than 3.1 Å and three conserved aromatic residues at the surface of each molecule contribute to interface stabilization.
CC1, we have mutated the CC1 cysteines (Cys341 and Cys356) individually to serine or alanine, respectively. Removal of the disulfide bond leaves the structural integrity of the mutant proteins unaffected (see supplemental Fig. S4). Importantly, the enzymatic activity of the mutant proteins is up to 10-fold lower when compared with wild-type BjGCL (Vmax 19 ± 7% for C341S, 12.6 ± 2.6% for C356A and 9.4 ± 0.9% for a C341S/C356A double mutant, respectively; Vmax 100% for wt-BjGCL, 3.33 ± 114 nmol min⁻¹ mg⁻¹; n = 5). In contrast, the binding affinities for all three substrates were largely unaffected (C341S, 12.6 ± 1.4% for CC1, we have mutated the CC1 cysteines (Cys341 and Cys356) individually to serine or alanine, respectively. Removal of the disulfide bond leaves the structural integrity of the mutant proteins unaffected (see supplemental Fig. S4). Importantly, the enzymatic activity of the mutant proteins is up to 10-fold lower when compared with wild-type BjGCL (Vmax 19 ± 7% for C341S, 12.6 ± 2.6% for C356A and 9.4 ± 0.9% for a C341S/C356A double mutant, respectively; Vmax 100% for wt-BjGCL, 3.33 ± 114 nmol min⁻¹ mg⁻¹; n = 5). In contrast, the binding affinities for all three substrates were largely unaffected (C341S, 12.6 ± 1.4% for L-Cys 0.18 ± 0.03 mM, Km L-Glu 8.3 ± 0.5 mM, Km ATP 1.1 ± 0.5 mM; n = 5). Combining these results and considering the large distance of CC1 from the active site, we suggest that reduction of CC1 reorients the β-hairpin so that it shields the entry to the substrate binding sites, thereby slowing down binding of new substrates and release of products.

In trying to study the role of the second disulfide bridge (CC2) by site-directed mutagenesis we obtained only heavily aggregated protein, suggesting that the mutation causes improper folding of the enzyme (see supplemental Fig. S4). To still dissect the role of CC2 in plant GCL regulation we monitored the effect of reducing agents on the CC1 mutant proteins (C356A and C341S), in which the first disulfide bond (CC1) has been disrupted (see Fig. 1). As these mutants are properly folded and show catalytic activity (see above and supplemental Fig. S4) we could specifically address the role of CC2 in the redox regulation of plant GCL. The mutant proteins were dialyzed against a reducing agent containing buffer (Table 3; see “Experimental Procedures”), as it has been described previously for wild-type AtGCL (23), and then assayed for enzymatic activity.

Comparing oxidized and reduced BjGCL in analytical size-exclusion chromatography, we observed a peak shift for the reduced protein (both CC1 mutant and wild-type; Fig. 4, A and B) that has been previously described also for wild-type AtGCL (23). In contrast to that analysis where large conformational changes in monomeric GCL were proposed to be responsible for the peak shift (23), we found the different retention times on the column to be consistent with oxidized dimeric and reduced monomeric forms of BjGCL, respectively (Fig. 4A).

The change in elution profile of BjGCL in response to reducing conditions is reversible, consistent with previous experiments using AtGCL (23). Testing different reducing agents, the activity of the reduced CC1 mutant protein (C356A) was significantly (up to 5-fold) decreased when compared with the already depressed levels of the oxidized mutant (Table 3). Finally, the specific activity of reduced monomeric wt-BjGCL was 207 ± 64 nmol min⁻¹ mg⁻¹, and that of the re-oxidized dimer was 1908 ± 38 nmol min⁻¹ mg⁻¹, suggesting that reduction of CC1 and CC2 can reversibly inhibit GCL activity.

The existence of an oxidized dimeric form of BjGCL is supported by the arrangement of the oxidized GCL molecules in the crystals: (i) The asymmetric unit of crystal form A (Table 1) is composed of four highly similar BjGCL dimers (largest root mean square deviation comparing all corresponding Ca atoms was 0.4 Å); we found the same dimer in crystal form B between symmetry-related molecules; (ii) A relatively large dimer interface (1500 Å²; Fig. 3, C and D) assembles contact residues in a zipper-like arrangement and, most importantly, is in close proximity to the disulfide bridge CC2 (highlighted in yellow in Fig. 3B).

The crystal structure of oxidized BjGCL thus provides a possible explanation as to why the reduced protein shows a decreased enzymatic activity. The CC2 disulfide bridge joins two helical elements in BjGCL that extensively contribute to the dimer interface in the oxidized enzyme (Fig. 3, B and C). We speculate that reduction of CC2 distorts this interface by unzipping these helical elements, a process that leads to the dissociation of dimers. The associated decrease in activity could be accounted for by structural alterations in the ATP binding site, which is in close proximity of the dimer interface (Fig. 3B).

### Table 3

| Concentration | β-Mercaptoethanol | DTT | TCEP | GSH |
|---------------|------------------|-----|------|-----|
| mM            | % activity*      |     |      |     |
| 0             | 100              | 100 | 100  | 100 |
| 1             | 112 ± 9.1        | 87 ± 11.8 | 70 ± 10.0 | 100 ± 21 |
| 2             | 105 ± 7.6        | 65 ± 6.7  | 55 ± 2.6  | 88 ± 17  |
| 5             | 90 ± 2.5         | 47 ± 4.0  | 23 ± 1.6  | 46 ± 13  |
| 10            | 76 ± 1.6         | 31 ± 4.0  | 18 ± 0.6  | 18 ± 9   |

*Where 100% correspond to 225 ± 12 nmol of γ-glutamylcysteine min⁻¹ mg⁻¹.

The catalytic machinery of plant GCL has significant structural similarity with the enzyme from *E. coli* yet harbors a completely novel regulatory mechanism that involves two intramolecular disulfide bonds. Reduction of CC1 triggers structural rearrangements in plant GCL that hinder the access of substrates to the catalytic site (Fig. 3A). The second disulfide bridge CC2 stabilizes a homodimeric configuration of the active oxidized enzyme (Fig. 3B). Interestingly, the native molecular mass of AtGCL directly extracted from *Arabidopsis thaliana* chloroplasts is consistent with a dimeric rather than a monomeric configuration of the enzyme in planta (49).

### Fast and Slow Redox Response in Plant GCL—When dialyzing oxidized wt-BjGCL in a buffer containing 5 mM TCEP, more and more dimeric GCL dissociates into the reduced monomeric form as a function of time. The specific activities of dimer and monomer fractions of wt-BjGCL (dimer: 1908 ± 38 nmol min⁻¹ mg⁻¹; dimer + 5 mM TCEP: 480 ± 30 nmol min⁻¹ mg⁻¹ (1) in Fig. 4B); monomer + 5 mM TCEP: 207 ± 64 nmol min⁻¹ mg⁻¹ (2) in Fig. 4B) suggest that the surface-exposed CC1 disulfide bridge (Figs. 1 and 3A) is quickly reduced, whereas dimer dissociation occurs on a rather large time scale in the range of several hours (also see Ref. 23). A likely explanation for this slow reduction of CC2 in the presence of a very potent reducing agent is that CC2 is buried in the dimeric interface of oxidized BjGCL (Fig. 3B). Therefore, it may not be prone to direct reduction from the surrounding buffer. Alternatively, the...
differences observed in the kinetics of CC1 and CC2 reduction may be linked to differences in the redox potentials of the two disulfide bridges.

Notably, CC1 and CC2 do not only respond to reducing agents in a distinct way but also show a different degree of sequence conservation. Although CC1 is present in the well studied *Brassicaceae* (AtGCL (23) and BjGCL) and *Legumino-sae* proteins, it appears to be absent in some plant families (supplemental Fig. S2). In contrast, CC2 seems to be well conserved not only among all known plant GCL but also in the related bacterial sequences (supplemental Fig. S2).

**Compartment-specific Regulation of Plant GCL**—There is strong evidence that GCL proteins in the *Brassicaceae* family are exclusively targeted toward the plastids (16), organelles that allow for the formation of disulfide bonds and that contain GSH in high concentrations. The surface-exposed position of CC1 in

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**FIGURE 4.** Redox controlled monomer-dimer transition in plant GCL. A, a 280 nm absorbance trace of an analytical size-exclusion chromatography (SD 200 HR10/30; see “Experimental Procedures”). The C356A mutant elutes as a dimer under non-reducing (black line) and as a monomer (in red) under reducing conditions (5 mM TCEP; dialyzed overnight). Void ($V_v$) and total volume ($V_t$) are shown together with the elution volumes of molecular weight standards (A, aldolase, MW 158,000; B, bovine serum albumin, MW 67,000; C, chymotrypsinogen A, MW 25,000). The estimated molecular weight values of the BjGCL dimer and monomer are 95,000 and 45,000, respectively. The calculated monomer molecular weight is 51,017. B, a size-exclusion chromatography (SD 75 HR10/30) of wt-BjGCL samples dialyzed against 50 mM HEPES (pH 8.0), 50 mM NaCl, 50 mM l-Glu, and 5 mM TCEP for 0, 3, and 8 h, respectively. C, SDS-PAGE analysis of fractions corresponding to peaks 1 and 2 obtained using BjGCL dialyzed against 5 mM TCEP for 3 and 8 h, respectively. Molecular mass markers are indicated.
the BjGCL structure would certainly allow for a direct reduction by the plastidic GSH pool. In contrast, the slow dissociation of BjGCL dimers in the presence of a strong reductant indicates that in vivo a catalyst, e.g. a protein/cofactor, may be required to control the redox state of the buried CC2 disulfide bridge.

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