Arabidopsis thaliana Glutamate-Cysteine Ligase

FUNCTIONAL PROPERTIES, KINETIC MECHANISM, AND REGULATION OF ACTIVITY*

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In plants, glutathione accumulates in response to different stress stimuli as a protective mechanism, but only limited biochemical information is available on the plant enzymes that synthesize glutathione. Glutamate-cysteine ligase (GCL) catalyzes the first step in glutathione biosynthesis and plays an important role in regulating the intracellular redox environment. Because the putative Arabidopsis thaliana GCL (AtGCL) displays no significant homology to the GCL from bacteria and other eukaryotes, the identity of this protein as a GCL has been debated. We have purified AtGCL from an Escherichia coli expression system and demonstrated that the recombinant enzyme catalyzes the ATP-dependent formation of γ-glutamylcysteine from glutamate ($K_m = 9.1 \text{ mM}$) and cysteine ($K_m = 2.7 \text{ mM}$). Glutathione feedback inhibits AtGCL ($K_i \approx 1.0 \text{ mM}$). As with other GCL, buthionine sulfoximine and cystamine inactivate the Arabidopsis enzyme but with inactivation rates much slower than those of the mammalian, bacterial, and nematode enzymes. The slower inactivation rates observed with AtGCL suggest that the active site differs structurally from that of other GCL. Global fitting analysis of initial velocity data indicates that a random ter-

Regulation of the intracellular redox environment is critical in cellular physiology for influencing signaling pathways and cell fate in response to stress (1). In plants, as in other organ-

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† The abbreviations used are: GCL, glutamate-cysteine ligase (alternate name: γ-glutamylcysteine synthetase); AtGCL, Arabidopsis thali-

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Arabidopsis Glutamate-Cysteine Ligase

The plant GCLs are largely unexamined at the molecular level. Expression cloning isolated a cDNA from Arabidopsis that was unrelated to the mammalian, E. coli, or yeast GCL but complemented a GCL-deficient E. coli strain (14). However, doubts about the specificity of the assay used to measure GCL activity in cell lysates and the limited sequence similarity with other GCLs have challenged the identity of the AtGCL clone (4, 22). To characterize the biochemical properties of the putative AtGCL, recombinant enzyme was expressed in E. coli and purified to homogeneity. We demonstrate that the purified protein catalyzes the formation of γ-glutamylcysteine. Our analysis shows that the Arabidopsis enzyme shares some functional properties with the GCL from other species but is regulated differently than the GCL from either bacteria or non-plant eukaryotes.

EXPERIMENTAL PROCEDURES

Materials—Integrated DNA Technologies, Inc. synthesized all oligonucleotides used in this study. The pGEM-T Easy vector was obtained from Promega. E. coli Rosetta (DE3) cells were from Novagen. Ni²⁺-nitrilotriacetic acid (NTA)-agarose was bought from Qiagen. Benzamidine-Sepharose and the HiLoad 26/60 Superdex-75 FPLC column were from Amersham Biosciences. All other reagents were purchased from Sigma-Aldrich and were of ACS reagent quality or better.

Cloning and Generation of Expression Vectors—AtGCL (GenBank Z29490) (15) was amplified by PCR from an Arabidopsis cDNA library using 5′-dCTTCGATGGCGCTGTTCAAGCGG-3′ as the forward primer (NcoI site is underlined, the AtGCL start codon is in bold, and two E. coli optimized codons are in italic) and 5′-dTTTAGACACCTTGGTCACGCCATTCTC-3′ as the reverse primer (the putative AtGCL stop codon is in bold). The 1.6-kb PCR product was subcloned into the pGEM-T Easy vector (Promega). Automated nucleotide sequencing confirmed the fidelity of the PCR product (Washington University Sequencing Facility, St. Louis, MO). The pHIS8-AtGCL expression vector was constructed by digesting pGEM-T-AtGCL with NcoI and NotI and then ligating the fragment into a NcoI/NotI-digested pHIS8 vector (23). An expression construct of AtGCL with a truncated N terminus (pHIS8-AtGCL585) was generated by PCR using the appropriate oligonucleotides.

Expression in E. coli and Protein Purification—Expression constructs were transformed into E. coli Rosetta (DE3) cells. Transformed E. coli were grown at 37 °C in Terrific broth containing 50 μg/ml kanamycin, 340 μg/ml chloramphenicol until A600 nm = 0.6. After induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside, the cultures were grown at 18 °C for 6 h. Cells were pelleted by centrifugation and resuspended in lysis buffer (50 mM Tris (pH 8.0), 500 mM NaCl, 20 mM imidazole, 5 mM MgCl₂, 10% (v/v) glycerol, and 1% (v/v) Tween 20). After sonication and centrifugation, the supernatant was passed over a Ni²⁺-NTA column previously equilibrated with lysis buffer. His-tagged protein was eluted with elution buffer (wash buffer containing 250 mM imidazole). Incubation with thrombin during overnight dialysis at 4 °C against wash buffer removed the His-tag. DiaZyed protein was reloaded on a Ni²⁺-NTA column, and the flow-through was depleted of thrombin using a benzamidine-Sepharose column. The flow-through of this step was dialyzed overnight against 30% (v/v) glycerol, 25 mM HEPES (pH 7.5), 5 mM MgCl₂, and 100 mM NaCl then loaded onto a Superdex-75 FPLC column equilibrated in the same buffer without glycerol.

Enzyme Assays—The activity of AtGCL was determined spectrophotometrically at 25 °C by measuring the rate of ADP formation using a coupled assay with pyruvate kinase and lactate dehydrogenase. A standard reaction mixture (0.5 ml) contained 100 mM MOPS (pH 7.0), 150 mM NaCl, 20 mM MgCl₂, 10 mM cysteine, 20 mM sodium glutamate, 5 mM disodium ATP, 2 mM sodium phosphoenolpyruvate, 0.2 mM NADH, 5 units of type III rabbit muscle pyruvate kinase, and 10 units of type II rabbit muscle lactate dehydrogenase. Reactions were initiated by adding AtGCL (50 μg). The rate of decrease in A500 nm was followed using a Cary BioS00 spectrophotometer. Steady-state kinetic parameters were determined by initial velocity experiments. Measurements of the kcat and Kₘ values for glutamate (1–80 mM) were made at 20 mM ATP and 20 mM cysteine. Kinetic constants for cysteine (0.1–20 mM) were measured at 20 mM ATP and 80 mM glutamate. For determination of the kinetic constants for ATP (0.25–20 mM), 20 mM cysteine and 80 mM glutamate were used. Kinetic parameters were calculated to fit untransformed data to v = kcat[S]/(Kₘ + [S]) using Kaleidograp (Synertek, Inc. software).

Inhibition and Inactivation Assays—For glutathione inhibition of AtGCLΔ85, initial velocities were determined spectrophotometrically using the standard assay system. Enzyme activity was determined after addition of glutathione (0–5 mM) to assay solutions containing either varied glutamate (1–40 mM) or cysteine (0.5–20 mM). Global fitting analysis was then used to fit all data to the equation for non-competitive inhibition, v = Vₘₐₓ/[1 + [(I/Kₛ)(1 + Kₛ[S])]] in SigmaPlot (Systat Software, Inc.).

The time-dependent inactivation of AtGCLΔ85 by buthionine sulfoximine and cystamine was performed as follows. AtGCLΔ85 (125 μg) was incubated (37 °C) in 100 μl of 0.1 M MOPS (pH 7.0) and 20 mM MgCl₂ solution of either 0.5 mM cystamine or 50 mM buthionine sulfoximine. All incubations were initiated by the addition of the inactivator. Aliquots (20 μl) were withdrawn from the incubation mixture and diluted into the standard assay system and then the enzymatic activity remaining was determined. All inactivation experiments were monitored relative to a control sample without inactivator, which is set to 100% activity at each time point. Inactivation data were plotted as log (% initial enzyme activity) versus time. Semilog plots were fitted to the equation –dE/dt = k[I], where the disappearance of enzyme activity over time is related to the concentration of inactivator (I), multiplied by k, a rate constant. This allowed a determination of the half-life for inactivation (t½) at each [I]. A Kitz-Wilson analysis of the data was used to generate the limiting constant for inactivation (k₈50), and K₈ was plotted as log (k₈) versus 1/[I] (24).

Analysis of the Kinetic Mechanism—Analysis of the kinetic mechanism of AtGCLΔ85 used global curve fitting (25, 26). The reaction rates were measured as described above using a matrix of substrate concentrations (glutamate, 2–40 mM; ATP, 0.5–20 mM; cysteine, 1–20 mM). In this matrix, the rate measured for the concentration of one substrate is measured over the entire range of the other two substrates. SigmaPlot was used for curve fitting and modeling of the kinetic data to rapid equilibrium rate equations of the possible ter-reactant kinetic mechanisms (27).

Mass Spectrometry—An Applied Biosystems QSTAR XL hybrid quadrupole time-of-flight (TOF) mass spectrometry (MS) system equipped with a nanoelectrospray source (Proteomic X2 Mass Spectrometer) was used for an accurate molecular weight determination. The nanoelectrospray was generated from a PicoTip needle (New Objectives, Inc.) at 1200 volts. The sample flow rate was estimated to be 100 nl min⁻¹. The instrument m/z response was calibrated with standards from the manufacturer to provide molecular mass measurement accuracy of ~30 ppm for proteins up to 50 kDa and of ~5 ppm for lower mass peptides. For detection of protein complex species, data were acquired initially over the range m/z 900–10000 and later over a narrow mass range with intense signals. The accumulation time was 1 s, for 300 cycles. The two declustering potential parameters and focusing potential, i.e. DP, DP2, and FF, were 100, 15, and 300, respectively. To maintain the protein complexes in gas phase, the gas pressure in the collision cell was increased to 4.0 × 10⁻² Torr for ion cooling. For MS/MS analysis, data were acquired using the information-dependent acquisition feature in the Analyst Q5 software. TOF and tandem MS data were acquired over m/z ranges of 300–2200 and 65–2000, respectively. Every spectrum was accumulated for 1 s and was followed by three-product ion spectra (each for 3 s). The DP, DP2, FF settings were 50, 10, and 200, respectively, and the collision energy was dependent on the m/z values of the ions.

For identification of the reaction product by electrospray ionization (ESI)-TOF MS, scaled-up reactions (5-ml volume, 1 mg of protein) were performed using standard assay conditions. Reactions were quenched with 5% (v/v) acetic acid. The supernatant was evaporated to dryness and dissolved in water. Enzymatically synthesized γ-glutamylcysteine was compared with an authentic standard (Sigma-Aldrich).

RESULTS

Expression and Purification of AtGCL—The reported nucleotide sequence (15) was used to design oligonucleotides for amplifying AtGCL from an Arabidopsis library. Several clones were identical to each other but had a single base pair deletion (T1470) compared with the published sequence that shifts the reading frame at the C terminus. The sequence obtained was identical to other GenBank™ entries for AtGCL, including that from genome sequencing (NP_194041). The C-terminal amino acid sequence of our AtGCL clone was 70% identical to
other plant GCL sequences, suggesting that this is the correct sequence. Because the N-terminal region of AtGCL encodes a chloroplast transit signal (10), we generated a version of AtGCL (AtGCLΔ55) lacking the localization sequence based on comparison with the cytosolic GCL from Zea mays (28).

AtGCL and AtGCLΔ55 were overexpressed and purified by Ni²⁺-affinity and size-exclusion chromatography (Fig. 1). SDS-PAGE analysis of the purified proteins showed that AtGCL and AtGCLΔ55 migrated with molecular masses of 58 and 50 kDa, respectively, corresponding to their predicted masses. Full-length AtGCL was primarily insoluble, but 1 μg of size-exclusion purified protein (after thrombin digest).

Fig. 1. Expression and purification of AtGCLΔ55. SDS-PAGE samples were stained for total protein using Coomassie Blue. Molecular mass markers are indicated. Lanes A–C are as follows: A, 75 μg of sonicate; B, 10 μg of Ni²⁺-NTA purified protein (before thrombin digest); C, 10 μg of size-exclusion purified protein (after thrombin digest).

AtGCL catalyzed the ligation of glutamate to cysteine in the presence of ATP with a specific activity of 120 nmol min⁻¹ mg protein⁻¹. AtGCLΔ55 catalyzed the same reaction with a similar specific activity. Because the removal of the localization sequence improved solubility without altering enzymatic activity, we used AtGCLΔ55 for subsequent analysis. Substitution of α-amino butyrate for cysteine in these reactions resulted in 20-fold reductions in specific activity even when high concentrations (up to 100 mM) were used. Therefore, cysteine was employed as a substrate for all further enzyme assays.

Kinetic Analysis and Identification of the Reaction Product—Steady-state kinetic parameters (kcat and Km) for glutamate, cysteine, and ATP were determined for AtGCLΔ55 (Table I). The kcat and Km values were comparable with those reported for GCL purified from tobacco cell suspension (29). The reaction rate of recombinant purified AtGCLΔ55 was 50-fold higher than the tobacco enzyme likely because of the greater purity of these samples. Compared with other characterized GCL (17, 19–21, 30–33), AtGCL displays a Km value for glutamate 3–10-fold higher and a Kcat value for cysteine 0.5–10-fold different.

The reaction product of AtGCLΔ55 was analyzed by ESI-TOF mass spectrometry (Fig. 2). A TOF-MS survey scan showed a major component of m/z = 251.0894. The mass matches that of authentic γ-glutamylcysteine (MW 251.0841). Fragmentation of the precursor ion generated two major product ions corresponding to γ-glutamylcysteine cleaved at the peptide bond. The data demonstrated that the purified AtGCL catalyzed the production of γ-glutamylcysteine and is a GCL, even though it displays a low sequence homology with the GCL from other species.

Inhibition and Inactivation of AtGCL—Glutathione inhibits the activity of GCL from non-plant eukaryotes and bacteria (8, 17, 19–21). To determine whether similar inhibition occurs in plants, we examined the effect of increasing glutathione levels on AtGCLΔ55 (Fig. 3). Glutathione was determined to be a non-competitive inhibitor of GCL activity versus both glutamate (K1 = 0.72 ± 0.05 mM) and cysteine (K1 = 2.21 ± 0.14 mM). These values fall within the range of inhibition constants (0.1–8.2 mM) reported for other GCL (8, 17, 19–21, 30–33).

Buthionine sulfoximine (34) and cystamine (35) inactivate GCL. GCL catalyzes the ATP-dependent phosphorylation of buthionine sulfoximine to form a γ-glutamylphosphate intermediate that mimics the transition state bound at the active site (34). Cystamine is a thiol-specific inactivator of GCL that targets a cysteine within the active site (35).

We examined the inactivation of AtGCL by these compounds (Fig. 4). Both molecules inactivated AtGCLΔ55 with pseudo-first order kinetics (Fig. 4A and C). Inactivation by buthionine sulfoximine displayed a kina = 0.024 min⁻¹ (t½ = 28.7 min) and a K1 = 1.2 mM (Fig. 4B). Buthionine sulfoximine did not inactivate AtGCLΔ55 in the absence of ATP or MgCl2, confirming that inactivation requires phosphorylation of the inactivator. Inactivation by cystamine showed a kina = 0.028 min⁻¹ (t½ = 24.5 min) and a K1 = 11 mM (Fig. 4D). Overall, inactivation of AtGCL by both molecules occurs more slowly and with increased K1 values than inactivation of the bacterial, mammalian, or nematode enzymes (20, 21, 33, 35, 36). For example, E. coli GCL is inactivated by buthionine sulfoximine with kina = 0.3 min⁻¹ (t½ = 2.3 min) and K1 = 66 μM (21) and inactivation of rat GCL is even more rapid with kina = 3.7 min⁻¹ (t½ = 11 s) and K1 < 100 μM (34).

Kinetic Mechanism of AtGCL—To determine the kinetic mechanism of AtGCL, we obtained a complete matrix of kinetic data for the three substrates (25–27). Six families of data were generated in which one of the ligands is maintained at a saturating concentration whereas the other two substrates are varied. Kinetic data were first analyzed as double-reciprocal plots (Fig. 5). Because the plots for each family of data converged, potential ping-pong kinetic mechanisms for AtGCL were eliminated. Next, the initial velocity data were globally fit to the 16 possible rapid equilibrium rate equations describing a terreactant system (27), including random, ordered, and partially...
ordered/random mechanisms. Simultaneous fitting of the initial velocity data provides a more robust analysis than replotting reciprocal data parameters (37).

Based on global fits to the initial velocity data, the best results were obtained using the equation for a random ter-reactant system (Equation 1),

\[
\frac{V}{V_{\text{max}}} = \frac{[\text{ATP}][\text{Cys}][\text{Glu}]}{K_{\text{ATP}} + [\text{ATP}][\text{Cys}] + [\text{ATP}][\text{Glu}] + [\text{ATP}][\text{Cys}][\text{Glu}] + K_{\text{Cys}} + [\text{Cys}][\text{Glu}] + \frac{K_{\text{ATP}}K_{\text{Cys}}K_{\text{Glu}}}{K_{\text{ATP}}K_{\text{Cys}}K_{\text{Glu}}}}
\]

(Eq. 1)

where \(K_{\text{ATP}}, K_{\text{Cys}}, \text{ and } K_{\text{Glu}}\) are the equilibrium dissociation constants for the binding of substrate with the free enzyme and, \(\alpha, \beta, \text{ and } \gamma\) are the interactions factors between cysteine and glutamate, ATP and glutamate, and ATP and cysteine, respectively. These factors indicate how the dissociation con

FIG. 3. Inhibition of AtGCL by glutathione. Lines represent the global fit of all data \((n = 3)\) to the equation for non-competitive inhibition. A, double-reciprocal plot of the effect of glutamate on inhibition by glutathione. Initial velocities were determined as described under “Experimental Procedures” at 0 (closed circles), 0.1 (open circles), 0.25 (closed squares), 0.5 (open squares), 1 (closed triangles), and 2.5 mM (open triangles) glutathione. B, double-reciprocal plot of the effect of cysteine on inhibition by glutathione. Initial velocities were determined as described under “Experimental Procedures” at 0 (closed circles), 0.1 (open circles), 0.25 (closed squares), 0.5 (open squares), 0.5 (closed triangles), 1 (open triangles), and 5 mM (closed diamonds) glutathione.

FIG. 4. Inactivation of AtGCL by buthionine sulfoximine and cystamine. All inactivation incubations were performed \((n = 3)\) as described under “Experimental Procedures.” A, time- and concentration-dependent inactivation by buthionine sulfoximine. From top to bottom, 0, 1, 5, 10, 25, and 50 mM buthionine sulfoximine. B, Kitz-Wilson analysis of buthionine sulfoximine inactivation. C, time- and concentration-dependent inactivation by cystamine. From top to bottom, 0, 1, 2.5, 5, 10, and 50 mM cystamine. D, Kitz-Wilson analysis of cystamine inactivation.
stant for a ligand changes when other ligands are bound. Fits of the data are shown as lines in Fig. 5 for this kinetic model (Fig. 6). Table II summarizes the fitted parameters and the calculated substrate dependences.

The \( K_m \) values reported in Table I are in agreement with the results from global fitting of the initial velocity data (Table II). Because binding of substrates influences the binding of other substrates in a random kinetic mechanism, the \( K_m \) values (Table I) will not equal the dissociation constants calculated from fitting the data (27). Instead, the \( K_m \) values approximate a combination of the dissociation constant and interaction factors. For example, the \( K_m \) for cysteine (Table I) is similar to \( \alpha \gamma K_{Cys} \) calculated from fits of the initial velocity data (Table II).

**Redox Sensitivity of AtGCL**—Size-exclusion chromatography of full-length AtGCL (58 kDa) and AtGCLΔ85 (50 kDa) during their purification showed that each protein migrated as a 60–65-kDa species. However, incubation of AtGCLΔ85 with DTT shifted the elution volume to a corresponding molecular mass of ~30 kDa (Fig. 7A). SDS-PAGE analysis of peaks A (60 kDa) and B (30 kDa) from the size-exclusion column in buffer containing 1 mM DTT showed that AtGCLΔ85 (50 kDa) was present in both peaks (Fig. 7B). Similar size-exclusion chromatography and SDS-PAGE results were observed for full-length AtGCL. Assays for GCL activity demonstrated that peaks A and B had specific activities of 130 and 15 nmol min\(^{-1}\) mg protein\(^{-1}\), respectively. Size-exclusion chromatography after dialysis in the presence of either 10 mM \( \beta \)-mercaptoethanol or 10 mM glutathione had the same effect as DTT.
The change in elution profile of AtGCL in response to reducing environment is reversible (Fig. 7C). After incubation in the presence of 10 mM DTT, AtGCLΔ85 elutes from the size-exclusion column at a volume corresponding to 30 kDa with a specific activity of 15 nmol min⁻¹ mg protein⁻¹. Removal of DTT shifts the elution profile back to a 60-kDa species with a specific activity of 120 nmol min⁻¹ mg protein⁻¹. After incubation with DTT, AtGCLΔ85 was incubated with iodoacetamide to block free thiol groups on the protein (Fig. 7C, inset). Following dialysis, size-exclusion chromatography showed that the protein primarily eluted as the 30-kDa species indicating that blocking of free thiol prevents formation of the active monomer form. These results suggest that the plant GCLs are regulated differently than non-plant eukaryotic GCL (16, 17).

**DISCUSSION**

AtGCL has remained functionally uncharacterized (15) and the lack of sequence homology with other GCL and concerns with the original activity assays have caused controversy over the identity of this protein (4, 22). To resolve this issue and to provide a biochemical analysis of AtGCL, we have expressed (not shown). Because similar results were obtained in ammonium acetate buffer (not shown), the presence of an intermolecular interaction can be eliminated. The ESI-TOF MS conditions for determining the oligomerization state were optimized using bovine serum albumin and yeast enolase (38, 39).

**FIG. 7. Sensitivity of AtGCL to reductants.** A, size-exclusion chromatography of AtGCLΔ85 in the absence and presence of DTT. Before injection on a Superdex-75 26/60 FPLC column, purified protein was dialyzed overnight at 4 °C in buffer (25 mM HEPES (pH 7.5), 100 mM NaCl, and 5 mM MgCl₂) containing 0 (orange), 1 (black), or 10 (red) mM DTT. Peak A corresponds to 60 kDa, and peak B corresponds to 30 kDa using gel-filtration molecular weight standards. B, SDS-PAGE analysis of the fractions corresponding to peaks A and B obtained using AtGCLΔ85 incubated with 1 mM DTT. Arrowheads correspond to molecular mass markers as indicated. C, reversible dissociation of AtGCLΔ85. Purified protein was dialyzed for 4 h at 4 °C in 25 mM HEPES (pH 7.5), 100 mM NaCl, 5 mM MgCl₂, and 10 mM DTT. Approximately half of the sample was injected on a Superdex-75 26/60 FPLC column (black). The remaining sample was dialyzed overnight at 4 °C in buffer without DTT and then loaded onto the same column (red). Inset, iodoacetamide blocking of free thiols. After treatment with 10 mM DTT, AtGCL was incubated with 100 mM iodoacetamide. The elution from the Superdex-75 column is shown. Axis are the same as C.  

**TABLE II**

| Substrate dependences | Fitted parameters | AtGCLΔ85 |
|-----------------------|------------------|---------|
| | | V_max (nmol/min/mg protein) | 225 ± 12 |
| | | K_ATP (mm) | 6.4 ± 3.2 |
| | | K_Glu (mm) | 5.7 ± 1.3 |
| | | K_Cys (mm) | 95 ± 23 |
| | | α | 0.063 ± 0.022 |
| | | β | 0.38 ± 0.16 |
| | | γ | 1.7 ± 0.8 |

**Kinetic constants for a random ter-reactant model**  
K_ATP, K_Glu, and K_Cys are the equilibrium dissociation constants for the binding of substrate with the free enzyme. The interaction factors between cysteine and glutamate, ATP and glutamate, and ATP and cysteine are represented by α, β, and γ, respectively.
and purified the enzyme using an E. coli expression system. Our results demonstrate that AtGCL shares functional properties with the GCL of other families but is regulated differently than the GCL of other non-plant eukaryotes or bacteria.

Biochemical Evidence of GCL Function—Using purified recombinant protein, we have shown that AtGCL is a genuine GCL. The steady-state kinetic parameters of AtGCL are similar to those reported for GCL purified from tobacco cell suspensions (42), indicating that the GCL from Arabidopsis and tobacco are functionally similar. The steady-state kinetic values of AtGCL (Table I) are most similar to those of the isolated catalytic subunit of Drosophila GCL (K_m Gln = 2.9 mM, K_m Cys = 5.5 mM, V_max = 244 nmol min^{-1} mg protein^{-1}). In addition, ESI-TOF MS analysis confirms that γ-glutamylcysteine is the reaction product of AtGCL.

Although AtGCL is functionally similar to other GCL, inactivation studies suggest structural differences at the active site of AtGCL compared with members of the bacterial and non-plant eukaryotic GCL. The inactivation of AtGCL by both buthionine sulfoximine and cystamine occurs with slower k_{inact} rates and higher K values than observed for the mammalian, nematode, or bacterial GCL (20, 33–36). Importantly, the inactivation specificity of AtGCL compared with those of GCL from T. brucei (32), Ascaris suum (32), and Onchocerca volvulus (33) suggests that the design of anti-nematode compounds is possible. Although these nematodes are human parasites, the generation of inhibitors against nematodes that attack crop and non-crop plants can also be envisioned (40).

Kinetic Mechanism of AtGCL—The kinetic mechanism of GCL has been described as ping-pong (31, 41), ordered A (ATP), random BC (42, 43), or random ter-reactant (25) mechanisms. The kinetic data generated for AtGCL are not consistent with a ping-pong mechanism. In addition, evidence for the formation of different reaction intermediates does not support a ping-pong kinetic mechanism in the GCL of other organisms (36). The initial velocity line patterns for AtGCL are best fit to a random kinetic mechanism displaying a preferred order of substrate addition (25).

The interaction factors of AtGCL for a random mechanism (Table II) indicate that some substrate binding sites exhibit positive interactions with other ligand sites. For example, the binding of either ATP or glutamate increases the binding affinity for the other substrate 2.5-fold (β = 0.38). AtGCL also displays a positive interaction between the glutamate and cysteine binding sites (α = 0.063) with binding of either ligand increasing binding affinity for the other 16-fold. Similar cooperativity was described for T. brucei GCL. With α-aminobutyrate as a substrate, binding of either ATP or glutamate increased the binding affinity for the other 18-fold (25). Interestingly, the T. brucei GCL showed a negative interaction between the glutamate and α-aminobutyrate binding sites with a 6-fold decrease in binding affinity between these ligands. The differences between the Arabidopsis and T. brucei enzymes likely result from using different substrates for the kinetic analysis or may also reflect potential structural variations at the active site. For both AtGCL and the T. brucei enzyme, ATP and cysteine or α-aminobutyrate does not significantly alter the binding energies of each other.

Regulation of AtGCL Activity—In mammals and bacteria, glutathione regulates GCL as a feedback inhibitor (8, 21). This is a fundamental mechanism for controlling intracellular glutathione levels in response to the redox environment (2). Here we have shown that glutathione also inhibits AtGCL. However, our experimental results also suggest a mechanism for the regulation of GCL activity in plants that differs from other eukaryotes.

The GCL from mammals (18, 19), Drosophila (17), and A. suum (32) consist of catalytic and regulatory subunits. The reversible association of these two subunits in response to redox environment modulates GCL activity (18, 19). Fraser
et al. (44) demonstrated the importance of intersubunit disulfide bonds by the mutagenesis of cysteines in the regulatory subunit of the *Drosophila* GCL. Likewise, mutagenesis studies of the human GCL catalytic subunit showed a loss of redox sensitivity when a conserved cysteine in the C-terminal region of human GCL was mutated, implicating this residue in forming a disulfide linkage with the regulatory subunit (45). Interestingly, this cysteine is not found in the plant GCL because it is located beyond the C terminus of the plant enzymes.

Our results indicate that AtGCL is catalytically active as a monomeric protein that is sensitive to reducing agents. Hell and Bergmann (29) reported that treatment of the enzyme with DTT resulted in a loss of activity and suggested that the protein dissociated into 30-kDa subunits but never showed evidence of dissociation. We observed the same shift in the elution profile of AtGCL from a size-exclusion column following the addition of reductant; however, ESI-MS analysis before and after treatment with DTT indicates that the protein remains monomeric in both conditions.2 Although some dimer species are observed under non-reducing conditions, this species accounts for one-thirtieth of the total protein. Similar results were reported for the *T. brucei* GCL using analytical ultracentrifugation and likely represent a nonspecific interaction between monomers (46). These experiments suggest that monomeric AtGCL undergoes a conformational change in response to redox environment, modulating enzymatic activity.

Because this change is mediated by reducing agents, an intramolecular disulfide bond is likely involved. When the cellular environment is reducing (i.e., high glutathione levels), the disulfide bond is disrupted and AtGCL adopts a rod-like shape with attenuated activity. Thus, regulation of AtGCL would not require association with a regulatory subunit, as in the mammalian system, for activation. This model for controlling AtGCL activity is also consistent with observations that oxidative stimulus induces glutathione synthesis (47, 48). May et al. (48) noted that hydrogen peroxide treatment of a GCL-deficient yeast strain complemented with AtGCL yielded higher glutathione levels without increased gene expression and suggested that posttranslational modification of GCL occurred. Activation of GCL by a change in redox environment would also explain these results. Similar redox signal-induced conformational switching by disulfide formation of intracellular proteins has been described for other proteins, including Yap1 (49), thioredoxins (50), and OxyR (51).

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