Kinetic Mechanism of Glutathione Synthetase from Arabidopsis thaliana*

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Glutathione synthethase (GS) catalyzes the ATP-dependent formation of the ubiquitous peptide glutathione from \( \gamma \)-glutamylcysteine and glycine. The bacterial and eukaryotic GS form two distinct families lacking amino acid sequence homology. Moreover, the detailed kinetic mechanism of the bacterial and the eukaryotic GS remains unclear. Here we have overexpressed Arabidopsis thaliana GS (AtGS) in an Escherichia coli expression system and purified the recombinant enzyme for biochemical characterization. AtGS is functional as a homodimeric protein with steady-state kinetic properties similar to those of other eukaryotic GS. The kinetic mechanism of AtGS was investigated using initial velocity methods and product inhibition studies. The best fit of the observed data was to the equation for a random Ter-reactant mechanism in which dependencies between the binding of some substrate pairs were preferred. The binding of either ATP or \( \gamma \)-glutamylcysteine increased the binding affinity of AtGS for the other substrate by 10-fold. Likewise, the binding of ATP or glycine increased binding affinity for the other ligand by 3.5-fold. In contrast, binding of either glycine or \( \gamma \)-glutamylcysteine causes a 6.7-fold decrease in binding affinity for the second molecule. Product inhibition studies suggest that ADP is the last product released from the enzyme. Overall, these observations are consistent with a random Ter-reactant mechanism for the eukaryotic GS in which the binding order of certain substrates is kinetically preferred for catalysis.

Glutathione is a key modulator of the intracellular reducing environment that provides protection against reactive oxygen species and maintains protein thiols in a reduced state (1). In plants, metabolic pathways for the detoxification of herbicides (2), air pollutants such as sulfur dioxide and ozone (3), and heavy metals (4) also rely on glutathione. For example, glutathione is the metabolic precursor for the synthesis of phytochelatin peptides that sequester cadmium, mercury, and arsenic (5).

Glutathione biosynthesis occurs through a two-step pathway found in all organisms. In the first reaction, glutamate-cysteine ligase (EC 6.3.2.2) catalyzes the ATP-dependent formation of the dipeptide \( \gamma \)-glutamylcysteine from cysteine and glutamate. Next, glutathione synthetase (GS, EC 6.3.2.3) catalyzes the addition of glycine to the dipeptide (Scheme I).

\[
\text{GS} \quad \gamma \text{-Glutamylcysteine} + \text{glycine} + \text{ATP} \rightarrow \text{glutathione} + \text{ADP} + \text{P}_i
\]

In this reaction, transfer of the \( \gamma \)-phosphate group of ATP to the C-terminal carboxylate of \( \gamma \)-glutamylcysteine yields an acylyphosphate intermediate. Nucleophilic attack on the acylyphosphate intermediate by glycine leads to formation of glutathione with release of ADP and inorganic phosphate (1, 6).

GS from bacteria and eukaryotes form two distinct families that share no amino acid sequence homology (7). Detailed characterization of the Escherichia coli GS demonstrates that this enzyme is functional as a tetramer of \( \sim 300 \)-residue subunits (8–11), whereas the mammalian and yeast GS are active as dimers of \( \sim 470 \)-residue subunits (12–15). Despite the lack of sequence homology, different oligomeric organization, and molecular masses of these enzymes, the E. coli (10), human (14), and yeast (15) GS share a common three-dimensional fold and are part of the ATP-grasp structural family (16).

Here we report the expression and purification of AtGS and the basic biochemical characterization of this enzyme. To elucidate the kinetic mechanism of AtGS, we used a combination of initial velocity data collected as a function of varying substrate concentrations and product inhibition patterns to determine the order of substrate addition and product release remains to be established.

EXPERIMENTAL PROCEDURES

Materials—E. coli C41(DE3) cells were purchased from Avidis, Inc. N\(_i\)-nitroliotriacetic acid (NTA)-agarose was bought from Qiagen. Benzamidine-Sepharose and the HiLoad 26/60 Superdex-75 fast protein liquid chromatography column were from Amersham Biosciences. The BioMol Green reagent used for the detection of inorganic phosphate was purchased from BioMol Research Laboratories, Inc. All other reagents were purchased from Sigma and were of ACS reagent quality or better.

Cloning and Generation of Expression Vectors—AtGS (GenBank™ accession number U22359) (26) was amplified from a A. thaliana cDNA library using PCR with 5'-TDTCCATGGGATGGAATCACA-

Arabidopsis thaliana glutathione synthetase; NTA, nitroliotriacetic acid; \( \gamma \)-EC, \( \gamma \)-glutamylcysteine.

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1 The abbreviations used are: GS, glutathione synthetase; AtGS, Arabidopsis thaliana glutathione synthetase; NTA, nitroliotriacetic acid; \( \gamma \)-EC, \( \gamma \)-glutamylcysteine.
Expression in E. coli and Protein Purification—The pHISS-AtGS bacterial expression construct was transformed into E. coli C41(DE3) cells. Transformed E. coli were grown at 37 °C in Terrific broth containing 50 µg ml\(^{-1}\) kanamycin until \(A_{\text{600 nm}}\) = 0.8. After induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), the cultures were grown at 25°C for 4 h. Cells were pelleted by centrifugation and resuspended in lysis buffer (50 mM TRIS-HCl (pH 8.0), 500 mM NaCl, 20 mM imidazole, 5 mM MgCl\(_2\), 10% (v/v) glycerol, and 1% (v/v) Tween-20). After sonication and centrifugation, the supernatant was passed over a Ni\(^{2+}\)-NTA column previously equilibrated with lysis buffer. The column was then washed with wash buffer (lysis buffer without Tween-20). His-tagged protein was eluted with elution buffer (wash buffer containing 250 mM imidazole (pH 8.0)). Incubation with thrombin (1:2000 the amount of AtGS by weight) during overnight dialysis at 4 °C against wash buffer removed the His-tag. Dialyzed protein was reloaded on a mixed benzamidine-Sepharose/Ni\(^{2+}\)-NTA column. The flow-through of this step was dialyzed overnight against 30% (v/v) glycerol, 25 mM HEPES (pH 7.5), 50 mM MgCl\(_2\), and 100 mM NaCl, then loaded onto a HiLoad 16/60 Superdex-200 fast protein liquid chromatography column equilibrated in the same buffer without glycerol. Fractions containing AtGS were pooled, concentrated to 10 mg ml\(^{-1}\), and stored at −80 °C. Protein concentration was determined by the Bradford method (protein assay, Bio-Rad) with bovine serum albumin as standard.

Enzyme Assays—The activity of AtGS was determined spectrophotometrically at 25 °C by measuring the rate of formation of ADP using a coupled assay with pyruvate kinase and lactate dehydrogenase (8). A standard reaction mixture (0.5 ml) contained 100 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl\(_2\), 2.5 mM γ-glutamylcysteine, 10 mM glycine, 2.5 mM disodium ATP, 2 mM sodium phosphoenolpyruvate, 0.2 mM NaF, and 10 units of type II rabbit muscle pyruvate kinase, all at 200 mM. The reaction was started by adding AtGS (0.75 µg). The rate of decrease in \(A_{\text{340 nm}}\) was measured using a Cary Bio300 UV/Vis spectrophotometer. Steady-state kinetic parameters were determined by initial velocity experiments. Measurements of the \(k_{\text{cat}}\) and \(K_{\text{m}}\) values for γ-glutamylcysteine (0.002–2.5 mM) were made at 10 mM glycine and 2.5 mM ATP. Kinetic constants for glycine (0.1–10 mM) were measured at 2.5 mM γ-glutamylcysteine and 2.5 mM ATP. Determination of the kinetic constants for ATP (0.002–2.5 mM) used 2.5 mM γ-glutamylcysteine and 10 mM glycine. Steady-state kinetic parameters were calculated using Kaleidagraph (Synergy Software) to fit the untransformed data to the Michaelis-Menten equation:

\[
v = \frac{k_{\text{cat}}[S]}{K_{\text{m}} + [S]}
\]

Analysis of the Kinetic Mechanism—Analysis of the kinetic mechanism of AtGS used global fitting analysis (22–29). Reaction rates were measured using standard assay conditions in a matrix of substrate concentrations (γ-glutamylcysteine, 0.01–2.5 mM; ATP, 0.01–2.5 mM; glycine, 0.1–10 mM). Thus, the rate measured for any given substrate concentration was measured over the entire range of the other two substrates. Global curve fitting in SigmaPlot (Systat Software, Inc.) was used for modeling of the kinetic data to rapid equilibrium rate equations describing the possible Ter-reaction kinetic mechanisms (30).

Product Inhibition Assays—Initial velocities in the presence of glutathione or phosphate were determined spectrophotometrically using the standard assay system. Enzymatic activity was determined after the addition of AtGS (0.75 µg) to solutions containing inhibitor and varied concentrations of γ-glutamylcysteine (0.01–2.5 mM), glycine (0.1–10 mM), or ATP (0.01–2.5 mM). Variations of glutathione (0.5, 10, 25, or 50 mM) and potassium phosphate (0, 10, or 25 mM) were used. Assays with added phosphate (1.5 µg AtGS) contained a 1:1 ratio with ATP and no added MgCl\(_2\) to prevent precipitation of magnesium phosphate at high phosphate concentrations.

In the presence of added ADP, initial rates were measured by colorimetric determination of inorganic phosphate. Each 0.1-ml assay mixture contained 100 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl\(_2\), varied substrate concentrations (as above), MgCl\(_2\), and ADP (0, 0.1, 0.25, 1, 4, and 20 mM). The reaction was initiated by addition of AtGS (0.5 µg) and quenched at several time intervals (0–5 min) with 1 ml of BioMol Green reagent. Reaction rates were linear over the time course monitored. Control reactions were used to correct for background levels of inorganic phosphate in the reaction mixture. Color was measured at \(A_{\text{620 nm}}\) and quantified with a standard curve for inorganic phosphate (0–8 nmol).

Global fitting analysis in the kinetics module of SigmaPlot (Systat Software, Inc.) was used to simultaneously fit all data to the equations for competitive inhibition, \(v = \frac{V_{\text{max}}}{1 + (K_{\text{I}}/[S])}\), non-competitive inhibition, \(v = \frac{V_{\text{max}}}{1 + [K + K_{\text{I}}/[S]]}\), and uncompetitive inhibition, \(v = \frac{V_{\text{max}}}{1 + [I/K + K_{\text{I}}/[S]]}\).

RESULTS

Expression and Purification of AtGS—AtGS was overexpressed in E. coli C41(DE3) as octahistidine-tagged protein and purified to apparent homogeneity using Ni\(^{2+}\) affinity and size exclusion chromatography (Fig. 1). By SDS-PAGE analysis, purified recombinant AtGS migrated with a monomer weight of ~50 kDa, which corresponds to the predicted molecular weight (53.4 kDa). Matrix-assisted laser desorption ionization mass spectrometry of purified AtGS confirmed the identity of the protein and verified the molecular weight (not shown). AtGS eluted from the size exclusion column as a 90-kDa species representing the physiologic dimer. Unlike previous efforts to express AtGS in a bacterial expression system (18–19), we did not observe any protein degradation during purification. Purified recombinant AtGS had a specific activity of 7.5 µmol min\(^{-1}\) mg protein\(^{-1}\) using standard assay conditions. The recombinant protein was stable for three months at ~80 °C and for 24 h at 4 °C (with a loss of 25% activity after 72 h at 4 °C). Typical yields were ~2 mg of pure protein per liter of culture.

Steady-state Kinetic Analysis of AtGS—Steady-state kinetic parameters (\(k_{\text{cat}}\) and \(K_{\text{m}}\)) for γ-glutamylcysteine, glycine, and ATP were determined for AtGS (Table I). Similar values were obtained using the colorimetric assay for the detection of inorganic phosphate (not shown). Purified recombinant AtGS displayed \(K_{\text{m}}\) values similar to those reported for the partially purified GS (\(K_{\text{m}}\) γ::NC = 0.022 mM and \(K_{\text{m}}\) GSH = 0.31 mM) assayed in tobacco cell homogenates (17). However, the reaction rate was 2000-fold higher because of the greater purity of the AtGS sample. The \(k_{\text{cat}}\) and \(K_{\text{m}}\) values determined for each substrate of AtGS were equivalent to those reported for the yeast (12), human (22), and Plasmodium (30) enzymes. Compared with the E. coli GS (8), the \(K_{\text{m}}\) values of AtGS for both γ-glutamylcysteine and ATP were 10-fold lower, and the turnover rate was 10-fold slower. In place of magnesium, manganese (2.5 mM MnCl\(_2\)) supported enzymatic activity with 2- to 3-fold reductions in \(k_{\text{cat}}\) and no changes in \(K_{\text{m}}\) for each substrate compared with those determined with MgCl\(_2\).

Because different plants also synthesize a range of glutathione analogs in which glycine is substituted by another amino acid (4), we examined the substrate specificity of AtGS. Assays containing ATP, γ-glutamylcysteine, and either β-alanine, serine, or glutamate showed no enzymatic activity (10 and 50 mM substrate; 100 µg AtGS).
Initial Velocity Analysis of the Kinetic Mechanism—Initial velocity kinetic studies were performed by varying two of the three substrates while holding the third substrate constant under identical reaction conditions (24, 25, 28, 29). Six families of data were generated such that for a saturating concentration of one substrate the reaction rates were measured over the entire range of the other two substrates. Double reciprocal plots of the experimental data are presented in Fig. 2. Any potential ping-pong kinetic mechanisms were eliminated from further consideration because the reciprocal plots for each family of substrate data converge.

The data were then globally fit to the 16 rate equations describing the different Ter-reactant systems (25). These included six potential ordered mechanisms, nine possible partially ordered/random mechanisms, and a random kinetic mechanism. Computerized simultaneous fitting of the initial velocity data provides a robust analysis of the data compared with earlier reciprocal re-plotting approaches (24, 29, 30). Based on global fits to the initial velocity data, the best fit ($r^2 = 0.993$) was obtained using the equation of a random Ter-reactant system (Equation 1).

\[
\frac{v}{V_{\text{max}}} = \frac{[\text{ATP}][\text{Gly}][\text{EC}]}{1 + \frac{[\text{ATP}]}{K_{\text{ATP}}} + \frac{[\text{Gly}]}{K_{\text{Gly}}} + \frac{[\text{EC}]}{K_{\text{EC}}} + \frac{[\text{ATP}][\text{Gly}]}{\alpha \beta K_{\text{ATP}} K_{\text{Gly}} K_{\text{EC}}} + \frac{[\text{ATP}][\text{EC}]}{K_{\text{ATP}} K_{\text{EC}}} + \frac{[\text{Gly}][\text{EC}]}{\beta K_{\text{Gly}} K_{\text{EC}}} + \frac{[\text{ATP}][\text{Gly}][\text{EC}]}{\alpha K_{\text{ATP}} K_{\text{Gly}} K_{\text{EC}}} + \frac{[\text{ATP}][\text{Gly}][\text{EC}]}{\alpha \beta K_{\text{ATP}} K_{\text{Gly}} K_{\text{EC}}}}
\]

(Eq. 1)

In this equation, $K_{\text{ATP}}$, $K_{\text{Gly}}$, and $K_{\text{EC}}$ are the equilibrium dissociation constants for the binding of substrate to the free enzyme. The interaction factors between glycine and γ-glutamylcysteine, ATP and γ-glutamylcysteine, and ATP and glycine are represented by $\alpha$, $\beta$, and $\gamma$, respectively. The interaction factors are factors by which the dissociation constant for a given substrate varies as other ligand binding sites are occupied. Examination of the residual plots and the standard errors of the fitted parameters also indicated that a random Ter-reactant system best described the data.

Fits of the data to a random Ter-reactant kinetic model are drawn as lines in Fig. 2. The resulting kinetic model is shown in Fig. 3. Table II summarizes the fitted equation parameters and the calculated substrate dependencies. The fit of the data to a random mechanism was significantly better than the next best models, as follows: ordered B (ATP/random AC ($r^2 = 0.971$), ordered C (glycine/random AB ($r^2 = 0.941$), or ordered A (ATP)/random BC ($r^2 = 0.919$). The fit ($r^2 = 0.896$) to the previously proposed ordered mechanism (ATP/γ-glutamylcysteine/glycine) for E. coli GS (11) was worse than these partly ordered/random models.

The fitted kinetic parameters and the calculated substrate dependencies were in excellent agreement. For example, the apparent $K_{\text{Gly}}$ values for ATP, γ-glutamylcysteine, and glycine (Table I) correspond to $\gamma K_{\text{ATP}}$, $\alpha K_{\text{Gly}}$, and $\alpha \beta K_{\text{EC}}$, respectively (Table II). The model-derived dissociation constants are influenced by the interaction factors because the binding of some substrates will influence the binding of a second substrate. The interaction factors derived from fitting the data to a random Ter-reactant system (Table II) indicate that positive and negative interactions occur between different binding sites. Positive interactions occur between the ATP and γ-glutamylcysteine ($\beta = 0.10$) such that the binding of one substrate increases the affinity of AtGS for the other substrate 10-fold. Likewise, the prior binding of ATP increases the affinity for glycine (and vice versa) 3.5-fold ($\gamma = 0.29$). Negative interaction that reduces binding affinity 6.7-fold occurs between the glycine and γ-glutamylcysteine binding sites ($\alpha = 6.68$). Thus, although the order of substrates binding to the enzyme is random, interactions between binding sites minimize unfavorable interactions and enhance the binding of certain substrates.

Product Inhibition—Product inhibition studies were performed to determine the order of product release (31). The resulting $K_i$ values and the best fits of the data to the inhibition equations are summarized in Table III. ADP was found to be a competitive inhibitor with respect to ATP. ADP was a non-competitive inhibitor versus both γ-glutamylcysteine and glycine. Glutathione was a non-competitive inhibitor of ATP, γ-glutamylcysteine, and glycine. Inorganic phosphate was also found to be a non-competitive inhibitor with respect to each substrate.

DISCUSSION

GS catalyzes the ATP-dependent formation of glutathione, a ubiquitous cellular protection compound. Compared with the bacterial GS, the kinetic properties of the eukaryotic enzymes are not well characterized. Here we described the heterologous expression of AtGS and the steady-state kinetic characteriza-
previous studies and our data are most consistent with a random Ter-reactant kinetic mechanism displaying a preference for the order of substrate binding.

The kinetic data show that the initial velocity line patterns all intersect (Fig. 2). Because none of the patterns are parallel,
no kinetically irreversible steps occur between the binding of any of the substrates. This observation eliminates the possibility of a ping-pong mechanism and supports the hypothesis that the substrates form a ternary complex before catalysis.

Simultaneous global fitting of the initial velocity data to the equations for each of the 16 possible Ter-reactant kinetic mechanisms shows that a random mechanism best describes the observed data obtained with AtGS (Table II). However, the fitted parameters and calculated substrate dependencies indicate that there is a preferred but not necessarily required binding order for forming the central complex. The positive interaction between the binding of either the ATP/γ-glutamylcysteine or ATP/glycine substrate pairs (Fig. 3, dark lines) is in contrast to the binding of the glycine/γ-glutamylcysteine substrate pair, which reduces the affinity of the site for either ligand (Fig. 3, dotted lines). Interestingly, fitting of the data to the proposed fully ordered mechanism (11, 23, 32) in which ATP, γ-glutamylcysteine, and glycine sequentially bind yields a significantly lower correlation coefficient.

The results of the product inhibition experiments also suggest a random mechanism with a preferential order of substrate binding and product release for AtGS. The product inhibition patterns for a completely ordered reaction mechanism should all be uncompetitive except for the competitive inhibition pattern of the last product versus the first substrate (25, 31, 33). However, if the kinetic mechanism is completely random, then no abortive complexes form and competitive inhibition should be observed between any substrate and any product (31). These descriptions of the expected inhibition patterns for a completely ordered and a completely random kinetic mechanism do not match those observed with AtGS.

For AtGS, ADP is a competitive inhibitor versus ATP. Therefore, both ligands bind to the same enzyme state. However, non-competitive inhibition by ADP with respect to γ-glutamylcysteine and glycine suggests that ADP is released from an enzyme state that differs from the form that binds either substrate. These inhibition patterns suggest that ADP is the last product released with ATP as the first substrate to bind. Likewise, both inorganic phosphate and glutathione are non-competitive inhibitors with respect to each substrate. Therefore, the kinetic mechanism of AtGS is not completely ordered, which is consistent with the initial velocity data.

The appearance of non-competitive inhibition patterns implies that the reaction products are either randomly released, form dead-end complexes with free enzyme and/or an enzyme-substrate complex, or bind at an allosteric site on the enzyme (31, 33). The x-ray crystal structures of the E. coli (11), human (14), and yeast (15) enzymes formed in complex with different substrate and product combinations eliminate the possibility of an allosteric binding site on AtGS. These three-dimensional structures also suggest that the active site architecture would not prevent the binding of substrates and products, such as γ-glutamylcysteine and ADP, as dead-end complexes. Alternatively, based on the product inhibition profiles, the release of phosphate and glutathione from the active site may be random and occur before ADP leaves the enzyme.

The product inhibition profiles and the overall kinetic mechanism of AtGS are similar to those described for other ATP-dependent peptide ligases, including glutamate-cysteine ligase (28, 34), UDP-N-acetylmuramate-l-alanine ligase (29), glutamine synthetase (35), d-alanyl-d-alanine ligase (36), and UDP-N-acetylmuramate-tripeptide-d-alanyl-d-alanine-adding enzyme (37). Each of these enzymes catalyzes the transfer of the γ-phosphate group of ATP to the C-terminal carboxylate of one substrate to yield an acylphosphate intermediate. Subsequent nucleophilic attack on the acylphosphate intermediate by a second substrate leads to product formation with the release of phosphate, peptide product, and ADP. In each case, ATP is the first substrate to bind with ADP as the last product to leave. Inorganic phosphate is the first product released from glutamine synthetase (35) and d-alanyl-d-alanine ligase (36). For UDP-N-acetylmuramate-l-alanine ligase (29), the order for releasing phosphate and the peptide product was not distinguishable.

In conclusion, our results indicate that AtGS uses a random Ter-reactant kinetic mechanism with a preferred ordered pathway. Similar kinetic mechanisms have been proposed for glutamine synthetase (35), alcohol dehydrogenase (38) and hexokinase (39) in which alternate pathways for substrate binding/product release are available to the enzyme even though product inhibition patterns suggest an ordered mechanism. For GS, the three-dimensional structures of this enzyme (10, 11, 14, 15) indicate that the substrates may not be trapped on the enzyme as required by a compulsory sequential mechanism (31, 35), thus allowing for exchange of substrates on the kinetic pathway leading to formation of the active ATP-γ-glutamylcysteine-glycine central complex. Because AtGS shares approximately 40% amino acid sequence identity with the other eukaryotic GS (26), it is likely that this kinetic mechanism is conserved in those enzymes.

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