Trypanosoma brucei γ-Glutamylcysteine Synthetase
CHARACTERIZATION OF THE KINETIC MECHANISM AND THE ROLE OF CYS-319 IN CYSTAMINE INACTIVATION

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The parasitic protozoan Trypanosoma brucei utilizes a conjugate of glutathione and spermidine, termed trypanothione, in place of glutathione to maintain cellular redox balance. The first committed step in the biosynthesis of glutathione and thereby trypanothione, is catalyzed by γ-glutamylcysteine synthetase (γ-GCS). We have determined the kinetic mechanism for T. brucei γ-GCS. The kinetics are best described by a rapid equilibrium random ter-reactant mechanism, in which the model derived $K_d$ values for the binding of L-Glu, L-α-aminobutyrate, and ATP to free enzyme are 2.6, 5.1, and 1.4 mM, respectively. However, significant dependences exist between the binding of some of the substrate pairs. The binding of either ATP or L-Glu to the enzyme increases the binding affinity of the other by 18-fold, whereas the binding of L-Glu or L-α-aminobutyrate decreases the binding affinity of the other by 6-fold. Similarly to the mammalian enzyme, cystamine is a time-dependent, irreversible inhibitor of T. brucei γ-GCS. It has been suggested by several studies that cystamine labels an active site Cys residue essential for catalysis. Among the enzymes reported to be inactivated by cystamine, only one Cys residue is invariant (Cys-319 in T. brucei γ-GCS). Mutation of Cys-319 to Ala in T. brucei γ-GCS renders the enzyme insensitive to cystamine inactivation without significantly affecting the enzyme’s catalytic efficiency, kinetic mechanism, or substrate affinities. These studies suggest that cystamine inactivates the enzyme by blocking substrate access to the active site and not by labeling an essential active site residue.

The parasitic protozoa Trypanosoma brucei is the causative agent of African sleeping sickness. T. brucei is responsible for significant morbidity and mortality, yet the available anti-trypansomal drugs are limited in effectiveness by drug resistance, by high toxicity and by their lack of action against late stage disease (1). Major differences have been found in the utilization of the tripeptide thiol glutathione (GSH) between the parasite and the mammalian host. Mammals rely on GSH for protection against oxidative injury by peroxides or free radicals and for detoxification of xenobiotics (2). Trypanosomes maintain redox balance by synthesizing a conjugate of glutathione and spermidine, termed trypanothione (3). The reduced form of trypanothione is maintained by trypanothione reductase, a homolog of mammalian glutathione reductase (4).

The first step in the biosynthesis of glutathione, and thereby trypanothione, is catalyzed by γ-glutamylcysteine synthetase (γ-GCS). This enzyme catalyzes the ATP-dependent ligation of L-Cys and L-Glu to produce γ-glutamylcysteine. γ-GCS is the rate-limiting enzyme in the biosynthesis of glutathione in mammalian cells (2) and of trypanothione in the trypanosomatid Leishmania tarentolae (5). An enzyme-activated inhibitor of γ-GCS, buthionine sulfoximine, cures mice infected with T. brucei (6), implicating γ-GCS as a potential drug target. The finding that trypanosomes lack catalase (7), and consequently possess an intracellular hydrogen peroxide concentration 100 times that found in mammalian cells, may account for the selective toxicity of glutathione depletion on the trypanosome (8). A number of other studies also demonstrate that oxidative stress has detrimental effects on T. brucei viability. Deletion of even a single allele of trypanothione reductase in Leishmania donovani decreased survival in macrophages (9). Lysis of the cattle variant of T. brucei by haptoglobin in human serum is likely to be mediated by H$_2$O$_2$ (10). γ-GCS amplification in L. tarentolae cell lines resistant to antimonomals and arsenicals was an essential factor in the resistant phenotype (5).

Studies on mammalian γ-GCS suggest that the reaction proceeds through the generation of a γ-glutamylphosphate intermediate (11). This result is supported by the finding that buthionine sulfoximine is phosphorylated by γ-GCS in the presence of ATP to form a tight binding transition state analog (12, 13). Studies to delineate the kinetic mechanism of mammalian γ-GCS have provided conflicting results; the reaction has been proposed to proceed through an ordered ter-ter mechanism (11), an ordered A (ATP) random BC mechanism (14, 15), a random AB ordered C (L-Aba) mechanism (16), and various ping-pong mechanisms (17, 18).

Little is known about the active site or catalytically relevant residues of γ-GCS from any species. Mammalian, worm, and T. brucei γ-GCS have been demonstrated to be inactivated by cystamine (16, 19–21). Inactivation is proposed to proceed through formation of a disulfide bond to a Cys residue in the active site (19, 22), L-Glu protects the mammalian enzyme from cystamine inactivation, suggesting the labeled Cys residue may be in the L-Glu binding site (23). These data have been used to suggest a role for this Cys residue in catalysis. Several other inhibitors that inactivate the enzyme are also thought to react to the same Cys residue (24, 25). Comparison of the amino acid sequences for the enzymes that have been demonstrated to be inactivated by cystamine reveals only a single conserved Cys

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1 The abbreviations used are: γ-GCS, γ-glutamylcysteine synthetase; wt-TbγGCS, wild-type T. brucei γ-GCS; TbC319A, Cys-319 to Ala mutant T. brucei γ-GCS; l-Aba, L-α-aminobutyrate; PCR, polymerase chain reaction.
residue, *T. brucei* γ-GCS Cys-319, suggesting this residue is likely to mediate the response to cystamine. However, the role of this residue in catalysis is unknown.

We recently cloned *T. brucei* γ-GCS and reported preliminary enzyme characterization (20). In this paper, we address the kinetic mechanism of γ-GCS from *T. brucei* and characterize the role of Cys-319 in cystamine inactivation and catalysis. The *T. brucei* γ-GCS reaction mechanism is best described by a random ter-reactant mechanism. However, the mechanism also predicts that the binding affinities of some of the substrates are influenced by the prior binding of others. Our studies demonstrate that Cys-319 is the site of cystamine inactivation, but this residue does not play a significant role in substrate binding or catalysis. These studies suggest that cystamine inactivates the enzyme by blocking substrate access to the active site and not by labeling an essential active site residue.

**EXPERIMENTAL PROCEDURES**

**Materials**

Reagents for the enzyme assay were purchased from Sigma, Ni²⁺-agarose and pREP4 were purchased from Qiagen.

**Mutagenesis**

Site-directed mutagenesis was performed as described (37) using an oligonucleotide (5′-ATGTTAAGCTGAGGCGGTTGACGCC-3′) designed to incorporate a PstI site and to mutate Cys-319 to Ala (Fig. 1). Mutagenesis was performed in the Bluescript SK+ (Strategene) vector using helper phage B408 (Strategene) and Escherichia coli strain BO285. A 1-kilobase pair fragment containing the mutation was subcloned to the expression constructs and verified by DNA sequencing.

**Expression Constructs**

*pTbCtag—*We previously reported the expression of γ-GCS as an N-terminal His₆-tagged fusion protein under control of a T7 promoter (20). We found better expression levels and greater purification can be obtained using a C-terminal His₆ tag. The vector was constructed as follows. PCR primers were designed to insert a NsiI restriction site on the 5′ end of the gene and a TEV protease site followed by a SacII site on the 3′ end (sense primer 5′-AAATGATATCCCTTACAACCTGCGGCGACCAG; antisense primer 5′-AAACCCGGGCGCTGAAATAAGGATCTCGTCCGACGCTCTGGTTGCTTTTACAT). These primers were used to PCR the gene from pTB121 (20), and the resulting PCR fragment was subcloned into the NsiI and SacII sites of pLOD11 (26). An oligonucleotide linker, flanked with a SacII site on one side and a HindIII site on the other (sense oligonucleotide = 5′-GGGCTTACATCCGACCCTGGA; antisense oligonucleotide = 5′-AGCTGTCTGTTGAGCTGATGAGTGGTGGGCC) was next used to place a His₆ tag site at the C terminus of γ-GCS to yield the final expression construct, pTbCtag.

*pTb-GCSS319A—*The mutated fragment was cloned into the XhoI/SacI sites of pTbCtag, described above, to generate C-terminally tagged pTbC319A.

**Expression in E. coli and Purification**

wt-Tbγ-GCS and TbC319A γ-GCS were expressed and purified from *E. coli* cells as described previously, except the cells were cotransformed with pREP4 (Qiagen) to more tightly regulate the protein expression. Approximately 10,000 freshly transformed colonies were used to inoculate 6 liters of LB, which was incubated at 37°C. Protein expression was induced when cells reached 1 OD₆₅₀ by the addition of isopropyl-β-D-thiogalactopyranoside (0.2 mM) and the temperature was lowered to 27°C. Cells were harvested by centrifugation 6–8 h after induction, resuspended, and lysed in buffer A (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl₂, 1 mM β-mercaptoethanol), containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 4 μg/ml antipain, 20 μg/ml benzamidine, 2 μg/ml pepstatin, 2 μg/ml chymostatin), and 1 mg/ml lysozyme. After centrifugation (150,000 × g for 1 h at 4°C), the supernatant was applied to a Ni²⁺-agarose column (Qiagen) equilibrated in buffer A. The column was washed with buffer A containing fractions were dialyzed, concentrated in buffer A plus 1 mM β-octagulcoside, and applied to a HiLoad Superdex 200 16/60 gel filtration column (Amersham Pharmacia Biotech) in buffer A. The protein was determined to be greater than 98% pure by SDS-polyacrylamide gel electrophoresis analysis. The molecular mass of the purified enzyme is 77 kDa.

**Enzyme Assays and Kinetic Analysis**

All kinetic analysis was done on purified γ-GCS. γ-GCS activity was followed at 37°C using a spectrophotometric assay in which coupled ADP production to NADH oxidation as described (20). Buffer (100 mM Tris-HCl, pH 8.0, 150 mM KCl, 2 mM MgCl₂, 2 mM phenylephoinopyruvate, 0.27 mM NaDH) was mixed with type III rabbit muscle pyruvate kinase (5 units of 5–600 unit/mg of redissolved lyophilized powder; Sigma), type II rabbit muscle lactic acid dehydrogenase (10 units of 800–1200 unit/mg ammonium sulfate suspension; Sigma), and γ-GCS substrates to provide a final reaction volume of 0.1 ml. The assay was run for 1 h at 4°C, the supernatant was applied to a Ni²⁺-agarose column (Qiagen) in buffer A. The protein was determined by measuring the OD₅₆₀. The extinction coefficient for γ-GCS was determined to be 1.35 (mg/ml)⁻¹OD as described (27). Briefly, the extinction coefficient for denatured enzyme was determined based on its primary amino acid sequence by calculation using the ProtParam tool on the ExPASy web site. The enzyme was denatured in 6 M guanidinium-HCl, 0.02 M phosphate, pH 6.5, and the absorbance at 280 nm was determined. Using the extinction coefficient for the denatured enzyme, the protein concentration of the sample was determined. This concentration was used to determine the extinction coefficient of the native enzyme.

NADH has been reported to be a competitive inhibitor of ATP for the rat γ-GCS-catalyzed reaction (14). However, we observed no inhibitory effects of NADH on *T. brucei* γ-GCS in the range of 0.1–0.6 mM NADH. Thus, the concentration of NADH in the assay mix is not having an inhibitory effect on the enzyme.

**Cystamine Inhibition Studies**

The time-dependent inhibitor cystamine was incubated with wt-Tbγ-GCS and TbC319A γ-GCS in different times at various inhibitor concentrations. Aliquots of the reaction were removed and diluted 1/50 into a standard 0.5-mL reaction mixture, and the remaining activity was measured by the standard assay (above). β-Mercaptoethanol was removed from the enzyme preparation on a 10 × 30-cm Fast desalting column (Sephadex G-25; Amersham Pharmacia Biotech) prior to these studies. To demonstrate that cystamine inactivation was irreversible, cystamine-inactivated wt-Tbγ-GCS (incubated with 1 mM cystamine for 30 min) was run on the Fast desalting column to remove excess inhibitor prior to assay.

**Analysis of the Kinetic Mechanism**

Data to assess the kinetic mechanism was collected for wt-Tbγ-GCS and TbC319A γ-GCS at a range of substrate concentrations. A complete matrix of rates as a function of substrate concentration (L-Glu, 0.1–8 mM; ATP, 0.04–2 mM; l-Abu, 2.5–100 mM) were collected such that for any combination of any one substrate the rates were measured over the entire range of the other two substrates. Curve fitting and modeling of the kinetic data was based on the rapid equilibrium rate equations (28) and was performed using Sigma Plot (SPSS Inc.).

**RESULTS**

**The Role of Cys-319 in Cystamine Inactivation of γ-GCS—**

Cys-319 in *T. brucei* γ-GCS (Fig. 1) was mutated to Ala and purified as described under "Experimental Procedures." The purified wild-type *T. brucei* γ-GCS (wt-Tbγ-GCS) and the Cys-319 to Ala mutant enzyme (TbC319A) both have *kₗₐₜ* values of 2–3 s⁻¹ in the presence of saturating concentrations of all three substrates (standard assay conditions described under "Experimental Procedures"). Thus, mutation of Cys-319 to Ala had no effect on the catalytic rate of the enzyme under these conditions. The wild-type enzyme is irreversibly inactivated by cystamine in a time-dependent reaction. Incubation of the wild-type enzyme with as little as 10 μM cystamine for 10 min results in 75% loss in enzyme activity (Fig. 2).
Mechanism of *T. brucei* γ-GCS

The amino acid sequences were obtained from the published references: *T. brucei* (20), rat (33), *Saccharomyces cerevisiae* (34), *Schizosaccharomyces pombe* (35), and Caenorhabditis elegans (36).

A matrix of kinetic data was collected for *wt-Tb*-GCS and *TbC319A* γ-GCS using a range of substrate concentrations, such that one substrate was held constant while the other two were varied. The procedure was repeated until all combinations of fixed and variable substrates had been collected (Fig. 3). Inspection of the Lineweaver-Burk plots indicates that the substrates form a ternary complex in the enzyme active site. A ping-pong mechanism is ruled out because the Lineweaver-Burk plots for all substrate combinations had been collected (Fig. 3).

![Fig. 1. Amino acid sequence alignment of representative γ-GCSs in the region of Cys-319. The amino acid sequences were obtained from the published references: *T. brucei* (20), rat (33), *Saccharomyces cerevisiae* (34), *Schizosaccharomyces pombe* (35), and *Caenorhabditis elegans* (36).]

![Fig. 2. The effects of cystamine on the activity of *wt-Tb*-GCS and *TbC319A* γ-GCS. Enzymes were incubated with cystamine at the depicted concentration for either 10 min or 1 h before assay. Data are displayed as the percentage of the control activity (initial velocity with inhibitor/initial velocity without inhibitor) versus cystamine concentration (mM): ○, *wt-Tb*-GCS, 10-min incubation; ▲, *wt-Tb*-GCS, 1-h incubation; ■, *TbC319A* γ-GCS, 10-min incubation; ●, *TbC319A* γ-GCS, 1-h incubation.](image)

Kinetic Mechanism of γ-GCS—A matrix of kinetic data was collected for *wt-Tb*-GCS and *TbC319A* γ-GCS using a range of substrate concentrations, such that one substrate was held constant while the other two were varied. The procedure was repeated until all combinations of fixed and variable substrates had been collected (Fig. 3). Inspection of the Lineweaver-Burk plots indicates that the substrates form a ternary complex in the enzyme active site. A ping-pong mechanism is ruled out because the Lineweaver-Burk plots for all substrate combinations clearly converge (Fig. 3). Further variation of l-Glu and ATP in a constant ratio (1:10) versus varied l-Abu produces a series of Lineweaver-Burk plots (1/V versus 1/[l-Abu]) that converge (Fig. 4). Similar results were obtained when varying l-Glu and l-Abu at a constant ratio (1:20) versus varied ATP (data not shown). These results demonstrate that a product release step does not occur between the binding of ATP or l-Glu and l-Abu or between the binding of l-Glu or l-Abu and ATP (28). Thus our results are not consistent with the two possible ping-pong mechanisms in which: 1) ADP is released after phosphorylation of l-Glu but prior to binding of l-Abu or 2) ADP is released after generation of a phosphorylated enzyme intermediate prior to the binding of the other substrates.

The matrix of data obtained for either the wild-type or mutant enzyme was simultaneously fit to the rapid equilibrium rate equations (28), which describe the various ter-reactant systems. The quality of the fit was assessed by examination of Lineweaver-Burk plots, by examination of the residual plots and by statistical analysis of the standard error of the fitted parameters and the correlation coefficient ($R^2$). After excluding the possible ping-pong mechanisms, 16 possible models were tested, including random, ordered (all 6 possibilities), ordered A random BC (3 possibilities), ordered B random AC (3 possibilities), and ordered C random AB (3 possibilities).

The data for both *wt-Tb*-GCS and *TbC319A* γ-GCS was best fit by the equation below for a rapid equilibrium random ter-reactant system, where $K_{p1}$, $K_{p2}$, $K_{ATP}$, $K_{Glu}$, and $K_{Aba}$ are the equilibrium dissociation constants ($K_d$ values) for the binding of substrate with free $E$, and $\alpha$, $\beta$, and $\gamma$ are the interaction factors by which the dissociation constants of one substrate are influenced by the prior binding of one of the other substrates (Fig. 5). None of the other tested models provided a satisfactory fit to the data.

$$
\frac{V}{V_{max}} = \frac{[Glu][Aba][ATP]}{K_{p1} + [Glu][Aba] + [ATP]} + \frac{[Glu][Aba][ATP]}{K_{p2} + [Glu][Aba] + [ATP]} + \frac{[Glu][Aba][ATP]}{K_{ATP} + [Glu][Aba] + [ATP]} + \frac{[Glu][Aba][ATP]}{K_{Glu} + [Glu][Aba] + [ATP]} + \frac{[Glu][Aba][ATP]}{K_{Aba} + [Glu][Aba] + [ATP]}
$$

(Eq. 1)

Lineweaver-Burk plots for a representative data set of *wt-Tb*-GCS fit to Equation 1 are displayed in Fig. 3, and the model derived parameters are reported in Table I. The kinetic constants derived from the random ter-reactant model are very similar for *wt-Tb*-GCS and *TbC319A* γ-GCS (Table I). The specific activity ($V_{max}$) is identical for the two enzymes, and no significant change is observed in the substrate dissociation constants as a result of the mutation. For both *wt-Tb*-GCS and *TbC319A* γ-GCS, there is positive cooperativity between the binding sites of l-Glu and ATP ($\beta = 0.06$ for *wt-Tb*-GCS and 0.04 for *TbC319A* γ-GCS). Therefore, the prior binding of ATP or l-Glu to the enzyme increases the binding affinity of the enzyme for the other substrate by 18-fold for the wild-type enzyme and by 23-fold for the mutant enzyme. In contrast, there is negative cooperativity between the binding sites of l-Glu and l-Abu ($\gamma = 6.3$ for *wt-Tb*-GCS and 5.0 for *TbC319A*); thus, the binding of one decreases the binding affinity of the other by 6.3-fold to the wild-type enzyme and by 5.0-fold to the mutant enzyme. ATP and l-Abu do not significantly effect the binding energies of each other, for either enzyme ($\alpha = 1$).

In our previous paper, we reported for *wt-Tb*-GCS that the apparent $K_m$ values for l-Glu, l-Abu, and ATP were 0.24, 10, and 0.07 mM, respectively (20). These values were obtained by varying the concentration of one substrate, while holding the concentrations of the other two substrates at saturating levels. Given the random mechanism for catalysis by *Tb*-GCS, the $K_{app}$ values describe the interaction of a given substrate to enzyme which is bound to the other two substrates. In contrast, $K_{Glu}$, $K_{Aba}$, and $K_{ATP}$ are the model derived dissociation constants for the binding of each substrate to free enzyme. Because the prior binding of some substrates influences the binding affinity of others, the $K_{app}$ values will not equal the model derived dissociation constants but will instead be best approximated by a combination of the $K_d$ and the binding interaction factors.
factors (e.g., $K_m^{app}$ for l-Glu describes the binding of l-Glu to $E^*Aba^*ATP$ and should be similar to the model derived constant $\beta \gamma K_{Glu}$ (Fig. 5)). The model derived parameters $\beta \gamma K_{Glu}$, $\alpha \gamma K_{Aba}$, and $\alpha \beta K_{ATP}$ (Table I) are within the experimental errors of the reported $K_m^{app}$ values, providing good agreement between the data.

**FIG. 3.** Lineweaver-Burk analysis of a representative data set for wt-Tb$\gamma$GCS. Units are: velocity ($v$) in $\mu$mol/min and substrate concentrations in mM. The *closed circles* represent actual data points, and the *lines* represent the simultaneous fit of all displayed data to Equation 1, which describes a random ter-reactant mechanism. The parameters for the fit to this representative data set are: $V_{max} = 2.0 \pm 0.06$ $\mu$mol/min/mg, $K_{Glu} = 1.7 \pm 0.42$, $K_{Aba} = 4.0 \pm 1.1$, $K_{ATP} = 0.58 \pm 0.11$, $\alpha = 0.91 \pm 0.16$, $\beta = 0.070 \pm 0.010$, and $\gamma = 6.3 \pm 1.4$. Errors are the standard error of the fit. The square of the correlation coefficient ($R^2$) for the global fit of all displayed data points is 0.993.
The error is the standard deviation of the mean for three independent experiments. A representative fit to the model is displayed in Fig. 3. $K_{g\text{lu}}, K_{g\text{ab}},$ and $K_{ATP}$ are the equilibrium dissociation constants for the binding of substrate with free $E$. $\alpha$, interaction factor between L-Aba and ATP; $\beta$, interaction factor between L-Glu and ATP; $\gamma$, interaction factor between L-Glu and L-Aba.

The full kinetic profile of the Tb$\gamma$-GCS was obtained to determine the kinetic mechanism of the enzyme. The kinetic data collected for both wild-type and TbC319A $T. brucei \gamma$-GCS are best fit to the equation that describes a rapid equilibrium random ter-reactant mechanism in which significant dependences exist between the binding of some of the substrate pairs. There is a favorable interaction energy of 1.7 kcal/mol ($\Delta G$ interaction $= -RT\ln(\beta K_{Glu} or ATP/K_{Glu} or ATP)$) between the L-Glu and ATP binding sites, an unfavorable interaction energy of 1.1 kcal/mol between the L-Glu and L-Aba binding sites, and no interaction between the ATP and L-Aba binding sites. These results are consistent with what is known about the reaction mechanism. ATP transfers the $\gamma$-phosphate to L-Glu, and the activated L-Glu undergoes nucleophilic attack by the amino group of L-Aba (11). Thus, uniquely the L-Glu binding site must be positioned so that L-Glu can interact with both the other substrates, while ATP and L-Aba would not require direct interaction. It has been reported that low levels of ATP hydrolysis ($<1\%$ of the overall reaction) occurs in the absence of the other substrates and that L-Glu inhibits this reaction (29). The cooperativity between the L-Glu and ATP binding sites might be expected to reduce the extent of this unwanted side reaction.

Several possible kinetic mechanisms have been proposed for mammalian $\gamma$-GCS, including partially ordered ter-reactant (14–16) and ping-pong mechanisms (17, 18). Our data are not consistent with a ping-pong mechanism for $\gamma$-GCS catalysis. However, because the substrates exhibit dependences on each other for binding, in the absence of the modern robust fitting techniques that allow the entire data set to be fit to the model simultaneously, this mechanism would be difficult to distinguish from the previously proposed partially ordered mechanisms (e.g. ordered A, random BC (14, 15), or random AB, ordered C (16)). $T. brucei \gamma$-GCS could also exhibit species differences compared with mammalian $\gamma$-GCS, but we find that the recombinant human $\gamma$-GCS also follows a rapid equilibrium catalysis.

### DISCUSSION

Few studies have shed understanding on the nature of the $\gamma$-GCS active site. $\gamma$-GCS from several species, including $T. brucei$, is activated by cystamine. Prior studies on the mammalian enzyme suggested that the inactivation occurred by labeling of a Cys residue (19, 22). This residue was believed to be in the L-Glu binding site because L-Glu protects against inactivation, suggesting that the labeled Cys residue might be important for catalysis (29). A single Cys residue (Cys-319) is conserved among those enzymes reported to be sensitive to cystamine inactivation. Instead, these data suggest that cystamine inactivates the enzyme by blocking access of substrates to the active site. The amino acid sequence of $\gamma$-GCS from $L. donovani$ was recently reported, and it lacks Cys-319 (5). These data suggest that the $L. donovani$ enzyme will be resistant to cystamine inactivation.

### TABLE I

| \(\text{Kinetic constants derived for a random ter-reactant model of the} \) \(\gamma\text{-GCS mechanism}\) | \(\text{wt-TbGCS}\) | \(\text{TbC319A}\) |
|---|---|---|
| \(V_{\text{max}}\) (\(\mu\text{mol/min/mg}\)) | 2.0 ± 0.2 | 1.7 ± 0.29 |
| \(K_{g\text{lu}}\) (mM) | 2.6 ± 2.0 | 5.9 ± 3.3 |
| \(K_{g\text{ab}}\) (mM) | 5.4 ± 2.9 | 6.1 ± 1.8 |
| \(K_{ATP}\) (mM) | 1.4 ± 1.2 | 1.4 ± 0.33 |
| \(\alpha\) | 0.93 ± 0.16 | 1.5 ± 0.23 |
| \(\beta\) | 0.057 ± 0.024 | 0.044 ± 0.030 |
| \(\gamma\) | 6.3 ± 1.2 | 5.0 ± 1.8 |

### FIG. 4

Lineweaver-Burk analysis of the variation of L-Glu and L-ATP in a constant ratio (1:10) versus varied L-Aba concentrations. Units are: velocity (\(v\)) in \(\mu\text{mol/min}\) and substrate concentrations in mM. Displayed ratios are the actual concentrations of L-Glu:ATP in mM.

### FIG. 5

Model of the random Ter-reactant mechanism of $\gamma$-GCS catalysis. \(K_{g\text{lu}}, K_{g\text{ab}},\) and \(K_{ATP}\) are the equilibrium dissociation constants for the binding of substrate with free $E$. $\alpha$, interaction factor between L-Aba and ATP; $\beta$, interaction factor between L-Glu and ATP; $\gamma$, interaction factor between L-Glu and L-Aba.

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**DISCUSSION**

Few studies have shed understanding on the nature of the $\gamma$-GCS active site. $\gamma$-GCS from several species, including $T. brucei$, is activated by cystamine. Prior studies on the mammalian enzyme suggested that the inactivation occurred by labeling of a Cys residue (19, 22). This residue was believed to be in the L-Glu binding site because L-Glu protects against inactivation, suggesting that the labeled Cys residue might be important for catalysis (29). A single Cys residue (Cys-319) is conserved among those enzymes reported to be sensitive to cystamine inactivation. Instead, these data suggest that cystamine inactivates the enzyme by blocking access of substrates to the active site. The amino acid sequence of $\gamma$-GCS from $L. donovani$ was recently reported, and it lacks Cys-319 (5). These data suggest that the $L. donovani$ enzyme will be resistant to cystamine inactivation.

**TABLE I**

**Kinetic constants derived for a random ter-reactant model of the $\gamma$-GCS mechanism**

| \(\text{Kinetic constants derived for a random ter-reactant model of the} \) \(\gamma\text{-GCS mechanism}\) | \(\text{wt-TbGCS}\) | \(\text{TbC319A}\) |
|---|---|---|
| \(V_{\text{max}}\) (\(\mu\text{mol/min/mg}\)) | 2.0 ± 0.2 | 1.7 ± 0.29 |
| \(K_{g\text{lu}}\) (mM) | 2.6 ± 2.0 | 5.9 ± 3.3 |
| \(K_{g\text{ab}}\) (mM) | 5.4 ± 2.9 | 6.1 ± 1.8 |
| \(K_{ATP}\) (mM) | 1.4 ± 1.2 | 1.4 ± 0.33 |
| \(\alpha\) | 0.93 ± 0.16 | 1.5 ± 0.23 |
| \(\beta\) | 0.057 ± 0.024 | 0.044 ± 0.030 |
| \(\gamma\) | 6.3 ± 1.2 | 5.0 ± 1.8 |

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**FIG. 4**

Lineweaver-Burk analysis of the variation of L-Glu and L-ATP in a constant ratio (1:10) versus varied L-Aba concentrations. Units are: velocity (\(v\)) in \(\mu\text{mol/min}\) and substrate concentrations in mM. Displayed ratios are the actual concentrations of L-Glu:ATP in mM.

**FIG. 5**

Model of the random Ter-reactant mechanism of $\gamma$-GCS catalysis. \(K_{g\text{lu}}, K_{g\text{ab}},\) and \(K_{ATP}\) are the equilibrium dissociation constants for the binding of substrate with free $E$. $\alpha$, interaction factor between L-Aba and ATP; $\beta$, interaction factor between L-Glu and ATP; $\gamma$, interaction factor between L-Glu and L-Aba.
random mechanism.\textsuperscript{3} \textit{T. brucei} glutathionylspermidine synthetase, which catalyzes a similar reaction to \(\gamma\)-GCS, has also been reported to follow a rapid equilibrium random mechanism (30). These data suggest that a random kinetic pattern is common among ATP-dependent peptide ligases.

The model for the kinetic mechanism of \(\gamma\)-GCS reported in this paper provides a method to assess effects of mutations on the reaction as we have done for the TbC319A \(\gamma\)-GCS mutant. In addition, the activity of mammalian \(\gamma\)-GCS has been reported to be influenced by interaction with a regulatory subunit (31, 32). The regulatory subunit decreases the apparent \(K_m\) for \(L\)-Glu, but its effects on the reaction mechanism have not yet been addressed. A regulatory subunit has not yet been found associated with any of the non-mammalian enzymes, leaving open the possibility that differences in reaction mechanism may be present between the regulated mammalian enzyme and \textit{T. brucei} \(\gamma\)-GCS. In the future, characterization of differences between these two enzyme will aid in the design of species-selective \textit{T. brucei} inhibitors.

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