Structure Prediction and Active Site Analysis of the Metal Binding Determinants in γ-Glutamylcysteine Synthetase*

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γ-Glutamylcysteine synthetase (γ-GCS) catalyzes the first step in the de novo biosynthesis of glutathione. In trypanosomes, glutathione is conjugated to spermidine to form a unique cofactor termed trypanothione, an essential cofactor for the maintenance of redox balance in the cell. Using extensive similarity searches and sequence motif analysis we detected homology between γ-GCS and glutamine synthetase (GS), allowing these proteins to be unified into a superfamily of carboxylate-thiol that acts as a sulfhydryl buffer. It is present in mammalian cells (6) and of trypanothione in the trypanosome, which was previously poorly understood, was modeled using the known structure of GS. Two metal-binding sites, each ligated by three conserved active site residues (n1: Glu-55, Glu-83, Glu-100; and n2: Glu-53, Gln-321, and Glu-489), are predicted to form the catalytic center of the active site, where the n1 site is expected to bind free metal and the n2 site to interact with MgATP. To elucidate the roles of the metals and their ligands in catalysis, these six residues were mutated to alanine in the Trypanosoma brucei enzyme. All mutations caused a substantial loss of activity. Most notably, E93A was able to catalyze the l-Glu-dependent ATP hydrolysis but not the peptide bond ligation, suggesting that the n1 metal plays an important role in positioning l-Glu for the reaction chemistry. The apparent $K_m$ values for ATP were increased for both the E489A and Q321A mutant enzymes, consistent with a role for the n2 metal in ATP binding and phosphoryl transfer. Furthermore, the apparent $K_d$ values for activation of E489A and Q321A by free Mg$^{2+}$ increased. Finally, substitution of Mn$^{2+}$ for Mg$^{2+}$ in the reaction rescued the catalytic deficits caused by both mutations, demonstrating that the nature of the metal ligands plays an important role in metal specificity.

Glutathione (γ-glutamyl-cysteinyl-glycine) is a tripeptide thiol that acts as a sulfhydryl buffer. It is present in mammalian cells at high concentrations and plays an important role in many cellular processes, such as detoxification of oxidative species, protein and DNA synthesis, and cellular import of amino acids (1, 2). The parasitic protozoa Trypanosoma brucei causes African sleeping sickness, a disease responsible for significant morbidity and mortality in Africa. The identification of metabolic differences between parasite and host is an important step in the development of new anti-trypanosomal agents (3). Mammals and trypanosomes differ in their metabolism of glutathione. Trypanosomes utilize a unique molecule that is a conjugate of glutathione and spermidine, called trypanothione, in order to maintain reduced thiol pools in the cell (4). The first committed step in the biosynthesis of glutathione, and thereby trypanothione, is the formation of γ-glutamylcysteine, catalyzed by γ-glutamylcysteine synthetase (γ-GCS)$^1$ (5). γ-GCS is the rate-limiting enzyme in the synthesis of glutathione in mammalian cells (6) and of trypanothione in the trypanosomes, Leishmania tarentolae (7).

γ-GCS catalyzes the formation of a peptide bond between the carbonyl of l-Glu and the α-amino group of l-Cys. A number of acceptor amino acids have been tested in place of l-Cys and the enzyme was found to catalyze the reaction utilizing l-Aba with similar efficiency as l-Cys (8). The enzyme utilizes ATP to promote this reaction by first phosphorylating the carbonyl of l-Glu (Fig. 1). The phosphate group of this intermediate is a good leaving group for the subsequent attack by the α-amino group of l-Cys (5). Purified rat kidney γ-GCS is activated by both Mg$^{2+}$ and Mn$^{2+}$ (9). Mn$^{2+}$ activates the enzyme at 10-fold lower concentrations than Mg$^{2+}$, but the rate in the presence of Mn$^{2+}$ is 4-fold slower than with Mg$^{2+}$. Moreover, Mn$^{2+}$ lowers the specificity of the enzyme for the acceptor amino acid (8). γ-GCS has been identified in animals, bacteria, plants, fungi, and protozoa. However, the relationship between animal, bacterial, and plant enzymes was unclear due to the absence of pronounced sequence similarity between them (10). Despite extensive functional studies of γ-GCS in many organisms, our understanding of its structure and catalytic mechanism had been limited. A three-dimensional structure of γ-GCS is not available and the enzyme is structurally unrelated to mechanistically similar enzymes such as d-Ala d-Ala ligase and glutathione synthetase (11). However, with the rapid expansion of protein sequence data and the development of sensitive similarity search tools such as PSI-BLAST (12) and HMMer (13), the limits of sequence-based homology detection are being extended (14). Multiple sequence alignments contain information about the positional variation of amino acid usage that is the basis for profile generation (15). Profiles transformed into po-

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1 The abbreviations used are: γ-GCS, γ-glutamylcysteine synthetase; GS, glutamine synthetase; l-Aba, l-alanine; HPLC, high performance liquid chromatography; mutant enzymes are designated by single amino acid codes and residue number, eg. E55A, Glu-55 to Ala mutant T. brucei γ-GCS.
sition-specific scoring matrices (12, 16) or encoded in hidden Markov models (17) enhance the sensitivity of sequence similarity searches.

In the course of extensive similarity searches using PSI-BLAST (12), we were able to detect homology between animal, fungal, plant, and bacterial γ-GCS proteins. A multiple alignment was constructed from these γ-GCS sequences. Using the profile generated from this multiple sequence alignment, statistically significant sequence similarity between γ-GCS and glutamine synthetase (GS) was found. Homology between γ-GCS and GS was further supported by secondary structure predictions and conservation pattern analysis.

GS is a crucial enzyme in nitrogen metabolism that ligates ammonia to the γ-carboxyl of L-Glu, also in an ATP-dependent reaction (18, 19). The structure of GS with and without bound substrates has been reported (20) and thus provides a fold prediction for γ-GCS that yields insight into the molecular basis of its catalytic function. A partial model of the γ-GCS active site was constructed based on the GS structure allowing the Mg\(^{2+}\), MgATP, and L-Glu-binding sites to be modeled.

This model of the γ-GCS active site residues was used as a guide for experimental studies of the T. brucei γ-GCS, which has previously been kinetically characterized (21, 22). Based on the homology to GS, six conserved residues in γ-GCS are predicted to form two metal-binding sites (n1: Glu-55, Glu-93, Glu-100; and n2: Glu-53, Glu-489, Gln-321). The roles of these six amino acid residues were probed by site-directed mutagenesis. All six residues are crucial for enzyme activity.

The protein was fully sequenced in their coding regions to verify the construct. CGTTTCGTGTAATGGACG-3

GTCGCGAGCCTCCCCGGCAAGCC-3

GCCCAGCATATGGGAGTTTTATGG-3

GACGAAATTGCACACCAGCTCGTCCGC-3

The proposed reaction mechanism of γ-GCS catalyzed peptide bond ligation.

Multiple Sequence Alignments—Multiple sequence alignments were constructed using the T-COFFEE program (26) for each γ-GCS sequence group. These alignments were merged manually based on the conservation patterns and secondary structure predictions from the JPRED server (27). The merged multiple sequence alignment was used as input to generate a position-specific scoring matrix (-b option in the program blastpgp (12)) for a new round of PSI-BLAST searches starting from each individual γ-GCS sequence. Multiple alignments for representative sequences of the three types of GSs were also constructed by T-COFFEE (26), adjusted manually, and merged with the γ-GCS alignment. The complete full-length alignment is available through anonymous FTP from ftp://iole.swmed.edu/pub/GCS/gGCS.ali.doc.

Euclidian Space Mapping and Distance Diagram for the γ-GCS and GS Sequences—The conserved segments of the γ-GCS and GS multiple alignment shown in Fig. 2 were used to calculate pairwise identity fractions \(q_{ij}\) between each sequence pair \(i\) and \(j\). The identity fractions were converted to evolutionary distances with the formula,

\[
d_{ij} = -\ln(q_{ij} - q_{ij}^{-}\ln(1 - q_{ij}^{-})]
\]

where \(q_{ij}^{-}\) is an expected identity percentage of two random sequences with the same amino acid composition as the sequences \(i\) and \(j\). Each sequence was represented as a point in a multidimensional Euclidian space in such a way that Euclidian distances \(d_{ij}\) between the points optimally approximated the estimated distances \(d_{ij}\) between the sequences.

These points were grouped using the following procedure. Each point representing a sequence generated a Gaussian density in the Euclidian space having the following properties. The mean of each density was the point’s coordinates and the variance \(\sigma^2\) of each density was identical for all points. Starting from each point, the local maximum of the sum of such Gaussians was found. The points giving rise to the same local maximum were grouped together.

Site-directed Mutagenesis—Polymerase chain reaction-based mutagenesis of the T. brucei γ-GCS expression vector was performed using the QuickChange\textsuperscript{TM} site-directed mutagenesis kit from Stratagene as recommended by the manufacturer. 5′-3′ primers used to direct mutagenesis of the exact complement of the 6 metal-binding residues are listed as follows (the primers of other mutants that are not discussed in detail are not shown), with the replaced codon underlined. E35A, 5′-CGGTTTTCTATG-GGGAGCCGCAATGGAACACGGCTCGGCCG-3′; E55A, 5′-GGGGAACGTTGCACACAGCGTCTCGGCCG-3′; E93A, 5′-GGCAGATGGC-GCCGGACATAGGCTATGCGG-3′; E100A, 5′-GGGATCGCTTCT-GTCGGGACCTCCCCGAGACCG-3′; E321A, 5′-GGGCGTGCAACATGCGTCTCC-3′; Q321A, 5′-GGGCGTGCAACATGCGTCTCC-3′.

These DNA encoding mutant enzyme were fully sequenced in their coding regions to verify the construct.

Protein Expression and Purification—T. brucei γ-GCS was expressed as a N-terminal His\(_6\)-tag fusion in a T7 bacterial (BL21/DE3 cells co-transformed with pREP4) expression vector. Protein was purified over a Ni\(^{2+}\)-agarose column followed by gel filtration (22). Protein solutions were stored in 50 mM Tris, pH 8, 100 mM NaCl, 5 mM MgCl\(_2\) at 4 °C. Protein concentration was determined by absorbance at 280 nm with previously determined extinction coefficient (22).

Enzyme Kinetics Analysis of the γ-GCS Reaction by Spectrophotometric Assay—Kinetic analysis was done on purified γ-GCS at 37 °C with a spectrophotometric assay (17). The ATP used was with NADH oxidation (21). Buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 2 mM phosphonopyruvate, 0.27 mM NADH) was added with 10 units/ml type II rabbit muscle pyruvate kinase, 20 units/ml of type II rabbit muscle lactic acid dehydrogenase, 5–60 mM total MgCl\(_2\), and γ-GCS substrates. Unless otherwise stated, the following concentrations were used: 5 mM ATP, 100 mM L-Abu, and 10 mM L-Glu. L-Abu was substi-
tuted for L-Cys in all kinetic studies. Previous studies used KCl instead of NaCl but we found KCl to be inhibitory at high concentrations. The reaction was begun with the addition of γ-GCS. Enzyme concentration ranges were as follows: wild-type γ-GCS, 0.1–0.4 μM; E489A and Q321A, 30–60 μM with Mg²⁺ and 0.4–1.4 μM with Mn²⁺; E53A, E55A, E93A, and E100A, 4–30 μM. For the metal binding studies, the protein samples (10–20 ml) were dialyzed twice against 4 liters of buffer without divalent metals. Divalent metal in the form of MgCl₂ or MnCl₂ was added to the reaction over a range of free concentrations. The concentration of free metal was determined at a given total metal concentration and ATP concentration under the conditions of the assay with the program “Bound and Determined” (28). When using Mn²⁺, the above reaction buffer was modified to use 15 units/ml of type III rabbit muscle pyruvate kinase and 30 units/ml of type II rabbit muscle lactic acid dehydrogenase. Reaction rates were confirmed to be linear with enzyme concentration. All curve fitting and modeling of kinetic data was performed using Sigma Plot 5.0 (SPSS Inc.).

**RESULTS**

Identification of Homology among γ-GCS Proteins

To clarify the relationships among γ-GCS proteins from different organisms, we conducted PSI-BLAST searches starting from individual sequences from fungi, plants, and bacteria. Similarity searches initiated with plant sequences retrieved a hypothetical protein from *Synechosystis* spp. (gi|7496902, iteration 2, E-value 0.05). Using this protein as the query we detected many bacterial sequences as well as sequences from plants (e.g., gi|7489004, *L. esculentum*, iteration 3, E-value 4e-05), animals (e.g., gi|7494173, *Plasmodium falciparum*, iteration 3, E-value 0.044), and fungi (e.g., gi|885591, *Schizosaccharomyces pombe*, iteration 4, E-value 0.007), implying that these γ-GCSs are homologous. Searches with the *E. coli* γ-GCS sequence (gi|121661) converged within a separate bacterial group that could not be linked with other γ-GCSs using PSI-BLAST. However, analysis of conserved motifs and secondary structure
predictions in this group, and their comparison with other γ-GCSs, suggested homology among all known γ-GCS proteins (Fig. 2). Based on sequence similarities and phylogeny of organisms, we divided γ-GCS homologues into two eukaryotic groups (E-I and E-II) and three prokaryotic groups (P-I, P-II, and P-III) (Fig. 2).

Identification of GS as a Structural Homolog of γ-GCS

PSI-BLAST searches starting from γ-GCS sequences could not detect any proteins outside the γ-GCS family with significant E-values (<0.02). Because the sensitivity of profile-based similarity searches relies on the quality of the multiple sequence alignment, alignments produced automatically by PSI-BLAST might be of insufficient quality to detect remote homologues. Thus after automated PSI-BLAST iterations failed to detect γ-GCS homologues with statistically significant scores, we used the manually adjusted multiple alignment of all identified γ-GCS sequences to start profile-based searches. For some query sequences (for example, gi|11282603 from Xylella fastidiosa, Fig. 2), seeding PSI-BLAST with the γ-GCS alignment produced significant hits (E-value less than 0.02, bit score >40) to a protein outside the γ-GCS family. This Mycobacterium tuberculosis protein (gi|7478087) is annotated as a "probable glnA3 protein" and belongs to the type I GS family (Fig. 2). No other proteins were detected with E-values less than 0.02. The key residues for metal and substrate binding or catalysis in GS form highly conserved motifs that are mainly located in the central β-sheet (Fig. 3). An inspection of the multiple sequence alignments of γ-GCS and GS reveals consistent conservation of these key residues (Fig. 2). Additionally, hydrophilic and hydrophobic positions alternate along the predicted β-strands, and secondary structure prediction (27) of the aligned regions are similar (data not shown). Thus we conclude that γ-GCSs and GSs are homologous and unify them into a superfamily of carboxylate-amine/ammonia ligases.

Structural Features of the GS/γ-GCS Superfamily

The GS from Salmonella typhimurium has been thoroughly studied and its structure has been determined (19, 20). Only the C-terminal domain of GS is homologous to γ-GCS and it consists of a central anti-parallel β-sheet of 6 strands with several α-helices packed on one side of it (Fig. 3). The other side of the β-sheet accommodates the active site with the binding sites for metal ions and substrates. All of the core β-strands and α-helices of GS can be mapped to the γ-GCS sequences, among which are two hydrophobic α-helices buried between the β-sheet and outside helices (Fig. 2). Two divalent metal ions (termed n1 and n2, which may be Mg²⁺ or Mn²⁺) participate in substrate binding and catalysis by GS. The n1 metal interacts with the α-carboxyl group of L-Glu and the n2 metal interacts with ATP and is involved in phosphoryl transfer to the substrate. Each metal is chelated by three highly conserved residues: the n1 metal is liganded by Glu-131, Glu-212, and Glu-220 and the n2 metal is bound by Glu-129, His-269, and Glu-357 (Fig. 3). Five of the metal ligands are conserved throughout the γ-GCS and GS families, while His-269 is conservatively replaced with Gln in many γ-GCS proteins (Fig. 2). The conservation of metal-binding residues across the superfamily suggests that γ-GCS also binds two metal ions that are chelated similarly. The binding sites for L-Glu and ATP are also predicted to be similar between GS and γ-GCS, while the third substrate differs between the two (ammonia in GS versus L-Cys in γ-GCS) and the binding pockets are not expected to be conserved. The negative charges of the ATP phosphoryl groups in GS are mainly stabilized by the n2 metal and by Arg-344, which is conserved in both families (Fig. 2). His-271 also interacts with the phosphates of ATP in GS and is replaced by Asn or Thr in many of the γ-GCS sequences, while Ser-273, which forms a hydrogen bond to N-6 of the ATP purine ring in GS is replaced by Ser, Asp, or Gln in γ-GCS (Fig. 2). In GS two arginine residues confer the substrate specificity for L-Glu, Arg-321 interacts with the L-Cys carboxylate of L-Glu (Figs. 2 and 3). The corresponding positions in γ-GCS also contain a positively charged residue (typically Arg), suggesting that L-Glu binds to γ-GCS in a similar manner.

Diversity of γ-GCS and GS Superfamily

The difficulty in identifying the relationship between the γ-GCS and GS families results from the fact that this superfamily of carboxylate-amine/ammonia ligases is very diverse, e.g. with sequence identity ranging from 7 to 92% for the full-length sequences in Fig. 2. Thus, it is not straightforward to build a phylogenetic tree for the superfamily, the branching order in such a tree would be highly unreliable. To illustrate the relationships between the superfamly members, we have chosen to represent the sequences as points in Euclidian space with distances between the points approximating evolutionary distances. The two-dimensional projection of the space onto the plane of the largest scatter of points is shown (Fig. 4). The points were grouped according to the procedure described under "Experimental Procedures" resulting in 7 clusters that are colored differently on the distance projection plot (Fig. 4). Sequences that are more closely related group together on the plot. Eukaryotic group I (E-I) contains animal and fungal sequences; they share high sequence similarities. E-II/P-I consists of plant and prokaryotic sequences, which are similar to each other and relatively distant from animal or fungal sequences. The high similarity between plant and prokaryotic
sequences from this group might suggest horizontal transfer events between plants and bacteria/archaea. The distance plot shows that sequences of groups E-II/P-I and P-II are closer to each other than to any other group. In contrast, the P-III group is well separated from the rest and it includes the first experimentally characterized prokaryotic γ-GCS from *E. coli* (30). Finally, the GS sequences, which have been classified into three groups, are distant from all of the γ-GCS sequences and group together on the plot.

The sequences in the P-II group and many prokaryotic sequences in the E-II/P-I group are hypothetical proteins without functional annotations. Their detected homology to γ-GCS facilitates functional annotation and classification of these sequences. Two archaeal proteins (gi|11499888 from *Archaeoglobus fulgidus* and gi|10580892 from *Halobacterium sp.*) are among these hypothetical proteins. There have been no previous reports about the presence of γ-GCS in archaea. Our detection of γ-GCS in *Halobacterium sp.* is consistent with previous findings that γ-glutamylcysteine dipeptide is one of the major thiols in halobacteria in the absence of significant glutathione (31).

**Characterization of Metal Dependence by Wild-type T. brucei γ-GCS**

The finding that γ-GCS is a structural homolog of GS supports the conclusion that γ-GCS has two metal-binding sites. To evaluate this hypothesis experimentally, the dependence of the reaction rate of wild-type γ-GCS on free Mn$^{2+}$ was monitored. The concentrations of free metal and MgATP were calculated from total metal and ATP concentrations by using the computer program “Bound and Determined” (28). Saturating concentrations of MgATP, L-Glu, and L-Aha were used for the analysis. A hyperbolic dependence of the reaction rate on free Mn$^{2+}$ was observed (Fig. 5). The data were fitted to the Michaelis-Menten equation and the $K_{\text{app}}$ (apparent dissociation constant) for free Mn$^{2+}$ at 2 mM ATP was determined to be 0.50 ± 0.04 mM (Table I). Small variations in the concentration of MgATP (less than a 2-fold change occurs over the 35-fold range of free Mn$^{2+}$) did occur over the range of the titration but were not substantial enough to account for the rate activation by free Mn$^{2+}$. Thus, the data are consistent with the model derived from the GS structure in which the active enzyme species binds both Mn$^{2+}$ and MgATP at separate sites.

**Effects of Substituting Mn$^{2+}$ for Mg$^{2+}$**

Wild-type *T. brucei* γ-GCS is also activated by Mn$^{2+}$ (Fig. 5). The activity is stimulated at significantly lower concentrations of Mn$^{2+}$ ($EC_{50} = 6 \mu M$) than Mn$^{2+}$, however, the maximum catalytic rate is slightly lower. Total Mn$^{2+}$ concentrations above 0.75 mM are inhibitory. Unlike for Mg$^{2+}$, the dependence of rate on free Mn$^{2+}$ concentrations is sigmoidal and suggests multiple Mn$^{2+}$ ions may be binding cooperatively. Indeed the concentration of MnATP could not be held constant over the range of free Mn$^{2+}$ concentrations used in the titration, thus the data reflect contributions from the binding of both free Mn$^{2+}$ and MnATP to separate sites on the enzyme.

The kinetic constants for all three substrates were also measured in the presence of constant concentration of free Mn$^{2+}$. The $K_m$ values for l-Glu and Aba were similar to those measured in the presence of Mg$^{2+}$ and the $K_m$ for MnATP was 3-fold lower than for MgATP (Tables II and III).

**Steady-state Kinetic Analysis**

**Mutation of n1 Metal-binding Residues—**Based on the multiple sequence alignment of GS with γ-GCS family members, residues Glu-55, Glu-93, and Glu-100 of *T. brucei* γ-GCS were identified as the n1 metal ligands and would be expected to form the free Mn$^{2+}$-binding site (Fig. 2). Mutation of these residues to Ala drastically reduced $k_{\text{cat}}$ (Table II). The activities of the E55A and E100A mutants were not distinguishable from background rates in the presence of either Mg$^{2+}$ or Mn$^{2+}$. The $k_{\text{cat}}$ of E93A ($k_{\text{cat}} = 0.066 \pm 0.005 \text{ s}^{-1}$) is 60-fold lower than for wild-type γ-GCS based on the coupled spectrophotometric assay that follows ATP hydrolysis to ADP. Moreover, the $K_{m}$ for l-Glu rises 46-fold to
The dependence of the reaction rate on free Mn\(^{2+}\) concentration is hyperbolic suggesting that only a single site is involved in the titration. However, this titration may represent interactions at the n2 site and not the n1 site, since the concentration of MnATP also varies during the titration.

Interestingly, while the reaction catalyzed by E93A is l-Glu dependent, it is not dependent on l-Aba over a concentration range from 0 to 200 mM. Analysis of this reaction by HPLC shows that the dipeptide product, γ-glutamylaminobutyrate, was not formed during the course of the reaction catalyzed by the E93A enzyme (Fig. 6). In contrast for the wild-type enzyme, the rate of formation of γ-glutamylaminobutyrate determined by HPLC analysis exactly corresponds to the rate of ADP formation measured in the spectrophotometric assay. These data demonstrate that the mutant enzyme is capable of hydrolyzing ATP in a l-Glu-dependent reaction but cannot catalyze the peptide bond ligation between l-Glu and l-Aba.

**Mutation of n2 Metal-binding Residues**—The n2 metal-binding residues of GS align with residues Glu-53, Gln-321, and Glu-489 of *T. brucei* γ-GCS (Fig. 2). Mutation of these residues to Ala dramatically lowers the reaction rate catalyzed by the enzyme (Table II). E53A γ-GCS has no detectable activity, consistent with a role for this residue as a crucial ligand for the n2 metal site. The *k*\(_{cat}\) of the E489A enzyme falls 720-fold to 0.0053 ± 0.0005 s\(^{-1}\). In addition, the *K\(^{app}\)\(_{M}\)* for ATP rises 28-fold while negligible effects were observed on the other two substrates. The effects of mutating Gln-321 to Ala are focused on MgATP binding. The enzyme rate does not saturate with increasing MgATP concentrations up to the limit of the assay ([ATP] = 15 mM) and the *k*\(_{cat}\)/*K\(^{app}\)\(_{M}\)* for MgATP is 3.7 ± 0.4 M\(^{-1}\) s\(^{-1}\), a 14,000-fold decrease compared with the wild-type enzyme. The *K\(^{app}\)\(_{M}\)* values for the other two substrates were not changed, although it is important to note that these data could not be obtained at saturating MgATP levels. The kinetic analysis of E53A, E489A, and Q321A γ-GCS points to their role in binding the n2 metal and are consistent with the observation in GS that the n2 metal ion coordinates the γ-phosphate oxygen of ATP to allow phosphoryl transfer in the first step of the reaction (20, 32).

Higher concentrations of free Mn\(^{2+}\) are required for full activation of both the E489A (36-fold) and Q321A (26-fold) mutant enzymes (Table I, Fig. 5). The concentration of MgATP does not vary over the course of the titration and, similar to wild-type γ-GCS, the measured apparent *K*\(_{d}\) values most likely reflect binding of free Mn\(^{2+}\) to the n1 site. Thus, these data do not provide direct information about the interaction of MgATP at the mutated n2 site. Rather, the data suggest that the n2 and n1 sites either interact cooperatively or that if Mn and MgATP bind in an ordered mechanism, that mutation of the n2 site effects the apparent *K*\(_{M}\) by shifting the equilibrium.

Surprisingly, substitution of Mn\(^{2+}\) for Mg\(^{2+}\) in the enzyme assay rescued the activity of the E489A and Q321A mutant enzymes. In the presence of Mn\(^{2+}\), nearly wild-type γ-GCS rates were observed with both the E489A and Q321A mutant enzymes (Table I, Fig. 5). Similarly to the wild-type enzyme, significantly lower concentrations of Mn\(^{2+}\) are required for full enzyme activation compared with Mg\(^{2+}\) and again the titration represents the effects of changes in both free Mn\(^{2+}\) and MnATP. However, higher concentrations of Mn\(^{2+}\) (20–50-fold) are required to activate the mutant enzymes compared with wild-type γ-GCS and this result is at least in part likely to be a reflection of changes in binding of MnATP to the n2 site. For the E489A mutant the activation of the enzyme by Mn\(^{2+}\) is largely a *k*\(_{cat}\) effect, as the *K*\(_{m}\) for MnATP is as significantly changed from that observed for MgATP (Table III). The *K*\(_{m}\) values for the other two substrates, l-Glu and Aba, do not differ

**Active Site Analysis of γ-Glutamylcysteine Synthetase**

**FIG. 5.** Divalent metal activation of wild-type and mutant γ-GCS. The dependence of enzyme rate on free Mn\(^{2+}\) (●) or Mg\(^{2+}\) (▼). Free metal concentration was calculated from total metal concentration using the program “Bound and Determined.” Lines represent the fit of the displayed data to the Michaelis-Menten equation. The concentrations of ATP in the assays were: A, wild-type, 2 mM; B, E93A, 5 mM; C, E489A, 5 mM; and D, Q321A, 15 mM. Wild-type data with Mn\(^{2+}\) was fit to the Hill equation (n = 2.5) as a hyperbola did not fit the data (see “Results”). The square of the correlation coefficient (*r*\(^{2}\)) for the global fit of all displayed data are greater than 0.98. For panels C and D, the insets show an expanded view of the hyperbolic enzyme rate dependence on free Mn\(^{2+}\) concentration.

11 ± 2 mM, consistent with the proposed role for the n1 metal in l-Glu binding. The rate of ATP hydrolysis catalyzed by E93A γ-GCS is activated by free Mg\(^{2+}\) but requires 12-fold higher concentrations (*K*\(^{app}\)\(_{M}\) = 6.1 ± 0.9 mM; Table I) than for the wild-type enzyme to achieve the maximal rate under saturating substrate conditions (Fig. 5). The concentration of MgATP was held constant over the range of Mg\(^{2+}\) concentrations tested, supporting the conclusion that the titration represents Mg\(^{2+}\) binding to the n1 site. E93A γ-GCS is also activated by free Mn\(^{2+}\) (*K*\(^{app}\)\(_{M}\) = 16 ± 3 μM) at similar concentrations to the wild-type enzyme.
**Active Site Analysis of γ-Glutamylcysteine Synthetase**

Values were obtained by fitting rate data to the Michaelis-Menten equation. Errors are the standard error of the fit. Data were collected with the following substrate concentrations: 10 mM L-Glu; 100 mM L-Aba; and 2, 5, and 15 mM ATP in wild-type, E489A, and Q321A γ-GCS, respectively.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ MgATP</th>
<th>$K^{app}_{M}$ L-Glu</th>
<th>$K^{app}_{M}$ L-Aba</th>
<th>$k_{cat}$ MnATP</th>
<th>$K^{app}_{M}$ MnATP</th>
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</thead>
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<tr>
<td>Wild-type</td>
<td>3.8 ± 0.1</td>
<td>0.24 ± 0.02</td>
<td>10 ± 1</td>
<td>0.071 ± 0.010</td>
<td>53,000</td>
</tr>
<tr>
<td>E55A</td>
<td>ND</td>
<td>N.D.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E93A</td>
<td>0.066 ± 0.005</td>
<td>11 ± 2</td>
<td>N.D.</td>
<td>0.32 ± 0.08</td>
<td>210</td>
</tr>
<tr>
<td>E100A</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
<td></td>
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</tr>
<tr>
<td>Q321A</td>
<td>&gt;0.24</td>
<td>1.6 ± 0.3</td>
<td>18 ± 3</td>
<td>&gt;45</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>E489A</td>
<td>0.0053 ± 0.0005</td>
<td>1.1 ± 0.1</td>
<td>15 ± 2</td>
<td>2.0 ± 0.5</td>
<td>2.7</td>
</tr>
</tbody>
</table>

$^a$ The $k_{cat}$ value for MnATP is at least 10-fold higher than $k_{cat}$ for MgATP.

$^b$ The rate for the Q321A mutant did not saturate with MgATP concentration. $k_{cat}$ is the highest observed rate at saturating metal concentration and fixed substrate concentrations.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (MnATP)</th>
<th>$K^{app}_{M}$ L-Glu</th>
<th>$K^{app}_{M}$ L-Aba</th>
<th>$k_{cat}$ MnATP</th>
<th>$K^{app}_{M}$ MnATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>2.4 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>6.0 ± 0.5</td>
<td>0.022 ± 0.001</td>
<td>110,000</td>
</tr>
<tr>
<td>E489A</td>
<td>0.89 ± 0.003</td>
<td>0.65 ± 0.01</td>
<td>9.4 ± 0.7</td>
<td>0.1 ± 0.0</td>
<td>8,100</td>
</tr>
<tr>
<td>Q321A</td>
<td>1.5 ± 0.0</td>
<td>1.1 ± 0.1</td>
<td>14 ± 2</td>
<td>1.2 ± 0.1</td>
<td>1,300</td>
</tr>
</tbody>
</table>

$^a$ The $k_{cat}$ and $K^{app}_{M}$ values were obtained by fitting the rate data to the Michaelis-Menten equation. Errors are the standard error of the fit. The reported $k_{cat}$ is the fitted parameter obtained from varying metal concentration over its dynamic concentration range. One substrate was varied over a concentration range above and below $K_m$ while the other substrates were present at fixed concentration. The fixed substrate concentrations were: L-Glu (10 mM), L-Aba (100 mM), and ATP (1 mM for wild-type enzyme and 5 mM for E489A and Q321A mutant enzymes). Free Mn$^{2+}$ concentrations were kept constant in all experiments at: 0.025 mM in wild-type enzyme; 1.7 mM in the E489A γ-GCS, and 2 mM in the Q321A enzyme.

**DISCUSSION**

The data presented in this paper provides the first important insight into the three-dimensional structure of γ-GCS and into the composition of the enzyme active site. The structural prediction that γ-GCS is a homolog of the well-characterized enzyme GS opens the way for detailed mechanistic analysis of the enzyme to understand the roles of active site residues in catalysis. The GS/γ-GCS superfamly is composed of a diverse set of sequences that made identification of the homology between the family members difficult. However, the evidence for this homology is clear and compelling. PSI-BLAST analysis using optimized sequence alignments identified the connection between the families. The secondary structural elements determined for the GS structure are predicted to be conserved in the γ-GCS family members and the key residues for metal and substrate binding or catalysis identified in GS, form motifs that are highly conserved throughout the entire GS/γ-GCS superfamily. Finally, we have confirmed that the residues predicted to form the two metal-binding sites in γ-GCS play analogous roles in catalysis and substrate binding to those described for GS.
were observed for the activation of GS with both Mg²⁺ interacts with L-Glu during the reaction. Mutation of all three ligands Glu-55, Glu-93, and Glu-100 bind the free metal that are essential for catalysis. The rate of the rate dependence on Mn²⁺ is predicted to bind two metal ions, one as MgATP. Similar results have also been described for the n2 metal ligand mutants Q321A and E93A. Unlike the effects of Mg²⁺ on the rate dependence of the reaction, the effects of Mn²⁺ may result from interactions at either or both of the two metal-binding sites, since the concentrations of both free Mn²⁺ and MnATP change during the titration of the metal. The Mn²⁺ activation of the E489A mutant enzyme is clearly a kcat effect. In contrast, activation of Q321A γ-GCS by Mn²⁺ results from the fact that the Kapp for MnATP is significantly lower than for MgATP. Differential effects on catalytic efficiency between Mn²⁺ and Mg²⁺ have also been described for the n2 metal ligand mutants in GS (37, 38) and for phosphodiesterase-5 (40). Mn²⁺ forms bidentate carboxylate complexes with ligands whereas Mg²⁺ cannot (41). In the mutant enzymes, Mn²⁺ may be able to take advantage of this type of interaction with one of the remaining carboxylate ligands to retain proper orientation for effective catalysis and substrate binding. Mg²⁺ and Mn²⁺ also differ in their diameter, polarizability, and average distance of the metal-oxygen bond (39, 41). These varied properties may produce changes in the orientation of the reacting substrates. Small perturbations that affect the overlap of the reacting orbitals in the transition state have been demonstrated to have large effects on catalysis (42).

In summary, these data are a strong first step in understanding the active site of γ-GCS. We have elucidated two metal-binding sites, termed n1 and n2, and identified their amino acid ligands. Furthermore, these studies provide insight into the roles of both metals and their ligands in substrate binding and catalysis of γ-GCS. However, other important residues...
such as acid/base catalysts or those involved in substrate binding, especially l-Cys, remain to be defined.

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Structure Prediction and Active Site Analysis of the Metal Binding Determinants in γ-Glutamylcysteine Synthetase

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