Regeneration of catalytic activity of glutamine synthetase mutants by chemical activation: Exploration of the role of arginines 339 and 359 in activity

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Abstract

In order to understand the nature of ATP and L-glutamate binding to glutamine synthetase, and the involvement of Arg 339 and Arg 359 in catalysis, these amino acids were changed to cysteine via site-directed mutagenesis. Individual mutations (Arg → Cys) at positions 339 and 359 led to a sharp drop in catalytic activity. Additionally, the K_m values for the substrates ATP and glutamate were elevated substantially above the values for wild-type (WT) enzyme. Each cysteine was in turn chemically modified to an arginine “analog” to attempt to “rescue” catalytic activity by covalent modification; 2-chloroacetamidine (CA) (producing a thioether) and 2,2'-dithiobis (acetamidine) (DTBA) (producing a disulfide) were the reagents used to effect these chemical transformations. Upon reaction with CA, both R339C and R359C mutants showed a significant regain of catalytic activity (50% and 70% of WT, respectively) and a drop in K_m value for ATP close to that for WT enzyme. With DTBA, chemically modified R339C had a *greater* k_{cat} than WT glutamine synthetase, but chemically modified R359C only regained a small amount of activity. Modification with DTBA was quantitative for each mutant and each modified enzyme had similar K_m values for both ATP and glutamate. The high catalytic activity of DTBA-modified R339C could be reversed to that of unmodified R339C by treatment with dithiothreitol, as expected for a modified enzyme containing a disulfide bond. Modification of each cysteine-containing mutant to a lysine “analog” was accomplished using 3-bromopropylamine (BPA). The R339C mutant, upon modification with BPA, had a *greater* k_{cat} than WT enzyme; however, the R359C mutant did not show significant regeneration of activity with this reagent. The data are consistent with X-ray crystallographic studies showing Arg 339 and Arg 359 at the active site of glutamine synthetase (Liaw SH, Eisenberg D, 1994, *Biochemistry* 33:675–681) interacting with ATP, glutamate, and intermediates along the catalytic pathway. Because enzyme activity could be restored for the R339C mutant by monofunctional (amine) and bifunctional (amidine) reagents, Arg 339 most likely interacts with substrates in a monodentate fashion. Conversely, Arg 359 seems to interact bifunctionally with substrates because covalent modification of R359C with BPA did not lead to a significant regain of catalytic activity.

Keywords: active site arginines; mutant enzyme reactivation; substrate binding

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Abbreviations: BPA, 3-bromopropylamine; CA, 2-chloroacetamidine; DTBA, 2,2'-dithiobis(acetamidine); DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); GS, glutamine synthetase; HEPES, *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid); PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); R339C, mutant GS with Arg 339 replaced by cysteine; R359C, mutant GS with Arg 359 replaced by cysteine; R339C-CA, R339C modified with CA; R339C-BPA, R339C modified with BPA; R339C-DTBA, R339C modified with DTBA; R359C-CA, R359C modified with CA; R359C-BPA, R359C modified with BPA; R359C-DTBA, R359C modified with DTBA; WT, wild-type glutamine synthetase.

Glutamine is one of the most important compounds in nitrogen metabolism. The enzyme glutamine synthetase catalyzes the conversion of L-glutamate to L-glutamine in the presence of ammonia and ATP (Stadtman & Ginsburg, 1974). *Escherichia coli* GS is a dodecameric enzyme with a molecular weight of ca. 52 kDa per subunit. The crystal structure (Almassy et al., 1986; Yamashita et al., 1989) of GS from *Salmonella typhimurium*, which is 95% homologous to the enzyme from *E. coli*, reveals an active site between neighboring subunits (Kinemage 1). Two residues, R339 and R359, are close to the active site and are conserved in GS obtained from various sources (Wray & Fisher, 1988). The structural and sequence data suggest that both arginine residues

may be involved in the binding of ATP and L-glutamate and possibly in catalysis.

A means of probing the functional role of amino acids in enzyme catalysis by chemical modification of mutant residues was suggested by Smith and Hartman (1988) and Lukac and Collier (1988). Smith and Hartman mutated the lysines at positions 166 and 329 of ribulosebiphosphate carboxylase/oxygenase to cysteines and found that the mutants had very low, if any, catalytic activity. Treatment of the mutant proteins with 2-bromoethylamine partially restored enzyme activity in each case, presumably due to aminoethylation of the cysteine introduced through mutation. In other studies, Lukac and Collier (1988) modified a mutant cysteine residue in endotoxin A to a glutamate analog by carboxymethylation, and Planas and Kirsch (1991) studied the catalytic lysine of aspartate aminotransferase. More recently, Wynn and Richards (1993) chemically introduced unnatural cysteine disulfides into the hydrophobic core of *E. coli* thioredoxin by mutation of individual amino acids to cysteine. Additionally, chemical modification of lysine residues (introduced by mutagenesis) to homoarginine has been employed by 2 groups to explore the role of arginine residues in protein function (Beyer et al., 1987; Engler et al., 1992). No one has reported using modification of cysteine residues to explore the functional role of arginines in enzyme catalysis.

In this paper, we explored the role of arginines in catalysis and substrate binding of *E. coli* GS by generating R339C and R359C mutants. The results indicate that there was a substantial loss of activity upon mutation. The mutant cysteines were then modified to arginine "analogs" with either 2-chloroacetamide or 2,2'-dithiobis(acetamide) (Fig. 1). These reagents introduced an amidine group into the enzyme with a $pK_a \sim 11$ (Bolhofer et al., 1979), similar to that of the guanidino group of arginine ($pK_a = 12.5$). Also, the side chains of the modified cysteine residues varied slightly in length compared to arginine, which permitted an examination of the spatial requirements of arginine residues in substrate binding and catalysis. Additionally, the cysteine residues were modified with 3-bromopropylamine to produce a lysine "analog." The latter modification allowed an examination of the "monodentate" versus "bidentate" role of each arginine residue at the active site of GS. Steady-state kinetic data with the modified mutant enzymes are reported in this paper to assess the contributions of arginines 339 and 359 to substrate binding and catalysis.

Results

Kinetic studies

Mutant enzymes R339C and R359C were successfully prepared as described in Materials and methods. Using the glutamyl transferase assay as a measure of enzyme activity, the specific activities of R339C and R359C were 1.1% and 0.6%, respectively, that of the wild-type enzyme (Table 1). A more detailed kinetic analysis was conducted using the biosynthetic assay and the data are presented in Table 2. The K_{ATP} for R339C was found to be 59 times higher than that of WT enzyme and the K_{Glu} 24 times higher. The corresponding constants for R359C were 93 and 40 times higher, respectively. The k_{cat} for R339C was 1.4% that of WT enzyme, whereas the corresponding value for R359C was 0.7%.

Fig. 1. Structures of arginine, lysine, and modified cysteines. The structures are shown in extended conformation starting from the C $_{\alpha}$ position. The groups attached to the cysteines are named according to the molecules that were used as modifying agents: CA, 2-chloroacetamide; BPA, 3-bromopropylamine; DTBA, 2,2'-dithiobis(acetamide).

Chemical modification of mutant enzymes

The R339C and R359C GS mutants were modified with three reagents: two had an amidine functionality to mimic the guanidino group of arginine, and one had a propylamine group

Table 1. Specific activity using the glutamyl transferase assay^a

Type of <i>E. coli</i> GS	Specific activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)
WT	120
R339C	1.3
R339C-CA	80
R339C-BPA	38
R339C-DTBA	32
R359C	0.6
R359C-CA	37
R359C-DTBA	9.9

^a Glutamyl transferase assay as described in Materials and methods.

Table 2. Steady-state kinetic parameters for *E. coli* glutamine synthetase in the Mn^{2+} -supported biosynthetic reaction^a

Enzyme	K_{Glu} (mM)	K_{ATP} (μ M)	k_{cat} (s^{-1})	k_{cat}/K_{Glu} ($mM^{-1} s^{-1}$)	k_{cat}/K_{ATP} ($\mu M^{-1} s^{-1}$)
WT	0.070 ± 0.005	1.5 ± 0.3	2.0 ± 0.2	28	1.3
R339C	1.7 ± 0.1	89 ± 12	0.028 ± 0.003	0.016	0.00031
R359C	2.8 ± 0.1	140 ± 6	0.013 ± 0.001	0.0043	0.000093
R339C-CA	0.15 ± 0.02	6.6 ± 0.4	0.95 ± 0.1	6.3	0.14
R359C-CA	0.59 ± 0.06	3.2 ± 0.4	1.4 ± 0.1	2.4	0.44
R339C-DTBA	3.5 ± 0.3	28 ± 1	3.6 ± 0.4	1.0	0.13
R359C-DTBA	3.7 ± 0.2	26 ± 3	0.063 ± 0.006	0.017	0.0024
R339C-BPA	2.9 ± 0.2	46 ± 3	2.3 ± 0.3	0.79	0.05

^a Assay outlined in Materials and methods.

that mimics lysine (Fig. 1). The extent of enzyme modification by each reagent was followed to the point of optimal recovery of activity, as measured by the glutamyl transferase assay (Figs. 2, 3). Multiple experiments were conducted to ensure that reproducible results were obtained before quantitation of the extent of incorporation of each reagent was attempted.

The extent of modification of each enzyme was determined using Ellman's reagent (5,5'-dithio-bis(2-nitrobenzoic acid)). Following earlier experiments, we determined that WT GS has 4 cysteine residues that do not react with DTNB unless the enzyme is denatured (Stadtman & Ginsburg, 1974). As a control experiment, treatment of the WT enzyme with 50 mM DTBA for 30 min at room temperature led to no change in the enzyme's activity. Denaturation of this enzyme sample confirmed that 4 cysteines remained unmodified. WT enzyme was also treated with 0.2 M CA and BPA under the above conditions for 24 h, and again, there was no significant change in the number of unreacted cysteine residues.

The mutant enzymes, R339C and R359C, were denatured, and 5 cysteines per subunit were available for reaction with DTNB, as expected. The mutant enzymes were reacted with CA,

BPA, and DTBA, and R339C-DTBA, R359C-CA, R359C-BPA, and R359C-DTBA had only 4 available cysteines per subunit as determined by DTNB titration after denaturation. Thus, these 3 enzymes were fully modified, i.e., 12 groups were introduced per dodecamer. The modified mutants, R339C-BPA and R339C-CA, after "full reactivation" as determined by the glutamyl transferase assay, had 9/dodecamer and 8/dodecamer of their mutant thiol groups modified, respectively. Further incorporation of reagent could not be achieved.

Reactivation

The activities of the mutant enzymes increased significantly after chemical modification for almost all combinations of mutants and reagents. An exception was the attempt to reactivate R359C with BPA. Two reactions catalyzed by GS were followed for the reactivation experiments, the glutamyl transferase assay (Table 1; Figs. 2, 3), and the biosynthetic reaction (Table 2). The glutamyl transferase assay is a measure of a partial reaction catalyzed by GS (Ginsburg et al., 1970), whereas the biosynthetic reaction is a measure of the physiological formation of gluta-

Fig. 2. Chemical modification of R339C with 2-chloroacetamidine. The reaction solutions contained 0.4 mg/mL R339C in buffer at 25 °C with other conditions as described in Materials and methods. The concentration of CA was 0.2 M and the reactivation was followed using the glutamyl transferase assay (see Materials and methods).

Fig. 3. Chemical modification of R359C with 2-chloroacetamidine. The reaction solutions contained the indicated amount of enzyme (below) in buffer at 25 °C as described in Materials and methods. Other conditions were the same as those given in the legend to Figure 2, except for the length of time. ■, 0.4 mg/mL R359C, 0.2 M CA; ●, 0.1 mg/mL WT, 50 mM CA.

mine (Meek & Villafranca, 1980). The results in Figures 2 and 3 show that reactivation of R339C and R359C with CA increases with time and that a different amount of time is required for the activity to reach a plateau for each mutant. Because the reaction of cysteine with CA is a nucleophilic displacement reaction, the different reaction times may reflect different active site environments and/or pK_a values for cysteine 339 versus 359. A control experiment with WT GS (Fig. 3) shows that the reagents do not affect "normal" catalytic activity, and the thiol titration experiments (mentioned above) demonstrate that the 4 endogenous cysteines do not react with the reagents. Thus, the reactivation phenomenon is specific for the cysteines introduced by mutagenesis. The K_m values for substrates for most modified mutant enzymes were also much closer to the WT K_m values than to the values for the unmodified forms of these enzymes (Table 2).

A substantial increase in intrinsic tryptophan fluorescence (~50%) occurs upon ATP binding to GS (Atkins et al., 1991). The 2 mutants, R339C and R359C, showed very little change in fluorescence (~4–6% increase) upon titration with ATP. For the R359C series, the fluorescence changes correlated with enzyme activity; R359C-CA showed a large fluorescence change (~30% increase), whereas R359C-DTBA underwent a small change in fluorescence (~9% increase) upon titration with ATP.

Reversible reactivation

Because reaction of cysteine mutants with DTBA leads to disulfide bond formation, addition of thiols to DTBA-activated GS should lead to removal of the thioacetamide moiety due to the thiol/disulfide exchange reaction. Indeed, treatment of R339C-DTBA ($SA = 32 \mu\text{mol min}^{-1} \text{mg}^{-1}$) and R359C-DTBA ($SA = 9.9 \mu\text{mol min}^{-1} \text{mg}^{-1}$) with dithiothreitol led to a loss of activity; the R339C mutant activity dropped to $1.3 \mu\text{mol min}^{-1} \text{mg}^{-1}$, whereas the R359C mutant activity dropped to $0.6 \mu\text{mol min}^{-1} \text{mg}^{-1}$. A significant amount of this activity loss was regained on retreatment of the reduced enzymes with DTBA. The specific activity of R339C-DTBA increased to $23.0 \mu\text{mol min}^{-1} \text{mg}^{-1}$, whereas R359C-DTBA went to $6.8 \mu\text{mol min}^{-1} \text{mg}^{-1}$. Thus, the reaction of cysteine mutants with DTBA and thiols has all of the expected characteristics of enzyme modification through formation of a disulfide bond.

Discussion

All enzymes that utilize ATP in their catalytic reactions, and for which a crystal structure is known, have either arginine or lysine (or both) in their active sites. Glutamine synthetase catalyzes an ATP-dependent ligase reaction wherein the energy available in the β, γ -phosphoryl bond of ATP is utilized to drive synthesis of glutamine from glutamate and NH_4^+ . Chemical modification experiments with GS have implicated arginine residues in ATP binding and catalysis (Powers & Riordan, 1975; Colanduoni & Villafranca, 1985), and several arginines are conserved in GS sequences reported from many sources (see Kinemage 1; Wray & Fisher, 1988). Site-directed mutagenesis can be utilized to explore the role of individual amino acid residues in substrate binding and catalysis in several ways. The most obvious is to replace a residue like arginine with one that lacks charge and/or length of side chain. A more informative method uses mutagenesis to replace an amino acid with one that retains a

functional group, e.g., cysteine, so that chemical modification can be used to attempt to "rescue" activity by adding back chemical fragments that have functional groups. With this method, charge requirements as well as length of side chain can be investigated. The first reported application of this method was in the study of the function of a lysine residue in the reaction catalyzed by ribulosebisphosphate carboxylase/oxygenase (Smith & Hartman, 1988). In the present paper, we introduce a set of reagents to explore the function of arginine residues in GS.

The data reported in the tables and figures demonstrate that for 2 conserved arginine residues, R339 and R359, low levels of activity are observed upon replacement of arginine with cysteine. With these 2 mutant enzymes, both negatively charged substrates, ATP and glutamate, have elevated K_m values indicating a loss in the ability of GS to bind these molecules. Of course, K_m is a complex kinetic constant that reflects both binding and catalytic steps, so the interpretation that binding alone is attenuated by mutation must be viewed with caution. Nonetheless, binding of substrates (K_d) is a substantial fraction of K_m (Abell & Villafranca, 1991) for GS so part or all of the changes in K_m values reported in Table 2 can be attributed to be changes in binding interactions between side chains and substrates.

The activity of the GS mutants could be "rescued" by treatment with amidine-containing reagents that covalently react with the cysteine residues producing a positively charged "surrogate" for the guanidino group of arginine. The critical control experiments showed that the WT enzymes were unaffected by this chemical modification process. This fact coupled with the increase in activity of the mutant enzymes after chemical modification indicate that the cysteines being modified are indeed the mutated cysteines.

Modified mutant enzymes show increased k_{cat} and K_m values for substrates that approach the WT values (Table 2). For R339C, the unmodified enzyme has a $(K_{\text{Glu}})^{\text{mutant}}/(K_{\text{Glu}})^{\text{WT}}$ ratio of 24 and a $(K_{\text{ATP}})^{\text{mutant}}/(K_{\text{ATP}})^{\text{WT}}$ ratio of 60. Upon modification with CA, the ratios go down to 2 and 4, respectively, clearly demonstrating a regeneration of binding ability in the modified mutant enzyme. The k_{cat} value of the unmodified R339C is 1.4% that of the WT enzyme and modification with CA restores the k_{cat} to 43% that of the WT enzyme.

Figure 1 shows a schematic drawing of the extended side chains of arginine, lysine, and the modified cysteine residues. The lengths are calculated from the C_α carbon to the atom indicated by the bar. Addition of the CA or the DTBA group to cysteine gives a shorter or longer side chain, respectively, than that of arginine. For the R339C modified mutants, the difference in side-chain length produces measurable differences in all kinetic parameters. However, the CA and DTBA derivatives of the R359C mutant give enzymes with dramatic differences in k_{cat} values and modest differences in K_m values. Thus, the length of side chain at position 359 plays a significant role in the proper positioning of the substrates in the active site, but is an even more important determinant in catalysis.

During the reaction catalyzed by GS, the γ -phosphoryl group of ATP is transferred to the γ -carboxylate of glutamate; P_i is subsequently released after attack by ammonia on γ -glutamyl phosphate. Liaw and Eisenberg (1994) have recently crystallized several enzyme substrate and inhibitor complexes of GS. The structures reveal that 1 of the 2 guanidino nitrogens on R339 is in proximity to the γ -phosphoryl group that undergoes transfer between ATP and glutamate. Also, both guanidino nitrogens

of R359 are involved in substrate binding with interaction to the γ -carboxylate of glutamate throughout the reaction sequence; additional binding is observed to the γ -phosphoryl group undergoing transfer as described above. Overall, the crystal structure of GS complexes shows that both arginine 339 and 359 are intimately involved in binding the substrates ATP and glutamate, as well as the intermediates in the reaction sequence.

Because the crystal structures indicate that only one of the nitrogens on R339 is involved in substrate binding and electrostatic stabilization, the modification of R339C with both "bidentate" arginine and "monodentate" lysine analogs should lead to substantial recovery of activity. This is exactly what is observed in the kinetic studies. Conversely, because both nitrogens from R359 are engaged in electrostatic stabilization, an arginine analog introduced into R359C should show activity, whereas a lysine analog is expected to give less activity. This proposal is borne out by the observation that R359C is not reactivated by BPA, but it does show an increase in activity when modified by the arginine analogs.

In sum, the experiments described in this paper provide another tool with which to examine the role of individual amino acid side chains in an enzyme in both substrate binding and catalytic function. Through a combination of mutagenesis and chemical reactivation, different functional groups can be introduced at one amino acid position to interrogate the role of individual atoms in binding and catalysis.

Materials and methods

All restriction enzymes and other enzymes used in cloning and mutagenesis were purchased from New England Biolabs. [^{35}S - α -thio]ATP for sequencing was purchased from New England Nuclear Research Products. Oligonucleotides were purchased from National Biosciences. Oligonucleotides had no protecting trityl groups and were used without further purification. BPA was purchased from Sigma Chemical Company and used without further purification. CA and DTBA were synthesized according to published procedures (Schaefer & Peters, 1961; Westland et al., 1972).

R339C and R359C were generated using the method of Sayers et al. (1988) with a mutagenesis kit from Amersham Corporation. The following oligonucleotides were used: 5'-GCGCGTACCTGTTTCGCGTCTATC-3' for R339C and 5'-ATCGAAGTAIGTTTCCCGGACCCGGCA-3' for R359C. (The underlined letters represent the bases that were mutated.) To allow screening with restriction digests, silent mutations were introduced in the oligos to create an *Aha* II site (in the R339C oligo) and to remove a *Bam* HI site (from the R359C oligo). The vector for mutagenesis, pTZ-glnA was created by cloning the *Sal* I fragment from pglN35 (Backman et al., 1981) into pTZ18R, a phagemid vector capable of producing single-stranded DNA (Viera & Messing, 1987). Screening of the mutants was done by restriction enzyme digests followed by sequencing of the double-stranded mutant DNA.

Each fragment containing a mutation was subcloned into pglN35, and the corresponding mutant glnA gene was expressed to produce mutant GS. The clonings used *Bsu* 361 and *Bam* HI sites for R339C and *Sal* I for R359C. The mutations were confirmed by sequencing mutant pglN35 DNA before enzyme preparation. The pglN35 plasmid was transformed into the *E. coli* YMC21E cell strain, which has a deletion of the glnA gene. This

strain also lacks the glnE gene, which encodes for the enzyme ATP:*E. coli* GS adenylyltransferase. Hence, enzyme preparations provided the unadenylylated forms of mutant GS. The unadenylylated enzyme is the physiologically active form with Mg^{2+} (Ginsburg et al., 1970).

Cells were grown under sterile conditions. An overnight culture (37 °C) was grown with 9 mL of LB medium, 1 mL of 2 mM L-glutamine, 32 μL each of 25 mg mL^{-1} kanamycin and ampicillin, and about 50 μL of cells. This was used to inoculate 0.5 L minimal medium containing 400 mL aqueous solution with KH_2PO_4 (30 mM), K_2HPO_4 (30 mM), $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (17 mM), and MgSO_4 (1.7 mM) at pH 7.5; 50 mL of 5% dextrose (aqueous); 50 mL of aqueous solution with L-glutamine (5 mM), thiamine hydrochloride (1 $\mu\text{g mL}^{-1}$), and ampicillin (80 $\mu\text{g mL}^{-1}$); and 0.5 mL of 1 M MnCl_2 . The cells were grown in a shaker-incubator at 30 °C to an OD_{660} of 0.9–1. This solution was used to inoculate 8 L of minimal medium (with the same composition). Growth was continued at 30 °C till the OD_{660} reached 0.8–0.9, at which time the cells were harvested by centrifugation (6,000 rpm, 30 min) and after quantification, stored at –70 °C.

The isolation and purification of mutant GS was performed based on the procedure of Roseman and Levine (1987). The cells were suspended in a buffer containing 10 mM imidazole (pH 7.0), 50 mM MgCl_2 , and 1 mM MnCl_2 and were disrupted by sonication. Nucleic acids were precipitated with 20 mg mL^{-1} streptomycin sulfate (pH 5.85, 4 °C). GS was selectively precipitated with ZnSO_4 (pH 7.2, 37 °C, final Zn^{2+} concentration of 2.5 mM). The enzyme was resuspended in and dialyzed (3 $\times$) against a buffer containing 10 mM imidazole (pH 7.2), 100 mM KCl, 10 mM EDTA, and 20 mM MgCl_2 , followed by dialysis (3 $\times$) into a buffer containing 10 mM imidazole (pH 7.2), 100 mM KCl, and 10 mM MnCl_2 . The solution pH was now adjusted to 5.7 and the enzyme precipitated with acetone (4 °C, to a final concentration of 45% acetone, v/v). The precipitate was resuspended in a buffer containing 10 mM imidazole (pH 7.2), 100 mM KCl, and 1 mM MnCl_2 , and GS was precipitated by adding $(\text{NH}_4)_2\text{SO}_4$ (to 10%, w/v) and adjusting the pH to 4.4. The precipitated enzyme was resuspended in a buffer containing 10 mM imidazole (pH 7.2), 100 mM KCl, and 1 mM MnCl_2 and dialyzed (3 $\times$) against the same. The enzyme was stored in this buffer.

Enzyme concentrations were determined using the BCA Protein Assay kit from Pierce. Steady-state kinetic assays (biosynthetic activity) on the enzyme were performed according to the procedure of Meek and Villafranca (1980). The assays were based on the coupling reactions of the enzymes pyruvate kinase and lactic dehydrogenase, both obtained from Sigma Chemical Company. Each assay mixture contained 50 mM PIPES (pH 6.5), 100 mM KCl, 1 mM NH_4Cl , 1 mM phosphoenolpyruvate, 0.19 mM NADH, 66 μg each of the coupling enzymes, and 0.04 mg of GS. The decrease of A_{340} due to NADH consumption was monitored on a Cary 2200 spectrophotometer. After the metal ion (Mn^{2+}) concentration was optimized, profiles of reaction rate were obtained using varying concentrations of either L-glutamate or ATP while keeping the other saturating. For the γ -glutamyl transferase assay, the reaction mixture (24 mL) contained 50 mM HEPES (pH 7.5), 100 mM KCl, 42 mM $\text{NH}_2\text{OH} \cdot \text{HCl}$, 0.42 mM Na_2ADP (pH 7.0), 21 mM KH_2AsO_4 , and 150 mM L-glutamine. The pH of this reaction mixture was adjusted to 7.58 and 10 μL of 1 M MnCl_2 was added (final concentration of Mn^{2+} is 0.42 mM). Stop solution was prepared with $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (20 g), trichloroacetic acid (12 g), and 6 N

HCl (25 mL) made up to a final volume of 600 mL with distilled water. Aliquots (0.48 mL) of the reaction mixture were preincubated at 37 °C. GS (20 µL containing 0.6–0.8 µg) was added and the solution incubated at 37 °C for 20–30 min. Stop solution (2.0 mL) was added, and following vortexing, the absorbance at 540 nm was measured; a blank solution contained everything except the enzyme.

Chemical modifications were accomplished at room temperature. Each enzyme sample was partially reduced using 1 mM dithiothreitol. After removal of excess dithiothreitol on a Sephadex G-25 column, enzyme (0.1–0.4 mg/mL) in 10 mM imidazole, pH 7.2, 100 mM KCl, 1 mM MnCl₂, was treated with 0.1–0.2 M of CA, BPA, or DTBA in the same buffer as the enzyme. Reactivation of the enzyme was followed by monitoring the glutamyl transferase assay at 37 °C (Ginsburg et al., 1970). After maximal reactivation was reached (see Figs. 2, 3), excess reagent was removed on a Sephadex G-25 column and the enzyme was used for steady-state kinetic studies.

The number of thiol groups in each enzyme was determined using DTNB (Ellman, 1959). Each enzyme (approximately 6 µM) in 10 mM Tris (pH 8.5), 2 mM EDTA, and 6 M guanidine was treated with 10 µL of 10 mM DTNB in 10 mM Tris (pH 8.5) and kept at room temperature for 10 min, after which the absorbance at 412 nm was measured. The concentration of thiol groups was calculated using an extinction coefficient of 13,600. Each determination had ±5% error.

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References

- Abell LM, Villafranca JJ. 1991. Effect of metal ions and adenylation state on the internal thermodynamics of phosphoryl transfer in the *Escherichia coli* glutamine synthetase reaction. *Biochemistry* 30:1413–1418.
- Almassy RJ, Janson CA, Hamlin R, Xuong NH, Eisenberg D. 1986. Novel subunit-subunit interactions in the structure of glutamine synthetase. *Nature (Lond)* 323:304–309.
- Atkins WM, Stayton PS, Villafranca JJ. 1991. Time-resolved fluorescence studies of genetically engineered *Escherichia coli* glutamine synthetase. Effects of ATP on the tryptophan-57 loop. *Biochemistry* 30:3406–3416.
- Backman K, Chen YM, Magasanik B. 1981. Physical and genetic characterization of the *glnA-glnG* region of the *Escherichia coli* chromosome. *Proc Natl Acad Sci USA* 78:3743–3747.
- Beyer WF Jr, Fridovich I, Mullenbach GT, Hallewell R. 1987. Examination of the role of arginine-143 in the human copper and zinc superoxide dismutase by site-specific mutagenesis. *J Biol Chem* 262:11182–11186.
- Bolhofer WA, Habecker CN, Pietruszkiewicz AM, Torchiana ML, Jacoby HI, Stone CA. 1979. 2-Mercaptoacetamidines as gastric antisecretory agents. *J Med Chem* 22:295–301.
- Colandruoni JA, Villafranca JJ. 1985. Labeling of a specific arginine residue at the active site of glutamine synthetase (*E. coli*). *Biochem Biophys Res Commun* 126:412–418.
- Ellman GL. 1959. Tissue sulfhydryl groups. *Arch Biochem Biophys* 82:70–77.
- Engler DA, Campion SR, Hauser MR, Cook JS, Niyogi SK. 1992. Critical functional requirement for the guanidinium group of the arginine 41 side chain of human epidermal growth factor as revealed by mutagenic inactivation and chemical reactivation. *J Biol Chem* 267:2274–2281.
- Ginsburg A, Yeh J, Henning SB, Denton MD. 1970. Some effects of adenylation on the biosynthetic properties of the glutamine synthetase from *Escherichia coli*. *Biochemistry* 9:633–648.
- Liaw SH, Eisenberg D. 1994. A structural model for the reaction mechanism of glutamine synthetase, based on five crystal structures of enzyme-substrate complexes. *Biochemistry* 33:675–681.
- Lukac M, Collier RJ. 1988. Restoration of enzymatic activity and cytotoxicity of mutant, E553C, *Pseudomonas aeruginosa* exotoxin A by reaction with iodoacetic acid. *J Biol Chem* 263:6146–6149.
- Meek TD, Villafranca JJ. 1980. Kinetic mechanism of *Escherichia coli* glutamine synthetase. *Biochemistry* 19:5513–5519.
- Planas A, Kirsch JF. 1991. Reengineering the catalytic lysine of aspartate aminotransferase by chemical elaboration of a genetically engineered cysteine. *Biochemistry* 30:8268–8276.
- Powers SG, Riordan JF. 1975. Functional arginyl residues as ATP binding sites of glutamine synthetase and carbamyl phosphate synthetase. *Proc Natl Acad Sci USA* 72:2616–2620.
- Roseman JE, Levine RL. 1987. Purification of a protease from *Escherichia coli* with specificity for oxidized glutamine synthetase. *J Biol Chem* 262:2101–2110.
- Sayers JR, Schmidt W, Eckstein F. 1988. 5'-3' Exonucleases in phosphothiorate-based oligonucleotide-directed mutagenesis. *Nucleic Acids Res* 16:791–802.
- Schaefer FC, Peters GA. 1961. Base catalyzed reaction of nitriles with alcohols. A convenient route to imidates and amidine salts. *J Org Chem* 26:412–418.
- Smith HB, Hartman FC. 1988. Restoration of activity to catalytically deficient mutants of ribulosephosphate carboxylase/oxygenase by aminoethylation. *J Biol Chem* 263:4921–4925.
- Stadtman ER, Ginsburg A. 1974. The glutamine synthetase of *Escherichia coli*: Structure and control. *Enzymes* 10:755–807.
- Viera J, Messing J. 1987. Production of single-stranded plasmid DNA. *Methods Enzymol* 153:3–11.
- Westland RD, Merz MM, Alexander SM, Newton LS, Bauer L, Conway TT, Barton JM, Khullar KK, Devdhar PB, Grenan MM. 1972. 2-Mercaptoacetamidines and derivatives as antiradiation agents. *J Med Chem* 15:1313–1321.
- Wray LV, Fisher SH. 1988. Cloning and nucleotide sequence of the *Streptomyces coelicolor* gene encoding glutamine synthetase. *Gene* 71:247–256.
- Wynn R, Richards FM. 1993. Unnatural amino acid packing mutants of *Escherichia coli* thioredoxin produced by combined mutagenesis/chemical modification techniques. *Protein Sci* 2:395–403.
- Yamashita MM, Almassy RJ, Janson CA, Cascio D, Eisenberg D. 1989. Refined atomic model of glutamine synthetase at 3.5 Å resolution. *J Biol Chem* 264:17681–17690.