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LABELING OF A SPECIFIC ARGININE RESIDUE AT THE ACTIVE SITE OF GLUTAMINE SYNTHETASE (E.coli)*

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SUMMARY: Chemical modification of a specific arginine residue of Escherichia coli glutamine synthetase has been accomplished by the use of the arginine-specific reagents p-hydroxyphenylglyoxal, phenylglyoxal, and methylglyoxal. Modification of one arginine residue results in complete inactivation of the enzyme and the modified enzyme seems to be extremely stable since no reactivation is observed upon addition of free arginine or dialysis. Saturating levels of ATP but not L-glutamate, L-methionine sulfoximine, or inorganic phosphate provide substantial protection against inactivation of the enzyme suggesting the modified amino acid is at or near the ATP substrate binding site. However, an ATP affinity analog is not prevented from binding upon modification of the arginine residue indicating that the reduction in catalytic activity is not solely due to alteration in substrate binding but may also reflect a catalytic role for the arginine residue.

Glutamine synthetase [EC 6.3.1.2.] from Escherichia coli catalyzes the following reaction.

$$\text{Glutamate} + \text{ATP} + NH_3 \longrightarrow \text{Glutamine} + \text{ADP} + P_i$$

The enzyme has a molecular weight of 600,000 and is composed of twelve identical subunits (1). The enzyme exists in two forms, adenylylated, and unadenylylated. Adenylylation has been found to be a regulatory mechanism involving a cascade system where activity is halted upon covalent modification by ATP and a specific enzyme (ATase). The form of the enzyme used in this study was the unadenylylated form which is active with Mg^{2+} and is believed to be the normal biologically active form. The adenylylated enzyme which is active in the presence of Mn^{2+} is completely inactive with Mg^{2+} (1).

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The enzyme has been proposed to go through a γ-glutamyl phosphate intermediate followed by formation of a tetrahedral adduct when ammonia adds to the activated carbonyl group (2,3). However not much is known about the enzyme groups involved in catalysis and or binding at the active site of glutamine synthetase. Many enzymes with anionic substrates such as ATP have been found to have an arginine playing a role in catalysis and Riordon's group found that three arginines can be labeled with phenylglyoxal in the sheep brain glutamine synthetase (4). It is the purpose of this study to determine if any arginine residues are involved at the active site of glutamine synthetase from E. coli and to characterize the substrate binding of the modified enzyme.

MATERIALS AND METHODS

Glutamine synthetase was prepared from E. coli cells grown in a nitrogen limiting medium. The method for isolating the enzyme from the cells followed the procedure of Miller, Shelton and Stadtman (5). This procedure utilizes the ability to precipitate glutamine synthetase with Zn^{2+}. The adenylylation state and concentration of the enzyme were determined spectrophotometrically. The adenylylation state was determined to be 2.7 (1).

All nucleotides, other enzymes, substrates, phenylglyoxal, methylglyoxal, and buffers were purchased from Signma Chemical Co. p-Hydroxyphenylglyoxal and camphorquinone-10-sulfonate were purchased from Pierce Chemical Co.

The synthesis of 5'-p-fluorosulfonylbenzoyladenosine (5'-p-FSO_2BzAd) was performed using Colman's procedure (6).

The biosynthetic activity was monitored using the lactate dehydrogenase and pyruvate kinase coupling system as described in earlier work (3). Where saturated the substrates were 50 mM NH_4Cl, 200 mM L-glutamine, and 2 mM ATP. Assays were performed in 1.0 cm cuvettes with 1.00 mL total volume in a Beckman DU spectrophotometer equipped with a Gilford attachment at 25°C at 339 nm. Assays were initiated with 10 μL aliquots containing 1.5 to 10 μg of glutamine synthetase.

Incubations with the methylglyoxal, phenylglyoxal, p-hydroxyphenylglyoxal, and camphorquinone-10-sulfonate were performed at 25°C with 3.2-100 μM glutamine synthetase in 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) (pH 7.50), 100 mM KCl, and 15 mM $MgCl_2$. Aliquots were taken at different time periods and checked for activity. The stoichiometry experiments with p-hydroxyphenylglyoxal were performed in the same manner except that aliquots were quenched and unreacted inhibitors were removed by the Penefsky method (7). The stoichiometry with p-hydroxyphenylglyoxal was determined spectrophotometrically with a molar absorbtivity of 8825 $M^{-1}cm^{-1}$ determined at pH 7.50 in our system after gel permeaton chromatography.

Incubations of glutamine synthetase (5-200 μM) with 1.0 mM of 5'-p-FSO_2BzAd were performed in 10% DMF 50 mM Hepes (pH 7.50), 15 mM $MgCl_2$, 100 mM KCl at 25°C for six hours. The stoichiometry of the reaction was determined from equation 1 allowing for the adenylylation state of the enzyme.

A specific amino acid segment of the enzyme was removed by trypsin treatment by the addition of 1% (w/w) trypsin to 10-102 μM (.5-5.0 mg/ml) enzyme in 50 mM

Hepes (pH 7.0), 100 mM KCl, and 10 mM $MnCl_2$. The enzyme activity was checked by taking aliquots out of the incubation mixture. After inactivation from proteolysis was complete a two-fold excess of trypsin inhibitor was added to destroy the protease activity. The mixture was then passed down a G-120 Sephadex column equilibrated with 50 mM Hepes (pH 7.50, 100 mM KCl, and 15 mM $MgCl_2$. The residual enzyme activity was then checked with the standard biosynthetic activity except the glutamate level was 200 mM and the ATP level was 15 mM.

Fluorescence titrations of the perturbation of tryptophan residues due to substrate and inhibitor binding were performed on a Perkin Elmer Model MPF-44B fluorescence spectrophotometer at 25°C. The buffer used was 50 mM Hepes (pH 7.50), 100 mM KCl, and 15 mM $MgCl_2$. The excitation wavelength was 300 nm in order to only excite tryptophan residues. The emmision wavelength observed was 336 nm. The titrations were performed in 1 cm quartz fluorescence cuvettes. Glutamine synthetase was kept at 4 to 6.5 μM. Titrant was added to the enzyme or initial enzyme ligand complex with an adjustable micropipette and mixed. Enzyme or initial enzyme ligand complex was kept at the same concentration in the titrant as well as in the cuvette to keep the enzyme concentration constant and eliminate problems due to dilution. Titrant solutions were prepared by adjusting the ligand concentration to saturating levels. Then the ligand to be varied was added in aliquots to the cuvette.

RESULTS AND DISCUSSION

Four compounds that are potential modifying reagents for arginine residues were tested with glutamine synthetase. They were methylglyoxal, phenylglyoxal, p-hydroxyphenylglyoxal, and camphorquinone-10-sulfonate. All inhibited the enzyme except camphorquinone-10-sulfonate which did not inhibit at up to 100 mM from pH 6.7-9. The first order plots of activity versus time are shown in Figure 1. The data for phenylglyoxal and p-hydroxyphenylglyoxal are biphasic. The possibility that the reagent was unstable in the incubation solution was tested by incubating the compound alone for 6 hours and then adding the enzyme. Identical inactivation rates as those shown in Figure 1 were obtained following this procedure.

Since the inhibition was biphasic, enzyme was incubated with 4 mM phenylglyoxal for 5 minutes and then the incubation mixture was quenched with 130 mM L-arginine. Upon quenching, the activity of the enzyme remained constant for over two hours. The V_{max} value for this partially inactivated enzyme was 65 ± 3% the native enzyme's activity. However the K_m values were the same for the modified and native enzyme (71 μM for ATP and 3.3 mM for L-glutamate). Therefore the biphasic inactivation was not due to multiple site modification or cooperativity between subunits.

ATP at saturating concentrations (5 mM) was found to be the best compound to protect the enzyme against inactivation (Figure 2). Saturating levels of

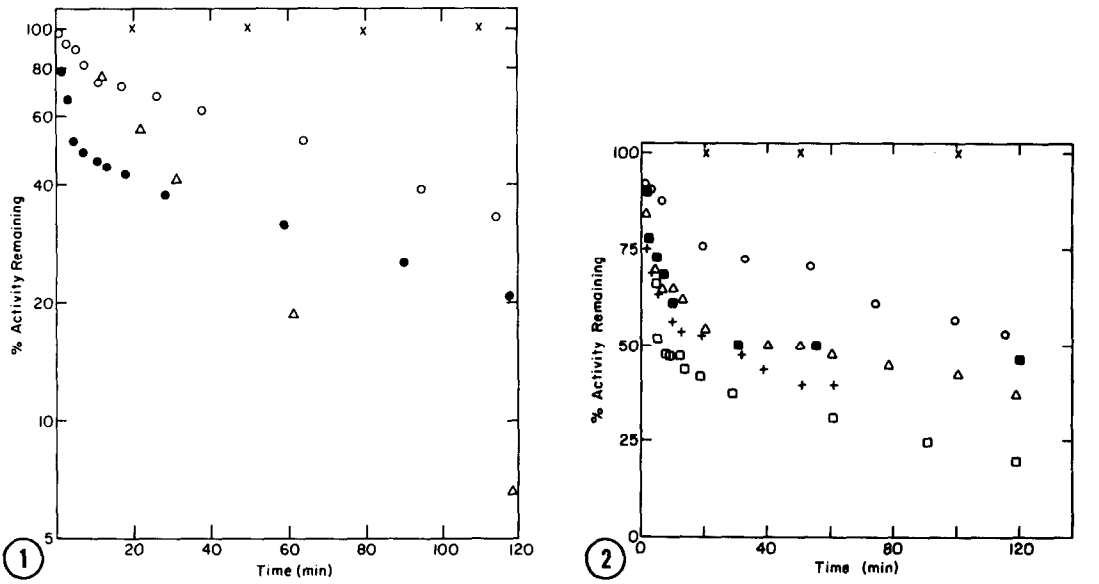
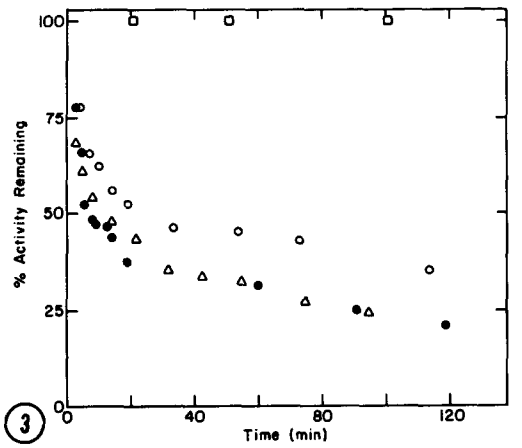


Figure 1. Time course of inhibition of glutamine synthetase by the following arginine specific reagents, 4 mM phenylglyoxal (●), 4 mM p-hydroxyphenylglyoxal (○), 36 mM methylglyoxal (△), and no inactivator present (×). See materials and methods for additional experimental details.

Figure 2. Time course of inactivation by 4 mM phenylglyoxal containing 5 mM ATP (○), 100 mM L-glutamate (△), 10 mM L-MSOX (+), 50 mM inorganic phosphate (■), phenylglyoxal only (□), and no phenylglyoxal present (×). See materials and methods for additional experimental details.

L-glutamate (100 mM), L-methionine-(RS)-sulfoximine (10 mM) (a transition state analog), and inorganic phosphate (50 mM) were found to be much less effective at protecting the enzyme against inactivation. These daia imply that ATP is near the arginine group being modified but ATP did not completely prevent inactivation by phenylglyoxal.

High concentrations of magnesium were found to increase inactivation by phenylglyoxal (Figure 3). At low magnesium (1.5 mM) the inhibition is much slower than at 15 mM or 100 mM concentrations. The enzyme has two divalent metal ion sites with the first site having the K_d of 50 μM for Mg^{2+}, (8) so at 1.5 mM this site would be saturated. The second metal ion site binds Mg^{2+} very weakly in the absence of ATP and the binding constant was estimated by kinetic experiments. Free Mg^{2+} exhibited competitive behaviour with ATP as the varied substrate in steady state kinetic experiments and a K_I of 50 mM for Mg^{2+} was determined. Therefore population of the second metal ion binding site could result in the arginine group being more accessible to the modifying reagent.



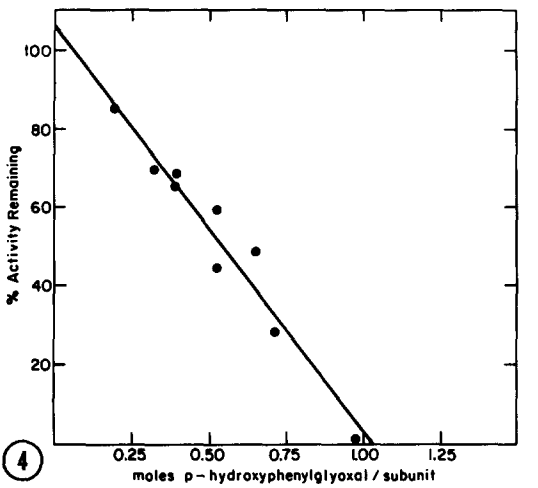


Figure 3. Time course of inactivation by 4 mM phenylglyoxal containing 1.5 mM $MgCl_2$ (○), 15 mM $MgCl_2$ (△), 100 mM $MgCl_2$ (●), and no phenylglyoxal present with 1.5 mM $MgCl_2$ (□). See materials and methods for additional details.

Figure 4. Plot of incorporation of p-hydroxyphenylglyoxal versus % activity remaining. The line drawn was determined by a least squares analysis of the data. See materials and methods for additional details.

The stoichiometry of incorporation of p-hydroxyphenylglyoxal was determined and shows that only one arginine residue has to be modified for complete inactivation of the enzyme (Figure 4). At longer incubation times (48 hours) with this reagent (4 mM) the stoichiometry ranges from 2.2 to 2.65 molecules per monomer of enzyme. This additional labeling results from other groups reacting at much slower rates than the arginine which is implicated in catalysis.

It is slightly unusual that modification of the group required for catalysis is not reversed at pH 7.50 after excess reagent has been removed. However dialysis for up to 48 hours in the same buffer as used in the incubation experiments does not lead to reactivation. The inhibitor-arginine complex must be excluded from solution or must be stabilized in some way.

Fluorescence titrations of phenylglyoxal-modified enzyme with ATP and L-glutamate do not show the fluorescence changes that occur with unmodified enzyme (9). L-methionine-(RS)-sulfoximine also does not produce a fluorescence change with the enzyme as compared to the native enzyme. Normally all give substantial fluorescence changes upon binding (9). Either the enzyme does not undergo the

same conformational change which results in the normal fluorescence change with these compounds or the modified enzyme does not bind these compounds at all.

In order to determine if ATP could bind to the enzyme modified with phenylglyoxal, 5'-p-FSO_2BzAd was added to previously inactivated enzyme. A stoichiometry of 1.02 molecules of this ATP affinity label was determined to be bound per monomer after inactivation by phenylglyoxal. This affinity label has been shown to label the ATP substrate site of the native enzyme and covalently bind to a lysine group (10). Evidently ATP or at least molecules with an adenosine moiety can still bind to the inactive enzyme. Therefore the enzyme still probably binds ATP. The modified enzyme may be inactive because one of the groups involved in catalysis, i.e. the arginine, is not available or because the substrates cannot bind in their proper conformations.

Enzyme which has undergone limited proteolysis by trypsin was labeled with p-hydroxyphenylglyoxal. Trypsin proteolysis under these conditions has been found to remove a peptide with 15 amino acid residues including one arginine (11). This peptide is thought to be part of the active site. The "clipped" enzyme with the small peptide removed by gel filtration and a sample of native enzyme were modified by incubation with 4 mM p-hydroxyphenylglyoxal for 8 hours. The native enzyme retained less than 1% of its original activity and was labeled with 0.95 molecules of p-hydroxyphenylglyoxal per monomer. The clipped enzyme was labeled with 1.01 molecules of the inhibitor per monomer. Evidently this peptide does not contain the arginine which is associated with the inactivation of glutamine synthetase.

The evidence here shows that the arginine involved in the inactivation by these arginine specific reagents is probably adjacent to the ATP binding site. Its role is possibly to neutralize some of the negative charge of the phosphate groups of ATP and perhaps to align the phosphate groups for catalysis. Alternatively, it could be involved in a proton transfer step in the enzymatic mechanism. The enzyme has been reported to exhibit slow conformational changes upon substrate, inhibitor, and product binding. The absence of any observable fluorescence changes due to perturbations of a tryptophan residue near the active

site along with the fact that p-FSO_2BzAd still labels the enzyme tends to show that ATP and possibly the other ligands can still bind but not in the normal manner. By kinetic experiments ATP has also been shown to be the first substrate to bind (12), and by not allowing the first substrate to bind correctly, inhibition of enzyme activity could result. The fact that magnesium increases inactivation by binding at the second metal ion site also indicates that the arginine is in the vicinity of these phosphate groups since the second metal ion site is the nucleotide metal ion site.

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