Labeling of Specific Lysine Residues at the Active Site of Glutamine Synthetase*

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Glutamine synthetase (Escherichia coli) was incubated with three different reagents that react with lysine residues, viz. pyridoxal phosphate, 5'-p-fluorosulfonylbenzoyladenosine, and thiourea dioxide. The latter reagent reacts with the ε-nitrogen of lysine to produce homoarginine as shown by amino acid analysis, nmr, and mass spectral analysis of the products. A variety of differential labeling experiments were conducted with the above three reagents to label specific lysine residues. Thus pyridoxal phosphate was found to modify 2 lysine residues leading to an alteration of catalytic activity. At least 1 lysine residue has been reported previously to be modified by pyridoxal phosphate at the active site of glutamine synthetase (Whitley, E. J., and Ginsburg, A. (1978) J. Biol. Chem. 253, 7017-7025). By varying the pH and buffer, one or both residues could be modified. One of these lysine residues was associated with ~81% loss in activity after modification while modification of the second lysine residue led to complete inactivation of the enzyme. This second lysine was found to be the residue which reacted specifically with the ATP affinity label 5'-p-fluorosulfonylbenzoyladenosine. Lys-47 has been previously identified as the residue that reacts with this reagent (Pinkofsky, H. B., Ginsburg, A., Reardon, L., Heinrikson, R. L. (1984) J. Biol. Chem. 259, 9616-9622; Foster, W. B., Griffith, M. J., and Kingdon, H. S. (1981) J. Biol. Chem. 256, 882-886). Thiourea dioxide inactivated glutamine synthetase with total loss of activity and concomitant modification of a single lysine residue. The modified amino acid was identified as homoarginine by amino acid analysis. The lysine residue modified by thiourea dioxide was established by differential labeling experiments to be the same residue associated with the 81% partial loss of activity upon pyridoxal phosphate inactivation. Inactivation with either thiourea dioxide or pyridoxal phosphate did not affect ATP binding but glutamate binding was weakened. The glutamate site was implicated as the site of thiourea dioxide modification based on protection against inactivation by saturating levels of glutamate. Glutamate also protected against pyridoxal phosphate labeling of the lysine consistent with this residue being the common site of reaction with thiourea dioxide and pyridoxal phosphate.

Glutamine synthetase from Escherichia coli catalyzes the following reaction.

\[
\text{Glutamate} + \text{ATP} + \text{NH}_2 \xrightleftharpoons{Mg^+} \text{glutamine} + \text{ADP} + P_i
\]

The enzyme is composed of 12 identical subunits of \( M_r = 50,000 \) (1) and exists in two forms, adenylated and unadenylated. The enzymatic mechanism has been proposed to proceed through formation of a γ-glutamyl phosphate intermediate to facilitate the next step of the reaction, i.e. NH₂ attack on the carbonyl carbon to produce glutamine (2–5). However, little is known about the groups involved in catalysis and/or binding at the active site of glutamine synthetase but several recent studies have indicated that lysine residues are present at the active site (6–8).

Powers and Riordan (9) found one reactive arginine residue at the ATP site of sheep brain glutamine synthetase. A recent study in our laboratory identified 1 arginine at the ATP site of the Escherichia coli enzyme which was not necessary for ATP binding. Modification of this residue resulted in inactivation of the enzyme, however (10). Pinkofsky et al. (7) found that the ATP analog 5'-p-fluorosulfonylbenzoyladenosine (5'-p-FSO₃BzAdo) is covalently bound to the enzyme through a lysine residue where 5'-p-FSO₃BzAdo was previously found to bind to the ATP site of the enzyme (6). Whitley and Ginsburg (8) also found that 5 lysine residues could be labeled with pyridoxal phosphate and that one or more may play a role in binding or catalysis. Based in part on previous work mentioned above, we designed experiments to modify and characterize amino acid residues on the enzyme that are potentially at the active site and to examine a new lysine modifying reagent, thiourea dioxide, that is a possible mimic of the γ-glutamyl phosphate intermediate in the enzymatic reaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glutamine synthetase was prepared from E. coli grown in a nitrogen limiting medium. The method for isolating the enzyme from the cells followed the procedure of Miller et al. (11) which takes advantage of the property that glutamine synthetase can be precipitated by Triton X-100.

1 The abbreviations used are: 5'-p-FSO₃BzAdo, 5'-p-fluorosulfonylbenzoyladenosine; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Chex, 3-cyclohexyamin-1-ethanesulfonic acid; TEA, triethanolamine.

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tated with Zn²⁺. The concentration of glutamine synthetase was determined by its UV spectrum. The adenylateylation state of the enzyme was determined spectrophotometrically to be 2.7 using the relationship in Equation 1.

\[ A = 15.3(A_{290}-A_{280}) - 13.6 \] (1)

All nucleotides, other enzymes, substrates, buffers, and inhibitors were purchased from Sigma. [¹³C]Thiourea was purchased from ICN Radiochemicals.

A molecular weight of 56,000 was used for bovine liver glutamate dehydrogenase and the enzyme concentration was determined spectrophotometrically at 278 nm using a molar absorbtivity of 48,000 M⁻¹ cm⁻¹ (12).

5'-p-FSO₂BuAdo was synthesized according to Wyatt and Coleman (13). The product was analyzed by thin layer chromatography on silica gel giving a single spot with an Rf value of 0.63 was obtained.

[¹³C]Thiourea dioxygen was synthesized by the addition of 1.5 g of thiourea (8000 $\mu$Ci/mol) to 25 ml of a 6% hydrogen peroxide solution in ice (14). Once the product fully precipitated the solid was washed with hot methanol and air dried.

**Enzyme Assays**—The glutamine synthetase bio-synthetic activity was monitored using the lactate dehydrogenase and pyruvate dehydrogenase coupling system. The assay mixture consisted of 50 mM Hepes (pH 7.50), 100 mM KCl, 15 mM MgCl₂, 1 mM phosphoenolpyruvate, 190 $\mu$M NADH, 33 $\mu$M of pyruvate kinase/ml, and 33 $\mu$M of L-lactate dehydrogenase/ml. For most assays, the substrate concentrations were 50 mM N&Cl, 200 mM L-glutamate, and 2 mM ATP. Assays were performed at 25 °C at 339 nm in 1-cm cuvettes with 1.00 ml total volume in a Beckman DU UV-VIS spectrophotometer equipped with a Gilford attachment. Reactions were initiated with 10-μl aliquots containing 1.5-10 μg of glutamine synthetase.

Glutamate dehydrogenase activity was measured spectrophotometrically at 340 nm at 25 °C in 1.0-cm cuvettes with 1.00 ml total volume with the above mentioned equipment. The assay contained 100 mM inorganic phosphate (pH 7.10), 1 mM NADP, and 200 mM l-glutamate. The assays were initiated with 10-μl aliquots of 0.1-2 units of enzyme.

5'-p-FSO₂BuAdo Labeling—Incubations of glutamine synthetase (5-200 μM) with 1.0 mM of 5'-p-FSO₂BuAdo were performed in 10% N,N-dimethylformamide, 50 mM Hepes (pH 7.50), 15 mM MgCl₂, 100 mM KCl at 25 °C for 6 h. The incubation mixture was then passed through a gel permeation column by the method of Penefsky (15) to remove unreacted 5'-p-FSO₂BuAdo. The stoichiometry of the reaction was determined using Equation 1 allowing for the initial adenylation state of the enzyme.

Thiouria Dioxide Incubations—Incubations of glutamine synthetase and glutamate dehydrogenase with thiourea dioxide were performed by incubating the enzyme (3-200 μM) with 10-100 μM of the inhibitor in 50 mM Hepes (pH 7.50), 100 mM KCl, and 15 mM MgCl₂. The concentration of thiourea dioxide was varied from 1 to 100 mM in these experiments over a time interval up to 24 h. Inactivation and Reactivation Experiments with Methionine Sulfoximine and ATP—Inactivation of glutamine synthetase by the formation of an enzyme methionine sulfoximine phosphate-ADP complex was conducted by the addition of 1 mM L-methionine sulfoximine to 5-200 μM of glutamine synthetase in 50 mM Hepes (pH 7.50), 100 mM KCl, and 15 mM MgCl₂. The concentration of O-methylisourea was varied from 1 to 100 mM in these experiments over a time interval up to 24 h.

Reductive Methylation—Glutamine synthetase was reductively methylated by the addition of a 10-fold excess/lysyl residue of NaCNBH₃ (0.1 M) and formaldehyde (0.10 M). The final concentration of enzyme in the reaction mixture was 1-2 mg/ml (20-40 μM subunits) and typical reaction conditions were as follows: 0.10 ml of enzyme (40 mg/ml) in buffer (50 mM Hepes (pH 7.50), 100 mM KCl, 15 mM MgCl₂ was diluted to 1.6 ml with a standard buffer solution followed by addition of 0.2 ml of a 0.1 M solution of recrystallized NaCNBH₃. The solution was mixed and then allowed to react for a period of several hours at room temperature. Enzyme activity was monitored periodically using the biosynthetic assay described previously.

**Specific Lysine Modification of Glutamine Synthetase**—A specific peptide fragment of glutamine synthetase was removed by trypsin treatment by the addition of 1% (w/v) trypsin to 10-102 μM (0.5-5.0 mg/ml) enzyme in 50 mM Hepes (pH 7.0), 100 mM KCl, and 10 mM MgCl₂ (21). The activity was checked by taking aliquots from the incubation mixture and assaying at various times. After the activity stopped decreasing, the trypsin was inactivated with bovine pancreatic trypsin inhibitor at a ratio of 2 to 1 with trypsin inhibitor in excess. The mixture was then passed through a G-120 Sephadex column equilibrated with 50 mM Hepes (pH 7.50), 100 mM KCl, and 15 mM MgCl₂. The residual enzyme activity was checked with the standard biosynthetic assay using 200 mM glutamate and 15 mM ATP.

**Fluorescence Binding Studies**—Fluorescence titrations at 25 °C were performed by following the perturbation of tryptophan residues of glutamine synthetase due to substrate and inhibitor binding. A Perkin-Elmer Model MPF-44B fluorescence spectrophotometer was used and the buffer solution was 50 mM Hepes (pH 7.50), 100 mM KCl, and 15 mM MgCl₂. Excitation at 300 nm was employed in order to only excite tryptophan residues and the emission wavelength used to monitor the titration was 336 nm. The titrations were performed in 1-cm quartz fluorescence cuvettes. Glutamine synthetase was kept at 3.4-5.5 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 concentrations in the titrant as in the cuvette in order to keep the enzyme concentration constant and eliminate dilution effects. In some experiments, the titrant solution consisted of enzyme plus a saturating level of the substrate or ligand that was being studied, and aliquots of the solution were added to a cuvette that contained enzyme only.

Fluorescence titration data were fit to the following equation:

\[ Q = X \cdot Q_{ES} + (1 - X) \cdot Q_E \] (2)

where Q is the measured signal, $Q_{ES}$ is the signal due to the binary enzyme complex, $Q_E$ is the signal due to uncomplexed enzyme, and X is the mole fraction of E in the complexed species. Once X is...
determined, the final relationship is

\[
X = \left( K_d + \frac{[E_i] + [L]}{2k_{inact}} \right) - \frac{\sqrt{4K_d + [E_i] + [L]}}{2k_{inact}} + \frac{[E_i] + [L]}{2k_{inact}} \frac{[E_i] + [L]}{2k_{inact}} \]
\]

where \( K_d \) is the dissociation constant, \( [E_i] \) is the total enzyme concentration, and \( [L] \) is the ligand concentration.

**Irreversible Inhibition Kinetics**—Irreversible inhibition of glutamine synthetase by thiourea dioxide was analyzed using the following model.

\[
K_d \quad E \xrightarrow{h_{inact}} \quad E \cdot A
\]

**SCHEME 1**

where \( E \) is native enzyme, A is thiourea dioxide, \( E \cdot A \) is the reversible enzyme-inactivator complex, \( E \cdot A \) is the inactivated enzyme complex, \( K_d \) is the apparent dissociation constant, and \( h_{inact} \) is the first order rate constant for the formation of the inactive complex.

By keeping \([A] \gg [E]\), the reaction should exhibit pseudo-first order kinetics (Equation 4) when observing the loss of activity versus time at a given concentration of \( A \). In Equation 4,

\[
\ln[E]/[E]_o = -k_{obs} \cdot t
\]

\([E]_o \) is the amount of initial active enzyme, \([E] \) is the amount of active enzyme at time \( t \), and \( k_{obs} \) is the pseudo-first order rate constant. \( h_{inact} \) is measured at several inhibitor concentrations and the data are analyzed by the following relationship.

\[
1/h_{inact} = (K_d/h_{inact})(1/[A]) + 1/k_{inact}
\]

When a competitive inhibitor that binds to \( E \) is present in the solution during inactivation, the following model is used.

\[
B \quad E \xrightarrow{h_{inact}} \quad A
\]

**SCHEME 2**

where \( B \) is a competitive inhibitor of inactivation. The equation for inactivation is now

\[
1/h_{inact} = (K_d/(h_{inact}(A)))(1 + [B]/K_d) + 1/k_{inact}
\]

where \( K_d \) in the apparent dissociation constant for the competitive inhibitor.

**Amino Acid Analyses**—Amino acid analyses were conducted at the University of Pennsylvania in Dr. Ruth Hogue-Angeletti’s laboratory. Standards were run that included homoaarginine in the amino acid mixture to establish the retention time of this amino acid. All enzyme samples were hydrolyzed in 6 M HCl by standard procedures.

**RESULTS**

**Inactivation of Glutamine Synthetase by Thiourea Dioxide**—Several reagents are known to react with lysine residues of enzymes and glutamine synthetase is known to have at least 1 lysine group at the ATP binding site (7, 8). Because of its structural similarity to the phosphoryl moiety of the \( \gamma \)-glutamyl phosphatase intermediate in the glutamine synthetase catalyzed reaction, thiourea dioxide was tried as an inhibitor of the enzyme. Thus thiourea dioxide was incubated with glutamine synthetase and was found to inactivate the enzyme in a pseudo-first order manner (Fig. 14). Glutamate, near saturating concentrations (100 mM), significantly protected the enzyme from inactivation, while ATP (5 mM) did not alter the inactivation rate.

Additional studies were performed with ammonia, P\(_2\), and methionine sulfoximine (a transition-state analog), to test if these compounds could protect against inactivation (Fig. 1B). Ammonia and phosphate had no effect on the inactivation rate while methionine sulfoximine (\( K_d = 250 \mu M \)) was found to protect less efficiently than glutamate (\( K_d \sim 20 \) mM) (22).

Inactivation with thiourea dioxide was an irreversible process since dialysis or gel filtration (as described under “Experimental Procedures”) did not lead to reactivation. Hydroxylamine or ammonia (up to 1 M) did not lead to reversal of the inactivation. \( \text{Na}_2\text{SO}_3 \) did not inactivate the enzyme at concentrations up to 0.20 M removing any possibility that \( \text{SO}_3^- \) produced by decomposition of thiourea dioxide leads to inactivation of glutamine synthetase.

A structurally similar reagent to thiourea dioxide, \( O \)-methylisourea was tested as an inactivator of glutamine synthetase. No inactivation was observed over a 24-h period at up to 0.10 M \( O \)-methylisourea under identical incubation conditions described for the experiments with thiourea dioxide.

**Reactions of Thiourea Dioxide**—Not much is known about the chemical reactivity of thiourea dioxide, except that it has been used to reduce ketones, azo compounds, and metal ions (14, 23). Magnesium has a high enough reduction potential to eliminate the possibility that it is being reduced by thiourea dioxide leading to inactivation of the enzyme. Also, apoglutamine synthetase is inactivated by thiourea dioxide, with an approximate half-life of 8 h demonstrating that the presence of Mg\(^{2+} \) is not absolutely necessary for the inactivation reaction.

By employing \(^1\)H and \(^13\)C NMR spectroscopy and mass spectrometry, we investigated the reaction of thiourea dioxide with numerous compounds containing sulphydryl (ethanethiol, and mercaptoethanol), phenolic (phenol, cresol), disulfide (cystine), guanidino (arginine, guanidine), indole (indole, skatole), amide (glutamine, acetamide), or carboxylate groups (propionate, acetate). The compounds with these functional groups did not react with thiourea dioxide under a variety of conditions of pH (4–14), metal ion concentrations (0–100 mM Mg\(^{2+} \)), and temperature (20–80 °C). However, several primary amines (methylamine, amino-n-caproic acid, lysine, ornithine) and imidazoles (imidazole, histidine) were found to react with thiourea dioxide under basic conditions. By mass spectrometry the product of the addition of methylamine with thiourea dioxide under basic conditions showed a parent molecular ion of 73 which is consistent with the formation of...
the guanidino product. The $^1$H NMR spectrum of the product gave a peak at 2.85 ppm which is in good agreement with the spectrum of an authentic sample. The $^1$H NMR spectrum of the product of incubation of ornithine and thiourea dioxide incubation under basic conditions contained peaks identical with arginine. However, with several free amino acids the $\alpha$-amino group was also found to react but only readily at higher temperatures (40–50°C) and pH values higher than approximately 12. Glutamate and methionine sulfoximine did not react with thiourea dioxide at 25°C from pH 7.5–12.5 under standard incubation conditions. Therefore the possibility of glutamate or methionine sulfoximine reacting with thiourea dioxide under the conditions of the protection experiments shown in Fig. 1 was eliminated. Mass spectrometry of the reaction mixture of thiourea dioxide with imidazole showed a mixture of products that were not further analyzed. However, since the inactivation was shown to be associated with modification of a lysine residue (see later), we assumed that this reaction was of less importance. 2-Methylimidazole did not react with thiourea dioxide.

**Stoichiometry of Incorporation of Thiourea Dioxide**—The stoichiometry of the inactivation reaction was studied by incubating glutamine synthetase with [14C]thiourea dioxide followed by removing the excess reagent at different time intervals by gel filtration. The residual enzymatic activity was measured as a function of incorporation of radioactive label and these data are presented in Fig. 2. The apparent stoichiometry is 3 groups/monomer but one molecule could be removed by dialysis after four 6-hour dialysis periods. Therefore one molecule of reagent must bind reversibly with a tight binding constant. Most importantly, however, is the fact that this reversible binding does not lead to a loss of activity. Therefore the effective stoichiometry of labeling is 2 molecules of inactivator bound per subunit under these conditions. At higher concentrations or longer incubation periods, it was found that more than 2 residues were labeled per subunit. Incubation with 50 mM thiourea dioxide for 24 h led to approximately 3 residues modified after dialysis. Incubation of inactive glutamine synthetase with up to 100 mM thiourea dioxide for 1 or 2 days after complete inactivation shows further incorporation of up to 4.9 ± 0.4 groups bound per subunit after dialysis. This is due most likely to other less reactive residues being modified by this reagent.

The inactivation reaction was followed at several inhibitor concentrations and the data are plotted in Fig. 3. Each line was fit to Equation 4 and a replot of the results is shown in Fig. 4. When the data in Fig. 4 were fit to Equation 5, the $K_a$ determined for thiourea dioxide was 31 ± 2 mM and $k_{inact}$ value was $2.1 \pm 0.3 \times 10^{-3}$ s⁻¹. Thiourea dioxide was tested as a competitive inhibitor of glutamine synthetase under initial rate conditions with glutamate as the varied inhibitor. The $K_{IS}$ determined from these experiments was 26 ± 3 mM at pH 7.5 and 150 ± 10 mM at pH 8.1. The inhibition pattern was competitive at both pH values.

The pH rate profile for inactivation was determined and is shown in Fig. 5. Nearly the same inactivation rate is found from pH 8.0 to 9.5 and the inactivation rate increases at lower pH values. Data were not taken at pH values lower than 7.0 because thiourea dioxide decomposes under these acidic conditions. Thus an accurate $pK_a$ value could not be determined. In a separate experiment we determined that the $pK_a$ value for thiourea dioxide was 9.5 ± 0.2 which means that a group on the enzyme with a $pK_a$ value of less than 7.0 must be protonated to increase the rate of inactivation by thiourea dioxide.

Since lysine and/or terminal amino acid residues are good candidates for reaction with thiourea dioxide, we conducted an experiment in which we blocked all the free amino groups
The stoichiometry of [14C]thiourea dioxide labeling was also determined with enzyme inactivated by methionine sulfoximine and ATP treatment. Methionine sulfoximine and ATP react to form an inactive complex in which ADP and methionine sulfoximine phosphate are essentially irreversibly bound to the active site (18, 19). The labeling was conducted under the same conditions as described for the previous experiment. The stoichiometry of thiourea dioxide bound to the enzyme was 1.9 ± 0.2 molecules bound per monomer after gel permeation and dialysis. Within experimental error 1 amino acid residue was unavailable for reaction with thiourea dioxide after protection of the active site by this procedure since the control performed under identical conditions showed 3.0 ± 0.2 residues modified.

**Binding Experiments with Thiourea Dioxide Inactivated Glutamine Synthetase**—Fluorescence titrations were used to monitor binding of ATP, glutamate, and methionine sulfoximine to the modified enzyme. Glutamate binding was measured in the presence of saturating ATP (2 mM) since the native enzyme does not exhibit a fluorescence change with glutamate binding alone. Glutamine synthetase was inactivated and labeled with thiourea dioxide up to a stoichiometry of 2.1 ± 0.2 and the enzyme was isolated by gel filtration and exhaustive dialysis. The binding constants were determined from the fluorescence changes upon effector binding and were compared to binding of the same effectors to native enzyme under identical conditions. The results are given in Table I. From the data in Fig. 1B and using Equation 6 the $K_B$ values determined for L-glutamate and methionine sulfoximine were 36 ± 5 and 0.57 ± 0.04 mM, respectively.

**Inactivation of Glutamine Synthetase by Pyridoxal Phosphate**—Pyridoxal phosphate was also used to inhibit the enzyme. Pyridoxal phosphate forms Schiff bases with reactive lysine residues of glutamine synthetase which can be reduced by NaBH₄ to form a relatively stable adduct which has a fluorescence maximum at approximately 396 nm at pH 7.50.

The results show that approximately 81% inactivation is achieved after incubation of glutamine synthetase with pyridoxal phosphate (4–5 mM) for up to 12 h at pH 7.50. The inactivation rate and the total extent of inactivation did not increase at pH 8.00 in Hepes buffer. The first order plot of inactivation by pyridoxal phosphate was nonlinear, therefore the kinetic behavior was not fully explored with this reagent. The inactivation rate was found to be the same whether the Schiff base was or was not reduced under our conditions for measuring the residual enzymatic activity. However, upon gel filtration, the unreduced pyridoxal phosphate labeled enzyme did slowly undergo reactivation. Therefore to study the extent of stoichiometric incorporation of pyridoxal phosphate versus

![Fig. 5. The pH rate profile of inactivation of glutamine synthetase by thiourea dioxide (10 mM).](image-url)

**Table I**

Fluorescence titrations of native, pyridoxal phosphate, and thiourea dioxide labeled glutamine synthetase

<table>
<thead>
<tr>
<th>Enzyme form, ligand and enzyme complex</th>
<th>Native</th>
<th>Thiourea dioxide</th>
<th>Pyridoxal phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme sample</td>
<td>2.1 ± 0.2 molecules of label/monomer of enzyme. See &quot;Experimental Procedures&quot; for details.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP + $E$</td>
<td>150 ± 20 μM</td>
<td>180 ± 10 μM</td>
<td></td>
</tr>
<tr>
<td>L-(DL)-Methionine</td>
<td>0.61 ± 0.02 mM</td>
<td>0.60 ± 0.05 mM</td>
<td>1.1 ± 0.2 mM</td>
</tr>
<tr>
<td>L-Glu + E-ATP</td>
<td>2.8 ± 0.3 mM</td>
<td>110 ± 20 mM</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ No fluorescence change observed upon addition of ligand.
enzymatic activity, the enzyme-inhibitor complex was reduced with NaBH₄.

Protection experiments showed that ATP and ammonia had no effect on the inactivation rate while inorganic phosphate and methionine sulfoximine exhibited some protection against inactivation. Glutamate protected the enzyme completely up to a period of 1 h (Fig. 6). Increasing the pH to 8.0 had essentially no effect on the rate of inactivation although the rate was slightly faster.

Experiments to determine the proportion of pyridoxal phosphate versus activity showed that 2.1 ± 0.2 mol of pyridoxal phosphate were incorporated after incubation with 8 mM pyridoxal phosphate for up to 24 h at 25 °C. In Fig. 7 are presented the data which show that both enzyme residues are being labeled at essentially the same rate resulting in a 80 ± 2% total loss of activity. However, using the previously published conditions (5) for pyridoxal phosphate labeling at pH 8.0 in 50 mM TEA acetate and 50 mM we found that 3.1 ± 0.3 mol of pyridoxal phosphate are bound per monomer after 12 h of incubation at 6 mM inactivator. By changing to these experimental conditions we were able to reproduce the stoichiometry of 3.2 mol of pyridoxal phosphate incorporated and produce enzyme that was totally inactivated (5). Evidently a third lysine residue is also required for catalysis or overall protein conformation.

Fluorescence Titration Experiments with Pyridoxal Phosphate Labeled Glutamine Synthetase—Fluorescence titrations were conducted with enzyme labeled with pyridoxal phosphate having a stoichiometry of 2.1 ± 0.2 mol bound per monomer. Two sets of experiments were conducted in which 1) the intrinsic fluorescence of the enzyme was monitored and 2) an excitation wavelength of 326 nm was used to excite the pyridoxal phosphate groups directly (a fluorescence emission maximum was observed at 396 nm). No change in the emission spectrum was observed upon addition of L-glutamate (100 mM) or ATP (5 mM) added alone or together in the experiment where the pyridoxal phosphate group was directly monitored. However, when the luminescence of the protein was monitored, the results shown in Table I demonstrate that the binding constants for ATP and methionine sulfoximine do not change appreciably upon modification of glutamine synthetase with pyridoxal phosphate. Glutamate binding could not be followed using this method since no fluorescence change was observed.

Labeling Experiments with both Thiourea Dioxide and Pyridoxal Phosphate—[14C]Thiourea dioxide labeling of glutamine synthetase was carried out on enzyme that was previously labeled with 2.1 ± 0.2 mol of pyridoxal phosphate, 50 mM [14C]thiourea dioxide was incubated with the pyridoxal labeled enzyme for 5 h in this experiment. The stoichiometry found was 1.0 ± 0.1 mol of thiourea dioxide/monomer after this incubation period while native enzyme labeled in a control experiment showed 2.2 ± 0.2 mol of [14C]thiourea dioxide were incorporated and was 98.5% inactive. Native enzyme that was labeled with thiourea dioxide to the extent of 2.2 ± 0.2 mol was further subjected to labeling with 4 mM pyridoxal phosphate for 24 h (pH 7.5). In this experiment, 0.95 ± 0.10 mol of pyridoxal phosphate were incorporated. Evidently both lysine modifying reagents react with 1 common residue that is responsible for loss of enzyme activity.

Differential Labeling Experiments with 5'-p-FSO₂BzAdo and Pyridoxal Phosphate—Pyridoxal phosphate (4 mM, 24 h, pH 7.50) was found to react with 2.0 ± 0.2 residues/monomer when incubated with enzyme that had previously been labeled with 0.98 ± 0.10 mol of 5'-p-FSO₂BzAdo. When the reverse order of labeling was carried out, 1.0 ± 0.1 molecules of 5'-p-FSO₂BzAdo were bound per monomer using enzyme previously labeled with 2.1 ± 0.2 mol of pyridoxal phosphate. When pyridoxal phosphate (4 mM, 30 h) was incubated at pH 8.0 in 50 mM TEA acetate and 50 mM MgCl₂, 2.3 ± 0.2 residues were modified using glutamine synthetase that was previously labeled with 0.98 ± 0.10 mol of 5'-p-FSO₂BzAdo. The control for this latter experiment using native enzyme resulted in 2.9 ± 0.3 mol of pyridoxal phosphate being incorporated per monomer.

In additional experiments glutamine synthetase was incubated with methionine sulfoximine (1.0 mM) and ATP (1.0 mM) to produce totally inactivated enzyme which was then incubated with pyridoxal phosphate (4 mM, 24 h, pH 7.5). The stoichiometry of pyridoxal phosphate bound per monomer was 1.3 ± 0.1 in this experiment.

Since L-glutamate protected against inactivation by pyridoxal phosphate, the enzyme was incubated with pyridoxal phosphate (4 mM, 1 h, pH 7.5) containing 100 mM L-glutamate in the incubation mixture. The stoichiometry of pyridoxal phosphate incorporated per monomer under these conditions was 0.71 ± 0.07. The amount of activity remaining was 95%.

Investigation of a Peptide Fragment Thought to be near the
Active Site of Glutamine Synthetase—Limited proteolysis with trypsin was carried out (see "Experimental Procedures") with glutamine synthetase according to the procedure of Monroe et al. (25). This results in the removal of a peptide containing 15 amino acid residues. Trypsin was inactivated by trypsin inhibitor and the mixture passed through a gel permeation column to separate the small peptide and trypsin from glutamine synthetase. The modified enzyme had 1.5% of the activity of the native enzyme.

The modified glutamine synthetase was then incubated with 4 mM pyridoxal phosphate and 100 mM [14C]thiourea dioxide separately for 24 h by the same procedures described above. A control using native enzyme was also run. The stoichiometry of the labels bound to the proteolyzed enzyme were the following: 2.0 ± 0.2 mol of pyridoxal phosphate were bound per monomer and 4.3 ± 0.4 mol of [14C]thiourea dioxide were bound per monomer. The control enzyme had 2.1 ± 0.2 and 4.0 ± 0.3 mol bound of each reagent, respectively. Therefore essentially no difference was found for the stoichiometry of each reagent with the native and proteolyzed enzymes.

Reactivation of Glutamine Synthetase Inactivated with ATP and Methionine Sulfoximine after Lysine Modification by Thiourea Dioxide and Pyridoxal Phosphate—Maurizi and Ginsburg (20) demonstrated that glutamine synthetase can be reactivated after the inactivation reaction produced by methionine sulfoximine and ATP. From earlier results presented in this paper we found that methionine sulfoximine and ATP inactivation resulted in the prevention of 1 lysine residue being labeled which was a common site at which both thiourea dioxide and pyridoxal phosphate react. In a control experiment enzyme that was totally inactivated with methionine sulfoximine and ATP could be reactivated to 100% of the native enzyme activity. In another experiment, enzyme inactivated with methionine sulfoximine and ATP was then incubated with 4 mM pyridoxal phosphate or 100 mM [14C]thiourea dioxide in separate mixtures for 24 h. 1.3 ± 0.1 mol of pyridoxal phosphate and 3.1 ± 0.3 mol of [14C]thiourea dioxide were incorporated per monomer. The controls showed 2.1 ± 0.2 and 4.0 ± 0.3 mol bound, respectively. Therefore, 1 lysine group was protected from reaction with each reagent. Both samples were then reactivated. The pyridoxal phosphate labeled enzyme regained ∼100% activity while the thiourea dioxide-labeled enzyme regained ∼86% activity. Both labeled enzymes were subjected to relabeling with the same experimental conditions. The pyridoxal phosphate labeled enzyme now incorporated a total of 2.4 ± 0.2 mol of pyridoxal phosphate/monomer and the thiourea dioxide-labeled enzyme had 4.4 ± 0.4 mol of total thiourea dioxide bound per monomer.

Amino Acid Analysis of Labeled and Unlabeled Glutamine Synthetase—Amino acid analyses were carried out on native glutamine synthetase and enzyme that had been modified by thiourea dioxide and pyridoxal phosphate as described under "Experimental Procedures." All enzyme samples were dialyzed into pH 7.50 ammonium bicarbonate (0.010 M) and then lyophilized. Each enzyme sample was then subjected to amino acid analysis (see "Experimental Procedures"). A homoaarginine control gave a retention time of 45.59–45.60 min while the modified enzyme gave a retention time of 45.60–45.63 min. A sample of native enzyme gave no peak near the homoaarginine retention time. Enzyme labeled under conditions where 2.1 ± 0.2 and 4.6 ± 0.4 mol of thiourea dioxide were incorporated showed the appearance of 0.5 ± 0.2 mol of homoaarginine/monomer for both preparations. The low estimate of homoaarginine arises due to the integration procedure in the amino acid analyzer. In addition, with the enzyme labeled to the extent of 4.6 mol with thiourea dioxide, there was a decrease of 4–5 histidine residues.

Enzyme labeled with 2.1 ± 0.2 mol of pyridoxal phosphate prior to thiourea dioxide labeling (25 mM thiourea dioxide for 5 h, <2% residual activity) did not contain homoaarginine. Therefore it seems that thiourea dioxide reacts with only 1 lysine residue but that several histidine residues are also modified. Since, the chemical behavior of thiourea dioxide with histidine has not been explored extensively, we did not attempt to identify the modified histidines in the amino acid chromatogram.

DISCUSSION

Thiourea dioxide when incubated with E. coli glutamine synthetase leads to inactivation by irreversible covalent modification. Homoaarginine is produced as a result of this reaction as determined by amino acid analysis. Model studies were conducted of the chemical reaction of thiourea dioxide with a variety of compounds containing functional groups commonly found as side chains in proteins. The products identified after reaction of thiourea dioxide with a primary amine contained a guanidino group. Thiourea dioxide does not readily react with protonated amino groups of amino acids (pH 7.5) in compounds such as L-glutamate and methionine sulfoximine under the conditions of the inactivation reaction with glutamine synthetase. Histidine modification may result due to formation of radicals in solution with thiourea dioxide since it is a reducing agent. However, at this time the nature of reaction of imidazole groups with thiourea dioxide has not been explained in detail.

Thiourea dioxide inactivated glutamine synthetase under conditions (pH 7.5), where O-methylisourea did not react with the enzyme. O-Methylation, structurally similar to thiourea dioxide, is a known lysine modifying reagent that converts the ε-amino group to a guanidino moiety although the typical pH range where this modification takes place is approximately 10.

The possibility that SO₃⁻ is formed by decomposition of thiourea dioxide under the incubation conditions and results in inactivation of the enzyme was ruled out since no inactivation was observed upon incubation of glutamine synthetase with Na₂S₃O₅. Apoenzyme was inactivated by thiourea dioxide, although at a much slower rate, eliminating the possibility of a metal complex being essential for inactivation. The increase in the rate of inactivation with the metal ion present is most likely due to structural differences between the holoenzyme and the apoenzyme. Alternatively, the metal ion may play a role in the reaction by binding to the sulfonic acid moiety of thiourea dioxide. Thiourea dioxide, as will be discussed later, seems to react at the glutamate binding site of the enzyme and the sulfonic acid group may bind in the region where the γ-carboxylate of glutamate binds.

Several lines of evidence (in addition to the identification of approximately 1 residue of homoaarginine being formed) lead to the conclusion that inactivation by thiourea dioxide is due to modification of 1 lysine residue. The experiments which showed a lack of incorporation of [14C]thiourea dioxide with enzyme in which all the amino groups had been reductively methylated by formaldehyde and NaCNBH₃ are consistent with the modification of a lysine residue. Reductively methylated glutamine synthetase still exhibits similar binding and

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1. The reactions of thiourea dioxide with several amines is being extensively studied with regard to stoichiometry of the reaction and understanding the reaction mechanism (E. Turov, S. Weinreb, J. Colandouni, and J. D. Villafranca, unpublished results).
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structural characteristics of the native enzyme but is inactive (24). Glutamate dehydrogenase\(^3\) was also found to be inactivated by thiourea dioxide and glutamate dehydrogenase has at least 3 lysine groups at its active site (12, 26, 27).

In order to determine which regions of the active site are modified by thiourea dioxide, we devised several experiments to protect the various amino acid residues with different combinations of substrates. L-Glutamate and methionine sulfonimine, a transition state analog for glutamine synthetase, were the only two reagents which protected against inactivation. ATP and ammonia did not provide any protection indicating that they did not hinder thiourea dioxide from reacting with the group(s) that are associated with the loss of enzymatic activity. These results strongly suggest that the glutamate substrate site seems to be nearest the site of thiourea dioxide modification.

Scheme 1 was used as the kinetic model for inhibition by thiourea dioxide, in which the initial binding of thiourea dioxide is considered reversible and in rapid equilibrium followed by a slower inactivation reaction. The inactivation data at several thiourea dioxide concentrations were fit to Equation 4 and found to exhibit pseudo-first order behavior as expected if the results were correct. The data were fit to Equation 5 and the equilibrium constant \(K_e\) was 31 ± 2 mM, and \(b_{\text{inact}}\) was 2.1 ± 0.3 × 10\(^{-3}\) s\(^{-1}\) at pH 7.5. As a second test of this model, thiourea dioxide was tested as a competitive inhibitor against L-glutamate in an initial velocity steady-state kinetic experiment. The inhibition was found to be competitive showing mutually exclusive binding and the \(K_a\) value was found to be 26 ± 3 mM at pH 7.5. This agrees well with the value of \(K_a\).

It was also possible to determine the equilibrium binding constants for L-glutamate and methionine sulfonimine by using these compounds to protect against inactivation by thiourea dioxide. When the data are fit to Equation 6, binding constants of 36 ± 5 and 0.57 ± 0.04 mM for L-glutamate and methionine sulfonimine, respectively, were obtained. The binding constant for L-glutamate has been reported to be approximately 20 mM (22) while the binding constant of L-glutamate in an initial velocity steady-state kinetic experiment. The enzyme is known to bind substrates and possibly inhibitors in a two-step model where there is a rapid binding followed by a slower conformational change. This model is shown in the following scheme.

\[
\begin{align*}
E + C & \rightarrow EC \\
& \overset{K_e}{\underset{K_c}{\rightleftharpoons}} EC^* \\
\text{SCHEME 3}
\end{align*}
\]

where \(E\) is free enzyme, \(C\) is free ligand, \(EC\) is the initial enzyme-ligand complex and \(EC^*\) is the enzyme-ligand complex after the conformational change. Therefore the binding constant from the fluorescence titrations experiments can either be smaller or greater than the value determined by the kinetic experiments depending on whether the partition ratio \(K_{c_e}\) between the EC and EC* complexes is greater than or less than one.

A group with a \(pK_a\) less than 7.0 was implicated in the inactivation reaction and when this group is protonated the inactivation rate increases. Thiourea dioxide has a \(pK_a\) of 9.5 which means that the group with a \(pK_a\) less than 7.0 must be an enzyme group. The increase in inactivation could either be due to an increase in the binding constant \(K_e\) of thiourea dioxide to the enzyme or an increase in the rate of the inactivation step \(b_{\text{inact}}\). This was tested by measuring the \(K_{c_e}\) value at a higher pH value than 7.5. Weaker binding was observed at higher pH values which is consistent with binding being increased by protonation of a residue on the enzyme. This is in accord with other experiments from our laboratory that show a requirement for a group on the enzyme to be protonated in order for L-glutamate to bind.\(^4\) However, it does seem that thiourea dioxide can still inactivate the enzyme with this group in the deprotonated form in a pH independent manner at higher pH values. At higher pH values (>8), the inactivation reaction may be a simple bimolecular reaction where reversible binding of thiourea dioxide to the enzyme does not occur.

The stoichiometry of labeling by thiourea dioxide indicates that 2 groups are modified on the enzyme at essentially the same rate over the time course of the inactivation reaction. Further modification of the enzyme occurs with longer incubation times than an original group which is protonated. Up to approximately a total of 5 residues can be modified after longer incubation periods. The modification of many residues was also observed for glutamate dehydrogenase upon incubation with thiourea dioxide over lengthy time periods.

The possibility that the lysine residue which is covalently modified by the affinity label 5′-p-PSO-BzAdo is the same residue as 1 of the 2 residues modified by thiourea dioxide was ruled out in experiments conducted with each reagent.

The question of whether both residues modified initially by thiourea dioxide were necessary for inactivation was investigated by inactivating glutamine synthetase with methionine sulfonimine and ATP. This was done in order to block the active site to see if protection of one or both residues resulted. The data showed that 1 residue was protected against thiourea dioxide labeling in this experiment. Reactivation of the enzyme containing the tightly bound methionine sulfonimine phosphate-ADP-enzyme complex led to approximately 85% recovery of activity. Therefore the single residue modified under conditions where the active site is protected is not essential for enzyme activity. This residue, however, reacts readily with thiourea dioxide.

As can be seen in Table I the binding of ATP is not affected significantly by thiourea dioxide modification but L-glutamate and methionine sulfonimine binding were weaker. This is consistent with the finding that the modification site is at or adjacent to the glutamate substrate site as seen in the protection and competition experiments with thiourea dioxide. Methionine sulfonimine binding was weaker by a factor of approximately 2 while L-glutamate binding was weaker by a factor of approximately 30. However, glutamate binding was measured in the presence of ATP so either glutamate binding or the formation of an enzyme-ATP-glutamate complex or enzyme-ADP-γ-glutamyl phosphate complex might be hindered by covalent labeling of thiourea dioxide at the active site.

Inactivation of glutamine synthetase by pyridoxal phosphate was dependent on pH and the buffer used in the inactivation experiments. In Hepes buffer at pH 7.5-8.0, the inactivation resulted in only a 20-30% drop of the initial activity. This loss of activity was associated with the labeling of 2 lysine residues/subunit. However, Ginsburg's group found that at pH 8.0 in TEA acetate buffer total

\(^3\)Total inactivation of bovine liver glutamate dehydrogenase under identical reaction conditions used with glutamine synthetase was accomplished after 36 h with an approximate half-life of 6 h. The stoichiometry of \(\text{[U]C}\) thiourea dioxide incorporation was found to be 11.2 mol of bound per monomer of glutamate dehydrogenase. This demonstrates that inactivation by thiourea dioxide is not unique to glutamine synthetase alone.

\(^4\)J. Colandozini, R. Nissan, and J. J. Villafranca, unpublished results.
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inactivation could be obtained after 3 lysine residues were labeled per subunit (5). We were able to repeat these experiments under similar conditions to those of the earlier report. Evidently in Hepes buffer only two of these Schiff base adducts are stabilized.

Substrate protection experiments against pyridoxal phosphate inactivation showed similar behavior as those conducted with thiourea dioxide. ATP and ammonia did not protect against inactivation by pyridoxal phosphate or thiourea dioxide at pH 7.5. However, inorganic phosphate protected against pyridoxal phosphate inactivation while it had no effect on the rate of inactivation with thiourea dioxide. This is reasonable since pyridoxal phosphate may bind partially to the phosphate product site of the enzyme. Glutamate protects against inactivation by pyridoxal phosphate as it did for thiourea dioxide. These data suggest that both reagents modify a common amino acid residue.

Pyridoxal phosphate labeling of enzyme (Hepes buffer, pH 7.5) previously labeled with 5'-p-FS\(_2\)BzAdo was identical to an experiment with native enzyme in that 2 amino residues are modified in each experiment. However, at pH 8.0 in the TEA acetate buffer there was competition for 1 lysine residue/monomer for each reagent. Evidently the third lysine residue modified under these conditions is the lysine residue that has reacted with 5'-p-FS\(_2\)BzAdo. This residue is necessary for activity. Our data are also consistent with Ginsburg's results where ATP protected against inactivation by pyridoxal phosphate in the pH 8.0 TEA acetate buffering system (5). At pH 7.5 in Hepes buffer where the third lysine residue is not labeled in our experiments, ATP does not protect since we did not observe any labeling at this third lysine residue which may be near the \(\gamma\)-phosphate position of ATP.

Pyridoxal phosphate labeling of enzyme which was inactivated by ATP and methionine sulfoximine showed one less lysine residue labeled as compared to experiments with thiourea dioxide. Once methionine sulfoximine phosphate and ADP were removed all activity was regained. This shows that only 1 lysine is associated with the 80% inactivation observed upon treatment with pyridoxal phosphate.

Competition experiments with thiourea dioxide and pyridoxal phosphate at pH 7.5 demonstrated that only one of the several lysine residues that react with these reagents can be modified by both reagents. Therefore the lysine residue which is protected by methionine sulfoximine phosphate and ADP is the residue in common. This residue may still be playing a role in catalysis when pyridoxal phosphate has modified the enzyme, whereas when homoarginine is formed by the thiourea dioxide reaction, all activity is lost. One possible explanation is that the high \(pK_a\) or bulk of the guanidino group alters enzyme activity while the secondary amine formed upon pyridoxal phosphate labeling is still able to function in catalysis although with diminished efficiency. The unmodified lysine group in the native enzyme may function in catalysis as a base to deprotonate ammonia during attack on the \(\gamma\)-glutamyl phosphate intermediate. Homoarginine has an approximate \(pK_a\) of 12 which may preclude this function in the normal catalytic reaction but the pyridoxal phosphate modified enzyme may still assist catalysis although very weakly.

In conclusion we have identified one or possibly 2 lysine residues that play an active role in catalysis with glutamine synthetase. In addition we have described a new lysine modifying reagent, thiourea dioxide. Thiourea dioxide may be useful as a glutamate analog for other enzymes where glutamate is a substrate and this reagent may find use in protein modification studies due to the formation of homoarginine from lysine at low pH. This change in the environment of the active site due to replacement of one positively charged residue by another with a higher \(pK_a\) value should alter binding and catalytic properties of many enzymes. Further work in our laboratory is currently underway with the aim of identifying the specific location in the protein sequence of the amino acid(s) that is modified.

REFERENCES