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Inactivation of *Escherichia coli* Glutamine Synthetase by Thiourea Trioxide[1]

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Incubation of *Escherichia coli* glutamine synthetase with thiourea trioxide resulted in partial inactivation of the enzyme. This reagent specifically modifies lysine residues to form homoarginine. By amino acid analysis 2.3 ± 0.3 residues of homoarginine are produced per enzyme subunit after treatment with thiourea trioxide. Previously we determined that thiourea dioxide totally inactivated glutamine synthetase and modified both lysine and histidine residues (J. Colanduoni and J. J. Villafranca (1985) *J. Biol. Chem.* **260,** 15,042–15,050). Thiourea trioxide reacted with the same lysine residues of glutamine synthetase as thiourea dioxide. The K_m values for the thiourea trioxide modified enzyme were determined and are 210 ± 30 μM and 10 ± 1 mM for ATP and glutamate, respectively. Both values are about threefold higher than for native enzyme assayed under the same conditions. Fluorescence titrations of native and thiourea trioxide labeled enzyme showed that ATP binding was virtually unchanged by the modification while glutamate and methionine sulfoximine bound about twofold more weakly to the modified enzyme.

INTRODUCTION

Glutamine synthetase of *Escherichia coli* catalyzes the formation of ADP, P_i, and glutamine from ATP, ammonia, and glutamate in the presence of divalent metal cations. The enzyme has a molecular weight of 600,000 and is composed of twelve identical subunits (*1*). The enzymic reaction has been proposed to go through a γ-glutamyl phosphate intermediate followed by the formation of a tetrahedral adduct when ammonia adds to the activated carbonyl group (*2, 3*).

Several studies to determine groups involved at the active site of glutamine synthetase have been made (*4–8*). Powers and Riordan (*4*) have found one arginine residue at the ATP site of ovine brain glutamine synthetase. Colanduoni and Villafranca have also identified an arginine residue at the ATP site of *E. coli* glutamine synthetase which is required for catalysis but not for ATP or amino acid binding (*5*). Whitley and Ginsburg (*6*) have identified at least one lysine residue required for catalysis with pyridoxal phosphate modification. Pinkofsky *et al.* (*7*) have identified Lys 47 to be the residue where the affinity label 5′-*p*-fluorosulfonyl-

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benzoyladenosine (FSBA)[3] covalently binds. Colanduoni and Villafranca (*8*) have also identified one lysine residue which appears to be at the glutamate site and is covalently modified by both pyridoxal phosphate and a new lysine modifying reagent thiourea dioxide. Another lysine residue is modified with both pyridoxal phosphate and FSBA at the ATP site (*8*). However, thiourea dioxide also reacts with other amino acids (*9*) and reacts with histidine residues (*8*).

Thiourea trioxide, a structurally similar reagent to thiourea dioxide was synthesized and studied in order to develop a reagent which would react more specifically with lysine residues under mild conditions and without the addition of other reagents. The results of these experiments are reported in this paper.

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 *et al.* (*10*). This procedure utilizes the ability to precipitate glutamine synthetase with Zn^{2+}. The adenylylation state and concentration of the enzyme were determined spectrophotometrically (*1*). The adenylylation state was determined to be 2.7.

[^{14}C]Thiourea was purchased from New England Nuclear. Peracetic acid was purchased from Pfalz and Bauer. All other substrates, enzymes, and reagents were purchased from Sigma Chemical Company.

The synthesis of thiourea dioxide was performed using Banett's procedure (*11*). The synthesis of thiourea trioxide and [^{14}C]thiourea trioxide was performed using the procedure of Walter and Randau (*12*) except that peracetic acid was added in 50% excess to insure full oxidation to the trioxide form. The specific radioactivity was found to be 131 cpm/nmol for the [^{14}C]thiourea dioxide prepared. In the radioactive synthesis 100 μCi of [^{14}C]thiourea dioxide was used. The synthesis of 5′-*p*-fluourosulfonylbenzoyladenosine was performed by the procedure of Wyatt and Colman (*13*).

The biosynthetic activity was monitored using the lactate dehydrogenase and pyruvate kinase coupling system. When saturating the substrate concentrations were 50 mM NH_4Cl, 200 mM L-glutamate, and 6 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 1.5–5 μg of glutamine synthetase.

^{1}H NMR samples for the model reactions were performed on a Bruker 360-MHz NMR. These samples contained 75 mM thiourea trioxide, 75 mM of the reagent being tested, and with and without 75 mM KOH in 50% deuterated water. These samples were allowed to sit for 24 h at 25°C.

Thiourea dioxide and thiourea trioxide labeling was performed in 50 mM Hepes (pH 7.50), 100 mM KCl, and 15 mM $MgCl_2$ at 25°C with 10 mM thiourea dioxide for

[3] Abbreviations used: FSBA, 5′-*p*-fluorosulfonylbenzoyladenosine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

9 h (or 10 mM thiourea trioxide for 9 h when used) with 3–60 μM glutamine synthetase. The enzyme was tested for biosynthetic activity to ensure full inactivation.

FSBA labeling was performed in 50 mM Hepes (pH 7.50), 100 mM KCl, and 15 mM $MgCl_2$ in 10% DMF at 25°C with 1.0 mM FSBA for 6 h with 3 to 60 μM glutamine synthetase. The enzyme was tested for biosynthetic activity to ensure full inactivation.

Methionine sulfoximine and ATP inactivation was performed by the addition of 2 mM L-methionine sulfoximine and 2 mM ATP in 50 mM Hepes (pH 7.50), 100 mM KCl, and 15 mM $MgCl_2$ at 25°C for 6 h with 3–60 μM glutamine synthetase. The enzyme was tested for biosynthetic activity to insure full inactivation.

Pyridoxal phosphate labeling was performed in 50 mM Hepes (pH 7.5), 100 mM KCl, 15 mM $MgCl_2$, and 8 mM pyridoxal phosphate at 25°C with 3 to 60 μM glutamine synthetase for 9 h. A 100-fold excess of sodium borohydride was added to reduce the Schiff base. A molar absorbtivity of 10,000 M^{-1} cm^{-1} liter at 326 nm was used to determine the stoichiometry of pyridoxal phosphate bound per enzyme subunit (*6*).

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 emmission 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. The binding data were analyzed using the method described previously (*8*).

Samples of thiourea trioxide labeled glutamine synthetase for amino acid analysis were prepared in the following manner. Glutamine synthetase (60 μM) was incubated with thiourea trioxide as described above. Another native sample was also prepared. These samples were then dialyzed twice against 10 mM ammonium bicarbonate and then lyophilized. Amino acid analyses were conducted at the University of Pennsylvania in Dr. Ruth Hogue-Angeletti's laboratory. Standards were run that included homoarginine in the amino acid mixture to establish the retention time of this amino acid. All enzyme samples were hydrolyzed in 6 N HCl by standard procedures.

RESULTS AND DISCUSSION

Thiourea trioxide was found to inactivate glutamine synthetase only partially (~25%) in contrast to thiourea dioxide which inactivates the enzyme completely

(*8*) as shown in Fig. 1. Incubations with thiourea trioxide for up to 24 h or additions of fresh aliquots of this compound at various times did not lead to further loss in activity. The coupling enzymes in the assay solution were uneffected by the levels of thiourea trioxide transferred into the assay solution. To determine which amino acid was modified by thiourea trioxide treatment, glutamine synthetase was incubated as described above followed by digestion in 6 N HCl. Amino acid analysis was performed and the results showed the appearance of 2.3 ± 0.3 mol of homoarginine per enzyme subunit in the modified enzyme.

To characterize this reaction more completely, we decided to incubate a simple primary amine with thiourea trioxide and monitor product formation by ^{1}H NMR. From these experiments, we found that methylamine reacts with thiourea trioxide to form methylguanidine (δ = 2.85 ppm) only in the presence of base. An authentic sample of methylguanidine had the same chemical shift. Thus the model reaction and the enzyme reaction are similar since primary amino groups reacted to form guanidino products. Thiourea dioxide previously was found to react with amino groups to form guanidino products and imidazole groups to form a mixture of products (*8*). Since thiourea dioxide can react as an electron transfer reagent and this reaction is thought to proceed through formation of radicals, it is quite possible that these radicals would react with imidazole containing substrates. However, thiourea trioxide does not react in this manner and in model reactions does not react with imidazole groups.

Steady-state kinetic studies were conducted to determine whether thiourea trioxide is a competitive inhibitor of ATP or glutamate in the biosynthetic reaction of glutamine synthetase. Thiourea dioxide was shown previously to be a competitive inhibitor versus glutamate (*8*). The results showed that thiourea trioxide is a mixed type inhibitor with both glutamate and ATP as the varied substrate. The K_{IS} and K_{II} values for thiourea trioxide with glutamate as the varied substrate are 11.0 ± 0.3 and 8.0 ± 0.2 mM, respectively while with ATP as the varied substrate the K_{IS} and K_{II} values are 11.0 ± 0.2 and 6.0 ± 0.1 mM, respectively. Therefore the interaction of thiourea trioxide with various enzyme complexes is more complicated than that found with the dioxide. The sulfonic acid portion of thiourea

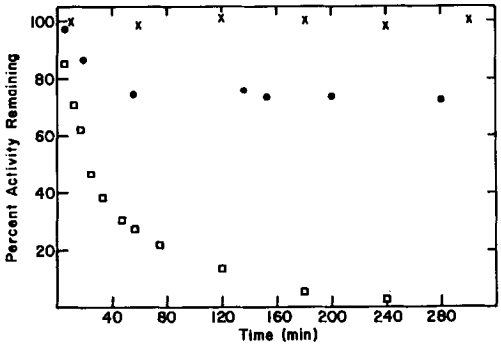

FIG. 1. Time course of inactivation of glutamine synthetase by thiourea dioxide and thiourea trioxide. Solutions contained 3 μM glutamine synthetase plus 10 mM thiourea dioxide (□), or 10 mM thiourea trioxide (●), or no inhibitor (×).

trioxide might bind to the enzyme at the γ-phosphate binding site of ATP and also at the glutamate γ-carboxyl site. These interactions may interfere with ATP binding and thus inhibit formation of an enzyme–ATP–thiourea trioxide complex.

Experiments were performed to determine the number of lysine residues which react with thiourea trioxide and also to determine if thiourea trioxide reacts with the same groups that react with pyridoxal phosphate and thiourea dioxide. Pyridoxal phosphate reacts with two lysine residues under the conditions used here (*8*). One residue was shown to react at a site not associated with activity loss or protection by substrates. The other lysine group is at the glutamate site and is associated with an approximate 80% loss in activity. [^{14}C]Thiourea dioxide also reacts with approximately two lysine residues (*8*) including one group that is not associated with activity loss or substrate protection. The second residue which reacts with thiourea dioxide is associated with the 100% inactivation. This latter group was found to be the same residue which is associated with 80% loss in activity with pyridoxal phosphate. It is possible that a histidine residue was also being modified by thiourea dioxide.

Enzyme modified with pyridoxal phosphate before [^{14}C]thiourea trioxide addition showed 1.2 ± 0.1 mol of [^{14}C]thiourea trioxide bound per enzyme subunit while 2.3 ± 0.2 mol of [^{14}C]thiourea trioxide were bound per enzyme subunit of native enzyme. This indicates that one group modified by pyridoxal phosphate is also labeled with thiourea trioxide. Enzyme modified with FSBA before addition of [^{14}C]thiourea trioxide had 2.6 ± 0.3 mol of radiolabel bound per monomer. This indicates that thiourea trioxide does not covalently bind to Lys-47. Enzyme labeled with thiourea dioxide before addition of [^{14}C]thiourea trioxide had 0.14 ± 0.06 mol of [^{14}C]thiourea trioxide bound per enzyme subunit indicating that thiourea dioxide and thiourea trioxide react with the same amino acid residues.

When glutamine synthetase is incubated with methionine sulfoximine and ATP, an enzyme–ADP–methionine sulfoximine phosphate complex is formed which is stable and inactive (*14, 15*). These conditions were used in prior experiments by us (*8*) to study modification by pyridoxal phosphate and thiourea dioxide. The results showed that formation of the enzyme–ADP–methionine sulfoximine phosphate complex protected against pyridoxal phosphate modification and partially protected against thiourea dioxide modification. Glutamine synthetase was incubated with methionine sulfoximine and ATP prior to the addition of [^{14}C]thiourea trioxide. Incorporation of 2.2 ± 0.2 mol of [^{14}C]thiourea trioxide per enzyme subunit was found. After methionine sulfoximine phosphate and ADP were released from the enzyme complex using the method of Maurizi and Ginsburg (*16*), a 24% loss of activity was observed compared to a control with no thiourea trioxide. Together these data indicate that the complete loss of activity due to incubation of glutamine synthetase with thiourea dioxide is not solely due to lysine modification.

In order to determine the change in the kinetic parameters for glutamine synthetase modified with thiourea trioxide, the K_m values for ATP and glutamate were determined. The K_m values for ATP and glutamate were 210 ± 30 μM and 10 ± 1 mM, respectively. The K_m values for ATP and glutamate with native enzyme were 70 ± 10 μM and 3.3 ± 0.2 mM, respectively. Since the K_m values for these two

substrates change by ~threefold, this argues that each subunit has been modified to the same extent by thiourea trioxide.

Fluorescence titrations of modified enzyme were performed to determine if the binding of glutamate, ATP, or methionine sulfoximine, were affected by reaction with thiourea trioxide. Glutamine synthetase was modified with 10 mM thiourea trioxide for 9 h followed by two dialysis changes (500-fold dilution) of 9 h each. The dissociation constants for the modified enzyme are 0.18, 7.1, and 0.81 mM for ATP, glutamate (in the presence of 2 mM ATP), and methionine sulfoximine, respectively. The native enzyme has been shown to have dissociation constants of 0.16, 2.8, and 0.41 mM for ATP, glutamate (in the presence of 2 mM ATP), and methionine sulfoximine, respectively, under identical conditions. Glutamate binding studies with thiourea trioxide modified enzyme gave a dissociation constant of approximately 5.4 mM. Therefore the binding of ATP is only slightly affected by thiourea trioxide modification. These data are in agreement with data obtained with both pyridoxal phosphate and thiourea dioxide modified enzymes (*8*). Methionine sulfoximine and glutamate binding are affected substantially by thiourea trioxide modification as was the case with pyridoxal phosphate and thiourea dioxide modified enzyme. This is the expected result since it was shown that pyridoxal phosphate, thiourea trioxide, and thiourea dioxide react with the same group in the vicinity of the glutamate binding site.

The above results are consistent with thiourea trioxide reacting with the same lysine residue at the active site of glutamine synthetase as thiourea dioxide and pyridoxal phosphate. Both thiourea trioxide and pyridoxal phosphate modified glutamine synthetase are partially active whereas the thiourea dioxide modified enyzme is completely inactive. This suggests that thiourea dioxide may react with another amino acid group, possibly a histidine, without forming a covalent complex. This is plausible because histidine oxidation results in total inactivation of glutamine synthetase (*17, 18*).

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