Metabolism of the Cysteine S-Conjugate of Busulfan Involves a β-Lyase Reaction


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Received February 2, 2008; accepted May 7, 2008

ABSTRACT:

The present work documents the first example of an enzyme-catalyzed β-elimination of a thiocysteine S-conjugate. β-(S-Tetrahydrothiophenium)-L-alanine (THT-A) is the cysteine S-conjugate of busulfan. THT-A slowly undergoes a nonenzymatic β-elimination reaction at pH 7.4 and 37°C to yield tetrahydrothiophene, pyruvate, and ammonia. This reaction is accelerated by 1) rat liver, kidney, and brain homogenates, 2) isolated rat liver mitochondria, and 3) pyridoxal 5′-phosphate (PLP). A PLP-dependent enzyme in rat liver cytosol that catalyzes a β-lyase reaction with THT-A was identified as cystathionine γ-lyase. This unusual drug metabolism pathway represents an alternate route for intermediates in the mercapturate pathway.

Busulfan is a bifunctional alkylating agent used for the treatment of hematological and other malignancies before stem cell transplantation (e.g., Iwamoto et al., 2004). Busulfan is converted to a glutathione S-conjugate [l-γ-glutamyl-β-(S-tetrahydrothiophenium)-L-alanylglutathione] by direct interaction with glutathione (Ritter et al., 1999) and enzymatic catalysis by glutathione S-transferases, especially glutathione S-transferase A1-1 (Czerwinski et al., 1996; Gibbs et al., 1996; Ritter et al., 1999, 2002). The busulfan-glutathione adduct undergoes a base-catalyzed β-elimination reaction yielding tetrahydrothiophene (Roberts and Warwick, 1961). Oxidation products of tetrahydrothiophene make up the majority of identified busulfan metabolites; although an enzymatic pathway leading to tetrahydrothiophene formation has not been elucidated.

A hypothetical pathway to tetrahydrothiophene from busulfan involved intermediates in the mercapturate pathway that convert glutathione S-conjugates to cysteine S-conjugates via the action of γ-glutamyltransferase and dipeptidase/cysteinyglycinase (Meister, 1989). The mercapturate pathway of busulfan metabolism was shown to occur in rats by the detection of the sulfonium mercapturate, N-acetyl-β-(S-tetrahydrothiophenium)-L-alanine in the urine (Hassan and Ehrrson, 1987). Therefore, it is probable that the busulfan-glutathione adduct is converted in vivo to the corresponding cysteine S-conjugate [β-(S-tetrahydrothiophenium)-L-alanine, THT-A] by enzymes of the mercapturate pathway (Fig. 1). Theoretically, THT-A may also be formed nonenzymatically by the condensation of busulfan and cysteine.

Because THT-A contains a good leaving group (nucleofuge), it is reasonable to predict a facile β-elimination reaction to yield pyruvate, ammonium, and tetrahydrothiophene. Although this reaction is likely to occur slowly nonenzymatically, we hypothesize that one or more cysteine S-conjugate β-lyases will accelerate the reaction. These lyses are pyridoxal 5′-phosphate (PLP)-dependent enzymes that catalyze β-elimination reactions with γ-cysteine S-conjugates [RSHCH(NH$_3^+$)CO$_2^-$]. The enzyme-catalyzed reaction generates an RSH fragment and aminoacrylate [CH$_2$=CH(NH$_3^+$)CO$_2^-$]. The aminoacrylate is an enamine that rapidly tautomerizes to the more stable ketimine tautomer, α-iminopropionate [CH$_2$CH(= NH$_3^+$)CO$_2^-$], followed by hydrolysis to pyruvate [CH$_3$C(O)CO$_2^-$] and ammonium. The net reaction is shown in eq. 1 [see Cooper and Pinto (2006) for a recent review].

$$\text{RSHCH(NH}_3^+\text{)CO}_2^- + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{C(O)CO}_2^- + \text{NH}_3^+ + \text{RSH}$$

(1)

Cystathionine γ-lyase (γ-cystathionase), although primarily a γ-lyase (Greenberg, 1962), also catalyzes β-elimination reactions. For example, rat liver cystathionine γ-lyase catalyzes 1) the formation of S-mercaptopentane S-conjugate from γ-cysteine (Cavalli, 1960), 2) β-elimination of alkane thiols from several cysteine S-conjugates containing nonhalogenated alkyl groups attached to the sulfur (Tomisawa et al., 1988), and 3) β-elimination of allyl/allyl thiol/sulfides from various allyl/allyl cysteine S-conjugates present in garlic extracts (Cooper and Pinto, 2005; Pinto et al., 2006). In the present study, we evaluated the ability of free sulfuric acid to catalyze the β-elimination of the cysteine S-conjugate of busulfan.
(i.e., nonprotein-bound) PLP, rat liver cystathionine γ-lyase, and rat tissue homogenates to catalyze a β-elimination reaction with THT-A.

**Materials and Methods**

**Reagents and Enzymes.** l-Homoserine, l-alanine, PLP, Nα-propargylglycine, Tris, 2,4-dinitrophenylhydrazine, NADH, ADP, bovine serum albumin, protease inhibitor mixture, busulfan, tetrahydrothiophene, and the sodium salts of pyruvate, α-ketoglutarate (KG) and α-keto-γ-methylbutyrate (KMB) were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). OmniSol acetonitrile was high-performance liquid chromatography (HPLC) grade and was obtained from EM Science (Gibbstown, NJ). THT-A was synthesized by adapting previously described methods (Roberts and Warwick, 1961). The positive ion electrospray ionization mass spectrum (Finnigan LCQ mass spectrometer, Thermo Electron Corporation, Waltham, MA) showed a molecular species consistent with the compound mass of THT-A (m/z 176). Tandem mass spectrometry fragmentation gave a daughter ion consistent with the neutral loss of tetrahydrothiophene. The synthesized THT-A used in the experiments contained about 4% molar equivalent of pyruvate as determined by the 2,4-dinitrophenylhydrazone procedure and about 4% tetrahydrothiophene as determined by a CoullArray detection procedure (see below). Aqueous stock solutions of THT-A (50 mM) were stored at −20°C. Some additional pyruvate formation was noted on storage of THT-A at −20°C, but the rate was slow (~1% conversion of THT-A to pyruvate per month). Stock solutions of tetrahydrothiophene (10 mM) were made immediately before use in 50% (v/v) acetonitrile/water.

Highly purified cystathionine γ-lyase (specific activity, 2.4 U/mg; 1.32 U/mg) was isolated from rat liver cytosol by the method of Pinto et al. (2006) and stored frozen at −20°C. SDS-polyacrylamide gel electrophoresis indicated a purity of >98%. The enzyme is stable in 10 mM potassium phosphate buffer, pH 7.4, at −20°C for at least 1 year and to repeated freeze/thawing. Bovine liver glutamate dehydrogenase (40 U/mg in 40% glycerol) was obtained from Boehringer Mannheim (Mannheim, Germany). Rat liver mitochondrial asparagine synthetase (mitAspAT) [1.35 mg/ml in 20 mM Tris-HCl buffer, pH 8.3, containing 0.1 mM EDTA, 150 mM NaCl, and 0.2% (w/v) sodium azide; 410 U/mg protein at 37°C] was a gift from Dr. Ana Iriarte, University of Missouri-Kansas (Kansas City, MO). Rat kidney glutamine transaminase K (GTK) (5 U/mg in 20% glycerol; 0.18 U/ml) was purified from the cytosolic fraction of rat kidneys as described previously (Cooper, 1978).

**Enzyme Assays.** Cystathionine γ-lyase was assayed by a slight modification of the procedure of Cooper and Pinto (2005) in which α-ketobutyrate formed from l-homoserine was measured as the 2,4-dinitrophenylhydrazone.

The standard reaction mixture (20 μl) contained 100 mM potassium phosphate buffer, pH 7.4, 20 mM l-homoserine, and enzyme. The blank contained enzyme but no l-homoserine. After incubation at 37°C, the reaction was terminated by the addition of 10 μl of 5 mM 2,4-dinitrophenylhydrazine in 2 M HCl. After a further 10-min incubation, 170 μl of 1 M NaOH was added, and the absorbance at 430 nm was read within 2 min of addition of alkali against a blank carried through the same procedure. The extinction coefficient of α-ketobutyrate 2,4-dinitrophenylhydrazone under these conditions is 15,000 M⁻¹cm⁻¹.

β-Lyase reactions with THT-A were measured in a reaction mixture (20 μl) containing 100 mM potassium phosphate buffer, pH 7.4, 5 mM THT-A, and enzyme. After incubation at 37°C the reaction was stopped by addition of 10 μl of 5 mM 2,4-dinitrophenylhydrazine in 2 M HCl. After an additional 10-min incubation, 170 μl of 1 M NaOH was added, and the absorbance at 430 nm was read within 2 min. The extinction coefficient of pyruvate 2,4-dinitrophenylhydrazone under these conditions is 16,000 M⁻¹cm⁻¹. The blank contained no enzyme or enzyme source added just before addition of 2,4-dinitrophenylhydrazine reagent. A blank containing no enzyme took into account the small amount of pyruvate formed nonenzymatically from 5 mM THT-A. We cannot rule out the possibility that some β-lyase activity toward THT-A was lost as a result of the initial freezing of the tissue or mitochondrial homogenates. However, subsequent freeze/thawing of rat liver cytosol and mitochondria had no significant effect on the β-lyase activity toward THT-A.

All the spectrophotometric measurements were carried out with a SpectraMax 96-well plate spectrophotometer (Molecular Devices, Sunnyvale, CA). A unit of enzyme activity (U) is defined as the amount of enzyme that catalyzes the formation of 1 μmol product/min at 37°C.

**Reaction of Cysteine with Busulfan.** Busulfan (10 μg) in acetonitrile (50 μl) was added to cysteine (2.0 mg) in 4.9 ml of 100 mM potassium phosphate buffer, pH 7.4. The mixture was vortexed for 1 min and incubated at 37°C. Aliquots (100 μl) removed at 0, 30, 60, 90, 120, and 180 min were diluted with 100 μl of acetonitrile containing [3H]busulfan (5 μg/ml; internal standard) and transferred to HPLC vials. Busulfan was measured according to the method described by Mürder et al. (2001). The chromatography system (Waters 2695, Waters, Milford, MA) used a Luna C8 analytical column (5-μm particle size, 150 × 2 mm i.d.; Phenomenex, Torrance, CA). Elution was carried out using a gradient of 10 mM ammonium acetate and 10 mM acetic acid, and acetonitrile at a flow rate of 0.4 ml/min. Gradients were programmed as follows: 15% acetonitrile at 0 min, increased to 45% over 7 min, decreased to 15% acetonitrile over 0.1 min, and then left at 15% acetonitrile for 3 min to re-equilibrate. Detection was carried out using a Waters Micromass ZMD mass spectrometer equipped with an electrospray source operating in the positive ion mode.
mode. Selected ion monitoring was used to detect the ammonium adduct of busulfan (mlc 264) and \( ^{[2} \text{H}_2 \text{busulfan} \) ammonium adduct (mlc 272).

The disappearance of busulfan with time was measured in an incubation mixture containing a 400-fold molar excess of cysteine over busulfan in 100 mM potassium phosphate buffer (pH 7.4, 37°C). Busulfan decomposed in solution slowly \( k_{\text{busulfan}} = 0.0014/min \) as determined by analysis of products resulting from hydrolysis and adduct formation \( k_{\text{hydrolysis}} = 0.0007/min \).

### Stability of THT-A

The stability of THT-A was evaluated by measuring its rate of disappearance from aqueous solutions (100 µg/ml) at pH 7.4 and 8.0 and 37°C. Aliquots (6 µl) were removed at 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 5, and 6 h and were diluted in 1.5 ml of distilled water, and 50-µl aliquots were injected into a liquid chromatograph/mass spectrometry system consisting of a Waters 2695 HPLC coupled to a Waters 996 photodiode array detector programmed to scan between 200 and 300 nm. Quantification of THT-A was performed using selected ion monitoring at m/z of 176. The mobile phase consisted of methanol/water (50:50, v/v) pumped at 0.4 ml/min through an Agilent Zorbax SB-NC.

### Specific activities (nmol/mg protein/min) of cystathionine γ-lyase in selected rat tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Homogenate</th>
<th>Cytosol</th>
<th>Mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>26.3 ± 0.8</td>
<td>88.6 ± 2.9</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.59 ± 0.09</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Brain</td>
<td>0.27 ± 0.03</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

### Experimental Procedures

**Preparation of Rat Tissue Homogenates.** All the experimental procedures involving preparations of rat tissues were performed in the Dementia Division of the Department of Neurology, Weill Medical College of Cornell University at the Burke Medical Research Institute. The protocols were approved by the Institutional Animal Care and Use Committee (Protocol 0505-367A). Six-month-old male Fisher × Brown Norway F1 rats were fed ad libitum and had full access to water. The rats were sacrificed by decapitation. Liver, kidneys, and forebrains were removed and separately immersed in 50 ml of ice-cold isolation buffer containing 300 mM sucrose, 10 mM HEPES, 0.5 mM EGTA, and 0.5% (w/v) fatty acid-free bovine serum albumin (pH adjusted to 7.4 with Tris base). The tissues were prechilled for ~5 to 6 min. Each tissue sample was separately cut into small pieces with scissors and homogenized in a ~10-fold volume of isolation buffer supplemented with protease inhibitor mixture (1/100 dilution). Liver and kidney tissues were homogenized using a Potter homogenizer. The brains were homogenized using a Dounce homogenizer. All the steps were conducted at 0 to 4°C. The homogenates were divided into several aliquots and stored at −20°C. In some experiments, the liver homogenate was fractionated into cytosolic and mitochondrial fractions and stored at −20°C (Krasnikov et al., 2005). The purity of the mitochondria was assessed by measuring the specific activity of the traditional cytosolic marker lactate dehydrogenase in the cytosolic and mitochondrial fractions of the rat liver. The specific activity of lactate dehydrogenase in preparations of rat liver mitochondria obtained by the above procedure in our laboratory is consistently 3 to 5% that of the cytosolic fraction. Compelling evidence presented by Brooks et al. (1999) indicates that this amount of enzyme is endogenous to mitochondria. By contrast, cystathionine γ-lyase is exclusively cytosolic in rat liver (Allsup and Watts, 1975). Cystathionine γ-lyase activity was not detected in the rat liver mitochondrial preparation used in the present study (Table 1). Thus, by this criterion, our rat liver mitochondrial preparation was essentially free of cytosolic elements.

### Results

**Cystathionine γ-Lyase Catalyzes β-Elimination of Tetrahydrothiophene from THT-A.** Two PLP-containing enzymes, previously shown to catalyze β-elimination reactions with cysteine S-conjugates containing halogenated, electron-withdrawing groups attached to the sulfur, namely, GTK and mitAspAT (Cooper and Pinto, 2006), were tested for their ability to catalyze a β-elimination reaction with THT-A. These enzymes serve as well established reference enzymes that catalyze cysteine S-conjugate β-lyase reactions. However, we were unable to detect any β-lyase activity toward THT-A with GTK and mitAspAT, even when 0.5 mM KMB or 0.5 mM KG was included in the reaction mixture. On the other hand, we showed that highly purified rat liver cystathionine γ-lyase is able to catalyze a β-lyase reaction with THT-A (see below).

The specific activity of the purified rat liver cystathionine γ-lyase...
obtained in the control reaction mixture that lacked enzyme. To pyruvate under the incubation conditions (change in absorbance caused by reductive amination of ammonium was measured by the glutamate dehydrogenase method). Note that there was a small amount of contaminating ammonia in the blank at the zero time. This value was subtracted to calculate net ammonia production. In the absence of this subtraction, the net change in absorbance caused by reductive amination of α-ketoglutarate in the reaction mixture containing enzyme at 1 and 6 h was about 1.9 and 5.4 times the values obtained in the control reaction mixture that lacked enzyme.

Cystathionine γ-Lyase Activity in Rat Tissue Homogenates. To determine whether cystathionine γ-lyase is the only (or predominant) enzyme able to catalyze a β-elimination reaction with THT-A in rat tissues, the specific activity of cystathionine γ-lyase in rat tissue fractions was compared with that of the β-lyase activity toward THT-A. The specific activity of cystathionine γ-lyase is relatively high in rat liver but lower in rat kidney and brain (Table 1). The specific activities of cystathionine γ-lyase in kidney and brain homogenates relative to that in the liver homogenate are about 3 and 1%, respectively (Table 1).

Propargylglycine, although not entirely specific for cystathionine γ-lyase, is nevertheless a potent inhibitor of this enzyme (Washtien and Abeles, 1977). Inclusion of 5 mM dl-propargylglycine in the standard reaction mixture (homoserine as substrate) resulted in no detectable cystathionine γ-lyase activity in rat liver homogenate or cytosol (Table 1).

TABLE 2

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Homogenate</th>
<th>Cytosol</th>
<th>Mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>4.01 ± 0.13*</td>
<td>4.01 ± 0.13*</td>
<td>4.01 ± 0.13*</td>
</tr>
<tr>
<td>No addition</td>
<td>0.69 ± 0.02</td>
<td>1.06 ± 0.29</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>+ 5 mM D,L-Propargylglycine</td>
<td>0.19 ± 0.04*</td>
<td>0.23 ± 0.11*</td>
<td>2.79 ± 0.17**</td>
</tr>
<tr>
<td>+ 0.5 mM KG</td>
<td>0.75 ± 0.06</td>
<td>1.34 ± 0.14</td>
<td>3.66 ± 0.52</td>
</tr>
<tr>
<td>+ 0.5 mM KMB</td>
<td>0.65 ± 0.06</td>
<td>1.23 ± 0.32</td>
<td>2.50 ± 0.33***</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.18 ± 0.09</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>No addition</td>
<td>1.18 ± 0.09</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Brain</td>
<td>0.23 ± 0.03</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>No addition</td>
<td>0.23 ± 0.03</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Significant differences between “no addition” and “addition” values are *p = 0.05, **p = 0.025, and ***p = 0.0025.
β-lyase reaction with THT-A (Table 2) is greater than the relative cystathionine γ-lyase specific activities (Table 1). These findings indicate that kidney and brain also possess enzymes in addition to cystathionine γ-lyase capable of catalyzing β-elimination reactions with THT-A.

The β-lyase activity toward THT-A was strongly, but not totally, inhibited by dl-propargylglycine in the rat liver homogenate and cytosolic fraction. dl-Propargylglycine also significantly inhibited the β-lyase activity in the mitochondrial fraction but proportionately less so than that in the cytosol and homogenate (Table 2). Because dl-propargylglycine completely inhibits cystathionine γ-lyase in rat tissue homogenates (Table 1), these data are also consistent with the hypothesis that enzymes in addition to cystathionine γ-lyase contribute to β-lyase activity with THT-A in rat. Thus, the combined activities of cystathionine γ-lyase and as-yet unidentified β-lyases in liver, kidney, and brain are expected to contribute substantially to busulfan pharmacokinetics and to the metabolic fate of THT-A in vivo.

The β-lyase activity toward THT-A was not significantly stimulated by added KMB or KG in any of the rat liver fractions (Table 2). Interestingly, there was a highly significant inhibition of the β-lyase reaction by 0.5 mM KMB catalyzed by the liver mitochondrial fraction (Table 2). The reason for this inhibition is not known. The cystathionine γ-lyase activity in the rat liver homogenates and cytosolic fraction is not inhibited by 0.5 mM KMB (Table 1). Purified cystathionine γ-lyase is also not inhibited by 0.5 mM KMB (data not shown). Taken together, these data show that rat liver mitochondrial contain at least one enzyme relatively unresponsive to DL-propargylglycine that catalyzes a β-lyase reaction with THT-A.

Stoichiometry of the Cystathionine γ-Lyase–Catalyzed β-Lyase Reaction with THT-A. Figure 2 shows the rate of formation of pyruvate and ammonium from THT-A in the presence and absence of cystathionine γ-lyase. A slow nonenzymatic β-elimination reaction of pyruvate and ammonium from THT-A was noted in the presence of 100 mM potassium phosphate buffer, pH 7.4, at 37°C (about 2–4 nmol/h/100 nmol THT-A) (Table 3). Figure 2 shows that a substantial increase in both pyruvate and ammonium occurs when cystathionine γ-lyase is included in the reaction mixture. The enzyme-catalyzed formation of these products is linear for at least 6 h. Moreover, the rate of enzyme-catalyzed ammonium generation is identical to that of pyruvate production within experimental error. After subtracting the blank, the rate of product (pyruvate, ammonium) formation is ~6.8 nmol/h/12 mU enzyme in the reaction mixture.

As we show below, free PLP can catalyze a β-elimination reaction with THT-A. The cystathionine γ-lyase preparation used in the experiment shown in Fig. 2 contained no added PLP, and no PLP was added to the reaction mixture. Rat liver cystathionase is a homotetramer (M_r of each subunit ~44,000) with each subunit containing tightly bound PLP (Oh and Churchich, 1973). To exclude the possibility that the pyruvate and ammonia formed in the upper curves in Fig. 2 is caused by a nonenzymatic reaction with extraneous PLP derived from the cystathionine γ-lyase preparation, several control experiments were performed. Table 3 shows that the amount of pyruvate in the reaction mixture containing 5 mM THT-A and active cystathionine γ-lyase was significantly greater than in reaction mixtures containing 1) THT-A, 2) THT-A plus boiled enzyme, 3) THT-A plus active enzyme plus 25 mM l-alanine, or 4) THT-A plus active enzyme plus 10 mM dl-propargylglycine. l-Alanine is a strong competitive inhibitor of rat liver cystathionine γ-lyase (K_i, 1.3 mM) relative to substrate L-homoserine (K_m, 20 mM) (Washtien et al., 1977), and as alluded to above, propargylglycine is a potent irreversible inhibitor of the enzyme (Washtien and Abeles, 1977).

Having unequivocally established that cystathionine γ-lyase catalyzes β-elimination of THT from THT-A, we then showed that the enzyme exhibits saturation kinetics. Double reciprocal plots indicated K_m and V_max values of ~2.4 mM and ~25 nmol/min/mg at 37°C in 100 mM potassium phosphate buffer, pH 7.4.

Nonenzymatic β-Elimination Reaction of THT-A in Phosphate Buffers and in the Presence of PLP. The rates of decomposition of 5 mM THT-A at pH 7.4 and 8.0 at 37°C were determined by an HPLC/mass spectrometry method measuring the disappearance of THT-A. Although the products of decomposition of THT-A were the same as those of the enzymatic process (i.e., pyruvate, ammonium, and tetrahydrothiophene), the rate of decomposition was slow at both pH values (<5%/h). Within experimental error, the rate of appearance of tetrahydrothiophene in 0.1 M potassium phosphate buffer, pH 7.4, at 37°C was similar to the rate of appearance of pyruvate and ammonium noted in the blanks shown in Fig. 2, A and B.

The nonenzymatic decomposition of THT-A to pyruvate in phosphate buffer, pH 7.4, was found to be significantly accelerated in the presence of 2.5 μM PLP (Table 4). This study confirmed the ability of free PLP by itself to enhance the β-elimination of THT from THT-A.

### Discussion

Glutathionylation of busulfan generates an unstable sulfonium ion adduct that is a metabolite along a pathway to the major intermediate metabolite, tetrahydrothiophene (Roberts and Warwick, 1961). We hypothesize that although a portion of the busulfan glutathione adduct could be converted nonenzymatically to tetrahydrothiophene, the major metabolic fate of the adduct in liver and other organs in vivo involves THT-A that is formed by the consecutive action of γ-glutamyltransferase and dipeptidase/cysteinylglycinase in the mercapturate pathway. THT-A is a cysteine S-conjugate that has structural

### Table 3

<table>
<thead>
<tr>
<th>Addition</th>
<th>Pyruvate (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THT-A only</td>
<td>15.4 ± 2.7</td>
</tr>
<tr>
<td>THT-A + boiled enzyme</td>
<td>16.1 ± 2.9</td>
</tr>
<tr>
<td>THT-A + active enzyme</td>
<td>22.7 ± 1.1</td>
</tr>
<tr>
<td>THT-A + active enzyme + 25 mM l-alanine</td>
<td>15.4 ± 0.1</td>
</tr>
<tr>
<td>THT-A + active enzyme + 10 mM dl-propargylglycine</td>
<td>15.3 ± 1.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different from the value obtained with boiled enzyme with p = 0.05.

<sup>b</sup> Significantly different from all the other determinations with p = 0.01.

### Table 4

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Pyruvate (nmol)</th>
<th>Ammonium (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>47.6 ± 1.0</td>
<td>48.8 ± 2.4</td>
</tr>
<tr>
<td>1</td>
<td>65.0 ± 2.6</td>
<td>63.0 ± 6.4</td>
</tr>
<tr>
<td>2</td>
<td>88.0 ± 4.0</td>
<td>84.8 ± 3.8</td>
</tr>
<tr>
<td>Plus PLP &lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>51.0 ± 1.0</td>
<td>60.4 ± 12.2</td>
</tr>
<tr>
<td>1</td>
<td>168 ± 1</td>
<td>185 ± 9</td>
</tr>
<tr>
<td>2</td>
<td>248 ± 3</td>
<td>264 ± 6</td>
</tr>
</tbody>
</table>

<sup>a</sup> The blank contained PLP and phosphate buffer but no THT-A.
properties similar to those of β-lyase substrates. Most mammalian cysteine S-conjugate β-lyases described thus far are PLP-containing aminotransferases that catalyze β-eliminations as side reactions (reviewed by Cooper and Pinto, 2006). For these enzymes, a transamination reaction usually competes with the β-lyase reaction. A proposed pathway for the PLP-catalyzed β-elimination of THT-A is shown in Fig. 3. Condensation of THT-A with enzyme-bound PLP forms an aldime intermediate. Loss of the α-proton of the amino acid produces an intermediate described as a quinonoid-carbanion structure (Metzler et al., 1988). Extending the conjugation of the system by elimination of tetrahydrothiophene is likely to be a process that is favored over imine hydrolysis. In the final step, the PLP catalyst is regenerated from the ketimine intermediate in Schiff base linkage to the ε-amino group of an active site lysine residue, yielding aminoacrylate (not shown), which is hydrolyzed to pyruvate and ammonium.

A half transamination results in the conversion of coenzyme PLP to its pyridoxaline 5'-phosphate form. The enzyme in the pyridoxaline 5'-phosphate form cannot support a β-lyase reaction. For the enzyme to catalyze a β-lyase reaction at an optimal rate, an α-keto acid substrate (or additional PLP) must be present in the assay mixture to ensure maximal presence of the PLP form of the coenzyme in the active site (Stevens et al., 1986; Cooper and Pinto, 2006). To determine whether β-lyase–catalyzed reactions with THT-A are dependent on an α-keto acid, in some experiments the β-lyase reaction mixture was supplemented with 0.5 mM KMB or 0.5 mM KG. KMB is a good α-keto acid substrate of GTK, an enzyme that catalyzes a strong β-lyase reaction with the halogenated cysteine S-conjugates, S-(1,1,2,2-tetrafluoroethyl)-l-cysteine and S-(1,2-dichlorovinyl)-l-cysteine (Stevens et al., 1986; Commandeur et al., 2000; Cooper and Pinto, 2006). KG is a good α-keto acid substrate of most other mammalian aminotransferases, including mitAspAT, an enzyme that also exhibits β-lyase activity toward S-(1,1,2,2-tetrafluoroethyl)-l-cysteine and S-(1,2-dichlorovinyl)-l-cysteine (Cooper et al., 2002). β-Elimination reactions with THT-A in the presence of rat tissue homogenates were not significantly stimulated by addition of KG or KMB (Table 2). Thus, either the enzymes responsible for the elimination reaction are not aminotransferases or THT-A possesses such a good leaving group that an aminotransferase reaction cannot compete effectively with a β-lyase reaction at the active site. Moreover, in the present work we were unable to detect β-lyase activity toward THT-A in the presence of GTK or mitAspAT. This finding eliminates GTK and mitAspAT as catalysts in the β-elimination of tetrahydrothiophene from THT-A in rat tissue homogenates. The finding does not, however, eliminate the possibility of other aminotransferases or other PLP-dependent enzymes in the β-elimination of tetrahydrothiophene from THT-A. Indeed, the present work shows that the PLP enzyme cystathionine γ-lyase contributes to the β-elimination of tetrahydrothiophene from THT-A and that as-yet unidentified enzymes in tissue homogenates also contribute to the overall β-elimination.

Rat liver cystathionine γ-lyase exhibits relatively high $K_m$ values toward L-homoserine and L-cystathionine (20 and 3 mM, respectively) (Greenberg, 1962; Washtien et al., 1977). These values are far higher than the concentrations of L-homoserine and L-cystathionine in most mammalian tissues. For example, the concentration of L-cystathionine in human brain is about 1 or 2 mM but is about 20 μM in human kidney and liver (Tallan et al., 1958). The concentration of L-homoserine in rat liver is also very low (Chatagner, 1964). Assuming that the concentration of homoserine in human liver is also low and that the properties of human cystathionine γ-lyase are similar to those of the rat liver enzyme, then the human enzyme is far from saturated with L-homoserine or L-cystathionine in the liver. As noted above, L-alanine binds to rat liver cystathionine γ-lyase with a $K_i$ of 1.3 mM (Washtien et al., 1977). The concentration of L-alanine in rat liver is ∼1 mM (Brosnan et al., 1970). Thus, assuming that human liver is similar to rat liver, some cystathionine γ-lyase active sites may be available for binding of THT-A in the liver of patients administered busulfan.

Because PLP catalyzes a β-elimination reaction with THT-A (Table 4), part of the propargylglycine-insensitive β-lyase activity with THT-A may be, in part, a result of the presence of free PLP in the rat tissue homogenates. However, our experiments with low-$M_i$ fractions obtained from ultramicrofiltrates of rat tissue preparations suggest that although some conversion of THT-A to tetrahydrothiophene may occur spontaneously and via catalysis by low-$M_i$ effectors (including perhaps free PLP), the predominant route for formation of tetrahydrothiophene from THT-A in vivo is enzymatic. In future work, it will be important to determine which enzymes, in addition to cystathionine γ-lyase, are responsible for formation of tetrahydrothiophene from THT-A.

Several biologically important reactions involve conversion of sulfonyl compounds to thioethers. Examples include 1) formation of S-adenosyl-l-homocysteine following transfer of methyl moieties to acceptor molecules, 2) formation of S'-methylthioadenosine from S-adenosyl-l-methionine catalyzed by Esherichia coli S-adenosyl-l-methionine cyclotransferase (Mudd, 1959), 3) spontaneous elimina-

![Fig. 3. Proposed intermediates in the PLP-catalyzed cystathionine γ-lyase mediated conversion of THT-A to tetrahydrothiophene, pyruvate, and ammonium. Rearrangement of the THT-A PLP Schiff base eliminates tetrahydrothiophene from the complex. Hydrolysis provides an enamine analog of pyruvate (not shown) that tautomizes and hydrolyzes to give pyruvate. PLP is bound to the enzyme (Enz) through a lysine residue.](image-url)
tation of S-adenosyl-L-methionine following transamination of S-adeno-
sylmethionine with 7-oxo-8-aminopelargonic acid (Stoner and
Eisenberg, 1975), 4) formation of 5′-methylthioadenosine from de-
carboxylated S-adenosyl-L-methionine during polyamine biosynthesis
(e.g., Wallace et al., 2003), and 5) enzyme-catalyzed or spontaneous
formation of biogeochemically important dimethylsulfide from S-
methyl-L-methionine and dimethylsulfoniopropionate (Cooper and
Hanson, 1994). To the best of our knowledge, however, the present
work is the first to document an enzyme-catalyzed β-elimination
reaction of a thioether from a sullumion cysteine S-conjugate. We
suggest that the enzyme-catalyzed β-elimination of tetrahydrothio-
phene (a thioether) from the cysteine S-conjugate of busulfan (a
sulfonium compound) is an example of a less well known drug
metabolism pathway that should be taken into consideration in the
development of new drugs that have the potential to form sullumion
cysteine S-conjugates.

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