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THE INHIBITION OF 3-MERCAPTOPYRUVATE  
SULFURTRANSFERASE BY THREE  $\alpha$ -KETO ACIDS

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94-08718

ABSTRACT

3-mercaptopyruvate sulfurtransferase (3-MPST: E.C. 2.8.1.2) is an enzyme located in the cytosol and mitochondria of cells and is believed to be involved in the endogenous detoxification of cyanide (CN) because it is capable of transferring sulfur from 3-mercaptopyruvate (3-MP) to CN, forming thiocyanate (SCN). In addition, 3-MPST activity is present in the erythrocyte and cyanide appears to be converted to SCN primarily in the blood, providing further evidence that 3-MPST may make a significant contribution to the endogenous detoxification of cyanide.

*In vitro* studies of 3-MPST were designed to determine the enzymatic rate of SCN production by purified 3-MPST. 3-MPST activity was measured in 220 mM 2-amino-2-methyl-1,3-propanediol buffer (pH 9.5) with 20 mM potassium cyanide and initial 3-MP concentrations ranging from 3.3-39.5 mM (0.10-20.0 mM when corrected for cyanohydrin formation). SCN production was determined by a colorimetric assay at 460 nm. The enzyme kinetic constants were determined to be  $K_m = 0.29 \pm$

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0.04 mM 3-MP and  $V_{max} = 2.12 \pm 0.05$   $\mu$ moles SCN/mg protein/min for 3-MPST (N=4).

Three  $\alpha$ -keto acids which have been proposed as CN antidotes were tested to determine their effect on the activity of 3-MPST. The activity of 3-MPST was determined as described above, except 1 and 3 mM  $\alpha$ -ketoglutarate ( $\alpha$ -KG), 5 and 10 mM  $\alpha$ -ketobutyrate ( $\alpha$ -KB), or 2.5 and 5 mM pyruvate were included in the assay. The effect of the  $\alpha$ -keto acids was examined as percent decrease from the control at 15 mM 3-MP, which is a substrate concentration at which 3-MPST would be at  $V_{max}$ . All of the  $\alpha$ -keto acids tested decreased the activity of 3-MPST. Specifically, 3-MPST activity was decreased 16.9% and 31.1% by 1 and 3 mM  $\alpha$ -KG (N=4), while 5 and 10 mM  $\alpha$ -KB decreased 3-MPST activity 34.2% and 71.4% (N=4), and 2.5 and 5 mM pyruvate decreased 3-MPST activity 25.4% and 34.7% (N=4), respectively. Continuing studies will determine the mechanism of inhibition by the three  $\alpha$ -keto acids in order to provide some indication of what factors are critical to the substrate specificity of 3-MPST. The data presented indicates that the  $\alpha$ -keto acids tested inhibit the CN detoxifying enzyme, 3-MPST. Thus, the results of these studies suggest that  $\alpha$ -keto acids, such as  $\alpha$ -KG,  $\alpha$ -KB and pyruvate, may not be appropriate or efficient prophylactic treatments for CN.

#### INTRODUCTION

The oxidation of cyanide (CN) to thiocyanate (SCN) is the primary *in vivo* biochemical pathway for CN detoxification (1). Two enzymes, thiosulfate:cyanide sulfurtransferase (rhodanese: E.C. 2.8.1.1) and 3-mercaptopyruvate sulfurtransferase (3-MPST: E.C. 2.8.1.2), are considered to be the enzymes primarily responsible for the enzymatic detoxification of CN (2,3). Rhodanese transfers sulfur from a sulfane-sulfur donor molecule to CN (4) while 3-MPST catalyzes the transfer of sulfur from 3-mercaptopyruvate to CN (5), both forming SCN (4,5). It has been suggested that CN toxicity could be mitigated by enhancing the endogenous cyanide detoxification rate via increasing the availability of sulfur donor molecules (6), the activity of enzymes involved in CN detoxification (7,8) or both.

Several models have been proposed for the endogenous CN detoxification process, and 3-MPST has a significant role in each of them. The simplest model proposes that 3-MPST directly transfers sulfur from 3-mercaptopyruvate to CN forming SCN (9). Alternatively, a recent proposal has suggested that 3-MPST transfers sulfur from 3-mercaptopyruvate to albumin in the liver, which then enters the circulation and is available to react with extracellular CN (10). 3-MPST activity is high in erythrocytes (11) and liver (12) in comparison to rhodanese. A pharmacokinetic study has determined that the conversion of CN to SCN appears to occur primarily in the blood or tissue areas in close proximity to blood (13). Thus, the results of this pharmacokinetic study is in agreement with either of these two models. Another proposal that has been made suggests that rhodanese and 3-MPST act in concert to detoxify cyanide, with 3-MPST producing sulfane-sulfur compounds which contribute to the cellular sulfane-sulfur

pool. These sulfane-sulfur compounds produced by 3-MPST, which may be polysulfides, are subsequently utilized by rhodanese as sulfur donors (10, 14). Although the exact role of 3-MPST in the endogenous detoxification process is not known, 3-MPST has a significant role in all of the models proposed for the endogenous CN detoxification process.

To develop new sulfur donor substrates for 3-MPST the factor(s) which determine the substrate specificity of 3-MPST must be determined. Thus, *in vitro* 3-MPST assays were conducted in the presence and absence of three structurally related compounds to 3-mercaptopyruvate (pyruvate,  $\alpha$ -KG and  $\alpha$ -KB) to begin to understand the substrate specificity of 3-MPST.

#### MATERIALS AND METHODS

##### Chemicals

2-oxobutyric acid and 2-oxoglutaric acid were obtained from Fluka. 2-oxopropionic acid, 2-amino-2-methyl-1,3-propanediol (AMPD) and bovine serum albumin (fraction V) were obtained from Sigma Chemical Company. Sodium 3-mercaptopyruvate was purchased from United States Biochemical Corporation. All other chemicals were of analytical grade.

##### Purified 3-Mercaptopyruvate Sulfurtransferase (E.C. 8.2.1.2)

Purified 3-MPST was prepared from bovine kidney under Army contract (DAAD 0589T2752) using a previously described procedure (5). The concentrated enzyme was stored at  $-80^{\circ}\text{C}$ . The concentrated enzyme was diluted prior to assay each day with enzyme dilution buffer (5 mM  $\text{KH}_2\text{PO}_4$ , 0.10 mg/ml BSA, pH 7.4) to give a concentration of approximately 1  $\mu\text{g}$  3-MPST/ $\mu\text{l}$ .

##### Protein Assay

Protein was determined using the BIO-RAD Protein Assay Kit (BIO RAD). Bovine serum albumin was used as standard.

##### 3-Mercaptopyruvate Sulfurtransferase Assay

3-MPST activity was determined by a modification of a previous procedure (7). The standard 3-MPST assay contains 220 mM AMPD (pH 9.5), 3-mercaptopyruvate concentrations ranging from 0.1 to 20 mM, 20 mM potassium cyanide and enzyme (10  $\mu\text{g}$  protein) in a total volume of 0.50 ml. The 3-mercaptopyruvate concentrations were corrected for cyanohydrin formation from the dissociation constant (15). All of the components of the assay except KCN were pre-incubated at  $30^{\circ}\text{C}$  for 15 min, and the assay was initiated by the addition of KCN. The reaction was allowed to proceed for 15 minutes, and was terminated by the addition of 0.25 ml of 38% formaldehyde. Thiocyanate formation due to non-enzymatic reaction was determined as described for the standard 3-MPST assay but enzyme was omitted from the assay. 3-MPST activity was corrected for non-enzymatic activity by subtracting the non-enzymatic from the enzymatic thiocyanate production. Thiocyanate production was determined as a ferric ion complex at 460 nm (7). The standard 3-MPST assay was modified to determine the effect of pyruvate,  $\alpha$ -ketobutyrate and  $\alpha$ -ketoglutarate on the activity of 3-MPST. The assay was performed

as described above, except pyruvate (2.5 and 5 mM),  $\alpha$ -ketobutyrate (2.5 and 5 mM) or  $\alpha$ -ketoglutarate (1 and 3 mM) was added to the assay mixture and pre-incubated as described above.

#### Enzyme Kinetic Analysis

Data from each experiment was fitted to a linear transformation of the Michaelis-Menten equation and estimates of  $V_{max}$  and  $K_m$  were found. These estimates were used as starting values in fitting the data from each experiment again using a nonweighted nonlinear regression on the Michaelis-Menten equation. Goodness-of-Fit was determined by convergence of the sums of squares and a significant F statistic for the nonlinear regression analysis. All analyses were conducted using the SAS system.

#### Statistical Analysis.

Differences in the enzyme kinetic parameters was analyzed by a one-way analysis of variance.

### RESULTS

As seen in Table 1, the activity of 3-MPST was decreased by all three of the  $\alpha$ -keto acids tested, indicating that all were inhibitors of 3-MPST. Specifically,  $\alpha$ -KB decreased both the  $K_m$  and  $V_{max}$  of 3-MPST (Figure 1) suggesting it was a uncompetitive inhibitor with respect to 3-mercaptopyruvate. The ratio of  $K_m/V_{max}$ , which represents the slope of the reciprocal plot ( $1/V$  vs.  $1/[S]$ ) lines, did not differ significantly ( $P>0.05$ ) from the control value of  $0.142 \pm 0.009$  and averaged  $0.159 \pm 0.007$  for 2.5 mM  $\alpha$ -KB and  $0.163 \pm 0.007$  for 5 mM  $\alpha$ -KB (Table 1). Thus, the slopes of the reciprocal plot lines are parallel, indicating that the mechanism of inhibition was uncompetitive (16). The  $K_i$  was calculated using the values of  $V_{max}$ ,  $V_{maxi}$  (apparent  $V_{max}$  in the presence of inhibitor) and the concentration of  $\alpha$ -KB (16) and was determined to be  $6.83 \pm 0.61$  mM.

The enzyme kinetic data for  $\alpha$ -KG was similar to  $\alpha$ -KB in that  $\alpha$ -KG decreased both the  $K_m$  and  $V_{max}$  of 3-MPST (Figure 2) suggesting it was a uncompetitive inhibitor with respect to 3-mercaptopyruvate. Further evidence for this mechanism was obtained from the ratio of  $K_m/V_{max}$ , which did not differ significantly ( $P>0.05$ ) from the control value of  $0.142 \pm 0.009$  and averaged  $0.139 \pm 0.004$  for 1 mM and  $0.149 \pm 0.014$  for 3 mM  $\alpha$ -KG (Table 1). The  $K_i$  was calculated using the values of  $V_{max}$ ,  $V_{maxi}$  and the concentration of  $\alpha$ -KG (16) and was determined to be  $8.07 \pm 1.88$  mM.

In contrast to the kinetic data for  $\alpha$ -KG and  $\alpha$ -KB, pyruvate did not affect the  $K_m$  but did decrease the  $V_{max}$  of 3-MPST (Figure 3), which is indicative of an inhibitor whose mechanism is noncompetitive (16). Specifically, at 2.5 mM pyruvate the  $K_m$  was determined to be  $0.300 \pm 0.013$  mM 3-MP and at 5 mM pyruvate it was  $0.286 \pm 0.009$  mM 3-MP, which did not differ significantly ( $P>0.05$ ) from the control value of  $0.310 \pm 0.014$  mM 3-MP. However, the  $V_{max}$  of 3-MPST was decreased when pyruvate was present, from the control value of  $2.214 \pm 0.062$   $\mu$ moles SCN/mg protein/min to  $1.578 \pm 0.094$   $\mu$ moles SCN/mg protein/min at 2.5 mM

Table 1. The Kinetic Constants  $K_m$ ,  $K_{m1}$ <sup>1</sup>,  $V_{max}$  and  $V_{max1}$ <sup>2</sup> for 3-MPST in the Presence and Absence of Three  $\alpha$ -Keto Acids.

TEST COMPOUND	[INHIBITOR] (mM)	$K_m$ or $K_{m1}$	$V_{max}$ or $V_{max1}$	$K_m/V_{max}$
Control <sup>3</sup>	0.0	0.310 $\pm$	2.214 $\pm$	0.142 $\pm$
		0.014	0.062	0.009
$\alpha$ -KG <sup>4</sup>	1.0	0.275 $\pm$	1.852 $\pm$	0.139 $\pm$
		0.004	0.036	0.004
$\alpha$ -KB <sup>5</sup>	2.5	0.222 $\pm$	1.503 $\pm$	0.149 $\pm$
		0.009	0.031	0.014
pyruvate <sup>6</sup>	2.5	0.271 $\pm$	1.703 $\pm$	0.159 $\pm$
		0.007	0.011	0.007
	5.0	0.183 $\pm$	1.127 $\pm$	0.163 $\pm$
		0.003	0.009	0.007
	2.5	0.300 $\pm$	1.578 $\pm$	0.192 $\pm$
		0.013	0.094	0.008
	5.0	0.286 $\pm$	1.377 $\pm$	0.209 $\pm$
		0.009	0.059	0.009

<sup>1</sup> $K_m$  in the presence of the inhibitor.

<sup>2</sup> $V_{max}$  in the presence of the inhibitor.

<sup>3</sup>Control data between inhibitor compounds were not significantly different ( $P > 0.05$ ) and thus the data were pooled ( $N=14$ ).

<sup>4</sup> $N=5$  at each concentration of  $\alpha$ -KG.

<sup>5</sup> $N=5$  at each concentration of  $\alpha$ -KB.

<sup>6</sup> $N=4$  at each concentration of pyruvate.

FIGURE 1. THE EFFECT OF  $\alpha$ -KETOBUTYRATE ON THE ACTIVITY OF 3-MPST

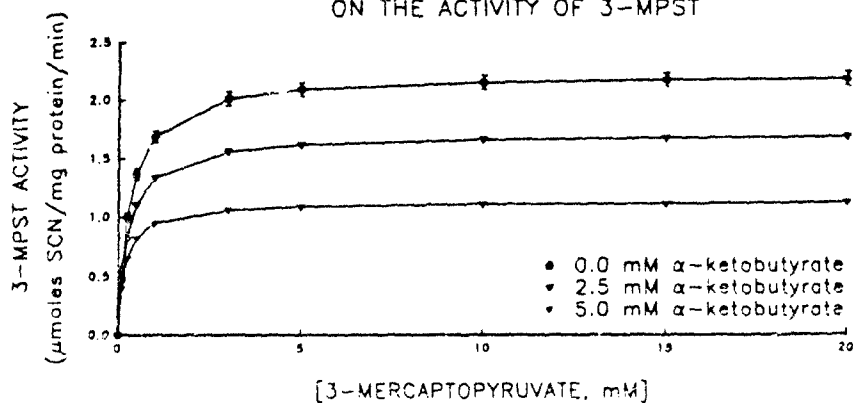


FIGURE 2. THE EFFECT OF  $\alpha$ -KETOGLUTARATE ON THE ACTIVITY OF 3-MPST

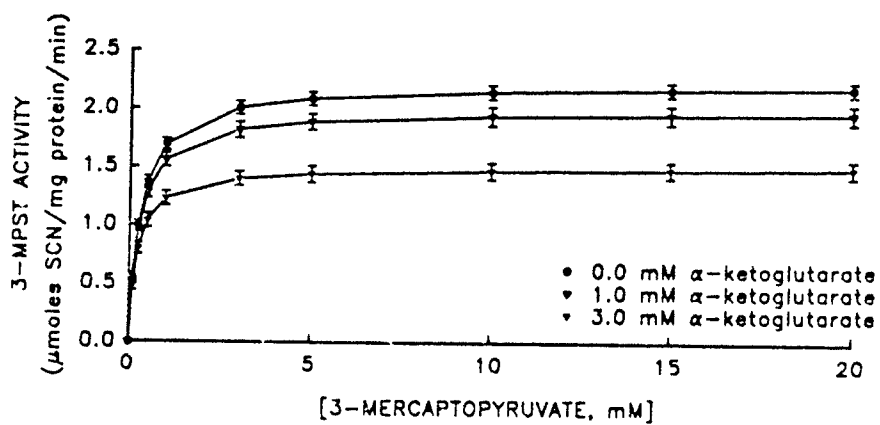
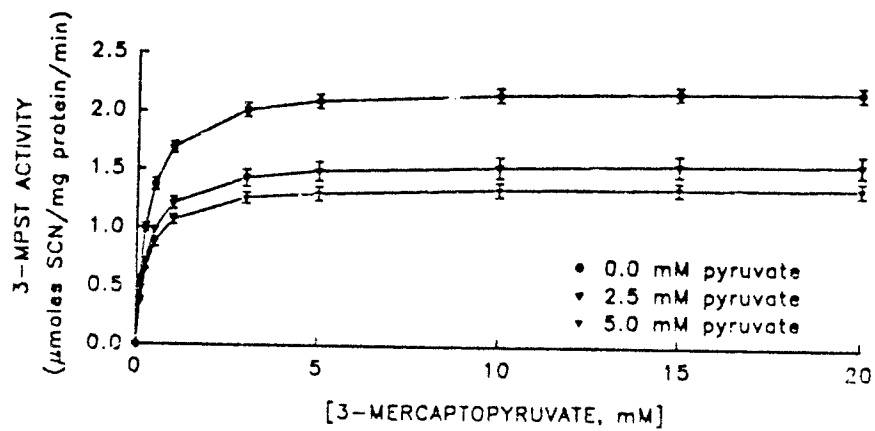


FIGURE 3. THE EFFECT OF PYRUVATE ON THE ACTIVITY OF 3-MPST



pyruvate and  $1.377 \pm 0.059$   $\mu$ moles SCN/mg protein/min at 5 mM pyruvate. The  $K_i$  was calculated using the values of  $V_{max}$ ,  $V_{maxi}$  and the concentration of pyruvate (16) and was determined to be  $7.72 \pm 0.91$  mM.

#### DISCUSSION

Although the exact role of 3-MPST in the endogenous detoxification process is not known, 3-MPST has a significant role in all of the models proposed for the endogenous CN detoxification process. Consequently, it is consistent with any of the proposed models that the inhibition of 3-MPST may have an adverse effect on the endogenous cyanide detoxification process.

The *in vitro* enzyme assays determined that all three of the  $\alpha$ -keto acids examined in this study inhibited 3-MPST. Both  $\alpha$ -KG and  $\alpha$ -KB were uncompetitive inhibitors with respect to 3-mercaptopyruvate. Uncompetitive inhibition is thought to occur when the binding of substrate produces a conformational change in a enzyme, producing and/or unmasking the inhibitor binding site followed by binding of the inhibitor to the enzyme and the subsequent formation of the inactive enzyme-substrate-inhibitor complex (16). Thus, the model of uncompetitive inhibition predicts that the inhibitor binding site is distinct from the substrate binding site. Consequently, the results suggest that  $\alpha$ -KG and  $\alpha$ -KB bind at site(s) independent of the 3-mercaptopyruvate binding site.

In contrast to  $\alpha$ -KG and  $\alpha$ -KB, pyruvate was determined to be a noncompetitive inhibitor with respect to 3-mercaptopyruvate. Two theoretical models can account for noncompetitive inhibition: one proposes that substrate and inhibitor binding are not mutually exclusive events, while the other model proposes that the inhibitor binds first and interferes with substrate binding through a conformational change in the enzyme or sterically blocking the substrate binding site (16). Both models predict that an inactive enzyme-substrate-inhibitor complex will form. Based on the determination that pyruvate is a noncompetitive inhibitor the data suggests that the pyruvate binding site is distinct from that of the binding site for 3-mercaptopyruvate.

In general, the results of the  $\alpha$ -keto inhibition studies indicate that the substrate specificity of 3-MPST for 3-mercaptopyruvate is quite high. This conclusion is in agreement with that of a previous study which examined the substrate specificity of 3-MPST using 16 different potential substrates for 3-MPST (17). More specifically, none of the three  $\alpha$ -keto acids directly competes with 3-mercaptopyruvate for binding to 3-MPST even though they are structurally very similar to 3-mercaptopyruvate. Particularly interesting is the determination that pyruvate, which is very similar to 3-mercaptopyruvate in structure, is a noncompetitive inhibitor and not a competitive inhibitor, suggesting that the thiol group of 3-mercaptopyruvate is an important determinant of the substrate specificity of 3-MPST.

The results of this study provide the first evidence of what factors are important in the substrate specificity of 3-MPST. Based on the mechanism of inhibition determined for the three  $\alpha$ -keto acids

tested, particularly pyruvate, it appears that the thiol group of 3-mercaptopyruvate is an important determinant of the substrate specificity of 3-MPST. Continuing studies will determine what additional factor(s) also affect the substrate specificity of 3-MPST. This information will allow a more rational approach to be used in the selection of potential CN antidotes whose mechanism will be via increasing the activity of the CN detoxifying enzyme 3-MPST.

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