INHIBITION OF γ-GLUTAMYLCYSTEINE SYNTHETASE BY CYSTAMINE; AN APPROACH TO A THERAPY OF 5-OXOPROLINURIA (PYROGLUTAMIC ACIDURIA)

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SUMMARY γ-Glutamylcysteine synthetase is strongly inhibited by cystamine; thus, 20 μM cystamine inhibited the activity by 50%. Inhibition is rapid and the inhibited enzyme is reactivated by dithiothreitol suggesting that cystamine reacts with an enzyme sulfhydryl group. Inhibition by cystamine is not prevented by MgATP, L-α-aminobutyrate, or L-glutamate suggesting that cystamine may not interact at the active site. Little or no inhibition was observed with N,N'-diacetyl cystamine, L-cystine, glutathione disulfide, 2-hydroxyethyl disulfide, and thioglycolate disulfide, whereas thiocholine disulfide produced moderate inhibition. Cystamine or an inhibitory analog of cystamine might be useful in the therapy of the disease 5-oxoprolinuria in which there is an overproduction of γ-glutamylcysteine.

Patients with 5-oxoprolinuria have a generalized deficiency of glutathione synthetase (1) and consequently of glutathione (GSH), which normally serves as a competitive feedback inhibitor of γ-glutamylcysteine synthetase (2,3). In the presence of markedly decreased intracellular levels of glutathione, γ-glutamylcysteine is overproduced in very large amounts and converted by γ-glutamyl cyclotransferase to 5-oxoproline and cysteine. The formation of 5-oxoproline exceeds the capacity of 5-oxoprolinase (4) to convert it to glutamate, and therefore 5-oxoproline accumulates. The symptoms of 5-oxoprolinuria are therefore considered to be due to the primary deficiency of glutathione and excessive formation of 5-oxoproline. Ideal treatment of this disease would consist of supplying intracellular glutathione, but such therapy is not yet available; GSH is not transported to a significant extent across cell membranes. However, a drug that would inhibit overproduction of 5-oxoproline (by inhibition of γ-glutamylcysteine synthetase or of γ-glutamyl cyclotransferase) might be helpful in alleviating episodes of severe acidosis, which occur in 5-oxoprolinuria (5,6). In previous studies (3) in which several compounds were tested as inhibitors of 5-oxoproline formation from glutamate in erythrocyte extracts, cysteamine was found to be a

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Table I. γ-Glutamyl Cyclotransferase Activity*

Compound Added	5-Oxoproline Formed nmol
None	687
Cystamine (1 mM)	687
Cysteamine (5 mM)	801
Dithiothreitol (5 mM)	795

*The reaction mixtures contained (final volume, 0.1 ml) 0.125 M Tris-HCl buffer (pH 8.0), 15 mM L-γ-[^{14}C]glutamyl-L-α-aminobutyrate, enzyme (0.008 Unit), and the indicated compound; the formation of 5-oxoproline was determined after incubation for 90 min at 37° (9).

Table II. Effect of Cystamine on γ-Glutamylcysteine Synthetase*

Cystamine Concentration	Pi Formed nmol	Inhibition %
None	335	[0]
20 μM	185	45
40 μM	114	66
100 μM	55	84
200 μM	30	91
400 μM	18	95
10 mM	2	100

*The reaction mixtures (final volume, 0.5 ml) contained enzyme (0.022 Unit), 0.1 M Tris-HCl buffer (pH 8.2), 10 mM ATP, 20 mM $MgCl_2$, 10 mM L-glutamate, 10 mM L-α-aminobutyrate, 0.1 mM EDTA, cystamine, and 0.05 mg of bovine serum albumin. The enzyme was added last. After incubation at 37° for 15 min, Pi formation was determined (8).

good inhibitor; under the conditions employed, about 50% inhibition of 5-oxoproline formation was observed with 30 μM cysteamine. In the present work, this interesting observation was pursued to learn whether cysteamine inhibits γ-glutamylcysteine synthetase or γ-glutamyl cyclotransferase. We have found that cystamine (produced by oxidation of cysteamine) is the actual inhibitor and that cystamine inhibits γ-glutamylcysteine synthetase. The striking inhibition of γ-glutamylcysteine synthetase by cystamine is of interest in relation to the mechanism of action and the control of the activity of this enzyme. The findings may have therapeutic significance in 5-oxoprolinuria.

EXPERIMENTAL Cystamine·di HCl, 2-hydroxyethyl disulfide, thioglycolic acid disulfide and cysteamine·HCl were obtained from Aldrich. L-cysteine, L-α-aminobutyric acid, L-cystine, dithiothreitol and glutathione disulfide were obtained from Sigma. Glutathione was obtained from CalBiochem. N,N'-Diacetylcystamine was prepared by acetylation of cystamine with acetic anhydride in glacial acetic

acid. Thiocholine disulfide was prepared by treating cystamine with excess CH_3I in aqueous methanol to which portions of NaOH were added to maintain alkalinity.

γ-Glutamyl cyclotransferase, prepared from sheep brain, was kindly supplied by Dr. Vaira Wellner. γ-Glutamylcysteine synthetase was purified from rat kidney through the DEAE-cellulose step (7). The enzyme activities were determined as indicated in the tables; Pi was determined essentially as described by Fiske and Subbarow (8). The conversion of L-[^{14}C]glutamate to 5-oxo-L-[^{14}C]proline catalyzed by cell-free extracts of erythrocytes was studied essentially as described (3).

RESULTS Previous studies showed that cell-free extracts of erythrocytes catalyze the conversion of L-[^{14}C]glutamate to 5-oxo-L-[^{14}C]proline in the presence of MgATP and either L-α-aminobutyrate or L-cysteine (3); the data indicated that this transformation was catalyzed by 2 reactions of the γ-glutamyl cycle, i.e., those catalyzed by γ-glutamylcysteine synthetase and γ-glutamyl cyclotransferase. The effect of cysteamine in this system is described in Fig. 1; in the presence of L-α-aminobutyrate, cysteamine produced substantial inhibition, whereas much less inhibition was found when L-α-aminobutyrate was replaced by L-cysteine. The mechanism by which cysteamine inhibits the formation of 5-oxoproline from glutamate in this system was examined in studies with purified preparations of γ-glutamylcysteine synthetase and γ-glutamyl cyclotransferase.

As shown in Table I, cysteamine did not inhibit γ-glutamyl cyclotransferase, but produced moderate activation as did dithiothreitol; under these conditions cystamine did not affect the activity. Activation of γ-glutamyl cyclotransferase by various thiols has been observed (10).

Experiments on the effect of cysteamine on γ-glutamylcysteine synthetase showed that this compound at concentrations from 20 μM to 1 mM were not inhibitory. On the other hand, cystamine inhibited markedly; as shown in Table II, 20 μM cystamine produced about 50% inhibition, and about 90% inhibition was observed with 0.2 mM cystamine. The findings are in general agreement with those found in the erythrocyte extract system (3), under the conditions of which (incubation at pH 7.5 and 37° for 2 h) much of the added cysteamine is oxidized to cystamine. In the present studies, the enzyme was preincubated with 100 μM cystamine in 0.1 M imidazole-HCl buffer (pH 7.2) for 10 min in the presence and

Table III. Effect of Cystamine and Other Disulfides on γ-Glutamylcysteine Synthetase*

Disulfide Added (0.2 mM)		Pi formed nmol	Inhibition %
None	(Control)	317	[0]
Cystamine	$H_2N-CH_2-CH_2-S-S-CH_2-CH_2-NH_2$	20	94
Thiocholine disulfide	$(CH_3)_3\overset{+}{N}-CH_2-CH_2-S-S-CH_2-CH_2-\overset{+}{N}(CH_3)_3$	203	36
N,N'-Diacetylcystamine	$CH_3\underset{\underset{O}{\|}}{C}NH-CH_2-CH_2-S-S-CH_2-CH_2NH\underset{\underset{O}{\|}}{C}CH_3$	308	3
Cystine	$H_2N-\underset{\underset{COOH}{\|}}{CH}-CH_2-S-S-CH_2-\underset{\underset{COOH}{\|}}{CH}NH_2$	315	0
2-Hydroxyethyl disulfide	$HO-CH_2-CH_2-S-S-CH_2-CH_2OH$	310	2
Thioglycolate disulfide	$HOOC-CH_2-S-S-CH_2-COOH$	285	10
Glutathione Disulfide	$(\gamma\text{-glu-cy}\underset{\underset{\|}{}}{S}\text{-gly})_2$	319	0

*The conditions were the same as given in Table II.

absence of (a) 5 mM L-glutamate, (b) 5 mM L-α-aminobutyrate, or (c) 5 mM ATP + 10 mM $MgCl_2$. Under these conditions, the added compounds did not protect against inactivation by cystamine. When the enzyme was incubated with 10 μM cystamine in imidazole buffer at pH 7.2, inhibition was complete with 30 sec. After 10 min, addition of 10 mM dithiothreitol to the completely inhibited enzyme led to restoration of 67% of the initial activity within 1 min; thereafter the activity declined slowly (about 1% per min) presumably due to inactivation by dithiothreitol (11).

Studies on the effects of various thiols and disulfides on γ-glutamylcysteine synthetase are summarized in Table III. It is notable that the N,N'-diacetyl derivative of cystamine did not inhibit, nor did cystine, glutathione disulfide, and 2-hydroxyethyl disulfide; only slight inhibition was found with thioglycolate disulfide. Under these conditions 10 mM cysteamine inhibited

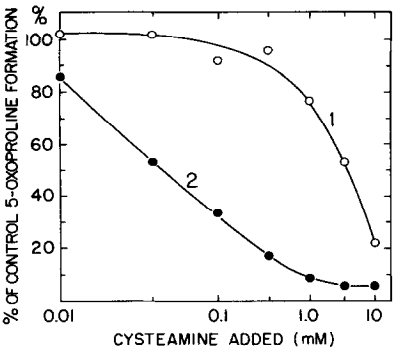

Figure 1. Effect of adding cysteamine on formation of 5-oxoproline from glutamate by cell-free extracts of erythrocytes. The reaction mixtures (0.5 ml) contained 2.5 mM L-[^{14}C]glutamate, 20 mM L-α-aminobutyrate (curve 1) or 4 mM L-cysteine (curve 2), 4 mM ATP, 4 mM $MgCl_2$, 50 mM Tris-acetate (pH 7.5) and erythrocyte extract (3); 5-oxoproline was determined after incubation for 2 h at 37° (3).

about 20%; this result may be ascribed in part to oxidation of this thiol to cystamine. Under these conditions moderate inhibition (15-22%) was found with 10 mM L-cysteine, 2-mercaptoethanol, and dithiothreitol (in general confirmation of earlier findings (11)); about 40% inhibition was observed with glutathione. It is of interest that the inhibition by cystamine is substantially greater than that found with glutathione, which is probably the physiologically significant feedback inhibitor of γ-glutamylcysteine synthetase (2).

DISCUSSION The findings show that cystamine inhibits γ-glutamylcysteine synthetase very strongly and evidently rather specifically. This effect seems to elucidate the observation (3) that addition of cysteamine decreases 5-oxoproline formation from glutamate in erythrocyte extracts. In support of this conclusion it was shown that when L-α-aminobutyrate was replaced by L-cysteine in the erythrocyte extract system, the efficiency of cysteamine as an inhibitor was decreased by about two orders of magnitude (Fig. 1). Several aspects of the inhibition by cystamine seem notable. Cystamine inhibits markedly at low concentrations. The inhibition is rapid, apparently instantaneous and the inhibited enzyme is reactivated by addition of dithiothreitol. The findings suggest that

cystamine interacts with the enzyme to yield a mixed disulfide whose formation reversibly decreases enzymatic activity. Inhibition by cystamine was not prevented by addition of MgATP, L-α-aminobutyrate, or L-glutamate suggesting that cystamine may not interact at the active site of the enzyme. The available data on the specificity of inhibition by cystamine suggest that inhibition is favored by the presence of two positively charged groups (Table III); thus, the corresponding N,N'-diacetyl derivative, cystine, 2-hydroxyethyl disulfide, and thioglycolate disulfide were relatively weak inhibitors or were not inhibitors, whereas thiocholine disulfide inhibited.

Further studies on the inhibition by cystamine and studies on its binding to the enzyme may be of significance in the elucidation of the structure-function relationships of this enzyme. Thus, it will be of importance to establish the mode, site, and stoichiometry of the binding of cystamine to the enzyme. It is conceivable that cystamine may play a role in the physiological control of the activity of γ-glutamylcysteine synthetase.

Understanding of the mechanism of 5-oxoproline overproduction in 5-oxoprolinuria makes it possible to consider rational approaches to the therapy of this disease. Thus, administration of a compound that would produce partial inhibition of γ-glutamylcysteine synthetase might be of value in decreasing production of 5-oxoproline. The present findings suggest that cystamine might be useful for such a purpose. However, a number of factors including, for example, the rate of *in vivo* reduction of cystamine to cysteamine, need to be evaluated. Derivatives that might yield cystamine *in vivo* such as the corresponding N,N'-diacetyl derivative might also be considered. It is also possible that certain analogs of cystamine might be useful as *in vivo* inhibitors of γ-glutamylcysteine synthetase.

Cysteamine and derivatives of cysteamine have been administered as protectants against ionizing radiation and alkylating reagents, and more recently as a therapy for paracetamol toxicity (see, for example 12-15). Such treatment is based on the premise that the administered thiol acts to supplement tissue thiols (chiefly GSH) in neutralizing toxicity. However, the present findings indicate that cysteamine

(via cystamine) can inhibit glutathione formation, a result suggesting that therapy with other thiols might be more advantageous.

Another possible approach to decreasing 5-oxoproline formation in 5-oxoprolinuria would be to inhibit γ-glutamyl cyclotransferase. This might be accomplished by administration of compounds such as β-glutamyl-α-aminobutyrate and D-γ-glutamyl-L-α-aminobutyrate, which have been found to inihibit γ-glutamyl cyclotransferase in vitro (9). Inhibition of γ-glutamyl cyclotransferase would be expected to lead to accumulation of γ-glutamylcysteine. Although it is unlikely that γ-glutamylcysteine would function fully as a physiological substitute for glutathione, this dipeptide can serve effectively in trenspeptidation reactions and its presence would increase the total intracellular concentration of thiols.

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