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On the active site thiol of γ-glutamylcysteine synthetase: Relationships to catalysis, inhibition, and regulation

(glutathione/cystamine/*Escherichia coli*/kidney/enzyme inactivation)

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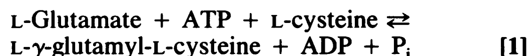
ABSTRACT **γ-Glutamylcysteine synthetase (glutamate–cysteine ligase; EC 6.3.2.2) was isolated from an *Escherichia coli* strain enriched in the gene for this enzyme by recombinant DNA techniques. The purified enzyme has a specific activity of 1860 units/mg and a molecular weight of 56,000. Comparison of the *E. coli* enzyme with the well-characterized rat kidney enzyme showed that these enzymes have similar catalytic properties (apparent K_m values, substrate specificities, turnover numbers). Both enzymes are feedback-inhibited by glutathione but not by γ-glutamyl-α-aminobutyrylglycine; the data indicate that glutathione binds not only at the glutamate binding site but also at a second site on the enzyme that interacts with the thiol moiety of glutathione but not with a methyl group. Both enzymes are inactivated by buthionine sulfoximine in the presence of ATP, suggesting a common γ-glutamyl phosphate intermediate. However, unlike the rat kidney enzyme that has an active center thiol, the bacterial enzyme is insensitive to cystamine, γ-methylene glutamate, and *S*-sulfo amino acids, indicating that it does not have an active site thiol. Thus, the rat kidney and *E. coli* enzymes share several catalytic features but differ in active site structure. If the active site thiol of the rat kidney enzyme is involved in catalysis, which seems likely, there would appear to be differences in the mechanisms of action of the two γ-glutamylcysteine synthetases.**

The first step in the synthesis of glutathione is catalyzed by γ-glutamylcysteine synthetase (glutamate–cysteine ligase; EC 6.3.2.2) [Reaction **1** (ref. 1, pp. 671–697)]. This reaction, usually the rate-limiting step in glutathione synthesis, is feedback-inhibited by glutathione (2). γ-Glutamylcysteine synthetase, like glutamine synthetase (ref. 1, pp 699–754, and refs. 3 and 4), is inactivated by methionine sulfoximine in the presence of ATP (5). Methionine sulfoximine and certain other sulfoximines, such as buthionine sulfoximine [which inhibits γ-glutamylcysteine synthetase but not glutamine synthetase (6–9)], are phosphorylated by ATP on the enzymes; the phosphorylated sulfoximines bind tightly, but noncovalently, to the enzymes, thus producing inhibition.



L-Glutamate + ATP + L-cysteine ⇄
L-γ-glutamyl-L-cysteine + ADP + P_i **[1]**

The presence of a thiol at the active site of γ-glutamylcysteine synthetase was suggested by the finding (10) that highly purified γ-glutamylcysteine synthetase from rat kidney is inactivated by low concentrations of L-2-amino-4-oxo-5-chloropentanoate (11), that such inactivation is associated with covalent binding of the inactivator, and that glutamate protects against inactivation. Subsequently it was found that the enzyme is strongly inactivated by cystamine (12–14) and that inactivation by cystamine is reversed by treatment with dithiothreitol, suggesting that cystamine forms a mixed disulfide between cysteamine and an enzyme thiol (15). Inactivation of the enzyme by the L- and D-isomers of 3-amino-1-chloro-2-pentanone, as well as that by cystamine, is prevented by L-glutamate (14). Treatment of the enzyme with cystamine prevents its interaction with the sulfoximines. Titration of the enzyme with 5,5′-dithiobis(2-nitrobenzoate) reveals that the enzyme has a single exposed thiol that reacts with this reagent without affecting activity (16). 5,5′-Dithiobis(2-nitrobenzoate) does not interact with the thiol that reacts with cystamine. Evidence has also been obtained that γ-methylene-D-glutamate, which inactivates the enzyme, binds in the glutamate binding site of the active center and forms a covalent linkage with the active site thiol through a Michael-type addition reaction (17). The enzyme is also inactivated by *S*-sulfocysteine and *S*-sulfohomocysteine; these inactivators are tightly but noncovalently bound and their interaction with the enzyme is postulated to involve effects of the active site thiol (18). These and earlier (14) observations have led to the suggestion that the active center thiol may play a role in the mechanism of action of this enzyme.

In contrast to the results summarized above obtained in studies on rat kidney γ-glutamylcysteine synthetase, the findings on the γ-glutamylcysteine synthetase of *Escherichia coli*, reported here, indicate that the bacterial enzyme has properties that do not reflect the presence of an active center thiol. Nevertheless, the bacterial enzyme exhibits a number of characteristics that closely resemble those of the mammalian enzyme, including, for example, high turnover number, feedback inhibition by glutathione, and inactivation by incubation with buthionine sulfoximine in the presence of ATP. A preliminary account of this work has been presented (*; ref. 19).

EXPERIMENTAL PROCEDURES

Materials. Agarose-hexane-ATP (N^6; type 2), CM-Sephadex C-50, Sephadex G-100, and Sephadex G-150 were purchased from Pharmacia. DE-52 was obtained from Whatman. L-Buthionine-(*SR*)-sulfoximine (6–8), *S*-sulfocysteine (18), *S*-sulfohomocysteine (18), and γ-methylene glutamate (17) were synthesized as described. Other compounds were obtained from Sigma.

Microorganisms. Previously a strain of *E. coli* enriched by recombinant DNA techniques in its content of the two synthetases required for glutathione synthesis was described (20, 21). The genes for the synthetases were isolated and inserted into a vector that was used to transform the original strain. The transformed strain, in an immobilized form, has been used for the synthesis of isotopically labeled glutathione (22) and for the synthesis of glutathione analogs (23). In the present work a bacterial strain enriched in its content

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of γ-glutamylcysteine synthetase (C600/pGS100, designated here as strain KM) was used; this was obtained by transforming the wild strain *E. coli* K-12 (C600, designated here as strain W) with a plasmid containing the gene for this enzyme. The bacterial strains were kindly provided by Kousaku Murata (Research Institute for Food Science, Kyoto University). *E. coli* strain KM was grown as described (22, 23) in the presence of chloramphenicol (20 μg/ml); the transforming vector confers resistance to this antibiotic. *E. coli* strain W was grown in the same medium lacking chloramphenicol.

γ-Glutamylcysteine Synthetase Activity. The formation of ADP was followed spectrophotometrically by coupled assay with pyruvate kinase and lactate dehydrogenase (24). The reaction mixture (final volume, 1.0 ml) contained Tris·HCl buffer (100 mM; pH 8.2), sodium L-glutamate (10 mM), L-α-aminobutyrate (10 mM), $MgCl_2$ (20 mM), Na_2ATP (5 mM), sodium phospho*enol*pyruvate (2 mM), KCl (150 mM), NADH (0.2 mM), pyruvate kinase (5 units), lactate dehydrogenase (10 units), and the enzyme sample. The rate of decrease in absorbance at 340 nm was followed at 37°C. The formation of γ-glutamyl-α-aminobutyrate under these conditions was confirmed by analysis using a Durrum amino acid analyzer. A unit of enzyme activity is defined as the amount that catalyzes formation of 1 μmol of product per hr. Specific activity is expressed as units/mg of protein. Protein was determined by the Bradford (25) method; bovine serum albumin was used as the standard.

Isolation of γ-Glutamylcysteine Synthetase. The enzyme was isolated by a procedure similar to that used for isolation of the enzyme from rat kidney (24, 26); an important step involves chromatography on ATP-agarose (26). All steps were carried out at 4°C. *Step 1*: Cells (40 g), obtained from 10 liters of medium, were suspended in 200 ml of Tris·HCl buffer (50 mM; pH 7.4) containing 5 mM $MgCl_2$. After disruption by sonication (Sonifier cell disruptor, model W185 D; 80 W; 3 min), the solution was freed of particulate material by centrifugation (15,000 × *g*; 30 min). *Step 2*: $(NH_4)_2SO_4$ was added to 40% of saturation (24.3 g/100 ml), and the precipitate was removed by centrifugation. $(NH_4)_2SO_4$ was added to 80% of saturation (31.8 g/100 ml). The precipitate was collected by centrifugation and dissolved in Tris·HCl buffer (50 mM; pH 7.4) containing 5 mM $MgCl_2$. This solution was dialyzed against four changes of 4 liters each of the same buffer. *Step 3*: Protamine sulfate (10%; pH 7) was added (50 μl/ml) and the precipitate that formed was removed by centrifugation. The supernatant solution was dialyzed against the same buffer. *Step 4*: The dialyzed enzyme was applied to a DE-52 column (2.4 × 20 cm) equilibrated with Tris·HCl buffer (50 mM; pH 7.4) containing 5 mM $MgCl_2$ and 5 mM L-glutamate. The column was washed with 120 ml of this buffer and the enzyme was eluted with a linear NaCl gradient established between 400 ml each of buffer and buffer containing 0.2 M NaCl. The flow rate was 60 ml/hr; fractions of 10 ml were collected. Fractions containing enzyme activity were combined and concentrated to about 5 ml by use of an Amicon concentrator. *Step 5*: This solution was applied to a Sephadex G-100 column (2.54 × 100 cm) equilibrated with 4-morpholinepropanesulfonic acid (Mops) buffer (50 mM; pH 7.0) containing 5 mM L-glutamate. The flow rate was 40 ml/hr; 5-ml fractions were collected. Fractions containing enzyme activity were pooled. *Step 6*: $MnCl_2$ was added to a final concentration of 5 mM and the solution was then applied to an ATP-agarose column (0.5 × 3.9 cm) equilibrated with Mops buffer (50 mM; pH 7.0) containing 5 mM L-glutamate and 5 mM $MnCl_2$. The flow rate was 30 ml/hr. After adding 24 ml of the starting buffer, 24 ml of this buffer containing 0.7 M NaCl and 10% glycerol was applied. Enzyme was eluted with Mops buffer (50 mM; pH 7.0) containing 5 mM $MgCl_2$, 0.2 M NaCl, and 1 mM ATP. Fractions containing activity were pooled and concentrated to 12 ml with an Amicon concentrator and then dialyzed against two changes of 4 liters each of imidazole buffer (10 mM; pH 8.4) containing 1 mM EDTA. The overall purification was 470-fold (Table 1). γ-Glutamylcysteine synthetase was isolated from *E. coli* (strain W) by following the procedure described above through the first three steps. The enzyme was then chromatographed on CM-Sephadex C-50; the active fractions were combined and applied to the ATP-agarose column as described above. After washing with 24 ml of starting buffer, the enzyme was eluted with Mops buffer (50 mM; pH 7.0) containing 5 mM $MgCl_2$ and 1 mM ATP. A high salt concentration was avoided because it was found to inactivate the enzyme. The enzyme, purified about 70-fold, exhibited a specific activity of 31.1 units/mg.

Properties of the Enzymes Isolated from *E. coli*. The purified enzymes from *E. coli* strain KM and *E. coli* strain W exhibited a molecular weight of 56,000 as determined by gel filtration; these findings are consistent with the calculated molecular weight (57,000) obtained from the gene sequence (27). Molecular weights were estimated by gel filtration on Sephadex G-150 by using standards consisting of catalase, bovine serum albumin, carbonic anhydrase, and cytochrome *c* (molecular weights, 250,000, 67,000, 29,000, and 12,000, respectively).

Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (28) using 7.5% gels in the presence or absence of $NaDodSO_4$. Slab gels (12 × 13.5 cm with 0.75-cm lanes) were run at 70 mA per slab gel in 25 mM Tris·HCl buffer (pH 8.3) containing 192 mM glycine with or without 0.1% $NaDodSO_4$. Protein bands were stained with Coomassie blue.

Amino Acid Analysis. The enzyme (100 μg) was oxidized with performic acid by dissolving it in 30 μl of methanol, 10 μl of formic acid, and 60 μl of performic acid (a mixture of 950 μl of 95% formic acid and 50 μl of 5% H_2O_2 that had been placed at room temperature for 2 hr). The mixture was incubated at −5°C for 2.5 hr. Performic acid-treated samples, together with untreated samples, were lyophilized and hydrolyzed with HCl containing 1% phenol at 110°C for 24 hr by using a vapor-phase system (Waters Associates). After removal of HCl, samples were derivatized with phenylisothiocyanate (29). Separation of the phenylthiocarbamoyl derivatives was achieved by a Pico-Tag amino acid analysis system (Waters Associates). Amino acids were quantitated by using a standard mixture of amino acids containing L-cysteic acid. The determined amino acid composition of the enzyme isolated from *E. coli* strain KM is consistent with that deduced from the gene sequence. The analyses for half-cystine indicated 8 mol/mol of enzyme (compared to 9 mol/mol as deduced from the gene sequence).

Kinetic Constants and Specificity. The apparent K_m values for L-glutamate, L-α-aminobutyrate, and L-cysteine (determined by double-reciprocal plots) for the enzyme isolated from *E. coli* strain W and *E. coli* strain KM were 0.7, 1.3, and 0.1 and 1.7, 1.4, and 0.2 mM, respectively. The corresponding values for the rat kidney enzyme (1, 24) are 1.6,

Table 1. Isolation of γ-glutamylcysteine synthetase from *E. coli* strain KM

Step	Volume, ml	Protein, mg	Activity, units	Specific activity, units/mg
1. Extract*	246	2760	10,900	3.95
2. $(NH_4)_2SO_4$	38	951	9,910	10.4
3. Protamine	38	401	9,180	22.9
4. DE-52	184	79	6,220	78.7
5. Sephadex G-100	60	19.7	4,930	250
6. ATP-agarose	21	2.3	4,270	1860

*From 40 g of packed cells (see text).

1.0, and 0.3 mM. The relative rates of reaction found with the enzyme isolated from *E. coli* strain KM when L-cysteine was replaced by L-α-aminobutyrate, β-chloro-L-alanine, S-methyl-L-cysteine, and L-serine were 0.85, 0.79, 0.70, and 0.18, respectively. The corresponding values for the enzyme isolated from *E. coli* strain W were 0.81, 0.99, 0.72, and 0.13. Less than 9% of the activity obtained with L-glutamate and L-cysteine was found with both enzyme preparations when L-glutamate was replaced with D-glutamate or L-aspartate and when L-cysteine was replaced by L-norvaline or L-methionine.

Inhibition Studies. The enzyme was incubated with cystamine (0.1–10 mM) in Tris·HCl buffer (50 mM; pH 8.2) at 37°C. A portion (10 μl) of the mixture was diluted into 0.99 ml of the standard assay solution. Inactivation by 4-methylene-DL-glutamate was carried out by preincubation of the enzyme with this amino acid (10 mM) in a buffer containing Tris·HCl (50 mM; pH 8.2) and 0.25 mM $MnCl_2$ for 1 hr at 37°C. After incubation, a 20-μl portion of the solution was diluted into 0.98 ml of the assay solution. Studies with S-sulfo-DL-homocysteine and S-sulfo-DL-cysteine were carried out in the same manner. In the experiments with buthionine sulfoximine, the enzyme was preincubated with 10 mM L-buthionine-(*SR*)-sulfoximine in Tris·HCl buffer (10 mM; pH 8.2) containing 20 mM $MgCl_2$ and 5 mM Na_2ATP at 37°C for 10 min. Then a 20-μl portion of the solution was diluted into 0.98 ml of assay solution. In the studies on inhibition by glutathione and γ-glutamyl-α-aminobutyrylglycine, the enzyme was added to the standard assay mixture containing 2–10 mM glutathione or γ-glutamyl-α-aminobutyrylglycine.

RESULTS

The enzyme isolated from *E. coli* strain KM (molecular weight, 56,000) was found to be homogeneous on polyacrylamide gel electrophoresis in the presence and absence of $NaDodSO_4$. The catalytic properties (apparent K_m values, substrate specificity) of this enzyme and those of the homogenous enzyme obtained from rat kidney (1, 24) are similar as noted above. The kidney enzyme (molecular weight, 104,000) is composed of two subunits (molecular weights, 73,000 and 27,700); the catalytic and feedback properties of the enzyme are associated with its heavy subunit. The isolated kidney enzyme exhibits a specific activity of about 1600 units/mg of protein, whereas the enzyme isolated from *E. coli* strain KM has a specific activity of about 1900 units/mg. The turnover numbers of the *E. coli* and kidney enzymes are calculated to be 2400 and 3130 min^{-1}, respectively.

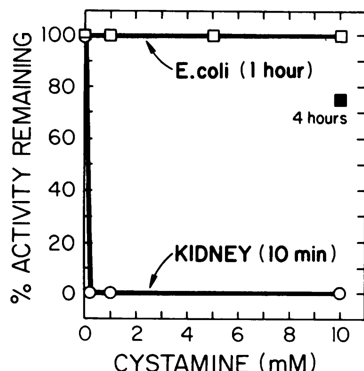

FIG. 1. Effect of cystamine on γ-glutamylcysteine synthetases. Purified γ-glutamylcysteine synthetase isolated from *E. coli* strain KM (6 units) and from rat kidney (4 units) was incubated at 37°C in 100 μl containing Tris·HCl buffer (50 mM; pH 8.2) and cystamine. A portion (0.01 ml) of each reaction mixture was removed and added to 0.99 ml of the standard enzyme assay solution. *E. coli* enzyme was assayed after treatment with cystamine for 1 hr or 4 hr; kidney enzyme was assayed after treatment with cystamine for 10 min. Inactivation of the kidney enzyme was completely reversed by treatment with 10 mM dithiothreitol, whereas the 25% inactivation observed after incubating the *E. coli* enzyme with 10 mM cystamine for 4 hr was not reversed by dithiothreitol.

Table 2. Effects of several compounds on *E. coli* and rat kidney γ-glutamylcysteine synthetases

	% activity remaining		
	E. coli		
Inhibitor	Strain KM	Strain W	Kidney
4-Methylene glutamate	98	100	41
S-Sulfocysteine	100	109	5
S-Sulfohomocysteine	99	100	10
Buthionine sulfoximine*	16	7.6	16

For details see text.
*No inhibition was observed when ATP was omitted from the preincubation mixture.

As shown in Fig. 1, the *E. coli* strain KM enzyme was not inactivated by cystamine; indeed, this enzyme, as compared to the kidney enzyme, is highly resistant to inactivation by cystamine. Thus, the *E. coli* strain KM enzyme was not inactivated after incubation with 10 mM cystamine for 1 hr, whereas the kidney enzyme was completely inactivated by incubation with 0.1 mM cystamine for 10 min. When the *E. coli* enzyme was incubated with 10 mM cystamine for 4 hr, there was about 25% inactivation; this was not reversed by treatment with 10 mM dithiothreitol. The *E. coli* strain W enzyme preparation was also resistant to inactivation by cystamine.†

4-Methylene glutamate, which inactivates the kidney enzyme by reaction with the active center thiol (17), did not inactivate the *E. coli* enzymes (Table 2). S-Sulfohomocysteine and S-sulfocysteine did not affect the activity of the *E. coli* enzyme under conditions in which the kidney enzyme was substantially inactivated. On the other hand, buthionine sulfoximine in the presence of ATP inactivated the enzyme preparations obtained from *E. coli* as well as the kidney enzyme (Table 2).

†Following our initial report (19), we became aware of the paper by Watanabe *et al.* (30), who reported purification of *E. coli* γ-glutamylcysteine synthetase and its inhibition by cystamine. Watanabe *et al.* obtained a protein that was homogeneous on polyacrylamide gel electrophoresis, but the specific activity of this enzyme calculated from their data is 21.6 units/mg (compared to 1860 units/mg found in the present work). The apparent discrepancy in specific activity may be ascribed to differences in the assay procedures used. Watanabe *et al.* assayed the enzyme in a mixture containing ATP, glutamate, cysteine, and relatively low levels of $MgCl_2$ and coupling enzymes. They used 7 μmol of $MgCl_2$, 0.98 unit of lactate dehydrogenase, and 0.90 unit of pyruvate kinase in a total volume of 0.7 ml; in the present work, the respective concentrations were 20 μmol, 10 units, and 5 units per ml. We have observed that although cystamine activates pyruvate kinase markedly, it inhibits lactate dehydrogenase; however, at the concentration of lactate dehydrogenase used in our assay, cystamine does not affect coupling and therefore does not prevent efficient measurement of the ADP formed in the γ-glutamylcysteine synthetase-catalyzed reaction. In our assay, we substituted L-α-aminobutyrate for L-cysteine. This substitution, as noted elsewhere (1, 24), is desirable because of the marked tendency of cysteine to undergo oxidation and to form mixed disulfides. Cystamine would be expected to be highly active in forming a mixed disulfide with cysteine under assay conditions. On the basis of our studies on the properties of the coupled assay system, we conclude that the reported (30) apparent inhibition of *E. coli* γ-glutamylcysteine synthetase by cystamine is a function of the assay system used rather than an effect of cystamine on γ-glutamylcysteine synthetase. (See *Note Added in Proof i.*)

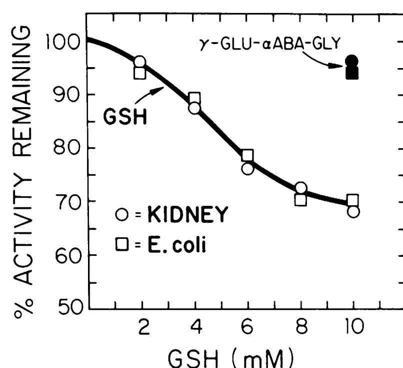

FIG. 2. Inhibition by glutathione of γ-glutamylcysteine synthetase activities of *E. coli* and rat kidney. Enzyme activity was determined as described in the text in the presence of various concentrations of glutathione (GSH) or of 10 mM L-γ-glutamyl-L-α-aminobutyrylglycine (γ-GLU-αABA-GLY).

Previous studies have shown that glutathione inhibits the activity of γ-glutamylcysteine synthetase of rat kidney in a manner that is competitive with glutamate (2). This is thought to provide a physiologically significant feedback inhibition mechanism, which explains, for example, the finding of 5-oxoprolinuria in severe congenital glutathione synthetase deficiency (31, 32). Glutathione inhibits the activity of γ-glutamylcysteine synthetase from *E. coli* strain KM to about the same extent as found with the rat kidney enzyme. Neither enzyme is inhibited appreciably by γ-glutamyl-α-aminobutyrylglycine.

DISCUSSION

The catalytic properties of the γ-glutamylcysteine synthetases from *E. coli* and rat kidney are very similar. This is evident from a comparison of the turnover numbers, substrate specificities, and apparent K_m values. Furthermore, both enzymes are inactivated to about the same extent when incubated with buthionine sulfoximine and ATP (Table 2). Another point of similarity concerns the inhibition of the enzyme activities by glutathione. As indicated in Fig. 2, the quantitative aspects of glutathione inhibition are virtually the same for the *E. coli* and rat kidney γ-glutamylcysteine synthetases. It is interesting to observe that such inhibition requires the thiol group of glutathione; thus, the analog γ-glutamyl-α-aminobutyrylglycine is ineffective in producing inhibition. Data previously published (2) led to the conclusion that inhibition by glutathione is "non-allosteric" because inhibition is competitive toward L-glutamate. However, glutathione, but not γ-glutamyl-α-aminobutyrylglycine, inhibits. This suggests that glutathione binds not only at the glutamate binding site of the active center but also at another site that interacts with the thiol moiety of glutathione. The latter site does not bind a peptide in which the thiol of glutathione is replaced by a methyl group.

In striking contrast to the several points of similarity between the two enzymes summarized above, properties of the kidney enzyme that may be attributed to the presence of an active site thiol are lacking in the *E. coli* enzyme. Thus, the *E. coli* enzyme is not inactivated by cystamine, 4-methylene glutamate, *S*-sulfohomocysteine, or *S*-sulfocysteine.

The finding of a thiol at or close to the active site of the kidney enzyme led to the previous suggestion that a thiol group is involved in the mechanism of the catalytic reaction, and thus a γ-glutamyl-*S*-enzyme intermediate was considered (14). The present findings on the *E. coli* enzyme indicate that a thiol is not involved in the reaction catalyzed by this enzyme; this may be taken as an indication that the thiol found at or close to the active center of the kidney enzyme does not play a role in catalysis. Accordingly, the observed reactions of the thiol of the kidney enzyme may simply serve to block the glutamate binding site. However, it is difficult to conceive that such a reactive thiol group that is located so close to the γ-carboxyl moiety of glutamate is not involved in catalysis. That a thiol moiety is important in the reactions catalyzed by both enzymes, but that it is markedly hindered (or "buried") in the active site of the *E. coli* enzyme, appears unlikely.‡ It is probable that the thiol plays a role in catalysis by the kidney enzyme and that this function is performed by another structure in the *E. coli* enzyme. An active center thiol seems not to be involved in inactivation by buthionine sulfoximine, a phenomenon associated with phosphorylation of buthionine sulfoximine by ATP that is presumably analogous to γ-glutamyl phosphate formation.

The possibility that the active center thiol of the kidney enzyme has a function apart from catalysis needs to be considered. The data indicate that such a function is unrelated to feedback inhibition by glutathione, but perhaps another metabolic phenomenon is connected with the action of kidney γ-glutamylcysteine synthetase; the nature of such a metabolic link is not now clear.

One must consider the idea that the rat kidney enzyme and the *E. coli* enzyme, although they catalyze the same overall reaction, perform their catalytic functions by use of different active center structures. Although bacterial and mammalian enzymes have commonly been found to have somewhat different properties, it is generally thought that the structures of the respective active sites are similar. This may not be the case for the *E. coli* and kidney enzymes considered here; thus, two different active sites might catalyze the same chemical reaction.

No information is currently available that relates to the evolution of γ-glutamylcysteine synthetase. If both enzymes arose from an ancestral γ-glutamylcysteine synthetase, possibly the kidney enzyme evolved to a form possessing an active site thiol. The *E. coli* enzyme seems to have evolved toward a more streamlined molecule that might be expected to have greater survival properties. Thus, the *E. coli* enzyme does not contain subunits, has a smaller molecular mass, and, importantly, cannot be inactivated by reactions involving an active center thiol. (See *Note Added in Proof ii.*)

‡The kidney enzyme is substantially inhibited by incubation for 5 min with 5 mM iodoacetamide [partially protected by L-glutamate (10, 33)] and *p*-hydroxymercuribenzoate and *p*-chloromercuribenzenesulfonate at concentrations of 0.002 mM and 0.02 mM, respectively (33). The *E. coli* strain KM enzyme is not affected by incubation for 10 min with 5 mM iodoacetamide or 10 mM *N*-ethylmaleimide; after incubation for 10 min with 1 mM *p*-hydroxymercuribenzoate, it was about 65% inhibited in the presence or absence of 10 mM L-glutamate.

Notes Added in Proof. (*i*) We have recently learned from K. Murata (personal communication) that he has made observations that agree with these conclusions—i.e., that the apparent inhibition of the *E. coli* enzyme by cystamine does not reflect the presence of an enzyme thiol but is related to the method of assay. (*ii*) We have recently established that the antigenic determinants of the *E. coli* and kidney enzymes are different; polyclonal antibodies obtained from rabbits immunized against the purified enzymes do not cross-react on Ouchterlony plates.

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