Cystamine-Sepharose

A PROBE FOR THE ACTIVE SITE OF γ-GLUTAMYL CYSTEINE SYNTHETASE

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γ-Glutamylcysteine synthetase, previously known to be potently inhibited by cystamine, has been found to bind covalently to cystamine-Sepharose. ATP facilitates, whereas glutamate plus magnesium ions inhibit, binding of the enzyme to cystamine-Sepharose. A large fraction of the enzyme applied to columns of cystamine-Sepharose binds by forming a disulfide bond between cystamine-C Sepharose and a sulfhydryl group at or near the active site of the enzyme. The enzyme may be released by treatment with dithiothreitol. Some of the enzyme applied to such columns is inactivated and not bound covalently to the column. That the enzyme does not bind to columns of S-(S-methyl)cysteamine-Sepharose, whereas free S-(S-methyl)cysteamine is a potent inhibitor, indicates that a cysteamine-S disulfide moiety derived from the external cysteamine residue of cystamine-Sepharose is the critical group recognized by the enzyme. The observed partitioning of the enzyme on columns of cystamine-Sepharose between covalently column-bound enzyme and nonbound inactivated enzyme suggests that the reactive enzyme sulfhydryl group forms a disulfide linkage with the sulfur atom at the immobilized end of cysteamine to link the enzyme to the column and to liberate free cysteamine, and also that the enzyme interacts with the external cysteamine moiety of the bound cystamine. The latter may occur if the free cysteamine released is spontaneously oxidized to free cysteamine followed by its inhibition of the enzyme, or if there is a direct reaction between the enzyme-reactive sulfhydryl group and the sulfur atom of the external cysteamine moiety of cystamine-Sepharose.

γ-Glutamylcysteine synthetase, which catalyzes the first step in the synthesis of glutathione (Reaction 1 (1, 2)), is very potently inhibited by the disulfide cystamine (3–5). Treatment of the inhibited enzyme with reducing agents reverses the inhibition. This observation and other data indicate that interaction between the enzyme and cystamine leads to the formation of a mixed disulfide between cysteamine and an enzyme sulfhydryl group (3, 5). The inability of certain cysteamine analogs and other disulfides to inhibit the enzyme (3) suggested that cystamine may uniquely fulfill rather stringent requirements for inhibition of this enzyme. However, we demonstrate here that γ-glutamylcysteine synthetase binds covalently to a modified cystamine which is immobilized through one amino group by attachment to a large Sepharose bead, and that such binding is significantly facilitated by the presence of ATP. The immobilized enzyme can subsequently be cleaved from the matrix by treatment with dithiothreitol. L-Glutamate and magnesium ions, which enhance the recovery of the enzymatic activity retrieved from the cystamine-Sepharose column, protect the enzyme against inhibition by both free and immobilized cystamine. The enzyme does not bind to S-(S-methyl)cysteamine, similarly immobilized by attachment through its only amino group to Sepharose, a finding which provides insight into the nature of the interaction of the enzyme with cystamine.

EXPERIMENTAL PROCEDURES

Materials and Methods—Activated CH-Sepharose 4B (6-carbon spacer arm) was obtained from Pharmacia. Sodium dodecyl sulfate was obtained from BDH Biochemicals, and acrylamide was purchased from Bio-Rad. The substrates, buffers, cystamine, 5,5'-dithio(bis)nitrobenzoic acid, dithiothreitol, and cysteamine-agarose, were obtained from Sigma. γ-Glutamylcysteine synthetase was purified (6) from frozen rat kidneys (Pel-Freeze) and kidneys of rats purchased from Taconic Farms; the purified preparations gave a single band on gel electrophoresis (6) and had specific activities in the range of 900–1400 units/mg.

Preparation of Cystamine-Sepharose—CH-Sepharose 4B (5 g) was suspended in 1 mM HCl to give 15 ml of swelled gel. The gel was rinsed with 1 liter of 1 mM HCl. A 10-fold excess of ligand (3.5 g of cystamine) was dissolved in 25 ml of 0.1 M NaHCO3 buffer containing 0.5 M NaCl (coupling buffer) and mixed in an Erlenmeyer flask with the washed gel. The flask was shaken gently at 4 °C overnight. Excess ligand was washed from the gel with 1 liter of coupling buffer, and any remaining active groups were blocked by washing with 1 M ethanolamine at pH 9.0. The resin was then washed successively with 300 ml of 0.1 M sodium acetate buffer (pH 4.0) containing 1 M NaCl, and 300 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 1 M NaCl for a total of three times each, and then stored in the Tris buffer. The amount of bound cystamine (quantitated after reduction by reaction with 5,5'-dithio(bis)nitrobenzoic acid) was estimated to be about 1.8 μmol/ml. Prior to use, the cystamine-Sepharose was equilibrated with 10 mM imidazole buffer (pH 8.2).

Preparation of S-(S-Methyl)cysteamine-Sepharose—Cysteamine-agarose (2.0 ml) was washed extensively with water. It was then suspended in a mixture containing 400 μl of water, 400 μl of methanol, and 40 μl of methyl methaneethiosulfonate, and allowed to react at 24 °C for 30–45 min. It was washed again with copious amounts of water to remove excess reagents, and was then equilibrated in 10 mM imidazole buffer (pH 8.2). The amount of bound S-(S-methyl)cysteamine, determined by reaction with 5,5'-dithio(bis)nitrobenzoic acid before and after reductive cleavage of the disulfide, was estimated to be 0.2 μmol of ligand/ml.

Assay—γ-Glutamylcysteine synthetase activity was determined from the rate of formation of ADP (assumed to be equal to the rate of oxidation of NADH) as calculated from the change in absorbance.
RESULTS

Effect of Substrates on Inactivation of γ-Glutamylcysteine Synthetase by Cystamine—Incubation of the enzyme with 162 μM cystamine at 4 °C for 30 min led to a loss of about 60% of the initial activity (Table I). Addition of ATP to the preincubation mixture led to complete loss of enzyme activity under these conditions.1 On the other hand, the addition of magnesium chloride and L-glutamate (separately or together) partially protected against inactivation, probably because cystamine binds at or close to the glutamate binding site of the enzyme where it reacts with an enzyme sulfhydryl group (5). L-α-Aminobutyrate did not protect the enzyme against inactivation by cystamine. When the enzyme samples inhibited by preincubation with cystamine under the conditions given in Table I were subsequently treated with 50 mM dithiothreitol, there was virtually complete restoration of enzyme activity.

Chromatography of Purified γ-Glutamylcysteine Synthetase on Cystamine-Sepharose—In these studies we found that the enzyme is effectively bound by cystamine-Sepharose and that preincubation of the enzyme with ATP markedly increases binding. Fig. 1A illustrates a typical chromatography profile. In this study, the enzyme was preincubated with ATP and then applied to a column of cystamine-Sepharose. Some protein eluted with the starting buffer and a larger amount eluted with 0.5 M sodium chloride (Fig. 1A, arrow a). Enzyme activity equivalent to about 60% of that applied remained covalently bound; this was released by elution with a buffer containing 50 mM dithiothreitol, 10 mM magnesium chloride, and 10 mM L-glutamate (arrow b). The noncovalently bound protein, which eluted with 0.5 M sodium chloride was inactive but could be completely reactivated by treatment with dithiothreitol. The total recovery of the applied enzyme activity in this chromatography was about 90%. Similar results were obtained when this experiment (Fig. 1A) was done with a preincubation mixture that contained 5 mM manganese chloride in addition to ATP.

Fig. 1B shows that the covalent interaction between enzyme and cystamine-Sepharose is substantially diminished when the enzyme is not preincubated with ATP. Most of the protein does not bind covalently to the column under these conditions; about 87% of the activity was eluted with a buffer containing 0.5 M sodium chloride (Fig. 1B, arrow a). After treatment of this fraction with dithiothreitol, an equivalent amount of enzyme activity was found. About 16% of the applied enzyme activity became covalently linked to the column in this study; this was eluted with buffer containing dithiothreitol, magne-

1 Although this effect of ATP was observed in the absence of added magnesium ions, it is likely that magnesium ions used in the purification are present in the enzyme preparation. The kinetics of the effect of ATP on cystamine inactivation will be addressed in detail in a subsequent paper.

| Additions to preincubation mixture | Rate of inactivation3 | Activity remaining3 % |
|-----------------------------------|-----------------------|----------------------|
| None                              | 31.6                  | 40                    |
| L-Glutamate (7.8 mM) + MgCl2      | 3.753                 | 90                    |
| MgCl2 (7.8 mM)                    | 3.73                  | 90                    |
| Na2ATP (8.3 mM)                   | 204.0                 | 0                     |
| L-α-Aminobutyrate (8.3 mM)        | 25.9                  | 45                    |
| Controls†                         | 5093                  | 100                   |

*Pseudo-first order rate constant (× 10⁻⁴) min⁻¹ for inactivation of enzyme.

γ-Glutamylcysteine synthetase (12.8 units) was incubated at 4 °C in 10 mM imidazole buffer (pH 8.2) containing 162 μM cystamine and the compounds indicated above (Table I, column, 120 μl). After 30 min, 10-μl portions were removed (containing 1.06 unit of enzyme and 13.5 μmol of cystamine), and assayed at 37 °C in reaction mixtures containing 10 mM ATP, 100 mM MgCl2, 10 mM L-glutamate, 10 mM L-α-aminobutyrate, 150 mM KCl, 2 mM Na2EDTA, 0.2 mM NADH, 17 μg of pyruvate kinase, and 17 μg of lactate dehydrogenase (total volume, 1.0 ml).

† Controls were treated in an identical manner except cystamine was omitted. Each of the controls retained 100% of the initial activity.
Binding of Cystamine to γ-Glutamylcysteine Synthetase

Figure 1. Elution profile of γ-glutamylcysteine synthetase on cystamine-Sepharose. A, a 500-μl portion (67.5 units) of the enzyme was incubated for 10 min with 1 mM ATP. The mixture was then applied to a column (0.75 × 4.1 cm) which had previously been equilibrated with 10 mM imidazole buffer (pH 8.2) containing 10 mM L-glutamate, and 10 mM MgCl₂. B, a 500-μl portion (350 units) of the enzyme was applied to a column (0.75 × 4.2 cm) which had previously been equilibrated with 10 mM imidazole buffer (pH 8.2) containing 10 mM L-glutamate and then placed on a cystamine-Sepharose column for purification of the enzyme. C, a 500-μl portion (67 units) of the enzyme was applied to a column (0.75 × 4.2 cm) which had been equilibrated with 10 mM imidazole buffer (pH 8.2) with a flow rate of 40 ml/hr. At arrow a, the buffer was changed to include 0.5 M NaCl. At arrow b, the buffer was changed to 10 mM imidazole buffer containing 50 mM dithiothreitol, 10 mM L-glutamate, and 10 mM MgCl₂. D, a 500-μl portion (350 units) of the enzyme was applied to a column (0.75 × 4.1 cm) which had been equilibrated with 10 mM imidazole buffer (pH 8.2) with a flow rate of 40 ml/hr. At arrow a, the buffer was changed to include 0.5 M NaCl. At arrow c, the buffer was changed to 10 mM imidazole buffer containing 50 mM dithiothreitol. At arrow d, 10 mM imidazole buffer containing 50 mM dithiothreitol, 10 mM L-glutamate, and 10 mM MgCl₂ was used. This is a typical elution profile for enzyme that had been preincubated with either (a) 47.6 mM dithiothreitol and then applied to a column (0.75 × 3.0 cm) previously equilibrated with 10 mM imidazole buffer (pH 8.2) with a flow rate of 60 ml/hr, or (b) 1 mM L-glutamate (8.3 mM) and 8.3 mM MgCl₂ and then applied to a column (0.75 × 3.2 cm) previously equilibrated with 10 mM imidazole buffer (pH 8.2) containing 10 mM L-glutamate and 10 mM MgCl₂ with a flow rate of 60 ml/hr. In each case, at arrow a, buffer was changed to include 0.5 M NaCl. At arrow b, 10 mM imidazole buffer (pH 8.2) buffer containing 50 mM dithiothreitol, 10 mM L-glutamate, and 10 mM MgCl₂ was used. 

Figure 2. Elution profile of γ-glutamylcysteine synthetase on S-(S-methyl)cysteamine-Sepharose. Enzyme (250 μl; 74.5 units) was incubated with 10 μl of a 50 mM ATP solution for 10 min. It was then placed on a S-(S-methyl)cysteamine-Sepharose column (0.75 × 4.1 cm) previously equilibrated with 10 mM imidazole buffer (pH 8.2) with a flow rate of 60 ml/hr. At arrow a, the buffer was changed to contain 0.5 M NaCl. At arrow b, the buffer was changed to contain 50 mM dithiothreitol, 10 mM L-glutamate, and 10 mM MgCl₂.

Chromatography of Impure γ-Glutamylcysteine Synthetase on Cystamine-Sepharose—The potential usefulness of cystamine-Sepharose for purification of the enzyme was explored using enzyme purified through step 4 of the isolation procedure (6); this material contains about 10% active enzyme. A solution of the enzyme in 10 mM imidazole buffer (pH 8.2) was applied to a column containing 2 ml of cystamine-Sepharose previously equilibrated with the same buffer. Fig. 3 shows the chromatography profile obtained; about 10% of nonactive protein did not bind, but eluted with the starting buffer. Buffer containing 0.5 M NaCl (Fig. 3, arrow a) eluted additional protein and activity. Elution with dithiothreitol, magnesium chloride, and L-glutamate (Fig. 3, arrow b) led to prompt appearance of about 50% of the initial activity applied to the column. The total recovery of enzyme activity was 90%; the material eluted from the column (Fig. 3, arrow b) was about 70% pure as determined by spectrophotometric analysis.

S-(S-Methyl)cysteamine is a very effective inhibitor of the enzyme; detailed studies on the kinetics of inactivation by this compound will be reported later.
Previous studies, which showed that cystamine inhibits at least five other enzymes (phosphorylase phosphatase (16, 17), guanylate cyclase (18), indoleamine N-acetyltransferase (19), glycogen synthetase (20), and transglutaminase (21)), and that cysteamine does not, it seems probable that the enzyme recognizes cystamine through the internal disulfide bond and the sulfur atom of cystamine (see the text). Examination of the literature reveals that glutamate and cysteamine bind to cystamine-Sepharose, and that some cystamine analogs may thus provide an alternative approach to the inhibition of glutathione synthesis, and be of value in vivo inhibition of glutathione synthesis, and be of value in 5-oxoprolinuria (12-15).

Such inhibition. Presumably, these inhibitors as well as cysteamine might be effective inhibitors. As discussed elsewhere (3, 12), partial in vivo inhibition of γ-glutamylcysteine synthetase may be of therapeutic value in 5-oxoprolinuria (12-15). Cystamine analogs may thus provide an alternative approach to in vivo inhibition of glutathione synthesis, and be of value in experimental work on the function of glutathione.

Examination of the literature reveals that γ-glutamylcysteine synthetase is not unique among enzymes in being potently inhibited by cystamine. Thus, there are published reports indicating that cystamine inhibits at least five other enzymes (phosphorylase phosphatase (16, 17), guanylate cyclase (18), indoleamine N-acetyltransferase (19), glycogen synthetase (20), and transglutaminase (21)), and that it activates...
at least two enzymes (fructose diphosphatase (22) and acetyl coenzyme A hydrolase (23)). The published data indicate that the specificity of cystamine in producing these effects varies considerably, but that in most cases, the effects can be reversed by treatment of the enzyme with a thiol. It seems likely that the cystamine-Sepharose matrix described here may therefore be useful as an affinity chromatography procedure for the study of the active sites of other enzymes and possibly also in their purification.

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G F Seelig and A Meister

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