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Utilization of L-Cystine by the γ-Glutamyl Transpeptidase–γ-Glutamyl Cyclotransferase Pathway

(γ-glutamyl cycle/amino-acid transport/L-cysteine/glutathione/γ-glutamylcystine)

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ABSTRACT **L-Cystine is a good acceptor of the γ-glutamyl group of γ-glutamyl donors in the reaction catalyzed by γ-glutamyl transpeptidase. The product of the enzymatic reaction and an authentic sample of γ-glutamylcystine were shown to exhibit identical chromatographic and electrophoretic behaviors; acid hydrolysis gave equimolar amounts of cystine and glutamate. In studies with two γ-glutamyl donors, apparent K_m values in the neighborhood of 0.3 mM were found for L-cystine; these values are not far from the concentrations of L-cystine in mammalian blood plasma. At an amino-acid acceptor concentration of about 0.5 mM, L-cystine is somewhat more active than L-glutamine, and much more active than L-cysteine. L-γ-Glutamyl-L-cystine was found to be a good substrate of γ-glutamyl cyclotransferase. These observations thus indicate that L-cystine is a very active substrate of the γ-glutamyl transpeptidase–γ-glutamyl cyclotransferase pathway. In relation to the hypothesis that the γ-glutamyl cycle functions in amino-acid transport, it may be significant that glutathione (which is the most abundant intracellular form) is a much better γ-glutamyl donor than glutathione disulfide, while the predominant extracellular form—cystine—is a much better γ-glutamyl acceptor substrate than cysteine.**

Previous studies in this laboratory have been concerned with the properties of γ-glutamyl transpeptidase (1–4), one of the enzymes involved in the γ-glutamyl cycle (5–7), a series of reactions which accounts for the synthesis and degradation of glutathione (for a recent review, see ref. 8). γ-Glutamyl transpeptidase catalyzes the transfer of the γ-glutamyl moiety of glutathione (and of other γ-glutamyl compounds) to amino-acid acceptors:

Glutathione + amino acid →
γ-glutamyl-amino acid + cysteinylglycine [1]

Studies on highly purified preparations of rat kidney γ-glutamyl transpeptidase have shown that a wide variety of amino acids are active as acceptors of the γ-glutamyl moiety. In a recent survey of the amino-acid acceptor specificity of this enzyme carried out with 2.5 mM γ-glutamyl-*p*-nitroanilide and 5 mM glutathione as γ-glutamyl donors, and 20 mM L-amino acid as acceptors, it was found that glutamine, methionine, cysteine, serine, alanine, glutamate, and glycine were among the most active acceptors (1); the relatively insoluble amino acids tyrosine and cystine were not included in this study.

In the present work, we have examined the acceptor activities of several less soluble amino acids at much lower concentrations and have found that while tyrosine is slightly active, cystine is very active at low concentrations which approximate those found *in vivo*. Under these conditions it was found that cystine is more active than glutamine, and also that the apparent K_m value for cystine is relatively low. Evidence was also obtained that γ-glutamylcystine is a substrate of γ-glutamyl cyclotransferase. The findings thus indicate that cystine is a good substrate of the γ-glutamyl transpeptidase–γ-glutamyl cyclotransferase pathway, which may function in the *in vivo* utilization of this amino acid.

EXPERIMENTAL

Materials

Glycylglycine, L-γ-glutamyl-*p*-nitroanilide, glutathione disulfide, carboxypeptidase A, and L-cysteine hydrochloride were obtained from Sigma Chemical Co. D-Cystine, L-tyrosine, *meso*-lanthionine, and *meso*-α,ε-diaminopimelic acid were products of Schwarz/Mann. L-Glutamine, glutathione, L-cystine, and DL-cystathionine were from Calbiochem. Dithiothreitol was obtained from RSA Corp. L-Cystathionine was obtained from Cyclo Chemical Co. Chloroacetophenone was obtained from Aldrich Chemical Co. L-γ-Glutamyl-L-α-aminobutyric acid and *S*-acetophenone glutathione were prepared as described (1).

γ-Glutamyl transpeptidase (form III) was isolated from rat kidney as described (3); the preparation used in these studies exhibited a specific activity of 1360 μmol/min per mg in the assay with L-γ-glutamyl-*p*-nitroanilide (3). γ-Glutamyl cyclotransferase was isolated from ovine brain by a modification of the procedure previously used (9); we are indebted to Ronald Sekura of this laboratory for this preparation.

L-γ-Glutamyl-L-cysteine disulfide was obtained by treatment of glutathione disulfide with carboxypeptidase A (10). L-γ-Glutamyl-L-cystine was prepared by treating L-γ-glutamyl-L-cysteine disulfide with dithiothreitol at pH 8 in an atmosphere of nitrogen followed by addition of twice the molar quantity of L-cysteine hydrochloride; the pH was kept at 8.0 by addition of ammonium hydroxide. After the solution was stirred in air for 2 hr, it was applied to a column of Dowex 1-acetate and eluted with water to remove cystine. Elution was then continued with 0.5 N acetic acid, and the product was obtained by lyophilization of this effluent. After oxidation with performic acid (11) and hydrolysis *in vacuo* in 6 N hydrochloric acid at 110° for 18 hr, amino-acid analysis on a Durrum model 500 amino-acid analyzer indicated a ratio of cysteic acid to glutamic acid of 2.2.

Methods

Amino-acid analyses were performed on a Durrum model 500 amino-acid analyzer; we thank Lawrence Refolo for

these studies. Protein was determined by the method of Lowry *et al.* (12).

γ-Glutamyl transpeptidase activity was determined by following the increase in absorbance at 305 nm or at 410 nm using *S*-acetophenone glutathione or γ-glutamyl-*p*-nitroanilide as the substrates, respectively, essentially as described (1). The reaction mixtures (final volume, 1.005 ml) contained enzyme (0.14 μg), 0.5 mM γ-glutamyl donor and various concentrations of acceptor in 0.01 M Tris·HCl buffer (pH 8.0). The apparent K_m values were obtained from the reciprocal plots of the data (after subtraction of the blank values), and the best straight line was determined by the method of least squares.

γ-Glutamyl cyclotransferase activity was determined by measurement of the absorbance due to 5-oxoproline at 205 nm after deproteinization (9), or by amino-acid analysis on the Durrum amino-acid analyzer. When the latter method was used, both appearance of free amino acid and the disappearance of the substrate were determined after deproteinization with 5% sulfosalicylic acid.

Paper chromatography was carried out on Whatman 3 MM paper in the descending direction with a solvent consisting of *n*-butanol, pyridine, and water (1:1:1). The R_F values (×100) were: 95, 70, 19, 12, 8.5, 6, and 5, for *p*-nitroaniline, γ-glutamyl-*p*-nitroanilide, glutamic acid, cystine, γ-glutamylcystine, γ-glutamylcysteine disulfide, and glutathione, respectively.

Paper electrophoresis was carried out on Whatman 3 MM paper in a buffer consisting of 105 g/liter acetic acid adjusted to pH 3.5 by addition of pyridine. A potential of 3.5 V/cm was applied for 2 hr at 4°. The mobilities for γ-glutamylcystine, γ-glutamylcysteine disulfide, and glutathione were, respectively, 2.5, 5.7, and 1.3 cm (toward the anode); *p*-nitroaniline, γ-glutamyl-*p*-nitroanilide, glutamic acid, and cystine moved, respectively, 3.2, 3.2, 1.3, and 2.5 cm toward the cathode.

RESULTS

The data given in Table 1 indicate that L-cystine is very active as an acceptor substrate in transpeptidation with γ-glutamyl-*p*-nitroanilide and *S*-acetophenone-glutathione. These studies, which were carried out with acceptor amino acid concentrations of about 0.5 mM, show that amino acids of closely related structure, i.e., *meso*-lanthionine, L-cystathionine, and DL-allocystathionine are also good acceptor substrates. In agreement with previous findings, glutamine and glycylglycine were found to be active. Relatively lower activities were observed with L-cysteine, L-tyrosine, and D-cystine.

Evidence that the product formed in the reaction between the γ-glutamyl donor and L-cystine is γ-glutamylcystine was obtained by paper chromatographic and electrophoretic studies. Thus, it was shown that a product with the same R_F and mobility as authentic γ-glutamylcystine was formed in reaction mixtures containing γ-glutamyl-*p*-nitroanilide and L-cystine. The enzymatically formed product was eluted from the paper chromatogram, hydrolyzed in 6 N HCl *in vacuo* at 110° for 18 hr, and then subjected to amino-acid analysis; glutamic acid and cystine were found in a ratio of 1.0.

In studies in which the concentration of L-cystine was varied, activity values (corrected) of 1.47, 2.67, 3.85, and 5.34 nmol/min were obtained, respectively, at L-cystine concentrations of 0.06, 0.125, 0.25 and 0.45 mM in experiments with *S*-acetophenone glutathione. From these data, an apparent K_m value of 0.32 mM was calculated for L-cystine. An apparent K_m value of 0.24 mM was obtained in analogous studies with γ-glutamyl-*p*-nitroanilide. The respective apparent V_{max} values were 65.0 and 47.3 μmol/min per mg.

TABLE 1. *Activities of various amino acids as acceptors of the γ-glutamyl group**

Amino acid (mM)	γ-Glutamyl donor: *S*-Acetophenone-glutathione (nmol/min)	γ-Glutamyl donor: γ-Glutamyl-*p*-nitroanilide (nmol/min)
None	1.47	6.50
L-Cystine (0.45)	6.81	10.6
L-Cysteine (0.50)	1.86	6.84
L-Glutamine (0.50)	4.07	9.57
Glycylglycine (0.50)	9.47	18.4
L-Tyrosine (0.45)	1.74	6.84
meso-Lanthionine (0.50)	6.54	10.6
L-Cystathionine (0.54)	6.27	10.3
DL-Allocystathionine (0.65)	5.34	8.54
meso-α,ε-Diaminopimelic acid (0.45)	2.80	7.86
D-Cystine (0.45)	2.41	6.50

* The experimental details are given under *Methods*.

γ-Glutamyl cyclotransferase activity was determined with L-γ-glutamyl-L-cystine prepared as described under *Methods*. Values based on measurement of the decrease in the concentration of γ-glutamylcystine and on the appearance of cystine with time were in close agreement. The initial rate of reaction was 11.7 nmol/min in reaction mixtures containing 4 mM L-glutamyl-L-cystine. Under the same conditions but with 25 mM L-γ-glutamyl-L-α-aminobutyrate as substrate, the initial reaction rate was 20 nmol/min

DISCUSSION

The findings indicate that L-cystine is a good acceptor substrate of γ-glutamyl transpeptidase and that this amino acid is a better substrate at low concentrations than L-cysteine. It seems of interest to consider these results in relation to the γ-glutamyl cycle, to the proposal that this cycle plays a role in amino-acid transport, and also to data available on the state of oxidation of cyst(e)ine and its derivatives in body fluids and in cells.

Blood plasma contains much higher concentrations of cystine than cysteine, while within the cells, the concentration of cysteine is considerably higher than that of cystine. Thus, studies on the intracellular ratio of cysteine and cystine in various tissues (rat kidney cortex, diaphragm, jejunum, liver, and brain) showed a predominance of the reduced form, while the blood plasma contains more of the disulfide form (13, 14). There seems to be little or no glutathione in blood plasma, and it is well known that the intracellular concentration of glutathione is very much greater than that of glutathione disulfide. It thus appears that the reduced forms of cysteine and glutathione predominate over the oxidized forms within the cell; this seems to be related to the very highly active glutathione reductase system (15–19) and the occurrence of transhydrogenase reactions (20–25).

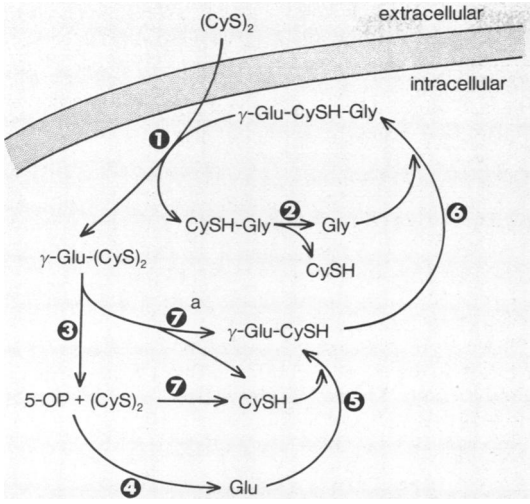

FIG. 1. Pathways of L-cystine transport and metabolism (see the *text*). Enzymes: (1) γ-glutamyl transpeptidase; (2) cysteinylglycinase; (3) γ-glutamyl cyclotransferase; (4) 5-oxoprolinase; (5) γ-glutamylcysteine synthetase; (6) glutathione synthetase; (7) and (7ª) reduction pathways (see the *text*). 5-OP = 5-oxoproline.

These considerations suggest that in its function in the γ-glutamyl cycle, the transpeptidase may be specially adapted to the physiological states of oxidation of glutathione and cystine. The enzyme is relatively specific for cystine (as compared to cysteine)—the putative extracellular γ-glutamyl acceptor—and for glutathione [as compared to glutathione disulfide (1)]—the intracellular γ-glutamyl donor substrate. Further, in view of the predominance of reduced forms of cysteine derivatives within the cell, it would seem that the action of the transpeptidase on glutathione initially yields cysteinylglycine (rather than the corresponding disulfide) and that this dipeptide is hydrolyzed to its constituent amino acids, glycine and cysteine. [Enzymatic activity capable of splitting the disulfide form of cysteinylglycine seems also to be present (26).] While the present results demonstrate the formation of γ-glutamylcystine by transpeptidation and the susceptibility of this substrate to the action of γ-glutamylcyclotransferase, it is also possible that γ-glutamylcystine is reduced to γ-glutamylcysteine. Such reduction might occur by a transhydrogenase reaction involving glutathione of the type first observed by Racker (27):

$$2\ \text{glutathione} + \gamma\text{-glutamylcystine} \rightarrow \text{glutathione disulfide} + \gamma\text{-glutamylcysteine} + \text{cysteine} \quad [2]$$

In such a reaction, both γ-glutamylcysteine and cysteine would be formed (Fig. 1; reaction 7ª). A number of studies (22) suggest that the reduction of disulfides may be coupled with the reaction catalyzed by glutathione reductase:

$$\text{R—S—S—R} + 2\ \text{glutathione} \rightleftharpoons \text{glutathione disulfide} + 2\text{RSH} \quad [3]$$

$$\text{glutathione disulfide} + \text{TPNH} + \text{H}^+ \rightarrow 2\ \text{glutathione} + \text{TPN}^+ \quad [4]$$

$$\text{Sum: R—S—S—R} + \text{TPNH} + \text{H}^+ \rightarrow 2\text{RSH} + \text{TPN}^+ \quad [5]$$

Although the enzymology of disulfide reduction requires clarification, there is good evidence that a number of cell types can reduce cystine to cysteine; thus, the reduction of cystine as well as that of γ-glutamylcystine have been included in the scheme given in Fig. 1 (reactions 7). The reduction of γ-glutamylcystine would yield directly a substrate of glutathione synthetase as well as one for γ-glutamylcysteine synthetase. Rat kidney glutathione synthetase utilizes γ-glutamylcystine at a much lower rate than γ-glutamylcysteine,* suggesting that synthesis of the mixed disulfide between glutathione and cysteine by glutathione synthetase is probably not a major pathway. Rat kidney γ-glutamylcysteine synthetase exhibits about 1% of its full activity when L-cysteine is replaced by L-cystine.†

A number of studies have been carried out on two human diseases that are associated with defects of cystine metabolism or transport. In cystinosis there is extensive deposition of cystine in the tissues, while in the milder disease cystinuria, there is evidently a defect of cystine (and often also lysine, arginine, and ornithine) transport in the kidney and intestine; excellent reviews of cystinosis (28) and cystinuria (29) are available. Consideration of the pathways given in Fig. 1 suggests that cystinosis is not associated with a defect in the γ-glutamyl transpeptidase–γ-glutamyl cyclotransferase pathway.‡ It seems more likely that in this disorder there is a defect in the reduction of cystine to cysteine, as has been suggested previously (28, 30). The present work suggests an additional possibility, i.e., a defect in the reduction of γ-glutamylcystine to γ-glutamylcysteine and cysteine (reaction 7ª). The transport defect in cystinuria might possibly be more closely related to the function of the transpeptidase or conceivably to a genetic deficiency of amino-acid binding proteins from which amino acids might be transferred to the transpeptidase (5). Earlier studies have indicated that cystine and cysteine are transported by different mechanisms (31, 32), and that slices of neonatal rat kidney cortex exhibit impaired ability to transport cystine (in contrast to cysteine) (32). It may be significant that neonatal kidney has very little γ-glutamyl transpeptidase activity, and that the cystine transport system (32) and the transpeptidase (3) develop soon after birth.§

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* Unpublished studies in this laboratory by Dr. Vaira P. Wellner.
† Unpublished studies in this laboratory by Ronald Sekura.
‡ Recent work in this laboratory by Dr. F. J. Lombardi has shown that cultured skin fibroblasts from a patient with cystinosis (kindly supplied by Dr. J. Oshima, Dept. of Pediatrics, University of California School of Medicine, La Jolla) had levels of γ-glutamyl transpeptidase similar to those found in normal controls.
§ A preliminary report by Schulman *et al.* (33) has appeared stating that cystine is a potent activator of the γ-glutamyl transpeptidase of human tissues, and that no abnormalities were found in this enzyme in cells from patients with cystinosis.

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