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CYSTINE LYASE: β-CYSTATHIONASE FROM TURNIP ROOTS

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Key Word Index—*Brassica rapa*; Cruciferae; turnip root; cystine lyase; β-cystathionase; cystine degradation; homocysteine formation.

Abstract—Cystine lyase (EC 4.4.1.-) was purified 277-fold by a combination of ammonium sulfate precipitation, chromatography on calcium phosphate and DEAE-cellulose with a 6% recovery. The MW as measured by gel filtration on Biogel P-300 was *ca* 150 000. The enzyme catalysed the pyridoxal phosphate-dependent degradation of cystine to pyruvate, ammonia and cysteine persulfide. Cysteine persulfide normally degraded spontaneously to elemental sulfur and cysteine, that further reacted to yield cystine and H_2S. Pyridoxal phosphate stabilized the enzyme. The K_m value for cystine was 0.94 mM. The enzyme was insensitive to thiol reagents but was inhibited by some thiols (which may have reduced the cystine). Cystine lyase degraded many compounds having the L-α-amino propionic acid group with a thioether or disulfide linkage attached to the β-carbon but was inactive towards D-configuration at the α-carbon or L-homocystine. The cystine lyase was also a β-cystathionase as indicated by (1) a constant ratio of β-cystathionase activity to cystine lyase activity throughout a 277-fold purification, (2) the inhibition of cystine lyase activity by cystathionine and inhibition of β-cystathionase activity by cystine and (3) similarity in sensitivity to heat, cyanide and hydroxylamine. Using DL-cystathionine as substrate, the K_m value was 4 mM.

In studies on *S*-methylcysteine biosynthesis [1], the administration of radioactive cystine to radish leaves not only labelled methylcysteine but also labelled alanine. Investigation showed that the cysteine degradation was caused by a cystine lyase. Since this enzyme interfered with cysteine synthesis studies [2,3], we purified it from turnip roots. Characterization revealed that the cystine lyase may also be the cystathionase responsible for homocysteine and methionine formation in higher plants.

RESULTS

Purification of enzyme

Cystine lyase was purified 277-fold from turnip roots (*Brassica rapa* L. cv purple top) in a 5 step procedure. All operations were carried out at 0–4°. All solutions contained 5×10^{-5} M pyridoxal phosphate to stabilize enzymatic activity (see below).

1. *Preparation of crude extract.* Chilled, chopped turnip roots (1 kg) were ground with 1.5 l. of 0.1 M K phosphate buffer (pH 7.5) in a Waring blendor at top speed for 2 min. The crude extract was the supernatant obtained after 20-min centrifugation at 10 000 *g*.

2. *Ammonium sulfate fractionation.* Solid enzyme-grade ammonium sulfate (277 mg/ml) was dissolved in the crude extract. The suspension was stirred for 30 min, then centrifuged for 30 min at 10 000 *g*. To each ml of the supernatant was added 112 mg of ammonium sulfate. The supernatant from a 20-min centrifugation (10 000 *g*) was discarded. The protein precipitate was dissolved in 10 mM K-phosphate buffer (pH 7.5) and the solution was dialysed against three changes (4 hr each) of K-phosphate buffer (10 mM, pH 7.5) (1 l.). The dialysed solution was clarified by centrifugation and the volume adjusted to make a solution of 5.3 mg of protein per ml. A lyophilized powder obtained from this solution retained enzymic activity indefinitely when stored dry at 4°.

3. *Chromatography on calcium phosphate adsorbed on cellulose.* Calcium phosphate adsorbed on cellulose was prepared by the procedures of Price and Greenfield [4] except that the ratio of cellulose to calcium phosphate was doubled. The dialysate from step 2 (50 ml) was applied to a 2.4 × 30 cm column of calcium phosphate on cellulose pre-equilibrated with 10 mM K phosphate buffer (pH 6.8). The equilibration buffer was pumped into the column at the rate of 50 ml/hr and the effluent was monitored by absorbance at 280 nm. When the absorbance had levelled off at a low value, 0.1 M buffer was pumped through the column until the absorbance reached a minimum. Then 0.5 M K phosphate buffer was pumped through the column while 9 ml fractions were collected. The fractions containing most enzyme activity were combined.

4. *Ammonium sulfate precipitation.* The combined fractions from step 3 were stirred with 243 mg of $(NH_4)_2SO_4$ per ml for 30 min and the resultant precipitate was removed by centrifugation. Ammonium sulfate (186 mg/ml) was dissolved in the supernatant and stirred for 30 min. The protein was recovered by centrifugation, then dissolved in a minimal volume of 0.1 M Na pyrophosphate at pH 8. The resultant solution was dialysed 8 hr against a 100-fold excess of the same buffer with one

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Table 1. Purification of cystine lyase from 1 kg of turnip roots

Step	Fraction	Volume	Protein concentration	Total protein	Total activity	Recovery of activity	Specific activity	Relative purity
		(ml)	(mg/ml)	(mg)	(kat × 10^9)	(%)	kat × 10^9 / mg protein	
1	K phosphate extract	1140	2.0	2360	387	100	0.164	1
2	$(NH_4)_2SO_4$ fraction	50	5.3	265	247	64	0.931	5.7
3	Ca phosphate column eluate	40	0.44	17.8	89.2	23	5.07	31
4	Second $(NH_4)_2SO_4$ fraction	5.2	1.0	5.25	61.8	16	11.8	72
5	DEAE-cellulose column eluate	8	0.065	0.51	23.2	6	45.4	277

change after 2 hr. The protein concentration was adjusted to 1 mg/ml.

5. *Chromatography on a column of DEAE-cellulose.* DEAE-cellulose (Cellex D from Biorad Corp.) was purified according to Peterson and Sober [5]. A 1.5 × 20 cm column was packed with DEAE-cellulose that had been equilibrated with 10 mM Na pyrophosphate at pH 8. The solution (6 ml) from step 4 was chromatographed with a linear NaCl gradient from 0 to 0.2 M prepared from 100 ml of 10 mM Na pyrophosphate buffer (pH 8) and 100 ml of this buffer containing 0.2 M NaCl. When 130 ml of buffer solution had emerged from the column (≃0.13 M NaCl), the eluant was changed to buffer containing 1 M NaCl. Ten 4 ml fractions were collected and assayed for enzymic activity. The fractions with the most activity (usually 3 or 4) were combined and dialysed against the pyrophosphate buffer.

The results of purification are summarized in Table 1. The purest preparation contained 6% of the original activity and was 277-fold purified. Because of the limited amount of the most highly purified enzyme and its liability, most tests were made on enzyme purified through steps 3 or 4.

Table 2. Intracellular location of cystine lyase of turnip root and cabbage leaf

Plant tissue	Fraction	Cystine lyase activity Units* (g fr. wt)	Cystine lyase activity Units (mg protein)
Turnip root	crude	0.92	0.36
Turnip root	particulate	0.028	0.083
Turnip root	soluble	0.72	0.47
Cabbage leaf	crude	1.72	0.36
Cabbage leaf	particulate	0.056	0.15
Cabbage leaf	soluble	1.50	0.36

*Unit = 10^{-9} kat.

Tissue homogenate was incubated with 70 μmol of Tris–acetate (pH 8.5), 7.5 μmol of L-cystine and 30 nmol of pyridoxal phosphate in a total volume of 2 ml for 30 min at 30°. The reaction was stopped with 0.3 ml of 20% trichloroacetic acid. The pyruvate formed was measured as its 2,4-dinitrophenylhydrazone.

Gel electrophoresis of step 5 protein showed three bands of equal intensity. We were unable to correlate bands with cystine lyase activity or to purify further by preparative gel electrophoresis.

MW of cystine lyase. The MW of cystine lyase was estimated by gel filtration [6] on Biogel P-300, with 4 pure proteins as standards. The MW of cystine lyase was *ca* 150 000.

Characteristics of assay method. Thiophenol formation and pyruvate formation were linear with time up to 30 min. However, when Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid)) was omitted from the standard incubation mixtures (see Experimental), the production of pyruvate decreased with time. The non-linearity was attributed to inhibition by cysteine formed because *N*-ethylmaleimide, a sulfhydryl trapping reagent, would prevent the non-linearity (data not shown).

Intracellular location of cystine lyase. Using the procedures of Tishel and Mazelis [7], we tested the location of cystine lyase in turnip roots and cabbage leaves. Most of the turnip and cabbage enzyme was in the cytosol (Table 2). Possibly the enzyme is particulate but readily solubilized. However, this possibility seems unlikely because the cabbage particulate enzyme remained firmly bound even after washing [7].

Properties of the cystine lyase

Nature of the reaction. We wanted to identify the substrate and the products. Under normal assay conditions, both cysteine and cystine were substrates indicating that the enzyme could be catalysing a cysteine desulfhydrase reaction (equation 1) or a cystine lyase reaction (equation 2). There was a strong possibility that cysteine was being oxidized to cystine during the incubation because cysteine is readily oxidized at the incubation pH of 8.

$$\text{Cysteine} + H_2O \longrightarrow \text{pyruvate} + H_2S + NH_3 \quad (1)$$

$$\text{Cystine} + H_2O \longrightarrow \text{pyruvate} + \text{cysteine} + S^0 + NH_3 \quad (2)$$

(S^0 signifies elemental sulfur.)

Incubation under anaerobic conditions showed that cystine was a far more effective substrate than cysteine. With cystine as substrate, 0.130 μmol of pyruvate was formed, but with cysteine only 0.012 μmol was formed

Table 3. Effect of pyridoxal phosphate on inhibition of cystine lyase by cyanide and hydroxylamine

Inhibitor	Pyridoxal phosphate concentration (mM)	% Inhibition
None	0	0
KCN	0	100
KCN	0.5	50
KCN	1.0	36
NH_2OH	0	100
NH_2OH	0.5	40
NH_2OH	1.0	32

KCN (0.5 mM) or NH_2OH (0.5 mM) were added to cystine lyase (0.23 units of step 5 enzyme) in 0.25 ml of 50 mM Na pyrophosphate buffer at pH 8.5 and pre-incubated for 15 min at 30°. Pyridoxal phosphate was added and pre-incubation was continued for 15 min at 30°. Untreated control produced 0.129 μmol of pyruvate.

(0.5 unit of step 2 enzyme tested under standard conditions with 3 mM substrate), indicating that the enzyme was a cystine lyase. This conclusion was supported by the stoichiometry of the reaction and the nature of the products. In one experiment with step 4 enzyme, the ratio of pyruvate to cysteine to ammonia to sulfur was 1:1.09:0.90:0.94. These results showed that the reaction catalysed was a cystine lyase reaction.

After consideration of the nature of the reaction and the work of others [8, 9], we assumed that cysteine persulfide would be intermediate in the reaction.

$$\text{Cystine} + H_2O \longrightarrow \text{pyruvate} + NH_3 + \text{cysteine persulfide}$$
$$\text{cysteine persulfide} \longrightarrow \text{cysteine} + S^0$$

Evidence for cysteine persulfide as an intermediate was obtained in two ways. First, an incubation was carried out in the presence of iodoacetate. If cysteine persulfide were formed during the incubation, it would react with iodoacetate to form cysteine–thioglycolic acid mixed disulfide [8]. Using TLC followed by colour formation with ninhydrin or cyanide–nitroprusside to detect amino acids and disulfides, respectively, we showed unambiguously the presence of cysteine–thioglycolic acid disulfide in the incubation mixture. Secondly, incubation under anaerobic conditions produced a compound that reacted with cyanide to form thiocyanate as measured as ferric thiocyanate [10]. Furthermore, in 30 min with 1.2 units (1 unit = 10^{-9} kat) of step 4 protein, incubation under anaerobic conditions produced 2.2 μmol of pyruvate and 2.4 μmol of persulfide (as measured by formation of thiocyanate).

Table 4. Effect of various compounds on cystine lyase activity

Compound (0.5 mM)	Relative activity (% of control)*
Cyanide	51
Hydroxylamine	60
p-Chloromercuribenzoate	96
Iodoacetate	109
N-Ethylmaleimide	117
β-Mercaptoethanol	65
Dithioerythritol	96
EDTA	106

*17 nmol pyruvate produced per min.

Step 4 enzyme (1.2 units) was incubated under standard conditions. Pyruvate produced was measured as 2, 4-dinitrophenylhydrazone (see Experimental).

Effect of pH on reaction rate and on stability. Cystine lyase had a pH optimum at pH 8.5 in 50 mM K phosphate buffer with half maximal activity at pH 7 and 9.5. The effect of pH on stability was determined by incubation for 15 min at 24° at different pHs. Stability was maximum at pH 7 with half maximum stability at pH 4 and 9.5.

Effect of pyridoxal phosphate on reaction rate and on stability of the enzyme. A consideration of the reaction catalysed indicates that cystine lyase should require pyridoxal phosphate as a cofactor, and strong evidence for pyridoxal phosphate participation in the catalysis was obtained. Step 3 enzyme was dialysed for 24 hr against 10 mM Tris–HCl (pH 8.5). The addition of pyridoxal phosphate (0.5 mM) to the dialysed preparation increased the activity 4.2-fold. Similarly, preincubation of step 5 enzyme with either 0.5 mM NaCN or 0.5 mM hydroxylamine for 15 min at 30° abolished enzymic activity. Addition of pyridoxal phosphate at 0.5 mM resulted in restoration of enzymic activity to 50 and 60%, respectively (Table 3). Mazelis *et al.* [7, 11] found a pyridoxal phosphate requirement in cystine lyase preparations from some plants but not in others.

Activity of L-*cystine.* When the effect of L-cystine concentration on pyruvate formation with step 3 enzyme under standard conditions was tested, the reaction rate gave a typical rectangular hyperbola. When these data were subjected to the double reciprocal plot of Lineweaver–Burk [12], the K_m for L-cystine was 0.94 mM, which approximates the K_m (1 mM) of Mazelis *et al.* [11] for cystine lyase from rutabaga.

Effect of various compounds on enzymic activity. We pointed out that aldehyde-binding compounds, such as cyanide and hydroxylamine, inhibit cystine lyase because they bind pyridoxal phosphate (Table 3). Thiol-binding compounds, such as *p*-chloromercuribenzoate, iodoacetate and *N*-ethylmaleimide, had no marked inhibitory effects at 0.5 mM showing that thiol groups were not essential for enzymic activity (Table 4). β-Mercaptoethanol (0.5 mM) inhibited enzymic activity 35% but dithioerythritol (0.5 mM) inhibited only 4%. The inhibition by mercaptoethanol could be attributed to reduction of cystine to cysteine but it is not clear why both thiols were not equally effective. The absence of any effect of ethylene-diaminetetraacetate (0.5 mM) on enzymic activity indicates that metal ions are not essential for activity.

Substrate specificity. A number of compounds similar to cystine were tested for their activity in the cystine lyase assay. Table 5 shows that several sulfur-containing amino acids are substrates. D-Cystine was not a substrate showing that configuration at the α-carbon is crucial. The activity of L-cysteine is probably due to its oxidation to cystine. Apparently the enzyme catalyses only a β-cleavage because methionine and homocystine are not substrates. The enzyme has activity toward thioethers (e.g. cystathionine), sulfoxides (e.g. *S*-methylcysteine

Table 5. Effect of various compounds as substrates for cystine lyase

Compound	Relative activity
L-Cystine	100*
D-Cystine	0
L-Cysteine	14
D-Cystine	0
S-Sulfo-L-cysteine	86
DL-Cystathionine	22
S-Methyl-L-cysteine	32
(+)-*S*-methyl-L-cysteine sulfoxide	46
(−)-*S*-methyl-L-cysteine sulfoxide	29
S-Ethyl-L-cysteine	21
L-Methionine	0
L-Homoserine	0
L-Serine	0
L-Djenkolic acid	16
L-Homocysteine	0
L-Homocystine	0

*5 μmol of each compound was incubated with 1.2 units of step 4 enzyme under standard conditions. With L-cystine as substrate, 0.98 μmol of pyruvate was formed. The assay measured keto acid formed.

sulfoxide) and thiosulfonates (e.g. *S*-sulfocysteine). The enzyme had no dehydratase activity towards serine or homoserine.

Cystine lyase as a β-cystathionase

The activity of cystine lyase on cystathionine (in contrast to other reported cystine lyases [7, 11] and the lack of a good explanation for a cystine lyase led us to consider that cystine lyase functions *in vivo* as a β-cystathionase. Several pieces of evidence indicate that the turnip cystine lyase is a β-cystathionase.

The activity of cystine lyase on cystathionine (in

Cleavage of cystathionine. The important function of a β-cystathionase in higher plants would be the cleavage of cystathionine to yield homocysteine, a normal precursor of methionine. The cystine lyase was tested at several stages of purification for its ability to catalyse a β- or a γ-cleavage of cystathionine. Table 6 shows that a crude extract catalyses both β- and γ-cleavages but that more highly purified preparations promote only a β-cleavage.

Table 6. β- and γ-cleavage of cystathionine by cystine lyase at two stages of purification

Enzyme preparation	Position of labelled carbon in cystathionine	Radioactivity in keto acid* (dpm)
Step 1	3-carbon moiety	20 300
Step 1	4-carbon moiety	10 900
Step 1	3-and 4-carbon moiety	30 000
Step 3	3-carbon moiety	8400
Step 3	4-carbon moiety	460
Step 3	3-and 4-carbon moiety	8600

*Corrected for boiled control.

Cystine lyase (0.15 unit) was incubated with 850 000 dpm of radioactive cystathionine under standard conditions (where cystathione replaced cystine).

Table 7. Effect of cystathionine on cystine lyase activity and of cystine on β-cystathionase activity

Substrate	Competitor	Competitor concentration (M)	Relative activity
Cystine	Cystathionine	—	100*
Cystine	Cystathionine	10^{-6}	94.8
Cystine	Cystathionine	10^{-5}	90.8
Cystine	Cystathionine	10^{-4}	83.5
Cystathionine	Cystine	—	100†
Cystathionine	Cystine	10^{-6}	72
Cystathionine	Cystine	10^{-5}	47
Cystathionine	Cystine	10^{-4}	19

*72 400 dpm.

†14 160 dpm.

One μmol of radioactive cystine or cystathionine (0.1 μCi of each) was incubated with 0.30 units of step 5 enzyme for 15 min, 20 μmol of Na pyrophosphate buffer (pH 8.5) and 10 nmol of pyridoxal phosphate in 0.5 ml for 15 min at 37°.

With a crude estract, the β- and γ-cleavage activities were additive indicating that they were due to different enzymes.

Effect of cystine lyase purification on cystathionase activity

A test for the identity of cystine lyase and β-cystathionase is their behaviour throughout purification. The ratio of cystine lyase activity to β-cystathionase activity changes little during 277-fold purification. This ratio was 4.2, 5.4, 6.0, 6.1 and 5.8 in preparations from purification steps 1 to 5, respectively. The change in ratio was largest between steps 1 and 2 indicating that the crude extract contained other β-cystathionase activity that was absent after the first step.

Effect of cystathionine on cystine lyase activity and effect of cystine on cystathionase activity

Table 7 shows that cystathionine inhibits cystine lyase activity and that cystine inhibits β-cystathionase activity in the most highly purified enzyme preparation. The greater inhibition by cystine than by cystathionine may be related to the lower K_m values of cystine (see below). These results indicate that cystine and cystathionine bind at the same site on the enzyme.

Table 8. Effect of cyanide, hydroxylamine and heat on cystine lyase and β-cystathionase activities of purified preparations

Deviation from control treatment	Cystine lyase Relative activity	β-Cystathionase Relative activity
None	100*	100†
Incubation with 0.5 mM cyanide	51	48
Incubation with 0.5 mM hydroxylamine	60	62
Enzyme preheated at 55° for 2 min	78	81
Enzyme preheated at 55° for 3 min	55	55
Enzyme preheated at 55° for 5 min	11	9

* 18 nmol pyruvate formed/mg protein/min.

† 3.1 nmol pyruvate formed/mg protein/min.

Step 5 enzyme (0.30 units) was incubated for 30 min at 37° under standard assay conditions. Pyruvate formed was measured with purified lactic dehydrogenase.

Effect of inhibitors and heat on cystine lyase and β-cystathionase activities

We previously demonstrated (Table 4) that cyanide and hydroxylamine were inhibitors of cystine lyase, and wanted to see whether those inhibitors had parallel effects on β-cystathionase activity. In Table 8, the effects of cyanide, hydroxylamine and heating at 55° on enzymic activities are presented. The data show that inhibitions were very similar for both cystine lyase and β-cystathionase activities.

Properties of β-cystathionase

When β-cystathionase was measured by pyruvate formation (by use of lactic dehydrogenase), the amount of pyruvate produced was proportional to time and quantity of enzyme. The pH optimum was 8 in pyrophosphate buffer (0.1 M) with half maximal activity at pH 7 and 8.8. The pH optimum of 8 is close to that for cystine lyase activity. The K_m value for cystathionine was 4 mM.

DISCUSSION

The most important question raised by this work, whether the purified turnip cystine lyase is the β-cystathionase involved in methionine biosynthesis, cannot be answered conclusively. Cystine lyase can utilize cystathionine as a substrate, but the activity is lower than that with cystine. Cystine inhibits activity toward cystathionine and vice versa indicating that they both bind at the same site on the enzyme. The latter conclusion is reinforced by a similarity in the inhibitions of cystine lyase and β-cystathionase by cyanide and hydroxylamine and similar inactivation by heat. Cystine lyase and β-cystathionase activities purified together throughout a 277-fold purification.

Tishel and Mazelis [7] found a particulate cystine lyase from cabbage that exhibited no activity towards cystathionine or *S*-methylcysteine and low activity towards D-cystine. Because the turnip enzyme we prepared is soluble, differences in substrate specificity were not unexpected. However, a soluble cystine lyase that was purified 6-fold from rutabaga root (*Brassica napobrassica*) [11] also displayed no activity towards cystathionine or *S*-methylcysteine, but did degrade *S*-methylcysteine sulfoxide and *S*-sulfocysteine. The pronounced difference in substrate specificity between the enzymes of two closely related plant species was unexpected. The data in Table 6 indicate that turnip may have a second cystine lyase, and that it might be comparable to the cystine lyase studied by Mazelis *et al.* [11]. Rutabaga should have a β-cystathionase, because this enzyme is probably required for methionine biosynthesis in higher plants [13].

Giovanelli and Mudd [13] purified from spinach leaves a β-cystathionase that is presumably involved in methionine biosynthesis. In contrast to the turnip enzyme, the spinach enzyme showed greater activity towards cystathionine than towards cystine, exhibited lower activity towards *S*-methylcysteine, and was more sensitive towards thiols. The turnip and spinach enzymes were similar with respect to pH optima and requirement for pyridoxal phosphate. The differences between turnip and spinach enzymes may reflect only species differences.

Flavin and Slaughter [8, 14] extensively purified from *Neurospora* a β-cystathionase that is required for methionine biosynthesis because it is not found in methionine auxotrophs [15]. This authentic β-cystathionase showed similar activity on both cystathionine and cystine. In contrast, the γ-cystathionase from *Neurospora* [16] promoted the degradation of cystathionine *ca* 5 times as fast as that of cystine. This is reasonable because the γ-cystathionase cleaves on the α-aminobutyric acid side of the sulfur atom, while cystine has only α-aminopropionic acid groups next to the sulfur atoms.

Possibly neither cystine nor cystathionine is the natural substrate. But in the absence of contrary evidence, the possibility that the natural function is as a cystine lyase must be considered. If so, what role does it play? Cystine lyase could provide a first step in regenerating cysteine that had been oxidized to cystine. However, this *raison d'être* for cystine lyase would be invalid if turnip has a cystine reductase similar to that in pea seeds [17]. Alternatively, cystine lyase could be a degradative enzyme whose role is to initiate the breakdown of cystine. The latter possibility seems unlikely because higher plants are not known to store sulfide. The evolution of H_2S from plants under certain conditions [18] is unrelated to cystine metabolism as it is correlated with a light-dependent reduction of sulfate. Since the cystine lyase also acts on compounds like *S*-methylcysteine sulfoxide [19], the enzyme may exist to recover sulfur from such compounds.

The cystine lyase described here is not a cysteine desulfhydrase. The low activity with cysteine as substrate is undoubtedly an artifact due to contaminating cystine or to oxidation of cysteine. One product of cystine lyase is cysteine persulfide that was readily and non-enzymically converted to cysteine and elemental sulfur. Possibly cystine lyase is an enzyme that catalyses a β-elimination of compounds having a sulfur-connected substituent on the β-carbon of a L-α-aminopropionic acid group. This could explain why the enzyme has such a wide range of substrates.

EXPERIMENTAL

Assay for cystine lyase. Cystine lyase was measured in several ways. The standard incubation mixture included the enzyme soln (0.1–0.3 ml) with 0.5 ml L-cystine (10 μmol/ml of 0.5 M Tris–acetate, pH 8.5; see prepn below), 0.1 ml pyridoxal phosphate (0.5 mM), 0.02 ml Ellman's reagent [20] (4 mM) (5,5′-dithiobis(2-nitrobenzoic acid)) in 50 mM K-Pi buffer, pH 7.3 and 0.1 ml M Tris–acetate, pH 8.5 in a total vol. of 1 ml. Incubations were carried out at 37° for 30 min. (Cystine is so insoluble in H_2O that a soln was prepared immediately before use by dissolving 24 mg L-cystine in 4 ml M Tris, adjusting the pH to 8.5 with 6 N HOAc and diluting to 10 ml. The reaction could be measured by following the increase in *A* at 412 nm due to the formation of *p*-nitrothiophenol [20]. This method was unsuitable in the presence of coloured materials. Pyruvate formed was measured as the 2,4-dinitrophenylhydrazone by a modification of the method of ref. [21]. After incubation, 1 ml 0.1% 2.4-dinitrophenylhydrazine in 2 N HCl was added to the incubation mixture. After 5 min at room temp., 2 ml 95% EtOH were added with thorough mixing. NaOH (5 ml of 2 N) was added and the mixture vigorously shaken for 30 sec. Pyruvate was measured 10–30 min later by the *A* of its phenylhydrazone at 415 nm.

Pyruvate was also measured with two methods by its ability to oxidize NADH in the presence of lactic dehydrogenase. In one procedure, the reaction was stopped by heating the incubation mixture at 100° and the denatured protein was removed by

centrifugation. An aliquot was mixed with 100 μmol K-Pi, pH 7.3, 0.1 μmol NADH and 50 μg of crystalline heart lactic dehydrogenase (Sigma Chemical Co.) in a total vol. of 1 ml. The lactic dehydrogenase was added last and the reaction was complete in 3 min at 37°; *A* was measured at 340 nm. In the second procedure, 5 μmol cystine in 100 mM Na-PPi buffer (pH 8.5), 0.5 μmol pyridoxal phosphate, 0.1 μmol NADH and 50 μg lactic dehydrogenase were made up to 1 ml and mixed. Then 0.1 ml cystine lyase soln was added with mixing and *A* at 340 nm was monitored at 37° for 10 min.

Cystine lyase activity was measured as the amount of enzyme producing 1 mol of pyruvate per sec at 37° (1 kat). A unit of activity is defined as 10^{-9} kat.

Measurement of products and stoichiometry of the cystine lyase reaction. The Sullivan–Hess procedure for determination of cystine [22] was modified to provide a more reproducible method. An incubation mixture was deproteinized by heating at 100° for 2 min, and then centrifuging. A 1 ml aliquot of the deproteinized incubation mixture was mixed with 0.2 ml 10% NaCN in N NaOH. After 10 min, 0.2 ml 1,2-naphthoquinone-4-sulfonate soln (1% in H_2O) was added and was immediately mixed on a vortex mixer for 15 sec. Immediately thereafter, 0.5 ml Na_2SO_3 soln (10% in 0.5 N NaOH) was added with vigorous mixing for 10 sec. The mixture was allowed to stand for 30 min, and 0.25 ml 8% $Na_2S_2O_4$ soln was added with mixing; *A* at 500 nm was read 10–30 min later. The Na_2SO_3 and $Na_2S_2O_4$ solns were prepared just prior to use.

Ammonia was measured by the phenol hypochlorite colorimetric method [23] after distillation of NH_3 into 0.1 N H_2SO_4 in a Conway vessel [24]. Elemental sulfur was converted to H_2S by reaction with 0.4 M cysteine in 2 N HCl for 30 min in a Conway vessel. The H_2S was collected in 1% zinc acetate soln in 0.4 N NaOH and measured by the method of ref. [25].

Detection of cysteine persulfide. L-Cystine (20 μmol), 100 μmol iodoacetate, 100 μmol K-PPi buffer (pH 8.5), 1 μmol pyridoxal phosphate and 17 units of step 4 enzyme in 2 ml were incubated for 1 hr at 37°. The reaction was stopped by boiling and the precipitated protein was removed by centrifugation. The supernatant was dried in moving air and the residue was dissolved in 100 μl of H_2O. Aliquots (10 μl) were subjected to TLC along with standard samples (0.1 μmol) of cystine, carboxymethylcysteine, thioglycolic acid disulfide and cysteine–thioglycolic acid disulfide. Two complete sets of incubation mixtures and standards were chromatographed on the same plate. TLC plates of Si gel (250 μm thick) were prepared by the Camag system [26]. The solvent used was methylethylketone–Py–H_2O–HOAc (70:15:15:2). Half of the plate was sprayed with 0.1% ninhydrin in MeOH and heated at 70° for 5 min to locate the compounds with amino groups. The other half of the plate was sprayed with cyanide–nitroprusside reagent [27] to locate disulfides.

Thioglycolic acid disulfide and cysteine-thioglycolic acid mixed disulfide were prepared by the procedures of ref. [8].

Assay for β- and γ-cleavage of cystathionine. Radioactive cystathionine labelled in either the 3-carbon or the 4-carbon moiety was prepared by the procedures of ref. [28] by use of partially purified rat liver cystathionine synthetase and serine-[UL-^{14}C] (0.9 μCi/μmol) or homocysteine-[1-^{14}C] (0.02 μCi/μmol). Radioactive cystathionine replaced cystine in the standard assay procedure. After the reaction was stopped by heating to 100° for 2 min, the protein was removed by centrifugation. An aliquot of the supernatant was applied to the top of a 0.5 × 5 cm column of Dowex 50-H^+ (8% divinylbenzene, 200–400 mesh). The resin was washed with enough H_2O to make a total vol. of 5 ml. The effluent from the resin was collected in a scintillation vial. Bray's [29] soln (15 ml) was added and the radioactivity in α-keto acids was measured by liquid scintillation.

Assay for β-cystathionine activity. β-Cystathionase activity was measured by the standard procedures for cystine lyase except that cystine was replaced by 10 μmol of DL-cystathionine.

Gel electrophoresis. Gel electrophoresis and location of protein bands were carried out by the procedures of ref. [30].

Protein determination. Protein was measured by the method of ref. [31] with BSA as standard.

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