Glutathione Synthetase of *Aspergillus niger*: Structural Properties of the Enzyme in Prokaryotes and Eukaryotes

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Glutathione synthetase isolated from a mold, *Aspergillus niger*, had a molecular weight of 110,000 and consisted of two apparently identical subunits, each with a molecular weight of 55,000. The enzyme was most active at pH 8.5. It specifically utilized glycine and ATP, and required Mg^{2+} or Mn^{2+} for its catalytic function. A comparison of glutathione synthetases from various sources indicated that the enzyme of eukaryotes (mammals, molds and yeasts) differ from those of prokaryotes (*Escherichia coli* B and *Proteus mirabilis*) in molecular structure, although the enzymes from both types of organisms contain an active site thiol and catalyze the same reaction.

Glutathione synthetase [EC 6.3.2.3] is the second enzyme in the glutathione synthetic pathway and catalyzes the condensation of γ -L-glutamyl-L-cysteine with glycine in the presence of adenosine 5'-triphosphate (ATP). Glutathione synthetase has been studied, from mammalian sources (pigeon liver,^{1,2)} human and bovine erythrocytes^{3,4)} and ciliary epithelium⁵⁾, higher plants (wheat germ⁶⁾), yeast (*Saccharomyces cerevisiae*^{7,8)}) and bacteria (*Escherichia coli*,⁹⁾ *Escherichia coli* B¹⁰⁾ and *Proteus mirabilis*¹¹⁾), and its physical properties have been well documented. The most highly purified glutathione synthetase was obtained from rat kidney.¹²⁾ It has a molecular weight of $118,000 \pm 4,000$ and is composed of two identical subunits. On the other hand, the enzyme from *E. coli* B has a molecular weight of 140,000 and is cleaved into four identical subunits.¹⁰⁾ A molecular weight of 147,000 has been reported for the *Proteus mirabilis* enzyme, but its molecular structure is not yet clear.¹¹⁾ However, there is a lack of information regarding the molecular structure of glutathione synthetases from eukaryotic microbial cells, except for the yeast enzyme, of

which the molecular weight has been shown to be 123,000.¹³⁾ We therefore present the physical properties of glutathione synthetase from a mold, *Aspergillus niger*, and compare the properties of the eukaryote and prokaryote enzymes.

Materials and Methods

Chemicals. Glutathione reductase and γ -L-glutamyl-L-cysteine were purchased from Nakarai Chemical Co., Ltd., Kyoto, Japan.

Assay for glutathione synthetase. Glutathione synthetase was assayed in a 1.0 ml reaction mixture containing 15 mM γ -L-glutamyl-L-cysteine, 10 mM glycine, 10 mM ATP, 10 mM $MgCl_2$ and 50 mM Tris-HCl buffer (pH 7.5) at 25°C. The reaction was initiated by adding the enzyme and terminated by immersing the test tube in a boiling water bath for 3 min. Glutathione and/or inorganic phosphate formed in the reaction mixture were determined by the method of Tietze¹⁴⁾ and Fiske and Subbarow,¹⁵⁾ respectively. Activity was expressed as μ mol of glutathione or inorganic phosphate formed per hr per mg of protein, which was determined by the method of Lowry *et al.*¹⁶⁾

Purification of glutathione synthetase

1) **Preparation of cell extracts.** *Aspergillus niger* ATCC 9642 was cultured in a nutrient medium (2.0% malt

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extract, 0.1% peptone, 2.0% glucose, 0.02% yeast extract, tap water) at 30°C with reciprocation for 48 hr. The cells (122 g wet wt.) from 9 l of culture were harvested through cheesecloth, resuspended in 100 ml of 10 mM Tris-HCl buffer (pH 7.5) and then homogenized in a Dyno-Mill with the aid of glass beads for 5 min at 0°C. The homogenate was centrifuged at 25,000 $\times g$ for 20 min and the resultant supernatant was used for purification of the enzyme.

2) *Ammonium sulfate fractionation.* To the clear supernatant (4,410 mg protein, 500 ml), 113 g of powdered ammonium sulfate was added, followed by stirring for 30 min at 0°C. The precipitate was separated by centrifugation as above. To the supernatant, another 64 g of ammonium sulfate was added, followed by stirring for 30 min at 0°C and then centrifugation as above. The precipitate was dissolved in 40 ml of 10 mM Tris-HCl buffer (pH 7.5) and then dialyzed against the same buffer at 4°C overnight.

3) *Ion-exchange chromatography.* The dialysate (1,750 mg protein, 55 ml) was applied to a DEAE-cellulose column (6.0 $\times$ 50 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.5). The proteins were eluted with a linear gradient of KCl (0–0.9 M; volume, 9,000 ml) in the same buffer at the flow rate of 48 ml/hr. Fractions of 3.2 ml were collected, and the active fractions, which were eluted at 0.25 M KCl, were combined and then concentrated by ultrafiltration using an Amicon PM10 membrane to about 5 ml.

4) *1st exclusion chromatography.* The concentrate (195 mg protein, 5 ml) was applied to a Sephadex G-150 column (2 $\times$ 90 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.5) and then the proteins were eluted with the same buffer. The flow rate was 32 ml/hr and fractions of 3.2 ml were collected. The active fractions (Nos. 39–49) were pooled and then concentrated to 3 ml as above.

5) *Ion-exchange/exclusion chromatography.* The concentrate (52.8 mg protein, 3 ml) was applied to a DEAE-Sepharose CL-6B column (1.5 $\times$ 95 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.5). The proteins were eluted with a linear gradient of KCl (0–0.6 M; volume, 350 ml) at

the flow rate of 53 ml/hr. Fractions of 3.5 ml were collected, and the active fractions, which were eluted at about 0.26 M KCl, were combined and then concentrated to 3 ml as above.

6) *2nd exclusion chromatography.* The concentrate (6.72 mg protein, 3 ml) was applied to a Sephadex G-150 column (1.5 $\times$ 90 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.5) and then the enzyme was eluted with the same buffer at the flow rate of 18 ml/hr. Fractions of 1.5 ml were collected and the active fractions (Nos. 32–37) were pooled.

7) *Hydrophobic chromatography.* The enzyme solution (2.71 mg protein, 9 ml) was saturated with ammonium sulfate (30%) and then applied to a Butyl-Toyopearl 650M column (1.0 $\times$ 3.0 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.5) saturated with ammonium sulfate (30%). The enzyme was eluted with a linear gradient of ammonium sulfate (30–0%; volume, 30 ml) in the same buffer. The flow rate was 30 ml/hr and fractions of 0.7 ml were collected. The active fractions, which were eluted at approximately 5% ammonium sulfate, were combined and then dialyzed against 10 mM Tris-HCl buffer (pH 7.5) at 4°C overnight. The dialysate was concentrated to about 2 ml as above and then used for characterization of the glutathione synthetase.

Determination of the molecular weight. The molecular weight of the enzyme was determined by gel filtration on a calibrated column of Sephadex G-150 (1.5 $\times$ 95 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.5), the proteins being eluted with the same buffer. Polyacrylamide gel electrophoresis in the presence or absence of sodium dodecyl sulfate was performed according to the method of Laemmli.¹⁷⁾

Results and Discussion

Glutathione synthetase was purified approximately 920-fold from a cell extract of

Table I. SUMMARY OF THE PURIFICATION OF *A. niger* GLUTATHIONE SYNTHETASE

Step	Total protein (mg)	Specific activity (μ mol/hr/mg-protein)	Total activity (μ mol/hr)	Yield of activity (%)	Fold
1. Cell extract	4410	15.3	67470	100	1.0
2. Ammonium sulfate	1750	32.1	56180	83.3	2.1
3. DEAE-cellulose	195	278	54210	80.3	18.2
4. 1st Sephadex G-150	52.8	990	52270	77.5	64.7
5. DEAE-Sepharose CL-6B	6.72	7140	47980	71.1	467
6. 2nd Sephadex G-150	2.71	13200	35770	53.0	863
7. Butyl-Toyopearl 650M	1.33	14100	18750	27.8	962

The purification procedures are described under **Materials and Methods**. The activity was determined by measuring the glutathione formed.

Fig. 1. Molecular Weight Determination of Glutathione Synthetase by Gel Filtration.

The Column conditions are given under **Materials and Methods**. V_e/V_o was plotted as a function of molecular weight, where V_e is the elution volume of protein and V_o the void volume of the column. The elution position of the purified glutathione synthetase is indicated by a closed circle.

Fig. 2. Polyacrylamide Gel Electrophoresis of Glutathione Synthetase in the Presence of Sodium Dodecyl Sulfate.

Lane 1: standard proteins (from top; myosin (H-Chain), phosphorylase *b*, bovine serum albumin, ovalbumin, α -chymotrypsinogen, β -lactoglobulin and lysozyme).

Lane 2: purified glutathione synthetase.

A. niger, with a 30% activity yield (Table I). The purified enzyme exhibited a single band on polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulfate (data not shown). Determination of the molecular weight by gel filtration on a Sephadex G-150 column gave a value of 110,000 (Fig. 1). On the other hand, gel electrophoresis in the presence of sodium dodecyl sulfate indicated a

Fig. 3. Effect of pH on Glutathione Synthetase Activity.

The reaction was carried out under the conditions given under **Materials and Methods**, except for the pH of the reaction mixture. Activities were determined by measuring the glutathione formed. Δ , acetate buffer; $\blacktriangle$, potassium phosphate buffer; $\bullet$, Tris-HCl buffer; $\circ$, borate-NaOH buffer.

subunit molecular weight of 55,000 (Fig. 2), thus indicating that the mold glutathione synthetase consists of two apparently identical subunits. As explained under **Introduction**, the glutathione synthetase of the mold resembled that of mammals and yeast in molecular weight and structure [although the subunit structure of the yeast enzyme has not been reported¹³⁾], but was quite different from the bacterial enzymes.

The purified enzyme from *A. niger* exhibited a pH optimum in the range 8.0~8.5 (Fig. 3). The apparent K_m values of the enzyme for γ -L-glutamyl-L-cysteine, glycine and ATP were calculated to be 0.8, 1.3 and 0.7 mM, respectively, from Lineweaver-Burk plots.¹⁸⁾ The enzyme showed no detectable ATP-hydrolyzing activity in the presence or absence of the substrate (γ -L-glutamyl-L-cysteine or glycine; data not shown). The enzyme was strictly specific to glycine. L-Amino acids (L-glutamate, L-glutamine, L-aspartate, L-asparagine, L-leucine, L-isoleucine, L-methionine, L-proline, L-tryptophan, L-threonine, L-arginine, L-histidine, L-lysine, L-cysteine and L-tyrosine) were not utilized. The enzyme from the mold was almost completely specific to ATP. Other nucleotide triphosphates (CTP, GTP, ITP, TTP and UTP) could not substitute for ATP, although GTP gave barely detectable activity. In the absence of divalent cations, the mold

Table II. EFFECTS OF VARIOUS CHEMICALS ON GLUTATHIONE SYNTHETASE ACTIVITY

Addition (mM)		Relative activity (%)
None		100
Dithiothreitol	(10)	350
Dithioerythritol	(10)	290
2-Mercaptoethanol	(10)	190
L-Cysteine	(10)	65.5
Iodoacetamide	(5.0)	51.3
Acetamide	(5.0)	67.8
N-Ethylmaleimide	(5.0)	50.5
p-Chloromercuribenzoate	(0.1)	0
HgCl ₂	(1.0)	0
Glutathione (reduced)	(1.0)	85.1
	(5.0)	70.4
	(10)	59.7
Glutathione (oxidized)	(1.0)	97.6
	(5.0)	93.7
	(10)	90.9

The reactions were carried out under the conditions given under **Materials and Methods**, except that the various chemicals were added. Activities were determined by measuring inorganic phosphate formed. The activity with no addition was taken as 100%

enzyme showed no activity. Mg^{2+} and Mn^{2+} were preferentially required for the catalytic function, with almost the same efficiencies. Other metal ions (Co^{2+} , Cu^{2+} , Ni^{2+} , Ba^{2+} , Ca^{2+} , Fe^{2+} , Sr^{2+} and Li^{2+}) showed no appreciable effects on the enzyme activity. The potassium ion did not activate the mold enzyme, though the ion is required for the maximum activity of the glutathione synthetase from rat kidney¹²⁾ and yeast.¹⁹⁾ In analogy with the case of the enzymes from rat kidney,¹²⁾ *P. mirabilis*¹¹⁾ and *E. coli B*,¹⁰⁾ the activity of the mold enzyme was completely inhibited by 1.0 mM $HgCl_2$ or 0.1 mM p-chloromercuribenzoate (Table II), thus suggesting that it also contains an active site thiol. On the other hand, reducing agents activated the mold enzyme, two- to three-fold increases in activity being observed in the presence of 10 mM dithiothreitol, dithioerythritol or 2-mercaptoethanol (Table II). Similar results have been reported in the case of the enzyme from *P. mirabilis*.¹¹⁾ The activity of glutathione syn-

thetase from *E. coli B* is feedback-inhibited by the oxidized form of glutathione, but not by the reduced form,¹⁰⁾ the inhibition being more than 90% in the presence of 10 mM oxidized glutathione. However, the mold enzyme was rather insensitive to both types of glutathione, the inhibition being 40% and 10% in the presence of 10 mM of the reduced and oxidized forms of glutathione, respectively (Table II).

Thus, the results obtained indicate that the molecular structure of glutathione synthetase is quite different between eukaryotes and prokaryotes, although the enzymes from both types of organisms contain an active site thiol and catalyze the same reaction. The glutathione synthetases of eukaryotes (mold, yeast and rat kidney) have molecular weights in the range 110,000~120,000 and consist of two apparently identical subunits. On the other hand, the enzyme of prokaryotes (*E. coli B* and *P. mirabilis*) have molecular weights of about 150,000 and the *E. coli B* enzyme consists of four identical subunits. Comparison of the base sequences of the genes for glutathione synthetases of mammals, mold (or yeast) and bacterial cells would be important for elucidation of the evolution of glutathione synthetase.

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