PURIFICATION AND PROPERTIES OF CYSTATHIONINE SYNTHETASE FROM RAT LIVER : SEPARATION OF CYSTATHIONINE SYNTHETASE FROM SERINE DEHYDRATASE

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Cystathionine synthetase (E.C.4.2.1.21) in higher animals has been regarded as a multifunctional enzyme, with serine dehydratase (E.C.4.2.1.13) and threonine dehydratase (E.C.4.2.1.16) activities (Selim and Greenberg, 1959, Nagabhushanam and Greenberg, 1959).

Our previous communication (Nakagawa et al., 1967a) indicated, however, that cystathionine synthetase might be different from serine dehydratase in rat liver for the following reasons : 1) the regulation of the two enzymes differed in various hormonal and dietary conditions (Suda, 1967), and 2) the anti-serine dehydratase serum (Nakagawa et al., in preparation) inhibited serine dehydratase activity, while it did not inhibit cystathionine synthetase activity (Nakagawa et al., 1967b). It was recently established, moreover, that serine dehydratase crystallized from rat liver exhibited no cystathionine synthetase activity (Kimura et al., in preparation). It was also shown by Brown et al. (1966) that cystathionine synthetase could be separated from serine dehydratase using a hydroxylapatite column.

The present communication is on the purification of cystathionine synthetase completely devoid of serine dehydratase, thus establishing that the two enzyme are entirely different. It is also reported that cystathionine synthetase requires pyridoxal phosphate as coenzyme.

Experimental

Cystathionine synthetase was assayed by the method of Mudd et al. (1965a), except that the incubation time was 15 minutes, the pH was 8.6 and the solvent used for eluting cystathionine from a Dowex 50 column was 1N pyridine in place of ammonia.

The incubation system contained 5 μmoles of L-serine-u-C^{14} (10,000 c.p.m. /μmole), 20 μmoles of L-homocysteine, 50 mμmoles of pyridoxal phosphate, 50 μmoles of Tris-HCl buffer, pH 8.6, 0.5 μmole of EDTA and enzyme in a final volume of 0.5 ml.

One unit of the enzyme was defined as the amount which catalyzed the formation of 1 μmole of cystathionine per minute. Specific activity was expressed as units per milligram of protein. Protein was determined by the method of Lowry et al. (1953).

Results

Purification of Cystathionine Synthetase

Step 1, Preparation of Crude Extract One hundred male Wistar rats, weighting 150 to 250 g, were sacrificed by decapitation and their livers were pooled and homogenized with 3 volumes of buffer A (0.05 M potassium phosphate buffer, pH 7.2, containing 0.15 M KCl and 10^{-3} M EDTA). The homogenate was centrifuged for 10,000 x g. All procedures were carried out at 2°, unless otherwise specified.

Step 2, Acid Treatment The supernatant was adjusted to pH 5.5 by dropwise addition of 5% acetic acid and then allowed to stand for 30 minutes with mechanical stirring. The precipitate formed was removed by centrifugation for 20 minutes and the supernatant was adjusted to pH 7.2 with 2N NaOH. This procedure was not highly effective for purification of the synthetase but very effective in removing serine dehydratase which was unstable at acidic pH values.

Step 3, Ammonium Sulfate Fractionation The above supernatant was fractionated with ammonium sulfate. The fraction precipitated at between 25 and 37.5% satu-

ration of ammonium sulfate was collected by centrifugation for 20 minutes at 10,000 x g and dissolved in one tenth volume of buffer B (0.01 M potassium phosphate buffer, pH 7.8, and 10^{-3}M EDTA). The fraction was dialyzed against 2 changes of 10 volumes of buffer B. Most of the cystathionase activity was removed by this procedure.

Step 4, 1st DEAE-cellulose Column Chromatography The dialyzed fraction was applied on a DEAE-cellulose column (5 x 20 cm) equilibrated with buffer B. The column was washed with 1,000 ml of buffer B and eluted with 1,000 ml of 0.05M potassium phosphate buffer, pH 7.8, containing 10^{-3}M EDTA followed by 1,000 ml of 0.1M potassium phosphate buffer, pH 7.8, containing 10^{-3}M EDTA. The last eluates, containing cystathionine synthetase, were pooled and brought to 40% saturation with solid ammonium sulfate. The pH was adjusted to 7.2 with 10% ammonium hydroxide solution. After standing for 30 minutes with mechanical stirring, the precipitate which formed was collected by centrifugation and dissolved in a small volume of buffer A.

Step 5, Treatment with P-cellulose The fraction was applied on a column of Sephadex G-25 (4.5 x 44 cm) which had been equilibrated with 0.01M maleate buffer, pH 5.5, containing 10^{-3}M EDTA, to remove ammonium sulfate. The column was eluted with the same buffer. The fraction of protein was located spectrophotometrically, and the eluate in this fraction was pooled (approximately 250 ml) and mixed with 100 ml (10g dry weight) of p-cellulose which had been previously equilibrated with 0.01 M maleate buffer, pH 5.5, containing 10^{-3}M EDTA. The mixture was allowed to stand for 30 minutes with mechanical stirring and then centrifuged for 20 minutes at 10,000 x g. The precipitate was washed once with 200 ml of the same buffer by centrifugation. The supernatant and washing were combined and brought to 40% saturation with ammonium sulfate. The precipitate was collected as described above and dissolved in a small volume of buffer A.

Step 6, Sephadex G-100 Column Chromatography The above fraction was applied on a Sephadex G-100 column (3 x 90 cm) which had been equilibrated with buffer

Table I

Summary of Purification of Cystathionine Synthetase

	Total volume (ml)	Total units	Total protein (mg)	Specific activity x 10^3	Yield (%)
Crude extract	2960	354	69,000	5.1	100
Acid treatment	2890	286	53,200	5.4	80.8
$(NH_4)_2SO_4$ Fr.	265	190	6,350	30.0	53.7
1st DEAE-cellulose $(NH_4)_2SO_4$ Fr.	24	76	500	152	21.4
P-cellulose $(NH_4)_2SO_4$ Fr.	7.0	15.9	33.5	473	4.5
Sephadex G-100	56.0	7.7	10.0	704	2.2
2nd DEAE-cellulose	30.0	4.0	3.1	1212	1.1

B. The column was eluted with the same buffer. Fractions of 5 ml were collected in a fraction collector and active fractions were combined.

Step 7, 2nd DEAE-cellulose Column Chromatography The pooled active fraction was applied to a DEAE-cellulose column (1.2 x 5 cm) which had been equilibrated with buffer B. The column was eluted by lineár gradient elution. The mixing vessel contained 35 ml of buffer B and the reservoir contained 35 ml of 10^{-3}M EDTA in 0.2 M potassium phosphate buffer, pH 7.8. The active fractions were combined and concentrated as described above. The enzyme was purified 240 fold by these 7 procedures. The purified enzyme could be stored for at least 1 month at -20° without loss of activity.

Properties of Cystathionine Synthetase

The purified enzyme has no serine dehydratase activity. In confirmation of previous findings that crystalline serine dehydratase exhibited no cystathionine synthetase activity, the data indicate clearly that cystathionine synthetase is entirely different from serine dehydratase. The molecular weight was determined using a Sepharose 4B column with blue dextran (1,000,000), catalase (250,000) and pyruvate kinase type M (250,000) (Tanaka et al., 1967) as marker proteins. The activity peak of the synthetase was

Table II

Effect of Hydroxylamine and Pyridoxal Phosphate on Cystathionine Synthetase

Additions	Cystathionine synthetase mμmoles/min	Activity (%)
None	12.5	100
NH_2OH, 1×10^{-3}M	0	0
NH_2OH, 4×10^{-4}M	3.3	27
NH_2OH, 4×10^{-4}M + Pyridoxal phosphate, 2×10^{-3}M	12.0	96

The purified enzyme (10.3 μg of protein) was incubated for 5 minutes at 37° with pyridoxal phosphate and/or hydroxylamine at the concentrations indicated above and then the reaction was started by addition of serine-u-C^{14}. Other conditions are described in the text.

found between the two marker enzymes which were eluted closely together. This indicates that the molecular weight of cystathionine synthetase is approximately 250,000. In the previous communication, it was reported that the molecular weight of serine dehydratase from rat liver was estimated to be 64,000 by equilibrium ultracentrifugation. However, the data so far obtained exclude the possibility that cystathionine synthetase is formed from serine dehydratase by a conformational change.

As shown in Table II, the purified synthetase was inhibited by hydroxylamine and the inhibition was reversed by pyridoxal phosphate. This suggests that pyridoxal phosphate is a cofactor of cystathionine synthetase.

The Km values for L-serine and L-homocysteine were 1.6×10^{-3}M and 1.1×10^{-2}M, respectively.

The enzyme exhibited the highest activity at pH 8.6 with a sharp decrease in activity toward alkaline pH values.

The difference between cystathionine synthetase and serine dehydratase are summarized in Table III.

Table III

Characteristics of Serine Dehydratase and Cystathionine Synthetase

	Serine Dehydratase	Cystathionine Synthetase
Molecular weight	64,000	250,000
Cofactor	Pyridoxal phosphate	Pyridoxal phosphate
Km for L-serine	5.9×10^{-2}M	1.6×10^{-3}M
for L-homocysteine		1.1×10^{-2}M
Neutralization with anti-SDH* serum	+	-
Distribution	Liver, Kidney	Various tissues (a)
Regulation	Hormonal regulation substrate induction (b)	Feedback repression by cystine (c, d)
Others	Cystathionine synthetase (-) TDH (+)	SDH (-) TDH*(-)

*SDH, serine dehydratase and TDH, threonine dehydratase
(a) Mudd et al., 1965a; (b) Nakagawa et al., in preparation; (c) Suda, 1967; (d) Finkelstein et al., 1967

Since the two enzymes differ, it is readily understood why the regulations of cystathionine synthetase, a key enzyme of cystine formation from methionine, and serine dehydratase, a key enzyme of gluconeogenesis from amino acids should differ (Ishikawa et al., 1965). This also gives a consistent explanation for a report of a patient with homocystinuria whose liver had threonine dehydratase (serine dehydratase) but no cystathionine synthetase (Mudd et al., 1965b).

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