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STUDIES ON SERINE HYDROXYMETHYLASE ISOENZYMES FROM RAT LIVER*

YOSUKE NAKANO, MOTOJI FUJIOKA AND HIROSHI WADA

Department of Biochemistry, Osaka University School of Medicine and Department of Surgery, Research Institute for Microbial Diseases, Osaka University, Osaka (Japan)

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SUMMARY

Serine hydroxymethylase (L-serine:tetrahydrofolate 5,10-hydroxymethyltransferase, EC 2 1 2 1) was found to occur in the soluble and mitochondrial fractions of rat liver. The enzymes were purified from the two fractions and some of their properties were investigated

1 The soluble fraction serine hydroxymethylase had a rather sharp pH optimum at 8 0, whereas the mitochondrial enzyme showed a broad pH optimum centering at 7 4.

2 There was no significant difference in the Michaelis constants for substrates between the soluble fraction and mitochondrial serine hydroxymethylases

3 The mitochondrial enzyme was more stable than the soluble fraction enzyme

4 The soluble fraction serine hydroxymethylase was more anionic than the mitochondrial enzyme as evidenced by the affinity for DEAE-cellulose and electrophoretic mobility.

INTRODUCTION

Serine hydroxymethylase (L-serine tetrahydrofolate 5,10-hydroxymethyltransferase, EC 2 1 2.1) which catalyzes the interconversion of serine and glycine

$$\text{Serine} + \text{tetrahydrofolate} \underset{b}{\overset{a}{\rightleftharpoons}} \text{glycine} + \text{5,10-methylenetetrahydrofolate} + H_2O \qquad (1)$$

according to Eqn 1 is reported to occur in a variety of mammalian, avian and plant tissues as well as in microorganisms[1] As to the intracellular localization of the enzyme, however, no detailed study was made

The present communication reports on the occurrence of serine hydroxymethylase in the soluble and mitochondrial fractions of rat liver, and some of the properties of the isoenzymes from the two fractions.

A preliminary account of this work has appeared[2].

* This work was taken from the thesis submitted to the Graduate School of Osaka University by Y Nakano, in the partial fulfillment for the degree of Doctor of Medical Science

MATERIALS AND METHODS

L(±)-Tetrahydrofolic acid was prepared by hydrogenation of folic acid as described by HATEFI *et al*.[3] Other chemicals were from commercial sources. Male albino rats of Wistar strain weighing approx 200 g were used in the present study The animals were fasted for 24 h prior to sacrifice and they were killed by decapitation. The freshly excised livers were homogenized in 0 25 M sucrose and subcellular fractionation was carried out essentially as described by HOGEBOOM AND SCHNEIDER[4] The mitochondrial pellets obtained were washed twice with 0 25 M sucrose and kept frozen until use. The nuclei were isolated by the procedure of HOGEBOOM, SCHNEIDER AND STIEBECK[5]. Protein was estimated by the method of LOWRY *et al*.[6] with bovine serum albumin as the standard Inorganic phosphate was determined according to the method of FISKE AND SUBBAROW[7].

5,10-Methylenetetrahydrofolate dehydrogenase was obtained from *Achromobacter eurydice* grown on phenylalanine or glutamate as the sole source of carbon The enzyme was purified from the sonic extracts of the bacterium through protamine sulfate treatment, $(NH_4)_2SO_4$ fractionation, heat treatment and DEAE-cellulose chromatography The details of the purification procedure will be published elsewhere The purified preparation migrated as a single band on polyacrylamide-gel electrophoresis and was completely devoid of serine hydroxymethylase activity (M. FUJIOKA AND Y. NAKANO, unpublished results)

Serine hydroxymethylase activity was determined by measuring the rate of reaction in either forward (Reaction 1a) or reverse direction (Reaction 1b).

Assay 1. Assay with glycine and 5,10-methylenetetrahydrofolate as substrates (Reaction 1b) The rate of Reaction 1b was determined by the method of SCRIMGEOUR AND HUENNEKENS[8] with modifications. The standard reaction mixture, in a final volume of 0 5 ml, contained 50 μmoles of potassium phosphate (pH 7 3), 0 1 ml of neutralized tetrahydrofolate–formaldehyde mixture (4 μmoles of (±)-tetrahydrofolate dissolved in 1 ml of 12 5 mM formaldehyde), 12 5 μmoles of glycine and an appropriate amount of the enzyme After incubation at 37° (routinely for 10 min), the reaction was terminated by the addition of 0 1 ml of 15% trichloroacetic acid 2 ml of the Nash reagent were then added and the mixture was incubated again at 37° to allow the color to develop. After 20 min of incubation, 0 8-ml aliquot of the mixture was diluted with 5 ml of H_2O, and the absorbance at 410 mμ was measured in a Bausch–Lomb 340 colorimeter. Under these assay conditions, the disappearance of formaldehyde was linear with respect to time and enzyme concentration until 40% of the compound was consumed A unit of the enzyme activity was defined as the amount of enzyme causing the disappearance of 1 μmole of formaldehyde per min under these conditions and the specific activity was expressed as units per mg of protein

Assay 2. Assay with serine and tetrahydrofolate as substrates (Reaction 1a). The rate of formation of 5,10-methylenetetrahydrofolate in Reaction 1a was estimated by the rate of NADPH formation in the presence of excess $NADP^+$ and 5,10-methylenetetrahydrofolate dehydrogenase To a 1 5-cm cuvette (1-cm light path) were added 150 μmoles of potassium phosphate (pH 7 3), 0 4 μmole of (±)-tetrahydrofolate dissolved in 0.02 M potassium phosphate (pH 7 3) containing 0.1 M 2-mercaptoethanol, 0 25 μmole of $NADP^+$, 0 3–0 6 unit* of 5,10-methylenetetrahydrofolate dehydrogen-

* A unit of 5,10-methylenetetrahydrofolate dehydrogenase is the amount of the enzyme which produces 1 μmole of NADPH per min

ase and the enzyme in a total volume of 1 4 ml. The reaction was started by the addition of 0.1 ml of 0.1 M L-serine The absorbance measurement was made with a Shimadzu QB 50 spectrophotometer Under these conditions, the increase in the absorbance at 340 mμ was proportional to time and enzyme concentration

RESULTS

Subcellular distribution of serine hydroxymethylase activity

As Table I shows, serine hydroxymethylase activity was found in the soluble, mitochondrial and nuclear fractions. No activity was found in the microsomal fraction. Since the nuclear fraction was contaminated with mitochondria and unbroken cells, no definite conclusion is drawn as to the occurrence of the enzyme in this fraction. The mitochondrial and soluble fractions were almost free from cross contamination

TABLE I

SERINE HYDROXYMETHYLASE ACTIVITY IN SUBCELLULAR FRACTIONS

Serine hydroxymethylase activity was determined by Assay 1

Fraction	*Specific activity (units/mg protein)*	*Total activity (units/g liver)*	*Total protein (mg/g liver)*
Homogenate	0 0095	2 02	213 0
Nuclear	0 0154	0 44	28 4
Mitochondrial	0 0136	0 71	52 0
Microsomal	0 0000	0 00	23 5
Soluble	0 0110	0 94	84 9

Purification of serine hydroxymethylase from soluble fraction

Since there was no activity associated with the microsomes, 10 000 $\times$ *g* supernatant of the liver homogenate was used as the starting material for purification of serine hydroxymethylase from the soluble fraction.

Step 1 The supernatant solution (1500 ml) was brought to 43% saturation with solid $(NH_4)_2SO_4$ After stirring for 30 min, the precipitate was removed by centrifugation. $(NH_4)_2SO_4$ was again added to the supernatant to 53% saturation, and the precipitated protein was collected by centrifugation The precipitate was dissolved in 0.05 M potassium phosphate (pH 7 1) and dialyzed overnight against 0 02 M potassium phosphate (pH 7 1) containing 2 μg per ml of pyridoxal phosphate

Step 2 The dialysate was diluted with the same buffer to give the protein concentration of about 20 mg per ml To the solution were added 0.2 vol of 0 5 M potassium phosphate (pH 6 5) and L-serine to a final concentration of 10 mM. The mixture was then heated at 70° for 3 min and, after rapid cooling, the denatured protein was discarded by centrifugation Heating under the same conditions but in the absence of added L-serine resulted in a loss of 75% of the activity.

Step 3 The supernatant solution from Step 2 was treated with $(NH_4)_2SO_4$ in the same manner as in Step 1 The precipitate obtained was taken up in a small amount of the buffer, and dialyzed against 0 05 M potassium phosphate (pH 6.5) containing 2 μg per ml of pyridoxal phosphate for 12 h

Step 4. The dialyzed enzyme containing approx. 240 mg of protein was poured over a column (2 cm × 16 cm) of DEAE-cellulose which had been equilibrated with 0.05 M potassium phosphate (pH 6 5) The column was then washed with the same buffer until no protein was eluted A gradient elution was then carried out with 600 ml of 0 05 M potassium phosphate (pH 6 5) in the mixing vessel and 0 4 M of the same buffer in the reservoir. The activity usually appeared at a phosphate concentration of 0 1 to 0 17 M The fractions having high specific activity were combined and concentrated by $(NH_4)_2SO_4$ precipitation

A summary of the purification procedure is given in Table II

TABLE II

PURIFICATION OF SERINE HYDROXYMETHYLASE FROM THE SOLUBLE FRACTION

Serine hydroxymethylase activity was determined by Assay 1

Fraction	*Volume (ml)*	*Protein content (mg/ml)*	*Total units*	*Specific activity (units/mg protein)*	*Yield (%)*
10 000 × *g* supernatant	1500	12 8	143 0	0 007	100
1st $(NH_4)_2SO_4$	62	53 2	118 0	0 036	82 7
Heat treatment, 70°	238	3 8	111 0	0 125	78 0
2nd $(NH_4)_2SO_4$	11 3	21 2	74 0	0 39	51 7
Chromatography on DEAE-cellulose	82 0	0 27	42 8	1 94	30 0
3rd $(NH_4)_2SO_4$	0 7	5 0	15 15	4 30	10 8

Purification of serine hydroxymethylase from mitochondrial fraction

Step 1 A suspension of mitochondria was diluted with water to give a protein concentration of about 2%. L-Serine and pyridoxal phosphate were added to the final concentration of 10 mM and 2 μg per ml, respectively, and the mixture was heated at 65° for 3 min. The denatured protein was discarded by centrifugation

Step 2 The heated extract was treated with $(NH_4)_2SO_4$ and the protein precipitating between 43 and 53% saturation was dissolved in 0 05 M potassium phosphate (pH 7 1) The solution was then dialyzed against 0 005 M potassium phosphate (pH 7 1) containing 2 μg per ml of pyridoxal phosphate for 12 h

Step 3 The dialyzed enzyme was adsorbed on a column of brushite (2 cm × 14 cm) which had been equilibrated with 0 005 M potassium phosphate (pH 7 1) Washing was made with the same buffer until no protein was detected in the eluate. Serine hydroxymethylase was eluted from the column by raising the concentration of the buffer to 0 03 M

Step 4 The fractions having high specific activity were pooled, diluted 2-fold with water and then poured over a 1 cm × 4 cm column of DEAE-cellulose. Under these conditions, serine hydroxymethylase was held by the cellulose as a sharp band on the top of the column The enzyme was eluted with 0 1 M potassium phosphate (pH 7 1) By this procedure the enzyme was concentrated and an additional 2-fold purification was usually achieved.

The purification procedure of the mitochondrial serine hydroxymethylase is summarized in Table III

TABLE III

PURIFICATION OF SERINE HYDROXYMETHYLASE FROM THE MITOCHONDRIAL FRACTION

Serine hydroxymethylase activity was determined by Assay 1

Fraction	*Volume (ml)*	*Protein content (mg/ml)*	*Total units*	*Specific activity (units/mg protein)*	*Yield (%)*
Mitochondrial suspension	70	52 5	26 6	0 007	100
Heat treatment, 65°	132	2 0	22 0	0 08	82 5
$(NH_4)_2SO_4$	2	22 0	15 0	0 36	58 9
Chromatography on brushite	13	0 21	7 8	2 83	29 0
Chromatography on DEAE-cellulose	0 5	1 5	4 0	5 50	15 0

Properties of soluble fraction and mitochondrial serine hydroxymethylases

pH optimum The effect of pH on serine hydroxymethylase activity was determined in 0.1 M potassium phosphate or potassium pyrophosphate. A rather steep, nearly symmetrical curve was obtained for the soluble fraction serine hydroxymethylase with the optimum at pH 8 0. The mitochondrial enzyme showed a broad pH optimum and was most active between pH 7.0 and 7.7 (Fig. 1)

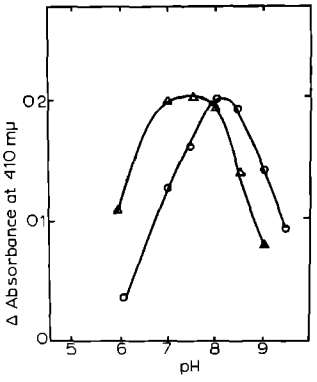

Fig 1 Effect of pH on serine hydroxymethylase activity The purified preparations of soluble fraction (○—○) and mitochondrial (△—△) serine hydroxymethylases were used Activity measurement was made by Assay 1

Michaelis constants for substrates The Michaelis constants for substrates were estimated from double reciprocal plots in the usual manner[9] As shown in Table IV, the K_m value for serine was smaller than that for glycine, and the K_m for tetrahydrofolate was smaller than that for 5,10-methylenetetrahydrofolate There was, however, no significant difference in affinities of substrates for serine hydroxymethylases from both fractions.

Affinity for DEAE-cellulose It was found during enzyme purification that the mitochondrial serine hydroxymethylase came off the DEAE-cellulose column with a buffer of lower ionic strength than that required for the elution of the soluble fraction enzyme. To ascertain the difference in the affinity of the both enzymes for DEAE-

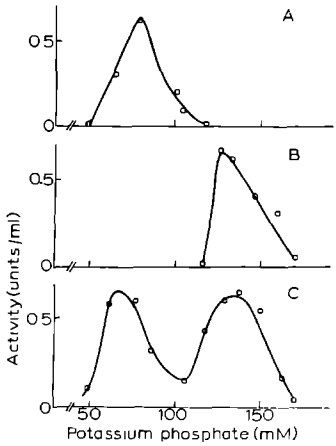

Fig 2 Affinity of soluble fraction and mitochondrial serine hydroxymethylases for DEAE-cellulose The purified preparation of serine hydroxymethylase was adsorbed on a 1 cm × 10 cm column of DEAE-cellulose Elution was carried out as described in the text Enzymic activity was determined by Assay 1 A, the mitochondrial serine hydroxymethylase, B, the soluble fraction serine hydroxymethylase, C, the mixture of A and B

cellulose, an experiment described in Fig 2 was performed. The purified preparations of soluble fraction and mitochondrial serine hydroxymethylases were adsorbed on DEAE-cellulose columns which had been equilibrated with 0 02 M potassium phosphate at pH 6 5 Fig. 2 shows the plot of serine hydroxymethylase activity against phosphate concentration of the eluates. The mitochondrial enzyme was eluted at the phosphate concentration of 0 05 to 0 1 M (the activity peak at 0 07 M), whereas the enzyme from the soluble fraction came off the column at the concentration of 0 1 to 0 17 M (the peak at 0 13 M) Chromatography of the mixture of both enzymes revealed two activity peaks centering at phosphate concentrations of 0.07 and 0 14 M

Electrophoresis Electrophoresis of the soluble fraction or the mitochondrial serine hydroxymethylase on cellulose acetate strip revealed a single protein band in each case To examine whether the band represents serine hydroxymethylase, the strip was cut into small pieces of the same size and each piece was eluted separately with 0 02 M potassium phosphate buffer (pH 7 1) The enzymic activity coincided with

TABLE IV

K_m VALUES FOR SUBSTRATES

Determination of K_m values for L-serine and tetrahydrofolate was made by Assay 2, and those for glycine and 5,10-methylenetetrahydrofolate by Assay 1

Substrate	*Soluble fraction serine hydroxymethylase (μM)*	*Mitochondrial serine hydroxymethylase (mM)*
L-Serine	540	0 54
L(±)-Tetrahydrofolate	7 2	0 010
Glycine	1200	1 8
L(±)-Methylenetetrahydrofolate	130	0 13

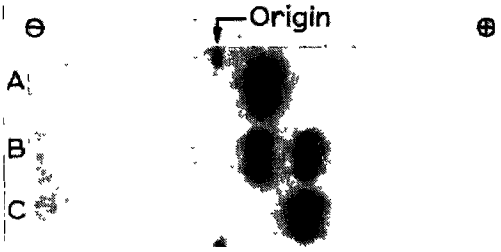

Fig 3 Electrophoresis pattern on cellulose acetate The mitochondrial serine hydroxymethylase (A), the soluble fraction serine hydroxymethylase (C), and the mixture (B) were applied on a cellulose acetate strip Electrophoresis was performed for 1 h at 20° with 0 07 M potassium phosphate (pH 8 0) as the buffer A constant current of 1 mA per cm of the strip was applied Protein was stained with the Ponseau 3R reagent[10]

the protein band Fig 3 shows the result of a typical experiment The soluble fraction serine hydroxymethylase travelled further to the anode than the mitochondrial enzyme under these conditions

Stability The purified preparation of soluble fraction serine hydroxymethylase was more unstable than that from the mitochondrial fraction For example, when the dilute solutions of serine hydroxymethylases (300 μg of protein per ml of 0 05 M potassium phosphate (pH 7.1)) were allowed to stand for 72 h at 23°, the soluble fraction serine hydroxymethylase lost 85% of the activity, whereas the mitochondrial enzyme only 20%

The addition of serine or glycine to the soluble fraction serine hydroxymethylase was effective in preventing the loss of activity that occurred during enzyme storage Sulfhydryl compounds such as 2-mercaptoethanol and dithiothreitol at concentrations of 1 and 0 1 mM, respectively, were partially effective On the contrary, EDTA, pyridoxal phosphate or serum albumin did not exert any protection against inactivation of the enzyme (Table V)

TABLE V

PROTECTIVE EFFECT OF VARIOUS COMPOUNDS ON SOLUBLE FRACTION SERINE HYDROXYMETHYLASE ACTIVITY

The purified preparation of soluble fraction serine hydroxymethylase was employed after dialysis for 12 h against 0 05 M potassium phosphate (pH 7 1) Enzyme activity was determined by Assay 1 after allowing the enzyme to stand for 48 h at 23° in the presence of each compound tested The protein concentration of the mixture was 300 μg per ml

Addition	*Concn (mM)*	*Initial enzyme activity (%)*
None		43 5
Glycine	10	88 5
L-Serine	10	100 0
Pyridoxal-*P*	0 1	44 0
EDTA	1	44 8
Dithiothreitol	0 1	61 0
2-Mercaptoethanol	1	70 3
Bovine serum albumin	1%*	42 0

* g per 100 ml

DISCUSSION

Among isoenzymes many examples are now known which differ in their localization within the cell Among those occurring in the soluble and mitochondrial fractions of mammalian tissues are isoenzymes of glutamate aspartate transaminase[11–13], glutamate alanine transaminase[14], kynurenine transaminase[15], malate dehydrogenase[16,17], isocitrate dehydrogenase[18], fumarase[19], creatine kinase[20], *etc* The data described in this communication indicate that there occur, in rat liver, isoenzymes of serine hydroxymethylase in the soluble and mitochondrial fractions The occurrence of serine hydroxymethylase in these cell fractions has also been demonstrated in livers of other species such as ox, mouse, pig and rabbit Homogeneous preparations of isoenzymes have recently been obtained from rabbit liver in this laboratory. The isoenzymes from this source had very similar properties with respect to pH optimum, electrophoretic mobility and stability, but they were distinct immunochemically[2]

The serine hydroxymethylase isoenzymes of rat liver differ in the pH optimum, stability and in net electric charge of the protein as evidenced by the difference in affinity for DEAE-cellulose and in electrophoretic mobility The isoenzymes, however, showed a marked similarity in K_m values for substrates This fact and the ready reversibility of the reaction suggest that the direction of the reaction catalyzed by these isoenzymes *in vivo* is largely dependent on the availability of substrates in particular cell components It seems impossible at present to assign any role to these isoenzymes in cellular metabolism because of the lack of information on the steady-state concentration of substrates in each cell compartment

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