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Purification and Characterization of Serine Hydroxymethyltransferase from Mitochondria of *Euglena gracilis* z

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Mitochondrial serine hydroxymethyltransferase, L-serine: tetrahydrofolate 5,10-hydroxymethyltransferase (EC 2.1.2.1), (m-SHMT) was extracted and highly purified from *Euglena gracilis* z. The specific activity increased from the crude extract with 10% yield up to 580-fold through the following steps: ammonium sulfate fractionation, DEAE-cellulose column chromatography and rechromatography, and affinity chromatography with L-lysine-Sepharose 4B. The molecular weight of the purified m-SHMT was 88,000 by gel filtration through Sephadex G-200, and 44,000 by SDS–PAGE. One mol of the purified enzyme contained two mol of pyridoxal 5′-phosphate (PLP), indicating that the enzyme is a dimer. Characteristics of the enzyme were examined and compared with SHMTs of other origins. The m-SHMT of *Euglena gracilis* z had L-threonine aldolase activity as did s-SHMT of the same origin in addition to the usual SHMT activity.

Key word: serine hydroxymethyltransferase

Serine hydroxymethyltransferase (SHMT), L-serine; tetrahydrofolate 5,10-hydroxymethyltransferase (EC 2.1.2.1), catalyzes the mutual transfer between L-serine and glycine,[1] and also the conversion of β-hydroxyamino acids, such as L-threonine or L-allothreonine, to glycine and corresponding aldehydes.[2–4] We have reported that SHMTs of *Euglena gracilis* z could be classified into three types, s-SHMT from cytosol, m-SHMT from mitochondria, and c-SHMT from chloroplasts.[5] To analyze the physiological function of the enzyme in *Euglena gracilis* z, we highly purified the s-SHMT,[6] and found that the enzyme catalyzed the cleavage of L-threonine, conversion of L-threonine to glycine and acetaldehyde, as well as both L-allothreonine into glycine and acetaldehyde, and L-serine into glycine and formaldehyde. We have reported that the L-threonine aldolase activity of s-SHMT does not exist in SHMTs from animal liver[7,8] or germinative plants.[7,9]

This paper deals with the purification of m-SHMT and the comparison of characteristics with highly purified s-SHMT.

Materials and Methods

Materials. Sephadex G-200 and CNBr-activated Sepharose 4B were obtained from Pharmacia Fine Chemicals. Yeast 5,10-methylenetetrahydrofolate (5,10-methylen H_4-folate) dehydrogenase [EC 1.5.1.5] and (+)-s-tetrahydrofolic acid (H_4-folate) were from Sigma Chemical Co; DEAE-cellulose (DE-52), from Whatman; $NADP^+$ and NADPH, from Oriental Yeast Co.; pyridoxal 5′-phosphate (PLP), from Wako Chemicals. L- and D-allothreonines were supplied by courtesy of the Department of Organic Chemistry, Institute for Protein Research, Osaka University. Other chemicals were commercial products of an analytical reagent grade.

Preparation of adsorbent. L-Lysine-Sepharose 4B was prepared as reported previously.[10]

Purification of m-SHMT. All processes were done at 0–4°C, unless specified.

Step 1. Crude preparation. Euglena gracilis z was cultured in Koren-Hutner medium[11] at 27°C under 2000 lux of artificial light for three days as reported previously. Since the content of m-SHMT in the cells was 1/3 of s-SHMT, we started with twice the amount of cells than those in the previous report.[6] Approximately 360 g of wet cells of *Euglena gracilis* z were washed by suspending them in 2 liters of water, followed by centrifugation at $1000 \times g$ for 5 min. The washing was repeated three times. The washed cells were suspended in 360 ml of 20 mM potassium phosphate buffer (pH 6.8) containing 20 μM PLP, 10 μM diisopropylfluorophosphate (DIFP), 1 mM β-mercaptoethanol, and 1 mM EDTA (B-1). The cells were disrupted by sonication (10 kHz, 3 min × 5). The ruptured cell suspension was centrifuged at $12,000 \times g$ for 30 min and the supernatant was dialyzed against 10 liters of B-1 for approximately 20 h, followed by a second centrifugation. The clear supernatant was used for further purification as the crude preparation.

Step 2. Ammonium sulfate fractionation. The 30–60% saturation of ammonium sulfate fraction was isolated, and dialyzed against 6 liters of B-1 at 4°C for approximately 20 h.

Step 3. Fist DEAE-cellulose column chromatography. The dialyzed preparation of Step 2 was put on a DEAE-cellulose column, 2.5 × 40 cm, equilibrated with 20 mM potassium phosphate buffer (pH 6.8) containing 20 μM PLP, 1 mM β-mercaptoethanol, and 1 mM EDTA (B-2), and the column was washed with 1.5 liters of B-2 solution. The elution was done with a linear gradient system consisting of 500 ml of B-2 and 500 ml of 0.5 M potassium phosphate buffer (pH 6.8) containing 20 μM PLP, 1 mM β-mercaptoethanol, and 1 mM EDTA. Ten-ml fractions were collected. The enzyme was eluted at 0.2 M potassium phosphate buffer (Fraction I) and at 0.35 M potassium phosphate buffer (Fraction II). As reported previously, the enzyme in Fraction I originated from cytosol, and that in Fraction II was from mitochondria.[5,6] The yield of s-SHMT was three times more than m-SHMT, and therefore, the latter fraction contaminated the former. Fraction II was dialyzed against 6 liters of B-2 for 20 h.

Step 4. Second DEAE-cellulose column chromatography. The dialyzed preparation was put on a DEAE-cellulose column (2.5 × 40 cm), followed

Abbreviations: SHMT, serine hydroxymethyltransferase; m-SHMT, mitochondrial serine hydroxymethyltransferase; s-SHMT, cytosolic serine hydroxymethyltransferase; c-SHMT, chloroplastyl serine hydroxymethyltransferase; H_4-folate, tetrahydrofolic acid; PLP, pyridoxal 5′-phosphate; DIFP, diisopropylfluorophosphate; B-1, 20 mM potassium phosphate buffer (pH 6.8) containing 20 μM PLP, 10 μM diisopropylfluorophosphate (DIFP), 1 mM β-mercaptoethanol, and 1 mM EDTA; B-2, 20 mM potassium phosphate buffer (pH 6.8) containing 20 μM PLP, 1 mM β-mercaptoethanol, and 1 mM EDTA.

by washing with 1.5 liters of B-2. The elution was done under the same conditions as described in Step 3. Fraction II eluted at 0.35 M potassium phosphate buffer was dialyzed against 6 liters of B-2.

Step 5. L-*Lysine-Sepharose 4B affinity chromatography*. The dialyzed preparation was put on an affinity column (1.0 × 35 cm), and the column was washed with 200 ml of B-2. The elution was done with a linear gradient system consisting of 200 ml of B-2 and 200 ml of 0.3 M potassium phosphate buffer, pH 6.8, containing 20 μM PLP, 1 mM β-mercaptoethanol, and 1 mM EDTA. Two-ml fractions were collected. The enzyme activity and protein content were measured for every fraction. The SHMT activity was eluted in the fractions at around 0.15 M potassium phosphate buffer, and the enzyme activity and protein peaks were parallel. The active fractions were combined, and dialyzed against 2 liters of B-2 solution at 4°C for 20 h.

Measurement of SHMT activity. The enzyme activity using either L-allothreonine or L-threonine as the substrate was measured by the diffusion method reported previously.[8,9] A reaction mixture containing 20 mM L-allothreonine (or L-threonine), 20 μM PLP, 20 mM potassium phosphate buffer (pH 6.8), and enzyme in a total volume of 1 ml was reacted in a 20-ml closed round flask at 37°C for 30 min. The reaction was stopped by cooling at −70°C. The reaction flask was then connected to a cooled (−70°C) test tube with a side arm containing 0.2 ml of 2 mM semicarbazide, and was put under a vacuum. Acetaldehyde formed by the reaction diffused into the side test tube followed by relocating it to a chamber warmed up to 30°C for 10 min. The amount of the product, semicarbazone, was measured by OD at 222 nm.

The SHMT activity using L-serine as the substrate was measured as described by Ogawa *et al.*[12] Reaction mixture containing 20 mM L-serine, 20 μM PLP, 0.2 mM H_4-folate, 1.4 mM $NADP^+$, 0.5 IU 5,10-methylene H_4-folate dehydrogenase, 20 mM potassium phosphate buffer (pH 6.8), and enzyme in a total volume of 1 ml was incubated at 37°C for 30 min. Absorbance increase resulted from NADPH formed thus was measured at 340 nm.

One unit of the enzyme was defined as that decomposing 1 μmol of the substrate in 1 min, and the specific activity was expressed as units per milligram of protein.

Measurement of molecular weight. The molecular weight of m-SHMT was measured by the gel filtration method[13] using a Sephadex G-200 column (2.5 × 95 cm), equilibrated with 20 mM potassium phosphate buffer (pH 6.8) containing 20 μM PLP, and 0.2 mM EDTA. A mixture of m-SHMT preparation and standard proteins was put on the column and eluted with the same buffer solution at the flow rate of 5 ml/h, and 1-ml fractions were collected. The enzyme activity and protein content in each fraction were measured.

Polyacrylamide gel electrophoresis. Ten % acrylamide gels were used for SDS-PAGE as described by Laemmli.[14] Coomassie brilliant blue R-250 was used for the staining of protein bands. Bovine serum albumin (68,000), hen egg albumin (45,000), and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36,000) were used as the standard proteins.

Content of PLP in m-SHMT. The bound PLP in m-SHMT was measured by the method of Wada and Snell.[15] The purified m-SHMT preparation was well dialyzed against 10 mM potassium phosphate buffer containing 1 mM EDTA. One ml of the dialyzed enzyme solution (1 mg protein/ml) was mixed with 1 ml of 1 M H_2SO_4 solution containing 2% phenylhydrazine. The precipitate was removed by centrifugation, and the absorbance at 410 nm of the supernatant was measured.

Protein content. Protein in the samples was measured by the absorbance at 280 nm and by the method of Lowry *et al.*[16] using bovine serum albumin as the standard.

Results

Purification of m-SHMT

The specific activity and the yield of m-SHMT in every step of purification are shown in Table I. Total and specific activities of SHMT in the crude preparation were 166.4 units and 0.01146 unit/mg protein, respectively. The portion of m-SHMT in the crude preparation was 20.7%, as reported previously.[5,6] Therefore, the m-SHMT in the crude preparation was a total of 34.4 units and 0.0024 unit/mg protein. The m-SHMT was purified approximately 580-fold over the original crude preparation with 1.39 unit/mg protein of specific activity. The yield was approximately 10%. The preparation showed a single protein band by SDS-polyacrylamide gel electrophoresis (Fig. 1).

Molecular weight of m-SHMT and its subunit

The molecular weight of m-SHMT was 88,000 by gel filtration with Sephadex G-200. On the other hand, the molecular weight of the subunit of the enzyme was 44,000, implying m-SHMT is a dimer of a single peptide.

Absorption spectrum

The absorption spectrum of m-SHMT indicated two peaks, one was at 278 nm, which is characteristic of protein, and the other at 415 nm, which was apparently the absorption of protein-bound PLP (Fig. 2). The ratio of A_{278}/A_{415} was 10.

PLP content

PLP was measured by calculating from the molecular weight of 88,000 of m-SHMT. One mol of m-SHMT contained 1.8 mol of PLP, indicating that a subunit binds one mol of PLP.

Optimum pH

The optimum pH of m-SHMT was 6.8 using L-allothreonine as the substrate.

Substrate specificity

L-Serine, L-threonine, and L-allothreonine were all broken up by m-SHMT, but the enzyme did not react with D-serine, D-threonine, and D-allothreonine (Table II). Although breakdown of L-threonine and L-allothreonine needed only PLP as a coenzyme, that of L-serine required H_4-folate as another cofactor in addition to PLP. Both V_{max} and K_m for L-serine, L-threonine, and L-allothreonine are also shown in Table II. The ratio of the catalytic constant, V_{max}/K_m, among the three substrates, L-serine/L-threonine/L-allo-

Table I. Typical Purification of m-SHMT from *Euglena gracilis* z

Enzyme activity was measured under the standard assay conditions with L-allothreonine as a substrate.

Purification step	Volume (ml)	Total protein (mg)	Total units (units)	Specific activity (units/mg protein)	Yields (%)
Cell extract	1,000	14,520	34.4	0.0024	100
Ammonium sulfate fractionation	200	2,420	28.3	0.0117	82.3
DEAE-Cellulose chromatography	200	592	18.0	0.0304	52.3
DEAE-Cellulose rechromatography	80	48.4	5.63	0.1163	16.4
L-Lysine-Sepharose 4B affinity chromatography	36	2.45	3.40	1.3878	9.9

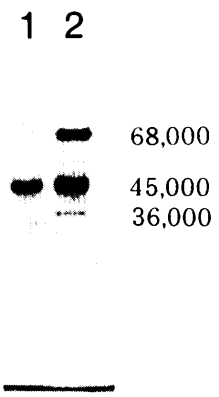

Fig. 1. SDS–PAGE of the Purified m-SHMT.

SDS-PAGE was done in 10% gel, 0.25 M Tris–HCl buffer, pH 8.3, containing 0.2 M β-mercaptoethanol; 3 μg of protein; staining, 0.25% Coomassie brilliant blue R-250 in EtOH–AcOH–H_2O (9 : 2 : 9). Lane 1, purified enzyme; lane 2, bovine serum albumin (68,000), hen egg albumin (45,000), and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36,000).

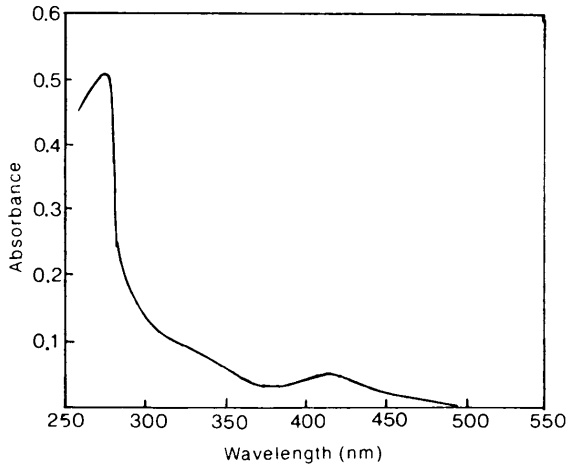

Fig. 2. Absorption Spectrum of the m-SHMT.

The solution contained 0.5 mg/ml of the enzyme in 10 mM potassium phosphate buffer (pH 7.2).

Table II. The Substrate Specificity

Substrate	Specific activity (units/mg protein)	K_m (mM)	V_{max} (mol/min/mol enzyme)
L-Serine	0.048	65.3	17.6
D-Serine	0		
L-Threonine	0.150	35.7	35.2
D-Threonine	0		
L-Allothreonine	1.388	4.35	160.2
D-Allothreonine	0		

K_m and V_{max} for L-serine, L-threonine, and L-allothreonine were estimated from Lineweaver–Burk plots.

threonine, was 1/3.7/136.4. These result showed that L-allothreonine had a high reaction velocity to the enzyme.

Effects of amino acids and their derivatives to m-SHMT

The inhibitory effects of amino acids and their derivatives on m-SHMT were examined using L-allothreonine as a substrate, as shown in Table III.

Of the amino acids tested, glycine, L-serine, L-cysteine, L-α,β-diaminopropionate, and β-alanine showed a strong inhibitory effect, especially L-serine, L-cysteine, and L-α,β-diaminopropionate, while L-alanine weakly inhibited the

Table III. Effects of L-Amino Acids and Their Analogues on L-Allothreonine Aldolase Activity of m-SHMT

Enzyme solution was incubated in the presence of 10 mM test compounds for 30 min at 30°C, and the reaction was started at 37°C for 30 min at pH 6.8 by the addition of an equal volume of L-allothreonine.

Inhibitor	Relative activity (%)	K_i (mM)
None	100	
Glycine	62.5	3.7
L-Alanine	81.4	
L-Valine	95.3	
L-Leucine	100	
β-Alanine	65.6	
γ-Aminobutyric acid	88.4	
L-Serine	37.6	1.7
L-Homoserine	100	
β-Hydroxybutyric acid	100	
L-Aspartic acid	98.6	
L-Glutamic acid	100	
L-α,β-Diaminopropionate	23.2	0.9
L-Ornithine	100	
L-Lysine	95.4	
L-Cysteine	20.9 (2 mM)	0.25
L-Methionine	100	
β-Mercaptopropionic acid	100	
Ethylenediamine	100	
Ethanolamine	100	
β-Mercaptoethanolamine	100	

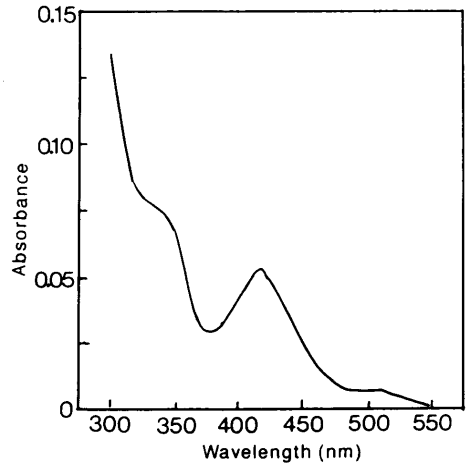

Fig. 3. Absorption Spectrum of the m-SHMT in the Presence of L-Allothreonine.

The mixture contained 0.5 mg of purified m-SHMT, 20 mM L-allothreonine, and 0.06 M potassium phosphate buffer (pH 7.2) in a total volume of 1 ml.

enzyme. Other amino acid had no effect. The inhibition by these amino acids for m-SHMT was all of the competitive type (Table III).

Other related compounds such as β-mercaptoethanolamine did not show any effect.

Absorption spectra of m-SHMT in the presence of L-allothreonine or L-threonine

The absorption spectrum of a mixture of m-SHMT (0.5 mg/ml) and L-allothreonine (20 mM) at pH 7.2 showed a peak at 505 nm other than the peaks at 278, 340, and 415 nm (Fig. 3). A similar spectrum was also observed in

the presence of L-threonine. These results suggest that m-SHMT decomposes L-allothreonine and L-threonine into glycine and acetaldehyde through an intermediate.

Discussion

We had purified m- and s-SHMTs from rat liver,[8] SHMT from corn germ,[9] and s-SHMT from *Euglena gracilis* z[6] using affinity chromatography with L-lysine-Sepharose 4B and characterized. This paper added another example of effective purification on m-SHMT of *Euglena gracilis* z using the same adsorbent.

The molecular weight of m-SHMT of *Euglena gracilis* z was 88,000 by Sephadex G-200 gel filtration, and that of the subunit was 44,000 by SDS–PAGE. One mol of m-SHMT contained 2 mol of PLP. These results strongly support that the m-SHMT is a dimer, and the fact was similar to s-SHMT of *Euglena gracilis* z as reported previously.[6] SHMT of *Hypomicrobium methylovorum*[17] is reported to be molecular weight of 90,000 and also is a dimetric structure. This enzyme is similar to m-SHMT from *Euglena gracilis* z.

The effects of various amino acids on the L-allothreonine aldolase activity of the m-SHMT investigated, and the results closely resembled those for s-SHMT. On the alkylated amino acids, the extent of inhibitory effect was glycine > L-alanine > L-valine. Thus, the inhibition of m-SHMT was affected by the length of the ($-CH_2-$) in the inhibitor, the inhibition decreasing with increases in the length of the ($-CH_2-$) group.

The inhibitory effects of the different functional groups at β-carbon of the amino acids were as follows; L-cysteine > L-α,β-diaminopropionate > L-serine > L-alanine, that is, $-SH > -NH_2 > -OH > -H$.

Some amino acids, L-cysteine, L-serine, and glycine were competitive inhibitors. In the reactions catalyzed by SHMT, the product was 5,10-methylene H_4-folate, which was formed from formaldehyde from L-serine and H_4-folate, while product by the L-allothreonine aldolase reaction was free acetaldehyde. Using L-serine as a substrate, since the formation of 5,10-methylene H_4-folate was expected to be the rate-limiting step, the apparent K_m for L-serine tended to be large. On the other hand, using L-allothreonine as the substrate, since free acetaldehyde was dissociated in the absence of H_4-folate, the rate-limiting step was not observed unlike with L-serine. The effects of L-serine on L-allothreonine aldolase activity were measured in the absence of H_4-folate. Accordingly it was suggested that L-serine showed a strong inhibitory effect, with a small K_i for L-serine. The kinetic mechanisms on action of SHMT should be further investigated.

The purified m-SHMT of *Euglena gracilis* z decomposes serine into glycine and "C_1" unit in the presence of PLP and H_4-folate, and also breaks down both L-allothreonine and L-threonine to glycine and acetaldehyde. When the m-SHMT was mixed with a saturating level of L-allothreonine, three distinct absorption bands were observed at 340, 415, and 505 nm, suggesting the interconversion due to three distinct enzyme-L-allothreonine complexes. The species showing absorption at 340 nm appeared to be the geminal diamine of L-allothreonine[18] formed on the reaction of L-allothreonine with the enzyme, the complex showing absorption at 415 nm was a hydrogen-bonded Schiff base between L-allothreonine and PLP in the enzyme, and the complex showing absorption at 505 nm was formed by the removal of acetaldehyde from L-allothreonine leading to the formation of a quinoid intermediate.[18] The absorption spectrum of the enzyme in the presence of L-threonine is similar to those of the addition of L-allothreonine to the enzyme. These results suggest that the m-SHMT can cleave L-threonine as well as L-allothreonine into glycine and acetaldehyde.

m-SHMT and s-SHMT from *Euglena gracilis* z had very similar catalytic properties; substrate specificities, and optimun pH, but differed in the ionic strength required for their elution from a DEAE-cellulose column, m-SHMT was eluted with about 0.35 M potassium phosphate, while s-SHMT was eluted with about 0.2 M. The specific activities of m-SHMT were about half of those of s-SHMT. Hereafter, we are trying to compare the structures of these enzymes with the gene level.

In contrast with SHMTs of animal liver[7,8] and germs of plants,[7,9] those are reported not to react with L-threonine. *Euglena gracilis* z m-SHMT catalyzes the conversion from L-threonine to glycine and acetaldehyde, L-threonine aldolase reaction, as well as the conversion of L-serine into glycine and "C_1" unit. This enzyme, therefore, seems to have some important physiological roles in L-serine, glycine, and L-threonine metabolisms.

Physiologically the function of the enzyme in *Euglena gracilis* z still remains to be investigated.

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