Bioscience, Biotechnology, and Biochemistry

ISSN: 0916-8451 (Print) 1347-6947 (Online) Journal homepage: https://www.tandfonline.com/loi/tbbb20

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To cite this article: Masako Sakamoto, Tsutomu Masuda, Yukio Yanagimoto, Yoshihisa Nakano & Shozaburo Kitaoka (1993) Effects of D-Amino Acids on Cytosolic Serine Hydroxymethyltransferase from *Euglena gracilis* z, Bioscience, Biotechnology, and Biochemistry, 57:3, 506-508, DOI: 10.1271/bbb.57.506

To link to this article: https://doi.org/10.1271/bbb.57.506

Published online: 12 Jun 2014.

Biosci. Biotech. Biochem., **57** (3), 506—508, 1993

Note

Effects of D-Amino Acids on Cytosolic Serine Hydroxymethyltransferase from *Euglena gracilis* z

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Received October 2, 1992

Serine hydroxymethyltransferase [L-serine: tetrahydrofolate 5,10-hydroxymethyltransferase, EC 2.1.2.1] (SHMT) from *Englena gracilis* z is known to involve three isozymes[1]; they are the cytosolic (s-SHMT), mitochondrial (m-SHMT), and chloroplastic fractions (c-SHMT).

We[2] purified s-SHMT from *Euglena gracilis* z and investigated its physical and chemical properties. We[2] also studied the inhibitory effects of L-amino acids on this enzyme, focussing on the functional group bound to the β-carbon atom in L-amino acids. It was found that the hydrophilic functional groups, such as –SH, $-NH_2$, and –OH, contribute to the effective inhibition of this enzyme, while the –COOH, $(-CH_2-)n$, and other groups lessen the inhibitory effect. Furthermore, the inhibitory effects of L-amino acids on this enzyme were found to change inversely with the length of the $(-CH_2-)n$ chain.[2]

The pyridoxal enzyme, the coenzyme of which is pyridoxal phosphate, forms a Schiff base with L-amino acids (alanine, valine, aspartate) which has a typical absorption spectrum with peaks near 280 nm, 340 nm, and 415 nm.[3] On the other hand, the complexes with glycine, L-threonine, and L-allothreonine have an absorption spectrum with a peak at 505 nm in addition to the above three peaks.[1,4]

There are only a few papers[3,5] reporting on the inhibitory effects of D-amino acids on SHMT and on the absorption spectrum of SHMT-D-amino acid complex. This paper reports on the inhibitory effects of D-amino acids on s-SHMT from *Euglena gracilis* z and the absorption spectra of its complexes with D-amino acids.

CNBr-activated Sepharose 4B was from Pharmacia Fine Chemicals; DEAE-cellulose (DE-52) from Whatman; pyridoxal phosphate from Wako Chemicals; L-allothreonine, D-valine, D-lysine, D-methionine, D-phenylalanine, D-leucine, D-aspartate, and D-glutamate were supplied by courtesy of the Department of Organic Chemistry, Institute for Protein Research, Osaka University. Other chemicals were commercial products of reagent grade.

Preparation of the s-SHMT. The s-SHMT from *Euglena gracilis* z was prepared as described previously.[2]

Enzymatic activity assay. The cleavage of L-allothreonine into glycine and acetaldehyde was measured by assaying acetaldehyde by the method reported previously.[2] A mixture (1.0 ml) containing 20 mM L-allothreonine, 20 mM pyridoxal phosphate, 20 mM phosphate buffer (pH 6.8), and an appropriate amount of enzyme was allowed to react in a tight round bottom flask (20 ml) at 37°C for 30 min, after which the flask was immediately cooled to −70°C, connected to a test tube containing 0.2 ml of 2 mM semicarbazide solution (pre-cooled to −70°C), evacuated, and sealed tight. Then, the flask alone was warmed to 30°C to diffuse over about two-thirds of the reaction broth into the test tube. Under these conditions, acetaldehyde in the flask was completely converted to semicarbazone. The contents of the tube were cooled to room temperature, and then the concentration of semicarbazone was measured by the absorbance at 222 nm. The amount of enzyme which yielded 1 μmol of acetaldehyde per min at 37°C was defined as 1 unit of enzyme activity. Specific activity was expressed as units per mg protein.

Protein was measured by the method of Lowry *et al.*[6] with bovine serum albumin as a standard.

α-Keto acids, the reaction products formed from D-alanine, D-serine, and other D-amino acids, were detected by thin-layer chromatography[5] on a silica gel plate (Silicagel HF, produced by Merck). A reaction mixture containing 0.1 M D-alanine (D-serine), 20 mM pyridoxal phosphate, 20 mM phosphate buffer (pH 6.8), and 1.0 mg of s-SHMT was allowed to react at 37°C for 1 h, after which 0.2 ml of 0.4% 2,4-dinitrophenylhydrazine dissolved in 2 N HCl was added. After vigorous shaking, the reaction products were extracted five times with 2-ml portions of ether. The extracts were combined, concentrated to 0.1 ml, put on a precoated plate of Silicagel HF, and analyzed by thin-layer chromatography with *n*-butanol–ethanol–12% aqueous ammonia (7:1:2) as a developing solvent.

Inhibition by D-amino acids of s-SHMT was investigated with L-allothreonine as the substrate. The results are shown in Table. Of the amino acids tested, D-cysteine and D-serine inhibited the enzyme, while D-alanine was a weak inhibitor. On the other hand, D-cystine, D-methionine, D-valine, and others were not effective. The modes of action of D-amino acids on s-SHMT were analyzed by Lineweaver–Burk plots[7];

Table Effects of D-Amino acids on s-SHMT from *Euglena gracilis* z

Inhibitor	Concentration (mM)	Relative activity (%)	K_i (mM)
None		100	
D-Alanine	30	83.9	13.0
	50	77.3	
D-Valine	30	97.5	
	50	95.4	
D-Leucine	30	98.2	
	50	96.0	
D-Serine	30	66.1	8.0
	50	53.1	
D-Threonine	30	92.6	
	50	88.1	
D-Aspartic acid	30	100	
	50	100	
D-Glutamic acid	30	100	
	50	100	
D-Lysine	30	100	
	50	100	
D-Cysteine	1	54.8	0.35
	2	35.6	
D-Methionine	30	100	
	50	100	
D-Cystine	30	100	
	50	100	
D-Phenylalanine	30	90.3	
	50	78.0	

Enzyme solution was incubated with the D-amino acids for 5 min at 30°C before the reaction and then incubated at 37°C for 30 min at pH 6.8 with an equal volume of L-allothreonine.

Abbreviations: SHMT, serine hydroxymethyltransferase; s-SHMT, cytosolic serine hydroxymethyltransferase; m-SHMT, mitochondrial serine hydroxymethyltransferase; c-SHMT, chloroplastic serine hydroxymethyltransferase.

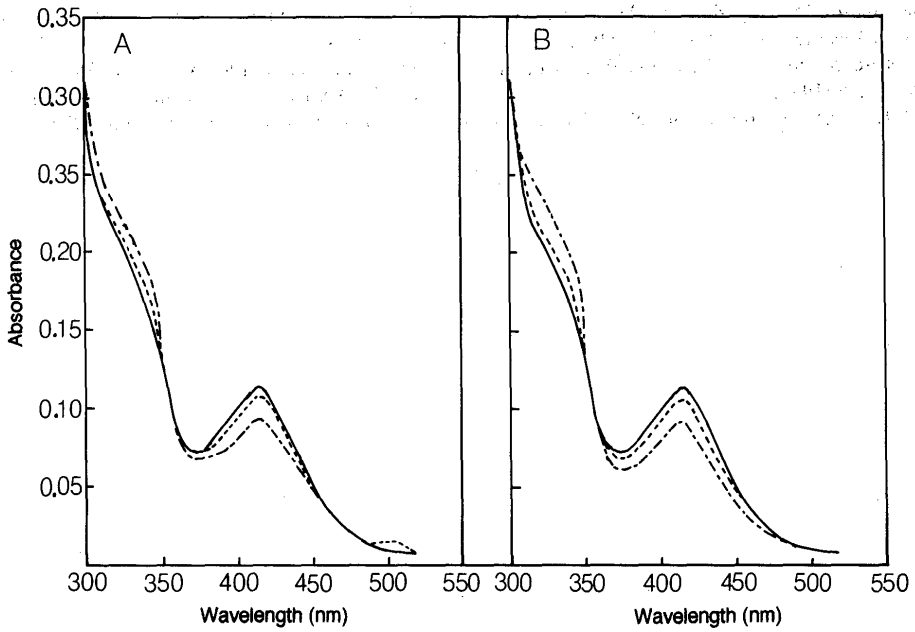

Fig. Absorption Spectra of s-SHMT in the Presence of D-Alanine and D-Serine.

A: ——, spectrum of s-SHMT (1.0 mg per 1 ml of 0.02 M phosphate buffer, pH 7.2, containing 1 mM β-mercaptoethanol and 1 mM EDTA); -----, spectrum of s-SHMT (1.0 mg per ml of 0.02 M phosphate buffer, pH 7.2, containing 1 mM β-mercaptoethanol, 1 mM EDTA, and 0.1 M D-alanine) after incubating for 60 min at 37°C; —·—, spectrum taken after incubating with D-alanine for 90 min.
B: ——, spectrum of s-SHMT (1.0 mg per 1 ml of 0.02 M phosphate buffer, pH 7.2, containing 1 mM β-mercaptoethanol and 1 mM EDTA); -----, spectrum of s-SHMT (1.0 mg per ml of 0.02 M phosphate buffer, pH 7.2, containing 1 mM β-mercaptoethanol, 1 mM EDTA, and 0.1 M D-serine) after incubating for 60 min at 37°C; —·—, spectrum taken after incubation with D-serine for 90 min.

D-alanine, D-serine, and D-cysteine were competitive inhibitors.

These results show that D-α-amino acids, which have hydrophilic functional groups bound to their β-carbon atoms, inhibit the enzyme effectively, such as the –SH and –OH group, as in the case of L-α-amino acids, and that the activity change inversely with the length of chain of ($-CH_2-$) groups.

The absorption spectrum of the s-SHMT–D-alanine complex was analyzed. s-SHMT was dialyzed for 24 h against a 20 mM phosphate buffer (pH 7.2) containing 1 mM β-mercaptoethanol and 1 mM EDTA. The visible absorption spectrum of the dialyzate (enzyme content, 1.0 mg/ml) was recorded after addition of 0.1 M D-alanine (Fig. A). The absorption spectrum obtained 60 min later had a shoulder around 340 nm, and peaks suggesting a Schiff base at 415 nm and the formation of a carbanion-quinoid resonance structure at 505 nm. Ninety min after addition of D-alanine, the peak at 505 nm disappeared, the 415 nm peak decreased from that obtained in 60 min after addition of D-alanine, and the 340 nm peak increased.

Dunathan[8] has postulated that in the pyridoxal enzyme, the C_α Schiff base bond with the greatest probability of undergoing cleavage would be the one that is perpendicular to the plane containing the imine nitrogen and the pyridine ring. Furthermore, the complex showing absorption at 505 nm seems that pyruvic acid formed by transamination of D-alanine leads to the formation of a quinoid intermediate in the catalytic center of the enzyme. The analysis of the product in the reaction mixture of thin-layer chromatography[5] found a small amount of pyruvic acid.

The absorption spectrum of s-SHMT–D-serine complex was analyzed. The results are shown in Fig. B. No peak appeared at 505 nm after addition of D-serine, the 340-nm absorption increased, while the 415-nm absorption decreased along with the time as in the case of the s-SHMT–D-alanine complex.

α-Keto acid could not be detected in the reaction mixture of D-serine with s-SHMT. These findings suggest that the decrease in the 415-nm absorption is caused by a decrease of the holoenzyme, but the mechanism remains unknown.

It has already been reported[9] that the reaction of s-SHMT with L-cysteine causes a decrease in the 415-nm absorption assigned to a Schiff base formed by the lysine residue of the enzyme and 4-position aldehyde group of pyridoxal phosphate *via* the L-amino group. Subsequently the Schiff base, upon aldimine transfer, forms thiazolidine between the L-cysteine and the pyridoxal phosphate, resulting in an increase in 340-nm absorption assigned to thiazolidine. Adding D-cysteine to *Euglena gracilis* z s-SHMT results in severe decrease in the 415-nm absorption and increase in the 340-nm. Accordingly, the absorption spectrum of s-SHMT–D-cysteine complex was found to be the same as that of s-SHMT–L-cysteine complex. The reaction of s-SHMT with D-cysteine resembles closely that of s-SHMT–L-cysteine, while the spectrum differs from the one of D-cysteine and s-SHMT from rat liver[5] of which the 270—280-nm absorption increases with time after addition of D-cysteine. These results suggest that the s-SHMT from *Euglena gracilis* z is different from that of rat liver in the stereochemical configuration of active cavity of the enzyme.

On the other hand, 1.0 mg of s-SHMT, which was dissolved in 1.0 ml of 20 mM phosphate buffer (pH 6.8) containing 1 mM β-mercaptoethanol and 1 mM EDTA, was incubated for 5 min at 37°C, and then 5 mM D-cysteine were added before sealing. The mixture was incubated at 37°C for an additional 30 min. After incubation, it was dialyzed 3 times against 500 ml of 20 mM phosphate buffer (pH 6.8) containing 1 mM β-mercaptoethanol and 1 mM EDTA, and then the enzyme activity was assayed using L-allothreonine as a substrate; the enzyme was inactive. When 20 mM pyridoxal phosphate was added to it, the enzyme retained more than 85% of its original activity, indicating almost complete recovery. These results suggest that the enzyme is inactive by irreversible formation of thiazolidine, which has the absorption peak at 340 nm as in the case of L-cysteine. Inactivation of the enzyme was closely related with these spectral changes. The compound with absorption at 340 nm, probably the thiazolidine of pyridoxal phosphate, was removed by dialysis and then the enzyme was almost recovered to the original activity.

This evidence is consistent with the results of SHMT from rabbit liver[9] and *Candida humicola*,[10] which catalyzed the cleavage of threonine into glycine and acetaldehyde, and it appears more likely that pyridoxal phosphate is necessary in maintaining the structure of s-SHMT from *Euglena gracilis* z.

Even when D-leucine, D-cystine, D-threonine, or D-aspartate was added to s-SHMT, no change occurred, leaving the same absorption spectrum as that of the holoenzyme.

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