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PURIFICATION AND CHARACTERIZATION OF SERINE TRANSHYDROXYMETHYLASE FROM *SACCHAROMYCES CEREVISIAE*

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SUMMARY

Serine transhydroxymethylase (serine:tetrahydrofolate 5, 10-transhydroxymethylase, (EC 2.1.2.1) was purified 180–200-fold from *Saccharomyces cerevisiae*. The isolated enzyme is very labile, but can be stabilized by storage in 30% (v/v) glycerol. It has a molecular weight of 180 000–200 000, and the activity is stimulated by divalent and monovalent cations. Purified serine transhydroxymethylase has an absolute requirement for tetrahydrofolic acid and pyridoxal phosphate for activity. The apparent Michaelis constants are $2.5 \cdot 10^{-4}$ M for tetrahydrofolic acid, $1.1 \cdot 10^{-4}$ M for pyridoxal phosphate, and $7.0 \cdot 10^{-4}$ M for L-serine.

INTRODUCTION

Serine transhydroxymethylase catalyzes the transfer of the β-carbon of serine to tetrahydrofolate yielding N^5, N^{10}-methylene tetrahydrofolate. This reaction serves as the first step in providing a number of one-carbon units at the oxidation level of formaldehyde and formate[1], thus involving it in the biosynthesis of purines and thymine. The reaction has also been implicated in supplying the methyl group of methionine[2–4], thereby relating it to numerous cellular transmethylation reactions *via* S-adenosylmethionine (SAM). In addition, the reversibility of the serine transhydroxymethylase reaction means it may serve as the last step in serine biosynthesis with glycine as the immediate precursor.

Serine transhydroxymethylase from *Escherichia coli* has a molecular weight of 170 000, is repressed strongly by methionine, but exhibits little or no susceptibility to feedback inhibition by methionine or SAM[5]. Serine transhydroxymethylase from rabbit liver has a molecular weight of 215 000 (ref. 6), is inhibited *in vitro* by folate coenzymes[7], guanosine, and thymidine[8], but no repression has been reported. In yeast, the regulation of serine transhydroxymethylase appears to differ from that in

Abbreviations: PLP, pyridoxal 5'-phosphate; SAM, S-adenosylmethionine.

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both *E. coli* and rabbit liver. The yeast enzyme is repressed slightly by methionine, and is inhibited *in vitro* by methionine and SAM[2].

Because of the potentially great importance of serine transhydroxymethylase as a control step for a variety of biosynthetic pathways, and because of differences observed for the enzyme from different sources, this study was undertaken to determine characteristics of the enzyme from *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Organisms and cultural conditions

Experiments utilized *S. cerevisiae* 3701B, a haploid yeast requiring uracil. Cells were grown in a medium containing 1% tryptone, 2% dextrose, and 0.5% yeast extract as previously described[2]. For large scale purification of serine transhydroxymethylase, cells were grown in a New Brunswick Fermacell Fermentor (Model CF-50) with stirring at 500 rev./min and vessel aeration at 2 l/min. Cells were grown for 18–20 h before being harvested, washed, and resuspended in 35 l of Wickerham's complete defined medium minus methionine[9]. Aeration was continued for an additional 6 h before harvesting.

Serine transhydroxymethylase assay

Serine transhydroxymethylase was assayed by the method of Taylor and Weissbach[4] with minor modifications. These included using 0.1 M sodium bicine buffer (pH 8.5) in the reaction mixture, and terminating the reaction with 0.5 ml of 1.0 M sodium acetate buffer (pH 4.5). The various parameters of the assay have been described previously using potassium phosphate buffer[2], and are unchanged by the use of bicine buffer. Sufficient DL-serine was added to the DL-[3-^{14}C] serine to give a final specific activity of 10^6 dpm/μmole. For the determination of apparent Michaelis constants, the tetrahydrofolic acid was partially purified on a DEAE-cellulose column using the method of Schirch and Jenkins[10], and the concentration was determined spectrophotometrically at 295 nm.

Purification of serine transhydroxymethylase

Step 1. Preparation of crude extract. Crude cell extracts were prepared in 0.1 M potassium phosphate buffer (pH 7.3), in a Bronwill MSK Cell Homogenizer as previously described[11]. After centrifugation, glycerol was added to the 25 000 $\times$ *g* supernatant to make it 30% (v/v) with respect to glycerol.

Step 2. Heat treatment. The 30% glycerol solution was heated at 55 °C in a water bath shaker for 15 min. It was then cooled to 4 °C and centrifuged for 30 min at 25 000 $\times$ *g*.

Step 3. Protamine sulfate. A 1% solution of protamine sulfate was added dropwise to the heat treated supernatant to give a final ratio of 0.1 mg protamine sulfate to 1.0 mg protein. This was centrifuged immediately at 20 000 $\times$ *g* for 30 min. The supernatant was dialyzed against 10–15 vol. of 0.1 M potassium phosphate buffer (pH 7.3), for 6 h. During this period the buffer was changed 4 times.

Step 4. $(NH_4)_2SO_4$ fractionation: Solid $(NH_4)_2SO_4$ was utilized, and percent saturation was defined at 4 °C. The 35–55% fraction was centrifuged, and the resultant precipitate was dissolved in 0.05 M potassium phosphate buffer (pH 7.3). This

solution was then dialyzed for 14–18 h against 200 vol. of the same buffer with five changes (200 vol. each) of buffer.

Step 5. DEAE-Sephadex. The dialyzed solution from Step 4 was layered on a 2.5 cm × 90 cm column of DEAE-Sephadex A-50 which had been equilibrated in 0.05 M potassium phosphate buffer (pH 7.3). The column was eluted with this buffer until the eluate showed an absorbance of less than 0.1 at 280 nm. The remaining protein was eluted with a linear KCl gradient (0–0.4 M). 5-ml fractions were collected, and the tubes containing high specific activity were pooled. The pooled samples were concentrated with a Diaflo ultrafiltration unit equipped with an XM-50 membrane.

Step 6. Sephadex G-200. The protein from Step 5 was added to an upward flow Sephadex G-200 column (2.5 cm × 90 cm) which had been equilibrated with 0.1 M potassium phosphate buffer (pH 7.3). Three-ml fractions were collected, and the tubes containing high specific activity were pooled and concentrated by ultrafiltration as above. The enzyme was stored at — 10 °C in 30% glycerol (v/v).

The temperature throughout the purification was maintained at 0–4 °C except where noted. All buffers, unless otherwise stated, were supplemented with $5 \cdot 10^{-8}$ M pyridoxal phosphate and $5 \cdot 10^{-6}$ M dithiothreitol. Protein determinations were made according to the method of Lowry *et al.*[12], using bovine serum albumin as the standard.

Enzyme homogeneity

The homogeneity of the purified enzyme was determined by polyacrylamide disc gel electrophoresis using the method of Davis[13]. Cross linking of 5.5% and pH of 9.5 were utilized, and polymerization was allowed to occur at room temperature. Protein was stained using a 1% Amido Black solution in 7% acetic acid, and destaining was carried out overnight in 5% acetic acid.

Adsorption spectrum

The absorption spectrum of the purified serine transhydroxymethylase apoenzyme and holoenzyme was determined with a PMQ II Zeiss spectrophotometer. SAM was converted from the iodide salt to the chloride salt by passage through a short column of Dowex-1 (Cl^-) before addition to the holoenzyme. The concentration of SAM was determined spectrophotometrically[14].

Molecular weight determinations

Molecular weight estimates were made by sucrose density centrifugation according to the method of Martin and Ames[15], and by Sephadex G-200 chromatography according to the method of Andrews[16]. Yeast alcohol dehydrogenase, mol.wt 150 000 (ref. 17), and beef liver catalase, mol.wt 250 000 (ref. 18), were employed as standards.

Chemicals, enzymes and isotopes

Pyridoxal 5'-phosphate (PLP), L-tetrahydrofolic acid, yeast alcohol dehydrogenase, and beef liver catalase were obtained from the Sigma Chemical Co. *S*-adenosyl-L-methionine was obtained from Calbiochem, and DL-[3-^{14}C] serine was obtained from Amersham/Searle Corp.

RESULTS

Purification of serine transhydroxymethylase

A 180–200-fold purification of serine transhydroxymethylase was accomplished following the purification scheme presented in Table I. The elution profile from the DEAE-Sephadex column exhibited three major protein peaks, with the peak containing serine transhydroxymethylase being eluted at KCl concentrations between 0.10 and 0.15 M. The elution of the enzyme from Sephadex G-200 was totally within the first protein peak.

TABLE I

PURIFICATION OF SERINE TRANSHYDROXYMETHYLASE FROM *S. cerevisiae*

Fraction	*Volume (ml)*	*Protein (mg)*	*Spec. act. (units/mg)*	*Yield (%)*
Crude extract	250	7150	0.36	100
Heat treatment	228	3920	0.59	90
Protamine sulfate	220	2200	0.83	71
$(NH_4)_2SO_4$	10	400	3.91	60
DEAE-Sephadex chromatography	6	52	20.6	42
Sephadex G-200 chromatography	5	4.5	66.3	12

Polyacrylamide disc gel electrophoresis of the purified enzyme exhibited one major and one minor band when stained with Amido Black dye.

Properties of serine transhydroxymethylase

Stability. Serine transhydroxymethylase is quite unstable, especially in the purified state where a 50% loss of activity could occur over a 10-h period when the enzyme was stored at 4 or — 10 °C. Serine, pyridoxal phosphate, and dithiothreitol provided only a small degree of protection. Furthermore, the inclusion of EDTA or phenylmethylsulfonyl fluoride to inhibit protease activity did not aid in the stabilization of the enzyme. The stability was greatly increased, however, by storage in 30% (v/v) glycerol, *i.e.* the purified enzyme could then be stored frozen for up to 1 month without significant loss of activity. Glycerol also stabilized serine transhydroxymethylase against heat inactivation during the heat treatment step in purification as can be seen in Fig. 1.

Molecular weight. Sucrose density gradient sedimentation and Sephadex G-200 chromatography were utilized to estimate the molecular weight of serine transhydroxymethylase. Values obtained range from 180 000–200 000 by both methods using either crude or purified enzyme preparations.

pH optima. The enzyme exhibited comparable activities at pH values ranging from 8.0 to 9.5 when sodium bicine was used as the buffer as opposed to the sharp pH optimum at 8.5 previously reported in phosphate buffer[2].

Effect of cations. Serine transhydroxymethylase activity was stimulated by Mg^{2+}, Mn^{2+}, Ca^{2+} and to a lesser extent by K^+ and Na^+. The optimal concentrations for divalent and monovalent cations were 0.5 and 10 mM, respectively. Though the

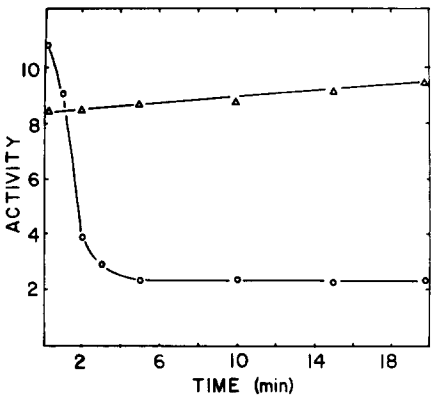

Fig. 1. Effect of glycerol on heat inactivation of serine transhydroxymethylase. Enzyme source was a dialyzed crude extract. Heating was done in a water bath shaker at 55 °C using 10 ml of extract. 1-ml samples were removed. ○, no glycerol; △, 30 % (v/v) glycerol.

extent of stimulation was variable in different preparations, the maximum stimulation ever achieved was about 3-fold. Unaccountably, purified enzyme showed less stimulation by cations with the maximum stimulation ever achieved being less than 2-fold. Dialysis against EDTA had no effect on the activity of serine transhydroxymethylase.

Cofactor requirements. Crude extracts and the purified enzyme had an absolute requirement for tetrahydrofolic acid. When PLP was not used as a supplement in the buffer during purification, the purified enzyme also had an absolute requirement for PLP. This dissociation of PLP from the enzyme did not occur in the crude extract by simple passage through a Sephadex G-25 column. The crude enzyme retained 30–40% of the activity of the control (PLP added) when no additional PLP was added to the reaction mixture.

Enzyme kinetics. Apparent Michaelis constants for the various reaction components were $7.0 \cdot 10^{-4}$ M for L-serine, $1.1 \cdot 10^{-4}$ M for PLP, and $2.5 \cdot 10^{-4}$ M for tetrahydrofolic acid. All values represent an average of several determinations.

Enzyme regulation. Serine transhydroxymethylase was previously reported to be inhibited *in vitro* by SAM and, to a lesser extent, by methionine, though the maximum inhibition appeared to be less than 35% (ref. 2). Various forms of the purines and thymine were tested to determine whether they had any effect on serine transhydroxymethylase activity. No significant inhibition of activity was shown by guanine, guanosine, thymine, thymidine, adenine, adenosine, or adenosine di- or triphosphate either alone or in combination with methionine.

Absorption spectrum. The absorption spectrum of purified serine transhydroxymethylase apoenzyme exhibited a single absorption peak at 280 nm. The holoenzyme (apoenzyme + PLP) exhibited an additional adsorption peak at about 440 nm, presumably due to PLP bound to the enzyme. As can be seen in Fig. 2, when SAM (0.5 mM) was added to the serine transhydroxymethylase–PLP holoenzyme, there was a decrease and blue shift of the 440-nm absorption peak. Addition of cysteine to the SAM-treated holoenzyme resulted in a further decrease in the 440-nm peak and the appearance of a new peak at 300 nm. Methionine had no effect on the absorption spectrum of the holoenzyme at concentrations up to 10 mM.

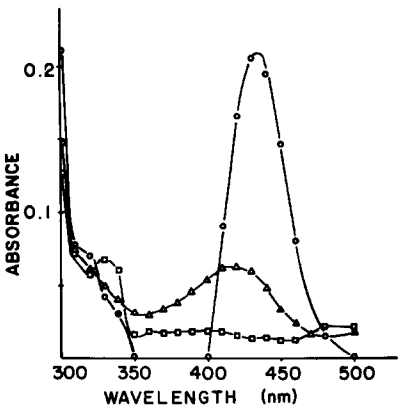

Fig. 2. Effect of SAM on the absorption spectrum of serine transhydroxymethyase. ○, spectrum of the purified holoenzyme (2.3 mg/ml); △, spectrum after addition of 0.5 mM SAM; □, spectrum after further incubation with 0.5 mM cysteine.

DISCUSSION

Previous work in this laboratory established some regulatory properties of serine transhydroxymethylase from *S. cerevisiae*[2]. It was found that the enzyme was slightly repressed by methionine, and was subject to inhibition by methionine and SAM, though to a limited extent. Studies on the physical properties of serine transhydroxymethylase were limited because of the labile nature of the isolated enzyme. However, the discovery during the present study of the stabilization of the enzyme by storage in 30% (v/v) glycerol has facilitated a closer examination of the physical properties of the enzyme.

The molecular weight of the enzyme, found to be between 180 000 and 200 000, suggests that it is slightly larger than serine transhydroxymethylase isolated from *E. coli* (mol.wt 170 000 (ref. 5)), and slightly smaller than the rabbit liver enzyme (mol.wt 215 000 (ref. 6)). However, the molecular weight reported for the purified rabbit liver enzyme has varied greatly[10,20], possibly due to loss of subunits during purification. To be sure that no loss of subunits occurred with the purification procedure utilized with *S. cerevisiae*, the molecular weight of serine transhydroxymethylase was determined with crude preparations as well as with the purified enzyme. The results were unchanged.

The yeast enzyme was stimulated by cations, unlike the enzyme from rabbit liver[7]. However, there was not an absolute requirement, as the inclusion of EDTA during dialysis did not lower enzyme activity any further. Such an absolute requirement for cations has been reported for serine transhydroxymethlase from *Nicotiana rustica*[21]. As with *N. rustica*, the stimulation of the *S. cerevisiae* enzyme was greatest for divalent cations, and was at a significantly lower concentration than that required for monovalent cations.

The apparent K_m for serine is almost identical to that reported for serine transhydroxymethylase from *E. coli*[5] and rabbit liver[22]. The enzyme differs from the rabbit liver enzyme, however, in the ease of resolution of the PLP cofactor. In yeast, the cofactor is dissociated from the apoenzyme during purification, while the rabbit

liver enzyme requires incubation with cysteine followed by dialysis to remove the PLP[8]. However, like the rabbit liver enzyme, a portion of the PLP appears to be dissociated quite easily while some is bound tightly, *i.e.* a crude preparation of serine transhydroxymethylase from *S. cerevisiae* retains 30–40% of its activity even after passage through a Sephadex G-25 column. The cofactor has been shown to be dissociated even more easily from the enzyme from *Clostridium cylindrosporum*, where a crude extract shows very little activity without preincubation in PLP[23].

Changes is the absorption spectrum of serine transhydroxymethylase caused by SAM suggest an interaction between the PLP cofactor and the enzyme inhibitor. Cysteine, which is known to form a covalent linkage (thiazolidine ring) with PLP[24], also inhibits serine transhydroxymethylase *in vitro*[25]. The fact that cysteine and SAM both elicit an alteration of the serine transhydroxymethylase spectrum, implied that an interaction between SAM and PLP might contribute to the inhibition of the enzyme. The possibility of SAM acting as a generalized inhibitor of PLP-dependent enzymatic reactions is pursued in the subsequent paper[26]. It should be noted that although inhibition of serine transhydroxymethylase by SAM may be due (at least in part) to interference with the functioning of the cofactor, such a mode of inhibition is apparently not the case for methionine. Methionine also inhibits serine transhydroxymethylase[2], but this amino acid had no effect on the absorption spectrum of the holoenzyme, even at concentrations 20 times higher than SAM. It seems likely, therefore, that typical end-product inhibition is the case for methionine.

It is apparent that the physical characteristics of serine transhydroxymethylase are variable, depending on the source of the enzyme. Whether these differences are of physiological significance in a particular system has not been established, but as the enzyme is characterized from additional sources, such information may become available.

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