Serine hydroxymethyltransferase from Escherichia coli: Purification and Properties

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Serine hydroxymethyltransferase from Escherichia coli was purified to homogeneity. The enzyme was a homodimer of identical subunits with a molecular weight of 95,000. The amino acid sequence of the amino and carboxy-terminal ends and the amino acid composition of cysteine-containing tryptic peptides were in agreement with the primary structure proposed for this enzyme from the structure of the glyA gene (M. Plamann, L. Stauffer, M. Urbanowski, and G. Stauffer, Nucleic Acids Res. 11:2065-2074, 1983). The enzyme contained no disulfide bonds but had one sulfhydryl group on the surface of the protein. Several sulfhydryl reagents reacted with this exposed group and inactivated the enzyme. Spectra of the enzyme in the presence of substrates and substrate analogs showed that the enzyme formed the same complexes and in similar relative concentrations as previously observed with the cytosolic and mitochondrial rabbit liver isoenzymes. Kinetic studies with substrates showed that the affinity and synergistic binding of the amino acid and folate substrates were similar to those obtained with the rabbit liver isoenzymes. The enzyme catalyzed the cleavage of threonine, allothreonine, and 3-phenylserine to glycine and the corresponding aldehyde in the absence of tetrahydrofolate. The enzyme was also inactivated by the transamination of the active site pyridoxal phosphate to pyridoxamine phosphate. This substrate specificity was also observed with the rabbit liver isoenzymes. We conclude that the reaction mechanism and the active site structure of E. coli serine hydroxymethyltransferase are very similar to the mechanism and structure of the rabbit liver isoenzymes.

Serine hydroxymethyltransferase catalyzes the reversible interconversion of serine and glycine, with tetrahydrofolate serving as the C1 acceptor (2). The physiological direction of the reaction is the cleavage of serine to glycine and 5,10-methylenetetrahydrofolate. This reaction is the major source of C1 groups used in the biosynthesis of compounds containing methyl groups, purine ring biosynthesis, and thymidylate (3). The enzyme has been purified and characterized from the livers of several mammals (14). The mammalian enzymes all contain pyridoxal phosphate at the active site, which forms unique absorption bands upon the addition of substrates and substrate analogs. These spectral absorption bands have aided in elucidating aspects of the mechanism of the enzyme, specifically the role of pyridoxal phosphate (18). These studies have shown that the enzyme has broad substrate and reaction specificities. It cleaves many 3-hydroxy amino acids to glycine and an aldehyde, decarboxylates amino malonate, and transaminates d-alanine. Cytosolic and mitochondrial isoenzymes have been purified from rat and rabbit livers and have been shown to have very similar structures and properties (18). In addition to a lysyl residue which forms a Schiff base with pyridoxal phosphate, a sulfhydril group has been implicated as being at the active site (8).

Serine hydroxymethyltransferase is also widely distributed in plants and procaryotes (3). The enzyme has been partially purified and characterized from Clostridium cylindrosporum and Escherichia coli (10, 22). However, most studies with the procaryotic enzyme have centered on the expression and control of the activity in vivo (5, 6). Recently, Plamann et al. have cloned the glyA gene from E. coli into a high-copy-number plasmid. The nucleotide sequence for the gene, which codes for serine hydroxymethyltransferase, has been determined, and an amino acid sequence has been proposed for the enzyme (11). The sequence of many tryptic and chymotryptic peptides from the rabbit liver cytosolic and mitochondrial isoenzymes are highly homologous with the amino acid sequence proposed for the E. coli enzyme (1). This includes the active site region, which has the lysyl group that binds pyridoxal phosphate.

Our previous work on this enzyme has been exclusively with the rabbit liver isoenzymes. The role of this enzyme is believed to have the same function in bacteria as in mammals. Since the enzyme has not been well characterized from any bacterial source, we have had an interest in purifying the enzyme from E. coli and comparing its properties with those of the rabbit isoenzymes. George Stauffer kindly provided us with the E. coli strain containing the plasmid with the glyA gene (20). This organism produces a 26-fold increase in the enzyme over the wild-type strain. This permitted us to develop a simple purification procedure which resulted in large quantities of pure enzyme. Our goal was to verify the amino acid sequence proposed for the E. coli enzyme, determine its quaternary structure, compare its mechanistic properties with those of the rabbit enzymes, and investigate the role of sulfhydril groups in the mechanism of the enzyme.

MATERIALS AND METHODS

Materials. The following materials were obtained from Sigma Chemical Co.: glycine, serine, NADH, tetrahydrofolate, alcohol dehydrogenase, threonine, d-alanine, iodoacetate, and 5,5'-dithiobis-(z-nitrobenzoic acid (DTNB). Allotheonine was obtained from ICN Pharmaceuticals. [1-13C]iodoacetic acid, 16 mCi/mmol, was purchased from New England Nuclear Corp. Methylenetetrahydrofolate dehydrogenase was purified from rabbit liver as previously described (13). All reagents for amino acid sequence determination and analysis were obtained from Pierce Chemical Co.

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**Assays.** Several procedures were used to determine the activity of the enzyme. With serine as the substrate, the rate of formation of the product 5,10-methylenetetrahydrofolate was determined by oxidizing it to 5,10-methylenetetrahydrofolate and NADPH with methenyltetrahydrofolate dehydrogenase and NADPH (15). With threonine and allothreonine as substrates, the rate of formation of the product acetaldehyde was determined by reducing it to ethanol with alcohol dehydrogenase and NADH (15). 3-Phenylserine cleavage to glycine and benzaldehyde was monitored spectrophotometrically by observing the increase in absorbance of benzaldehyde at 290 nm (4). The rate of transamination of D-alanine was determined by observing the increase in absorbance of pyridoxamine phosphate at 325 nm and the loss of enzyme-bound pyridoxal phosphate by the decrease in absorbance at 423 nm (16). Aposerine hydroxymethyltransferase was made by incubating the enzyme with L-cysteine as previously described for the rabbit enzyme (17).

**Molecular weight determination.** The molecular weight was determined by molecular sieve chromatography of the enzyme and protein standards on a TSK-3000 high-pressure liquid chromatography (HPLC) column (70 cm by 7.5 mm) and a Sephadex G-150 column (1.5 by 150 cm). The buffer for each of these experiments was 20 mM sodium N,N-bis (2-hydroxyethyl)-2-aminoethanesulfonate (BES) containing 100 mM sodium chloride, pH 7.0. Protein standards used to calibrate the columns were rabbit serine hydroxymethyltransferase, muscle aldolase, aspartate aminotransferase, alkaline phosphatase, bovine serum albumin, ovalbumin, carboxic anhydrase, and trypsinogen.

The molecular weight of the native enzyme was also determined by the meniscus depletion method of Edelstein and Schachman in a Beckman model E analytical ultracentrifuge (7). Centrifugations were performed at 18,000 rpm in a Beckman AN-D rotor at 20°C. Sample volume was 120 µl at a protein concentration of 0.2 mg/ml in 10 mM potassium phosphate–100 mM potassium chloride, pH 7.0. Equilibrium was achieved in 15 h. The $s_{20,w}$ was calculated from sedimentation velocity experiments at protein concentrations ranging from 1.0 to 18.0 mg/ml in the same buffer. The carboxy-terminal sequence of the *E. coli* enzyme was determined by incubating 10 nmol of the enzyme in 10 mM sodium phosphate–0.01% sodium dodecyl sulfate, pH 6.2, at 50°C for 1 h. To the denatured enzyme, 10 µg of carboxypeptidase Y in an equal volume of buffer without sodium dodecyl sulfate was added, and the mixture was incubated at 37°C. At 15-min periods, samples corresponding to 1 nmol of the enzyme were removed, and the released amino acids were determined by analysis on a Durrum MBB amino acid analyzer as previously described (1). The amino-terminal sequence was determined by the manual Edman degradation method of Tarr (21). The released phenylthiodyantoin amino acids were identified by HPLC as previously described (1).

**Modification of sulfhydryl groups.** The total number of sulfhydryl groups was determined by reacting the enzyme after denaturation in sodium dodecyl sulfate with DTNB (8). The number of sulfhydryl groups reacting with iodoacetate was determined by incubating the enzyme with 5 mM iodoacetate at 30°C. The excess iodoacetate was removed by gel filtration, and the number of remaining sulfhydryl groups was determined by the DTNB method (8). The rate of inactivation of aposerine hydroxymethyltransferase by iodoacetate and methyl-methanethiosulfonate was monitored by removing samples from a reaction mixture containing the thiol reagent and diluting into an assay buffer containing 0.2 mM pyridoxal phosphate. After incubation at 30°C for 15 min, the activity was determined by using the allothreonine assay.

Tryptic peptides containing cysteine residues were isolated by the same procedure previously used in our study of cytosolic and mitochondrial serine hydroxymethyltransferases (1). About 6 mg of the *E. coli* enzyme in 20 mM BES buffer, pH 7.8, was denatured by the addition of 5.5 M guanidine hydrochloride. To this solution was added 50 µCi of [1-¹⁴C]iodoacetate, which gave a final concentration of 1.5 mM. After 30 min at 30°C, additional iodoacetate was added to a final concentration of 50 mM. After 3 h, the solution was dialyzed overnight to remove the guanidine hydrochloride. The precipitated protein was collected by centrifugation and suspended in 1 ml of 0.1 M ammonium bicarbonate. Trypsin (0.1 mg) was added, and the digestion was allowed to proceed for 3 h at 37°C. A second sample of trypsin was added, and the solution was left overnight at room temperature. The tryptic peptides were separated on a C-18 reverse-phase HPLC column (7.5 mm by 30 cm) by a linear gradient from 0 to 100% solvent B in 2 h; solvent A was 50 mM phosphoric acid, pH 2.85, and solvent B was acetonitrile. The radioactive peptides were detected by passing the column effluent through a β-analytical radioactive monitor, model 504. Three major radioactive peaks were found. These were collected, evaporated to dryness, and then subjected to a second purification on a C-18 HPLC column. The same linear gradient was used, except that solvent A was 0.1% trifluoroacetic acid and solvent B was 0.1% trifluoroacetic acid in acetonitrile. For this column, peptides were detected by absorbance at 215 nm. Only three of the peptides isolated from this second column were radioactive. These were dried and hydrolyzed with HCl for amino acid analysis.

**Determination of kinetic constants.** The $K_m$ values for serine and tetrahydrofolate were determined by double-reciprocal plots of initial velocity versus substrate concentration. Tetrahydrofolate concentrations were varied from 0.005 to 0.1 mM at fixed concentrations of 0.1, 0.2, 0.5, and 1.0 mM. The $V_{max}$ was determined for the reaction velocity at infinite serine and tetrahydrofolate concentrations.

Dissociation constants for glycine, tetrahydrofolate, 5-methyltetrahydrofolate, and 5-formyltetrahydrofolate were determined by a spectrophotometric method as previously described for the rabbit enzyme (19). Like the rabbit isoenzymes the *E. coli* serine hydroxymethyltransferase forms a ternary complex with glycine and tetrahydrofolate which absorbs intensely at 500 nm. By determining the absorbance at 500 nm as a function of glycine concentration at different fixed concentrations of tetrahydrofolate, it is possible to determine the dissociation constants for these substrates. Since both 5-formyltetrahydrofolate and 5-methyltetrahydrofolate give this same ternary complex, it is also possible to obtain the dissociation constants for these two folate analogs.

**Bacterial cultures.** The bacterial strain used for the isolation of serine hydroxymethyltransferase was *E. coli* GS245 (11). Strain GS245 is a derivative of *E. coli* K-12 and is pheA905 araD139 ΔlacU169 ΔglyA rpsL thi. Host bacteria were transformed with plasmid pGS29 by the CaCl₂ procedure of Mandel and Higa (9). pGS29 is a derivative of pBR322 and contains the *E. coli* g1yA gene on a 3.3-kilobase SalI-EcoRI fragment (11). Transformant bacteria were
screened for ampicillin resistance and complementation of the glyA deletion. Bacterial cultures were maintained as smears on Luria broth agar slants containing 100 μg of ampicillin per ml at 4°C. Host bacteria and plasmid DNA were kindly supplied by George Stauffer.

**Purification procedure.** For the isolation of serine hydroxymethyltransferase, bacteria from the agar slants were grown in Luria broth containing 100 μg of ampicillin per ml and then restreaked on Luria broth agar plates containing ampicillin. The plate was then incubated at 37°C for 8 h. A single colony was chosen and incubated overnight at 37°C with constant shaking in 500 ml of Luria broth containing 100 μg of ampicillin per ml. The 500-ml overnight culture was used as the inoculum for 30 liters of Luria broth containing 25 mM potassium phosphate (pH 7.4) and 0.4% glucose. Bacteria were incubated at 37°C with vigorous aeration. Samples were taken at 1-h intervals for the measurement of optical density at 600 nm. Bacteria were harvested as they left the logarithmic phase of growth by centrifugation at 13,000 × g for 12 min. The cell pellet was frozen at −70°C overnight. On the following day, cells were thawed by the addition of 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA. Cell lysis was accomplished by the addition of 1 mg of lysozyme per g of packed E. coli cells. Lysozyme was freshly prepared as a 20-mg/ml solution in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA and then added to the thawed E. coli cell pellet with constant stirring. Incubation was continued for 30 min at room temperature. Cells were then sonicated on ice for 30 min, using a Branson Sonifier model 185 cell disruptor at an output control setting of 4. This extract was centrifuged at 13,000 × g for 30 min at 4°C. The precipitate was discarded.

(i) **Ammonium sulfate precipitation.** Solid ammonium sulfate was added to the cell extract to 50% of saturation (313 g/liter). The solution was centrifuged at 4°C at 13,000 × g for 30 min, and the precipitate was discarded. Additional ammonium sulfate was added to the supernatant to 75% of saturation (176 g/liter). After stirring at 4°C for 15 min, the precipitate was dissolved in 100 ml of 20 mM potassium phosphate, pH 7.2, and dialyzed against two 4-liter changes of this buffer. The dialysis buffer contained 0.1 mM pyridoxal phosphate and 1 mM EDTA.

(ii) **DEAE column.** The enzyme solution from step i was added to a DEAE-Sephadex column (12 by 18 cm) which had been equilibrated with 20 mM potassium phosphate, pH 7.2. The column was washed with the equilibrating buffer until the absorbance at 280 nm was below 0.2. The enzyme was then eluted with a linear salt gradient. The mixing chamber contained 2 liters of 20 mM potassium phosphate, pH 7.2, and the reservoir contained 2 liters of 200 mM potassium phosphate with 300 mM sodium chloride, pH 6.4. The enzyme moved down the column as a yellow band. The fractions containing activity were pooled, and the protein was precipitated by the addition of ammonium sulfate to 75% of saturation. The precipitate was dissolved in 20 mM BES buffer, pH 7.0, and dialyzed exhaustively against this buffer containing 0.1 mM pyridoxal phosphate.

(iii) **Hydroxylapatite column.** One-half of the enzyme from step ii was loaded onto a hydroxylapatite column (5 by 6 cm) which had been washed extensively with 20 mM BES buffer, pH 7.0. The column was then washed with 50 ml of the BES buffer to remove protein that did not stick to the column. The absorbed enzyme was eluted with a linear potassium phosphate gradient. The mixing chamber contained 250 ml of 20 mM BES buffer, pH 7.0, and the reservoir contained 250 ml of 100 mM potassium phosphate, pH 7.0. The enzyme eluted very early in the gradient. The enzyme which eluted at the beginning of the peak was pure as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, the protein which eluted on the back side of the peak contained some minor contaminants. Those fractions which contained activity were pooled, and the enzyme was precipitated by the addition of ammonium sulfate to 75% of saturation. The precipitate was dissolved and dialyzed against an appropriate buffer. This procedure was repeated for the remaining one-half of the enzyme from the DEAE column.

(iv) **TSK 3000 HPLC column.** Aliquots (500 μl) of the enzyme were loaded onto a TSK-3000 HPLC column (70 cm by 7.5 mm) which had been equilibrated with 100 mM potassium phosphate, pH 7.0. The enzyme was eluted at a flow rate of 0.5 ml/min at 23°C.

**RESULTS**

**Purification.** To our knowledge serine hydroxymethyltransferase has not been purified to homogeneity and characterized from any procaryotic organism. We have made several attempts to purify the E. coli enzyme without success. The problems were eliminated by the cloning of the glyA gene into a pBR322 plasmid. As reported by Plamann et al., the use of the cloned gene gives a 26-fold increase in the concentration of the enzyme (11). Although the properties and structure of the enzyme were very similar to those of the two rabbit liver isoenzymes, the purification procedure was very different. The E. coli enzyme did not stick to either blue or orange Sepharose, and the pl appeared to be less than 6.0 since it did not bind to carboxymethyl-Sephadex at this pH and low ionic strength. The enzyme also did not bind tightly to hydroxylapatite. To use this column as a purification step, the enzyme and column must be free of phosphate. The enzyme which was eluted from this column was better than 90% pure as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, the leading edge of the enzyme from the column appeared to be homogeneous. For several experiments, the enzyme was purified further by molecular sieve chromatography on a TSK-3000 HPLC column. The overall purification was 65-fold with a 49% yield (Table 1).

**Molecular weight.** The molecular weight of E. coli serine hydroxymethyltransferase was established through the use of three independent techniques. The retention time of the
FIG. 1. Equilibrium ultracentrifugation of purified E. coli serine hydroxymethyltransferase. Samples were centrifuged in H2O and 99% 2H2O at 18,000 rpm at 20°C in 10 mM KPi-100 mM KCl, pH 6.8. The protein concentration was 0.2 mg/ml in a volume of 120 μl.

native enzyme was determined on a TSK-3000 molecular sieve column and on a Sephadex G-150 column. The sedimentation equilibrium technique of Edelstein and Schachman (7) was the third method employed. From the DNA sequence, a protein with an Mr of 47,000 was predicted. Our enzyme preparation on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gave one homogeneous band with an Mr of 46,000. The values obtained from the HPLC system and the G-150 column were 115,000 and 100,000, respectively. The results of the sedimentation equilibrium experiments indicate that the enzyme has a partial specific volume of 0.734 ml/g and an Mr of 96,000. Figure 1 shows the data from the sedimentation equilibrium study for the enzyme in H2O and 2H2O. These results suggest that the enzyme is a dimer of identical subunits. An S20w of 5.5 was also determined from sedimentation velocity experiments at enzyme concentrations varying from 1 to 18 mg/ml. The constant value of S20w showed that the enzyme did not change its quaternary structure over this range of enzyme concentration.

**Amino acid sequence.** From the nucleotide sequence of the gene, a proposed amino acid sequence has been given for the enzyme (11). We have previously shown that 30% of the residues are homologous with the sequence of tryptic and chymotryptic peptides from the cytosolic and mitochondrial isoenzymes of rabbit liver (1). This sequence homology was located in the amino-terminal two-thirds of the sequence. This raised the possibility that the proposed carboxy-terminal sequence might be in error. To verify the proposed sequence we determined the amino- and carboxy-terminal sequence of the enzyme and the amino acid composition of the cysteine-containing tryptic peptides. The proposed amino acid sequence had only three cysteine residues, and two of these were near the carboxy-terminal end of the enzyme. After reacting the enzyme with [1-14C]iodoacetate and digestion with trypsin, three radioactive peptides were found after chromatography on an HPLC C-18 column in agreement with the prediction that the enzyme contains only three cysteine residues. Table 2 shows the amino acid compositions of the three radioactive peptides and their comparison to the predicted amino acid composition of the three tryptic peptides containing cysteiny1 residues. Our results demonstrated that all three radioactive peptides contained carboxymethyl cysteine and that their amino acid compositions were in agreement with those of the predicted peptides. The native enzyme was also reacted with [1-14C]iodoacetate. Only a single radioactive peptide was isolated. Its amino acid composition was identical to peptide 1 (Table 2).

We were able to determine the sequence three steps from the amino-terminal end of the molecule and found the sequence to be Met-Leu-Lys. From the carboxy-terminal end, the first three amino acids released by digestion with carboxypeptidase Y suggested the sequence -Val-Tyr-Ala. Both of these results were in agreement with the proposed structure of the enzyme (11).

**Properties of sulfhydryl groups.** Denaturation of the enzyme in sodium dodecyl sulfate and titration with DTNB showed the presence of three sulfhydryl groups per subunit. When the native enzyme was titrated with DTNB, only one sulfhydryl group reacted. This suggests that E. coli serine hydroxymethyltransferase has no disulfide bonds and that there is one exposed and two buried sulfhydryl groups. When DTNB reacted with the one exposed sulfhydryl group, there was a complete loss of activity. Incubation of the inactive enzyme with dithiothreitol completely restored activity, suggesting that the loss of activity was due to either a conformational change or blocking of a critical sulfhydryl group at the active site. Methyl methanethiosulfonate also inactivated the enzyme by reacting with a single sulfhydryl group. Serine protected the enzyme against inactivation. Iodoacetate reacted very slowly with the enzyme and did not

**TABLE 2. Amino acid composition of cysteine-containing tryptic peptides**

<table>
<thead>
<tr>
<th>Residue</th>
<th>Peptide 1 Calculated for Residues 402-412</th>
<th>Residue 2 Calculated for Residues 64-81</th>
<th>Residue 3 Calculated for Residues 381-401</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM-Cys</td>
<td>0.43</td>
<td>0.53</td>
<td>0.68</td>
</tr>
<tr>
<td>Asp</td>
<td>0.79</td>
<td>1.83</td>
<td>3.20</td>
</tr>
<tr>
<td>Ser</td>
<td></td>
<td>2.54</td>
<td>0.67</td>
</tr>
<tr>
<td>Glu</td>
<td></td>
<td>2.12</td>
<td>2.02</td>
</tr>
<tr>
<td>Gly</td>
<td></td>
<td>2.21</td>
<td>1.21</td>
</tr>
<tr>
<td>Ala</td>
<td>0.98</td>
<td>1.04</td>
<td>1.90</td>
</tr>
<tr>
<td>Val</td>
<td>1.20</td>
<td>1.25</td>
<td>1.44</td>
</tr>
<tr>
<td>Met</td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>Ile</td>
<td>0.91</td>
<td>0.96</td>
<td>2.22</td>
</tr>
<tr>
<td>Leu</td>
<td>0.90</td>
<td>0.96</td>
<td>2.22</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>1.00</td>
<td>1.00</td>
<td>1.27</td>
</tr>
</tbody>
</table>

* Three carboxymethyl (CM) peptides were isolated from a C-18 HPLC column as described in the text. The peptides were numbered according to their elution from the column.

* Taken from the proposed amino acid sequence given in reference 11.
completely inactivate the enzyme even after several hours. Substrates partially protected the enzyme against inactivation by iodoacetate.

Spectral properties. Mammalian serine hydroxymethyltransferases which have been purified contain a tightly bound pyridoxal phosphate group at the active site (14). The absorption properties of this coenzyme have played an important role in elucidating the mechanism of this enzyme. The E. coli enzyme was pale yellow and exhibited an absorption peak at 423 nm (Fig. 2). This absorption band is characteristic of an internal hydrogen-bonded Schiff base between the aldehyde group of pyridoxal phosphate and an amino group on the enzyme. The spectrum of the enzyme did not change over the pH range of its stability, which is from 6.0 to 8.5. The 280/423 nm absorbance ratio was 7.0 for the pure enzyme.

Saturation of the rabbit liver isoenzymes with glycine resulted in the accumulation of enzyme-substrate complexes. These were external Schiff bases of the pyridoxal phosphate with the substrate glycine. Many of these complexes have unique absorption properties which have been useful in determining their structure (14, 19). As with the rabbit isoenzymes when the E. coli enzyme was saturated with glycine, three spectral maxima absorbing at 343, 425, and 495 nm were observed. The absorption peaks at 343 and 495 nm were small, suggesting that these complexes are present in low concentrations. The major peak was at 425 nm, which was interpreted to be the hydrogen-bonded Schiff base between glycine and pyridoxal phosphate (14). The complex absorbing at 343 nm is believed to be the geminal diamine in which the aldehyde carbon of the coenzyme is bonded by amino groups from the enzyme and substrate (12). The addition of tetrahydrofolate caused a dramatic shift in the spectrum of the enzyme (Fig. 2). The absorbance at 425 nm decreased with a large increase in the absorbance at 500 nm. The complex absorbing at 500 nm is believed to be a quinoid species in which the glycine has lost an α proton to form a carbanion which is in resonance with the pyridoxal phosphate ring (12, 19). It is not possible to determine what happens to the absorbance of the 343-nm band in the presence of tetrahydrofolate because of the intense absorption of minor contaminants in the folate solution which absorb at this wavelength. Two folate analogs, 5-methyltetrahydrofolate and 5-formyltetrahydrofolate, when added to the enzyme-glycine complex, also produced this same spectral shift. The results with 5-methyltetrahydrofolate are shown in Fig. 2.

Several substrate analogs bind at the active site and produce spectral shifts in the spectrum of the bound pyridoxal phosphate. Saturation of the enzyme with D-alanine resulted in the formation of complexes which absorb at 425 and 500 nm. The addition of L- and D-cysteine to the enzyme resulted in complexes absorbing at 425 and 335 nm. Diazotization of the L-cysteine enzyme solution resulted in removal of the pyridoxal phosphate as a thiazolidine complex with L-cysteine leaving the colorless apoenzyme. This served as a convenient method for resolving the enzyme-enzyme complex.

Substrate and reaction specificity. Kinetic constants for L-serine and tetrahydrofolate were determined by initial velocity studies. Double-reciprocal plots of velocity versus substrate concentration gave converging lines, showing that the enzyme mechanism is sequential addition of substrates. Kinetic constants were defined according to the scheme shown in Fig. 3, where $k_{a1}$ and $k_{a2}$ represent $K_m$ values for the substrates at infinite concentration of the cosubstrate. The $K_m$ values for serine and tetrahydrofolate decrease with increasing concentration of the alternate substrate, showing that there is synergistic binding with these compounds (Table 3).

The affinity of glycine and tetrahydrofolate compounds for the enzyme can be determined spectrophotometrically by monitoring the absorbance at 500 nm with increasing concentrations of the two ligands. This method is based on the observation that only the enzyme-glycine-folate ternary

![FIG. 2. Spectra of serine hydroxymethyltransferase in the absence and presence of substrates. (A) Spectrum of the enzyme (0.8 mg/ml) in 20 mM KPi at pH 7.3. (B) Spectrum of the solution recorded in curve A after the addition of glycine (20 mM) and 5-methyltetrahydrofolate (0.18 mM).](http://jb.asm.org/)

![FIG. 3. Kinetic scheme for the binding of amino acid substrates (A) and folate compounds (B) to serine hydroxymethyltransferase. The ternary complex E - A - B absorbed at 500 nm when the amino acid was glycine and B was tetrahydrofolate, 5-methyltetrahydrofolate, or 5-formyltetrahydrofolate. Under these conditions there was no product formation.](http://jb.asm.org/)
TABLE 3. Kinetic constants for serine hydroxymethyltransferases (SHMT)*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Constant</th>
<th>Concn (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cSHMTa</td>
<td>mSHMTb</td>
</tr>
<tr>
<td>L-Serine</td>
<td>K_m</td>
<td>aK_m</td>
</tr>
<tr>
<td></td>
<td>aK_m</td>
<td>0.4</td>
</tr>
<tr>
<td>H_4-folate</td>
<td>K_m</td>
<td>aK_m</td>
</tr>
<tr>
<td>Glycine</td>
<td>K_m</td>
<td>aK_m</td>
</tr>
<tr>
<td>5-CHO-H_4-folate</td>
<td>K_d</td>
<td>aK_d</td>
</tr>
<tr>
<td>Allothreonine</td>
<td>K_m</td>
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</tr>
<tr>
<td>L-Threonine</td>
<td>K_m</td>
<td>40</td>
</tr>
<tr>
<td>L-Serine</td>
<td>k_cat</td>
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</tr>
<tr>
<td>Allothreonine</td>
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<tr>
<td>L-Threonine</td>
<td>k_cat</td>
<td>30</td>
</tr>
</tbody>
</table>

*Constants are defined according to the scheme shown in Fig. 3.
* Taken from references 18 and 19: c, cytosolic; m, mitochondrial.
* Units are micromoles of product formed per minute per micromole of active site.

The cloning of the E. coli glyA gene into a pBR322 plasmid permitted the rapid purification of serine hydroxymethyltransferase, which is coded for by this gene (11, 20). We compared the structural and mechanistic properties of the E. coli enzyme with the cytosolic and mitochondrial serine hydroxymethyltransferase isoenzymes purified from rabbit liver. In this study we confirmed that the proposed amino acid sequence for the E. coli enzyme is correct since the composition of cysteine-containing tryptic peptides, the amino-terminal sequence, and the carboxy-terminal sequence were as predicted. The mitochondrial and cytosolic isoenzymes from rabbit liver contained six and eight cysteine residues per subunit, respectively. The E. coli enzyme had only three cysteine residues. Like the rabbit enzymes, the E. coli enzyme contained no disulfide bonds. The cysteine corresponding to position 410 in the sequence appeared to be the only exposed sulfhydryl group since it was the only one labeled with iodoacetate in the native enzyme (Table 3). As observed with the rabbit isoenzymes, substrates protected the E. coli enzyme from inactivation by sulfhydryl reagents. There was a difference in the quaternary structure between the rabbit isoenzymes, which are tetramers of identical subunits, and the E. coli enzyme, which is a dimer of identical subunits.

Much of our current understanding about the mechanism of rabbit serine hydroxymethyltransferase comes from studies on the spectral properties of the enzyme-substrate complexes (14). Many of these studies were repeated with the E. coli enzyme and are reported above. The spectral properties of the enzyme with glycine and tetrahydrofolate were essentially identical to what was observed with the rabbit isoenzymes complexes absorbing at 343, 425, and 495 nm (12, 19). These results showed that the E. coli enzyme forms the same enzyme-substrate complexes with glycine and tetrahydrofolate and in relative concentrations similar to those of the rabbit liver isoenzymes. For the rabbit liver cytosolic serine hydroxymethyltransferase, kinetic studies showed that these enzyme-substrate complexes are intermediates in the reaction. This suggests that these complexes in the E. coli enzyme are also intermediates and that the enzyme catalyzes the reaction by the same mechanism. Further evidence that the active sites of the E. coli enzyme and of the rabbit enzymes must be very similar came from the similar spectral properties of the enzymes in the presence of the substrate analogs D- and L-cysteine, D-alanine, 5-formyltetrahydrofolate, and 5-methyltetrahydrofolate.

The most convincing evidence that the active site of the E. coli enzyme is very similar to the active site of the rabbit liver isoenzymes came from the kinetic studies (Table 3). The k_cat for the conversion of serine and tetrahydrofolate to absorbing at 325 nm appeared. This compound was not bound to the enzyme and had the spectral characteristics of pyridoxamine phosphate in acid and base. Addition of pyridoxal phosphate completely restored enzyme activity. The rate of disappearance of the complex absorbing at 500 nm was first order, with a rate constant of 0.02/min. These spectral changes were identical to those observed with the rabbit enzymes in the presence of D-alanine. Mechanistic studies with these enzymes showed that the products were pyridoxamine phosphate, pyruvate, and apoenzyme. The inactivation was the result of a half-transamination reaction. The rate of this inactivation by D-alanine with the E. coli enzyme was about twofold slower than that observed for the rabbit mitochondrial enzyme under similar conditions.

**DISCUSSION**

complex absorbed intensely at 500 nm (Fig. 2). As noted previously, the absorbance data at 500 nm can be treated as a velocity determination, and one can determine dissociation constants for glycine and the tetrahydrofolate compound according to the scheme shown in Fig. 3 (19). The results of this study for glycine, tetrahydrofolate, 5-methyltetrahydrofolate, and 5-formyltetrahydrofolate are shown in Table 3. The data showed that there was synergistic binding between glycine and all three folate compounds.

Several 3-hydroxy amino acids were tested as substrates for E. coli serine hydroxymethyltransferase. In addition to L-serine, the enzyme also cleaved allothreonine and L-threonine to acetaldehyde and glycine. The addition of tetrahydrofolate did not affect the rate of catalysis of these two substrates by the enzyme. The K_m and k_cat values for these two substrates are recorded in Table 3. The rate with L-threonine is so slow and the K_m is so large that we were unable to determine these constants. 3-Phenylserine was also tested as a substrate. Spectroscopic studies showed that the enzyme cleaves this amino acid to a product with a λ_{max} at 290 nm and glycine. The product absorbing at 290 nm is assumed to be benzaldehyde as found with the rabbit liver enzyme. A concentration dependency of rate versus substrate concentration indicated that the K_m for this amino acid was greater than 50 mM, and because 3-phenylserine has a limited solubility in water, we were not able to determine the V_{max} and K_m for this substrate.

Incubation of the enzyme with D-alanine slowly inactivated the enzyme. The addition of D-alanine resulted in the rapid appearance of enzyme-substrate complexes which absorb at 500 and 425 nm. During the course of inactivation these two complexes decreased, and a new compound
products is the same within experimental error to the value for the rabbit isoenzymes. The $K_m$ values for substrates and the existence of synergistic binding between the amino acid and folate substrate pair were very close to those found for the rabbit liver enzymes. The substrate specificity studies showed that both the prokaryotic and eukaryotic forms of the enzyme can use allothreonine, threonine, and 3-phenylserine as substrates in the absence of tetrahydrofolate. With respect to the threonine pair of substrates, the E. coli enzyme prefers the erythro configuration between the 2-amino and 3-hydroxy groups. Similar observations were found for the rabbit enzymes. One of the most interesting reactions catalyzed by the rabbit enzymes is the transamination of d-alanine to pyruvate and pyridoxamine phosphate (16). The E. coli enzyme was also inactivated by this reaction but at a slightly slower rate.

The observation that synergistic binding between the amino acid and folate substrate pairs is conserved in the E. coli and rabbit isoenzymes suggests that this property may be an important regulatory feature of the reaction. Another possible regulatory feature is that all three enzymes show a high affinity for 5-methyltetrahydrofolate. This is the major folate metabolite in the cell and could serve as a feedback inhibitor of the enzyme.

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LITERATURE CITED