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cDNA cloning, overexpression in *Escherichia coli*, purification and characterization of sheep liver cytosolic serine hydroxymethyltransferase

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A sheep liver cDNA clone for the cytosolic serine hydroxymethyltransferase (SHMT) was isolated and its nucleotide sequence determined. The full-length cDNA of SHMT was placed under the control of T7 promoter in pET-3C plasmid and expressed in *Escherichia coli*. The overexpressed enzyme, present predominantly in the soluble fraction, was catalytically active. The recombinant SHMT was purified to homogeneity with a yield of 10 mg/l bacterial culture. The recombinant enzyme was capable of carrying out tetrahydrofolate-dependent and tetrahydrofolate-independent reactions as effectively as the native enzyme. The K_m values for serine (1 mM) and tetrahydrofolate (0.82 mM) were similar to those of the native enzyme. The recombinant enzyme had a characteristic visible spectrum indicative of the presence of pyridoxal 5′-phosphate as an internal aldimine. The apoenzyme obtained upon removal of the cofactor was inactive and could be reconstituted by the addition of pyridoxal 5′-phosphate demonstrating that the recombinant SHMT was functionally very similar to the native SHMT. This overexpression of eukaryotic tetrameric SHMT in *E. coli* and the purification and characterization of the recombinant enzyme should thus allow studies on the role of specific amino acids and domains in the activity of the enzyme.

Keywords. Sheep serine hydroxymethyltransferase; cDNA clone; overexpression; recombinant enzyme.

Serine hydroxymethyltransferase (SHMT) is a pyridoxal-5′-phosphate(pyridoxal-*P*)-dependent enzyme catalyzing the reversible conversion of serine and tetrahydrofolate (H_4-folate) to glycine and 5,10-methylene-H_4-folate. This enzyme is a component of thymidylate cycle, along with thymidylate synthase and dihydrofolate reductase. The latter two enzymes are not only targets for cancer chemotherapy but also have served as model systems to study the structure/function relationships.

Earlier studies from our laboratory on the mechanism of interaction of substrates and substrate analogues with sheep liver SHMT has led to the design of active-site-directed inhibitors of SHMT [1–5]. The chemical modification studies with the sheep liver enzyme, indicated that histidine, cysteine and arginine residues were essential for activity [6], and the involvement of Arg269 in the binding of H_4-folate was demonstrated [7]. In spite of the successful isolation of the gene from several eukaryotic systems [8–12], the overexpression of this gene has not been reported. However, for understanding the structure/function relationship of the enzyme, it would be necessary to have an overexpressing clone of SHMT. In this communication, cloning, sequencing, and overexpression of the cytosolic sheep liver SHMT gene in *E. coli* is reported. We also describe the purification and characterization of the recombinant SHMT.

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Abbreviations. SHMT, serine hydroxymethyltransferase; H_4-folate, 5,6,7,8-tetrahydrofolate; ORF, open reading frame; pyridoxal-*P*, pyridoxal 5′-phosphate.

Enzyme. Serine hydroxymethyltransferase (EC 2.1.2.1).

Note. The novel nucleotide sequence data reported here have been submitted to the Genebank™/EMBL sequence data bank and are available under accession number X80024.

EXPERIMENTAL PROCEDURES

Materials. [α-^{32}P]dATP (3000 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol) were obtained from Board of Radiation and Isotope Technology, Unit of Department of Atomic Energy, Government of India, Bombay, India. [^{35}S]dATP[α-S] (600 Ci/mmol), L-[3-^{14}C]serine (55 mCi/mmol), were obtained from Amersham International. Restriction endonucleases, DNA-modifying enzymes were purchased from New England Biolabs, Perkin-Elmer Cetus and Amersham International. Cibacron Blue-agarose was from Bethesda Research Laboratories. CM-Sephadex C-50, Sephacryl S-200 were obtained from Pharmacia Fine Chemicals. All other biochemicals used in this study were obtained from Sigma Chemical Company. H_4-Folate was prepared by the method of Hatefi et al. [13]. Sheep liver cDNA 5′stretch (SWAJ-2) library was purchased from Clonetech Laboratories, Inc. The oligonucleotide primers were synthesized by Oligonucleotide Synthesis Facility, Centre for Genetic Engineering, Indian Institute of Science, Bangalore, India and National Biosciences, Plymouth MN, USA.

Bacterial strains and growth conditions. *E. coli* strain DH5α (Bethesda Research Laboratories) was the recipient for the plasmids used in subcloning and sequencing. Strain LE392 (obtained from Clonetech laboratories) was the recipient strain for plating the phage. BL21(DE3) [14] strain was used for the bacterial expression of pET-SHMT construct.

E. coli strains containing the plasmids were grown in Luria-Bertani medium or in terrific broth as described by Sambrook et al. [15].

Plasmid and λDNA isolation, DNA modifications and bacterial transformation. Plasmid preparations were by the alkaline lysis procedure as described by Sambrook et al. [15]. λDNA was isolated by the plate lysate method [15] with the following modifications. Before poly(ethylene glycol) precipitation, the phage was passed through a DEAE-cellulose column. DNA from the phage particles was released upon proteinase-K treatment. Restriction endonuclease digestions, nuclease digestions and ligations were carried out according to the manufacturer's instructions. The preparation of competent cells and transformation were carried out by the method of Alexander et al. [16].

PCR amplification of SHMT gene-specific cDNA fragment. Based on the amino acid sequence of sheep cytosolic SHMT determined by Usha et al. [17], two degenerate oligonucleotides DSH1 (5′-CAGAGRGTSGGSCTSGAGCTSATCG-CYAGCGAGAACTT-3′) and DSH2 (5′-GCCYCTSAGGG-TYTTRTGGGTGGTGGTGGTSACSACRTG-3′) were synthesized. The degenerate positions are indicated by R for purines, Y for pyrimidines, and S for G or C. PCR amplification was performed using AmpliTaq (Perkin-Elmer Cetus) as described in Perkin-Elmer Cetus protocol for DNA amplification with the following modifications. λDNA (0.1 μg) isolated from the pool of a sheep liver cDNA library and the oligonucleotides DSH1 and DSH2 (1 μM each) were added to a 20 μl reaction mixture. The samples were incubated in a thermal cycler for 30 cycles with 1 min denaturation (94 °C), 1 min annealing (55 °C), and 2-min chain elongation (72 °C). After 30 cycles, the reaction was continued for another 10-min elongation at 72 °C. The PCR product was gel purified using Geneclean II kit (BIO-101). Using *Eco*RV-digested pBlueseript (Stratagene), T-vector was prepared by the method of Marchauk et al. [18]. The PCR product was cloned into this T-vector and designated as pPSH.

Isolation of a sheep liver SHMT cDNA clone. Plaques were transferred to Duralon-UV™ membranes (Stratagene) and the DNA was fixed to the membrane by ultraviolet cross-linking. The PCR product was random-primer labeled by the method of Feinberg and Vogelstein [19] and purified through the Sephadex G-50 spun column as described by Sambrook et al. [15]. The membranes were washed as described by Sambrook et al. [15]. The prehybridization (with 50% formamide, 10% dextran sulfate, 1% SDS, 0.9 M NaCl and 200 μg of salmon sperm DNA), hybridization (denatured probe at 500000 cpm/ml prehybridization solution) and posthybridization [with 0.1×NaCl/Cit (NaCl/Cit, 0.15 M NaCl, 15 mM sodium citrate, pH 7), 0.1% SDS at 65 °C] conditions used were as described in the Stratagene instruction manual for Duralon-UV™ membranes.

ExonucleaseIII/S1 nuclease deletions and DNA sequencing. For deletions, supercoiled plasmid was prepared by the method of Wang et al. [20]. Deletions were performed using ExoIII/S1 nuclease as described in the Promega protocols and applications guide. DNA sequencing was performed by Sanger's dideoxynucleotide chain-termination method [21] using Circum-Vent™ thermal cycle sequencing kit (New England Biolabs).

Construction of pET-SHMT. SHP1 (sense oligonucleotide), with one extra T addition at the 5′terminus of the oligonucleotide (5′-TATGGCAGCTCCAGTCAAC-3′) and SHM1 (antisense olignucleotide) with a TA addition at the 5′ terminus of the oligonucleotide (5′-TATCTAGAAGCCAGGCAGG-3′) spanning the start and stop codons, respectively, were synthesized. PCR was performed using Vent DNA polymerase as described in New England Biolabs product specifications with the following modifications. For a 100-μl reaction, 6 ng of the full-length sheep SHMT cDNA clone (pSH11) DNA, 0.16 μM of each primer and 0.4 mM of each dNTP were used. The sample was incubated in a thermal cycler at 94 °C for 4 min, then kept for 25 cycles of amplification. Each cycle included 1 min denaturation (94 °C), 1 min annealing (50 °C), and 1.5 min chain elongation (72 °C). After 25 cycles, the reaction was extended for an additional 5 min at 72 °C. The 1.45-kb PCR product was purified from the agarose gel using Geneclean II kit.

*Nde*I-compatible ends were generated at the 5′ and 3′ ends of 1.45-kb Vent polymerase PCR product using the dinucleotide and trinucleotide sticky-end cloning (DISEC-TRISEC) method of Dietmaier et al. [22] with some modifications. The PCR product was treated with T4 DNA polymerase in the presence of 0.2 mM each of dGTP, dCTP for 30 min at 12 °C, then dATP was added to 0.2 mM concentration and incubated for 10 min at 37 °C. After performing reactions as above, DNA was extracted with phenol/chloroform and kept for alcohol precipitation. This DNA fragment was cloned at the *Nde*I site of pET-3C plasmid [23]. The presence of the insert in the proper orientation with respect to the T7 promoter was confirmed by sequencing.

The expression of SHMT in *E. coli* BL21 (DE3) and purification of the recombinant SHMT. *E. coli* BL21 (DE3) strain cells harboring the pET-SHMT plasmid were grown in 1 l Luria-Bertani medium containing 50 μg/ml ampicillin and 0.2% glucose. After absorbance at 600 nm reached 0.8, isopropyl-1-thio-β-D-galactopyranoside (0.4 mM) was added to induce the enzyme. 2 h later, the cells were harvested and the cell pellet was resuspended in 100 ml buffer A (50 mM sodium phosphate, pH 7.4, containing 2 mM EDTA, 1 mM DL-dithiothreitol and 100 μM pyridoxal-*P*) and frozen at −20 °C for overnight. To the thawed cells, 5 mg lysozyme and 1 mM phenylmethylsulfonyl fluoride were added and incubated at room temperature for 30 min. The cell suspension was sonicated until it became optically clear. The sonicate was centrifuged at 27000×*g* for 30 min. The supernatant was made up to 25% ammonium sulfate saturation and the precipitate was discarded. The supernatant was raised to 65% ammonium sulfate saturation by the addition of solid ammonium sulfate. The pellet obtained after the second ammonium sulfate fractionation was dissolved in 20 ml buffer B (20 mM sodium phosphate pH 7.4, containing 1 mM 2-mercaptoethanol, 1 mM EDTA and 50 μM pyridoxal-*P*) and dialyzed against the same buffer (2 l with two changes). The dialyzed ammonium sulfate extract was loaded on to a Cibacron Blue agarose column (1.75 cm×12 cm). The column was washed with 200 ml buffer C (50 mM sodium phosphate, pH 7.4, containing 1 mM 2-mercaptoethanol, 1 mM EDTA and 50 μM pyridoxal-*P*) and the enzyme was eluted with a 0 to 1-M KCl gradient in buffer C. 2-ml fractions were collected and active fractions were pooled (25 ml). The Cibacron Blue agarose eluant was concentrated by 0−65% ammonium sulfate precipitation and the precipitate was dissolved in 5 ml buffer C, loaded onto a Sephacryl S-200 column (3.0 cm×100 cm previously equilibrated with the buffer D (200 mM sodium phosphate, pH 7.4, containing 1 mM 2-mercaptoethanol, 1 mM EDTA and 50 μM pyridoxal-*P*) and 6-ml fractions were collected. The active fractions were pooled (45 ml), concentrated to 1.6 ml and used as the source of the enzyme in further characterization of the recombinant SHMT.

The recombinant apoenzyme was prepared from the holoenzyme using D-alanine as the substrate [24].

Native sheep liver SHMT was purified as described by Baskaran et al. [2].

SHMT was assayed using [3-^{14}C]serine and H_4-folate as substrates [25, 26]. Protein was estimated using BSA as a standard [27].

Protein sequencing. The recombinant SHMT was analyzed on SDS/PAGE [28], transferred to Immobilon™-P membrane (Millipore), stained with Ponceau-S and the appropriate band was cut out. The cut membrane containing the protein was destained and loaded on to a Shimadzu gas-phase sequenator, PSQ-1 [29].

RESULTS

Isolation of a cDNA clone for sheep liver SHMT. A cDNA fragment of 0.65 kb was amplified by PCR using primers DSH1 and DSH2. DSH1 has a partly degenerate sequence coding for the amino acids, QRVGLELIASENF, residues 43−55 in the sheep liver cytosolic SHMT [17], while DSH2 is also partly degenerate, codes for HVVTTTTHKTLRG, residues 248−260 of the sheep liver SHMT. The PCR product was cloned in T-pBluescript KS(+) vector. Plasmid (pPSH) containing the insert was identified and sequenced from both the ends. The predicted amino acid sequence encoded by insert DNA was homologous to the sheep cytosolic SHMT amino acid sequence [17]. The sheep liver cDNA library (70000 plaques) was screened using the 650-bp PCR product and 10 positive clones were identified after three successive rounds of screening. The maximum size of the insert from all these clones was only 1.2 kb instead of the expected 1.45 kb for the full-length clone. The pPSH contained additional 135 bp at the 5′ end compared to the 1.2-kb partial clones. This 135-bp fragment was used to screen the cDNA library (300000 plaques) to identify full-length clones. 12 positive clones were identified after four successive rounds of screening and the inserts from these positive clones were in the range of 1.3−1.7 kb.

Subcloning and sequencing. The inserts were released from the λ clones by partial *Eco*RI and complete *Xba*I digestion and cloned in pBluescript at *Eco*RI and *Xba*I sites. The plasmid clones with inserts of more than 1.2 kb were selected. The clones pSH1, pSH2 and pSH8 of 1.5 kb and pSH11 (1.7 kb) were used for further characterization. ExoIII/S1 nuclease deletions were performed using pSH11 in both directions and all the deletion clones were sequenced. pSH11 has an open reading frame (ORF) with 1452 bp and 198 bp 5′ non-coding sequence in which the most 5′ 172 bp were identical with the actin gene. This is probably a cloning artifact produced during the preparation of the cDNA library. However sequencing of the 5′ and 3′ ends of pSH1 and pSH8 clones showed the presence of a 1452-bp ORF and a 20-bp 5′ non-coding sequence, whereas, the pSH2 clone indicated the presence of a 26-bp non-coding sequence in addition to ORF. Sheep cDNA library was constructed by cloning the cDNA at *Eco*RI and *Xba*I sites of λSWAJ-2 vector without protecting the internal *Xba*I sites. Due to the presence of an internal *Xba*I site at the stop codon, none of the clones contained the 3′ non-coding sequence. The complete nucleotide sequence of sheep cytosolic SHMT obtained from pSH11 is shown in Fig. 1. The deduced amino acid sequence showed 98% identity with sheep liver cytosolic SHMT sequence [17], 90.5% identity with rabbit [10, 30] and human [11] SHMT.

Overexpression of sheep SHMT. The cDNA encoding SHMT, from Met1 through the TAG stop codon, was placed under the control of a strong T7 promoter (for details, see Experimental Procedures and Fig. 2). To avoid errors in the PCR amplification, we have used Vent DNA polymerase which, has a 5−15-fold more efficient proof-reading activity compared with *Taq* polymerase [31, 32]. The bacterial cells containing the SHMT expression plasmid (pET-SHMT) were induced with isopropyl-1-thio-β-D-galactopyronoside for various time periods (0−5 h) and analyzed on SDS/PAGE. There was no further increase in the level of expression beyond 2 h of isopropyl-1-thio-β-D-galactopyronoside induction (data not shown). Densitometric scanning of the gel (Fig. 3) showed that 22% of the total cellular protein was due to the overexpression of SHMT. More than 90% of this protein was present in the soluble fraction.

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GGATCGGGTGCCTCTGAACCAGCACAATGGCAGCTCCAGTCAACAAGGCACCCAGAGATG      60
                          M  A  A  P  V  N  K  A  P  R  D  A       12
CCGATTTGTGGTCCTTGCATGAGAAGATGCTGGCACAGCCCCTGAAGGACAACGATGTCG     120
 D  L  W  S  L  H  E  K  M  L  A  Q  P  L  K  D  N  D  V  E      32
AGGTTTACAACATCATTAAGAAGGAGAGTAACCGGCAGAGGGTTGGACTGGAGCTGATCG     180
 V  Y  N  I  I  K  K  E  S  N  R  Q  R  V  G  L  E  L  I  A      52
CCTCCGAGAACTTTGCCAGCCGGGCTGTTCTGGAGGCCCTAGGCTCTTGCCTGAACAACA     240
 S  E  N  F  A  S  R  A  V  L  E  A  L  G  S  C  L  N  N  K      72
AGTACTCTGAGGGGTACCCAGGCCAGAGGTACTATGGTGGGACGGAATTCATCGATGAGC     300
 Y  S  E  G  Y  P  G  Q  R  Y  Y  G  G  T  E  F  I  D  E  L      92
TAGAGGTCCTCTGTCAGAAGCGAGCGCTGCAGGTCTATGGCCTGGACCCCGAGTGCTGGG     360
 E  V  L  C  Q  K  R  A  L  Q  V  Y  G  L  D  P  E  C  W  G     112
GGGTTAACGTCCAGCCTTACTCAGGCTCCCCAGCAAATTTCGCAGTGTACACGGCCCTGG     420
 V  N  V  Q  P  Y  S  G  S  P  A  N  F  A  V  Y  T  A  L  V     132
TGGAGCCCCATGGCCGCATCATGGGCCTGGACCTGCCGGATGGGGGCCACCTGACCCATG     480
 E  P  H  G  R  I  M  G  L  D  L  P  D  G  G  H  L  T  H  G     152
GGTTCATGACTGATAAGAAGAAGATTTCTGCCACGTCCATCTTTTTTGAATCTATGCCTT     540
 F  M  T  D  K  K  K  I  S  A  T  S  I  F  F  E  S  M  P  Y     172
ACAAGGTGAATCCCGATACCGGCTACATCAACTACGACCAGCTGGAGGAGAACGCCCGCC     600
 K  V  N  P  D  T  G  Y  I  N  Y  D  Q  L  E  E  N  A  R  L     192
TCTTCCACCCGAGGCTGATCATTGCAGGGACTAGCTGCTACTCCCGGAACCTGGACTACG     660
 F  H  P  R  L  I  I  A  G  T  S  C  Y  S  R  N  L  D  Y  A     212
CTCGGCTGCGCAAGATCGCTGACGACAATGGGGCGTACCTCATGGCTGACATGGCACATA     720
 R  L  R  K  I  A  D  D  N  G  A  Y  L  M  A  D  M  A  H  I     232
TCAGCGGGCTGGTGGCGGCCGGCGTGGTGCCTTCCCCCTTCGAGCACTGCCACGTGGTGT     780
 S  G  L  V  A  A  G  V  V  P  S  P  F  E  H  C  H  V  V  S     252
CCACCACCACCCACAAGACCCTGCGGGGCTGCCGCGCCGGCATGATCTTCTACAGCAAGG     840
 T  T  T  H  K  T  L  R  G  C  R  A  G  M  I  F  Y  R  K  G     272
GAGTGCGCAGTGTGGACCCCAAGACAGGCAAAGAGACTCGCTACAACCTGGAGTCGCTCA     900
 V  R  S  V  D  P  K  T  G  K  E  T  R  Y  N  L  E  S  L  I     292
TCAACTCTGCTGTGTTCCCAGGCCTGCAAGGGGGACCTCACAACCACGCCATTGCTGGGG     960
 N  S  A  V  F  P  G  L  Q  G  G  P  H  N  H  A  I  A  G  V     312
TCGCGGTTGCTCTGAAGCAGGCCATGACTCCGGAGTTCAGAGCCTACCAGCGCCAGGTTG    1020
 A  V  A  L  K  Q  A  M  T  P  E  F  R  A  Y  Q  R  Q  V  V     332
TGGCCAACTGCAGGGCTCTGGCTGAGGCCTTGATGGGACTGGGCTACAGAGTGGTCACAG    1080
 A  N  C  R  A  L  A  E  A  L  M  G  L  G  Y  R  V  V  T  G     352
GGGGTTCTGACAACCACTTGATCCTCGTGGACCTCCGCTCCAAAGGCACAGATGGCGGCA    1140
 G  S  D  N  H  L  I  L  V  D  L  R  S  K  G  T  D  G  G  R     372
GGGCAGAGAAGGTGCTGGAAGCCTGCTCCATCGCCTGCAACAAGAATACCTGCCCAGGTG    1200
 A  E  K  V  L  E  A  C  S  I  A  C  N  K  N  T  C  P  G  D     392
ACAAAAGCGCACTACGGCCCAGTGGCTTGCGCCTGGGGACCCCAGCACTGACCTCCCGAG    1260
 K  S  A  L  R  P  S  G  L  R  L  G  T  P  A  L  T  S  R  G     412
GACTCTTGGAAGAAGATTTCCGAAAGGTTGCCCATTTCATTCACAGAGGCATAGAGCTGA    1320
 L  L  E  E  D  F  R  K  V  A  H  F  I  H  R  G  I  E  L  T     432
CCCTGCAGATCCAGGATGCTGTAGGAGTGAAGGCCACCCTGAAGGAGTTCATGGAGAAGC    1380
 L  Q  I  Q  D  A  V  G  V  K  A  T  L  K  E  F  M  E  K  L     452
TGGCAGGGGCCGAGGAGCACCAGCGGGCTGTGACAGCCCTCAGAGCAGAGGTGGAGAGCT    1440
 A  G  A  E  E  H  Q  R  A  V  T  A  L  R  A  E  V  E  S  F     472
TCGCCACCCTATTCCCGCTGCCTGGCCTGCCTGGCTTCTAGA                      1482
 A  T  L  F  P  L  P  G  L  P  G  F  *                          484
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Fig. 1. The nucleotide sequence of sheep liver cytosolic SHMT cDNA and derived amino acid sequence. The amino acid sequence underlined was confirmed by direct N-terminal sequencing of the purified recombinant SHMT. The residues indicated by (•) are diffirent from the reported sheep liver cytosolic SHMT [17].

Purification and characterization of the recombinant SHMT. The specific activity of the recombinant protein in the crude extract was 0.6 U (μmol · HCHO · min^{-1} · mg^{-1}) compared with the value of 0.02 U in sheep liver a 0.03 U in *E. coli* cells [BL21(DE3) strain].

The recombinant enzyme was purified by ammonium sulfate fractionation, Cibacron Blue agarose chromatography and Sephacryl S-200 gel filtration (Table 1 and Fig. 3). This procedure resulted in a 5.5-fold purification and 27% recovery. The recombinant enzyme eluted on a Sephacryl S-200 column at the same position as the native enzyme suggesting that it is a tetramer like the native SHMT (data not shown). The enzyme was found to be homogeneous on SDS/PAGE (Fig. 3) and had a subunit molecular mass of 53 kDa. The single band obtained was transferred onto a poly(vinylidene difluoride) membrane and the N-terminus was sequenced. The N-terminal sequence of the first 14 amino acids, AAPVNKAPRDADLW was identical to the deduced sheep cytosolic SHMT sequence (Fig. 1). The kinetic

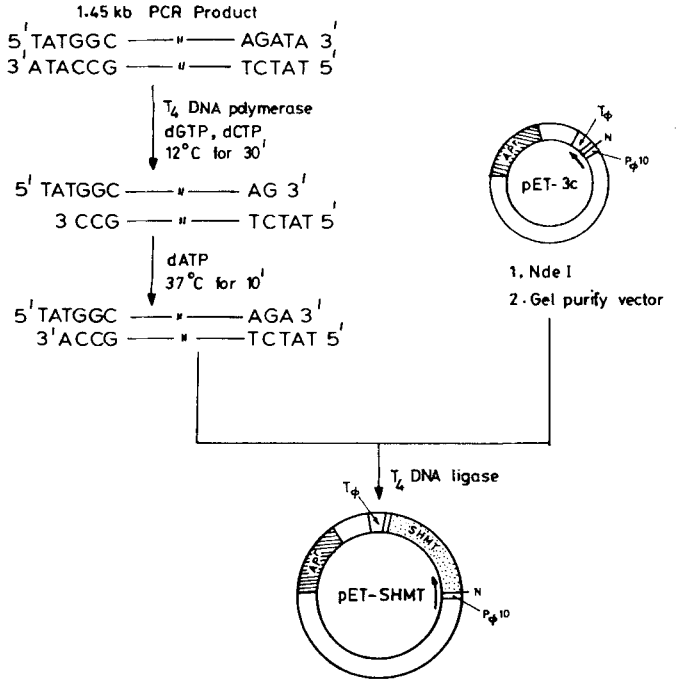

Fig. 2. The construction of pET-SHMT plasmid. The steps used to ligate the SHMT-encoding PCR-amplified fragment into the expression vector (pET-3C), yielding the pET-SHMT plasmid are shown (see Experimental procedures for details). $P_\phi 10$, T7 promoter; T_Φ, T7 terminator; Ap^r, ampicillin-resistance gene; Npb = *Nde*I.

Table 1. Purification of the recombinant SHMT. For total activity 1 U = 1 μmol HCHO produced in 1 min at 37°C.

Purification step	Total protein	Total activity	Specific activity	Yield	Purification
	mg	U	U/mg protein	%	-fold
Crude extract	202	120	0.6	100	1.0
Ammonium sulfate (25%–65%) fractionation	102	110	1.1	91.6	1.8
Blue agarose	19	50	2.7	41.6	4.5
Sephacryl S-200	10	32	3.20	26.7	5.4

characterization of recombinant SHMT revealed that the enzyme had a K_m of 1 mM for serine, 0.82 mM for H_4-folate and a V_{max} of 4.6 μmol HCHO · min^{-1} · mg^{-1} protein.

The enzyme had an absorbance maximum at 423.5 nm, a characteristic feature of pyridoxal-*P* present as an internal aldimine (Fig. 4). The bound pyridoxal-*P* could be removed by incubating the enzyme with D-alanine (200 mM) for 3 h at 37°C followed by dialysis to remove the pyridoxamine phosphate and pyruvate formed in the reaction. The apoenzyme had no spectrum in the visible region (Fig. 4) and was enzymically inactive. The apoenzyme could be reconstituted into the holoenzyme by the addition of pyridoxal-*P* (100 μM) as evidenced by the absorption spectrum with a maximum at 423.5 nm (Fig. 4) and the regaining of enzyme activity. The specific activity of the reconstituted enzyme was 3.0 μmol · min^{-1} · mg^{-1} compared with the value of 3.2 μmol · min^{-1} · mg^{-1} of the enzyme before removal of the pyridoxal-*P*.

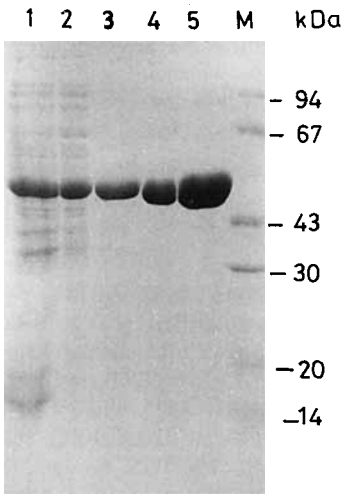

Fig. 3. The purification of recombinant sheep cytosolic SHMT from *E. coli*. Lane 1, crude extract (35 μg); lane 2, 25–65% ammonium sulfate fraction (17 μg); lane 3, Cibacron blue agarose eluant (6 μg); lane 4, Sephacryl S-200 fraction (6 μg); lane 5, Sephacryl S-200 fraction (14 μg); M_r molecular-mass markers (Pharmacia); markers used were phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactalbumin.

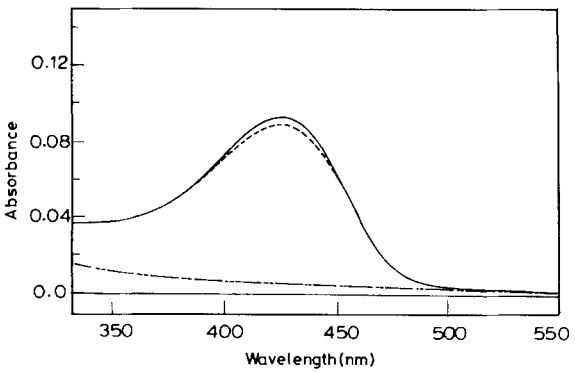

Fig. 4. The visible absorption spectrum of recombinant SHMT. The visible absorption spectrum of holo (——), apo (— — —) and reconstituted (– – –) SHMT. Protein (0.8 mg) was used for recording the spectrum.

DISCUSSION

Although extensive biochemical investigations on the mechanism of action of mammalian SHMT have been carried out previously [33, 34], a major limitation for a further understanding of the structure/function relationship has been the non-availability of an overexpressing clone of the eukaryotic SHMT. The results presented in this communication demonstrate the overexpression of an eukaryotic SHMT (from sheep liver) in *E. coli*. An SHMT overexpression vector (pET-SHMT) was constructed in which the sheep liver cytosolic SHMT encoding cDNA fragment was placed under the control of a strong T7 promoter, inducible by isopropyl-1-thio-β-D-galactopyranoside. The enzyme was not only expressed at high levels, i.e., 20-fold above the basal level in *E. coli* but was also present predominantly in the soluble fraction (Fig. 3). This protein constituted 22% of the total protein in the cell extracts. In contrast, the expression of rabbit liver cytosolic SHMT in COS-1 cells was only marginally above the basal level [10].

The recombinant enzyme was purified to homogeneity with a yield of 10 mg/l bacterial culture (Table 1). The native and subunit molecular mass of the recombinant enzyme was

213 kDa and 53 kDa, respectively. Thus the recombinant SHMT is also a homotetramer like the cytosolic sheep SHMT [26]. However the recombinant enzyme from *E. coli* was shown to be a dimer [35, 36], whereas, the wild-type enzyme was a tetramer [37]. It would be interesting to examine the differences in the nature of subunit interactions that lead to different states of assembly of subunits in the *E. coli* and sheep liver recombinant enzymes. The N-terminal sequence of the recombinant enzyme was identical to the deduced sequence although, the N-terminal methionine was lacking. This is not surprising if we consider that the N-terminal methionine in the recombinant SHMT is followed by alanine. It has been observed that in 96% of the cytosolic *E. coli* proteins, in which the second residue was alanine, the N-terminal methionine was excised [38]. A comparison of the deduced amino acid sequence of sheep cytosolic SHMT clone with the primary structure of the sheep liver cytosolic SHMT [17] revealed that there were differences in the amino acid sequence at 10 positions as indicated in Fig. 1. Those differences might be attributable to the differences in the genetic variety of sheep used for preparing cDNA library and for the isolation of the enzyme. In addition a few residues (A3/T, P4/A, W111/F, V115/Y, G136/A, P171/A and R196/K were misidentified in the protein sequence and have now been corrected. Despite small sequence differences between the enzyme purified from sheep liver and the recombinant enzyme, we did not detect any differences in the properties of the two enzyme preparations. The expressed SHMT effectively interacted with antibodies raised against sheep liver cytosolic SHMT indicating that the antigenic epitopes are well retained in the recombinant enzyme (data not shown) The enzyme was capable of using serine or β-phenylserine as the substrate, demonstrating its ability to carry out H_4-folate-dependent or independent reaction. The predicted secondary structure of the recombinant enzyme was identical to that of the native enzyme (data not shown). The purified recombinant enzyme had the characteristic visible absorption spectrum indicative of the presence of pyridoxal-*P* as an internal aldimine which could be removed and reconstituted in a manner similar to that of native sheep liver SHMT. The only difference in the properties of the recombinant enzyme and the native enzyme is the ability to bind CM-Sephadex which could have arisen from the absence of acetylation at the N-terminus or replacement of a few charged residues. The amino acid residues essential for activity are unchanged in the recombinant protein [7, 17]. The results presented in this study convincingly demonstrated that the recombinant SHMT was functionally identical to the native enzyme. The availability of a homogeneous and active recombinant SHMT in abundant amounts has laid the foundation for critical studies aimed at elucidating the role of the amino acid residues involved in the catalysis and binding of the substrates and substrate analogues.

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