

# Overexpression and characterization of dimeric and tetrameric forms of recombinant serine hydroxymethyltransferase from *Bacillus stearothermophilus*

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Serine hydroxymethyltransferase (SHMT), a pyridoxal-5'-phosphate (PLP) dependent enzyme catalyzes the interconversion of L-Ser and Gly using tetrahydrofolate as a substrate. The gene encoding for SHMT was amplified by PCR from genomic DNA of *Bacillus stearothermophilus* and the PCR product was cloned and overexpressed in *Escherichia coli*. The purified recombinant enzyme was isolated as a mixture of dimer (90%) and tetramer (10%). This is the first report demonstrating the existence of SHMT as a dimer and tetramer in the same organism. The specific activities at 37°C of the dimeric and tetrameric forms were 6.7 U/mg and 4.1 U/mg, respectively. The purified dimer was extremely thermostable with a  $T_m$  of 85°C in the presence of PLP and L-Ser. The temperature optimum of the dimer was 80°C with a specific activity of 32.4 U/mg at this temperature. The enzyme catalyzed tetrahydrofolate-independent reactions at a slower rate compared to the tetrahydrofolate-dependent retro-aldol cleavage of L-Ser. The interaction with substrates and their analogues indicated that the orientation of PLP ring of *B. stearothermophilus* SHMT was probably different from sheep liver cytosolic recombinant SHMT (scSHMT).

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## 1. Introduction

Serine hydroxymethyltransferase (SHMT) is identified as a possible target for cancer chemotherapy, as it provides the methylene group for the synthesis of thymidylate, a key precursor for DNA synthesis (Harish Kumar *et al* 1976; Snell 1984; Matthews *et al* 1998; Appaji Rao *et al*

2000). It is also a key enzyme, in the industrial production of amino acids (Hasiuo *et al* 1988), in linking amino acid and nucleotide metabolism (Schirch 1982) and in photorespiration of plants (McClung *et al* 2000). SHMT, a pyridoxal-5'-phosphate (PLP) dependent enzyme catalyzes the reversible conversion of L-Ser and Gly in the presence of tetrahydrofolate ( $H_4$ -folate) to yield 5,10-

**Keywords.** Oligomeric structure; orientation of pyridoxal-5'-phosphate; serine hydroxymethyltransferase; thermal stability

Abbreviations used: AAA, aminooxyacetic acid; bsSHMT, *Bacillus stearothermophilus* recombinant SHMT; CD, circular dichroism; DSC, differential scanning calorimetry; eSHMT, *Escherichia coli* recombinant SHMT;  $H_4$ -folate, 5,6,7,8-tetrahydrofolate; hcSHMT, human liver cytosolic recombinant SHMT; MA, methoxyamine; mcSHMT, murine liver cytosolic recombinant SHMT; 2-ME, 2-mercaptoethanol; N5,N10  $CH_2$ - $H_4$ -folate, N5,N10-methylene tetrahydrofolate; PLP, pyridoxal 5'-phosphate; rcSHMT, rabbit liver cytosolic recombinant SHMT; scSHMT, sheep liver cytosolic recombinant SHMT; SHMT, serine hydroxymethyltransferase.

methylene H<sub>4</sub>-folate (5,10-CH<sub>2</sub>-H<sub>4</sub>-folate). Like the other PLP dependent enzymes, SHMT also catalyzes a variety of side reactions, such as transamination, racemization, decarboxylation, etc. (Schirch 1982; Appaji Rao *et al* 2000). It has been suggested that enzymes from thermophilic organisms can more often be crystallized readily than from mesophilic sources (Panasik *et al* 2000; Vali *et al* 1980). An interesting property of SHMT is its conversion from an 'open' to 'closed' form in the presence of L-Ser, thereby conferring specificity to the physiological reaction. This conformational change is accompanied by increased thermal stability (Schirch *et al* 1991).

Although the recombinant enzyme from *Bacillus stearothermophilus* (bsSHMT) has been obtained earlier (Ide *et al* 1992), it has not been characterized in sufficient detail. In this paper, we report the cloning, overexpression and characterization of the dimeric and tetrameric forms of the recombinant SHMT from *B. stearothermophilus*.

## 2. Materials and methods

### 2.1 Materials

L-[3-<sup>14</sup>C]-Serine, restriction endonucleases and modifying enzymes were obtained from Amersham Pharmacia Biotech Ltd., Buckinghamshire, England. Deep Vent polymerase was purchased from New England Biolabs, Beverly, MA, USA. Taq DNA polymerase was purchased from Bangalore Genei Pvt. Ltd., Bangalore. DEAE-Cellulose, Sephacryl S-200, Gly, L-Ser, D-Ala, 2-mercaptoethanol (2-ME), folic acid, rubidium chloride, PLP, IPTG and EDTA were obtained from Sigma Chemical Co., St. Louis, MO, USA. Platinum oxide was purchased from Loba Chemie, Mumbai. All other chemicals used were of analytical reagent grade.

### 2.2 Bacterial strains, growth conditions and DNA manipulations

*Escherichia coli* strain DH5a (Bethesda Research Labs, USA) was the recipient for all the plasmids used for DNA isolations and subcloning. BL21 (DE3) pLysS strain (Studier and Moffat 1986) was used for the expression of scSHMT and bsSHMT. Luria-Bertani (LB) medium or terrific broth (24 g of yeast extract, 12 g of tryptone, 4 ml of glycerol, 2.31 g of KH<sub>2</sub>PO<sub>4</sub> and 12.54 g K<sub>2</sub>HPO<sub>4</sub> per litre) with 50 µg/ml of ampicillin was used for growing *Escherichia coli* cells containing the plasmids at 37°C. Plasmids were prepared by the alkaline lysis procedure described by Sambrook *et al* (1989). The genomic DNA of *B. stearothermophilus* was isolated

using the protocol described in Sambrook *et al* (1989). The DNA fragments were eluted from QIAquick gel extraction kit from Qiagen.

### 2.3 PCR amplification of bsSHMT gene from genomic DNA

Based on the sequence of bsSHMT available in the database (Acc No: E02190), the following primers were designed.

Sense primer: 5' GGGGGAGCTACATATGAACTACTTGCCAC 3'

Antisense primer: 5' GAGCGGAAACGGATCCGTCAAAGCGGCGAC 3'

Underlined nucleotides represent the *Nde*I site in the *B. stearothermophilus* sense primer sequence and *Bam*HI in antisense primer. The template genomic DNA (150 ng), *B. stearothermophilus* sense primer (50 pmol) and antisense primer (50 pmol) were added to PCR tubes containing 0.2 mM dNTPs, 1 mM MgSO<sub>4</sub>, 1.5 U Deep Vent DNA polymerase and Taq DNA polymerase along with the buffer provided with the Deep Vent DNA polymerase at 1X concentration. Amplification reaction was carried out in a Techne-PCR machine using the following cycling conditions: denaturation of the template at 95°C for 4 min followed by 25 cycles at 94°C for 1 min (denaturation) 54°C for 1 min (annealing) and 72°C for 2 min (extension). The reaction was continued for 10 min at 72°C to complete the extension. The PCR product obtained was extracted from agarose gel using QIAquick gel extraction kit from Qiagen. This DNA fragment was digested with *Nde*I and *Bam*HI and the digested product was gel eluted as before. This fragment was ligated to pRSET C, a T7-promoter based expression vector, which was previously double digested with *Nde*I and *Bam*HI. The colonies were screened by restriction analysis and the positive colonies were sequenced by the ABI Prism DNA automated sequencer.

### 2.4 Overexpression and purification of bsSHMT

The plasmid containing bsSHMT gene was transformed into *E. coli* BL 21 (DE3) pLys S strain. A single colony was inoculated in 50 ml of LB medium containing 50 µg/ml ampicillin and grown overnight at 30°C. These cells grown overnight were inoculated at 4% concentration into 1 litre of terrific broth medium containing 50 µg/ml ampicillin. After 4 h of growth at 30°C, the cells were induced with 0.3 mM IPTG for 5 h. The cells were then harvested, resuspended in 60 ml of buffer A

(50 mM potassium phosphate buffer pH 7.4, containing 5 mM 2-ME, 1 mM EDTA and 100  $\mu$ M PLP) and sonicated until it was optically clear (~ 30–40 min). The supernatant was subjected to 0–65% ammonium sulphate precipitation; the pellet obtained was resuspended in 20–30 ml of buffer B (20 mM potassium phosphate buffer pH 8.0, containing 1 mM 2-ME, 1 mM EDTA and 50  $\mu$ M PLP) and dialyzed for 24 h against the same buffer (1 litre with two changes). The dialyzed sample was loaded on to DEAE-cellulose, which was previously equilibrated with buffer B. The column was washed with 500 ml of buffer B and the bound protein was batch eluted with 50 ml of buffer C (200 mM potassium phosphate buffer, pH 6.4 containing 1 mM EDTA, 1 mM 2-ME, 50  $\mu$ M PLP). The eluted protein was precipitated at 65% ammonium sulphate saturation. The pellet was resuspended in buffer D (200 mM potassium phosphate buffer, pH 7.4 containing 1 mM EDTA, 1 mM 2-ME, 50  $\mu$ M PLP) and subjected to chromatography using Sephacryl S-200 column. Fractions with considerable enzyme activity were pooled and precipitated at 65% ammonium sulphate and the pellet was resuspended in buffer E (50 mM potassium phosphate buffer, pH 7.4 containing 1 mM EDTA, 1 mM 2-ME) and dialyzed against the same buffer (2 litres, with two changes) for 24 h. Protein was estimated using BSA as a standard (Lowry *et al* 1951)

Enzyme assays, spectroscopic methods, the method for determining the apparent  $T_m$  values and the procedure used for differential scanning calorimetry were as described by Krishna Rao *et al* (2000).

### 2.5 N-terminal sequencing

bsSHMT was subjected to 12% SDS PAGE and the band corresponding to Mw of 45 kDa was transferred on to a PVDF membrane and stained with Ponceau-S. The protein band was cut out, destained and loaded on a Shimadzu-gas phase sequenator PSQ-1 to determine the N-terminal amino acid sequence. The dimeric and tetrameric forms were subjected to 12% non-denaturing PAGE for 28 h and the proteins were transferred to PVDF membranes and sequenced as described above.

## 3. Results and discussion

### 3.1 Cloning, overexpression and purification of bsSHMT

The SHMT gene sequence obtained is nearly identical to that described earlier by Miyamoto and Nagaya (Acc. No. E02190) except for a few minor differences (N2K and F161L). These changes could be due to the difference in the strains used for the isolation of the gene (Ide *et al*

1992). An additional 17 amino acid residues were added at the C-terminus from position 398 due to the cloning strategy employed. These changes did not affect the expression of gene or the catalytic properties of the recombinant enzyme. The deduced amino acid sequence showed 60.8% and 40% identity with eSHMT (Plamann *et al* 1983) and scSHMT (Jagath-Reddy *et al* 1995, 1997), respectively. The enzyme contained 1 mol of PLP per mol of subunit. The purification described here (table 1) yielded 100 mg of the enzyme from cells grown in one litre of terrific broth.

When bsSHMT (table 1, step 4) was subjected to size-exclusion chromatography on a calibrated Superose-12 column attached to Pharmacia FPLC system, it eluted as a major peak (~ 90%) corresponding to a Mw of 90 kDa and a minor peak with a Mw of 180 kDa (figure 1a). bsSHMT (table 1, step 4) was subjected to non-denaturing PAGE followed by activity staining and two enzymatically active bands were observed (figure 1b). This observation suggested that the enzyme was probably isolated as a mixture of dimer and tetramer. It was, therefore of interest to characterize the two forms of the enzyme further. It was observed during the Sephacryl-200 (table 1, step 4) step of purification that several earlier fractions contained a small amount of enzyme activity, which were not collected previously. All these fractions were subjected to non-denaturing PAGE analysis followed by staining of the gels for enzyme activity (Ulevich and Kallen 1977). It was observed that these fractions contained the enzyme with Mw 180 kDa or a mixture of Mw 180 kDa and Mw 90 kDa forms of bsSHMT (data not shown). The fractions containing the dimer or the tetramer were pooled separately and concentrated by centricon filtration, whereas the fractions containing both the forms were discarded. While the pooled dimer fraction gave a single band upon 12% SDS-PAGE analysis, the pooled tetramer fraction indicated the presence of trace amounts of other contaminating proteins along with bsSHMT. It was, therefore necessary

**Table 1.** Purification of recombinant bsSHMT. *E. coli* cell pellet obtained from one litre of terrific broth media was used as the starting material for the purification of bsSHMT.

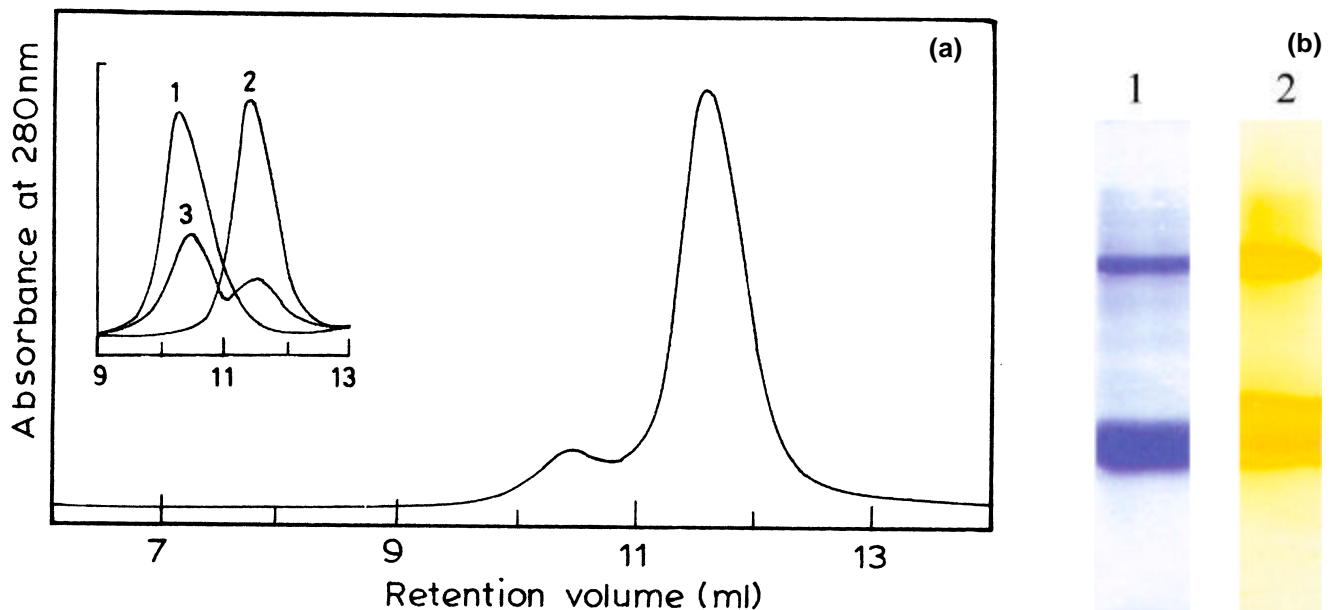
Purification step	Total activity (U*)	Specific activity (U*/mg)	Yield (%)	Purification fold
Crude extract	3063	1.1	100	1
Ammonium sulphate	2809	1.9	91	2
DEAE-cellulose	2662	5.2	87	5
S-200 gel filtration	504	6.7	17	6

\*1 U = 1  $\mu$ mol of HCHO formed per min at 37°C.

to subject this pooled fraction to further purification. It is well known that L-Ser stabilizes SHMT against heat denaturation and has been used as a step of purification in earlier studies (Nakano *et al* 1968). The contaminating proteins were removed by heating the tetrameric fraction at 60°C for 5 min in the presence of L-Ser (100 mM). This pooled fraction was loaded on to Superose-12 column, attached to Pharmacia FPLC system and absorbance at 280 nm was monitored. The peak fractions corresponding to a Mw of 180 kDa were collected and concentrated by centricon filtration and used as the tetrameric form of bsSHMT in these studies. Upon rechromatography on a calibrated Superose-12 column, the tetrameric form eluted as a single symmetrical peak corresponding to a Mw of 180 kDa (figure 1 inset, curve 1). The protein from this peak when heated for 5 min at 60°C and rechromatographed on a calibrated Superose-12 column eluted as a mixture of 180 kDa and 90 kDa protein (figure 1 inset, curve 3). The dimeric and tetrameric forms of bsSHMT gave a single band on SDS-PAGE (Mw ~ 45 kDa) and were identical in N-terminal sequence i.e. MKYLPQQDPQVFAA. Upon native PAGE followed

by staining for activity, they gave a single band with different mobilities (data not shown).

It is interesting to recall that in all prokaryotes, SHMT was present as a dimer (Schirch *et al* 1985; Scarsdale *et al* 1999), whereas it occurred as a tetramer in eukaryotes (Nakano *et al* 1968; Jagath-Reddy *et al* 1995; Renwick *et al* 1998; Scarsdale *et al* 1999; Appaji Rao *et al* 2000; Szebenyi *et al* 2000). Recently, it was shown that the *Trypanosoma cruzi* SHMT was present as a catalytically active monomer (Capelluto *et al* 2000). It has so far not been possible to obtain a fully active dimeric form of the eukaryotic enzyme either by mutation or dissociation. On the other hand, the enzyme from prokaryotes is invariably present as a dimer, although there is some evidence to suggest that a tetrameric form is present in the crystal structure of the ternary complex of eSHMT with Gly and 5-formyl H<sub>4</sub>-folate (Scarsdale *et al* 2000). The observation that bsSHMT can be present as a tetramer and dimer, possibly in inter-convertible forms provides a unique opportunity to examine some of the features of the enzyme such as the forces stabilizing the tetrameric and dimeric forms of the enzyme as well as



**Figure 1.** (a) Size exclusion chromatography profile of the purified bsSHMT (300 µg/ml) on Superose-12 column attached to a Pharmacia FPLC system. The column was calibrated with standard molecular weight markers, i.e. apoferritin (440,000), *b*-amylase (200,000), yeast ADH (150,000), bovine serum albumin (66,000) and carbonic anhydrase (29,000). The FPLC runs were performed in buffer E and the absorbance was monitored at A<sub>280</sub> nm at 0.02 AU sensitivity. *Inset*: Dimeric and tetrameric forms of SHMT. Curve 1: Fractions eluting in the Mw range 200 kDa in Sephacryl-200 gel filtration column for bsSHMT were pooled and heated at 60°C for 5 min in the presence of L-Ser (100 mM), concentrated by centricon filtration and rechromatographed on a calibrated Superose-12 column. Curve 2: The fraction corresponding to 90 kDa obtained from the Sephacryl-200 column was subjected to rechromatography as in curve 1. Curve 3: The peak fraction of curve 1 was heated at 60°C for 5 min and rechromatographed as above. It eluted as a mixture of dimer and tetramer. (b) Non-denaturing PAGE (10%) analysis of purified bsSHMT. The enzyme (40 µg) was subjected to 10% PAGE in duplicate. Lane 1, stained with comassie; lane 2, stained for activity with *b*-phenyl serine as substrate and benzaldehyde formed was detected by reaction with 2,4-dinitrophenyl-hydrazine (DNPH).

the organization of the active site in the two forms of the enzyme. Although it was possible to convert the tetramer to dimer by heating, preliminary efforts at converting the dimer to tetramer were not successful.

### 3.2 Catalytic properties of dimer and tetrameric forms of bsSHMT

The bsSHMT dimer and tetramer catalyzed the H<sub>4</sub>-folate dependent retro-aldol cleavage of L-Ser with a specific activity of 6.7 U/mg and 4.1 U/mg, respectively (table 2). The  $K_m$  values for L-Ser for the dimer and tetramer were 0.9 mM, 0.7 mM and  $k_{cat}$  values were 5.0 s<sup>-1</sup> and 3.6 s<sup>-1</sup> respectively (table 2). These values were comparable to that for eSHMT. bsSHMT catalysed the tetrahydrofolate-independent retroaldol cleavage of L-allothreonine at rates slower than the tetrameric mammalian enzymes (table 2). Both the tetrameric and dimeric forms of bsSHMT catalyzed the transamination of D-Ala very sluggishly. A comparison of the spectral changes in the reaction of D-Ala with the bsSHMT dimer and scSHMT is given in figure 2a,b. bsSHMT spectra showed an isosbestic point at 350 nm whereas it was absent in scSHMT indicating the presence of single and multiple intermediates in the reaction pathway respectively. The enzyme also catalyzes the H<sub>4</sub>-folate independent retro aldol cleavage of L-*allo*Thr (table 2) and *b*-phenyl serine reactions. A comparison of kinetic constants (table 2) and spectral changes (figure 2a,b) suggested that the bsSHMT had a higher specificity compared to scSHMT for the physiological reaction than for side reactions.

It was earlier reported that methoxyamine (MA) and aminoxyacetic acid (AAA) react with PLP-Schiffs base generating characteristic intermediates and provide a good handle to monitor the differences in the accessibility of the internal aldimine at the active site of SHMT (Acharya *et al* 1991). It can be seen from the figure 2c that scSHMT generates a characteristic intermediate with AAA absorbing at 380 nm (curve 1) before the formation

of the oxime. On the other hand, bsSHMT did not yield any intermediate prior to the formation of the oxime product (figure 2d). It can also be seen that the concentration of oxime at 1 min is much larger in bsSHMT compared to scSHMT. This observation suggested that the rate of conversion of the intermediate generated by AAA and bsSHMT to the final product was probably much faster than in scSHMT. It was earlier observed that the reaction of hydroxylamine derivatives with SHMT was substantially decreased in the absence of carboxy groups (Baskaran *et al* 1989; Acharya *et al* 1991), for example AAA was more reactive than MA. In the reaction of MA with scSHMT and bsSHMT, the intermediate absorbing at 388 nm was seen (figure 2e,f). The time course of the conversion of the intermediate to the oxime was similar in both bsSHMT and scSHMT. However, the isosbestic point in the conversion of intermediate to the final oxime product shifted from 364 nm to 355 nm (figure 2e,f). This difference could be attributed to the differences in the accessibility of PLP-Schiffs base to the inhibitors. It is interesting to recall that in the non-enzymatic conversion of PLP-N-*a*-acetyl Lys-Schiffs base to oxime by AAA/MA, no intermediate was seen suggesting that the formation of intermediate reflect the orientation of internal aldimine at the active site (Acharya *et al* 1991). Additional support for the hypothesis that the orientation of PLP in bsSHMT and scSHMT are different was the interaction of the substrates, L-Ser and Gly as monitored by visible CD measurements. The reaction of Gly with scSHMT in addition to quenching the CD intensity at 425 nm yielded a spectrum with a maximum CD intensity at 343 nm suggesting the formation of the geminal diamine (figure 3a). Although, addition of glycine to bsSHMT caused a reduction in CD intensity at 425 nm, there was no evidence for the formation of a geminal diamine (figure 3b, curve 1). This observation suggested that the equilibrium mixture of bsSHMT and Gly contained different proportions of the expected intermediates namely, geminal diamine and external aldimine. Another important difference in the interaction

**Table 2.** Kinetic parameters of scSHMT and dimeric and tetrameric SHMT using either L-Ser or L-*allo*Thr as substrate.

Substrate/ enzyme	L-Ser			L- <i>allo</i> Thr	
	Specific activity (U*/mg)	$k_{cat}$ ** (s <sup>-1</sup> )	$K_m$ (mM)	$K_{cat}$ ** (s <sup>-1</sup> )	$K_m$ (mM)
scSHMT	4.2	4.3 ± 0.3	1.0 ± 0.04	3.7 ± 0.4	0.7 ± 0.01
bsSHMT-dimer	6.7	5.0 ± 0.6	0.9 ± 0.03	0.9 ± 0.02	0.4 ± 0.009
bsSHMT-tetramer	4.1	3.6 ± 0.04	0.7 ± 0.04	0.7 ± 0.02	0.7 ± 0.013

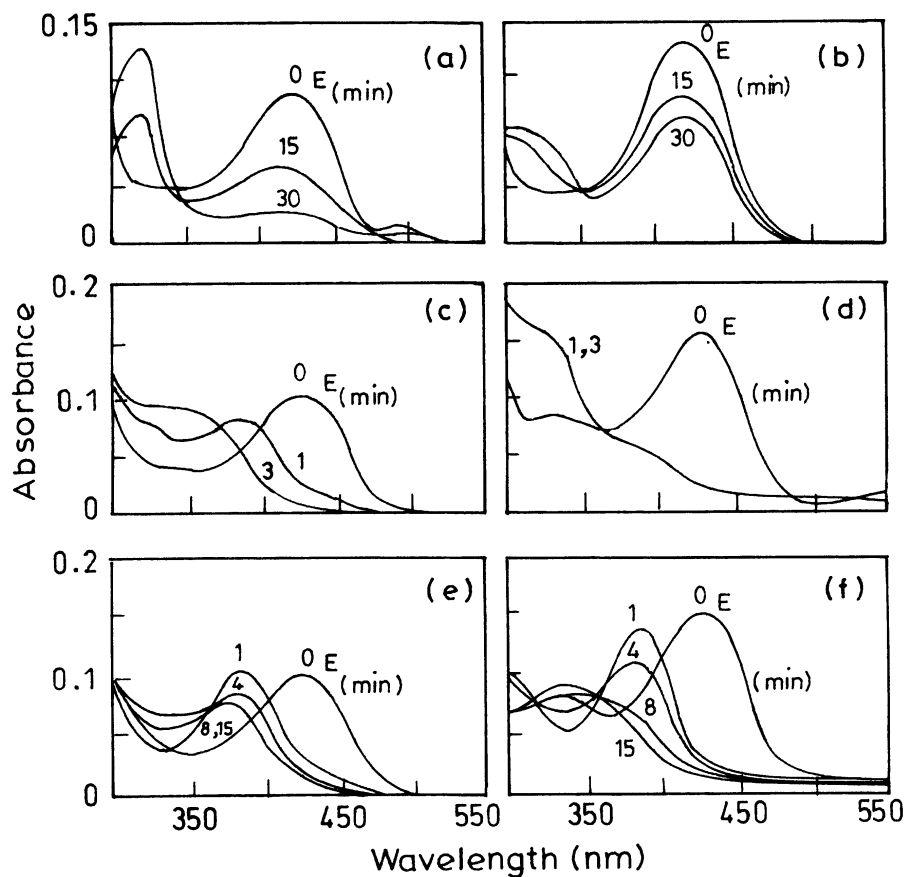
\*1 U = 1 μmol of HCHO formed per min at 37°C and pH 7.4.

\*\*Calculated per mol of subunit.

of L-Ser with PLP in the two enzymes was the observation that while Gly and L-Ser caused a similar amount of quenching in CD intensity in the case of scSHMT (figure 3a), L-Ser quenching of bsSHMT CD at 425 nm was larger than the quenching with Gly (figure 3b). These observations suggested that the symmetry of the external aldimine of bsSHMT is probably different from that in scSHMT.

It can be seen from figure 3d that the absorbance spectra obtained upon the addition of Gly to bsSHMT (figure 3d, curve 1) is very similar to that obtained in the absence of the substrate. On the other hand, comparison of this spectrum with that of scSHMT + Gly showed that there was a decrease in the absorbance at 425 nm with a concomitant increase at 343 nm, indicating the formation

of a geminal diamine intermediate (figure 3c, curve 1). These results support our suggestion that PLP in two enzymes might be interacting with the substrates differently. Addition of H<sub>4</sub>-folate to a mixture of scSHMT and Gly gave a spectrum with a characteristic absorbance at 495 nm suggesting the formation of a quinonoid intermediate (figure 3c, curve 2). A similar quinonoid intermediate having the maximum absorbance at 495 nm was also formed in the case of bsSHMT upon the addition of H<sub>4</sub>-folate to Gly and bsSHMT mixture (figure 3d, curve 2). The amount of quinonoid intermediate formed with bsSHMT was calculated to be 0.67 μmol/μmol of subunit, which is comparable to that observed with scSHMT (figure 3). Addition of L-Ser or formaldehyde decreased the intensity of quinonoid intermediate for both enzymes



**Figure 2.** Comparison of the spectral changes observed upon the interaction of scSHMT and bsSHMT with D-Ala, aminoxyacetic acid (AAA) and methoxyamine (MA). In all cases 1 mg of scSHMT/bsSHMT in buffer E was used and spectra were recorded from 300–550 nm (curve E). The absorption spectra were recorded in a Shimadzu UV-160A spectrophotometer in buffer E (a–f). (a, b) To scSHMT (a) D-Ala (100 mM) was added and the spectra were recorded at 37°C at time intervals of 0, 15 and 30 min. Spectra recorded in a similar conditions for bsSHMT (b). (c, d) AAA (50 μM) was added to either scSHMT (c) or bsSHMT (d) and spectra recorded after 1 and 3 min. (e, f) MA (50 μM) was added to scSHMT (e) or bsSHMT (f) and spectra recorded at time intervals of 0, 1, 5, 8 and 15 min.

indicating that it was part of kinetic mechanism of bsSHMT and scSHMT and the enzyme was catalysing the reaction in a similar manner (data not shown).

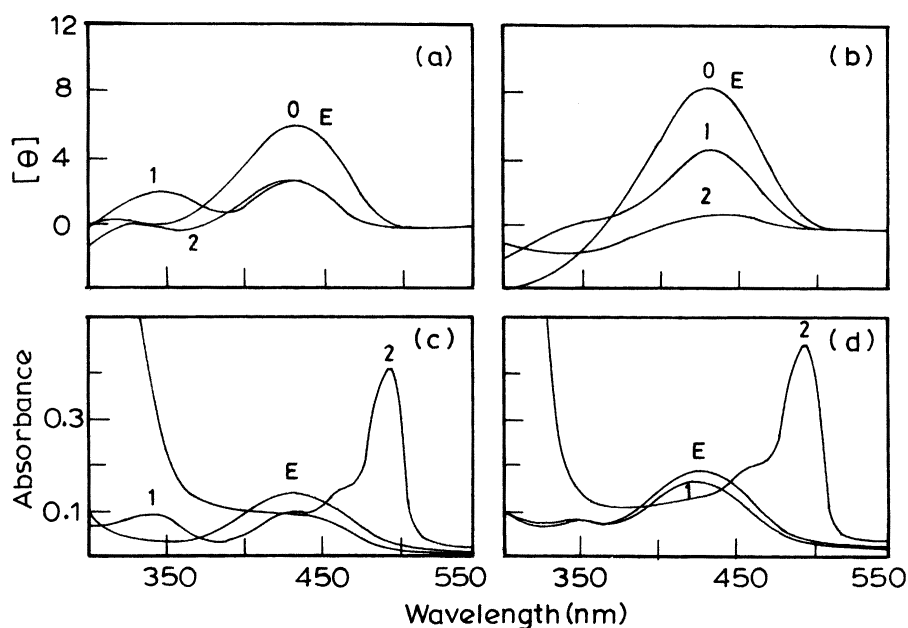
These observations emphasized once again the possible difference in the orientation of the PLP-Schiffs base in bsSHMT and scSHMT. This suggestion was supported by the analysis of crystal structure of bsSHMT (Subramanya H S, personal communication).

### 3.3 Thermal stability

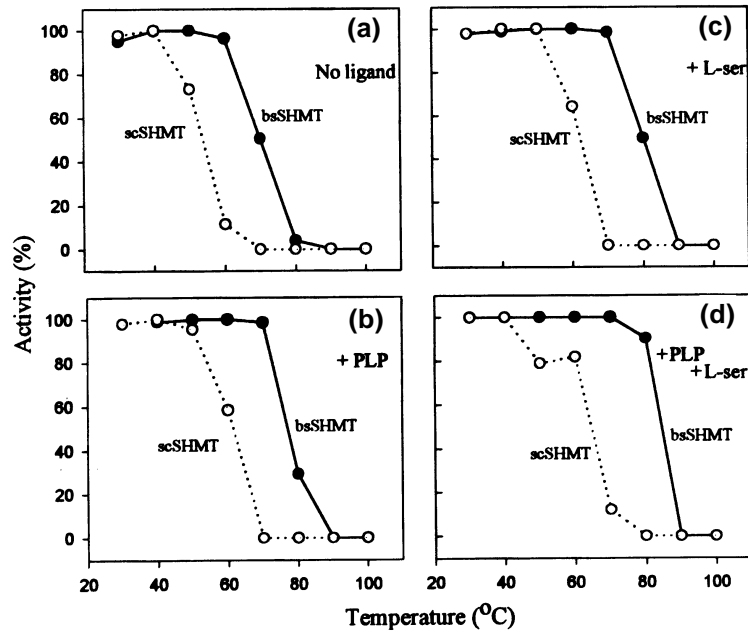
A characteristic feature of the enzymes from thermophilic organisms is their stability at higher temperatures (Okubo *et al* 2000). It can be seen from the figure 4 that bsSHMT is stable up to 60°C and the 50% activity is retained even at 70°C in the absence of any ligand. On the other hand, scSHMT is stable only up to 50°C and at 60°C, almost all the activity is lost (figure 4a). It was observed earlier with scSHMT and alanine racemase that PLP increases the thermal stability without enhancing their catalytic activity (Krishna Rao *et al* 1999; Okubo *et al* 2000). It was therefore, of interest to explore the effect of PLP (500 µM) on bsSHMT. It can be seen that

the mid point of inactivation was shifted from 65 to 78°C, whereas in the case of scSHMT it was shifted from 55 to 62°C (figure 4b). It is well known that L-Ser converts the enzyme from an 'open' to 'closed' form characterized by increased thermal stability (Schirch *et al* 1991; Bhaskar *et al* 1994). It can be seen from the figure 4c that there was no loss of activity of bsSHMT even at 70°C in the presence of L-Ser, while scSHMT lost most of its activity. The effect of PLP and L-Ser was additive and the bsSHMT was almost fully active at even 80°C, whereas scSHMT lost 90% of its activity at 70°C (figure 4d). These results clearly indicated that bsSHMT was thermostable and L-Ser and PLP enhanced its thermal stability. The temperature optimum of bsSHMT was 80°C with a specific activity of 32.8 U/mg compared to its specific activity of 6.7 U/mg at 37°C.

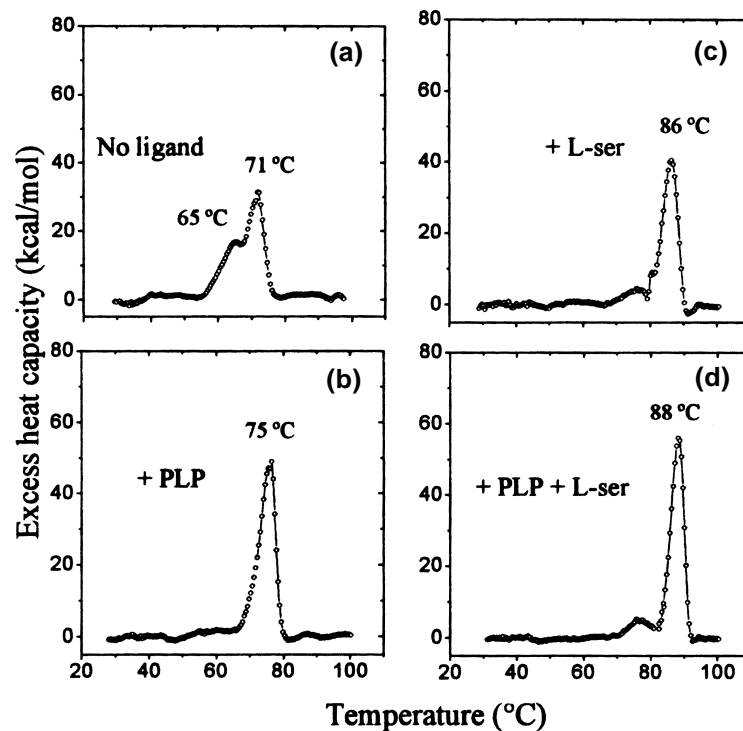
A direct measure for thermal stability is the determination of  $T_m$  values. PLP or L-Ser enhanced the thermal stability of scSHMT and bsSHMT by 10–12°C (table 3). The addition of L-Ser and PLP together to the enzymes enhanced its thermal stability even further (from 76°C to 85°C). Gly or D-Ala did not enhance their  $T_m$  value suggesting that these amino acids were unable to induce a



**Figure 3.** Interaction of substrates with sc and bsSHMT. (a, b) Visible CD spectra of scSHMT/bsSHMT recorded in a Jasco-500A automated recording spectropolarimeter. Visible CD spectra of scSHMT (a, curve E) and bsSHMT (b, curve E) with 100 mM Gly (curve 1) or with 100 mM L-Ser (curve 2). (a) Curve E, scSHMT; curve 1, scSHMT + Gly; curve 2, scSHMT + Gly + H<sub>4</sub>-folate. (b) Curve E, bsSHMT; curve 1, bsSHMT + Gly; curve 2, bsSHMT + Gly + H<sub>4</sub>-folate. (c, d) The spectral intermediates generated upon the addition of glycine (100 mM) and H<sub>4</sub>-folate. (c) curve E, scSHMT; curve 1, scSHMT + Gly; Curve 2, scSHMT + Gly + H<sub>4</sub>-folate. (d) Curve E, bsSHMT; curve 1, bsSHMT + Gly; curve 2, bsSHMT + Gly + H<sub>4</sub>-folate.



**Figure 4.** Protection of scSHMT and bsSHMT activity against heat inactivation. In all experiments 1  $\mu$ g of the bsSHMT (—●—)/scSHMT (---○---) in buffer E was heated at temperatures indicated in the figure for 5 min either in the absence of any ligand (a) or in the presence of 500  $\mu$ M PLP (b) or in the presence of 3-6 mM L-Ser (c) or in the presence of both PLP and L-Ser (d). The residual activity of the enzyme was assayed as described in §2. Activity assayed at 37°C was normalized to 100% and residual activity was expressed as a percent of this value.



**Figure 5.** Differential scanning calorimetry thermograms of bsSHMT (3.5 mg/ml) in the absence and presence of ligands. (a) No ligand; (b) + PLP (500  $\mu$ M); (c) + L-Ser (100 mM); (d) + PLP (500  $\mu$ M) + L-Ser (100 mM).



**Table 3.** Apparent  $T_m$  values ( $^{\circ}\text{C}$ ) of scSHMT and dimeric and tetrameric forms of bsSHMT in the absence and presence of ligands.

Substrate/ enzyme	No		PLP +			
	ligand	L-Ser	PLP	L-Ser	Gly	D-Ala
ScSHMT	55	66	66	76	55	55
BsSHMT-dimer	65	79	76	85	65	66
BsSHMT-tetramer	66	81	ND	85	ND	ND

ND, Not determined.

thermostable form of the enzymes (table 3). It is interesting to record that while additional PLP enhances the  $T_m$  value it has no effect on the catalytic activity, whereas L-Ser enhances the specific activity.

The thermal stability of scSHMT and bsSHMT in the presence of L-Ser was also monitored by using differential scanning calorimetry (DSC). bsSHMT gave a bimodal pattern (figure 5a) suggesting that one of the domains denatures earlier than the other. One of the domains, which starts denaturing at lower temperatures gave a  $T_m$  value of  $65^{\circ}\text{C}$  and the second one gave the  $T_m$  of  $71^{\circ}\text{C}$ . The addition of PLP resulted in the enzyme denaturing as a single species with the domain denaturing at lower temperature merging with the main peak with  $T_m$  of  $75^{\circ}\text{C}$  (figure 5b). Addition of L-Ser resulted in the formation of a stable form of the enzyme-serine complex, which denatured with a  $T_m$  value of  $86^{\circ}\text{C}$  (figure 5c). Addition of PLP and L-Ser together caused a marginal increase in stability ( $T_m = 88$ ) (figure 5d).

The results presented in this paper suggest that SHMT can exist in two active oligomeric states *in vivo*. It has been suggested that the evolution of tetramer confers cooperativity in interactions with  $\text{H}_4$ -folate and its analogues, which are absent in the dimers (Harish Kumar *et al* 1976; Szebenyi *et al* 2000). The presence of dimeric and tetrameric forms in the same organism could facilitate an examination of this hypothesis, especially when conditions for their interconversions become available. The orientation of the PLP at the active site of bsSHMT is different from that in scSHMT reflected by changes in the reaction rates with alternate substrates and analogues. This highly thermostable enzyme and its complexes with substrates and substrate analogues can be conveniently used to elucidate the three-dimensional structures of SHMT and its mutants for understanding the architecture and the role of specific amino acid residues and regions of the molecule involved in reaction specificity, catalysis, oligomeric state, etc.

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