

Identification of amino acid residues, essential for maintaining the tetrameric structure of sheep liver cytosolic serine hydroxymethyltransferase, by targeted mutagenesis

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Serine hydroxymethyltransferase (SHMT), a pyridoxal 5'-phosphate (PLP)-dependent enzyme, catalyses the transfer of the hydroxymethyl group from serine to tetrahydrofolate to yield glycine and *N*⁵,*N*¹⁰-methylene-tetrahydrofolate. An analysis of the known SHMT sequences indicated that several amino acid residues were conserved. In this paper, we report the identification of the amino acid residues essential for maintaining the oligomeric structure of sheep liver cytosolic recombinant SHMT (scSHMT) through intra- and inter-subunit interactions and by stabilizing the binding of PLP at the active site. The mutation of Lys-71, Arg-80 and Asp-89, the residues involved in intra-subunit ionic interactions, disturbed the oligomeric structure and caused a loss of catalytic activity. Mutation of Trp-110 to Phe was without effect, while its mutation to Ala resulted in the enzyme being present in the insoluble fraction. These results suggested that

Trp-110 located in a cluster of hydrophobic residues was essential for proper folding of the enzyme. Arg-98 and His-304, residues involved in the inter-subunit interactions, were essential for maintaining the tetrameric structure. Mutation of Tyr-72, Asp-227 and His-356 at the active site which interact with PLP resulted in the loss of PLP, and hence loss of tetrameric structure. Mutation of Cys-203, located away from the active site, weakened PLP binding indirectly. The results demonstrate that in addition to residues involved in inter-subunit interactions, those involved in PLP binding and intra-subunit interactions also affect the oligomeric structure of scSHMT.

Key words: oligomeric structure, pyridoxal 5'-phosphate, sequence comparison, site-directed mutagenesis, subunit interaction.

INTRODUCTION

Serine hydroxymethyltransferase (SHMT) catalyses a key step linking amino acid and nucleotide metabolism. It transfers the hydroxymethyl group of L-Ser to 5,6,7,8-tetrahydrofolate (*H*₄-folate) to yield Gly and 5,10-methylene-*H*₄-folate, an important intermediate in amino acid and nucleotide metabolism [1,2]. It serves as a source for 1-carbon fragments for a variety of end products. SHMT has been suggested as a potential target for cancer chemotherapy [3–6]. The gene for the enzyme has been isolated from a variety of pro- and eu-karyotic sources and overexpressed in *Escherichia coli* (reviewed by Appaji Rao et al. [6]). It was considered worthwhile to examine all the sequences of SHMT (<http://www.ncbi.nlm.nih.gov/Entrez/>) and identify the conserved residues with functional group(s) that could participate in catalysis or pair with an oppositely charged residue to stabilize the structure. The availability of the three-dimensional structures of SHMT from three eukaryotic [5,7,8] and two prokaryotic [9,10] sources could facilitate an understanding of the role of some of these residues in the structure and function of SHMT. This paper describes an analysis of mutation of several conserved amino acid residues essential for maintaining the oligomeric structure of the sheep liver cytosolic recombinant SHMT (scSHMT). The results suggest that the mutation of crucial residues involved in either intra- or inter-subunit interactions or in pyridoxal 5'-phosphate (PLP) binding cause disruption of the tetrameric structure, loss of PLP and a consequent decrease in catalytic activity.

EXPERIMENTAL

Materials

[α -³²P]dATP (3000 Ci/mmol) was obtained from the Board of Radiation and Isotope Technology, Government of India, Trombay, India. L-[3-¹⁴C]Ser, restriction endonucleases, Sequenase™ version 2.0 sequencing kit and DNA-modifying enzymes were obtained from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Deep Vent polymerase was purchased from New England Biolabs (Beverly, MA, U.S.A.). CM-Sephadex, Sephacryl S-200, Gly, L-Ser, D-Ala, 2-mercaptoethanol, folic acid, PLP, isopropyl β -D-thiogalactoside and EDTA were obtained from Sigma (St. Louis, MO, U.S.A.). Platinum oxide was purchased from Loba Chemie (Mumbai, India). Centricon filters were obtained from Amicon (Millipore, Beverly, MA, U.S.A.). All other chemicals were of analytical-reagent grade. The mutant oligonucleotides were purchased from Bangalore Genei Pvt. (Bangalore, India). *H*₄-Folate was prepared as described by Hatefi et al. [11].

Bacterial strains, growth conditions and DNA manipulations

E. coli strain DH5 α (Bethesda Research Labs, Bethesda, MD, U.S.A.) was the recipient of all the plasmids used for DNA isolation and subcloning. *E. coli* BL21(DE3) pLysS strain [12] was used for the expression of scSHMT [13] and the mutant SHMT clones. Luria–Bertani medium or terrific broth (24 g of

Abbreviations used: SHMT, serine hydroxymethyltransferase; scSHMT, sheep liver cytosolic recombinant SHMT; hcSHMT, human liver cytosolic recombinant SHMT; PLP, pyridoxal 5'-phosphate; *H*₄-folate, 5,6,7,8-tetrahydrofolate.

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yeast extract, 12 g of tryptophan, 4 ml of glycerol, 2.31 g of KH_2PO_4 and 12.54 g of K_2HPO_4 /l) with 50 $\mu\text{g}/\text{ml}$ ampicillin was used to grow *E. coli* cells containing the plasmids at 37 °C. Plasmids were prepared by the alkaline lysis procedure described by Sambrook et al. [14]. The DNA fragments were eluted from low-melting-point agarose gels after electrophoresis [15].

The co-ordinates for human liver cytosolic recombinant SHMT (hcSHMT; PDB code 1BJ4) [5] were analysed using the PROCHECK program to evaluate the secondary structure and accessibility (results not shown). These data were used to describe the position of the residues mutated in this study.

Site-directed mutagenesis

The R80A, D89N, R98A, W110A, W110F and C203S mutants were constructed by a PCR-based megaprimer method [15–17]. The first PCR product (megaprimer) was obtained using the mutant oligonucleotide and M13 (–24) universal reverse primer (5'-GGAAACAGCTATGACCATG-3'). pUCSH (pUC19 containing the SHMT cDNA fragment lacking 227 bp at the 5' end) was used as a template. The full-length PCR product was obtained using three primers, i.e. the megaprimer (first PCR product), SHP1 (5'-TATGGCAGCTCCAGTCAAC-3', at the start of the gene) and the M13 (–24) universal reverse primer using pETSH (containing a full-length cDNA for SHMT [13]), as a template. The full-length PCR product, obtained after two rounds of PCR, was subcloned into pUC19 at the *KpnI* and *BamHI* sites. This clone was digested with *KpnI* and *PmaCI* restriction enzymes to obtain the 520 bp fragment. The entire 520 bp fragment was sequenced using the Sequenase™ version 2.0 DNA sequencing kit to confirm the presence of the mutation and to rule out the presence of non-specific mutations. The 520 bp fragment containing the mutated region was gel-purified and swapped with the wild-type clone, i.e. pETSH, and the mutation was reconfirmed by sequencing the expression construct.

The other mutants used in the study were constructed using the sense/antisense primer method as described earlier [18]. Briefly, the wild-type template (pRSH; 80 ng), and sense and antisense primers (50 pmol) were added to PCR tubes containing 0.4 mM dNTPs, 1 mM MgSO_4 and 2.5 units of Deep Vent DNA polymerase along with the buffer provided with the enzyme at 1 \times concentration. The amplification reaction was carried out in a PerkinElmer PCR machine using the following cycling conditions: denaturation of the template at 95 °C for 4 min followed by 20 cycles at 94 °C for 45 s (denaturation), 52 °C for 1 min (annealing) and 72 °C for 5 min (extension). The reaction was continued for 20 min at 72 °C to complete the extension. The PCR-amplified mixture was treated with *DpnI* (10 units) at 37 °C for 1 h to digest the methylated DNA (template DNA) and transformed into DH5 α competent cells. The presence of the mutation was confirmed by sequencing the plasmid DNA by Sanger's dideoxy chain-termination method [19] using Sequenase™ version 2.0 (results not shown) or by ABI Prism automated DNA sequencer. K71Q, Y72F, Y81F, S202C and D227N were constructed using this procedure.

The H304A, H306A and H356A mutants were constructed using the sense/antisense primer method with minor modifications. The megaprimer (obtained by PCR using the mutant oligonucleotide and SHM1, 5'-TATCATGAAGCCAGGCAG-G-3', the C-terminal primer of the full-length SHMT gene) was used as sense and antisense primers on pRSH template. The mutant oligonucleotides used to construct site-specific mutants are given below: R80A, 5'-AC CCA GGC CAG GCC TAC TAT GGT GGG-3'; D89N, 5'-GA ATT CAT CAA TGA GCT

AGA G-3'; R98A, 5'-TC TGT CAG AAG GCA GCG CTG CAG G-3'; W110A, 5'-G GAC CCC GAG TGC GCG GGG GTT AAC G-3'; W110F, 5'-TG GAC CCC GAG TGC TTC GGG GTT AAC G-3'; C203S, 5'-GGG ACT AGC TCC TAC TCC CG-3'; H304A, 5'-AA GGG GGA CCG GCC AAC CAC GCC ATT-3'; H306A, 5'-GA CCT CAC AAC GCC GCC ATT GCT G-3'; H356A, 5'-TCT GAC AAC GCA TTA ATC CTC GTG GAC-3'; K71Q (sense), 5'-CTG AAC AAC CAG TAC TCT GAG-3'; K71Q (antisense), 5'-CTC AGA GTA CTG GTT GTT CAG-3'; Y72F (sense), 5'-AAC AAC AAG TTC TCT GAG GGG-3'; Y72F (antisense), 5'-CCC CTC AGA GAA CTT GTT GTT-3'; S202C (sense), 5'-GCA GGG ACT TTG TGC TAC TCC-3'; S202C (antisense) 5'-GGA GTA GCA CAA AGT CCC TGC-3'; D227N (sense), 5'-CTC ATG GCT AAC ATG GCA CAT-3'; D227N (antisense), 5'-ATG TGC CAT GTT AGC CAT GAG-3'.

Expression and purification of mutant enzymes

pETSH and mutant plasmids were transformed into the *E. coli* BL21(DE3) pLysS strain. scSHMT and mutants were grown at 30 °C and purified using the protocol standardized for scSHMT earlier [18,20]. The enzyme pellet was dialysed against buffer A (50 mM potassium phosphate buffer, pH 7.4/1 mM 2-mercaptoethanol/1 mM EDTA) for 24 h with two changes (1 litre) prior to use. Protein was estimated by the method of Lowry et al. [21].

Enzyme assays

Hydroxymethyltransferase reaction

The SHMT-catalysed aldol cleavage of L-Ser with H_4 -folate to form Gly and 5,10- CH_2 - H_4 -folate was monitored using L-[3- ^{14}C]Ser and H_4 -folate as substrates as described earlier [22,23]. The reaction mixture (0.1 ml) contained 400 mM potassium phosphate buffer, pH 7.4, 1.8 mM dithiothreitol, 1 mM EDTA, 50 μM PLP, 1.8 mM H_4 -folate, 3.6 mM L-Ser and an appropriate amount of the enzyme (determined in each case from an enzyme concentration versus velocity curve at different concentrations of the mutant or wild-type enzyme). A time course of the reaction was measured in all cases to ensure that the initial velocity was determined. One unit of enzyme activity was defined as the amount of enzyme that catalysed the formation of 1 μmol of formaldehyde by the decomposition of 5,10- CH_2 - H_4 -folate/min at 37 °C and pH 7.4. The specific activity was expressed as units/mg of protein. For the determination of K_m and k_{cat} values, the assay was carried out at various concentrations of L-Ser (0–4 mM) after ensuring that the initial velocity was being measured, and the kinetic parameters were calculated by non-linear regression analysis using Sigmaplot software.

Transaminase activity

SHMT catalyses the transamination of D-Ala to give pyruvate and the bound PLP is converted into pyridoxamine phosphate [24]. scSHMT and mutants were taken separately in 900 μl of buffer A and absorbance was measured at 425 nm against the same buffer blank in a Shimadzu UV-160A spectrophotometer after pre-incubation at 37 °C for 3 min. The reaction was started by the addition of 100 μl of 1 M D-Ala to both the cuvettes. Absorbance change at 425 nm was monitored with increasing periods of incubation and the pseudo-first-order rate constants were calculated as described earlier [20].

Spectroscopic methods

Absorption spectra

Absorption spectra of the enzyme preparations were recorded with a Shimadzu UV-160A spectrophotometer in buffer A. The spectra were recorded at 25 ± 2 °C against buffer A and a concentration of 1 mg of protein/ml was used in all cases.

Far-UV CD measurements

Far-UV CD spectral measurements were made in a Jasco J-500A spectropolarimeter using a cuvette of 1 mm pathlength. The spectra were recorded at 25 ± 2 °C in buffer A (Millipore-filtered) from 250 to 195 nm using 0.5 μ M SHMT. The data were plotted as molar ellipticity (θ_{ME}) assuming a relative subunit molecular mass of 52900 Da for scSHMT and the site-specific mutants:

$$\theta_{ME} = (\theta \times 100 \times M_r) / C \times d \quad (1)$$

where θ is the observed molar ellipticity (in degrees), M_r is the subunit molecular mass, C is the concentration of protein in mg/ml and d is the pathlength in decimetres.

Preparation of the apo-enzyme

Apo-enzyme of SHMT was prepared as described earlier [24] with minor modifications. D-Ala (200 mM) was added to the holo-enzyme in 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM 2-mercaptoethanol, 1 mM EDTA and 200 mM $(NH_4)_2SO_4$, and incubated at 37 °C for 4 h. The reaction mixture was rapidly dialysed in a Centricon tube at 4 °C for 1 h using buffer A, but not containing PLP. The pyruvate and pyridoxamine phosphate formed in the reaction were removed by this procedure and the apo-enzyme had no absorbance at 425 nm.

Size-exclusion chromatography

The native molecular masses of scSHMT and the site-specific mutant enzymes were determined using a Superose-12 HR 10/30 analytical gel-filtration column attached to an Amersham Biosciences FPLC system. The column was equilibrated with buffer A (with or without PLP) and calibrated with standard molecular-mass markers, i.e. apoferritin (440000 Da), β -amylase (200000 Da), yeast alcohol dehydrogenase (150000 Da), BSA (66000 Da) and carbonic anhydrase (29000 Da). The protein peaks were monitored by measuring the absorbance at 280 nm.

RESULTS

Analysis of sequences of SHMTs to identify conserved residues

A commendable job of aligning the sequences of α -class PLP enzymes, to which SHMT belongs, has been carried out by Grishin et al. [25]. In the present study, we have compared 55 sequences of SHMT (28 from prokaryotes, 17 from eukaryotes and 10 from archaeobacteria) using FASTA program (GCG package). A total of 60 amino acid residues are completely conserved between at least 54 of the known sequences of SHMT. Among these, there are several conserved amino acid residues with polar functional groups, which could participate in catalysis or in stabilizing the structure. A list of such conserved residues is given in Table 1. Some of these residues were mutated and characterized earlier. The properties of the following mutants of scSHMT are described and discussed in this paper: K71Q, Y72F, R80A, D89N, R98A, W110A, W110F, S202C, C203S, D227N, H304A, H306A and H356A.

Table 1 Highly conserved functional residues in 55 SHMT sequences

The numbers in bold represent the amino acids mutated in this study.

Amino acid	Residue number (based on scSHMT)
Arginine	80, 98 , 262, 401
Aspartate	89, 227
Glutamate	39, 74, 92
Histidine	147, 150, 230, 304, 306, 356
Lysine	71 , 256
Serine	52, 118, 202
Threonine	251, 252, 253, 254, 257
Tyrosine	72 , 82, 204
Tryptophan	110

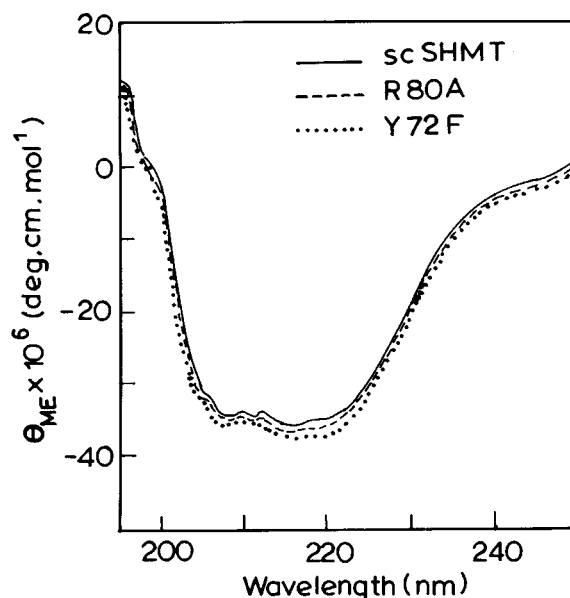


Figure 1 Representative far-UV CD spectra of scSHMT and its mutants

The spectra were recorded in the range 195–250 nm using a Jasco J-500A spectropolarimeter using 100 μ g/ml scSHMT or its mutants. Solid line, holo-scSHMT (tetramer); broken line, R80A (dimer); dotted line, Y72F (a mixture of dimer and tetramer). Similar spectra were obtained with K71Q, W110F, S202A, C203S, H304A, H306A and H356A scSHMT (data is not shown).

Properties of the mutant enzymes

scSHMT and the mutant enzymes were overexpressed and the recombinant enzymes were purified as described in the Experimental section. Figure 1 shows the far-UV CD spectra of scSHMT, R80A and Y72F, typical of a tetramer, dimer or mixture of tetramer and dimer, respectively. All the other mutants discussed in this study gave similar results (not shown). The far-UV CD spectrum of mutants was identical with that of the wild-type enzyme, suggesting that there were no changes in the secondary structure due to the mutation(s) (Figure 1).

The specific activities and the kinetic parameters of H_4 -folate-dependent hydroxymethyltransferase reaction catalysed by the mutant enzymes are listed in Table 2. It can be seen from Table 2 that there was near-complete loss of activity in K71Q, R80A, H304A and D227N SHMT mutants, a partial loss of activity in Y72F, S202C, H306A and H356A and no loss of activity in

Table 2 The kinetic properties of scSHMT and its mutant enzymes

Specific activity is given as μmol of HCHO formed/min per mg of protein at 37 °C and pH 7.4. The kinetic parameters were determined from six independent estimations using non-linear regression analysis, employing Sigmaplot software. ND, not determined as the activity was very low or the enzyme could not be solubilized.

Enzyme	Specific activity	K_m (mM)	k_{cat} per subunit (s^{-1})	k_{cat}/K_m ($\text{mM} \cdot \text{s}^{-1}$)
scSHMT	4.2 ± 0.1	0.93 ± 0.13	4.08 ± 0.2	4.39
K71Q	0.0002	ND	ND	ND
Y72F	0.032 ± 0.02	0.99 ± 0.23	0.06 ± 0.02	0.06
R80A	0.0002	ND	ND	ND
R98A	ND (insoluble)	ND	ND	ND
W110A	ND (insoluble)	ND	ND	ND
W110F	4.3 ± 0.1	0.94 ± 0.14	4.42 ± 0.18	4.7
S202C	$0.2 \pm .01$	ND	ND	ND
C203S	4.2 ± 0.2	1.03 ± 0.15	3.32 ± 0.35	3.2
D227N	0.0002	ND	ND	ND
H304A	0.0004	ND	ND	ND
H306A	1.88 ± 0.1	0.58 ± 0.25	3.1 ± 0.25	5.3
H356A	0.82 ± 0.15	0.94 ± 0.01	1.6 ± 0.24	1.7

Table 3 The oligomeric structure and PLP content of scSHMT and its mutants

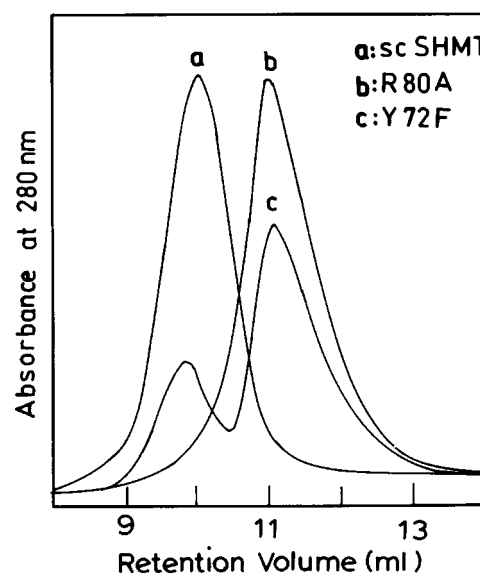
PLP content was determined using a molar absorption coefficient of 6600 M^{-1} for PLP. The errors were calculated from four different enzyme preparations. The retention volumes were determined using a calibrated Superose 12 column, and are means from at least two independent preparations. The volume 10.1 ml corresponds to the tetramer molecular mass (approx. 210 kDa) and 11.1 ml corresponds to the dimer molecular mass of approx. 110 kDa. ND, not determined as they were present in the insoluble fraction.

Enzyme	PLP content ($\mu\text{mol}/\text{subunit}$)	Holo-enzyme (retention volume; ml)
ScSHMT	0.9 ± 0.2	10.1
K71Q	0	11.2
Y72F	0.11	10.1, 11.2
R80A	0	11.1
Y81F	1.0 ± 0.1	10.2
R98A	ND	ND
W110A	ND	ND
W110F	1.1 ± 0.2	10.1
S202C	0.2 ± 0.05	10.1, 11.2
C203S	0.98 ± 0.1	10.2
D227N	0	11.2
H304A	0	11.2
H306A	0.53 ± 0.15	10.1, 11.2
H356A	0.63 ± 0.15	10.1, 11.2

C203S and W110F (Table 2). In the case of W110A and R98A SHMT, although good expression was observed, the mutant proteins were present in the insoluble fraction. All attempts at solubilizing the expressed proteins were unsuccessful. It can be seen from Table 2 that the mutations which led to partial loss of activity did not cause a change in the K_m value for L-Ser, although the k_{cat} and specific activity values were altered, thereby affecting the catalytic efficiency of the mutant enzymes (Table 2).

Physico-chemical properties of scSHMT and its mutants

scSHMT and all the mutants listed in Table 2 gave a single band with a molecular mass of 53 kDa on SDS/PAGE (results not shown). scSHMT gave a single symmetrical peak with a retention volume of 10.1 ml upon FPLC using a calibrated Superose 12 column (Table 3 and Figure 2), which confirmed that scSHMT is a tetramer with a molecular mass of 210 kDa (Figure 2, trace a). C203S and W110F SHMT mutants behaved in an identical

**Figure 2** Size-exclusion chromatography profiles of scSHMT and its mutants

The wild-type and mutant SHMT enzymes (150 μg) were loaded on to a Superose 12 size-exclusion chromatography column attached to an Amersham Biosciences FPLC system with an online UV detector. The column was calibrated using standard protein molecular-mass markers (see the Experimental section). The column was equilibrated with buffer A with or without PLP prior to the experiments. The flow rate was 1 ml/min. Trace a, scSHMT (tetramer); trace b, R80A SHMT (dimer); trace c, Y72F (a mixture of dimer and tetramer).

manner, showing that they were tetramers (Table 3). On the other hand, Y72F, S202C, H306A and H356A gave two peaks (retention volume, 10.1 and 11.2 ml) corresponding to dimer and tetramer (Table 3; data only for Y72F is presented in Figure 2). The percentages of dimer and tetramer in mutants Y72F, Y81F, S202C, H306A and H356A were 30:70, 20:80, 55:45, 60:40 and 80:20 (calculated from the area under the peaks upon gel-filtration analysis similar to that described in Figure 2, trace c, for Y72F) respectively. Gel filtration at different KCl concentrations up to 1 M did not alter the shape of the elution patterns or the area under the peaks. On the other hand, K71Q, R80A, H304A and D227N eluted as a single symmetrical peak corresponding to

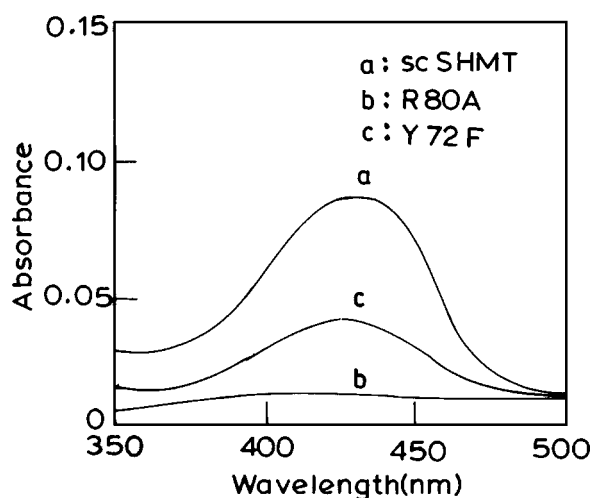


Figure 3 Absorbance spectra of scSHMT and its mutants

The visible absorbance spectra of the enzymes (1 mg/ml) in buffer A recorded on a Shimadzu UV/visible spectrophotometer from 350 to 500 nm at 25 °C. Trace a, scSHMT (tetramer); trace b, R80A SHMT (dimer); trace c, Y72F (a mixture of dimer and tetramer).

a dimer (Table 3 and Figure 2, trace b; data are shown for R80A only). A peak corresponding to a monomer was not seen with scSHMT or its mutants under any of the experimental conditions described above (results not shown).

The visible absorbance spectra of scSHMT and its mutants showed that the mutations caused no, partial or complete loss of PLP (Figure 3). The amount of PLP was calculated using a molar absorption coefficient of 6600 M^{-1} and is shown in Table 3 for all the enzymes. The concentration of PLP in scSHMT and its mutants correlated very well with the oligomeric status and percentage of dimer (no PLP) and tetramer in some of the mutant enzymes (results not shown).

DISCUSSION

The three-dimensional structures of the α -class of PLP-dependent enzymes are remarkably similar [5]. The availability of the crystal structures of hcSHMT [5], rabbit liver cytosolic recombinant SHMT [7], murine liver cytosolic recombinant SHMT [8], *E. coli* recombinant SHMT [9] and *Bacillus stearothermophilus* recombinant SHMT [10] has enabled a more critical examination of the role of amino acids in the structure and function of SHMT. The three-dimensional structures of SHMTs can be overlaid on each other to a large extent; SHMT is a highly conserved protein [26]. For these reasons, drawing analogies from hcSHMT's structure [5] to explain the observations with scSHMT is justified.

Intra-subunit interactions stabilizing the tetrameric structure of scSHMT

The N-terminal methionine residue is cleaved during expression of the scSHMT gene in *E. coli*. For this reason, there is a difference of one residue in the numbering of scSHMT and hcSHMT. For example, D89N in scSHMT is equivalent to D90N in hcSHMT.

Asp-89 and Arg-80

In an earlier study it was shown that the mutation of Asp-89 to Asn in scSHMT lead to the formation of a mixture of dimers and

tetramers [17]. The tetramer of D89N SHMT was fully active when isolated in the presence of excess PLP ($500 \mu\text{M}$). However the apo-enzyme, prepared by removal of PLP [24], and which was present only as a dimer, could not be reconstituted by addition of PLP [17]. Asp-89 interacts only with Arg-80 and it has no additional interactions and therefore the effect of this mutation was mild. An examination of the position of Arg-81 in the hcSHMT crystal structure (Arg-80 in scSHMT) revealed that this residue is positioned in β -sheet 2 and stabilizes α -helix 4 by the following interactions. (i) The guanidino NH1 group of the Arg-81 is within hydrogen-bonding distance of the carbonyl group of the main polypeptide chain and the hydroxy group of Ser-74 (Ser-73 in scSHMT). (ii) The second NH2 group is within hydrogen-bonding distance of the carbonyl group of Glu-93 (Glu-92 in scSHMT) and Asp-90 (Asp-89 in scSHMT; Figure 4a). It is therefore not surprising that the mutation of Arg-80 to Ala (in scSHMT) resulted in a dislocation of multiple interactions. This led to a drastic effect on the enzyme architecture, resulting in disruption of the tetrameric structure to a near-complete loss of enzyme activity and ability to bind PLP (Tables 2 and 3 and Figures 2 and 3).

Lys-71

In addition to Lys-256 in scSHMT (Lys-257 in hcSHMT), which anchors PLP, another Lys residue, Lys-71 in scSHMT (Lys-72 in hcSHMT) is located close to the active site. Lys-72 of hcSHMT stabilizes the tetrameric structure by interacting with the carbonyl group of a peptide bond involving Gly-84 and Gly-85 in hcSHMT (Gly-83 and Gly-84 in scSHMT; not shown) [27]. These interactions stabilize the intra-subunit interactions, which enable generation of the correct geometry for the binding of PLP at the active site. Mutation of the corresponding residue in scSHMT, Lys-71 to Gln, resulted in the formation of inactive dimers (Tables 2 and 3). These results once again emphasize the importance of intra-subunit interactions in maintaining the oligomeric structure of the enzyme.

Trp-110

Chemical modification studies had indicated that Trp residues were essential for the activity of the hcSHMT [28]. Trp-111 in hcSHMT is located at the beginning of β -sheet 4. The mutation of the corresponding residue, Trp-110, in scSHMT to Ala resulted in the enzyme being present in the insoluble fraction (Table 2). Trp-110 is conserved in all eukaryotic enzymes, whereas this is not the case in prokaryotes. An examination of the three-dimensional structure of hcSHMT revealed that Trp-111 was surrounded by hydrophobic residues Leu-106, Phe-245 and Phe-268 (Figure 4b). It is therefore possible that the mutation of Trp-110 to Ala in scSHMT disturbs this hydrophobic pocket, which is crucial for the proper folding of the enzyme. This suggestion was confirmed by the mutation of Trp-110 to Phe. The W110F SHMT mutant had properties similar to the wild-type enzyme (Tables 2 and 3). It also generated a geminal diamine with Gly and H_4 -folate, as in the wild-type enzyme (results not shown) [13].

Inter-subunit interactions stabilizing the tetrameric structure

Arg-98 and Asp-29

Arg-98 of scSHMT is conserved in all eukaryotic SHMTs and in 21 out of 28 prokaryotic sequences (Table 1). The corresponding residue Arg-99 in hcSHMT is located at α -helix 4 and interacts with Asp-30 (located at α -helix 2) of the neighbouring subunit (across the subunits of the tight dimer; Figure 4c). In theory, this

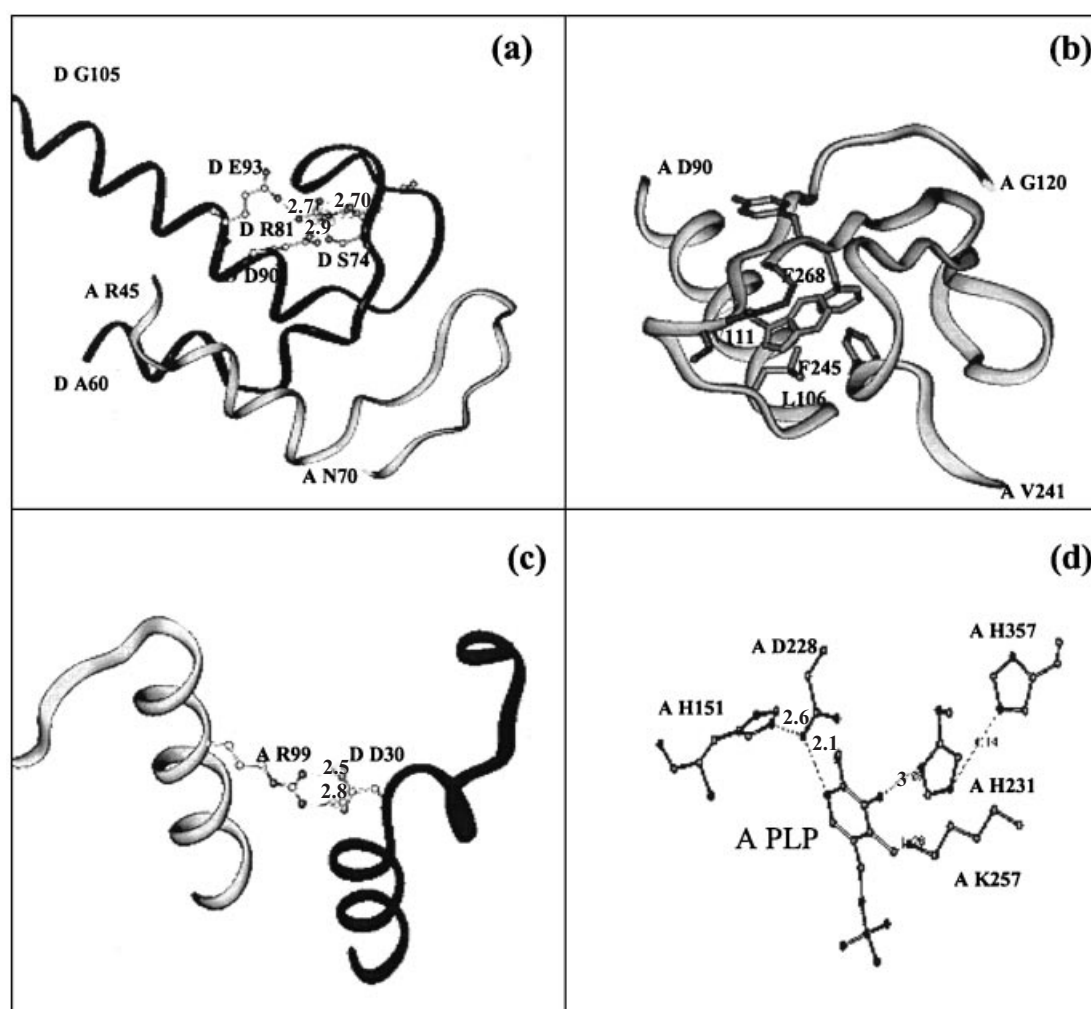


Figure 4 Residues involved in the intra-subunit interactions

The diagrams were generated using Insight II 97.2 software using hcSHMT co-ordinates (PDB code 1BJ4). Grey-coloured helix indicates the A subunit and black indicates the D subunit; A–D subunits make a tight dimer. Where there are two letters, the first indicates the subunit and the second is the amino acid code followed by the residue number. (a) Position of Ser-74, Arg-81, Glu-93 and Asp-90 and their interactions in hcSHMT. (b) The position of Trp-111, surrounded by the hydrophobic cluster in hcSHMT. (c) The position of A-subunit Arg-99 and its interaction with D-subunit Asp-30 in hcSHMT, representing inter-subunit interactions. (d) Interaction of His-151, Asp-228, His-231, Lys-257 and His-357 with PLP at the active site.

mutation should have led to the formation of loose dimers. However, the R98A SHMT mutant, although very well expressed, was present in the insoluble form, suggesting that the disruption of interaction between Arg-98 and Asp-29 (Arg-99 and Asp-30 in hcSHMT) at the tight dimer interface also affected folding of scSHMT (Tables 2 and 3).

His-304 and Ser-118

His-304 is conserved in eukaryotes but not in prokaryotic organisms (Table 1) [27], suggesting a possible role for the residue in the evolution of the tetrameric enzyme. The corresponding residue, His-305 in hcSHMT (His-304 in scSHMT), is positioned to interact with Ser-119 (Ser-118 in scSHMT) from a neighbouring subunit and is within hydrogen-bonding distance of the phosphate group of PLP (results not shown) [27]. It is therefore possible that the mutation of His-304 to Ala in scSHMT disrupts this interaction, leading to the formation of inactive dimers (Tables 2 and 3). The three-dimensional structure of

hcSHMT revealed that PLP is present at the tight dimer interface. Therefore, the disruption of interactions at the tight dimer interface leads to the formation of inactive dimers, which are incapable of binding PLP (Tables 2 and 3). On the other hand, mutation of His-134 involved in the interactions at the loose dimer interface lead to formation of partially active dimers with bound PLP [29].

Apart from the residues discussed above, the N-terminal regions clasp across the 'tight dimers', conferring stability on the tetramer [5,7,8]. Earlier work on the deletion mutants from the N-terminus indicated that the N-terminal arm stabilized the quaternary structure along with PLP [20], confirming their role in maintenance of the oligomeric structure.

Role of amino acids interacting with PLP in maintaining the tetrameric structure

Asp-228 in hcSHMT (Asp-227 in scSHMT), which is H-bonded to the pyridinium N (Figure 4d), is conserved not only in SHMT

but in all PLP-dependent fold type I enzymes [25]. The mutation of the corresponding residue, Asp-227, in scSHMT affects the binding of PLP, and consequently causes the disruption of tetrameric enzyme. D227N SHMT mutant did not give the characteristic absorbance at 425 nm and was present as an inactive dimer (Tables 2 and 3).

Similarly, mutation of Lys-256 in scSHMT, which anchors the PLP as an internal aldimine, to Gln/Arg resulted in the formation of inactive dimer with no PLP bound to it [30]. On the other hand, mutation of the corresponding residue, Lys-229, in the dimeric *E. coli* recombinant SHMT to His/Arg resulted in a loss of ability to bind PLP and a loss of catalytic activity with no change in the oligomeric structure. The K229Q mutant of *E. coli* recombinant SHMT was able to bind external aldimine and catalyse a single turnover [31].

The other residues that are involved in the stabilization of the negative charge on N1 of PLP, in addition to Asp-227 in scSHMT, are His-150, His-230 and His-356. The corresponding residues, Asp-228, His-151, His-231 and His-357, in hcSHMT form a charge relay system as shown in Figure 4(d). Among these residues, His-231 is directly linked to PLP O-3, whereas His-357 is hydrogen-bonded to His-231, and His-151 is hydrogen-bonded to Asp-228 (Asp-227 in scSHMT). It has been shown previously that His-150 in scSHMT participates in the proton-abstraction step of catalysis [29] and that His-230 facilitates the proton abstraction [32]. It could be predicted that disruption of these interactions leads to loss of oligomeric structure and catalysis. The results presented in Tables 2 and 3 substantiate the above prediction.

Ser-202 along with His-230 and Lys-256 in scSHMT are within hydrogen-bonding distance of O-3 of PLP at the active site. The decreased catalytic activity of S202C in scSHMT and its isolation as a mixture of tetramers and dimers (Tables 2 and 3) further confirms that Ser-202 interactions are not as crucial as those of Asp-227, Lys-256 or His-230 for maintaining PLP at the active site in scSHMT.

Tyr-72 in scSHMT (Tyr-73 in hcSHMT) is a very well conserved residue among all SHMT sequences and its hydroxy group interacts with O-2 of the phosphate group of PLP. Isolation of the Y72F SHMT mutant as a mixture of tetramers and dimers with marginal activity highlighted the importance of these interactions and the role of PLP in maintaining the oligomeric structure.

Cys-204 is positioned at the beginning of random coil 17 in hcSHMT and is conserved in all SHMTs. It is pertinent to recall that earlier studies reported the loss of enzyme activity in the presence of thiol inhibitors [33] and it was also predicted that Cys-204 could be involved in the thio-hemiacetal mechanism in hcSHMT [5]. C203S scSHMT had identical properties to the wild-type enzyme in its PLP content and H₄-folate-dependent reactions (Table 2). The mutant enzyme generated characteristic intermediates with Gly and H₄-folate (results not shown). However, the reaction with D-Ala was faster by an order of magnitude (0.26 min⁻¹ for scSHMT and 3.21 min⁻¹ for C203S). The apo-enzyme of C203S was a dimer and could only be partially reconstituted (30%) with PLP, whereas the apo-scSHMT exists as a tetramer and can be reconstituted fully (results not shown). This observation is in support of the suggestion that this Cys residue interacts indirectly with PLP [26] and may not be participating in the thio-hemiacetal mechanism. These results discussed above revealed that although Tyr-72, Asp-227, Lys-256, Ser-202, Cys-203 and His-356 in scSHMT are not involved in subunit interactions, their mutation affected PLP binding, leading to a loss of oligomeric structure. The observations reported in this paper identify amino acid residues essential for

maintaining the oligomeric structure through intra- and inter-subunit interactions and the interactions with PLP at the active site.

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