

## His230 of serine hydroxymethyltransferase facilitates the proton abstraction step in catalysis

Rashmi Talwar, Junutula R. Jagath, N. Appaji Rao and H. S. Savithri

Department of Biochemistry, Indian Institute of Science, Bangalore, India

The three-dimensional structures of rabbit and human liver cytosolic serine hydroxymethyltransferase revealed that H231 interacts with the O3' of pyridoxal-5'-phosphate and other residues at the active site such as S203, K257, H357 and R402 (numbering as per the human enzyme). This and the conserved nature of H231 in all serine hydroxymethyltransferases highlights its importance in catalysis and/or maintenance of oligomeric structure of the enzyme. In an attempt to decipher the role of H230 (H231 of the human enzyme) in the catalytic mechanism and/or maintenance of oligomeric structure of sheep liver serine hydroxymethyltransferase, the residue was mutated to arginine, phenylalanine, alanine, asparagine or tyrosine. Our results suggest that the nature of the amino acid substitution has a marked effect on the catalytic activity of the enzyme. H230R and H230F mutant proteins were completely inactive, dimeric and did not bind pyridoxal-5'-phosphate. On the other hand, mutation to alanine and asparagine retained the oligomeric structure and ability to bind pyridoxal-5'-phosphate. These mutants had only 2–3% catalytic activity. The side reactions like transamination and 5,6,7,8-tetrahydrofolate independent aldol cleavage were much more severely affected. They were able to form the external aldimine with glycine and serine but the quinonoid intermediate was not observed upon the addition of 5,6,7,8-tetrahydrofolate. Mutation to tyrosine did not affect the oligomeric structure and pyridoxal-5'-phosphate binding. The H230Y enzyme was 10% active and showed a correspondingly lower amount of quinonoid intermediate. The  $k_{cat}/K_m$  values for L-serine and L-allothreonine were 10-fold and 174-fold less for this mutant enzyme compared to the wild-type protein. These results suggest that H230 is involved in the step prior to the formation of the quinonoid intermediate, possibly in orienting the pyridine ring of the cofactor, in order to facilitate effective proton abstraction.

**Keywords:** His230; proton abstraction; pyridoxal-5'-phosphate; serine hydroxymethyltransferase.

Serine hydroxymethyltransferase (SHMT, E.C.2.1.2.1), a pyridoxal-5'-phosphate dependent enzyme, catalyzes the conversion of serine and 5,6,7,8-tetrahydrofolate (H<sub>4</sub>-folate) to glycine and 5,10-methylene-H<sub>4</sub>-folate (5,10-CH<sub>2</sub>-H<sub>4</sub>-folate) [1]. Like other PLP-dependent enzymes, SHMT exhibits broad substrate and reaction specificity, as it also catalyzes H<sub>4</sub>-folate-independent aldolytic cleavage, decarboxylation, racemization and transamination reactions. The X-ray structure of the human liver cytosolic SHMT (hcSHMT) [2] revealed that the tetrameric protein was a 'dimer of dimers', stabilized by the N-terminal arm as suggested earlier by mutational studies on sheep liver cytosolic SHMT (scSHMT) [3]. PLP is bound to K257 as a Schiff's base linkage and is present at the interface of the tight dimers. The protonated N1 nitrogen of PLP is hydrogen bonded to D228, which, in turn, is bonded to H151. H231 is involved in hydrogen bonding interactions with the O3' of PLP. In addition, it is within hydrogen-bonding distance of S203, K257, H357 and R402. The phosphate group of PLP is hydrogen bonded to various residues including Y73 and G303 from the neighboring

subunit. More recently, Scarsdale *et al.* [4] reported the three-dimensional structure of rabbit liver cytosolic SHMT (rcSHMT), which confirms these features in the structure of SHMT. They have also examined in detail the implications of the structure to the catalytic mechanism of the reaction.

Histidine, at position 230 of scSHMT (H231 of hcSHMT), is completely conserved in the primary structure of all SHMTs known so far [5]. The three-dimensional structure of aspartate aminotransferase (AATase) showed that a tyrosine was present at the equivalent position and was hydrogen bonded to the O3' of PLP [6]. However, Y225 was conserved only in  $\approx 70\%$  of the AATase sequences known so far. H207 of 8-amino-7-oxononanoate synthase [7], A186 of serine-pyruvate aminotransferase, T319 of ornithine decarboxylase and Q246 of dialkylglycine decarboxylase [9] occupy positions equivalent to Y225 of AATase. The role of this residue in catalysis has not been examined in any of the above mentioned enzymes, except for AATase, in which a Y225R mutation led to a complete loss of transaminase activity [10]. It was therefore of interest to mutate H230 to arginine in analogy with the studies on AATase; to alanine, a conservative choice in mutational studies; to tyrosine, which mimics the situation in AATase; to phenylalanine, in order to decipher the role of the hydroxyl group; or to asparagine, which is a conservative mutation with steric effects similar to those of histidine but has a neutralized charge. The results obtained in this study clearly demonstrate that mutation of H230 leads to a dramatic reduction in the catalytic activity of SHMT and also changes in its oligomeric structure depending on the nature of the substitution.

Correspondence to H. S. Savithri, Department of Biochemistry, Indian Institute of Science, Bangalore-560012, India.

Fax: +91 80 360 683, +91 80 3341814, Tel.: +91 80 360 0814, +91 80 360 1561, E-mail: bchss@biochem.iisc.ernet.in

**Abbreviations:** AATase, aspartate aminotransferase; hcSHMT, human liver cytosolic SHMT; H<sub>4</sub>-folate, 5,6,7,8-tetrahydrofolate; MA, methoxyamine; PLP, pyridoxal-5'-phosphate; scSHMT, sheep liver recombinant SHMT; SHMT, serine hydroxymethyltransferase; TSC, thiosemicarbazide.

(Received 30 September 1999, revised 12 November 1999, accepted 11 January 2000)

## EXPERIMENTAL PROCEDURES

Glycine, L-serine, D-alanine, NADH,  $\beta$ -phenylserine, 2-mercaptoethanol, folic acid, PLP, EDTA, aminooxyacetic acid, methoxyamine (MA), L-aspartate,  $\alpha$ -ketoglutarate and L-allothreonine were obtained from Sigma Chemicals Co. St. Louis, MO, USA. [ $\alpha$ - $^{32}$ P]dATP (3000 Ci·mmol $^{-1}$ ) and L-[3- $^{14}$ C]serine (55 mCi·mmol $^{-1}$ ), restriction endonucleases (*Kpn*I, *Bam*HI and *Pml*I), Sequenase Version 2 DNA sequencing kit and DNA modifying enzymes ( $T_4$  DNA ligase, Klenow fragment) were obtained from Amersham Pharmacia Biotech, Bucks, UK. [2- $^3$ H]Glycine (41.1 Ci·mmol $^{-1}$ ) was purchased from NEN Life Science Products, Inc. Boston, MA, USA. *Pfu* polymerase was purchased from Stratagene and Deep Vent polymerase from New England Biolabs, Inc., Beverly, MA, USA. Sephacryl S-200, Superose 12 HR 10/30 were purchased from Pharmacia, Uppsala, Sweden. The oligonucleotide primers were custom synthesized by Bangalore Genei Pvt. Ltd, India. H $_4$ -Folate was prepared by the method of Hafezi *et al.* [11]. All other biochemicals used in this study were of the highest purity available.

### Bacterial strains and growth conditions

*Escherichia coli* DH5 $\alpha$  was the recipient strain for the plasmids used in subcloning and sequencing. Strain BL21(DE3) pLysS [12] was used for bacterial expression of pET and pRSET constructs. CJ236 was the strain of choice for preparation of single strand DNA. Luria–Bertani medium or terrific broth with 50  $\mu$ g·mL $^{-1}$  of ampicillin was used for growing *E. coli* cells.

### DNA manipulations

Plasmids were prepared by the alkaline lysis method as described by Sambrook *et al.* [13]. Restriction analysis and DNA ligations were carried out according to the manufacturer's instructions. Competent cells were prepared by the procedure of Alexander [14].

### Site-directed mutagenesis

All the H230 mutants except H230R were constructed by the PCR based megaprimer method [15,16]. The oligonucleotides used for the generation of mutants are as follows: H230A, 5'-GAC ATG GCA GCG ATA TCC GGG CTG GTG GC-3'; H230F, 5'-C ATG GCA TTT ATC AGC GG-3'; H230N, 5'-AC ATG GCA AAT ATT AGC GGG CTG-3'; H230R, 5'-G GCT GAC ATG GCG CGC ATC AGC GGG C-3'; H230Y, 5'-AC ATG GCA TAT ATC AGC G-3'; M13 universal reverse primer (19-mer), 5'-GGA AAC AGC TAT GAC CAT G-3'; and SHP1 (19-mer), 5'-T ATG GCA GCT CCA GTC AAC-3'.

The full length PCR product was subcloned into pUC19 at *Kpn*I and *Bam*HI sites. The clone was double digested with *Kpn*I and *Pml*I to obtain the 0.5 kb fragment that was swapped with the corresponding 0.5 kb fragment of the wild-type clone, i.e. pETSH (SHMT gene in pET 3c). The entire 0.5 kb region was sequenced using Sequenase Version 2 DNA sequencing kit to confirm the presence of the desired mutation and the absence of any nonspecific mutation(s). The H230R mutation was generated by the conventional Kunkel's method [17] using pGSH [SHMT gene subcloned in pGEM 3z f(-) vector] as a template. The product was similarly subcloned to obtain the mutant in the expression vector.

### Expression and purification of H230 mutant proteins

The scSHMT and the mutant enzymes were purified by the protocol described earlier [3]. Briefly, the BL21(DE3) pLysS extracts were subjected to ammonium sulphate fractionation, CM-Sephadex and Sephacryl S-200 column chromatography. The fractions were pooled and precipitated with 65% ammonium sulphate and the pellet was resuspended in Buffer A (50 mM phosphate buffer, pH 7.4, 1 mM EDTA and 1 mM 2-mercaptoethanol). It was dialyzed against the same buffer with two changes and was used in all further studies. The amount of protein was estimated by the method of Lowry *et al.* [18].

### Enzyme assays

Aldol cleavage in the presence of H $_4$ -folate was monitored using L-[3- $^{14}$ C]serine by the method of Manohar *et al.* [19]. Aldolytic cleavage of L-allothreonine was monitored as described by Jagath *et al.* [20]. The rates of transamination of D-alanine were determined as described earlier [21].

### Proton exchange studies

The proton exchange studies were carried out using tritiated glycine as described by Schirch and Jenkins [22].

### Spectral measurements

The absorbance spectra of the wild type and mutant enzymes (5  $\mu$ M) were recorded in a Shimadzu UV 160 A spectrophotometer, both in the presence and absence of ligands and at different time intervals, when necessary. The oligomeric status of the proteins was determined using a calibrated Superose 12 HR 10/30 column attached to a Pharmacia FPLC system.

### Determination of association constants

The substrate induced quenching of fluorescence of enzyme bound PLP was monitored using a Jasco FP 777 spectrofluorimeter using a modified version of the protocol reported earlier [23].

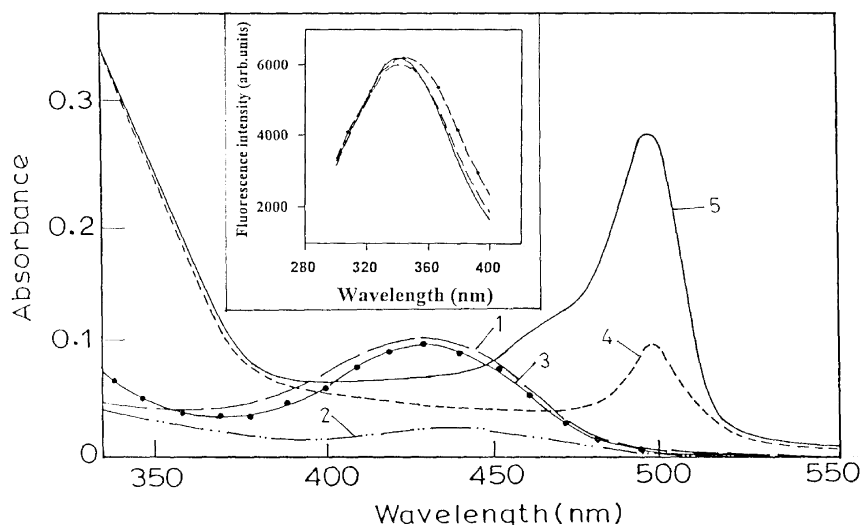
## RESULTS AND DISCUSSION

Biochemical characterization of the H230 mutants of sheep liver SHMT showed that the nature of the substitution affects catalysis and oligomeric status to different extents. It is evident

**Table 1.** Catalytic efficiency and binding constants of the H230 mutants of scSHMT. Specific activity is defined as the amount of HCHO ( $\mu$ mol) formed per min at 37 °C and pH 7.4. Binding constants of H230 mutants for L-serine and glycine were determined by quenching of the fluorescence of the enzyme bound PLP

Protein	Specific activity (U·mg $^{-1}$ )	% Activity	L-Serine (mM)	Glycine (mM)
scSHMT	4.8	100	0.98	12.0
H230A	0.11	2	0.85	11.3
H230N	0.12	2	0.94	12.0
H230F	0.005	0.001	–	–
H230R	0.005	0.001	–	–
H230Y	0.52	11	0.86	12.2

**Fig. 1. The visible absorption spectra of scSHMT and H230 mutants.** Visible absorption spectra of scSHMT and the H230A, H230N and H230Y mutants (line 1), and the H230F and H230R mutants (line 2) were recorded in a Shimadzu UV-160 A spectrophotometer using 5  $\mu\text{M}$  protein in buffer A. The spectra were recorded upon addition of 100 mM glycine (line 3) to scSHMT and the H230A, H230N and H230Y mutants followed by addition of 1.8 mM  $\text{H}_4$ -folate (line 4, H230A, H230N and H230Y mutants; line 5, scSHMT). Inset, intrinsic tryptophan fluorescence of H230 mutants. Protein (0.5  $\mu\text{M}$ ) in buffer A was excited at 280 nm and the emission spectrum was recorded between 300 and 400 nm on a Jasco FP 777 spectrofluorimeter at 25 °C. scSHMT (—); H230A, H230F, H230N and H230R mutants (—); H230Y mutant (- · -).



from Table 1 that both H230A and H230N showed only 2–3% activity compared to scSHMT, and H230F and H230R exhibited a marked decrease (a thousand-fold) in activity. The H230Y mutant, however, showed  $\approx 12\%$  catalytic activity compared to scSHMT. Staining for activity upon native PAGE using  $\beta$ -phenylserine and 2,4-dinitrophenylhydrazine gave the characteristic yellow-orange band [24] only with scSHMT and not with the mutants (data not shown). Although the H230Y mutant exhibited  $\approx 12\%$  catalytic activity with serine as substrate, it did not give the yellow-orange band with  $\beta$ -phenylserine, probably because the method of activity staining is not sensitive enough to pick up small amounts of benzaldehyde formed.

### Spectral and catalytic properties of the H230 mutant enzymes

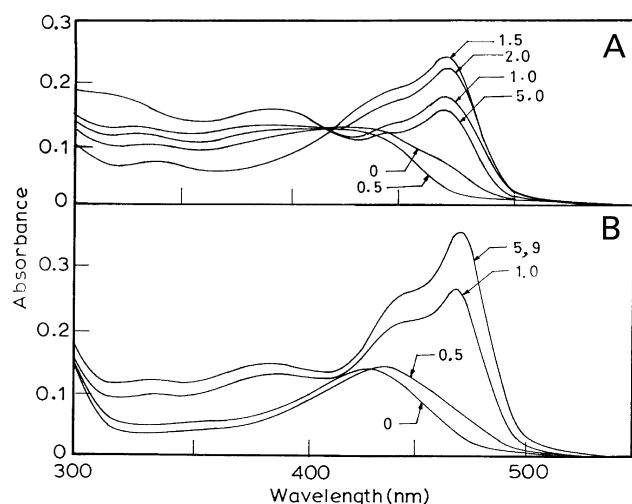
The far UV-CD spectra of all the mutant enzymes were essentially similar to scSHMT, indicating that the mutations may not have caused major alterations in the secondary structure (data not shown). The intrinsic tryptophan fluorescence emission spectra of all the H230 mutants, except H230Y, were similar to that of scSHMT (Fig. 1, inset). The H230Y protein showed a red shift in the emission maximum compared to scSHMT, suggesting that a subtle change in the environment around the tryptophan residues may have occurred as a consequence of the mutation. The H230A, H230N and H230Y mutants exhibited characteristic absorbance at 425 nm (Fig. 1, curve 1), whereas the H230R and H230F mutants showed very little absorbance at 425 nm when an equal amount of the protein (5  $\mu\text{M}$ ) was used to record the spectra (Fig. 1, curve 2). Like scSHMT, the H230A, H230N and H230Y mutants contained one mol of PLP bound per subunit. The addition of glycine to scSHMT resulted in the formation of a geminal

diamine (343 nm), external aldimine (425 nm) and a very small amount of the quinonoid intermediate (495 nm). The addition of glycine to H230Y also showed a small amount of geminal diamine (data not shown). The addition of  $\text{H}_4$ -folate to a mixture of scSHMT and glycine (Fig. 1, curve 5) and the H230Y mutant and glycine (Fig. 1, curve 4) gave the characteristic quinonoid intermediate, but the amount of this with the H230Y mutant was less. However, unlike scSHMT, the H230A and H230N mutants did not exhibit the characteristic spectral intermediates upon the addition of glycine (100 mM) followed by  $\text{H}_4$ -folate (1.8 mM). This observation suggests that a step prior to the formation of the quinonoid intermediate was probably affected in these two mutants. To identify this step, the catalytic mechanism was examined in detail. The formation of the external aldimine upon addition of the substrate serine/glycine is an early step in catalysis. The association constants for binding of serine and glycine to scSHMT and the H230A, H230N and H230Y mutant proteins were determined by measuring the substrate-induced fluorescence quenching of the enzyme-bound PLP (Table 1). The  $K_A$  values for serine ( $\approx 1$  mM) and glycine ( $\approx 10$  mM) for all the mutants were similar to that for scSHMT and indicated that they were capable of binding the substrates. The  $K_A$  values for the H230R and H230F mutant proteins could not be determined as they did not contain PLP.

The abstraction of the proton from the external aldimine to generate the quinonoid intermediate is a stereospecific reaction and is enhanced by  $\text{H}_4$ -folate [22]. Proton exchange studies carried out with tritiated glycine revealed that whereas the H230Y mutant showed 15% exchange compared to scSHMT, the H230A and H230N mutants showed only  $\approx 2\%$  exchange at 60  $\mu\text{M}$   $\text{H}_4$ -folate (data not shown), which correlates well with their observed physiological activity. Due to limitations of the spectral absorbance measurements, the formation of a small

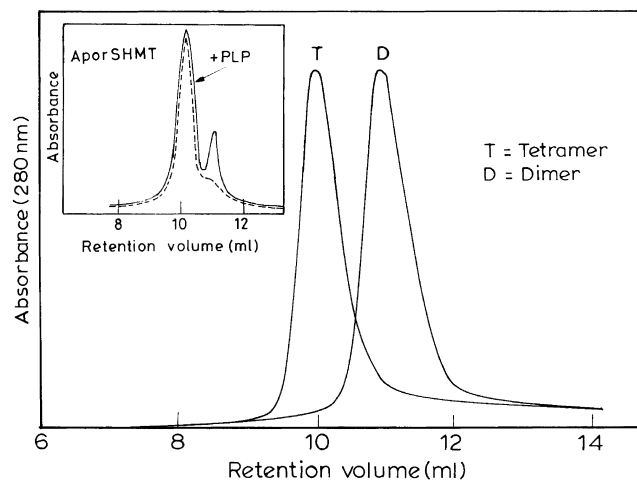
**Table 2. Kinetic parameters for the  $\text{H}_4$ -folate dependent and independent aldolytic cleavage catalyzed by H230Y SHMT.** The kinetic constants were evaluated by a double reciprocal plot of the rate of the reaction vs. varying substrate concentrations.

Protein	$K_m$ (mM)		$k_{\text{cat}}$ ( $\text{s}^{-1}$ )		$k_{\text{cat}}/K_m$ ( $\text{mM}^{-1}\cdot\text{s}^{-1}$ )	
	scSHMT	H230Y	scSHMT	H230Y	scSHMT	H230Y
L-Serine	1.0	0.9	4.23	0.43	4.23	0.48
L-Allothreonine	1.25	3.0	4.15	0.058	3.32	0.019



**Fig. 2.** Interaction of scSHMT and the H230Y mutant with TSC. Absorbance spectrum of the H230Y mutant (A) and scSHMT (B) ( $5 \mu\text{M}$ ) upon addition of TSC ( $2 \text{ mM}$ ) was recorded at 30 s intervals for 10 min at  $37^\circ\text{C}$ . Representative spectra recorded at time intervals indicated are shown in the figure.

amount of quinonoid intermediate with the H230A and H230N mutants, if any, cannot be ruled out. The R401A mutant enzyme, which does not catalyze proton exchange [25], was used as a negative control. The amount of proton exchange and the concentration of the quinonoid intermediate formed with the H230Y mutant (Fig. 1) correlates well with the observed activity of the mutant, emphasizing that the affected step of



**Fig. 3.** Size-exclusion chromatography profiles of scSHMT and H230Y mutants. The proteins ( $0.125 \text{ mg}\cdot\text{mL}^{-1}$ ) were analyzed on a calibrated Superose 12 HR 10/30 column attached to a Pharmacia FPLC system. The inset shows the gel filtration profile of apo scSHMT (—) prepared by incubating the enzyme with  $10 \text{ mM}$  L-cysteine followed by dialysis. The apoenzyme was reconstituted with  $50 \mu\text{M}$  PLP at room temperature for 5 min (---). 'T' represents the tetrameric form of scSHMT and the H230A, H230N and H230Y mutants, and 'D' represents the dimeric form of the H230F and H230R mutant proteins. The standard molecular weight markers used for calibration were: apoferritin ( $440\,000$ ),  $\beta$ -amylase ( $200\,000$ ), yeast alcohol dehydrogenase ( $150\,000$ ), bovine serum albumin ( $66\,000$ ) and carbonic anhydrase ( $29\,000$ ). The column was equilibrated with buffer A containing  $0.1 \text{ M}$  KCl at a flow rate of  $0.3 \text{ mL}\cdot\text{min}^{-1}$ . The eluent was monitored at  $280 \text{ nm}$ .

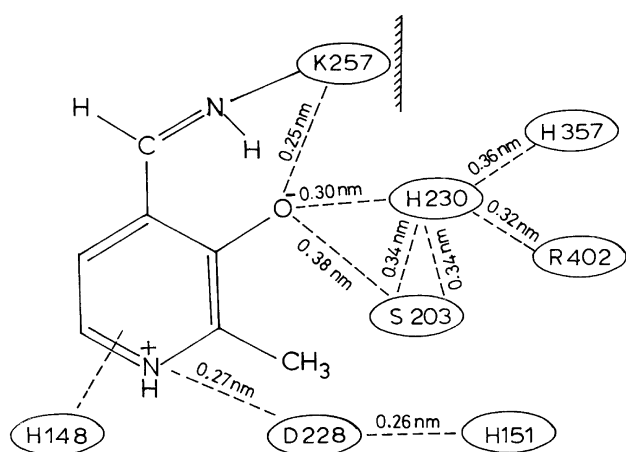
catalysis, namely proton abstraction, is partially restored. These observations would suggest that the proton transfer step of catalysis is affected but not fully abolished as a consequence of the H230A, H230N or H230Y mutations.

The effect of mutation on the alternative reactions such as  $\text{H}_4$ -folate-independent aldolytic cleavage of L-allothreonine is shown in Table 2. The  $K_m$  values for L-allothreonine remained unaltered but the  $k_{\text{cat}}$  values were reduced by two orders of magnitude. On the other hand, the rate of transamination of D-alanine ( $200 \text{ mM}$ ) was reduced by 50% for the H230Y mutant. The pseudo first-order rate constants for scSHMT and the H230Y mutant were  $0.0028 \text{ s}^{-1}$  and  $0.0012 \text{ s}^{-1}$ , respectively. The H230A and H230N proteins were unable to use D-alanine or L-allothreonine as substrates, implying a role for a well hydrogen-bonded H230 residue in the catalytic cycle of SHMT.

The observation that the proton abstraction step of catalysis and the side reactions were significantly affected by the mutation of H230 prompted an examination of the reactivity of PLP at the active site using well characterized inhibitors of SHMT such as MA [26] and thiosemicarbazide (TSC) [27]. The interaction of MA with scSHMT and H230Y was monitored spectrally. The pseudo first-order rate constants for the reaction of MA ( $20 \mu\text{M}$ ) with scSHMT and the H230Y mutant were  $0.052 \text{ min}^{-1}$  and  $0.026 \text{ min}^{-1}$ , respectively. Similar reduction in the rate of the reaction was observed with aminooxyacetic acid (data not shown) suggesting that there was an alteration in the reactivity of the PLP-Schiff's base in H230Y.

These findings were further corroborated when the interaction of the proteins with TSC was examined. It was shown earlier that TSC is a slow but tight binding inhibitor of sheep liver SHMT [27]. An early intermediate generated upon the interaction of TSC with sheep liver SHMT had a spectral absorbance at  $464 \text{ nm}$  and  $440 \text{ nm}$  and the concentration of this intermediate reached a maximal value after  $\approx 15 \text{ min}$  of the reaction [27]. Thereafter, the intermediate decomposed slowly to yield the thiosemicarbazone. It was established that this intermediate was present at the active site and the proton abstraction at the C2 position was an important step in its formation [27]. Upon reaction with TSC, scSHMT showed a profile (Fig. 2B) similar to that observed earlier for the native sheep liver SHMT [27]. Compared to scSHMT, the rates of formation of the TSC–quinonoid intermediate and its decomposition were rapid in the case of the H230Y mutant (Fig. 2A). It was not possible to determine the rate constants for these reactions, with accuracy, using conventional spectroscopic methods. There was no change in the absorbance at  $464 \text{ nm}$  for scSHMT, upto 10 min of the reaction; however, after 5 min, the concentration of the intermediate had decreased substantially (80%) in case of the H230Y mutant. These observations led to the conclusion that there is probably a change in the orientation of the PLP ring upon mutation of H230 to tyrosine that leads to an alteration in the reaction of the enzyme with different ligands, analogous to the situation for AATase.

An alteration in the  $T_m$  values of the protein in the presence of serine has been correlated to the conversion of the enzyme from an 'open' to a 'closed' conformation [28]. There was no change in the  $T_m$  values of the mutant enzymes upon addition of serine ( $54 \pm 2^\circ\text{C}$ ), unlike the wild-type enzyme, for which the addition of  $100 \text{ mM}$  L-serine to the holoenzyme enhanced its  $T_m$  value from  $54^\circ\text{C}$  to  $63^\circ\text{C}$ . These results indicate that the mutant enzymes, although capable of binding the substrates (Table 1), are unable to bring about the required conformational change, suggesting that they may be present in a partially closed conformation. The crystal structure of the Y225R mutant



**Fig. 4.** Hypothetical charge relay system operating at the active site of scSHMT formulated on the basis of the X-ray structure of hcSHMT (PDB accession number 1BJ4). H230 corresponds to H231 of hcSHMT. The graphics visualization program o was used for measuring the distances [31].

of AATase also showed the enzyme to be present in a partially closed conformation [10].

#### Oligomeric status of the mutant proteins

It was shown earlier that mutation of residues involved in PLP interaction lead to the dissociation of the tetrameric structure of scSHMT [29]. The observation that the H230F and H230R mutants did not have bound PLP whereas the H230A, H230N and H230Y mutants contained stoichiometric amounts of PLP (Fig. 1) necessitated an examination of the oligomeric structure of the mutant proteins. The oligomeric status of the freshly purified mutant enzymes was determined on a calibrated Superose 12 HR 10/30 analytical gel filtration column. scSHMT and the H230A, H230N and H230Y mutants eluted as a single symmetrical peak corresponding to a  $M_r$  of  $\approx 210$  kDa, suggesting that these proteins were tetramers (Fig. 3). This was confirmed by estimating the amount of PLP per subunit of the enzyme. The H230F and H230R mutant proteins, however, eluted as symmetrical peaks with a  $M_r$  corresponding to the dimeric form ( $\approx 110$  kDa) of the enzyme. Removal of the bound cofactor from the H230A, H230N and H230Y mutant proteins by reaction with L-cysteine gave the corresponding apoenzymes, which eluted as dimers, unlike the apoenzyme for scSHMT, which was predominantly a tetramer (Fig. 3, inset, solid trace). The mutant apoenzymes could not be

**Table 3.** Length of potential hydrogen bonds between cofactor and active site residues in hcSHMT (PDB accession number 1BJ4).

Protein group(s)	Hydrogen bond length (nm)
PLP O3'-K257 NZ	0.256
PLP O3'-H231 N $\delta$ 1	0.309
PLP O3'-S203 O $\gamma$	0.381
H231 Ne2-S203 O	0.344
H231 N $\delta$ 1-S203 O	0.344
H231 O-H357 Ne2	0.363
H231 Ne2-R402 NH1	0.327
D228 O $\delta$ 2-H151 Ne2	0.265
D228 O $\delta$ 2-PLP N1	0.276

reconstituted to tetramers upon exogenous addition of PLP (50–500  $\mu$ M) whereas apo scSHMT was converted to a tetramer that was fully active (Fig. 3, inset, dashed trace).

Enzymes are known to exert their functions not only through the chemical properties of the amino acid sidechains present at the active site but also through residues proximal to it. Charge distribution throughout the active site also facilitates catalysis by electrostatically guiding the substrate molecule into the active site. This theme has been observed recurrently not only in PLP-dependent enzymes [2,7–9,10] but also in other enzyme systems like pyruvate decarboxylase [30]. In the case of hcSHMT, it has been reported that H151 stabilizes, via hydrogen bonding, the negative charge on D228, which is crucial for determining the basicity of the N1 of PLP [2]. The interaction of PLP with the active site residues of scSHMT is depicted in Fig. 4, based on the available crystal structure coordinates (PDB accession number 1BJ4) of hcSHMT [2]. The X-ray structure of hcSHMT [2] revealed the presence of a large network of hydrogen bonding interactions at the active site (Table 3). H231 (H230 in scSHMT) is within hydrogen bonding distance of four residues within the same subunit, namely, O3' of PLP (0.309 nm), the NH group of the internal aldimine of K257 (0.256 nm), S203 (0.344 nm) and H357 (0.363 nm). R402, the residue binding the carboxyl group of serine, lies 0.32 nm away from H231. The active site milieu is highly polar as it is surrounded by charged residues such as arginine, aspartic acid, histidine, etc. (Fig. 4). It is obvious that H230 of scSHMT, which is equivalent to H231 of hcSHMT, along with the pyridine ring of PLP, D227 and H150, forms a charge relay system for effective proton abstraction from the external aldimine. The mutation of H230 to alanine, phenylalanine, asparagine, arginine or tyrosine disturbs the charge relay by affecting the hydrogen-bond interaction with O3' of PLP and altering the orientation of the PLP ring, thus affecting catalysis to various extents.

An examination of the X-ray structure of hcSHMT revealed that mutation of H230 of scSHMT to arginine would lead to a strong repulsion between the newly introduced positive charge of arginine and the R402 present nearby at the active site. This would probably result in the disruption of the tetrameric structure. Biochemical studies reported in this paper are in conformity with this explanation (Fig. 3).

The introduction of a large hydrophobic residue like phenylalanine in the highly polar active site pocket would affect the overall environment leading to the dissociation of the tetramer, explaining the dimeric nature of the H230F mutant. In both these mutants, i.e. H230F and H230R, loss of tetrameric structure leads to loss of catalytic activity and PLP binding.

Replacement of the H230 with asparagine, which is structurally similar to histidine, does not interfere with the active site interactions, although the hydrogen bond between O3' of PLP and amide nitrogen of H230N is probably weakened. This, in turn, could seriously affect the electron withdrawing ability of the pyridinium nitrogen, thus leading to loss of catalytic activity. In the case of the H230A mutant, as alanine is a small residue it does not sterically interfere with the residues at the active site. Both H230A and H230N mutants retain the oligomeric structure; however, H230A would be unable to form a hydrogen bond with O3' of PLP, thus affecting the charge relay system, leading to a drastic reduction in the catalytic efficiency.

The H231 in hcSHMT (PDB code 1BJ4) was mutated to tyrosine using the program o [31] and the resulting contacts were measured. Replacement of H230 with tyrosine establishes a hydrogen-bonding interaction between the O3' of PLP and the OH group of tyrosine. This hydrogen bond is stronger than that

with H230 as the distance between the two atoms decreases to 0.246 nm compared to 0.309 nm in the wild-type protein. However, this interaction decreases the bond distance between O3' of PLP and the OH group of S203 (0.137 nm compared to 0.381 nm in wild-type protein). In order to accommodate a tyrosine residue more effectively in the active site, S203 had to be rotated away from the O3' of PLP, disrupting the hydrogen bonding originally present in the wild-type enzyme. An important consequence of such a change could be that the basicity of N1 of PLP would be altered, thereby partially disrupting the charge relay system and affecting catalysis. Confirmation of these explanations, however, has to await the elucidation of the three-dimensional structure of the H230 mutants of SHMT.

The results presented above demonstrate that H230, which is hydrogen bonded to O3' of PLP and is part of a charge relay system, facilitates effective proton abstraction, an important event in the catalytic cycle of SHMT.

## ACKNOWLEDGEMENTS

This work was funded by the Department of Biotechnology, New Delhi, India and the CSIR Emeritus Scientist grant awarded to N. A. R. We thank Prof. M. R. N. Murthy and Mr S. Parthasarathy of MBU, IISc. for help with the modeling studies. The DBT protein and peptide sequencing facility is gratefully acknowledged for the N-terminal sequence analysis. Thanks are also due to Mr Jomon Joseph, Mr J. V. Krishna Rao and Ms. Kirthi Narayanswamy for helpful discussions.

## REFERENCES

- Schirch, L. (1982) Serine hydroxymethyltransferase. *Adv. Enzymol. Relat. Areas Mol. Biol.* **53**, 83–112.
- Renwick, S.B., Snell, K. & Baumann, U. (1998) The crystal structure of human cytosolic serine hydroxymethyltransferase: a target for cancer chemotherapy. *Structure* **6**, 1105–1116.
- Jagath, J.R., Sharma, B., Bhaskar, B., Datta, A., Appaji Rao, N. & Savithri, H.S. (1997) Importance of the amino terminus in maintenance of oligomeric structure of sheep liver cytosolic serine hydroxymethyltransferase. *Eur. J. Biochem.* **247**, 372–379.
- Scarsdale, J.N., Kazanina, G., Radaev, S., Schirch, V. & Wright, H.T. (1999) Crystal structure of rabbit cytosolic serine hydroxymethyltransferase at 2.8 Å resolution: mechanistic implications. *Biochemistry* **38**, 8347–8358.
- Appaji Rao, N., Talwar, R. & Savithri, H.S. (2000) Molecular organization, catalytic mechanism and function of serine hydroxymethyltransferase: a potential target for cancer chemotherapy. *Intl. J. Biochem. Cell Biol.* in press.
- McPhalen, C.A., Vincent, M.G. & Jansonius, J.N. (1992) X-ray refinement and comparison of three forms of mitochondrial aspartate aminotransferases. *J. Mol. Biol.* **225**, 495–517.
- Alexeev, D., Alexeeva, M., Baxter, R.L., Campopiano, D.J., Webster, S.P. & Sawyer, L. (1998) The crystal structure of 8-amino-7-oxononanoate synthase: a bacterial PLP-dependent, acyl-CoA-condensing enzyme. *J. Mol. Biol.* **284**, 401–419.
- Momany, C., Ernst, S., Ghosh, R., Chang, N. & Hackert, M.L. (1995) Crystallographic structure of a PLP-dependent ornithine decarboxylase from *Lactobacillus* 30a to 3.0 Å resolution. *J. Mol. Biol.* **252**, 643–655.
- Toney, M.D., Hohenester, E., Cowan, S.W. & Jansonius, J.N. (1993) Dialkylglycine decarboxylase structure: bifunctional active site and alkali metal sites. *Science* **261**, 756–759.
- Graber, R., Kasper, P., Malashevich, V.N., Sandmeier, E., Berger, P., Gehring, H., Jansonius, J.N. & Christen, P. (1995) Changing the reaction specificity of a pyridoxal-5'-phosphate-dependent enzyme. *Eur. J. Biochem.* **232**, 686–690.
- Hatefi, Y., Talbert, P.T., Osborn, M.J. & Huennekens, F.M. (1959) Tetrahydrofolic acid-5,6,7,8-tetrahydropteroyl-glutamic acid. *Biochem. Prep.* **7**, 89–92.
- Studier, F.W. & Moffatt, B.A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**, 113–130.
- Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.
- Alexander, D.C. (1987) An efficient vector-primer cDNA cloning system. *Methods Enzymol.* **154**, 41–64.
- Datta, A.K. (1995) Efficient amplification using 'megaprimer' by asymmetric polymerase chain reaction. *Nucleic Acids Res.* **23**, 4530–4531.
- Jagath-Reddy, J., Appaji Rao, N. & Savithri, H.S. (1996) 3' non templated 'A' addition by *Taq* DNA polymerase: an advantage in the construction of single and double mutants. *Curr. Sci.* **71**, 710–712.
- Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl Acad. Sci. USA* **82**, 488–492.
- Lowry, O.H., Rosenbrough, N.J., Farr, A.L. & Randall, R.J. (1951) Estimation of proteins by Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.
- Manohar, R., Ramesh, K.S. & Appaji Rao, N. (1982) Purification, physicochemical and regulatory properties of serine hydroxymethyltransferase from sheep liver. *J. Biosci.* **4**, 31–50.
- Jagath, J.R., Sharma, B., Appaji Rao, N. & Savithri, H.S. (1997) The role of His-134 -147, and -150 residues in subunit assembly, cofactor binding, and catalysis of sheep liver cytosolic serine hydroxymethyltransferase. *J. Biol. Chem.* **272**, 24355–24362.
- Schirch, L. & Jenkins, W.T. (1964a) Serine transhydroxymethylase-transamination of D-alanine. *J. Biol. Chem.* **239**, 3797–3800.
- Schirch, L. & Jenkins, W.T. (1964b) Serine transhydroxymethylase-properties of the enzyme substrate complexes of D-alanine and glycine. *J. Biol. Chem.* **239**, 3801–3807.
- Schirch, L. & Diller, A. (1971) Serine hydroxymethyltransferase: affinity of the active site for substrates, substrate analogues, and anions. *J. Biol. Chem.* **246**, 3961–3966.
- Ullevitch, R.J. & Kallen, R.G. (1977) Purification and characterization of pyridoxal-5'-phosphate dependent serine hydroxymethyltransferase from lamb liver and its action upon β-phenylserine. *Biochemistry* **16**, 5342–5350.
- Jagath, J.R., Appaji Rao, N. & Savithri, H.S. (1997) Role of Arg-401 of cytosolic serine hydroxymethyltransferase in subunit assembly and interaction with the substrate carboxy group. *Biochem. J.* **327**, 877–882.
- Acharya, J.K., Prakash, V., Appu Rao, A.G., Savithri, H.S. & Appaji Rao, N. (1991) Interactions of methoxyamine with pyridoxal-5'-phosphate-Schiff's base at the active site of sheep liver serine hydroxymethyltransferase. *Indian J. Biochem. Biophys.* **28**, 381–388.
- Acharya, J.K. & Appaji Rao, N. (1992) A novel intermediate in the interaction of thiosemicarbazide with sheep liver serine hydroxymethyltransferase. *J. Biol. Chem.* **267**, 19066–19071.
- Schirch, V., Shoshtak, K., Zamora, M. & Gautam-Basak, M. (1991) The origin of reaction specificity in serine hydroxymethyltransferase. *J. Biol. Chem.* **266**, 759–764.
- Talwar, R., Jagath, J.R., Datta, A., Prakash, V., Savithri, H.S. & Appaji Rao, N. (1997) The role of lysine-256 in the structure and function of sheep liver recombinant serine hydroxymethyltransferase. *Acta Biochim. Pol.* **44**, 679–688.
- Dobritzsch, D., König, S., Schneider, G. & Lu, G. (1998) High resolution crystal structure of pyruvate decarboxylase from *Zymomonas mobilis*. *J. Biol. Chem.* **273**, 20196–20204.
- Kleywegt, G.J. & Jones, T.A. (1996) Efficient rebuilding of protein structures. *Acta Crystallogr.* **D52**, 829–832.