Serine hydroxymethyltransferase (SHMT), a pyridoxal-5'-phosphate (pyridoxal5'-containing enzyme catalyzes a physiologically important reaction, namely the transfer of the bond that is being cleaved. The three-dimensional structures of human and rabbit liver cytosolic recombinant serine hydroxymethyltransferases (hcSHMT and rcSHMT) revealed that E75 and Y83 (numbering according to hcSHMT) are probable candidates for proton abstraction and Cα-Cβ bond cleavage in the reaction catalyzed by serine hydroxymethyltransferase. Both these residues are completely conserved in all serine hydroxymethyltransferases sequenced to date. In an attempt to decipher the role of these residues in sheep liver cytosolic recombinant serine hydroxymethyltransferase (scSHMT), E74 (corresponding residue is E75 in hcSHMT) was mutated to Q and K, and Y82 (corresponding residue is Y83 in hcSHMT) was mutated to F. The specific activities using serine as the substrate for the E74Q and E74K mutant enzymes were drastically reduced. These mutant enzymes catalyzed the transamination of d-alanine and 5,6,7,8-tetrahydrofolate independent retroaldol cleavage of L-allo threonine at rates comparable with wild-type enzyme, suggesting that E74 was not involved directly in the proton abstraction step of catalysis, as predicted earlier from crystal structures of hcSHMT and rcSHMT. There was no change in the apparent Tm value of E74Q upon the addition of L-serine, whereas the apparent Tm value of scSHMT was enhanced by 10 °C. Differential scanning calorimetric data and proteolytic digestion patterns in the presence of L-serine showed that E74Q was different to scSHMT. These results indicated that E74 might be required for the conformational change involved in reaction specificity. It was predicted from the crystal structures of hcSHMT and rcSHMT that Y82 was involved in hemiacetal formation following Cα-Cβ bond cleavage of L-serine and mutation of this residue to F could lead to a rapid release of HCHO. However, the Y82F mutant had only 5% of the activity and failed to form a quinonoid intermediate, suggesting that this residue is not involved in the formation of the hemiacetal intermediate, but might be involved indirectly in the abstraction of the proton and in stabilizing the quinonoid intermediate.

Keywords: active site residues; conformational change; proton abstraction; serine hydroxymethyltransferase; spectral intermediates.
oligomeric structure [12], as this Lys residue is located at the interface of the tight dimers [5,8,9]. From the three-dimensional structure of hcsHMT and rcsHMT, it is apparent that the side chains of E74 and Y82 are positioned to facilitate further reaction. Geminal diamine (II) in which both the amino group of the substrate and e-amino group of K256 are linked to pyridoxalP. This intermediate absorbs at 343 nm. The external aldimine, which absorbs at 425 nm (III) of pyridoxalP and the substrate, is formed by breaking the bond between e-amino group of K256 and pyridoxalP. The abstraction of the proton from the hydroxy group of l-serine possibly by E74 and/or Y82 results in the formation of the quinonoid intermediate (IV), absorbing at 492.5 nm. H4-Folate enhances the formation of the quinonoid intermediate and N5,N10-CH2-H4-folate. PyridoxalP-glycine external aldimine (V) absorbing at 425 nm is converted to the corresponding geminal diamine (VI) (absorbing at 343 nm) by reaction with e-amino group of K256. Release of the product glycine results in the formation of internal aldimine (I) for the next round of catalysis. Adapted from Schirch [3].

It was suggested from analysis of modeled ternary complex of serine and H4-folate of the rcsHMT that the pteridine ring of H4-folate is too far displaced (<8 Å) from the active site to directly accept the C3-hydroxy group of l-serine to its N5,N10 positions. The phenolic group of Y82, which interposes between the hydroxymethyl group of the l-serine and the N5,N10 positions of H4-folate, could form a hemiacetal with the formyl group produced by the deprotonation of the hydroxy group of serine by E74 [8]. It was also proposed that the short hydrogen bond between Y82 and exocyclic O4 of the pteridine ring could activate the Y82 hydroxy group for the formation of such an intermediate. This would place the hemiacetal group <5.2 Å from N5 of the folate to which it is to be transferred. It was therefore suggested that Y82 could serve as a shuttle for the hydroxymethyl group between l-serine-external aldimine and the folate cofactor or form the hemiacetal directly by a SN2 displacement at the Cα-bond. It was postulated that if this hypothesis was true, the Y82F mutant should cleave serine rapidly and release formaldehyde [8].

In order to examine the functions of Y82 and E74 as postulated above [5,8], Y82 was mutated to F and E74 to Q and K in scsHMT. Our results show that E74 is not involved in abstracting the proton from hydroxymethyl group of l-serine but participates in converting the enzyme from an open to closed form upon the addition of l-serine. It is also clear that Y82 is not involved in the formation of a hemiacetal intermediate but has a role in stabilizing the quinonoid intermediate. The mutation of this residue may indirectly affect the formation
of the quinonoid intermediate, thereby decreasing the catalytic efficiency.

**MATERIALS AND METHODS**

Materials

\[ \alpha^-{\text{32P}}\text{dATP (3000 Ci·mm}^{-1}\text{)} \] was obtained from the Board of Radiation and Isotope Technology (Government of India, Trombay, India). L-[\text{-3,14C]}-Serine, restriction endonucleases, Sequenase\textsuperscript{TM} version 2.0 sequencing kit and modifying enzymes were obtained from Amersham Pharmacia Biotech. Deep Vent polymerase was purchased from New England Biolabs (Beverly, MA, USA). CM-Sephadex, Sephacryl S-200, glycine, \text{-serine, \text{-alanine, NADH, \text{-threonine, 2-mercapto- ethanol, folic acid, rubidium chloride, pyridoxalP, isopropyl thio-}\text{-\text{-d-galactoside and EDTA were obtained from Sigma Chemical Co. (St Louis, MO, USA). Platinum oxide was purchased from Loba Chemie (Bombay, India). Centricron filters were obtained from Amicon, Millipore Corp. (Beverly, MA, USA). All other chemicals were of the analytical reagent grade. The mutant oligonucleotides were purchased from Bangalore Genei Pvt. Ltd (Bangalore, India). H\text{-Folate was prepared as described by Hatefi et al. [13].}

**Bacterial strains, growth conditions and DNA manipulations**

*E. coli* strain DH5\textalpha\ (Bethesda Research Labs, USA) was the recipient for all the plasmids used for DNA isolations and subcloning. BL21 (DE3) pLysS strain [14] was used for the expression of scSHMT [15], E74Q, E74K and Y82F SHMT mutant clones. Luria–Bertani medium or terrific broth (24 g of yeast extract, 12 g of tryptone, 4 mL of glycerol, 2.31 g of KH\textsubscript{2}PO\textsubscript{4} and 12.54 g of K\textsubscript{2}HPO\textsubscript{4} per L) with 50 \mu\text{-mL}^{-1} of ampicillin was used to grow *E. coli* cells containing the plasmids at 37 °C. Plasmids were prepared using the alkaline lysis procedure described by Sambrook et al. [16], DNA fragments were eluted from low-melting point agarose gels after electrophoresis [16].

**Subcloning of SHMT gene from pET vector to pRSET ‘C’ vector and site-directed mutagenesis**

The full-length gene for scSHMT (1452 bp) was released from pETSH (pET-3e vector containing the full length gene of scSHMT) [15] by double digestion using *NdeI* and *BamHI* restriction enzymes. This fragment was ligated to pRSET ‘C’, a T7 promoter-based expression vector previously double digested with *NdeI* and *BamHI*. The positive clones (pRSH, pRSET ‘C’ vector containing the full-length gene of scSHMT) were confirmed by restriction digestion and DNA sequencing. The smaller size (2.9 kb) of pRSET ‘C’ enabled easy generation of mutants directly in this expression vector. The PCR conditions used to polymerize the complete vector were as follows. The wild-type template (pRSH; 80 ng), sense and antisense primers (50 pmol) were added to PCR tubes containing 0.2 mm dNTPs, 1 mm MgSO\textsubscript{4}, 2.5 U of Deep Vent polymerase along with the buffer provided with the enzyme at 1× concentration. Amplification was carried out in a Perkin–Elmer 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. Sense and antisense primers for E74Q (sense: 5'-AAAGTACTCTCAG-

**Expression and purification of mutant enzymes**

pETSH, E74Q, E74K and Y82F plasmids were transformed into *E. coli* BL21(DE3) pLys S strain, scSHMT, E74Q, E74K and Y82F mutants were grown at 30 °C and purified using the protocol standardized for scSHMT earlier [18]. The enzyme pellet was dialyzed against the buffer A (50 mm potassium phosphate buffer pH 7.4, 1 mm 2-mercaptoethanol, 1 mm EDTA) for 24 h with two changes (1 L) prior to use. Protein was estimated by measuring the absorbance at 280 nm, 1 absorbance unit was equivalent to 1.2 mg·mL\textsuperscript{-1} [19].

**Enzyme assays**

Hydroxymethyltransferase reaction. SHMT catalyzed aldol cleavage of \text{-serine with H\text{-folate to form glycine and N\textsuperscript{5},N\textsuperscript{10}-CH\textsubscript{2}-H\textsubscript{4}-folate were monitored using L-[\text{-3,14C]}-serine and H\text{-folate as substrates as described previously [20,21]. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 \mu mol of formaldehyde by the decomposition of N\textsuperscript{5},N\textsuperscript{10}-CH\textsubscript{2}-H\textsubscript{4}-folate per min at 37 °C at pH 7.4. Specific activity was expressed as units per mg of protein. To determine \(K_m\) and \(k_{cat}\) values, the assay was carried out at varying concentrations of serine (0–4 mm) and the kinetic parameters were calculated by least square fitting of the data on double reciprocal plots.

SHMT-catalyzed H\text{-folate independent aldol cleavage of \text{-threonine to glycine and acetaldehyde [22] was monitored at 340 nm by the NADH-dependent reduction of acetaldehyde to ethanol and NAD\textsuperscript{+} by alcohol dehydrogenase present in an excess amount in the reaction mixture as described previously [23]. The NADH consumed in the reaction was calculated using a molar extinction coefficient of 6220 M\textsuperscript{-1}·cm\textsuperscript{-1} [24]. \(k_{cat}\) was defined as mol of NADH consumed per mol of subunit per s. The kinetic constants \(K_m\) and \(k_{cat}\) were calculated using double reciprocal plots.

**Transaminase activity.** SHMT catalyzes the transamination of \text{-alanine to give pyruvate and the bound pyridoxalP is converted to 5-pyridoxamine 5\'-phosphate [25]. scSHMT, E74Q and Y82F (1 mg·mL\textsuperscript{-1}) were taken separately in 900 \mu L of buffer A and the 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 \mu L of 1 mm \text{-alanine to both cuvettes. Absorbance change at 425 nm was monitored and the pseudo-first order rate constants were calculated as described previously [18].

**Spectroscopic methods**

Absorption spectra. Absorption spectra of the enzymes were recorded in a Shimadzu UV-160A spectrophotometer in
buffer A. All the spectra were recorded at 25 ± 2 °C against buffer A and the protein concentration used was 1 mg·mL⁻¹.

**CD spectra.** CD measurements were made in Jasco J-500A automated recording spectropolarimeter. Spectra were collected at a scan speed of 10 nm·min⁻¹ and a response time of 16 s. Far-UV CD spectra were recorded at 25 ± 2 °C in buffer A using the same buffer as the blank from 250 to 195 nm. The protein concentration corresponding to A₂₈₀ value of 0.1 per mL⁻¹ was used for far-UV CD studies. The cuvettes used for far-UV CD measurements had path length of 1 mm. The CD spectra obtained were plotted as molar ellipticity (θ<sub>280</sub>) assuming a relative subunit M<sub>r</sub> of 52 900 Da for the enzymes.

$$\theta_m = (\theta \times 100 \times M_r/c \times d)$$

where θ is the observed ellipticity (in degrees), M<sub>r</sub> is the concentration of the protein in mg·mL⁻¹ and d is the path length in dm (0.01 nm).

Visible CD spectra were recorded from 500 to 300 nm using 1 mg·mL⁻¹ protein concentration in buffer A with or without substrates (l-serine/glycine)

**Intrinsic tryptophan fluorescence spectra.** Intrinsic fluorescence measurements were made at 25 °C using Jasco FP 777 spectrofluorimeter. The protein concentration corresponding to an A₂₈₀ value of 0.1 per mL was used. The excitation wavelength was 280 nm and emission spectra were recorded from 300 to 400 nm.

**Thermal stability**

Thermal denaturation of scSHMT, E74Q and Y82F was performed in a Gilford Response II spectrophotometer from Ciba Corning Diagnostics (Oberlin, OH, USA) as described by Jagath et al. [18]. The absorbance change was monitored at 287 nm. The first derivative of the thermal denaturation profile obtained using the software supplied with the instrument, was used to evaluate the apparent transition temperatures for the proteins. The apparent denaturation temperature (apparent T<sub>m</sub>) is defined as the temperature at which the value of the fraction of the protein in the denatured state was 0.5.

**Differential scanning calorimetric measurements**

Differential scanning calorimetric (DSC) experiments were carried out on a Micro Cal MC-2 Ultra sensitive DSC, Micro Cal Inc. (MA, USA). Protein samples were dialyzed against buffer A either with 100 mM CaCl₂, or without CaCl₂, or with l-serine or without l-serine for 12 h at 4 °C with three changes of 250 mL buffer A. The final dialysate was used in the reference cell. A protein concentration of 3.5 mg·mL⁻¹ (0.066 M) was used in all experiments. All the protein solutions and buffer were degassed with gentle stirring under vacuum before being loaded into the calorimeter. Experiments were performed over a range of temperatures (30–90 °C) at a scan rate of 1.5 °C·min⁻¹. Normalized heat capacity (Cₚ) data were corrected for buffer baseline. Raw data from the DSC run were analyzed using ORIGIN™ (version 2.9) scientific plotting software. The DSC heat capacity was curve fitted using non-two-state fit.

**Proteolytic cleavage**

Proteolytic digestions of scSHMT and E74Q mutant enzymes were carried out using 5% trypsin (w/w) in 250 μL reaction mixture. One milligram of the protein (either with or without 50 mM l-serine/glycine) and 50 μg of trypsin were incubated at 37 °C for 1 h in buffer A. Extracts were analyzed on 15% SDS/PAGE.

**RESULTS**

**Physico-chemical and catalytic properties of E74Q, E74K and Y82F SHMTs**

scSHMT and mutant proteins, E74Q, E74K and Y82F were overexpressed in E. coli BL21(DE3) pLys S strain. Considerable quantities of the expressed proteins were present in the soluble fractions. The yields of the purified mutant proteins were ≈ 10 mg·L⁻¹ compared with 40–50 mg of scSHMT per L of medium. The purified proteins showed a single band on SDS/PAGE analysis corresponding to a M<sub>r</sub> of 53 kDa indicating homogeneity (data not shown). Mutant proteins were eluted from a calibrated Superose-12 HR 10/30 gel filtration column with a M<sub>r</sub> ≈ 220 kDa (Fig. 1A), indicating that all were homotetramers. This observation suggested that mutations did not lead to alteration in the oligomeric structure of these mutant SHMTs. The far-UV CD spectra of scSHMT and the mutants overlapped with each other indicating that the mutations also did not result in any gross change in the secondary structure. The intrinsic tryptophan fluorescence spectra of mutants were also similar to that of scSHMT (data not shown). The mutants contained 1 μmol of pyridoxalP per μmol of subunit. E74Q and E74K SHMT had a specific activity of 0.012 and 0.004 U·mg⁻¹, respectively, compared with 4.2 U·mg⁻¹ observed with scSHMT (Table 1) suggesting that the mutations had resulted in a considerable loss of activity. The K<sub>m</sub> value of serine for E74Q was 1.5 mM, similar to that of scSHMT (Table 1). The k<sub>cat</sub>/K<sub>m</sub> value was decreased by 410-fold compared with that of scSHMT. Interestingly, the hydroxy-methyltransferase activity of the E74Q mutant enzyme in the absence of H₄-folate was 0.013 U·mg⁻¹ compared with no detectable activity in the case of scSHMT. Addition of H₄-folate had no effect on the activity of mutant, whereas the activity of scSHMT increased to 4.2 U·mg⁻¹. The mutation of Y 82 to F (Y82F) also resulted in a significant decrease in activity from 4.2 U·mg⁻¹ for scSHMT to 0.16 U·mg⁻¹. The K<sub>m</sub> for l-serine was decreased from 1 mM for scSHMT to 0.3 mM. The k<sub>cat</sub>/K<sub>m</sub> value for this mutant decreased by fivefold compared with that of scSHMT (Table 1).

**Spectral intermediates**

E74Q, E74K, Y82F and scSHMTs gave a spectrum characteristic of an enzyme containing pyridoxalP-internal aldimine [25] (Fig. 2, curve E). The visible CD spectra of the mutants in the range of 300–500 nm were also similar to that of scSHMT, indicating that the pyridoxalP at the active site was present in a similar micro-environment in the bulk solvent (data not shown). In view of the observation that the catalytic activities of E74Q, E74K were significantly lower than that of scSHMT (Table 1), it was of interest to determine which catalysis step was affected by the mutation. Addition of glycerine to scSHMT resulted in the formation of a geminal diamine (343 nm) and external aldimine (425 nm, Fig. 2A, curve 1), as shown by the spectroscopic data. Upon addition of H₄-folate to this mixture, a prominent peak at 492.5 nm, corresponding to the quinonoid intermediate, was observed (Fig. 2A, curve 2). Interestingly, when glycerine was added to either E74Q or E74K, a distinct peak of the quinonoid intermediate was observed even in the absence of H₄-folate (Fig. 2B,C, curve 1). Addition of H₄-folate enhanced the...
concentration of quinonoid intermediate when scSHMT, E74Q or E74K were used (Fig. 2A–C curve 2). It was observed that the $\lambda_{\text{max}}$ of the quinonoid intermediate generated upon addition of H4-folate to the E74Q SHMT–glycine complex was shifted to 496.5 nm from 492.5 nm. This probably reflects a change in the environment of the active site of the mutants, especially the orientation of pyridoxal $P$ ring. Addition of glycine to Y82F mutant resulted in the formation of geminal diamine (Fig. 2D, curve 1). However, addition of H4-folate did not result in formation of the quinonoid intermediate (Fig. 2D, curve 2), even though the mutant was more active than E74Q and E74K (Table 1).

Effect of L-serine and HCHO on the quinonoid intermediate
It has been suggested that H4-folate is involved in the rapid removal of HCHO generated at the active site and that the quinonoid intermediate is not readily seen when L-serine and H4-folate are added to the enzyme [3]. As mentioned earlier, mutation of E74 could affect the abstraction of the proton from external aldimine and/or Cα-Cβ bond cleavage, leading to the formation of HCHO. It was therefore important to examine the effect of HCHO and L-serine on the concentration of the quinonoid intermediate generated by addition of glycine and H4-folate to scSHMT and E74Q. It can be seen from Fig. 3 that addition of HCHO (10 or 60 mM) to a mixture of scSHMT, glycine and H4-folate resulted in a marked decrease in the concentration of the quinonoid intermediate (Fig. 3A). Similarly, addition of HCHO (10 and 60 mM) to quinonoid intermediate generated by the addition of glycine and H4-folate to E74Q mutant resulted in a decrease in absorbance at 496.5 nm (Fig. 3C). This observation suggested that the mutation had probably not affected the removal of HCHO from the active site. In order to examine the possibility of the mutation affecting the cleavage of the Cα-Cβ bond, HCHO was generated in situ by addition of serine to a mixture of glycine, H4-folate and scSHMT (Fig. 3B). It can be seen that the concentration of the quinonoid intermediate decreased with increasing concentration of L-serine and reached a basal value at 60 mM L-serine. Similarly, in the case of E74Q (Fig. 3D), the

Table 1. Kinetic parameters for scSHMT, E74Q and Y82F using L-serine as substrate. Calculated from six independent determinations.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity (U·mg$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$k_{\text{cat}}/K_m$ (s$^{-1}$·mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>scSHMT</td>
<td>4.20</td>
<td>1.0</td>
<td>4.10</td>
<td>4.10</td>
</tr>
<tr>
<td>E74Q</td>
<td>0.012</td>
<td>1.5</td>
<td>0.015</td>
<td>0.01</td>
</tr>
<tr>
<td>Y82F</td>
<td>0.160</td>
<td>0.3</td>
<td>0.219</td>
<td>0.88</td>
</tr>
</tbody>
</table>

a $1U = 1$ μmol of HCHO formed per min at 37 °C. b Calculated per mol of subunit.
absorbance at 496.5 nm decreased upon addition of $l$-serine and reached a minimal value. This value was identical to that observed when only glycine was added to E74Q (Fig. 3D curves 1 and 4).

**Reaction with D-alanine and L-allo threonine**

In addition to catalyzing retro aldol cleavage of $l$-serine in the closed form, the enzyme also catalyzes transamination with D-alanine [26] and H$_4$-folate independent retro aldol cleavage of $l$-allo threonine [22] in the open form [27]. In order to examine the effects of mutation on reaction specificity, transamination and retro aldol cleavage of $l$-allo threonine of scSHMT and mutants were monitored. It can be seen from Table 2 that the pseudo-first order rate constants for the reaction with D-alanine for the E74Q and Y82F mutants were similar to that of scSHMT, suggesting that the mutations did not affect this reaction.

The $k_{cat}$ value for the $l$-allo threonine reaction catalyzed by E74Q (3.17 s$^{-1}$) was similar to that of scSHMT (3.7 s$^{-1}$), although there was a 10-fold increase in the $K_m$ value. In the case of Y82F, both the $k_{cat}$ and $K_m$ values decreased and the
catalytic efficiency of Y82F decreased by ≈17-fold compared with that of scSHMT (Table 2).

Formation of apoenzyme and its reconstitution

The transamination reaction with d-alanine has been used to prepare apoenzyme of SHMT. Earlier observations from this laboratory indicated that removal of pyridoxal $P$ from several mutants led to dissociation of the tetramer to a dimer [18,28]. scSHMT, E74Q and Y82F were isolated as tetramers with bound pyridoxal $P$, as revealed by size-exclusion chromatography (Fig. 1A) and visible absorbance spectra (Fig. 2, curve E). Removal of pyridoxal $P$ from scSHMT and E74Q resulted in the formation of apotetramer (Fig. 1B) which could be reconstituted to the holoenzyme (Fig. 1C) with a characteristic absorbance of the internal aldimine (Fig. 1D). In contrast, removal of pyridoxal $P$ from Y82F mutant resulted in an apoenzyme, which was present predominantly as a dimer (Fig. 1B). Addition of pyridoxal $P$ to this mutant apoenzyme resulted in the partial conversion of the dimer to a holotetramer (Fig. 1C,D) with regain of the enzyme activity (0.06 U·mg$^{-1}$, corresponding to the amount of tetramer). These results suggest that the aposcSHMT, E74Q and Y82F can be reconstituted to the holoenzymes suggesting that these residues are not crucial for the maintenance of the oligomeric structure of SHMT.

Substrate-induced stability

It has been suggested that addition of l-serine to SHMT converts the enzyme from an open to a closed form in which the reaction with l-serine and H$_4$-folate occurs. This conversion is

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>No ligand</th>
<th>L-Serine (100 mM)</th>
<th>Glycine (100 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>scSHMT</td>
<td>55</td>
<td>65</td>
<td>58</td>
</tr>
<tr>
<td>E74Q</td>
<td>56</td>
<td>56</td>
<td>57</td>
</tr>
<tr>
<td>Y82F</td>
<td>53</td>
<td>68</td>
<td>57</td>
</tr>
</tbody>
</table>

Table 2. Kinetic parameters for H$_4$-folate independent reactions catalyzed by scSHMT, E74Q and Y82F alternate reactions.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_m$ (mm)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/k_m$ (mm$^{-1}$·s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>scSHMT</td>
<td>0.23</td>
<td>0.70</td>
<td>3.70</td>
</tr>
<tr>
<td>E74Q</td>
<td>0.28</td>
<td>10.60</td>
<td>3.17</td>
</tr>
<tr>
<td>Y82F</td>
<td>0.21</td>
<td>0.45</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Table 3. Apparent $T_m$ (°C) values calculated for scSHMT, E74Q and Y82F in the presence and absence of l-serine and glycine.

$a k_{cat}$ values (average of six independent determinations) calculated per mol of subunit.

Fig. 4. DSC thermograms of scSHMT (3.5 mg·mL$^{-1}$) and E74Q (3.5 mg·mL$^{-1}$) in the absence and presence of l-serine. (A) scSHMT, (B) E74Q, (C) scSHMT + 100 mM l-serine, (D) E74Q + 100 mM l-serine.
Fig. 5. SDS/PAGE (15%) analysis of the products of tryptic digestion of scSHMT and E74Q SHMT. Lane 1, markers; lane 2, scSHMT, 40 μg (without trypsin); lane 3, scSHMT, 80 μg + trypsin; lane 4, scSHMT, 40 μg + 50 mM l-serine (without trypsin); lane 5, scSHMT, 80 μg + 50 mM l-serine + trypsin; lane 6, E74Q, 40 μg (without trypsin); lane 7, E74Q, 80 μg + trypsin; lane 8, E74Q, 40 μg + 50 mM l-serine (without trypsin); lane 9, E74Q, 80 μg + 50 mM l-serine + trypsin; lane 10, markers.

accompanied by an increase in thermal stability as reported previously [27]. The apparent T_m value determined using Gilford spectrophotometer was 55 °C and increased to 65 °C upon addition of l-serine to scSHMT. However, addition of glycine to scSHMT did not increase the apparent T_m value, indicating the specific interaction of scSHMT with l-serine. In contrast, addition of l-serine or glycine to E74Q did not increase the apparent T_m value of 55 °C (Table 3). An increase in the apparent T_m value to 68 °C was observed when l-serine was added to Y82F, whereas the apparent T_m value remained at 55 °C upon the addition of glycine. These results clearly indicate that the thermal coagulation temperature (a temperature-dependent phenomena) is enhanced by the specific interaction of l-serine with scSHMT and Y82F, which is necessary for physiological reactions to occur.

In order to quantify these apparent T_m value changes, DSC studies were performed both with enzyme alone and in the presence of l-serine in the temperature range of 30–90 °C at increments of 1.5 °C/min. The DSC thermograms of scSHMT and E74Q in buffer A show a typical and symmetrical pattern with a few other domains also having enthalpic peaks (Fig. 4A,B). However, 95% of the peak area is represented by a sharp transition in the region of 59 ± 1 °C for scSHMT and E74Q. A similar pattern was observed upon addition of glycine to either of the enzymes (data not shown). scSHMT showed a bimodal pattern in the presence of l-serine with a leading peak followed by a trailing peak with apex values of 78 and 72 °C (Fig. 4C). Contrasting this observation, three peaks were observed with apex values of 44, 63 and 81 °C upon addition of l-serine to E74Q (Fig. 4D). It is not clear why DSC thermograms are bimodal for scSHMT and multimodal for E74Q in the presence l-serine. However, one plausible explanation could be that the conformational change induced by the binding of l-serine to scSHMT leads to a closed form and binding to E74Q leads to a structurally different entity. The DSC thermograms of Y82F were similar to scSHMT either in the presence or absence of l-serine (data not shown).

The structural differences in scSHMT and E74Q were also examined by monitoring the sensitivity of the enzyme to trypsin digestion in the presence and absence of substrates and analyzing the products on 15% SDS/PAGE. It can be seen from Fig. 5 that the proteolytic digestion pattern of scSHMT in the absence and presence of l-serine was similar (lanes 3 and 5, respectively). The pattern of digested fragments of E74Q was similar to that of scSHMT in the absence of l-serine (lane 7). It is interesting to observe that, in the case of E74Q, an additional band corresponding to a Mr = 35 kDa was seen in the presence of l-serine (lane 9). This suggested that the sensitivity of E74Q to trypsin in the presence of l-serine was different to that of scSHMT. However, identical patterns were observed when scSHMT and E74Q were digested with trypsin in the presence of glycine (data not shown).

DISCUSSION

A major objective in the study of any enzyme-catalyzed reaction is the elucidation of the role of specific amino acids in the proposed catalytic mechanism. Analysis of the structural features of hcSHMT and rcSHMT led to the proposition that E75 and E57 (equivalent residues) are involved in abstraction of the proton from the hydroxy group of l-serine [5,8]. Critical examination of the recently determined structure of eSHMT-5-formyl H_2-folate-glycine ternary complex suggested that this residue (E57) is in a protonated form and that the carboxyl oxygen is hydrogen bonded to a water molecule from which it must be accepting a proton. It is close to the protonated N^{10} atom of folate carrying the methylene group [9]. This analysis raises some doubts about the role of this residue as a primary proton acceptor from the hydroxy group of l-serine.

The sequence identity (94%) of scSHMT with hcSHMT suggested that it may have a similar three-dimensional structure. E74 corresponds to E75 and E57 in hcSHMT and rcSHMT, respectively. It can be seen from Table 1 that the mutation of E74 to Q led to a marked decrease in hydroxymethyltransferase activity (0.012 U·mg⁻¹). The residual activity could be monitored conveniently (Table 1). The similar K_m value for l-serine suggested that the interaction of the enzyme with l-serine was probably not seriously affected by mutation (Table 1). The characteristic CD intensity at 425 nm of scSHMT was decreased by ≈ 50% upon addition of 100 mM l-serine. A similar decrease in CD intensity was observed with E74Q, suggesting that the mutant was capable of binding serine as efficiently as scSHMT (data not shown). These results suggest that a step in catalysis beyond substrate binding may be affected. The observation that comparable amounts of the quinonoid intermediate (0.35 and 0.39 μmol per μmol of subunit of scSHMT and E74Q, respectively) were seen in the presence of glycine and H_2-folate with both E74Q and scSHMT suggested that the proton abstraction step was probably unaffected by the mutation. It was interesting to observe the peak corresponding to the quinonoid intermediate upon addition of glycine to E74Q, suggesting that a significant
amount of proton abstraction from the Cα-carbon occurs even in the absence of H₄-folate. The small amount of H₂-folate independent hydroxymethyltransfer could be due to the ability of the mutant to abstract the equivalent proton from the hydroxymethyl group of serine. The step following formation of the quinonoid intermediate is the removal of HCHO generated at the active site, when l-serine is used as a substrate. l-Serine and HCHO decreased the concentration of quinonoid intermediate in a similar manner (Fig. 3), indicating that the interactions of H₂-folate and removal of HCHO from the active site are not probably affected by the mutation.

The structure of eSHMT in the presence of N⁵-formyl H₂-folate and glycine has revealed that the carbonyl oxygen atom of E57 could form an H-bonding interaction with a water molecule. It is possible that the carbonyl oxygen atom in Q could carry out a similar function and enable proton abstraction by the E74Q mutant. However, the E74K mutant SHMT, which cannot participate in such interactions, had similar activity (0.1% of scSHMT). This mutant also generated the quinonoid intermediate upon addition of glycine. Furthermore, addition of H₂-folate increased the concentration of the quinonoid intermediate (Fig. 2C). These results suggest that the carboxy group of E74 residue may not function as a proton acceptor. Additional support for this suggestion is that H₂-folate independent reactions, such as transamination and retroaldol cleavage of l-allo threonine (Table 2), were unaffected, strongly suggesting that the mutation has probably incapacitated the enzyme to undergo the conformational change consequent to l-serine binding. Catalytic efficiency with l-serine as the substrate, however, decreased by 410-fold (Table 1). It is therefore possible that E74 is involved in the conversion of the enzyme from an open to a closed conformation which is required for physiological reactions to occur. As demonstrated previously with SHMT [18,27], conversion of the open form to the closed form of the enzyme by l-serine is characterized by its increased thermal stability. In a similar manner, the thermal stability of scSHMT is enhanced by addition of l-serine but not glycine (Table 3). However, the thermal stability of E74Q was not enhanced by the interaction with l-serine or glycine (Table 3).

This is further confirmed by DSC data which suggest the possibility of a few critical domains or patches in the protein becoming very fragile or very stable upon the interaction of scSHMT and its mutants with l-serine. Addition of l-serine may have modified E74Q in such a way that these domains behave rather independently, as suggested by peak values in which the total enthalpic contribution of the protein molecule is provided by two or more domains. It is likely that under ‘stress conditions’, such as binding of ligand, structural changes or additional patches could be induced in the protein resulting in new transitions. The DSC data of E74Q and scSHMT in the absence of ligand and the presence of glycine were, however, identical, suggesting that thermal unfolding of these enzymes was identical and no conformational change occurs in the presence of glycine.

The altered conformation upon binding of l-serine in the case of E74Q was also monitored by differential susceptibility to tryptic digestion. SDS/PAGE patterns of tryptic digests of scSHMT and E74Q in the presence or absence of glycine were very similar, as were the tryptic digestion patterns of scSHMT either alone or in the presence of l-serine were similar (Fig. 5, lanes 3 and 5). However, the appearance of an additional ~35 kDa band in the SDS/PAGE of E74Q in the presence of l-serine (Fig. 5, lane 9) suggested that this mutant was converted to a new conformation different to the closed form generated by the interaction of l-serine to scSHMT, as well as to the open form.

The following observations support the contention that E74 may be involved in conversion of the enzyme from an open to a closed form and that the mutation to Q or K hampers this conversion. E74Q was characterized by limited physiological reaction in the absence of H₂-folate, a prominent quinonoid intermediate peak upon the addition of glycine alone, inability to enhance thermal stability in the presence of l-serine, multimodal thermograms of DSC in the presence of l-serine and altered susceptibility to tryptic digestion in the presence of l-serine.

In addition to the role of E74 in the catalysis, it was postulated that Y82 was involved in the formation of hemiacetal intermediate with the formyl group produced by Co-–C介 bond cleavage [5,8]. Our results clearly show that mutation of this residue did not cause a marked decrease in activity either with serine or alternate substrates thereby implying that Y82 may not have a crucial role in catalysis. It was also postulated by Scarsdale et al. [8] that if hemiacetal hypothesis was correct then the Y82F mutant should cleave serine and rapidly release formaldehyde. A rapid release of HCHO after the addition of l-serine to Y82F was not observed. The absence of a quinonoid intermediate in the presence of glycine and H₂-folate may be due to the effect of this residue on the stability of the quinonoid intermediate.

The results presented here show that E74 is not involved in abstracting the proton from hydroxymethyl group of l-serine, but in the conversion of the enzyme from an open to a closed form. Y82, is not involved directly in proton abstraction or Co-–C介 bond cleavage, but may have a role in stabilizing the quinonoid intermediate.

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