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This paper is dedicated to Professor Włodzimierz Ostrowski

The role of lysine-256 in the structure and function of sheep liver recombinant serine hydroxymethyltransferase*

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The active site lysine residue, K256, involved in Schiff's base linkage with pyridoxal-5′-phosphate (PLP) in sheep liver recombinant serine hydroxymethyltransferase (rSHMT) was changed to glutamine or arginine by site-directed mutagenesis. The purified K256Q and K256R SHMTs had less than 0.1% of catalytic activity with serine and H_4folate as substrates compared to rSHMT. The mutant enzymes also failed to exhibit the characteristic visible absorbance spectrum (λ_{max} 425 nm) and did not produce the quinonoid intermediate (λ_{max} 495 nm) upon the addition of glycine and H_4folate. The mutant enzymes were unable to catalyze aldol cleavage of β-phenylserine and transamination of D-alanine. These results suggested that the mutation of the lysine had resulted in the inability of the enzyme to bind to the cofactor. Therefore, the K256Q SHMT was isolated as a dimer and the K256R SHMT as a mixture of dimers and tetramers which were converted to dimers slowly. On the other hand, rSHMT was stable as a tetramer for several months, further confirming the role of PLP in maintenance of oligomeric structure. The mutant enzymes also failed to exhibit the increased thermal stability upon the addition of serine, normally observed with rSHMT. The enhanced thermal stability has been attributed to a change in conformation of the enzyme from open to closed form leading to reaction specificity. The mutant enzymes were unable to undergo this conformational change probably because of the absence of bound cofactor.

Serine hydroxymethyltransferase (SHMT; EC 2.1.2.1) is a key enzyme in the pathway for the interconversion of folate coenzymes. This pyridoxal-5′-phosphate (PLP) dependent enzyme catalyzes the reversible conversion of serine and H_4folate to glycine and

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Abbreviations: SHMT, serine hydroxymethyltransferase; rSHMT, sheep liver recombinant serine hydroxymethyltransferase; H_4folate, 5,6,7,8-tetrahydrofolate; β-ME, 2-mercaptoethanol; PLP, pyridoxal-5′-phosphate; AATase, aspartate aminotransferase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; buffer A, 50 mM potassium phosphate buffer, pH 7.3, 1 mM EDTA, 1 mM β-ME.

5,10-methylene-H_4folate. As in other PLP dependent enzymes, the coenzyme is covalently linked to the ε-amino group of an active site lysine residue (K256) by a Schiff's base linkage to form an internal aldimine in mammalian SHMTs. During catalysis, the nucleophilic attack by the amino group of the incoming amino acid substrate causes the release of the ε-amino group and the formation of an external aldimine, i.e. a Schiff's base between the amino group of the substrate and the aldehyde group of the cofactor. This is followed by a 1,3-prototropic shift involving abstraction of a proton from the α-carbon of the amino acid and donation of a proton to the aldehydic carbon of the coenzyme *via* a quinonoid intermediate to yield a ketimine product (Schirch, 1984).

The aim of the present study was to determine the functional role(s) of the lysine residue that binds PLP. This lysine residue has been postulated to serve one or more roles in the enzymatic reactions, namely, cofactor binding, formation of enzyme-substrate intermediates, catalysis or product release. It has been changed to other amino acids by site-directed mutagenesis in several PLP-dependent enzymes (Smith *et al.*, 1989; Nishimura *et al.*, 1991; Toney & Kirsch, 1991; 1992; Grimm *et al.*, 1992; Planas & Kirsch, 1991; Schirch *et al.*, 1993; Ilag & Jahn, 1992; Ziak *et al.*, 1990, 1993; Lu *et al.*, 1993; Bhatia *et al.*, 1993). In many of the cases, the mutant enzymes were either completely inactive or showed very low levels of activity. Extensive investigations have been carried out with aspartate aminotransferase (AATase) where K258 has been mutated to arginine and the mutation was shown to stabilize the key quinonoid intermediate (Toney & Kirsch, 1991). In another study involving K258H mutation (Ziak *et al.*, 1990) the enzyme was found to retain partial enzymatic efficiency indicating that, apparently, internal aldimine linkage is not essential for the enzymatic transamination reaction and a histidine residue can substitute for K258 which is assumed to act as a proton donor/acceptor in the tautomerization step. However, more recently, studies carried out on the active site lysine in *Escherichia coli* SHMT have shown that K229 is not the base that removes a proton from the α-carbon of the substrate. However, it is essential for expelling the product from the external aldimine intermediate (Schirch *et al.*, 1993).

It was observed earlier in our laboratory that sheep liver SHMT exists as a homotetramer in the presence of PLP (Manohar *et al.*, 1982). However, removal of the cofactor to generate the apoenzyme yields a protein showing two peaks on gel filtration corresponding to the tetrameric and dimeric forms of the enzyme (Brahatheeswaran *et al.*, 1996). The present study deals with the mutation of active site K256 of sheep liver SHMT to Q or R and the characterization of the mutant enzymes in order to assess the functional and structural role of K256.

MATERIALS AND METHODS

Materials. [α-^{32}P]dATP (3000 Ci/mmol) and L-[3-^{14}C]serine (55 mCi/mmol), restriction endonucleases and DNA modifying enzymes were obtained from Amersham International. CM-Sephadex and Sephacryl S-200 were obtained from Pharmacia Fine Chemicals. Glycine, L-serine, D-alanine, β-phenylserine, β-mercaptoethanol (2-ME), folic acid, PLP, Ponceau-S and EDTA were obtained from Sigma Chemical Company. H_4Folate was prepared by the method of Hatefi *et al.* (1959). All other chemicals were of analytical reagent grade. The mutant oligonucleotides were synthesized by Bangalore Genei Private Ltd., Bangalore, India. Centricon filters were from Amicon, Inc. Altered Sites II *in vitro* mutagenesis kit was purchased from Promega.

Bacterial strains and growth conditions. *E. coli* strain DH5α (BRL) was the recipient for all the plasmids used in subcloning. The BL21(DE3) pLysS strain (Studier & Moffat, 1986) was used for the bacterial expression of pETSH (Jagath-Reddy *et al.*, 1995) and mutant constructs. Luria-Bertani (LB) medium or terrific broth with 50 μg/ml of ampicillin was used for growing *E. coli* cells containing the plasmids (Sambrook *et al.*, 1989).

DNA manipulations. Plasmids were prepared by the alkaline lysis procedure as de-

scribed by Sambrook *et al.* (1989). Restriction endonuclease digestions, Klenow filling and ligations were carried out according to the manufacturers instructions. Preparation of competent cells and transformation was carried out by the method of Alexander (1987). The DNA fragments were eluted by the low melting agarose gel method from agarose gel (Sambrook *et al.*, 1989).

Site-directed mutagenesis. K256Q mutant was constructed using a PCR based megaprimer method as described earlier (Jagath-Reddy *et al.*, 1996). This mutant was constructed from pUCSH (containing the SHMT cDNA clone lacking 227 bp at 5′ end) as a template. The mutant oligonucleotide (20 mer) 5′ C ACC ACC CAC CAG ACC CTG C 3′ was used for the construction of the K256Q mutant. The full length PCR product, obtained upon two rounds of PCR was subcloned into pUC19 at *Kpn*I and *Bam*HI sites. The clones obtained after the mutagenesis procedure were screened by sequencing the gene at the mutated region. K256R mutant was generated using the Altered Sites II *in vitro* mutagenesis system. This mutant was constructed using a 20 mer mutagenic primer (5′ ACC ACC CAC AGG ACC CTG CG 3′) and the pALSH clone (SHMT cDNA clone lacking 227 bp at 5′ end in pALTER-1 vector) as a template. Initially, the clones were screened using ampicillin selection and later confirmed by DNA sequencing. pUC19 and pAlter-1 plasmids containing the mutated SHMT cDNA were purified and digested using the *Pma*CI and *Bam*HI restriction enzymes flanking K→Q and K→R mutations. The 738 bp *Pma*C–*Bam*HI mutated DNA fragments were gel purified and swapped at the same sites of pETSH vector and the presence of the mutation was confirmed by DNA sequencing. The entire 738 bp *Pma*CI–*Bam*HI DNA fragments of the K256Q and K256R mutant clones were sequenced using Sequenase™ version 2.0 DNA sequencing kit to rule out the presence of other non-specific mutations.

Expression and purification of K256 mutants. The expression and purification of the wild type (rSHMT) and the mutant enzymes was carried out essentially according to Jagath *et al.* (1997). Briefly, pETSH, K256R and K256Q constructs were transformed into BL21(DE3) pLysS strain and one litre of terrific broth containing 50 μg/ml of ampicillin was inoculated with overnight grown cells. The cells were harvested 3.5 h after induction with IPTG (0.3 mM). The cell pellets were resuspended in 150 ml of extraction buffer (50 mM potassium phosphate buffer, pH 7.4 containing 5 mM β-ME, 1 mM EDTA and 100 μM PLP) and sonicated. The supernatant was subjected to 0%–65% ammonium sulfate fractionation followed by chromatography using CM-Sephadex. Subsequently, the eluate was concentrated by ammonium sulfate precipitation and loaded onto Sephacryl S-200 column. The enzyme containing fractions were pooled and precipitated at 65% ammonium sulfate. The pellet was resuspended in buffer A (50 mM potassium phosphate buffer, pH 7.3, 1 mM β-ME, 1 mM EDTA) and dialyzed against 1 litre of the same buffer (with two changes) or buffer A containing 1 mM PLP and centricon filtered against the same buffer. Purification of the mutant proteins was also carried out, in the presence of glycine and PLP. In such cases, 100 mM glycine and 100 μM PLP was present in the buffer used for purification at all stages.

Enzyme assay. The wild type protein as well as the mutant proteins were assayed using L-[3-^{14}C]serine and H_4folate as substrates (Taylor & Weissbach, 1965). One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmole of HCHO/min at 37°C. The enzyme activity was also localized on 8% non-denaturing PAGE, and activity staining was carried out using β-phenylserine as a substrate (Ulevitch & Kallen, 1977). Protein estimation was carried out by the Folin-Lowry method using bovine serum albumin as a standard (Lowry *et al.*, 1951).

N-terminal sequencing. The mutant and the wild type proteins were analyzed by 10% SDS-PAGE, transferred onto PVDF membrane and stained with Ponceau-S. The protein bands were cut out and, after destaining, loaded on a Shimadzu-gas phase sequenator PSQ-1 to determine the N-terminal amino-acid sequence.

Size exclusion chromatography. In order to determine the oligomeric status of the mutant proteins, gel filtration studies were carried out using Superose-6 HR10/30 column on a Pharmacia FPLC system. The column was equilibrated with buffer A in the presence of 0.1 M KCl and calibrated using standard molecular markers: apoferritin (440 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa).

Spectrophotometric measurements. Absorption spectra of the wild type and mutant proteins were recorded on a UV-VIS 160A Shimadzu spectrophotometer at 25 ± 2°C. The enzyme solution (1 mg/ml) in the appropriate buffer solution was placed in a 1 ml cuvette and changes in absorbance were recorded between 300–550 nm upon the addition of 50 mM glycine and 0.18 mM H_4folate.

Circular dichroism. The far UV-CD spectra were recorded in the range 205–250 nm using a Jasco Model J-500A spectropolarimeter at 25 ± 2°C. Protein (0.2 mg/ml) in buffer A was placed in a cuvette with a pathlength of 1 mm. The molar ellipticity θ_{ME} was calculated using the formula

$$\theta_{ME} = \theta \times 100 \times (M_m)/(L \times C)$$

where θ is the observed ellipticity in degrees, L is the optical pathlength in decimeter, C is the concentration of the enzyme in mg/ml and M_m is the subunit molecular mass in daltons (52900).

Fluorescence measurements. Intrinsic tryptophan fluorescence spectra were recorded for the wild type and the mutant enzymes using Shimadzu RF-5000 spectrofluorimeter. Protein (0.1 mg/ml) in buffer A was excited at 280 nm. The emission spectra in the range of 300–400 nm were recorded at 25 ± 2°C.

Thermal denaturation. Melting temperature studies were carried out using a Gilford Response II spectrophotometer as described by Bhaskar *et al.* (1994). A clear protein solution of 400 µg/ml of the protein in 50 mM potassium phosphate buffer, pH 7.3 containing 1 mM EDTA and 1 mM β-ME was prepared. The protein (300 µl) was placed in thermal quartz cuvettes with the appropriate blanks and equilibrated to 30 °C to obtain the baseline. The samples were heated from 30°C to 80°C at a rate of 1°C/min using a programmable thermal control unit. The absorbance change in each case was monitored at 287 nm. The first derivative of the denaturation profile was used to evaluate the apparent melting temperatures (app. t_m) using the software supplied by the manufacturer. The results were analysed according to the method of White & Olsen (1987). The app. t_m was also determined in the presence of ligands *viz.* 100 mM serine or 0.1 mM PLP, respectively.

RESULTS AND DISCUSSION

Construction and expression of mutants

In addition to the desired single mutants K256R and K256Q, a double mutant T252I-K256Q was also obtained (Jagath-Reddy *et al.*, 1996) by the 3′-non templated 'A' addition by *Taq* polymerase. The presence of the mutation at the desired positions was confirmed by DNA sequencing. No other non specific changes in the sequence of the proteins were observed. As shown in Fig. 1 the mutant proteins were expressed at as high levels as the wild type and most of the expressed protein was in the soluble fraction. The double mutant was also present in the soluble fraction. However, it was not used for further studies.

Purification and properties of K256 mutants

The results of a typical purification of the expressed mutants are presented in Table 1. Both mutants showed, < 0.1% catalytic activity compared to the wild type rSHMT. The purity of the expressed proteins was confirmed by SDS-PAGE analysis (not shown). N-Terminal sequencing gave the sequence AAPVN.....which is the N-terminal sequence of rSHMT (Jagath-Reddy *et al.*, 1995). The mutant proteins along with the wild type rSHMT were electrophoresed on 8% non-de-

Figure 1. Expression of pETSH, K256Q and K256R mutant enzymes.

The mutants were obtained as described in the methods section. *E. coli* cells containing these plasmids were grown in and induced with IPTG. The cells were lysed as described in the text. Cell free extracts containing equal amounts of protein were subjected to SDS-PAGE analysis and the elctrophoresed gels were stained with Coomassie Brilliant Blue R-250. Lane 1, K256R; lane 2, K256Q; lane 3, T252I-K256Q; lane 4, pETSH; lane 5, pET-3e (expression vector not containing the SHMT gene); lane 6, molecular mass markers.

naturing gel in duplicate and subjected to protein and activity staining. As shown in Fig. 2, K256Q SHMT moved faster compared to rSHMT due to mutation of a positively charged residue to a neutral amino acid (K to Q). Upon activity staining, only rSHMT gave the yellow band of phenyl hydrazone confirming that the lysine mutants were catalytically incapable of carrying out the H_4folate independent cleavage reaction.

The far UV-CD spectra of the mutant and the wild type enzymes were recorded in the range of 205–250 nm. The mutant proteins did not show any qualitative change in the spectrum. However, K256Q SHMT showed a marginal difference in optical intensity compared to the wild type enzyme (Fig. 3).

The intrinsic fluorescence spectra of the two mutants and rSHMT were recorded as described in the Methods section. The wild type enzyme showed a maximum at 343 nm. The mutants showed a slight red shift in the emission maxima without any change in fluorescence intensity indicating that tertiary structures of the recombinant and mutant proteins were similar (not shown).

Catalytic properties of K256 mutant enzymes

It is evident from the data presented in the previous section that the mutation of K256 did not result in a gross structural change in the enzyme. However, the mutants were catalytically inactive compared to the wild type enzyme and were unable to catalyze aldol cleavage of serine (Table 1), H_4folate independent cleavage of β-phenylserine (Fig. 2) or transamination of D-alanine (not shown). The assays were also carried out with the protein concentration increasing to

Table 1. Purification of rSHMT and lysine mutants

Steps	rSHMT		K256Q		K256R	
	Total activity (Units)	Specific activity (Units/mg)	Total activity (Units)	Specific activity (Units/mg)	Total activity (Units)	Specific activity (Units/mg)
Crude extract	635	0.37	39	0.0205	29	0.0157
CM-Sephadex	163.8	3.52	0.22	0.0079	0.243	0.0063
Sephacryl S-200	132.3	5.01	0.168	0.0058	0.175	0.0053

Units: μmoles of HCHO formed/min at 37°C and pH 7.3.

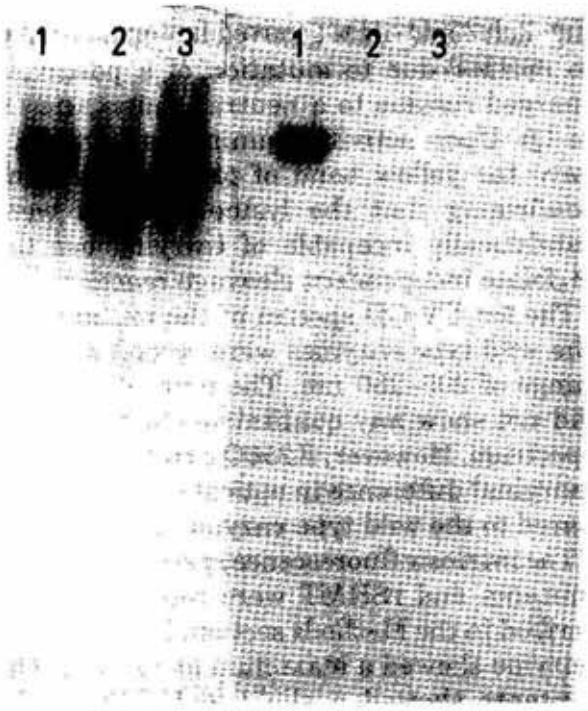

Figure 2. Native PAGE analysis of rSHMT, K256Q and K256R mutants.

The mutants were subjected to electrophoresis on 8% native gel for 36 h at 100 volts at 4°C. After electrophoresis, the gels were stained with Coomassie Brilliant Blue and a duplicate portion was stained using β-phenylserine. Lane 1, rSHMT; lane 2, K256Q SHMT; lane 3, K256R SHMT.

1 mg/ml to check the possibility of a slow turnover (Schirch *et al.*, 1993). The specific activities obtained were in the range of 0.005–0.008 units/mg indicating that the mutant enzymes were indeed catalytically crippled.

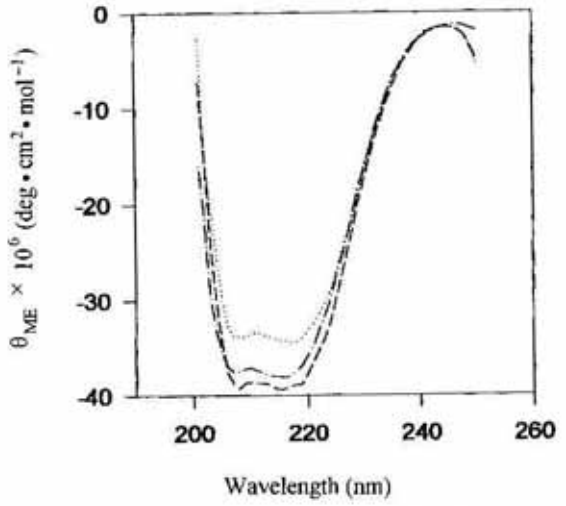

Figure 3. Far UV-CD spectrum of rSHMT and the lysine mutants.

The far UV-CD spectra were recorded using 0.2 mg/ml of the proteins as described in the text. Figure shows the spectra of rSHMT (—), K256R SHMT (– – –) and K256Q SHMT (· · ·).

A characteristic feature of all PLP-dependent enzymes is their ability to form the Schiff's base linkage with the cofactor *via* the ε-amino group of a lysine residue. The internal aldimine so formed shows an absorption maximum in the visible range. SHMT has an absorption maximum at 425 nm due to the presence of the internal aldimine. Upon addition of the substrate, glycine, three peaks corresponding to the geminal diamine (343 nm), the external aldimine (425 nm) and the quinonoid intermediate (495 nm) were observed. Addition of H_4folate led to a dramatic increase in absorbance at 495 nm (Usha *et al.*, 1992). As can be seen in Fig. 4a, rSHMT showed the characteristic 425 nm peak and the subsequent formation of the intermediates upon addition of glycine and H_4folate. However, K256R mutant protein failed to show the 425 nm peak indicating that the mutant enzyme was unable to bind PLP (Fig. 4a). Further addition of substrates did not give rise to any of the reaction intermediates, corroborating the activity data (Table 1). The above studies were also carried out with K256Q mutant protein and similar results were obtained (not shown). To test the possibility that sheep liver K256Q or K256R SHMT can also bind the external aldimine, the mutant proteins were purified and reconstituted in the presence of excess PLP (100 μM). However, no change in the spectra was observed except that a major peak corresponding to free PLP appeared at 390 nm. Even high amounts of protein (1 mg/ml) failed to produce any change in the enzymatic activity, unlike in the case of *E. coli* SHMT which was capable of carrying out one turnover of the reaction albeit slowly (Schirch *et al.*, 1993). The addition of other amines namely ammonium chloride, ammonium sulfate or methylamine did not facilitate the catalysis with serine and H_4folate, unlike it was in the case with D-amino acid transaminase (Futaki *et al.*, 1990).

In the case of K229Q mutant of *E. coli* SHMT, an absorbance peak at 425 nm was observed and was shown to be due to the formation of external aldimine with serine

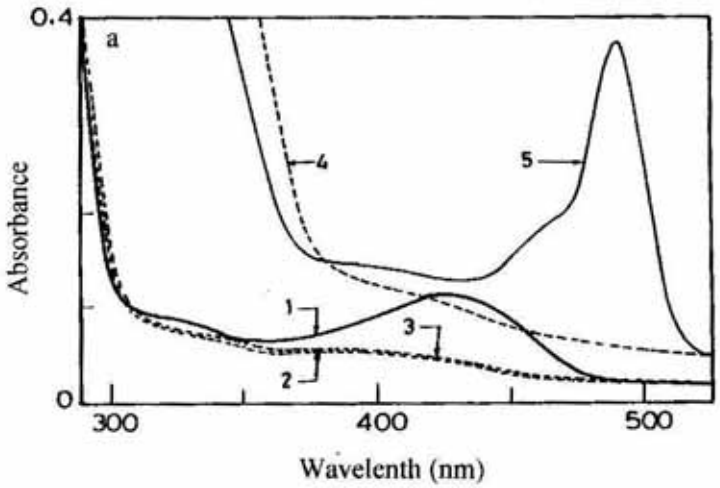

Figure 4a. Absorption spectra of K-256R, K256Q and rSHMTs.

The spectra of rSHMT (curve 1) K256R (curve 2) and K256Q (curve 3) in buffer A were recorded from 290 nm to 550 nm. Curves 4 and 5 show the effect of addition of 50 mM glycine and 0.18 mM H_4folate to K256R and rSHMT, respectively.

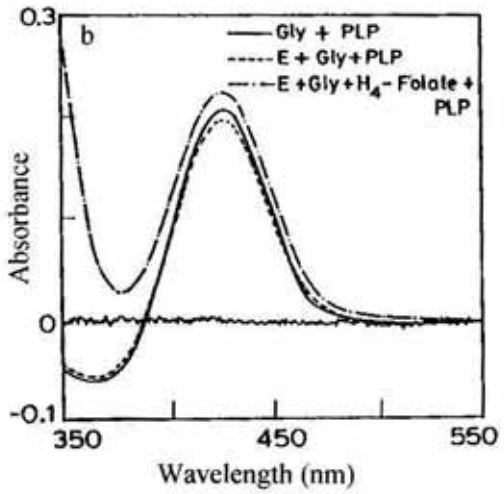

Figure 4b. Absorption spectra of K256R SHMT in the presence of non enzymatically formed external aldimine.

The reference cuvette contained PLP (100 µM) in buffer A. To the sample cuvette containing PLP in buffer A, 50 mM glycine was added and spectra recorded (—). K256R (1 mg/ml) was added to the sample cuvette and the spectra recorded (- - -). H_4Folate (0.18 mM) was added to the mixture of enzyme, glycine and PLP and spectra recorded (—·—).

and glycine (Schirch *et al.*, 1993). Efforts were also made to generate an external aldimine by carrying out the purification in the presence of 100 mM glycine and 100 µM PLP. However, in spite of the presence of the non-enzymatically formed external aldimine, the mutants failed to show catalysis upon addition of the substrate, emphasizing the absolute necessity of lysine residue at the active site for the catalysis to occur. In another experiment with K256R SHMT, first external aldimine (425 nm) was generated non-enzymatically by the addition of glycine and PLP as shown in Fig. 4b, then the mutant (1 mg) was added. No intermediate was detected suggesting that the mutant proteins were incapable of binding the external aldimine as well.

An important feature of reaction specificity of all PLP-dependent enzymes is the conversion from the open to the closed form following addition of the substrate. In the case of SHMT, the physiological reaction, namely, hydroxymethyl transfer with serine, is facilitated by the binding of serine to the active site and converting it from the open to the closed form. Table 2 depicts the thermal

Table 2. Thermal stability of rSHMT and lysine mutants in the presence of L-serine and PLP

Samples	Protein alone	Protein + 100mM serine	Protein + 0.1 mM PLP
rSHMT	56 ± 1°C	65 ± 1°C	57 ± 1°C
K256R	53 ± 1°C	55 ± 1°C	54 ± 1°C
K256Q	52 ± 1°C	55 ± 1°C	54 ± 1°C

melting temperatures of rSHMT and the mutant enzymes. It is evident that while serine enhanced the app. t_m of rSHMT, it was unable to cause a similar effect with K256Q and K256R SHMTs. The addition of PLP had no effect on the app. t_m of either rSHMT or the mutant enzymes. These results suggest that the mutant enzymes are incapable of undergoing the open to closed conformational change upon addition of ligands due to the absence of bound cofactor.

It was observed earlier in our laboratory that PLP plays an important role in the maintenance of oligomeric structure of SHMT (Brahatheeswaran *et al.*, 1996). Hence, it was of interest to examine the oligomeric status of the lysine mutants. The wild type and the mutant enzymes were subjected to gel filtration on a calibrated Superose 6 HR 10/30 column. The wild type rSHMT emerged as a single symmetrical peak with a retention volume of 15.2 ml, while K256R SHMT gave a broad peak with a retention volume of 15.56 ml and K256Q emerged as a single symmetrical peak at 16.2 ml. A plot of V_e/V_0 *vs* log molecular mass gave a value of about 212 kDa for rSHMT, consistent with its tetrameric nature while retention volume of 15.56 ml and 16.2 ml corresponded to a molecular mass of about 176 kDa and about 110 kDa, respectively. These results suggested that K256Q SHMT was in dimeric form in contrast to the tetrameric form of rSHMT. A value of about 176 kDa for K256R SHMT suggested that the mutation may have resulted in the formation of a mixture of tetramer and dimer which were in rapid equilibrium with each other. These studies were also carried out in buffer A containing 100 μM PLP. However, the presence of PLP failed to induce any change in the oligomeric structure of the proteins. It is therefore likely that mutation of lysine residue leading to the inability of the enzyme to bind to PLP could have resulted in the dissociation of the enzyme to the dimer rapidly in the case of K256Q and slowly in the case of K256R SHMT. The slow conversion of the tetrameric apoenzyme to reactivable and inactive dimers has also been observed with the sheep liver SHMT and also rSHMT (Jagath *et al.*, unpublished observations). It was therefore of interest to examine the stabilities of these proteins as a function of time. rSHMT (homotetramer) remained stable upon storage for a long time (> 2 months) as also was K256Q SHMT which was already a dimer upon purification. However, the K256R mutant enzyme dissociated gradually to the dimeric form upon storage (about 3 weeks).

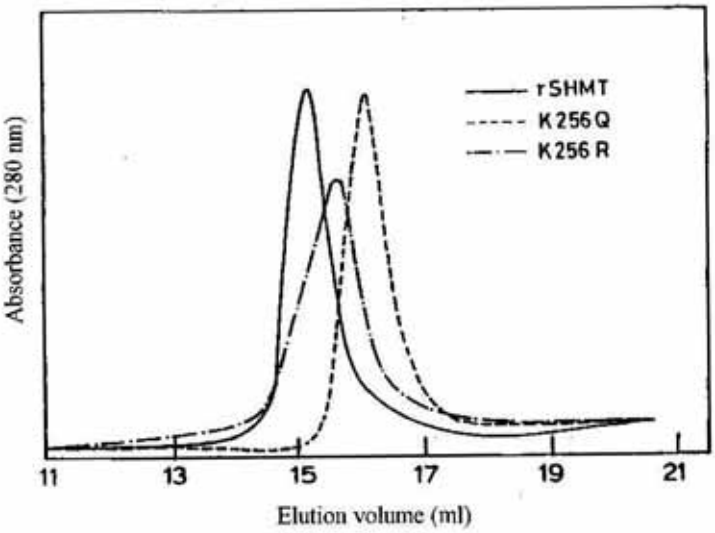

Figure 5. Gel permeation chromatography of rSHMT and lysine mutants.

rSHMT (–), K256Q SHMT (- - -) and K256R SHMT (—·—) were subjected to gel filtration on Superose 6 HR 10/30 column as described in the Methods section. 25 μg of protein in 200 μl was loaded on the column and the absorbance was measured at 280 nm.

Studies carried out on PLP dependent enzymes so far have implicated a role for PLP binding lysine either in proton abstraction (Lu *et al.*, 1993) or in expelling the product amino acid from the active site (Schirch *et al.*, 1993). The results presented in this paper demonstrate that the mutation of K256 in mammalian SHMT leads to loss of cofactor binding. As a consequence, the oligomeric structure of the enzyme is altered. The change in the oligomeric structure could, in turn, result in the inability of the mutant enzymes to bind to the external aldimine or to carry out one turnover reaction. Thus, neither the substrates nor the products bind to these mutant enzymes which are rendered completely inactive. These results emphasize the role of lysine-256 not only in binding to

PLP but also in maintaining the tetrameric structure of the enzyme essential for catalysis.

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