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Asp-89: a critical residue in maintaining the oligomeric structure of sheep liver cytosolic serine hydroxymethyltransferase

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Aspartate residues function as proton acceptors in catalysis and are involved in ionic interactions stabilizing subunit assembly. In an attempt to unravel the role of a conserved aspartate (D89) in sheep-liver tetrameric serine hydroxymethyltransferase (SHMT), it was converted into aspargine by site-directed mutagenesis. The purified D89N mutant enzyme had a lower specific activity compared with the wild-type enzyme. It was a mixture of dimers and tetramers with the proportion of tetramers increasing with an increase in the pyridoxal-5′-phosphate (PLP) concentration used during purification. The D89N mutant tetramer was as active as the wild-type enzyme and had similar kinetic and spectral properties in the presence of 500 μM PLP. The quinonoid spectral intermediate commonly seen in the case of SHMT was also seen in the case of D89N mutant tetramer, although the amount of intermediate formed was lower. Although the purified dimer exhibited visible absorbance at 425 nm, it had a negligible visible CD spectrum at 425 nm and was only 5 % active. The apo-D89N mutant tetramer was a dimer unlike the apo-form of the wild-type enzyme which was present predominantly as a tetramer. Furthermore the apo mutant dimer could not be reconstituted to the holo-form by the addition of excess PLP, suggesting that dimer–dimer interactions are weak in this mutant. The recently published crystal structure of human liver cytosolic recombinant SHMT indicates that this residue (D90 in the human enzyme) is located at the N-terminal end of the fourth helix of one subunit and packs against K39 from the second N-terminal helix of the other symmetry related subunit forming the tight dimer. D89 is at the interface of tight dimers where the PLP 5′-phosphate is also bound. Mutation of D89 could lead to weakened ionic interactions in the tight dimer interface, resulting in decreased affinity of the enzyme for the cofactor.

Key words: pyridoxal-5′-phosphate, site-directed mutagenesis, tetramer stability.

INTRODUCTION

Serine hydroxymethyltransferase (EC 2.1.2.1) (SHMT), a pyridoxal-5′-phosphate (PLP)-dependent enzyme, catalyses the reversible conversion of serine and tetrahydrofolate (H_4-folate) into glycine and 5,10-methylene H_4-folate [1]. This enzyme catalyses the first step in the pathway for the interconversion of folate co-enzymes and provides, almost exclusively (especially in mammalian systems), one-carbon fragments for the biosynthesis of a variety of end-products, such as DNA, RNA, ubiquinone, methionine etc. [2,3]. The importance of SHMT in DNA synthesis is reflected by the high levels of enzyme activity observed in rapidly proliferating cells compared with resting cells [4]. SHMT is a component of the thymidylate cycle along with dihydrofolate reductase and thymidylate synthase [2]. These features point to its potential as an alternate target for enzyme-directed cancer chemotherapy [2,5,6].

SHMT from mammalian sources is a homotetramer with a subunit molecular mass of $\sim$ 53 kDa and 4 mol of PLP per mol of enzyme [5–7], whereas the enzyme from prokaryotic sources is a homodimer [8]. The crystal structure of human liver cytosolic recombinant SHMT (hSHMT) showed that PLP is located at the tight dimer interface, as in the case of other PLP-dependent enzymes [6]. Alignment of SHMT sequences from a variety of sources with other PLP-dependent enzymes showed that it belongs to the group of fold type I PLP dependent enzymes [9]. Thus a comparative analysis of the properties of SHMT and its mutants from eukaryotic and prokaryotic sources would help in delineating the role of specific amino acids in catalysis, cofactor binding and maintenance of oligomeric structure. Mutation of K229 involved in forming a Schiff's base with PLP in *Escherichia coli* SHMT (eSHMT) [10] and of K256 in sheep liver cytosolic recombinant SHMT (rSHMT) [11] showed that this residue was essential for PLP binding and was not involved in proton abstraction [10,11], unlike in other PLP enzymes [12–15]. In addition, mutation of K256 leads to the dissociation of the tetramer to dimers in the case of rSHMT [11]. Mutation of the conserved histidine residue preceding the active-site lysine in eSHMT showed that this residue was involved not in proton abstraction but in reaction specificity [16]. Similarly, it was shown in eSHMT that the conserved T226 plays an important role in converting geminal diamine into the external aldimine [17]. It was shown that R363 in eSHMT and the corresponding residue R401 in rSHMT were essential for binding of substrate carboxy group [18,19]. Mutation of the conserved H134, H147 and H150 in rSHMT established their roles in the maintenance of oligomeric structure, cofactor binding and proton abstraction respectively [20]. Furthermore, the N-terminal arm was shown to be important for stabilizing the tetrameric structure of rSHMT [21]. These results are in conformity with the recently determined three-dimensional structure of hSHMT [6]. Asp-89 is a conserved residue among all the 35 SHMT sequences reported thus far and is located at the tight dimer interface [6]. We report, in this communication, that mutation of this residue in rSHMT results in the destabilization of the tetrameric enzyme and weakening of cofactor binding, leading to a decrease in activity.

Abbreviations used: SHMT, serine hydroxymethyltransferase; rSHMT, sheep liver cytosolic recombinant SHMT; hSHMT, human liver cytosolic recombinant SHMT; eSHMT, recombinant *Escherichia coli* SHMT; PLP, pyridoxal-5′-phosphate; H_4-folate, 5,6,7,8-tetrahydrofolate; 2-ME, 2-mercaptoethanol; PMP, pyridoxamine phosphate.

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EXPERIMENTAL

Materials

[α-^{32}P]dATP (300 Ci/mmol), L-[3-^{14}C]serine (55 mCi/mmol), restriction endonucleases, Sequenase™ version 2.0 DNA sequencing kit and T_4 DNA ligase were obtained from Amersham International (Little Chalfont, Bucks., U.K.). Sephacryl S-200, carboxymethyl (CM)-Sephadex, glycine L-serine, D-alanine, 2-mercaptoethanol (2-ME), folic acid, PLP, isopropyl β-D-thiogalactopyranoside and EDTA were obtained from Sigma Chemical Co (St. Louis, MO, U.S.A.). Platinum oxide was from Loba Chemie (Bombay, India). All other chemicals were of analytical-reagent grade. The oligonucleotides, custom synthesized, were purchased from Bangalore Genei Private Ltd (Bangalore, India).

Bacterial strains, growth conditions and DNA manipulations

E. col strain DH 5α (Bethesda Research Labs., Bethesda, MD, U.S.A.) was the recipient for all the plasmids used for DNA isolations and subcloning. BL 21 (DE3) pLysS [22] strain was used for the expression of rSHMT and D89N SHMT mutant clones. Luria–Bertani medium or terrific broth with 50 μg/ml ampicillin was used for growing *E. coli* cells containing the plasmids [23]. Plasmids were prepared by the alkaline lysis procedure described by Sambrook et al. [24]. The preparation of competent cells and transformation were performed by the method of Alexander [25]. The DNA fragments were eluted from low-melting-point agarose gels after electrophoresis [24].

Site-directed mutagenesis

The D89N SHMT mutant was constructed by a PCR-based megaprimer method [26,27]. The first PCR product (megaprimer) was obtained using the mutant oligonucleotide (5′GAA-TTCATCAATGAGCTAGAG 3′) and M13 (−24) universal reverse primer (5′GGAAACAGCTATGACCATG 3′). pUCSH (pUC19 containing the SHMT cDNA fragment lacking 227 bp at the 5′ end) was used as a template. The full-length PCR product was obtained using three primers, megaprimer (first PCR product), SHP1 (5′TATGGCAGCTCCAGTCAAC 3′, at the start of the gene) and the M13 (−24) universal reverse primer, using pETSH (containing a full-length cDNA for SHMT [23]) as a template. The full-length PCR product, obtained after two rounds of PCR, was subcloned into pUC19 at *Kpn*I and *Bam*HI sites. This clone was digested with *Kpn*I and *Pma*cI restriction enzymes to obtain the 520 bp fragment. The entire 520 bp fragment was sequenced using Sequenase™ version 2.0 DNA sequencing kit to confirm the mutation and to rule out the presence of non-specific mutations. The 520 bp fragment containing the mutated region was gel purified and swapped with the wild-type clone, i.e. pETSH, and the mutation was reconfirmed by sequencing of the expression construct.

Purification of wild-type and D89N SHMT

rSHMT (wild type) and D89N SHMT were overexpressed and purified as described previously [19]. Briefly, the purification procedure involved ammonium sulphate fractionation, CM-Sephadex chromatography followed by gel-filtration using a Sephacryl S-200 column. The wild-type and mutant enzymes were purified under identical conditions in buffers containing either 50 or 500 μM PLP. The final enzyme pellet was stored at 4 °C. When needed, the pellet was resuspended in buffer A (50 mM potassium buffer, pH 7.4/1 mM 2-ME/1 mM EDTA) dialysed against the same buffer. When the enzymes were purified in the presence of 500 μM PLP, the pellet was dissolved in buffer A containing 500 μM PLP and dialysed against the same buffer. The dialysed enzyme was used for further studies. Protein was estimated by measuring the absorbance at 280 nm, 1 absorbance unit was equivalent to 1.2 mg/ml [20].

Enzyme assay

The enzyme activity was estimated as described earlier [28,29], with minor modifications. Each 100 μl of assay mixture contained 400 mM potassium phosphate buffer, pH 7.4, with 1.8 mM dithiothreitol, no added PLP or 50 μM or 500 μM PLP, 1.8 mM H_4-folate and an appropriate amount of the enzyme. The reaction was started by the addition of 3.6 mM L-[3-^{14}C]serine (100 000 c.p.m.). After incubation for 15 min at 37 °C the reaction was stopped by the addition of 100 μl of dimedone (400 mM in 50 % ethanol). The reaction mixture was kept in a boiling-water bath for 5 min and the H-^{14}CHO-dimedone adduct was extracted into 3 ml of toluene. A 1 ml volume of toluene extract was added to 5 ml of scintillation fluid (0.6 % w/v 2,5-diphenyl oxazole in toluene) and radioactivity was measured in a Rack Beta 1209 liquid-scintillation counter (LKB Wallac, Wallac Oy, Finland). One unit of enzyme activity was defined as the amount of enzyme that catalysed the formation of 1 μmol of formaldehyde per min at 37 °C at pH 7.4. The specific activity was expressed as units per mg of protein. For the determination of K_m and k_{cat} values, the assay was carried out at various concentrations of serine (0–4 mM) and the kinetic parameters were calculated by least-squares fitting of the data on double-reciprocal plots.

Spectroscopy

The visible absorbance spectra were recorded at 25 °C in buffer A containing 500 μM PLP in a Shimadzu UV–visible (UV-160) spectrophotometer. The reference cuvette, when appropriate, also contained 500 μM PLP in buffer A. PLP has a non-specific absorbance at 425 nm. Therefore base-line correction was made before the addition of the enzyme. CD measurements were made with a Jasco-J-500A automated recording spectropolarimeter. The CD spectra were recorded at room temperature (25 °C) in buffer A containing 500 μM PLP, with the same buffer as blank.

Preparation of the apo-enzyme

Apo-enzyme of SHMT was prepared as described previously [29,30] with minor modifications. D-Alanine (200 mM) was added to the holo-enzyme in 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM 2-ME, 1 mM EDTA and 200 mM ammonium sulphate, and incubated at 37 °C for 4 h. The reaction mixture was rapidly dialysed in a Centricon tube at 4 °C for 1 h using buffer A not containing PLP. The pyruvate and pyridoxamine phosphate (PMP) formed in the reaction were removed by this procedure and the apo-enzyme had no absorbance at 425 nm.

Thermal stability

Thermal denaturation of rSHMT and D89N SHMT was performed in a Gilford Response II spectrophotometer from Ciba Corning Diagnostics (Oberlin, OH, U.S.A.) as described by Jagath et al. [21] with the following modifications. The protein samples (300 μl, 0.3 mg/ml) in buffer A, either without or with 500 μM PLP, were heated from 30 to 80 °C at the rate of 1 °C/min. The absorbance change in each case was monitored at 287 nm. The first derivative of the thermal-denaturation profile, obtained using the software supplied with the instrument, was

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used to evaluate the apparent transition temperature of both proteins. The apparent denaturation temperature (apparent T_m) is defined as the temperature at which the value of the fraction of the protein in the denatured state was 0.5. The thermal-denaturation profiles of rSHMT and D89N SHMT in the presence of serine in buffer A were similarly determined.

Size-exclusion chromatography

In order to determine the oligomeric status of the mutant, as well as to separate dimer and tetramer forms of the mutant, a calibrated TSK G 3000 SW gel-filtration column attached to a Shimadzu LC 6A HPLC system was used. Buffer A (with or without 500 μM PLP) was used as an eluent in this study.

RESULTS

Characterization of D89N SHMT

The visible CD spectra of rSHMT and D89N SHMT, purified through an S-200 gel-filtration column, are shown in Figure 1 (curves a and b). It is apparent that the visible CD spectrum at 425 nm (characteristic of the presence of PLP Schiff's base attached to the active site) of D89N SHMT was approx. 50 % of the value for rSHMT. One possible reason for this could be the loss of bound PLP during purification, as observed with other mutations such as H134 and H147 [20]. It was therefore of interest to compare the specific activities of the two enzymes purified in the presence of 50 or 500 μM PLP. D89N SHMT had a specific activity of 0.6 units/mg when purified in the presence of 50 μM PLP and assayed in buffers not containing PLP. When the concentration of PLP in the assay buffer was increased in the range 0–500 μM PLP, a maximal value of 1.6 units/mg was achieved at 50 μM and a higher concentration of PLP did not either enhance or decrease the specific activity. However, when D89N SHMT was purified in the presence of 500 μM PLP and assayed in buffer containing 500 μM PLP, the specific activity was 2.9 units/mg of protein. rSHMT, purified in the presence of 50 or 500 μM PLP, had a specific activity of 4 and 4.2 units/mg respectively (Table 1). These results suggested that the higher concentration of PLP (500 μM) is essential during the purification of the D89N SHMT for obtaining maximal activity of the enzyme; this was also reflected in the k_{cat} and k_{cat}/K_m values obtained from the corresponding double-reciprocal plots (Table 1).

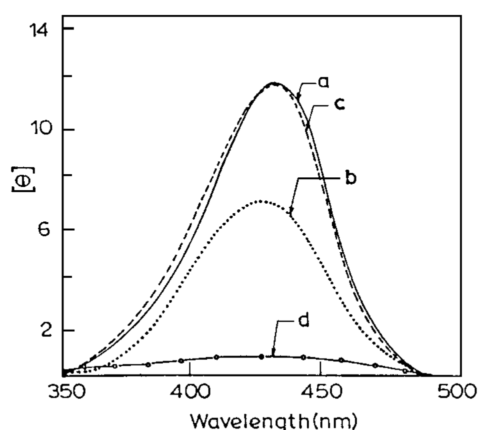

Figure 1 Visible CD spectra of rSHMT and tetramer and dimer of D89N SHMT

rSHMT and the mutant enzyme (subunit concentration, 18.8 μM) in buffer A containing 500 μM PLP (at 25 °C) were use to record the CD spectra from 300–500 nm using a Jasco J-500A automated recording spectropolarimeter. Curve a, rSHMT; curve b, D89N SHMT (as purified in the presence of 50 μM PLP, mixture of tetramer and dimer); curve c, D89N SHMT (tetramer); curve d, D89N SHMT (dimer).

Oligomeric status of D89N SHMT

The results presented in the previous section, as well as earlier observations on rSHMT and its mutants [19–21], prompted a more detailed examination of the oligomeric status of D89N SHMT. The wild-type and mutant enzymes were purified in the presence of 50 μM PLP as described in the Experimental section, and the enzymes, after S-200 column chromatography, were analysed on a calibrated HPLC TSK G-3000 column. rSHMT was eluted as a single symmetrical peak with a retention volume of 12.7 ml (Figure 2, curve a), corresponding to a molecular mass of 220 kDa (tetramer). On the other hand, the D89N SHMT mutant enzyme eluted as two peaks with retention volumes of 12.71 ml and 14.45 ml respectively, corresponding to tetrameric and dimeric forms of SHMT (Figure 2, curve b). When D89N SHMT was purified in an identical manner, but in the presence of 500 μM PLP, and subjected to HPLC, it gave two peaks, corresponding to tetrameric and dimeric forms (Figure 2, curve c). However, the tetramer was present in a higher proportion (70 %) compared with the dimer (30 %), suggesting that excess PLP may be stabilizing the tetrameric structure of D89N SHMT.

Activity measurements

Results presented in Figure 2 indicate that D89N SHMT is present as a mixture of tetramer and dimer. It was therefore of interest to separate the tetramer and dimer and examine their catalytic properties. The peak fractions corresponding to tetramer and dimer were collected and concentrated by Centricon (30 kDa cut-off) filtration. The concentrated samples were re-chromatographed on a TSK G-3000 SW gel-filtration column and were found to be homogeneous (Figure 2B inset: a, tetramer; b, dimer). rSHMT was similarly processed and served as a control. The specific activity of the purified D89N SHMT tetramer in the absence of PLP in the assay buffer was 2.03 units/mg and it increased to 4.05 units/mg in the presence of 500 μM PLP. The dimer had a specific activity of 0.05 units/mg and 0.2 units/mg in the absence and presence of 500 μM PLP respectively. The dimeric form was only 5 % as active as the wild-type enzyme. It was of interest to calculate the specific activity of the preparations, which contain dimers and tetramers in different proportions (Figure 2, curves b and c). When the mutant enzyme was purified in the presence of 50 μM PLP the tetramer and dimer were in the proportions 30 % and 70 %, with a specific activity of 1.6 units/mg; when the enzyme was purified in the presence of 500 μM PLP the ratio was 70:30 with a specific activity of 2.94 units/mg. The calculated specific activity of 1.6 units/mg and 2.94 units/mg corresponded to the amount of tetramer present in the two preparations. The K_m with respect to serine and k_{cat} for the D89N SHMT tetramer were similar to those of rSHMT (Table 1). As the activity of the dimer was very low and it was available in limited quantities, the kinetic parameters could not be evaluated with confidence. The far-UV CD spectra of tetrameric and dimeric D89N SHMT and rSHMT indicated that mutation of the D89 residue did not result in any significant change in the secondary structure of the protein (results not shown).

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Table 1 Kinetic properties of rSHMT (wild type) and D89N SHMT

Enzyme	Specific activity (units/mg)*		K_m (serine) (mM)		k_{cat} (s^{-1})†		k_{cat}/K_m (s^{-1} mM^{-1})	
	None‡	+PLP§	None	+PLP	None	+PLP	None	+PLP
rSHMT	4.00	4.20	0.90	0.92	4.20	4.32	4.70	4.70
D89N SHMT (purified in 50 μM PLP)	0.60	1.60	1.10	1.10	1.30	2.30	1.18	2.09
D89N SHMT (purified in 500 μM PLP)	–	2.94	1.10	1.10	–	3.00	–	2.70
D89N SHMT (tetramer)	2.03	4.05	1.10	1.10	2.13	3.90	1.19	3.50

* 1 unit = 1 μmole of HCHO formed per min at 37 °C.
† Calculated per mole of subunit.
‡ None, enzyme purified using buffers containing 50 μM PLP and dialysed against buffer A without PLP and assayed in the absence of PLP.
§ 500 μM PLP added in the assay buffer.

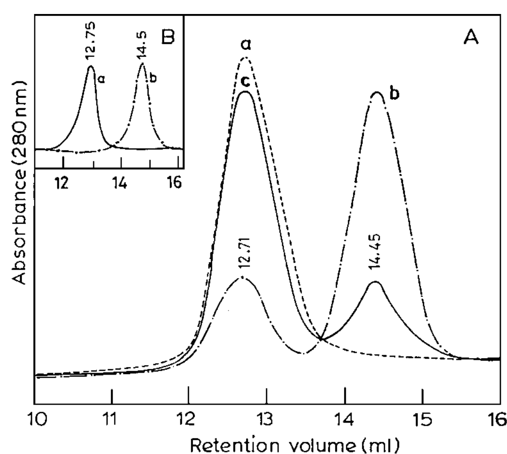

Figure 2 Size-exclusion chromatography profiles of rSHMT and D89N SHMT

(**A**) Wild-type and D89N SHMT enzymes (subunit concentration, 1.88 μM) after Sephacryl S-200 gel-filtration chromatography were loaded on to a TSK-G 3000 size-exclusion chromatography column attached to a Shimadzu LC 6A HPLC instrument with an on-line UV detector (SPD 6AV). The column was equilibrated with buffer A for curves a and b, and buffer A containing 500 μM PLP was used to generate curve c. The flow rate was 1 ml/min. The column was calibrated using standard protein molecular-mass markers. Curves a and b: rSHMT and D89N SHMT respectively, purified in the presence of 50 μM PLP and dialysed against buffer A not containing PLP. Curve c, D89N purified in the presence of excess PLP (500 μM) and dialysed against buffer A containing 500 μM PLP. (**B**) (inset) purified tetramer and dimer of D89N SHMT (1.88 μM) were re-chromatographed to check their purity. Curve a, tetramer; curve b, dimer.

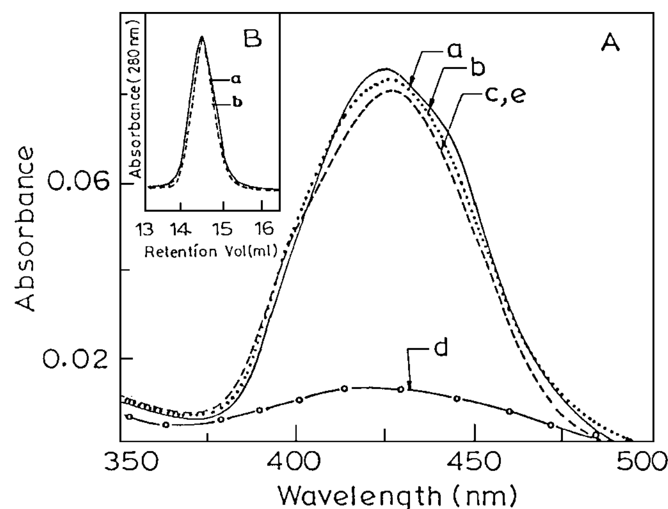

Figure 3 Visible absorbance spectra of rSHMT, D89N SHMT (tetramer) and D89N SHMT (dimer)

(**A**) Visible absorbance spectra of the proteins (subunit concentration, 9.4 μM) in buffer A containing 500 μM PLP were recorded within a Shimazdu UV–visible spectrophotometer at 25 °C. The reference cuvette contained 500 μM PLP in buffer A. Curve a, rSHMT; curve b, D89N SHMT (tetramer); curve c, D89N SHMT (dimer). Curve d, D89N SHMT (dimer) dialysed against buffer A not containing PLP. Curve e, enzyme preparation used in curve d + 500 μM PLP. (**B**) (inset) size-exclusion chromatography of D89N SHMT dimer on a TSK G-3000 column. Curve a, D89N SHMT dimer dialysed against buffer A not containing PLP (——) and loaded after pre-equilibration with buffer A. Curve b, D89N SHMT dimer after dialysis was reconstituted with excess PLP (500 μM) in buffer A (----) and loaded after pre-equilibration with buffer A containing 500 μM PLP. The protein concentration was 1.88 μM.

Visible absorbance and CD spectra

D89N SHMT tetrameric (Figure 3A, curve b) and dimeric (Figure 3A, curve c) forms isolated in the presence of 500 μM PLP showed visible absorbance maxima at 425 nm in buffer A containing 500 μM PLP, indicating the presence of an internal aldimine in both cases as well as in rSHMT (Figure 3A, curve a). When the dimer was dialysed against buffer A not containing PLP, the internal aldimine spectrum disappeared (Figure 3A, curve d), suggesting that the PLP was weakly bound. Similar dialysis experiments did not cause any change in the spectrum of rSHMT. However, dialysis of the D89N SHMT tetramer led to $\sim$ 50 % loss of absorbance at 425 nm, which was regained upon the addition of 500 μM PLP (results not shown). Addition of 500 μM PLP to the dialysed dimer also restored the absorbance at 425 nm (Figure 3A, curve e). As shown in Figure 1, curve a, the PLP bound at the active site of rSHMT gives a characteristic CD spectrum at 425 nm. The purified D89N SHMT tetramer also gave a visible CD spectrum, similar to that of rSHMT (Figure 1, curve c), whereas the dimer had a very negligible CD signal at

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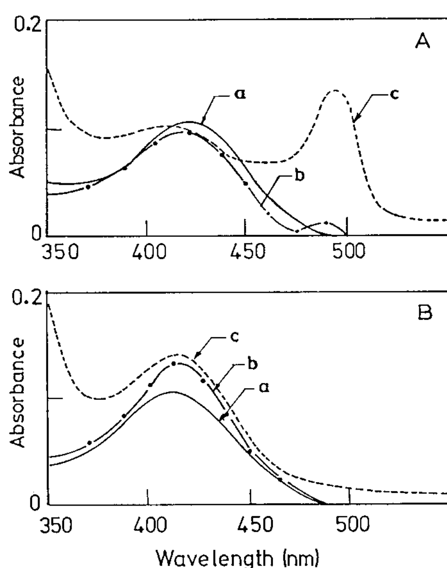

Figure 4 Spectral intermediates in the reaction of D89N SHMT (tetramer) and D89N SHMT (dimer) with glycine and H_4-folate

The spectral intermediates formed upon addition of ligands to the enzyme (subunit concentration, 9.4 μM) was recorded in buffer A containing 500 μM PLP with a Shimadzu UV–visible spectrophotometer at 25 °C from 350 nm to 550 nm. (**A**) D89N SHMT (tetramer): curve a, D89N SHMT (tetramer) enzyme only; curve b, enzyme + 200 mM glycine; curve c, enzyme + 200 mM glycine + 0.4 mM H_4-folate. (**B**) D89N SHMT (dimer): curve a, D89N SHMT (dimer) enzyme only; curve b, enzyme + 200 mM glycine; curve c, enzyme + 200 mM glycine + 0.4 mM H_4-folate.

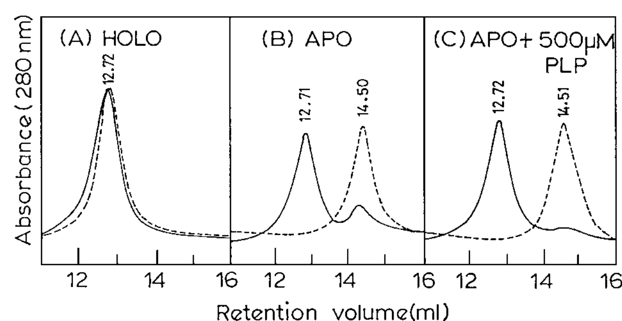

Figure 5 Gel-filtration profiles of rSHMT and D89N SHMT

Size-exclusion chromatography was performed by loading 1.88 μM enzyme on a TSK G3000 column as described in the legend of Figure 2. (**A**) Holo-enzymes of rSHMT (——) and D89N SHMT tetramer (----). (**B**) Apo-enzymes of rSHMT (——) and D89N SHMT tetramer (----) prepared by reaction with D-alanine. (**C**) Reconstituted rSHMT (——) and D89N SHMT tetramer (----) with 500 μM PLP.

425 nm (Figure 1, curve d). Dialysis of the D89N SHMT tetramer against buffer not containing PLP resulted in the loss of CD signal at 425 nm, which was regained upon the addition of PLP (500 μM) (results not shown). On the other hand, the dialysed dimer did not show a CD signal at 425 nm, even in the presence of 500 μM PLP, although the absorbance at 425 nm was restored (Figure 3, curve e). Furthermore, the oligomeric status of the D89N SHMT dimer was unaltered even in the presence of 500 μM PLP (Figure 3B: a, in the absence of PLP; b, in the presence of PLP).

Spectral intermediates

Whereas the D89N tetramer was fully active, the dimer had very little activity. This required an examination of the intermediates in the reaction to assess the effects of mutation on the enzyme. It can be seen from Figure 4(A) that the addition of glycine to the D89N SHMT tetrameric form of the enzyme (curve a) resulted in a marginal decrease in absorbance at 425 nm (curve b), with an indication of the formation of geminal diamine having absorbance at 343 nm (results not shown). The addition of H_4-folate to the above mixture resulted in an increase in the concentration of the quinonoid intermediate with absorbance at 495 nm (curve c). The molar absorption coefficient for the quinonoid intermediate was calculated to be 29255 $M^{-1} \cdot cm^{-1}$ for rSHMT (subunit concentration, 1 M) in the presence of 500 μM PLP. The amount of intermediate formed in the case of the D89N tetramer was calculated to be 0.48 μM/μM subunit using this value. Thus, the concentration of the intermediate is 50 % of that observed for rSHMT. However, the k_{cat} value for the D89N mutant tetramer (3.9 s^{-1}) was similar to that of the wild-type enzyme (4.32 s^{-1}), suggesting that the mutation has probably not significantly affected catalysis. When a similar experiment was carried out with the D89N SHMT dimer, the addition of glycine did not cause the expected decrease in absorbance at 425 nm, but resulted in an increase in absorbance (Figure 4B, curve b). The reasons for this increase is unclear. However, the addition of H_4-folate did not result in the expected increase in absorbance at 495 nm due to the formation of the quinonoid intermediate.

Thermal stability of mutant and wild-type enzymes

SHMT has been shown to undergo a change in conformation from an open to a closed form upon addition of serine, which results in an increase in the apparent T_m value by 5–10 °C [31]. Though the D89N SHMT tetramer is catalytically as active as rSHMT (Table 1), its apparent T_m value (54 °C) was lower than that of rSHMT (68 °C) in the presence of 500 μM PLP. The addition of serine to the D89N tetramer and rSHMT markedly increased the apparent T_m values to 68 °C and 74 °C respectively. There was no change in the apparent T_m values of the dimer in the presence or absence of serine, suggesting that serine probably failed to interact with the dimer.

Removal of PLP from D89N SHMT and its reconstitution

It has been well established that PLP plays a crucial role in maintaining the oligomeric structure of SHMT in addition to its function in catalysis [32]. SHMT catalyses the transamination of D-alanine to pyruvate and the bound PLP is converted into PMP, which can be removed by dialysis or by Centricon filtration to obtain the apo-enzyme. This reaction can be monitored by measuring the increase in absorbance at 325 nm due to the formation of PMP or by the decrease in absorbance at 425 nm due to disruption of the PLP Schiff's base at the active site. The rate constants, for reaction with D-alanine, monitored by the decrease in absorbance at 425 nm at 25 °C, were 0.31 min^{-1} and 0.18 min^{-1} for D89N SHMT tetramer and rSHMT respectively. When PLP from the D89N SHMT tetramer (Figure 5A, broken line) was removed by transamination with D-alanine, as described in the Experimental section, it was eluted at a position corresponding to the dimeric form of SHMT (Figure 5B, broken line), whereas rSHMT was eluted predominantly as a tetramer with a small amount of dimer (Figure 5B, solid line). Addition of PLP had no effect on the oligomeric status of apo-D89N SHMT mutant (Figure 5C, broken line), whereas the addition of PLP to

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apo-rSHMT led to conversion of even the small amounts of the dimer into the tetrameric form (Figure 5C, solid line).

DISCUSSION

Aspartate residues in several enzymes function to stabilize protein structure and are involved in interactions that lead to communication between subunits in an oligomeric protein [33]. They also function as proton acceptors and can bind to cofactors [34]. A comparison of primary sequences of SHMT isolated from a number of sources revealed that D89 was conserved among all the SHMTs, suggesting that it could have an important function either in maintaining the oligomeric structure or in catalysis. The recently published crystal structure of the tetrameric hSHMT [6] indicates that this residue (D90 in hSHMT) is located at the N-terminal end of the fourth helix and packs against K39 from the second N-terminal helix of a symmetry related subunit. Results presented in this paper demonstrate that mutation of D89 to N could lead to a weakening of the forces stabilizing the tetrameric structure, probably due to loss of this intersubunit ionic interaction. As the tetrameric structure is stabilized by additional interactions, especially by binding of the cofactor [6,35,36], the effect of the mutation can be partly overcome by increasing the concentration of PLP during the purification and assay. Observations in support of this suggestion are discussed below.

It is evident from Figure 2 that D89N SHMT, as isolated, is a mixture of tetramers and dimers, with the proportion of tetramer increasing when purified in the presence of 500 μM PLP, whereas rSHMT is purified as a tetramer at all concentrations of PLP with no indication of dimeric species. This suggests that the mutation of D89 affects the stability of the tetramer. The D89N SHMT mutant enzyme, purified in the presence of 50 μM PLP and assayed in the absence of PLP, was only 14 % as active as rSHMT (Table 1). However, when it was purified in the presence of 500 μM PLP and assayed in the presence of 500 μM PLP, its activity was increased to 70 % of that of rSHMT. To resolve the question of whether higher concentrations of PLP were required for increasing the activity or for enhancing the stability of the enzyme, the activity of D89N SHMT was measured at various concentrations of PLP. It was found that 50 μM PLP was sufficient for observing maximum activity (40 %). This would suggest that the mutant enzyme was probably saturated with PLP in this concentration range. A comparison of the activities of the separated tetramer and dimer would provide some clues to the role of PLP in stabilizing the enzyme or in catalysis. It is evident from the results (Table 1) that the D89N SHMT tetramer (in the presence of 500 μM PLP) was as active as rSHMT, whereas the dimer was only marginally (5 %) active. The k_{cat} and k_{cat}/K_m values of the D89N tetramer were comparable with those of rSHMT in the presence of 500 μM PLP (Table 1). These results suggest that a higher concentration of PLP was required both for catalysis and for stabilizing the tetrameric structure of the mutant enzyme. The orientation of PLP in the tetramer, as monitored by visible CD (Figure 1b), indicated that it was in the correct orientation for catalysis. The quinonoid spectral intermediate, commonly seen in the equilibrium mixture of SHMT, glycine and H_4-folate, was shown to have an absorption coefficient of 50000 $M^{-1} \cdot cm^{-1}$ in the case of rabbit liver [37] and pig liver [38] SHMTs. In the case of rSHMT the value was calculated to be 29255 $M^{-1} \cdot cm^{-1}$ in presence of 500 μM PLP. Based on this value, the concentration of the quinonoid intermediate in the D89N mutant tetramer was found to be 0.48 μM. This would suggest that the step subsequent to formation of the quinonoid intermediate is probably faster in the case of the mutant, although this may not be the rate-limiting step as the k_{cat} values for the mutant tetramer and wild-type enzymes are similar in the presence of 500 μM PLP. It is therefore evident that the mutation has probably not affected catalysis. The dimer was also capable of binding PLP in the correct stoichiometric amount (Figure 3c), but there was no significant visible CD signal (Figure 1d), suggesting that the PLP is probably not in the proper configuration. In situations where the dimer and tetramer were present (Figure 2), the activity of the mixture correlated very well with the concentration of the tetramer (Table 1).

Additional evidence in support of the suggestion that PLP binding is affected by mutation is provided by the decreased apparent T_m for the mutant in the presence of 500 μM PLP and serine, compared with rSHMT. In addition, the rate constant for the removal of PLP from the active site by reaction with D-alanine (0.31 min^{-1}) is higher than for rSHMT (0.18 min^{-1}). More convincing evidence for the role of PLP and D89 in maintaining the tetrameric structure was the observation that upon removal of PLP, the D89N tetramer dissociated to a dimer, whereas the rSHMT was present essentially as a tetramer. The small amount of dimer present along with tetramer in the case of apo-rSHMT was converted back into a fully active tetramer. On the other hand, the dimer formed from the D89N SHMT tetramer by removal of PLP could not be reconstituted upon the addition of PLP, demonstrating the role of D89 in maintaining the tetrameric structure of SHMT (Figure 5).

Although the molecular co-ordinates of the X-ray structure of SHMT [6] are not yet in the public domain, based on the published crystal structure of hSHMT it can be suggested that mutation of D89 in sheep liver SHMT could lead to disruption of ionic interaction with K38 from a symmetry related subunit, resulting in weakening of tight dimer interactions and consequently decreased affinity for PLP.

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