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Importance of the amino terminus in maintenance of oligomeric structure of sheep liver cytosolic serine hydroxymethyltransferase

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The role of the amino and carboxyl-terminal regions of cytosolic serine hydroxymethyltransferase (SHMT) in subunit assembly and catalysis was studied using six amino-terminal (lacking the first 6, 14, 30, 49, 58, and 75 residues) and two carboxyl-terminal (lacking the last 49 and 185 residues) deletion mutants. These mutants were constructed from a full length cDNA clone using restriction enzyme/PCR-based methods and overexpressed in *Escherichia coli*. The overexpressed proteins, des-(A1–K6)-SHMT and des-(A1–W14)-SHMT were present in the soluble fraction and they were purified to homogeneity. The deletion clones, for des-(A1–V30)-SHMT and des-(A1–L49)-SHMT were expressed at very low levels, whereas des-(A1–R58)-SHMT, des-(A1–G75)-SHMT, des-(Q435–F483)-SHMT and des-(L299–F483)-SHMT mutant proteins were not soluble and formed inclusion bodies. Des-(A1–K6)-SHMT and des-(A1–W14)-SHMT catalyzed both the tetrahydrofolate-dependent and tetrahydrofolate-independent reactions, generating characteristic spectral intermediates with glycine and tetrahydrofolate. The two mutants had similar kinetic parameters to that of the recombinant SHMT (rSHMT). However, at 55 °C, the des-(A1–W14)-SHMT lost almost all the activity within 5 min, while at the same temperature rSHMT and des-(A1–K6)-SHMT retained 85% and 70% activity, respectively. Thermal denaturation studies showed that des-(A1–W14)-SHMT had a lower apparent melting temperature (52 °C) compared to rSHMT (56 °C) and des-(A1–K6)-SHMT (55 °C), suggesting that N-terminal deletion had resulted in a decrease in the thermal stability of the enzyme. Further, urea induced inactivation of the enzymes revealed that 50% inactivation occurred at a lower urea concentration (1.2 ± 0.1 M) in the case of des-(A1–W14)-SHMT compared to rSHMT (1.8 ± 0.1 M) and des-(A1–K6)-SHMT (1.7 ± 0.1 M). The apoenzyme of des-(A1–W14)-SHMT was present predominantly in the dimer form, whereas the apoenzymes of rSHMT and des-(A1–K6)-SHMT were a mixture of tetramers (≈75% and ≈65%, respectively) and dimers. While, rSHMT and des-(A1–K6)-SHMT apoenzymes could be reconstituted upon the addition of pyridoxal-5′-phosphate to 96% and 94% enzyme activity, respectively, des-(A1–W14)-SHMT apoenzyme could be reconstituted only upto 22%. The percentage activity regained correlated with the appearance of visible CD at 425 nm and with the amount of enzyme present in the tetrameric form upon reconstitution as monitored by gel filtration. These results demonstrate that, in addition to the cofactor, the N-terminal arm plays an important role in stabilizing the tetrameric structure of SHMT.

Keywords: sheep cytosolic serine hydroxymethyltransferase; N-terminal deletion mutant; C-terminal deletion mutant; overexpression; subunit assembly.

Serine hydroxymethyltransferase (SHMT), a pyridoxal-5′-phosphate (pyridoxal-*P*)-dependent enzyme, plays a crucial role in one-carbon metabolism and provides precursors for the biosynthesis of purines, thymidylate, methionine, etc. [1]. The physiological reaction catalyzed by this enzyme is the reversible conversion of serine and 5,6,7,8-tetrahydrofolate (H_4-folate) to glycine and 5,10-methylene-H_4-folate. The enzyme exhibits broad substrate and reaction specificity [2]. The mammalian cytosolic enzyme is a tetramer of identical subunits of 53 kDa with 4 mol pyridoxal-*P*/mol enzyme [3]. The sheep liver cytosolic enzyme has been extensively characterized in our laboratory with respect to its primary structure [4], catalysis [5, 6], thermal stability [7] and interaction with inhibitors [8–10]. This enzyme has been cloned and overexpressed in *Escherichia coli* [11]. Recently, it has been shown that pyridoxal-*P* has an important role in the stabilization of quaternary structure [12].

In pyridoxal-*P*-dependent enzymes, limited proteolysis results in the formation of a core protein devoid of N-terminus which is either partially active [13], inactive [14] or fully active [15, 16]. In the case of porcine cytosolic aspartate aminotransferase (AAT), the deletion of nine amino acids from the amino-terminus by oligonucleotide-directed mutagenesis resulted in a striking decrease in its activity and thermal stability [17]. The

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Abbreviations. SHMT, serine hydroxymethyltransferase; H_4-folate, 5,6,7,8-tetrahydrofolate; pyridoxal-*P*, pyridoxal 5′-phosphate; pyridoxamine-*P*, pyridoxamine 5′-phosphate; rSHMT, sheep liver cytosolic recombinant SHMT; AAT, aspartate aminotransferase; buffer A, 50 mM potassium phosphate, pH 7.4, 1 mM 2-mercaptoethanol, 1 mM EDTA; buffer B, buffer A with 50 μM pyridoxal-*P*.

Enzymes. Serine hydroxymethyltransferase (EC 2.1.2.1); aspartate aminotransferase (EC 2.6.1.1).

N-terminal region was shown to play a role in the inter-subunit interactions of porcine cytosolic AAT [17]. It was evident from the crystallographic data that several pyridoxal-*P*-requiring enzymes have the same spatial fold as AAT even though these proteins have less than 15% sequence identity [18–20].

The alignment of SHMT sequences from several sources revealed that the N- and C-termini were less conserved compared to rest of the protein [4]. It was therefore of interest to examine the role of these terminal regions of the protein on its structural and functional properties. By limited proteolytic digestion, it was shown that the N-terminal 15 residues may not be important for the catalytic activity ([16] and Bhaskar et al., unpublished results). A disadvantage of the partial proteolysis procedure is the difficulty in obtaining pure preparations of the truncated protein as the cleaved peptides often associate non-covalently with the core molecule. To circumvent this problem, we decided to generate the deletions at 5′ and 3′ ends of the cDNA clone of SHMT and express these deletion clones. In this paper, we report the construction, expression, purification, characterization of deletion mutants of the sheep liver cytosolic SHMT and also show the importance of N-terminal arm in the stabilization of the dimer–dimer interactions which are crucial for tetramer formation and cofactor binding.

EXPERIMENTAL PROCEDURES

Materials. [α-^{32}P]dATP (3000 Ci/mmol) and L-[3-^{14}C]serine (55 mCi/mmol) were obtained from Amersham International. Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs and Amersham International. Blue-Sepharose, CM-Sephadex and Sephacryl S-200 were obtained from Pharmacia Fine Chemicals. All other biochemicals used in this study were obtained from Sigma Chemical Company. H_4-folate was prepared by the method of Hatefi et al. [21]. The oligonucleotides were synthesized by Bangalore Genei Private Ltd., (Bangalore, India). Centricon filters were from Amicon Inc.

Bacterial strains and growth conditions. *E. coli* strain DH5α (BRL) was the recipient for all the plasmids used in subcloning. The BL21(DE3), BL21(DE3) pLysS strains [22] were used for the bacterial expression of pETSH and deletion clones. Luria-Bertani medium or terrific broth with 50 μg/ml of ampicillin was used for growing *E. coli* cells containing the plasmids [23].

DNA manipulations. Plasmids were prepared by the alkaline lysis procedure as described by Sambrook et al. [23]. Restriction endonuclease digestions, Klenow filling and ligations were performed according to the manufacturer's instructions. The preparation of competent cells and transformation were performed by the method of Alexander et al. [24]. From the agarose gel, the DNA fragments (> 200 bp) were eluted by Gene Clean II Kit (Bio 101), < 200 bp in size by Mermaid Kit (Bio 101) or by low-melting agarose gel method [23].

Construction of N and C-terminal deletion clones. A common vector for the expression of mutant clones was prepared by the following method. pET-3c vector [25] has two *Nhe*I sites. To make a unique *Nhe*I site which is next to the ATG, the second *Nhe*I site was removed by deleting a 200-bp fragment by *Eco*RV digestion and self ligation. This vector was designated as pET-3e. The pET-3e plasmid was digested with *Nhe*I, Klenow-filled, then cut with *Bam*HI and gel purified as described in the previous section.

pETSH expression construct [11] was used for all the DNA manipulations. This pETSH clone was cleaved by *Nde*I, *Kpn*I and *Bam*HI into two fragments i.e. a 1.26-kb *Kpn*I-*Bam*HI as constant fragment and a 230-bp of *Nde*I–*Kpn*I as variable fragment. For convenient handling, the *Nde*I–*Kpn*I insert size was increased by releasing the insert either with *Sph*I–*Eco*RI (560 bp) or *Bgl*II–*Eco*RI (350 bp). The *Sph*I–*Eco*RI and *Bgl*II–*Eco*RI fragments were gel purified and used for further modifications. The *Sph*I–*Eco*RI fragment has an unique *Ava*II site at position 45 bp from the ATG codon. To obtain a 14-amino-acid deletion clone, the *Sph*I–*Eco*RI fragment was digested with *Ava*II, Klenow filled followed by *Kpn*I digestion. This *Ava*II–*Kpn*I 180-bp fragment along with *Kpn*I–*Bam*HI 1.26-kb constant fragment was ligated to pET-3e vector at *Bam*HI and end-filled *Nhe*I sites. This construct was designated as pSN2 (Δ14). Similarly, a *Bgl*II-*Eco*RI 370-bp fragment was used in the construction of pSN3 (Δ30) and pSN4 (Δ49) deletion clones. In order to obtain a large N-terminal deletion (Δ75), pETSH was digested with *Acc*65I, Klenow filled, followed by the *Bam*HI digestion. This 1.26-kb end-filled *Acc*65I–*Bam*HI fragment was cloned into the *Bam*HI and end-filled *Nhe*I sites of pET-3e vector (designated as pSN6). pSC2 (Δ185) C-terminal deletion clone was constructed by cloning the *Bgl*II–*Stu*I 1.0-kb fragment at the *Bgl*II and end-filled *Xba*I sites of pETSH vector. Two oligonucleotides were designed, namely SHP1a (5′ TG GCA CCC AGA GAT GCC GAT 3′) primer for a six amino-acid deletion (excluding Met1) and SHP1b (5′ TG GCT GTT CTG GAG GCC CTA 3′) primer for a 58-amino-acid deletion (excluding Met1). PCR was performed using Vent DNA polymerase as described in New England Biolabs product specifications with the following modifications. For a 100-μl reaction, 10 ng pETSH plasmid, 0.15 μM of each primer (SHP1a and SHM1 [11]) and 0.4 mM of each dNTP were used. The sample was incubated in a thermal cycler (MJ Research) at 94 °C for 4 min then kept for 25 cycles of amplification. Each cycle included 1-min denaturation (94 °C), 1-min annealing (50 °C) and 1.5-min chain elongation (72 °C). After 25 cycles, the reaction was extended for an additional 5 min at 72 °C. The 1.43-kb PCR product was gel purified from the agarose gel. This product was digested with *Kpn*I and the 210-bp fragment was gel purified and cloned at *Kpn*I and end-filled *Nde*I sites of pETSH vector, designated as pSN1 (Δ6). Similarly the PCR was performed using SHP1b and SHM1 primers and the 1.30-kb PCR product was gel purified. This was digested with *Kpn*I and the 52-bp fragment was cloned at the *Kpn*I and end-filled *Nde*I sites of pETSH vector, designated as pSN5 (Δ58). The clone pSC1 (Δ49) was constructed by using SHP1a and SHM2 (5′ TACAGGATCCTAGATCTGCAGGGTCA 3′) with a stop codon at 435th amino acid followed by a *Bam*HI site, as primers. The cloning junctions and the presence of deletion for all the above deletion clones were confirmed by restriction enzyme digestions and DNA sequencing [26].

Expression of N- and C-terminal deletion clones in BL21 (DE3) pLysS. BL21(DE3) pLysS strain with the deletion constructs, pETSH, pET-3e plasmids were grown in 25 ml Luria-Bertani medium containing 50 μg/ml of ampicillin and induced by addition of (0.3 mM) isopropyl 1-thio-β-galactopyranoside. The total, soluble and insoluble extracts were analyzed on SDS/PAGE [27].

Protein sequencing. The expressed proteins were analyzed on SDS/PAGE and transferred to a Hybond Poly(vinylidene difluoride) membrane. The proteins were stained with Ponceau S and the appropriate bands were cut out, loaded on to a Shimadzu gas-phase sequenator PSQ1 to determine the N-terminal sequence of the mutant proteins [28].

Purification of sheep liver cytosolic recombinant SHMT (rSHMT), des-(A1–K6)-SHMT and des-(A1–W14)-SHMT enzymes. pETSH, pSN1 and pSN2 plasmids were transformed into BL21 (DE3) pLysS strain. A single colony was inoculated

in 50 ml Luria-Bertani medium containing 50 μg/ml ampicillin and grown overnight at 37 °C. 4% of the cells were inoculated into 1 l terrific broth media containing 50 μg/ml ampicillin. After 3.5 h of growth at 37 °C, the cells were induced for 4 h with 0.3 mM isopropyl 1-thio-β-galactopyranoside. The cells were harvested and the cell pellet was resuspended in 150 ml extraction buffer (50 mM potassium phosphate, pH 7.4, 5 mM 2-mercaptoethanol, 1 mM EDTA and 100 μM pyridoxal-*P*). The cell suspension was left at room temperature for 1 h and sonicated until it was optically clear. The sonicate was centrifuged at 27000×*g* for 20 min. The supernatant was subjected to 25–65% ammonium sulfate fractionation, blue-Sepharose and Sephacryl S-200 column chromatography as described earlier [11]. The active Sephacryl S-200 fractions were pooled and subjected to 0–65% ammonium sulfate precipitation. The pellet obtained was resuspended in buffer A (50 mM phosphate, pH 7.4, 1 mM 2-mercaptoethanol, 1 mM EDTA) and extensively dialyzed against 1 l of the same buffer (with two changes) for 16 h. The dialyzed sample was used as the enzyme in these studies.

The determination of the isoelectric point (pI value) for rSHMT, des-(A1–K6)-SHMT and des-(A1–W14)-SHMT helped us in the design of a new purification protocol for the rSHMT and des-(A1–W14)-SHMT. The new protocol involved the use of CM-Sephadex instead of blue-Sepharose as reported earlier [11]. The crude extracts for rSHMT and des-(A1–W14)-SHMT were prepared as described before. The crude extracts were subjected to 0–65% ammonium sulfate precipitation, the pellet obtained was resuspended in 20 ml 20 mM potassium phosphate, pH 6.4, 1 mM 2-mercaptoethanol, 1 mM EDTA and 50 μM pyridoxal-*P* and then dialyzed for 24 h against the same buffer (1 l with two changes). The dialyzed sample was centrifuged at 27000×*g* for 10 min and the supernatant was loaded on to a CM-Sephadex column (2×20 cm) which was equilibrated with 20 mM potassium phosphate, pH 6.4, 1 mM 2-mercaptoethanol, 1 mM EDTA and 50 μM pyridoxal-*P*. The column was washed with same buffer (1 l) and the bound enzyme was eluted with 100 ml 200 mM potassium phosphate, pH 7.4, 1 mM 2-mercaptoethanol, 1 mM EDTA and 50 μM pyridoxal-*P*. The eluted sample was subjected to 0–65% ammonium sulfate precipitation. The pellet obtained was resuspended in 4 ml 200 mM potassium phosphate, pH 7.4, 1 mM 2-mercaptoethanol, 1 mM EDTA, 50 μM pyridoxal-*P* and subjected to Sephacryl S-200 column chromatography as described earlier [11]. Fractions containing enzyme were pooled and precipitated at 65% ammonium sulfate and the pellet was resuspended in buffer A and dialyzed against 1 l of the same buffer (with two changes) for 16 h and this enzyme preparation was used in these studies.

Enzyme assay. SHMT activity was assayed using [3-^{14}C] serine and H_4-folate as substrates [29, 30]. One unit (U) was defined as the amount of enzyme catalyzing the production of 1 μmol HCHO · min^{-1} at 37 °C. Proteins were analyzed on a 6% non-denaturing PAGE. SHMT activity staining was performed using β-phenylserine as substrate [31]. Protein was estimated using BSA as a standard [32] for the determination of the specific activity.

Isoelectric focussing. Isoelectric focussing was performed on 5% polyacrylamide gel plates between pH 3.5–10, at 2000 V, 50 mA and 30 W according to the manufacturer's instructions (Pharmacia Fine Chemicals). The gel was calibrated with the standard marker proteins (pI 3.5–10). The purified rSHMT, des-(A1–K6)-SHMT and des-(A1–W14)-SHMT samples were applied (20 μl of 1.5 mg/ml) on the gel. The proteins were fixed in 10% trichloroacetic acid and stained with Coomassie brilliant blue R-250 after subjecting to isoelectrofocussing.

Circular dichroism. Circular dichroic (CD) measurements were made in Jasco-J-500 A automated recording spectropolarimeter. All CD spectra were recorded at 22±2 °C in buffer A using the same buffer as blank. Far-ultraviolet CD studies were performed at a protein concentration corresponding to 0.1 A_{280} unit/ml (where 1 A_{280} unit is the amount giving an absorbance of 1 at 280 nm in 1 ml solution in a 1-cm light path). The CD spectra obtained were plotted as molar ellipticity assuming a relative subunit molar mass of 52900 g/mol for the rSHMT, 52319 g/mol for des-(A1–K6)-SHMT and 51622 g/mol for des-(A1–W14)-SHMT. The subunit molar masses were calculated from their respective amino acid sequences. $\theta_{ME} = (\theta m^\circ \times M)/c \times d$ where θm° = observed ellipticity, M = subunit molar mass, c = concentration of protein in mg/ml and d = path length in dm.

Visible CD spectra were recorded in a Jasco-J-20 C automated recording spectropolarimeter. The protein concentration corresponding to 1 A_{280} unit/ml in buffer A was used. The molar absorption coefficients for rSHMT, des-(A1–K6)-SHMT and des-(A1–W14)-SHMT were calculated from the amino acid sequence by the method of Gill and von Hippel [33] as 168325.2 M^{-1} cm^{-1}, 178968.8 M^{-1} cm^{-1}, 145320.5 M^{-1} cm^{-1}, respectively; the concentration of these proteins was calculated using the absorption coefficients.

Inactivation of the SHMTs by heat and urea. rSHMT, des-(A1–K6)-SHMT and des-(A1–W14)-SHMT (125 μg/ml) were incubated in buffer B (50 mM potassium phosphate pH 7.4, 1 mM 2-mercaptoethanol, 1 mM EDTA, 50 μM pyridoxal-*P*) at 50 °C or 55 °C. The aliquots (10 μl) were withdrawn at 0, 5, 10 and 15 min, cooled to 0 °C and assayed for residual enzyme activity [30]. They were also incubated at 12.5 μg/ml in buffer B with urea (0–2 M) at 30±2 °C for 2 h. An aliquot (80 μl) of this reaction mixture was used directly for enzyme assay by the addition of L-[3-^{14}C]serine (10 μl) and H_4-folate (10 μl) [30].

Thermal denaturation studies. The thermal denaturation of rSHMT, des-(A1–K6)-SHMT and des-(A1–W14)-SHMT was performed in a Gilford Response II spectrophotometer from Ms. Ciba Corning Diagnostics, as described by Bhaskar et al. [7] with the following modifications. The protein samples (250 μl of 0.3 mg/ml) in buffer A were heated from 30 °C to 80 °C at the rate of 1 °C/min using the software built into the instrument. The absorbance change in each case was monitored at 287 nm and data averaged from two experiments. The first derivative of the thermal denaturation profiles was used to evaluate the apparent transition temperatures of the three proteins. Similar conditions were used to monitor the thermal denaturation profiles of rSHMT and des-(A1–W14)-SHMT in the presence of serine, glycine and alanine (100 mM) and pyridoxal-*P* (50 μM) using the buffer blank in the reference cuvette with the same ligand concentration.

Apoenzyme preparation. Apoenzymes of rSHMT, des-(A1–K6)-SHMT and des-(A1–W14)-SHMT were prepared as described earlier [34] with minor modifications. D-Alanine (200 mM) was added to the holoenzyme (1 mg/ml) in 50 mM potassium phosphate pH 7.4, 10 mM 2-mercaptoethanol, 1 mM EDTA, 200 mM ammonium sulfate and incubated at 37 °C for 4 h. The pyruvate and pyridoxamine 5′-phosphate (pyridoxamine-*P*) formed during the reaction were removed by passing the sample through a 30-kDa Centricon filter.

Size-exclusion chromatography. To determine the native molecular mass of rSHMT, des-(A1–K6)-SHMT and des-(A1–W14)-SHMT, a Superose 6 HR 10/30 analytical gel filtration column was used on a Pharmacia FPLC system. The column was calibrated with the standard proteins apoferritin (440 kDa), sheep cytosolic SHMT (213 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa). The buffer used for this study was buffer A containing 0.1 M KCl.

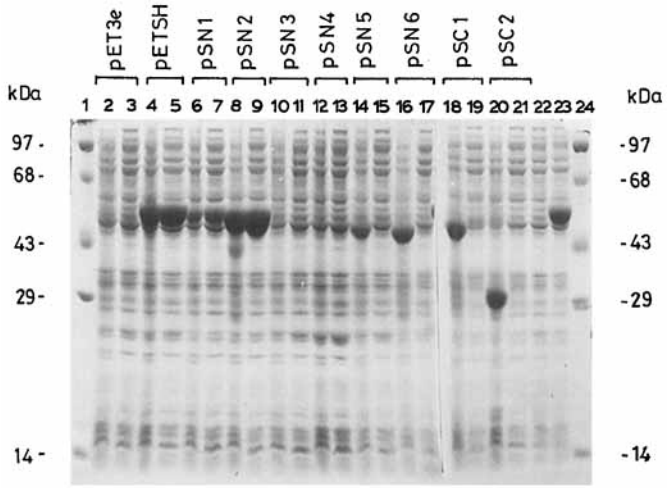

Fig. 1. The SDS/PAGE analysis of the expressed proteins of N- and C-terminal deletion clones of SHMT. Aliquots equivalent to 0.15 and 0.25 A_{600} units of *E. coli* cells were used for the analysis of total and soluble proteins, respectively. Lanes 1 and 24, molecular mass markers; lane 2, total protein and lanes 3 and 22 soluble proteins of pET-3e; lane 4, total protein and lanes 5 and 23, soluble proteins of pETSH; lanes 6, 8, 10, 12, 14, 16, 18, 20 total proteins and lanes 7, 9, 11, 13, 15, 17, 19, 21 soluble proteins of pSN1, pSN2, pSN3, pSN4, pSN5, pSN6, pSC1 and pSC2, respectively. The gels were stained with Coomassie brilliant blue R-250.

The molecular masses of the holo, apo and reconstituted holoenzymes of these proteins were determined on calibrated TSK-G 3000 SW gel filtration column connected to Shimadzu LC6A HPLC instrument with an on-line ultraviolet detector (SPD 6AV). The tetramer/dimer percentages were calculated by using area normalization method on HPLC-Chromatopac CR6A and the amount of sample in each peak was expressed as a percentage of the total. The column was equilibrated with buffer A containing 0.1 M KCl.

RESULTS

Construction and overexpression of N- and C-terminal deletion clones of SHMT. Using restriction enzyme and PCR-based methods, plasmids pSN1 (Δ6), pSN2 (Δ14), pSN3 (Δ30), pSN4 (Δ49), pSN5 (Δ58), pSN6 (Δ75) representing N-terminal deletions and pSC1 (Δ49), pSC2 (Δ185) of C-terminal deletions in the cDNA clone of sheep cytosolic SHMT were constructed. Restriction enzyme digestions and DNA sequencing of these clones confirmed the presence of appropriate deletion, ribosome binding site (Shine Dalgarno sequence), start (ATG) and stop (TAG) codons in all the deletion clones.

As shown in Fig. 1, des-(A1−K6)-SHMT (lane 7) and des-(A1−W14)-SHMT (lane 9) were present in the soluble extract. Although the expressed protein in the case of des-(A1−R58)-SHMT (lane 14), des-(A1−G75)-SHMT (lane 16), des-(Q435−F483)-SHMT (lane 18) and des-(L299−F483)-SHMT (lane 20) were present in the total extract, it could not be detected in the soluble fraction (lanes 15, 17, 19 and 21, respectively). These proteins were present in the insoluble fraction. The insoluble extracts were also enzymatically inactive. Unlike these mutant proteins, des-(A1−V30)-SHMT (lanes 10, 11) and des-(A1−L49)-SHMT (lanes 12, 13) were not present at detectable levels in any of the fractions and were barely detectable on western blots.

Protein sequencing. N-terminal amino acid sequencing showed that deletions of 6, 14, 58 and 75 amino acids had occurred in the protein expressed by pSN1, pSN2, pSN5 and pSN6 clones, respectively (Table 1). It can be seen that des-(A1−W14)-SHMT and des-(A1−G75)-SHMT had two additional amino acids (Ala-Arg) at the N-terminus compared to their expected deletions. These additional amino acids were derived from the vector during subcloning as indicated by DNA sequencing of the pSN1 and pSN6 clones.

Enzyme purification and characterization. rSHMT, des-(A1−K6)-SHMT and des-(A1−W14)-SHMT were purified as described [11] with the following modifications in the use of *E. coli* strain and growth medium. In this study, BL21(DE3) pLysS strain instead of BL21(DE3) was used [11] as this strain facilitated easy disruption of *E. coli* cells due to the presence of lysozyme. The terrific broth medium instead of Luria-Bertani medium was used for growing *E. coli*. The cells grown in 1 l terrific broth yielded 40−50 mg purified protein compared to only 10 mg protein from 1 l Luria-Bertani medium. As shown in Fig. 2B, compared to rSHMT, des-(A1−K6)-SHMT had a greater mobility and des-(A1−W14)-SHMT a lower mobility towards the anode on non-denaturing PAGE. The change in the net charge on the protein was confirmed by measuring the pI value by isoelectric focussing experiments, the values being 6.85, 5.85 and 7.12, respectively. The information on the pI values facilitated the standardization of the new protocol using CM-Sephadex at pH 6.4 for the purification of rSHMT and des-(A1−W14)-SHMT. The enzymes thus purified were homogeneous on non-denaturing PAGE, SDS/PAGE (Fig. 2) and amino acid sequencing. des-(A1−K6)-SHMT and des-(A1−W14)-SHMT were able to perform H_4-folate-dependent aldol cleavage of serine and H_4-folate, independent reactions with β-phenyl-

Table 1. N-terminal amino acid sequence of the deletion mutants of sheep liver cytosolic SHMT.

Protein	N-terminal amino acid sequence
rSHMT	AAPVNKAPRDADLWSLHEK.....RAVLE.....EGYPGQ..... 1 7 15 59 76
Des-(A1−K6)-SHMT	APRDADLWSLHEK.....RAVLE.....EGYPGQ..... 7 15 59 76
Des-(A1−W14)-SHMT	ARSLHEK.....RAVLE.....EGYPGQ..... 15 59 76
Des-(A1−R58)-SHMT	AVLE.....EGYPGQ..... 59 76
Des-(A1−G75)-SHMT	ARYPGQ..... 76

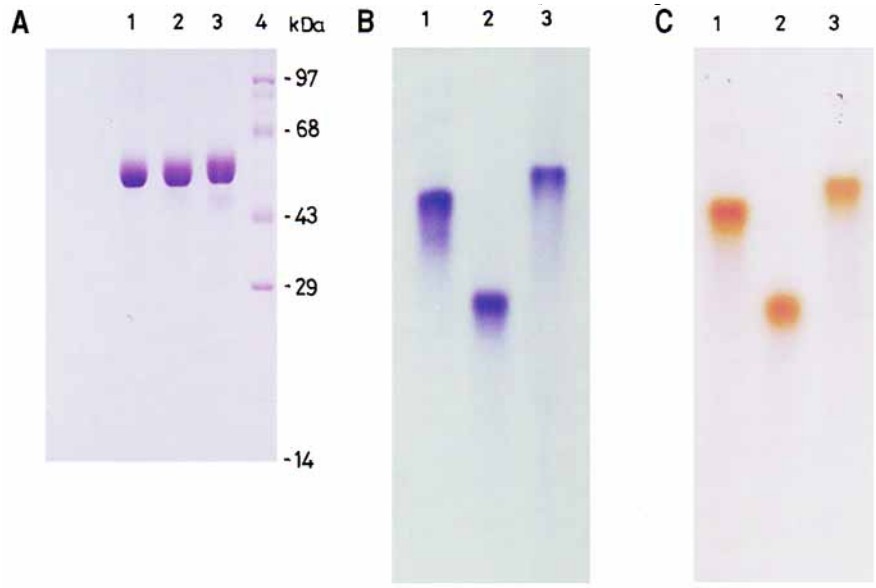

Fig. 2. SDS/PAGE and non-denaturing PAGE analysis of rSHMT, des-(A1–K6)-SHMT and des-(A1–W14)-SHMT. The proteins were purified as described in the Experimental Procedures. (A) SDS/PAGE analysis. Lane 1, des-(A1−W14)-SHMT; lane 2, des-(A1−K6)-SHMT; lane 3 rSHMT; all 30 μg. The non-denaturing PAGE of rSHMT (50 μg; lane 1), des-(A1−K6)-SHMT (50 μg; lane 2) and des-(A1−W14)-SHMT (45 μg; lane 3) in duplicate lanes was performed in 6% gels at 4°C for 30 h using 100 V current. A part of the gel (B) was stained with Coomassie and a second part (C) of the gel was stained for the enzyme activity as described in the Experimental Procedures.

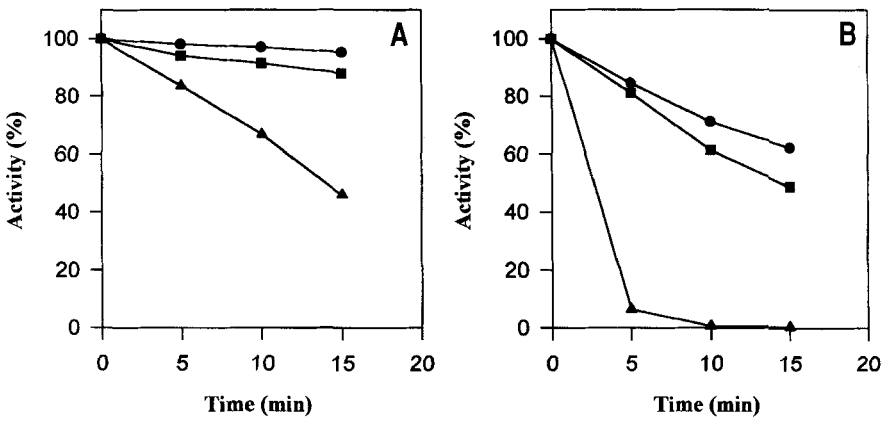

Fig. 3. Thermal inactivation of rSHMT (●), des-(A1–K6)-SHMT (■) and des-(A1–W14)-SHMT (▲). The three proteins (125 μg/ml) were incubated in buffer B at 50°C (A) or 55°C (B) for 5, 10, 15 min. Aliquots (10 μl) were withdrawn and immediately cooled to 0°C. The residual enzyme activity was assayed [30]. Activity at 0 time was normalized to 100%.

serine and D-alanine as substrates. The enzymes were capable of forming 343-nm-absorbing geminal diamine, 425-nm-absorbing external aldimine and 495-nm-absorbing quinonoid spectral intermediate species. Des-(A1−K6)-SHMT (0.9 mM, 0.95 mM) and des-(A1−W14)-SHMT (0.9 mM, 0.9 mM) had similar K_m values for serine and H_4-folate, respectively, compared to rSHMT (0.9 mM, 1.0 mM). They also had similar specific activities, i.e. 5.2, 5.5 and 4.9 units/mg, respectively. There were no marked differences in the secondary structure between the three proteins as monitored by CD spectra in the range of 195–250 nm.

Oligomeric structure. rSHMT, des-(A1−K6)-SHMT and des-(A1−W14)-SHMT proteins were subjected to chromatography on a Superose-6 HR 10/30 analytical gel filtration column, as described in Experimental Procedures and the native molecular mass of these proteins was calculated from a plot of relative elution volume versus the log (molecular mass) of standard protein markers. The values obtained were 225 kDa, 213 kDa and 190 kDa, respectively. These results suggested all these enzymes are homotetramers.

Temperature stability studies. rSHMT, des-(A1−K6)-SHMT and des-(A1−W14)-SHMT (125 μg/ml) were incubated at 50°C and 55°C for 0, 5, 10 and 15 min and the residual enzyme activity was determined. It is evident from Fig. 3A that incubation at 50°C for 15 min, resulted in a minimal loss of activity of rSHMT and des-(A1−K6)-SHMT, whereas des-(A1−W14)-SHMT lost >50% activity under similar conditions. When the temperature was increased to 55°C, the latter lost all its activity (within 5 min), whereas the other two retained more than 80% of their activity at this time interval, although increasing periods of incubation at 55°C resulted in a progressive loss of activity (Fig. 3B).

The thermal denaturation profile of the three enzymes showed that while rSHMT and des-(A1−K6)-SHMT behaved similarly, the midpoint of denaturation curve for des-(A1−W14)-SHMT was at lower temperature. The apparent melting

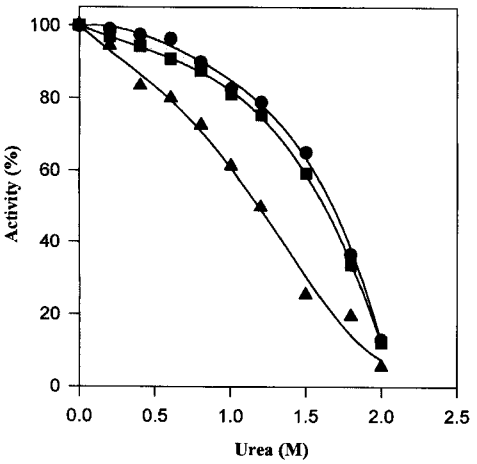

Fig. 4. Effect of urea on the oligomeric structure and activity of rSHMT (●), des-(A1–K6)-SHMT (■) and des-(A1–W14)-SHMT (▲). All the enzymes (12.5 μg/ml) were incubated in buffer B at urea concentrations indicated in the figure for 2 h at 30 ± 2 °C. Aliquots (80 μl) were withdrawn and assayed for residual activity by addition of 10 μl each of 36 mM L-[3-^{14}C]serine and 18 mM H_4-folate as described [30]. The enzyme activity determined in the absence of urea served as a control and this activity was normalized to 100%.

Table 2. Thermal stability of rSHMT and des-(A1–W14)-SHMT in the presence of ligand. The apparent melting points were determined from the first derivative plots of the denaturation curves (see text for details).

Ligand	Concn	Apparent melting point	
		rSHMT	des-(A1–W14)-SHMT
	mM	°C	
–		56	52
Serine	100	65	56
Glycine	100	58	55
D-Alanine	100	56	51
Pyridoxal-*P*	0.05	58	54

temperatures values calculated from the first derivative plot were 56 °C, 55 °C and 52 °C for rSHMT, des-(A1–K6)-SHMT and des-(A1–W14)-SHMT, respectively. It was observed that serine has a significant effect on the thermal stability of SHMT and shifted the enzyme from an 'open' to a 'closed' form, whereas other ligands such as threonine, alanine, etc., had no significant effect [7, 35]. It was therefore essential to compare the effect of the ligands on the thermal denaturation of these three proteins. Serine (100 mM) enhanced the apparent melting temperature from 56 to 65 °C for rSHMT, whereas it increased only from 52 °C to 56 °C for des-(A1–W14)-SHMT. Other ligands such as glycine (100 mM), D-alanine (100 mM) and pyridoxal-*P* (50 μM) caused either marginal increase or no change (Table 2).

Denaturation of rSHMT, des-(A1–K6)-SHMT and des-(A1–W14)-SHMT with urea. Urea-induced inactivation of these three proteins was performed as described in Experimental Procedures. Enzyme activity was measured after incubation with various concentrations of urea (0–2 M). The mid points of the inactivation for rSHMT, des-(A1–K6)-SHMT and des-(A1–W14)-SHMT were 1.8 ± 0.1 M, 1.7 ± 0.1 M and 1.2 ± 0.1 M, respectively (Fig. 4).

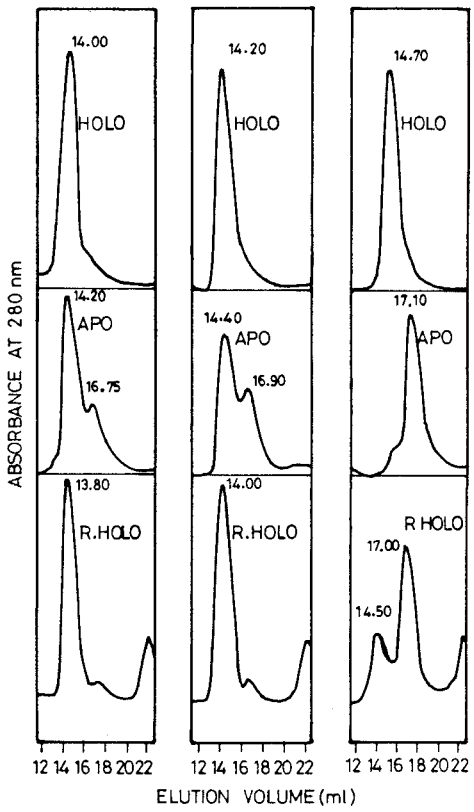

Fig. 5. Size-exclusion chromatography of holo, apo and reconstituted holo enzymes of rSHMT (left), des-(A1–K6)-SHMT (middle) and des-(A1–W14)-SHMT (right). All the enzyme forms (300 μg/ml) in buffer A were chromatographed on a TSK-G 3000 size-exclusion chromatography column attached to Shimadzu LC6A HPLC instrument with an on-line ultraviolet detector (SPD 6AV). The column was equilibrated with buffer A containing 0.1 M KCl. The same buffer was passed through the column at a flow rate of 1 ml/min. The elution profiles are shown in the figure. Peak I and peak II correspond to tetramer and dimer of SHMT, respectively.

The role of pyridoxal-*P* on the oligomeric structure of rSHMT, des-(A1–K6)-SHMT and des-(A1–W14)-SHMT. The apoenzymes were prepared by incubating the proteins with D-alanine (200 mM) for 4 h at 37 °C followed by Centricon filtration to remove pyridoxamine-*P* and pyruvate formed in the reaction. All three apoenzymes had no catalytic activity. The addition of pyridoxal-*P* (100 μM) to the apoenzymes resulted in the regain of 96% and 94% activity for rSHMT and des-(A1–K6)-SHMT, respectively. However, only 22% of the activity of des-(A1–W14)-SHMT was regained and increasing the concentration of pyridoxal-*P* to 1 mM did not increase the activity of the reconstituted protein. The oligomeric status of the three holo, apo and reconstituted holoenzymes were examined using TSK-G 3000 gel filtration column. As shown in Fig. 5, all three holoenzymes were present predominantly in the tetrameric form with elution volumes of 14.0 ml, 14.2 and 14.7 ml, respectively; however, the apoenzymes of rSHMT, des-(A1–K6)-SHMT were present as a mixture of tetramers (75% and 65%, respectively) and dimers, while the apoenzyme of des-(A1–W14)-SHMT was present predominantly as a dimer (95%). When pyridoxal-*P* (100 μM) was added to all the apoenzymes, rSHMT, and des-(A1–K6)-SHMT were present as >96% and 92% tetramer, respectively, while des-(A1–W14)-SHMT was only partially (20%) converted to tetramer.

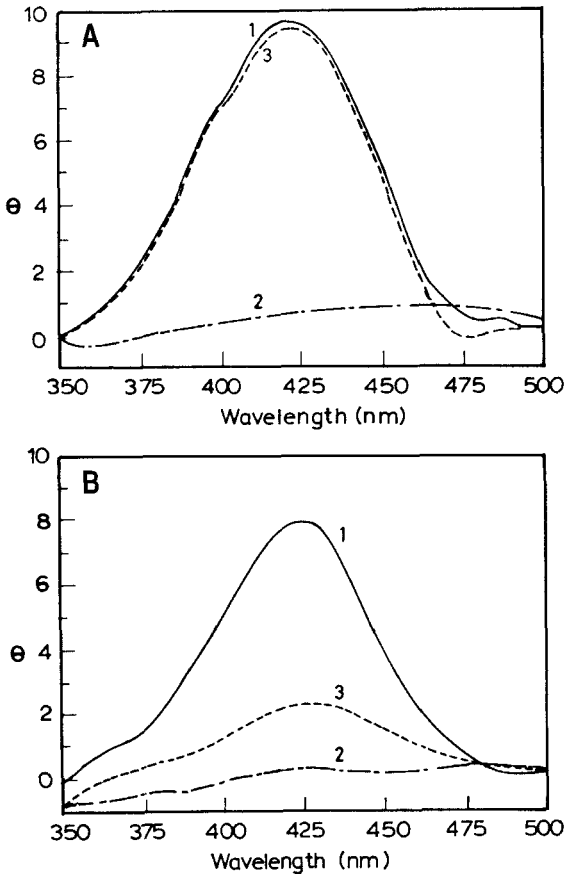

Fig. 6. Visible CD spectra of holo, apo and reconstituted holo enzymes of rSHMT (A) and des-(A1–W14)-SHMT (B). The protein concentration corresponding to 1 A_{280} unit/ml in buffer A was used for recording spectra in Jasco-J 20C Spectropolarimeter. (——) Holo, (– · – · –) apo and (– – –) reconstituted holoenzymes.

Additional evidence for the role of the N-terminal region in maintaining the tetrameric structure of the enzyme as indicated by the ability to form a schiff's base at the active site was monitored by measuring visible CD spectra. It is evident from Fig. 6A that the recombinant enzyme has the characteristic CD spectra with maximum ellipticity at 425 nm. The apoenzyme gave baseline ellipticity in the region 350–500 nm. Reconstitution of apoenzyme with pyridoxal-*P* yielded a holoenzyme preparation with a CD spectrum identical to that of the native holoenzyme. However, the apoenzyme of des-(A1–W14)-SHMT could only be partially reconstituted with pyridoxal-*P* (Fig. 6B).

DISCUSSION

The mammalian SHMT which is a homotetramer has been extensively characterized with respect to its catalytic mechanism [2, 5, 6]. However, there is little information available on the subunit interactions and stability of this tetrameric enzyme. Earlier in our laboratory, the cDNA for sheep cytosolic SHMT was cloned, overexpressed in *E. coli* and the physico-chemical as well as kinetic properties of the purified protein studied [11]. The availability of this clone facilitated an in-depth examination of the structure function relationship of the enzyme. As a first step in this direction, several clones with specified stretches of amino acids deleted at the N and C-termini were constructed and these enabled us to assign a role, if any, for these residues in catalysis, folding and assembly of the subunits of the enzyme. Using restriction-enzyme-based methods, pSN2, pSN3, pSN4, pSN6 and pSC2 deletion clones were constructed. pSN1, pSN5 and pSC1 were constructed by PCR method in which Vent DNA polymerase was used for the PCR amplifications to limit errors during amplification.

Table 3. Properties of apo, reconstituted holoenzymes of rSHMT, des-(A1–K6)-SHMT and des-(A1–W14)-SHMT. The holoenzymes had similar specific activities (4–5 units/mg, see text for details), were essentially in tetrameric form and exhibited characteristic maximum CD at 425 nm. These values were normalized to 100. The values given in the table are expressed as a percentage of their respective holoenzyme values. The three apoenzymes were catalytically inactive (< 1% compared to holoenzyme) and had negligible CD at 425 nm.

Enzyme	Property	Value for		
		rSHMT	des-(A1–K6)-SHMT	des-(A1–W14)-SHMT
		%		
Apoenzyme	tetramer	75	65	<4
	dimer	25	35	>95
Reconstituted holo	tetramer	>96	>92	20
	dimer	<3	<5	80
	SHMT activity	96	94	22
	visible CD (425 nm)	98	96	20

Under the experimental conditions described in Experimental Procedures, pSN1 and pSN2 were able to express soluble and catalytically active enzymes, similar to pETSH (Fig. 1). The low level of expression seen with pSN3 and pSN4 could be due to the possible secondary structure in the mRNA at the ribosome binding site and start codon [36, 37], or alternatively the deleted proteins des-(A1–V30)-SHMT and des-(A1–L49)-SHMT, might be unstable and therefore could not be detected on the SDS/PAGE gels. The expressed des-(A1–R58)-SHMT, des-(A1–G75)-SHMT, des-(Q435–F483)-SHMT and des-(L299–F483)-SHMT mutant proteins were present predominantly in the insoluble fraction (Fig. 1). The deletion of 14 N-terminal amino acids does not affect the enzyme activity, while deletion of a large number of amino acids from N and C-terminus resulted in the expression of proteins in the insoluble fraction.

Studies with porcine AAT revealed that the N-terminal region was involved in the inter subunit interactions [17]. The availability of the des-(A1–K6)-SHMT and des-(A1–W14)-SHMT facilitated an examination of the role of amino acids 1–14 in the subunit interactions and maintenance of oligomeric structure. Together with rSHMT, the enzymes were purified to homogeneity with a yield of 40–50 mg/l. The specific activity of rSHMT (4.9 U/mg) isolated in this study was comparable to the value reported earlier [11]. In addition to performing the physiological reaction, the mutant proteins were capable of using β-phenylserine as a substrate (Fig. 2C) thereby demonstrating their ability to perform H_4-folate-dependent as well as independent reactions.

A unique feature of the mutant enzymes was the significant change in their electrophoretic mobility on non-denaturing PAGE (Fig. 2B) and pI values. A comparison of the N-terminal sequences of the mutant enzymes (Table 1) showed that in des-(A1–K6)-SHMT one Lys (Lys 6) was deleted increasing the overall net negative charge, whereas, in des-(A1–W14)-SHMT one Lys, Arg and two Asp were removed and one Arg was added due to vector fusion, resulting in an increase in the overall net positive charge. These results indicated that the N-terminus in

SHMT contributed to the net charge carried by the enzyme. A comparison of the retention volumes of rSHMT, des-(A1−K6)-SHMT and des-(A1−W14)-SHMT (15.0 ml, 15.1 ml and 15.2 ml, respectively) suggested that removal of the N-terminus resulted in a change in the hydrodynamic volume of des-(A1−W14)-SHMT which is evidently less thermostable than the other two proteins (Fig. 3). It was observed that the ligands like serine convert the enzyme from an 'open' to a 'closed' form. One of the consequences of this change is the increased thermal stability of the enzyme. While the thermal stability of the rSHMT increased from 56°C to 65°C, upon the addition of serine, this change was only marginal (52°C to 56°C) with des-(A1−W14)-SHMT. Another criterion employed for the conversion of open to closed form is the aldol cleavage of serine which is supposed to occur only in the closed form [35]. The K_m and V_{max} values of des-(A1−W14)-SHMT were similar to those of rSHMT indicative of the fact that it was as capable as rSHMT in performing serine aldol cleavage. These observations taken together suggest that des-(A1−W14)-SHMT can be converted from an 'open' to 'closed' form and the absence of increased thermal stability in the presence of serine could be due to the deletion of the N-terminal arm. It is interesting to recall that in the case of porcine cytosolic AAT, a deletion of 9 amino-terminal residues resulted in the large decrease in the thermal stability [17]. Denaturation with urea is another convenient monitor of stability of the enzyme and it is evident that the concentration of urea required for 50% inactivation of the des-(A1−W14)-SHMT is low compared to rSHMT and des-(A1−K6)-SHMT (Fig. 4). The results strongly implicate the N-terminal 14 amino acids in the stabilization of the tetrameric SHMT.

Pyridoxal-*P* has been implicated in the maintenance of the oligomeric structure of sheep liver SHMT [12]. In view of the decreased stability of des-(A1−W14)-SHMT compared to rSHMT, it was necessary to examine the relationship between the N-terminal arm and pyridoxal-*P* in the stabilization of the tetrameric SHMT structure. Removal of pyridoxal-*P* from the rSHMT and des-(A1−K6)-SHMT holoenzymes resulted in partial dissociation of the tetramer to dimers. Immediate reconstitution with pyridoxal-*P* resulted in 90−95% recovery of enzyme activity and the formation of the tetramer (Fig. 5). The good correlation between the recovery of the enzyme activity, visible CD spectra (Fig. 6) and the tetramer concentration strongly suggested that the tetramer with bound pyridoxal-*P* is the enzymatically active form of the enzyme (Table 3). In the absence of pyridoxal-*P* which stabilizes the tetrameric SHMT, des-(A1−W14)-SHMT dissociated completely to dimers unlike rSHMT and des-(A1−K6)-SHMT. Addition of pyridoxal-*P* to apo des-(A1−W14)-SHMT resulted in reconstitution to active tetrameric species only partially (≈20%; Table 3). These results suggest that amino acid residues 7−14 are probably involved in dimer−dimer interactions. The presence of charged residues in this stretch suggests possible ionic interactions. The results presented in this paper provide evidence for the architecture of mammalian SHMT as a dimer of dimers and that this oligomeric structure is stabilized not only by the cofactor (pyridoxal-*P*) but also by the N-terminal arm of the protein.

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