

Role of Pro-297 in the catalytic mechanism of sheep liver serine hydroxymethyltransferase

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Serine hydroxymethyltransferase belongs to the α class of pyridoxal-5'-phosphate enzymes along with aspartate aminotransferase. Recent reports on the three-dimensional structure of human liver cytosolic serine hydroxymethyltransferase had suggested a high degree of similarity between the active-site geometries of the two enzymes. A comparison of the sequences of serine hydroxymethyltransferases revealed the presence of several highly conserved residues, including Pro-297. This residue is equivalent to residue Arg-292 of aspartate aminotransferase, which binds the γ -carboxy group of aspartate. In an attempt to change the reaction specificity of the hydroxymethyltransferase to that of an aminotransferase and to assign a possible reason for the conserved nature of Pro-297, it was mutated to Arg. The mutation decreased the hydroxymethyltransferase activity signifi-

cantly (by 85–90%) and abolished the ability to catalyse alternative reactions, without alteration in the oligomeric structure, pyridoxal 5'-phosphate content or substrate binding. However, the concentration of the quinonoid intermediate and the extent of proton exchange was decreased considerably (by approx. 85%) corresponding to the decrease in catalytic activity. Interestingly, mutant Pro-297 Arg was unable to perform the transamination reaction with L-aspartate. All these results suggest that although Pro-297 is indirectly involved in catalysis, it might not have any role in imparting substrate specificity, unlike the similarly positioned Arg-292 in aspartate aminotransferase.

Key words: proton abstraction, pyridoxal-5'-phosphate, reaction specificity, site-directed mutagenesis

INTRODUCTION

Serine hydroxymethyltransferase (SHMT, EC 2.1.2.1), a pyridoxal 5'-phosphate (PLP)-dependent enzyme, belongs to the α -class of PLP enzymes together with aspartate aminotransferase (AATase) and tryptophan synthase. Although the primary structures of the members of this family are different, they share a similar protein scaffold but catalyse different kinds of reaction [1].

SHMT catalyzes the retro-aldol cleavage of serine to glycine with tetrahydropteroylglutamate (H_4 -folate) as a coenzyme [2]. SHMTs isolated from both prokaryotic and eukaryotic sources are either homodimers or homotetramers with subunit molecular masses ranging from 45 to 54 kDa. The availability of the X-ray structures of human liver cytosolic SHMT (hcSHMT) [3] and rabbit liver cytosolic SHMT [4], coupled with site-directed mutagenesis studies on *Escherichia coli* SHMT (eSHMT) and sheep liver recombinant SHMT (rSHMT) [5], have enriched our understanding of the structure and function of this enzyme. Mammalian SHMTs are a 'dimer of dimers' that are stabilized by the N-terminal arm [3,4,6]. The active-site lysine residue (Lys-257 in hcSHMT) is present at the interface of tight dimers and binds PLP to form the internal aldimine [3]. Site-directed mutagenesis of Lys-256 in rSHMT (corresponding to Lys-257 in hcSHMT) revealed that this residue, in addition to binding PLP, is very important for maintaining the tetrameric structure of the enzyme [7]. In contrast, the active-site lysine residue, Lys-229, in eSHMT participates in product expulsion [8]. An analysis of mutants led to the identification of Arg-363 in eSHMT [9] and Arg-401 in rSHMT [10] (residues equivalent to each other) as being involved in interacting with the α -carboxy group of serine.

This was confirmed by the modelling studies of hcSHMT and rabbit liver cytosolic SHMT [3,4]. A detailed study of the catalytic mechanism of SHMT revealed that the addition of substrate, serine/glycine, generates an external aldimine that undergoes deprotonation at the α -carbon atom to form the quinonoid intermediate. Reprotonation, followed by hydrolysis and the release of product, generates the internal aldimine, which participates in a fresh cycle of catalysis. The intermediates have been identified spectrally and the rate constants for the different steps of catalysis have been determined [11].

As mentioned above, although the active-site geometries of SHMT and AATase are nearly superimposable [3], they catalyse different reactions, namely the H_4 -folate-dependent retro-aldol cleavage of serine and the transamination of L-aspartate respectively. This is probably achieved by the participation of different amino acid residues in substrate binding and catalysis. In addition, both the enzymes catalyse decarboxylation, racemization and transamination, for example, apart from their physiological reaction. Site-directed mutagenesis and X-ray crystallographic studies of AATase had identified Arg-386 as the residue binding the α -carboxy group of aspartate, whereas Arg-292 binds the γ -carboxy group [12]. A sequence comparison revealed that Arg-292 of AATase is equivalent to Pro-297 of rSHMT. This residue is conserved in all mammalian SHMTs reported so far, suggesting a possible role in hydroxymethyl transfer. The X-ray structure of hcSHMT shows Pro-297 to be present at the end of an α -helix away from the tight dimer interface, where PLP is bound [3]. Pro-297 was mutated to arginine in an attempt to change the substrate and/or reaction specificity of SHMT and to assess its importance in the catalytic mechanism of SHMT. The results presented have shown that it affects the proton abstraction step of catalysis.

Abbreviations used: AATase, aspartate aminotransferase; eSHMT, *Escherichia coli* SHMT; hcSHMT, human liver cytosolic SHMT; H_4 -folate, 5,6,7,8-tetrahydrofolate; 2-ME, 2-mercaptoethanol; MA, methoxyamine; PLP, pyridoxal-5'-phosphate; SHMT, serine hydroxymethyltransferase; rSHMT, sheep liver recombinant SHMT; T_m , denaturation temperature; TSC, thiosemicarbazide.

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EXPERIMENTAL

Materials

Glycine, L-serine, NADH, 2-mercaptoethanol, folic acid, PLP, EDTA, L-aspartate, α -oxoglutarate and L-allothreonine were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). [α - 32 P]dATP (3000 Ci/mmol), L-[3- 14 C]serine (55 mCi/mmol), restriction endonucleases, Sequenase Version 2 DNA sequencing kit and DNA-modifying enzymes were obtained from Amersham Pharmacia Biotech (Little Chalfont, Bucks, U.K.). [2- 3 H]Glycine (41.1 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA, U.S.A.). *Pfu* polymerase was purchased from Stratagene, and Deep Vent polymerase was from New England Biolabs (Beverly, MA, U.S.A.). Sephacryl S-200, Superose 12 HR 10/30 were purchased from Pharmacia (Uppsala, Sweden). The oligonucleotide primers were custom synthesized by Bangalore Genei Pvt. Ltd. (Bangalore, India). H₄-Folate was prepared by the method of Hatefi et al. [13]. All other biochemicals used were of the highest purity available.

Bacterial strains and growth conditions

E. coli DH5 α was the recipient strain for the plasmids used in subcloning and sequencing. Strain BL21(DE3) pLysS [14] was used for the bacterial expression of pET constructs. Luria-Bertani medium or terrific broth containing 50 μ g/ml ampicillin was used for growing *E. coli* cells.

DNA manipulations

Plasmids were prepared by the alkaline lysis method as described by Sambrook et al. [15]. Restriction analysis and DNA ligations were performed in accordance with the manufacturer's instructions. Competent cells were prepared by the procedure of Alexander [16].

Site-directed mutagenesis

The P297R mutant was constructed by the PCR-based method [17,18]. The oligonucleotides used for the generation of mutants were as follows: P297R (sense), 5'-GCT GTG TTC CGA GGC CTG CA-3'; P297R (anti-sense), 5'-TTG CAG GCC TCG GAA CAC AG-3'. The wild-type template pRSH (i.e. SHMT gene in pRSET C vector) along with the sense and anti-sense primers was amplified with 2.5 units of Deep Vent polymerase under optimal conditions. The PCR-amplified mixture was treated with *Dpn*I (10 units) at 37 °C for 1 h to digest the methylated DNA (template DNA) and transformed into *E. coli* DH5 α competent cells. The colonies obtained were sequenced by Sanger's dideoxy chain-termination method [19] with a SequenaseTM Version 2.0 DNA sequencing kit; the presence of the desired mutation was confirmed.

Expression and purification of rSHMT and Pro-297 mutant proteins

rSHMT and the mutant enzyme were purified by the protocol described previously [6]. In brief, the BL21(DE3) pLysS extracts harbouring the wild type or mutant construct, obtained after induction with isopropyl β -D-thiogalactoside, were subjected to fractionation with (NH₄)₂SO₄, followed by CM-Sephadex and Sephacryl S-200 column chromatography. The fractions were pooled and precipitated with 65% -satd (NH₄)₂SO₄ and the pellet was resuspended in buffer A [50 mM phosphate buffer (pH 7.4)/1 mM EDTA/1 mM 2-mercaptoethanol]. It was dialysed against the same buffer with two changes and was used in

all the studies. The amount of protein was estimated by the method of Lowry et al. [20].

Enzyme assays

The SHMT-catalysed aldol cleavage of serine in the presence of H₄-folate was monitored with L-[3- 14 C]serine by the modified method standardized by Manohar et al. [21]. The kinetic parameters for L-serine and H₄-folate and the aldolytic cleavage of L-allothreonine were monitored as described by Jagath et al. [22]. The rates of transamination of D-alanine were determined as described previously [23].

Proton-exchange studies

The proton-exchange studies were performed with tritiated glycine as described by Schirch and Jenkins [24].

Spectral measurements

The absorbance spectra of the wild-type and mutant enzymes (5 μ M) were recorded in a Shimadzu UV 160A spectrophotometer, in both the presence and the absence of ligands and at different time intervals, when necessary.

Size-exclusion chromatography

The oligomeric status of the proteins was determined with a calibrated Superose 12 HR 10/30 column attached to a Pharmacia FPLC system.

RESULTS

Expression and purification of P297R mutant SHMT

The P297R mutant and rSHMT were expressed to comparable levels and were present predominantly (more than 95%) in the soluble fraction. SDS/PAGE of the purified proteins showed that both the enzymes were essentially homogeneous and the Western blot analysis of the purified enzymes indicated that they were immunologically similar (results not shown). The N-terminal sequence of the mutant enzyme (AAVPN...) corresponded to that of rSHMT.

The P297R mutant had a specific activity of 0.6 ± 0.1 unit/mg compared with a value of 4.3 ± 0.2 units/mg for the wild-type enzyme. There was a slight increase in K_m and a significant decrease in k_{cat} for P297R in comparison with rSHMT (Table 1). Staining for enzyme activity, after native PAGE, with β -phenylserine and 2,4-dinitrophenylhydrazine gave the characteristic yellow-orange band only with rSHMT [25] and no detectable band with P297R SHMT, suggesting that the H₄-folate-independent retro-aldol cleavage reaction was also de-

Table 1 Kinetic parameters of rSHMT and P297R mutant

One unit of SHMT is defined as that amount that forms 1 μ mol of HCHO/min at 37 °C and pH 7.4. k_{cat} was calculated per mol of subunit. Values for K_m and k_{cat} were obtained from six replicate experiments. Values are presented as means \pm S.E.M.

Enzyme	Specific activity (units/mg)	K_m (mM)		k_{cat} (s ⁻¹)
		L-Ser	H ₄ -folate	
rSHMT	4.3 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	4.2 ± 0.1
P297R	0.6 ± 0.1	4.0 ± 0.1	2.1 ± 0.3	0.7 ± 0.1

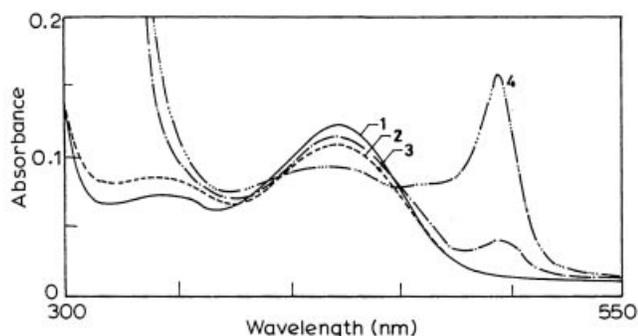


Figure 1 Spectral intermediates generated after the addition of glycine and H_4 -folate to rSHMT and P297R mutant

The visible absorption spectrum of P297R (spectrum 1) was recorded in a Shimadzu UV-160A spectrophotometer with $5 \mu\text{M}$ protein in buffer A. rSHMT gave a similar spectrum (results not shown). The spectrum was recorded after the addition of 50 mM glycine (spectrum 2) to P297R followed by the addition of 0.18 mM H_4 -folate (spectrum 3). Spectrum 4, rSHMT + glycine + H_4 -folate.

creased markedly. The failure to detect the band on reaction with β -phenylserine could also be due to the lack of sensitivity of the assay.

Spectral and catalytic properties of the P297R mutant enzyme

The far-UV CD and the intrinsic tryptophan fluorescence emission spectra of the mutant enzyme were identical with those of rSHMT (results not shown). It is evident from Figure 1 that P297R exhibited an absorbance spectrum similar to that of rSHMT [7] when an equal amount of protein (1 mg/ml) was used. Both the mutant enzyme and rSHMT contained 1 mol of PLP per subunit. The addition of glycine to rSHMT resulted in the formation of a gem diamine (343 nm), an external aldimine (425 nm) and a very small amount of quinonoid intermediate (495 nm) (results not shown). The addition of glycine to P297R also resulted in the formation of mixture of gem diamine (spectrum 2) and external aldimine. When glycine and H_4 -folate were added to rSHMT or P297R, an increased amount of quinonoid intermediate formation was observed (spectra 3 and 4). The increase in absorbance at 495 nm for P297R was much smaller (spectrum 3) than that of rSHMT (spectrum 4). This observation necessitated an examination of the different steps in the catalytic mechanism to identify the step being affected by the mutation of Pro-297. The formation of the external aldimine on the addition of the substrate, serine/glycine, is an early step in catalysis. The binding of serine and glycine to SHMT could induce the fluorescence quenching (50%) of enzyme-bound PLP. The addition of serine or glycine to rSHMT or P297R caused an appreciable quenching of fluorescence, whereas the addition of L-aspartate had no effect, suggesting that the conversion of Pro-297 to Arg had not induced an ability to bind Asp (results not shown). It was therefore not surprising that transamination of L-Asp was not observed with the mutant enzyme in a coupled assay system [26]. The effect of mutation on alternative reactions such as H_4 -folate-independent transamination of D-alanine was also examined. The transamination with D-alanine was decreased by almost 80% (results not shown).

The stereospecific removal of the proton from the α -carbon atom of the external aldimine leads to the generation of the quinonoid intermediate; the reaction is enhanced by H_4 -folate [24]. Proton-exchange studies performed with $[2\text{-}^3\text{H}]\text{glycine}$ (Figure 2) revealed that increasing concentration of H_4 -folate (0–100 μM) enhanced the exchange with rSHMT, whereas a

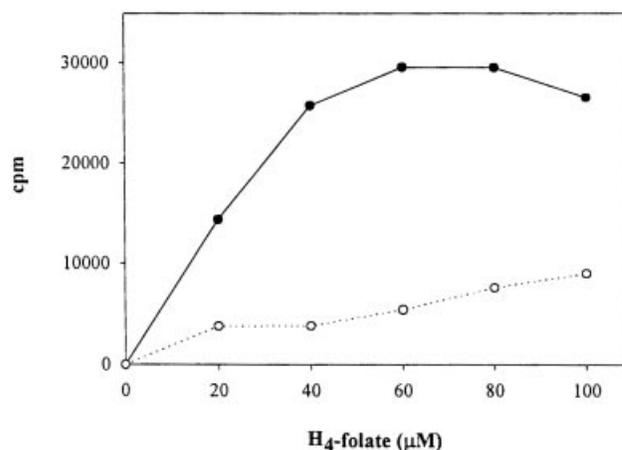


Figure 2 Abstraction of proton from $[2\text{-}^3\text{H}]\text{glycine}$ by rSHMT and P297R mutant proteins

rSHMT and P297R (60 μg) were incubated with 30 mM $[2\text{-}^3\text{H}]\text{glycine}$ (2.5×10^5 c.p.m.) at various concentrations of H_4 -folate (0–100 μM). The protons exchanged with the solvent were plotted as a function of H_4 -folate concentration for rSHMT (●) and P297R (○).

much smaller increase was observed with the P297R mutant. At 60 μM H_4 -folate the proton exchange with P297R was only 13–15% of that with rSHMT. The extent of exchange observed in P297R SHMT correlated well with the amount of quinonoid intermediate observed (Figure 1).

The observation that the proton-abstraction step of catalysis was significantly affected by the mutation prompted an examination of the reactivity of PLP at the active site of the enzyme with well-characterized inhibitors of SHMT such as methoxyamine (MA) [27] and thiosemicarbazide (TSC) [28]. The interaction of MA with rSHMT and P297R was monitored by measuring the decrease in A_{425} or the increase in A_{325} . The pseudo-first-order rate constants for the reaction of MA (20 μM) with rSHMT and P297R were 0.054 min^{-1} and 0.023 min^{-1} respectively. A similar decrease in the rate of the reaction was observed with amino-oxyacetic acid, suggesting that there was a change in the reactivity of the PLP Schiff base present at the active site of the P297R mutant protein.

These findings were corroborated when the interaction of the proteins with TSC was examined. On reaction with TSC, rSHMT yielded an intermediate absorbing at longer wavelengths (430 nm and 464 nm) whose concentration increased slowly and reached a maximal value in 5 min (Figure 3b). The intermediate was relatively constant in concentration up to 15 min, and it decomposed slowly thereafter: a 50% decrease in A_{464} occurred in approx. 8 h (results not shown). In contrast, with P297R (Figure 3a), the increase in A_{430} and A_{464} was not as large and reached a maximum in 1.5 min (the A_{464} was approx. $\sim 70\%$ of that in rSHMT) and then decreased rapidly. At 5 min, the value had decreased to approx. 50% of the maximum. A_{464} reached a basal value with the mutant, whereas with rSHMT a similar change was recorded in more than 24 h. This decrease was accompanied by the formation of the thiosemicarbazone absorbing at 340 nm.

Thermal denaturation studies performed in the absence and in the presence of serine showed very little change in the denaturation temperature (T_m) values of the mutant protein (mean \pm S.E.M. equal to 54 ± 2 °C). With the wild-type enzyme the addition of 100 mM L-serine to the holoenzyme enhanced its T_m from 54 ± 2 to 63 ± 2 °C, indicating its conversion from an 'open' to a 'closed' conformation.

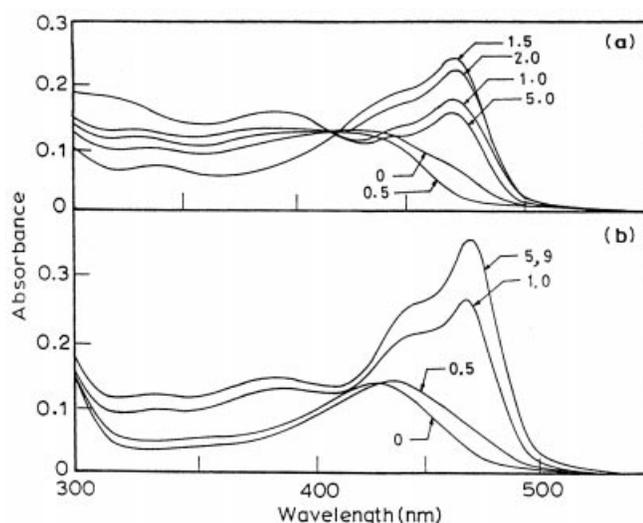


Figure 3 Interaction of rSHMT and P297R SHMT with TSC

Absorbance spectra of P297R (a) and rSHMT (b) ($5 \mu\text{M}$) after the addition of TSC (2 mM) were recorded at 30 s intervals for 10 min at 37°C . Representative spectra recorded at the time intervals indicated are shown.

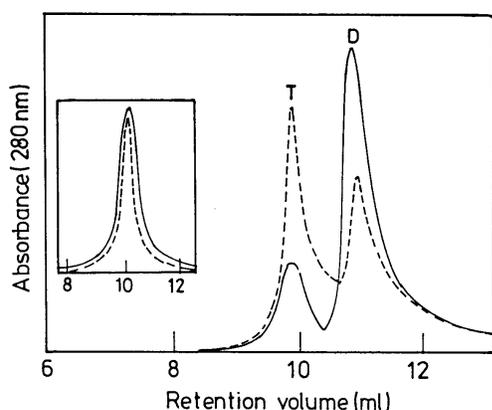


Figure 4 Size-exclusion chromatographic profiles of rSHMT and P297R SHMT proteins

The proteins (0.125 mg/ml) were analysed on a calibrated Superose 12 HR 10/30 column attached to a Pharmacia FPLC system. The inset shows the gel-filtration profile of holo rSHMT (solid line) and P297R SHMT (broken line). Main panel: the apoenzymes were prepared by incubating the enzymes with $200 \mu\text{M}$ MA followed by dialysis, and then reconstituted with $500 \mu\text{M}$ PLP at room temperature for 5 min. Solid line, apo P297R; broken line, reconstituted apo-P297R. The column was equilibrated with buffer A containing 0.1 M KCl at a flow rate of 0.3 ml/min . The eluate was monitored at 280 nm .

Oligomeric status of the mutant protein

It was shown previously that the mutation of residues of rSHMT involved in interaction with PLP led to the dissociation of the tetrameric structure of the protein [7,22]. The observation that P297R had decreased enzyme activity, although it contained stoichiometric amounts of PLP, prompted an examination of the effect of the removal and re-addition of PLP on the oligomeric status of the protein. The oligomeric structure of the freshly purified mutant enzyme was monitored on a calibrated Superose 12 HR 10/30 analytical gel-filtration column. Both rSHMT and P297R were eluted as a single symmetrical peak (10.5 ml) corresponding to a molecular mass of approx. 210 kDa , sug-

gesting that these proteins were tetramers (Figure 4, inset). Removal of the bound cofactor from the mutant protein by reaction with 10 mM L-cysteine yielded an apoenzyme that was eluted as a dimer. The addition of PLP to the mutant apoenzyme did not lead to a regaining of activity or reconstitution to a tetrameric enzyme (results not shown). The apo-rSHMT, in contrast, was predominantly a tetramer and the addition of PLP resulted in a regaining of activity [29]. However, when the mutant apoenzyme was prepared by reaction with $200 \mu\text{M}$ MA, it was predominantly a dimer (approx. 70%; Figure 4, solid line) and could be partly reconstituted to tetramers (approx. 70–75%; Figure 4, broken line) by the exogenous addition of PLP with a regaining of activity (7–8%) comparable with that of P297R.

DISCUSSION

Several reports in the recent past have reiterated the evolutionary relationship between PLP-dependent enzymes [1]. All these enzymes, although sharing the same protein scaffold, perform a diverse array of reactions. This alteration in reaction and/or substrate specificity of an enzyme is brought about by subtle changes in local conformations around the active site owing to changes in the primary structure of the proteins. As mentioned previously, although the active-site geometries of AATase and mammalian SHMT are quite similar [3,4], they catalyse different physiological reactions. AATase uses dicarboxylic amino acids as substrates and catalyses transamination, whereas SHMT is more efficient with monocarboxylic acids and transfers the hydroxymethyl group of serine to H_4 -folate [11]. In our attempts to analyse and attribute this difference in substrate and reaction specificity to specific amino acid residues, Pro-297, a highly conserved residue in SHMT and equivalent to Arg-292 in AATase, was mutated to Arg. Such a mutant exhibited a drastically decreased catalytic activity (Table 1) without any change in its oligomeric structure (Figure 4) and its ability to bind monocarboxylic acid substrates. The K_m values for L-serine and H_4 -folate were slightly higher than those obtained for rSHMT, suggesting that substrate binding was not significantly affected. Similarly, the K_m values for L-serine for H134N, H147N, H230Y and D89N mutant SHMTs were also not significantly altered and were 0.92 , 1.9 , 1.1 and 0.9 mM respectively, compared with 1 mM for rSHMT [22,30,31]. These mutants had significantly lower specific activity. Mutants such as K256Q, K256R and R401A were inactive [7,10]. The residue(s) involved in H_4 -folate binding have not been clearly defined by site-directed mutagenesis.

A stepwise examination of the reaction mechanism revealed that, whereas the mutant protein was able to form the external aldimine (monitored by fluorescence quenching studies of bound PLP), it was either unable to catalyse the deprotonation step efficiently or it was more efficient at reprotonating the quinonoid intermediate (Figure 2), as evidenced by the smaller amount of the intermediate formed on the addition of H_4 -folate and glycine to P297R (Figure 1, spectrum 3). Our results indicate that this residue is not directly participating in the proton-abstraction step of catalysis but is probably affecting the reactivity of the PLP Schiff base by altering the orientation of the PLP ring. This was monitored by using active-site-directed inhibitors such as MA and TSC. The suggestion was supported by the observation that the rate constant for the interaction of P297R with MA had decreased significantly (50%). More convincing was the observation that the reactivity with TSC was markedly changed. It was shown earlier that TSC interacted with native sheep liver SHMT as a slow tightly binding inhibitor, generating a quinonoid type of intermediate bound at the active site. The intermediate

reached a maximum concentration in 15 min and thereafter dissociated slowly (more than 24 h) to yield the apoenzyme and the PLP-thiosemicarbazone. This step in the reaction was extremely slow and a 50% decrease in absorbance occurred in 5–6 h. The rate constant for this step was calculated as approx. $4 \times 10^{-5} \text{ s}^{-1}$ [28]. rSHMT reacted similarly, yielding a quinonoid type of intermediate that decomposed slowly. However, the P297R mutant showed enhanced rates of formation and dissociation of the intermediate, probably due to an alteration in the reactivity of the PLP Schiff base at the active site caused by the introduction of a positive charge. These results suggest that the orientation of the PLP ring might have been altered as a consequence of the mutation, which would account for the decrease in the catalytic activity as a consequence of the decreased ability of P297R to abstract a proton.

An examination of the X-ray structure of hcSHMT revealed that Pro-297 was not interacting directly with any other residue within a radius of 8 Å [3,4]. Replacement of Pro-297 with Arg showed that the positive charge had not affected the network of interactions at the active site, nor had it provided an additional anchor for interacting with the γ -carboxy group of L-Asp. This, however, might not be an important requirement, because in AATase Arg-292 is brought into the active-site milieu after conformational changes subsequent to binding aspartate [12]. Our results suggest that this mutation is not sufficient to permit the binding of aspartate to P297R (results not shown). In an attempt to bring the active-site geometry of SHMT closer to that of AATase, His-230 of rSHMT was mutated to Tyr, the residue that interacts with O-3' of PLP and a double mutant, H230Y-P297R, was generated. Unfortunately, the mutant enzyme was present as a dimer and was unable to bind PLP. Therefore it was not possible to assay for transamination with L-aspartate. It is therefore apparent that additional factors have to be taken into consideration for the design of an active site of rSHMT to bring about the desired change in reaction specificity.

The results presented in this paper strongly suggest a role for Pro-297 in the catalytic mechanism of SHMT, probably by bringing about a change in the conformation of the active-site environment that affects the proton abstraction or re-addition step of catalysis. A confirmation of this hypothesis will have to await the elucidation of the three-dimensional structure of the wild-type and mutant enzymes in the absence and the presence of the substrate.

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