The Role of His-134, -147, and -150 Residues in Subunit Assembly, Cofactor Binding, and Catalysis of Sheep Liver Cytosolic Serine Hydroxymethyltransferase*

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In an attempt to unravel the role of conserved histidine residues in the structure-function of sheep liver cytosolic serine hydroxymethyltransferase (SHMT), three site-specific mutants (H134N, H147N, and H150N) were constructed and expressed. H134N and H147N SHMTs had K_m values for L-serine, L-*allo*-threonine and β-phenylserine similar to that of wild type enzyme, although the k_{cat} values were markedly decreased. H134N SHMT was obtained in a dimeric form with only 6% of bound pyridoxal 5′-phosphate (PLP) compared with the wild type enzyme. Increasing concentrations of PLP (up to 500 μM) enhanced the enzyme activity without changing its oligomeric structure, indicating that His-134 may be involved in dimer-dimer interactions. H147N SHMT was obtained in a tetrameric form but with very little PLP (3%) bound to it, suggesting that this residue was probably involved in cofactor binding. Unlike the wild type enzyme, the cofactor could be easily removed by dialysis from H147N SHMT, and the apoenzyme thus formed was present predominantly in the dimeric form, indicating that PLP binding is at the dimer-dimer interface. H150N SHMT was obtained in a tetrameric form with bound PLP. However, the mutant had very little enzyme activity (<2%). The k_{cat}/K_m values for L-serine, L-*allo*-threonine and β-phenylserine were 80-, 56-, and 33-fold less compared with wild type enzyme. Unlike the wild type enzyme, it failed to form the characteristic quinonoid intermediate and was unable to carry out the exchange of 2-S proton from glycine in the presence of H$_4$-folate. However, it could form an external aldimine with serine and glycine. The wild type and the mutant enzyme had similar K_d values for serine and glycine. These results suggest that His-150 may be the base that abstracts the α-proton of the substrate, leading to formation of the quinonoid intermediate in the reaction catalyzed by SHMT.

Serine hydroxymethyltransferase (SHMT)[1] catalyzes the reversible conversion of serine and 5,6,7,8-tetrahydrofolate (H$_4$-folate) to glycine and 5,10-methylene-H$_4$-folate (5,10-CH$_2$-H$_4$-folate) and plays a major role in one-carbon metabolism (1). This enzyme is a component of thymidylate cycle, along with thymidylate synthase and dihydrofolate reductase and has been suggested as an alternate target for cancer chemotherapy (2–4). SHMT contains covalently bound pyridoxal 5′-phosphate (PLP), which forms an internal aldimine with the ε-amino group of Lys-256 in the rabbit and sheep liver cytosolic SHMT (5, 6). An early step in the catalysis is the formation of an external aldimine, *i.e.* cofactor-substrate complex, which absorbs between 420–430 nm. This is followed by the formation of a resonance-stabilized carbanion (quinonoid intermediate) with an absorption maximum near 500 nm (7). The formation of the quinonoid intermediate requires the abstraction of an α-proton from the amino acid substrate. It has been suggested in several PLP enzymes that lysine, which forms an internal aldimine, is the likely candidate for the abstraction of this proton (8–12). In contrast to these observations, it has been suggested that in *Escherichia coli* SHMT, Lys-229 (equivalent to Lys-256 of rabbit and sheep liver cytosolic SHMTs) is not the base that removes the α-proton (13). SHMT has been classified by sequence alignments in the same family as aminotransferases for which crystal data are available (14–16). However, the mechanism of reaction catalyzed by SHMT is different from that of aspartate aminotransferase, since it involves a Cα–Cβ bond cleavage and removal of proton from β-hydroxyl group of serine in the aldol cleavage reaction. In the reverse reaction, with glycine and 5,10-CH$_2$-H$_4$-folate, the 2-S proton (Cα-H) is abstracted to form the quinonoid intermediate.

Chemical modification studies with sheep liver cytosolic SHMT suggested that Lys, Arg, His, and Cys residues are essential for catalysis (17). The alignment of SHMT sequences from several sources indicated that a few of the His residues are conserved among prokaryotic and eukaryotic SHMTs (18). As a first step in identifying the His residue(s) essential for enzyme activity, His-147, His-150, which were conserved among all SHMTs, and His-134, which was present only in eukaryotic SHMTs (Table I), were chosen for this study. An inspection of the alignment of the fold type I PLP-dependent enzymes showed that His-147 of sheep liver SHMT corresponds to conserved Trp-140 of aspartate aminotransferase, shown to be involved in PLP binding (19). In other members of the fold type I group, this position is occupied by Phe, Tyr, or His (16). However, SHMT His-150 is not conserved in other fold type I enzymes except aspartate aminotransferase and glutamate-1-semialdehyde aminotransferase.[2] This paper describes the construction, expression, and characterization of H134N, H147N,

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[1] The abbreviations used are: SHMT, serine hydroxymethyltransferase; rSHMT, sheep liver cytosolic recombinant SHMT; H$_4$-folate, 5,6,7,8-tetrahydrofolate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate.

[2] Grishin, N. V., Phillips, M. A., and Goldsmith, E. J. (1995) *Protein Sci.* **4** (Internet address: gopher://gopher.prosci.uci.edu: 70/00/PSvolumes/v4n7/SUPLEMNT/Grishin.SUP/Grishin.seq).
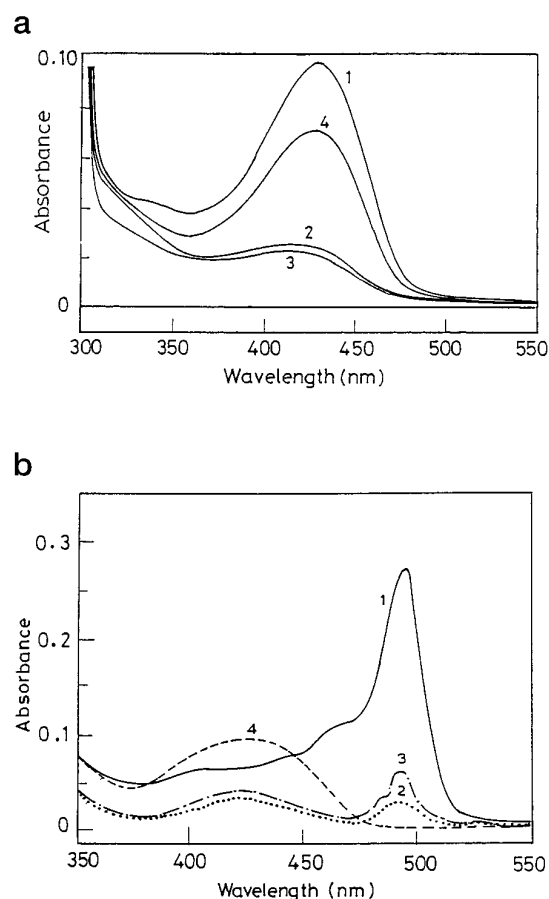
FIG. 1. **The absorption spectra of rSHMT and His mutant SHMTs.** *a*, visible absorption spectra. Absorption spectra of rSHMT (*line 1*), H134N (*line 2*), H147N (*line 3*), and H150N (*line 4*) were recorded in buffer A using a Shimadzu UV-160 A spectrophotometer at a protein concentration of 1.2 mg/ml. *b*, spectral intermediates. Glycine (200 mM) and H$_4$-folate (0.4 mM) were added to the enzyme samples, and the spectra were recorded as described for *panel a*. The reference cuvette contained all the components except the enzyme.

FIG. 2. **Gel filtration profiles of rSHMT and His mutant SHMTs.** rSHMT (0.1 mg) and His mutant SHMTs (0.1 mg) were analyzed on a calibrated Superose-12 HR 10/30 analytical gel filtration column on a Pharmacia FPLC system. The apoenzyme of rSHMT was prepared by incubating the rSHMT with D-alanine followed by dialysis to remove PMP and pyruvate. This apoenzyme (0.1 mg) was analyzed on a gel filtration column as described above. The *arrows* indicate the positions of elution of the standard marker proteins, *1*, apoferritin (440 kDa); *2*, sheep liver cytosolic SHMT (213 kDa); *3*, yeast alcohol dehydrogenase (tetramer, 150 kDa); *4*, yeast alcohol dehydrogenase (dimer, 75 kDa); *5*, carbonic anhydrase (29 kDa). *a*, rSHMT (— – —), H134N (——), H147N (– – – –), H150N (···); *b*, rSHMT holoenzyme (——), rSHMT apo (···), H147N (— – –).

and H150N mutants. A study of the oligomeric structure and the spectral and catalytic properties of these three mutants suggests that His-134 has a role in subunit interactions, His-147 in the cofactor binding, and His-150 in the proton abstraction step of catalysis.

EXPERIMENTAL PROCEDURES

Materials—[α-^{32}P]dATP (3000 Ci/mmol), L-[3-^{14}C]serine (55 mCi/mmol), restriction endonucleases, Sequenase™ version 2.0 DNA sequencing kit, and DNA-modifying enzymes were obtained from Amersham International. CM-Sephadex and Sephacryl S-200 were obtained from Pharmacia. Glycine, L-serine, D-alanine, NADH, β-phenylserine, 2-mercaptoethanol, folic acid, PLP, and EDTA were obtained from Sigma. L-*allo*-threonine was purchased from Fluka. H$_4$-Folate was prepared by the method of Hatefi *et al.* (21). All other chemicals were of analytical reagent grade. The mutant oligonucleotides were purchased from Bangalore Genei Private Ltd., Bangalore, India. Centricon filters were obtained from Amicon, Inc. The Altered Sites II *in vitro* mutagenesis system was purchased from Promega Corp.

Bacterial Strains and Growth Conditions—*E. coli* strain DH5α (Life Technologies, Inc.) was the recipient for all plasmids used in subcloning. The BL21(DE3) pLysS strain (22) was used for bacterial expression of pETSH (23) and His mutant constructs. Luria-Bertani medium or terrific broth (24) with 50 μg/ml of ampicillin was used for growing *E. coli* cells containing the plasmids (24).

DNA Manipulations—Plasmids were prepared by the alkaline lysis procedure as described by Sambrook *et al.* (24). Restriction endonuclease digestions, Klenow filling, and ligations were carried out according to the manufacturer's instructions. The preparation of competent cells and transformation was carried out by the method of Alexander (25).
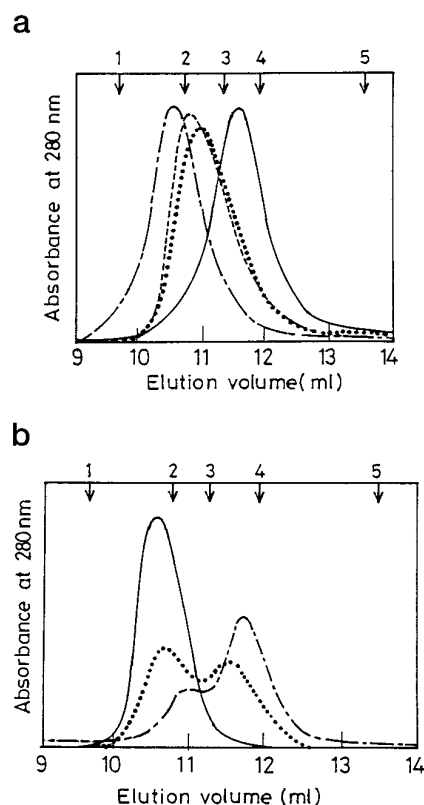
From the agarose gel, the DNA fragments were eluted by the low melting agarose gel method (24).

Site-directed Mutagenesis—The sheep liver cytosolic SHMT cDNA clone was isolated and overexpressed in *E. coli* (23). This clone was used for the preparation of site-specific mutants described in this paper. H134N and H147N mutants were constructed using a polymerase chain reaction-based megaprimer method as described earlier (26). These mutants were constructed from pUCSH (containing the SHMT cDNA fragment lacking 227 bp at the 5′-end in a pUC 19 vector) as a template. The mutant oligonucleotides, 5′-G GTG GAG CCC A̲AT GGC CGC A-3′ and 5′-G GAT GGG GGC A̲AC CTG ACC C 3′ were used for the construction of the H134N and H147N mutants, respectively. The full-length polymerase chain reaction products, obtained upon two rounds of polymerase chain reaction were subcloned into the pUC 19 vector at *Kpn*I and *Bam*HI sites. The clones obtained after the mutagenesis procedure were screened by sequencing the gene at the mutated region. The H150N mutant was generated using the Altered Sites II *in vitro* mutagenesis system from Promega. This mutant was constructed using a 20-mer mutagenic primer (5′-C CAC CTG ACC A̲AT GGG TTC A-3′) from the pALSH clone (SHMT cDNA clone lacking 227 bp at the 5′-end in pALTER-1 vector). Initially, the clones were screened by ampicillin selection and later by DNA sequencing according to the mutagenesis kit protocol. pUC 19 and pALTER-1 plasmids containing the mutated SHMT cDNA were purified and digested using the *Kpn*I and *Pma*CI restriction enzymes flanking all three histidine mutations. The 520-base pair *Kpn*I-*Pma*CI mutated DNA fragments were gel-purified and swapped at the same sites of pETSH vector (23). The clones obtained were screened by sequencing. The entire 520-base pair *Kpn*I-*Pma*CI DNA fragments were sequenced using Sequenase™ version 2.0 DNA sequencing kit in all three mutants to rule out the presence of other
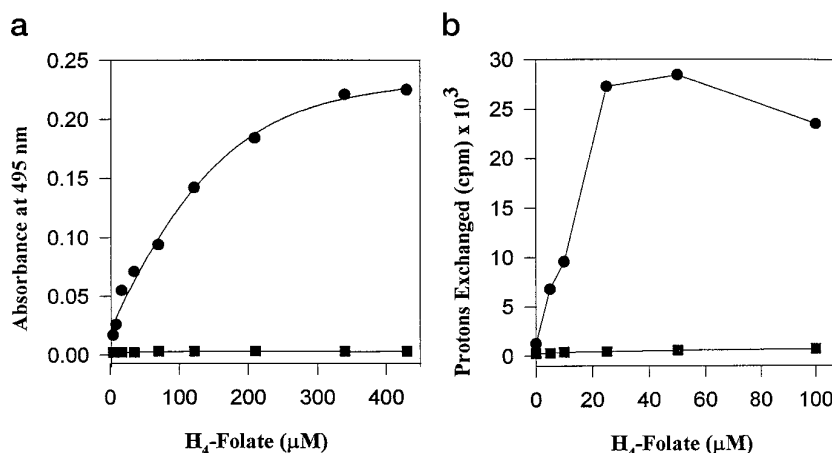
a

b

Fig. 3. **The role of His-150 in the formation of the quinonoid intermediate and proton abstraction.** *a*, rSHMT (1.2 mg/ml) and H150N SHMT (1.8 mg/ml) in buffer A were incubated separately with 100 mM glycine, and the spectra were recorded. H_4-folate (0–420 μM) was added to both rSHMT and H150N enzymes, and the spectra were recorded again. The reference cuvette had all the components except enzymes. The absorbance at 495 nm against H_4-folate concentration was plotted for rSHMT (●) and H150N SHMT (■). *b*, rSHMT (60 μg) and H150N (60 μg) were incubated in buffer A with 30 mM [^{3}H]glycine (2.2 × 10^5 cpm) with varying concentrations of H_4-folate (0–100 μM). The reaction was carried out as described under "Experimental Procedures." The protons exchanged with the solvent were plotted against H_4-folate concentration for rSHMT (●) and H150N SHMT(■).

nonspecific mutations.

Expression and Purification of His-134, -147, and -150 Mutant Enzymes—pETSH, H134N, H147N, and H150N mutant enzymes were purified as described by Jagath *et al.* by subjecting BL21 (DE3) pLys extracts to ammonium sulfate fractionation, CM-Sephadex, Sephacryl S-200 column chromatography (27). Sephacryl S-200 fractions containing SHMT were pooled and precipitated with 65% ammonium sulfate. The pellet was resuspended in buffer A (50 mM potassium phosphate buffer, pH 7.4, containing 1 mM 2-mercaptoethanol and 1 mM EDTA) and dialyzed against 1 liter of the same buffer (with two changes) for 24 h. This enzyme preparation was used in these studies.

Protein Estimation—One ml of purified rSHMT (10 A_{280}/ml) enzyme was passed through a Centricon filter by rinsing with 10 ml of double distilled water. After Centricon filtration, the absorbance was measured at 280 nm, and then the sample was lyophilized and weighed. The concentration at 1 A_{280} was found to be 1.2 mg with a molar extinction coefficient of 176,333 M^{-1} cm^{-1}. A similar value was obtained when the protein concentration was estimated by the method of Gill and von Hippel (28). This value was used for the estimation of all three mutant enzymes, since the absorbance at 280 nm did not change compared with wild type enzyme.

Enzyme Assays—The SHMT-catalyzed aldol cleavage of serine with H_4-folate to form glycine and 5,10-CH_2-H_4 folate was monitored using L-[3-^{14}C]serine and H_4-folate as substrates in the absence and presence of 500 μM PLP (29, 30). The SHMT-catalyzed aldol cleavage of L-*allo*-threonine to glycine and acetaldehyde was monitored at 340 nm by the NADH-dependent reduction of acetaldehyde to ethanol and NAD$^+$ in the presence of an excess amount of alcohol dehydrogenase as described earlier (31) with the following modifications. One ml of buffer A contained SHMT (1–70 μg), alcohol dehydrogenase (100 μg), NADH (250 μM), and 0–10 mM L-*allo*-threonine. The reference cuvette contained all the above components except L-*allo*-threonine. The reaction was monitored in the absence and presence of PLP (500 μM) at 37 °C for 10 min. The NADH consumed in the reaction was calculated using a molar extinction coefficient of 6220 M^{-1} cm^{-1} (32). The rate of cleavage of β-phenylserine to benzaldehyde was monitored at 279 nm for 5 min at 37 °C as described earlier (33). The amount of the enzyme (rSHMT and mutant enzymes) used in the reaction was in the range of 2.5–300 μg. The concentration of benzaldehyde formed in the reaction was calculated using a molar extinction coefficient of 1400 M^{-1} cm^{-1}. The rates of transamination of D-alanine to pyruvate and pyridoxamine 5′-phosphate (PMP) were determined by measuring the rate of increase in absorbance at 325 nm (34). The reaction was carried out in buffer A at 37 °C for 5 min. The amount of PMP formed in the reaction was calculated using a molar extinction coefficient of 8300 M^{-1} cm^{-1} (35). The concentration of the enzyme (rSHMT and H150N SHMT) used in the reaction was in the range of 0.35–1.5 mg.

Circular Dichroism—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. A protein concentration corresponding to 0.12 mg/ml was used for far-UV CD studies. Visible CD spectra were recorded in a Jasco-J-20 C automated recording spectropolarimeter at a protein concentration of 1.2 mg/ml in buffer A.

Size Exclusion Chromatography—To analyze the oligomeric structures of the His mutant enzymes, a Superose-12 HR 10/30 analytical gel filtration column attached to Pharmacia FPLC system was used. The column was calibrated with standard proteins such as 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 and 0.05% sodium azide.

Dissociation Constants—The dissociation constants of the enzyme-substrate complexes with rSHMT and H150N SHMT were determined by a slight modification of the earlier procedure (36). The substrate-induced quenching of enzyme bound PLP fluorescence was monitored using a Shimadzu RF-5000 spectrofluorimeter. The enzyme (1 mg/ml) sample was incubated with increasing concentrations of L-serine (0.1–50 mM) or glycine (0.5–100 mM) at 25 ± 2 °C, and the fluorescence was monitored at 450–550 nm after excitation at 425 nm. The K_d values were obtained from double reciprocal plots of the change in fluorescence units at 495 nm as a function of the ligand concentration. It was ensured that inner filter quenching was minimal and did not interfere with the measurements.
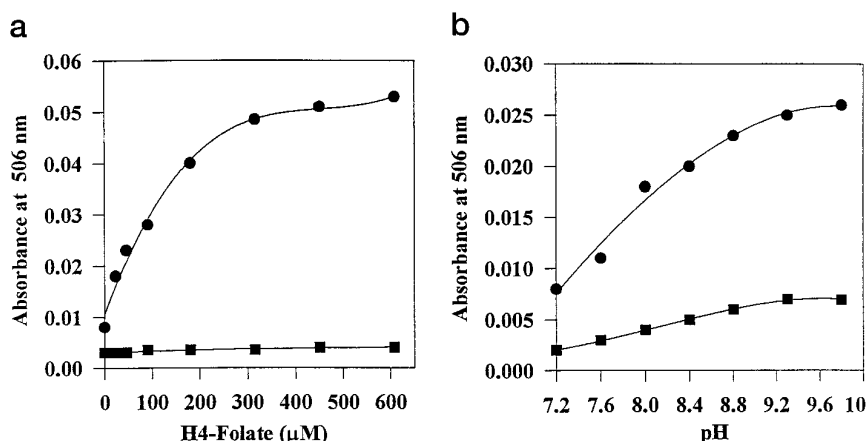
Proton Exchange Studies—[2-^{3}H]Glycine was purified on Dowex-50W-12 column packed in a 1-ml syringe as described (37). The rSHMT or H150N enzyme (60 μg) in HEPES buffer pH 7.4 was incubated with 30 mM [2-^{3}H]glycine (2.2 × 10^5 cpm) for 10 min at 37 °C. After the incubation, H_4-folate (0–100 μM) was added, and the reaction continued for an additional 1 min at 37 °C. The reaction was stopped by the addition of 10% trichloroacetic acid, and the denatured protein was removed by centrifugation. The supernatant was loaded onto a Dowex 50W-12 column that was previously equilibrated with 10 mM HCl. The column was washed with 5 ml of 10 mM HCl, the eluant was collected (0.5-ml fractions), and the radioactivity was measured.

Apoenzyme Preparation—Apoenzyme of rSHMT was prepared as described earlier (13) with minor modifications. D-Alanine (200 mM) was added to the holoenzyme in 50 mM potassium phosphate buffer, pH 7.4, containing 10 mM 2-mercaptoethanol, 1 mM EDTA and 200 mM ammonium sulfate and incubated at 37 °C for 4 h. The pyruvate and PMP formed during the reaction were removed by passing the sample through a 30-kDa centricon filter.

RESULTS

Expression and Purification of H134N, H147N, and H150N SHMTs—The conserved His-134, -147, and -150 residues were

FIG. 4. **Formation of quinonoid intermediate with D-alanine.** *a*, rSHMT (1.2 mg/ml) and H150N (1.8 mg/ml) enzymes were incubated with 400 mM D-alanine, and the spectra were recorded in buffer A from 300 to 550 nm. H_4-folate (0–600 μM) was added to both rSHMT and H150N SHMT, and the spectra were recorded again. The reference cuvette had all of the components except enzymes. The absorbance at 506 nm against the H_4-folate concentration was plotted for rSHMT (●) and H150N SHMT (■). *b*, in a similar experiment, instead of the addition of H_4-folate the reaction was carried out at different pH values (7.2–10) using 50 mM potassium phosphate buffer containing 1 mM 2-mercaptoethanol, 1 mM EDTA, and 400 mM D-alanine. The absorbance at 506 nm against pH was plotted for rSHMT (●) and H150N SHMT (■).

mutated to Asn by site-directed mutagenesis as described under "Experimental Procedures." The expression of the mutant constructs was as good, since the wild type clone (pETSH) and the expressed proteins were present predominantly (>90%) in the soluble fraction. The enzyme present in the soluble fraction was purified by a procedure identical to that used for the wild type enzyme, and yields of the enzymes were in the range of 40–50 mg/liter. The purified mutant proteins were homogeneous as indicated by a single band on native PAGE and SDS-polyacrylamide gel electrophoresis. The purified rSHMT (wild type), H134N, H147N, and H150N SHMTs were assayed using 0.6, 15, 30, and 30 μg of the enzyme, respectively. The H150N SHMT had the lowest specific activity of 0.06 units/mg, while H134N and H147N SHMTs had 0.18 and 0.09 units/mg, respectively, compared with a value of 4.8 units/mg for rSHMT.

Spectral Properties of H134N, H147N, and H150N SHMTs— The far-UV CD spectra of all the mutant enzymes were essentially similar to the wild type enzyme, suggesting that there were no alterations in the secondary structure upon mutation of the specified His to Asn residues. The presence of characteristic spectral intermediates in the catalytic process of SHMT has provided a convenient handle to examine the specific functions of identified amino acid residues in the structure and function of the enzyme. It can be seen from Fig. 1*a* that H150N has slightly reduced absorbance at 425 nm compared with rSHMT, while H134N and H147N SHMTs had much less absorbance when an equal concentration of protein (1.2 mg) was used for recording the spectrum. H134N and H147N SHMTs had very little CD in the visible region (350–500 nm), while H150N SHMT gave a visible CD spectrum characteristic of the presence of an internal aldimine at the active site. H134N, H147N, and H150N SHMTs had approximately 6.3, 3, and 64% of the visible CD (at 425 nm) that rSHMT had, respectively (data not shown). It can be seen from Fig. 1*b* that the quinonoid intermediate (495 nm) was observed when glycine and H_4-folate were added to H134N and H147N mutant enzymes. However, this intermediate was not seen with H150N SHMT. Even increasing the concentration of H150N SHMT from 1 to 5 mg did not result in the formation of this intermediate.

Oligomeric Structure—The oligomeric status of the enzymes immediately after the Sephacryl-S-200 column chromatography step of purification was examined by using a calibrated Superose-12 HR 10/30 analytical gel filtration column. The rSHMT, H147N, and H150N SHMTs eluted as single symmetrical peak corresponding to a molecular mass of ~220 kDa, indicating that they are in the tetrameric form. However, the H134N SHMT eluted as a single peak corresponding to the mass of the dimer (~100 kDa) (Fig. 2*a*). When the column was equilibrated with PLP (150 μM), the elution profiles were iden-
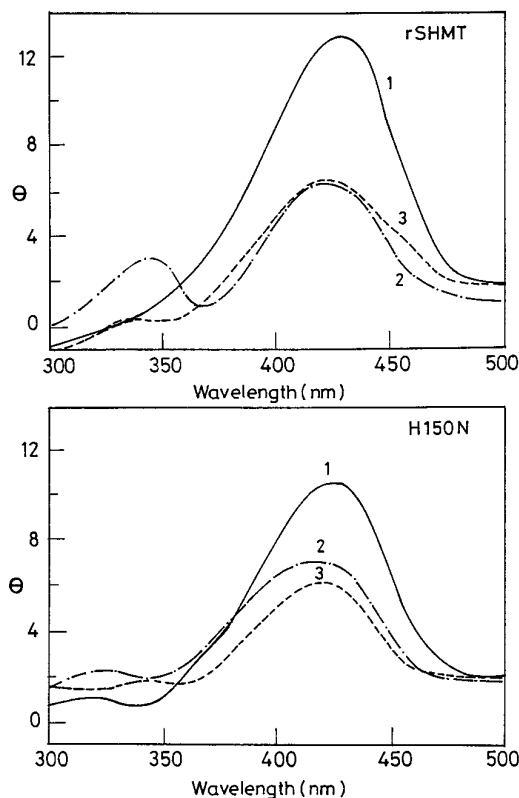

FIG. 5. **The formation of external aldimine with L-serine and glycine.** The rSHMT (1.2 mg/ml) and H150N SHMT (1.8 mg/ml) were mixed with 100 mM serine (*line 2*) or 100 mM glycine (*line 3*) or recorded without ligand (*line 1*), and the visible CD spectra were recorded from 300 to 500 nm in buffer A using a Jasco-J 20 spectropolarimeter.

tical. Dialysis of the H147N mutant enzyme against the buffer not containing the PLP resulted in the formation of apoenzyme that was predominantly in the dimeric form; in contrast, the rSHMT remained as a tetramer with bound PLP under similar conditions (Fig. 2*b*). Removal of PLP from rSHMT by transamination of D-alanine followed by dialysis for 24 h resulted in the partial dissociation of the tetramer to a dimer (Fig. 2*b*).

Catalytic Properties—A unique feature of SHMT is its ability to catalyze a variety of H_4-folate-dependent and -independent reactions (7). To assess the role of the mutated His residues in reaction specificity, some of these reactions were examined. Table II summarizes the K_m, k_{cat} and k_{cat}/K_m values determined for H134N, H147N, and H150N SHMTs using L-serine and L-*allo*-threonine as substrates in the absence and presence of added PLP (0.5 mM). K_m values of serine and H_4-folate (1.0 ±

TABLE I

A comparison of the amino acid sequences around His-134, -147, and -150 in sheep liver and other SHMTs

Pairwise alignment of sheep liver SHMT with all the other SHMTs was made using the GAP program of the Genetics Computer Group (GCG) package. Multiple alignment was made using the PRETTY program of the GCG package.

Source					Sequence											Reference
			134						147		150					
SHEEP cyt	E	P	H̲	A	R...G	G		H̲	L	T	H̲	G				18, 23
RAB cyt	E	P	H̲	G	R...G	G		H̲	L	T	H̲	G				5, 46
RAB mit	Q	P	H̲	D	R...G	G		H̲	L	T	H̲	G				47
HUM cyt	E	P	H̲	G	R...G	G		H̲	L	T	H̲	G				48
HUM mit	Q	P	H̲	D	R...G	G		H̲	L	T	H̲	G				48
PEA mit	K	P	H̲	D	R...G	G		H	L	S	H̲	G				49
NCRA cyt	P	V	H̲	G	R...G	G		H̲	L	S	H̲	G				50
YEAST cyt	K	P	H̲	E	R...G	G		H̲	L	S	H̲	G				51
ECOLI	E	P	G	D	T...G	G		H̲	L	T	H̲	G				52
BRAJA	Q	P	G	D	T...G	G		H̲	L	T	H̲	G				53
SALTY	Q	P	G	D	T...G	G		H̲	L	T	H̲	G				54
HYPME	Q	P	G	D	T...G	G		H̲	L	T	H̲	G				55
CAMJE	N	P	G	D	K...G	G		H̲	L	T	H̲	G				20

TABLE II

Kinetic properties of H134N, H147N, and H150N SHMT

k_{cat} values were calculated per mol of active site. The aldol cleavage of serine and L-*allo*-threonine reactions were monitored in the absence of externally added PLP or in the presence of (500 μM) PLP.

Enzyme	L-Serine		L-*allo*-threonine	
	No PLP	500 μM PLP	No PLP	500 μM PLP
rSHMT				
K_m (mM)	0.90	0.92	1.25	1.30
k_{cat} (s⁻¹)	4.23	4.32	4.15	4.65
k_{cat}/K_m (s⁻¹ M⁻¹)	4.7×10^3	4.7×10^3	3.32×10^3	3.57×10^3
H134N SHMT				
K_m (mM)	1.80	1.90	2.5	2.77
k_{cat} (s⁻¹)	0.235	1.20	0.393	0.49
k_{cat}/K_m (s⁻¹ M⁻¹)	0.13×10^3	0.63×10^3	0.157×10^3	0.157×10^3
H147N SHMT				
K_m (mM)	1.25	1.50	3.57	5.0
k_{cat} (s⁻¹)	0.084	0.75	0.363	0.807
k_{cat}/K_m (s⁻¹ M⁻¹)	0.067×10^3	0.50×10^3	0.101×10^3	0.16×10^3
H150N SHMT				
K_m (mM)	1.0	1.0	1.66	2.5
k_{cat} (s⁻¹)	0.059	0.073	0.098	0.109
k_{cat}/K_m (s⁻¹ M⁻¹)	0.059×10^3	0.073×10^3	0.059×10^3	0.044×10^3

0.2 mM) for the mutant enzymes were similar to that of wild type enzyme, whereas the k_{cat} values decreased significantly for all of the three mutant enzymes. A comparison of the k_{cat}/K_m values indicated that the H134N, H147N, and H150N SHMTs are 36-, 70-, and 80-fold less efficient compared with the wild type enzyme in the H_4-folate-dependent physiological reaction in the absence of added PLP, whereas in the presence of 500 μM PLP, the activities were 6.85-, 8.64-, and 64-fold less efficient. The K_m values for L-*allo*-threonine were similar in the absence and presence of added PLP for all of the mutant enzymes and rSHMT (Table II). However, the k_{cat} values were markedly decreased. The k_{cat}/K_m values for L-*allo*-threonine with the H134N, H147N, and H150N SHMTs were 21-, 32-, and 60 ± 4-fold less compared with wild type enzyme in the absence and presence of added PLP. The difference in the k_{cat} values of the mutants (H134N, H147N) in the H_4-folate-dependent and -independent reactions in the presence of excess PLP could be due to the effect of a large excess PLP or errors in the estimation of the activity due to the interference of PLP absorbance at 340 nm, the wavelength at which alcohol dehydrogenase activity was estimated. The mutant enzymes catalyzed the β-phenylserine cleavage very poorly compared with rSHMT. The K_m values for H134N, H147N, H150N, and rSHMT were 20, 25, 25, and 40 mM, respectively. The k_{cat} values for H134N, H147N, H150N, and rSHMT in the absence of added PLP were 0.187, 0.776, 0.218, and 11.65 s⁻¹, respectively. In the presence of 500 μM PLP, the β-phenylserine cleavage could not be monitored due to the interference of PLP in the assay.

Interaction of the PLP with H134N and H147N SHMTs—The low activity, the decreased absorbance (Fig. 1a), and visible CD at 425 nm indicated that these mutant enzymes contained only a small amount of bound PLP. A possible explanation for this could be an alteration in the affinity for the cofactor (PLP) upon mutation of these His residues. The apoenzyme of rSHMT was prepared as described under "Experimental Procedures." The hydroxymethyltransferase activity was monitored at increasing concentrations of PLP, and a maximal activity was obtained at about 60 μM PLP. From a replot of the data, a K_a value of 6 μM for PLP was obtained. Since the final enzyme preparations of H134N and H147N SHMTs had very little enzyme activity, attempts were made to enhance the activity by increasing the concentrations of PLP in the assay mixture. The activity of the H134N SHMT increased with increasing concentrations of PLP, and an apparent saturation was reached at 250 μM. An apparent K_a value of 60 μM was

calculated from a double reciprocal plot (data not shown). Similarly, increasing concentrations of the PLP enhanced the activity of H147N SHMT, and the apparent K_a value of 110 μM for PLP was calculated (data not shown). The k_{cat} values of the H134N and H147N SHMTs at 500 μM PLP were 1.20 and 0.75 s⁻¹, respectively, compared with 4.32 s⁻¹ for rSHMT (Table II).

Role of His-150 in Catalysis—Unlike H134N and H147N SHMTs, increasing concentrations of PLP did not increase the activity of H150N SHMT. It is interesting to recall that this mutant enzyme was unable to generate the quinonoid intermediate (Fig. 1b). Increasing concentrations of the H_4-folate from 0–450 μM did not result in the formation of quinonoid intermediate. However, under similar conditions, the concentration of quinonoid intermediate increased significantly with rSHMT (Fig. 3a).

Earlier investigations on the mechanism of catalysis involving D-alanine as the substrate had indicated the formation of the quinonoid intermediate (38). It can be seen from Fig. 4 that increasing concentrations of H_4-folate or increase in pH values enhanced the formation of the quinonoid intermediate with wild type enzyme in the presence of D-alanine. In the case of H150N SHMT, there was only a marginal increase in the quinonoid intermediate when pH was varied, but there was no change with increasing concentrations of H_4-folate.

A stereospecific proton (2-S) abstraction from the external aldimine is an integral part of the mechanism for the formation of the quinonoid intermediate, and the H_4-folate has been shown to enhance this proton abstraction from [2-³H]glycine (39). This step in catalysis has been conveniently monitored by exchange with the solvent protons and its enhancement upon the addition of H_4-folate. It can be seen from Fig. 3b that there was a large enhancement of proton exchange with wild type enzyme upon increase in H_4-folate concentration, while such an exchange was absent in the case of H150N SHMT. It was shown earlier that higher concentrations of H_4-folate inhibit the proton exchange reaction (37).

Formation of the External Aldimine—The formation of the external aldimine was monitored by recording the visible CD spectra in the presence of ligands (L-serine and glycine) or by monitoring the substrate induced fluorescence quenching as described under "Experimental Procedures." The K_d values for glycine obtained from the fluorescence quenching data were 10 and 13 mM for H150N SHMT and rSHMT, respectively. Similarly, the K_d values for serine were 1.54 and 1.0 mM for H150N SHMT and rSHMT, respectively. The binding of amino acid
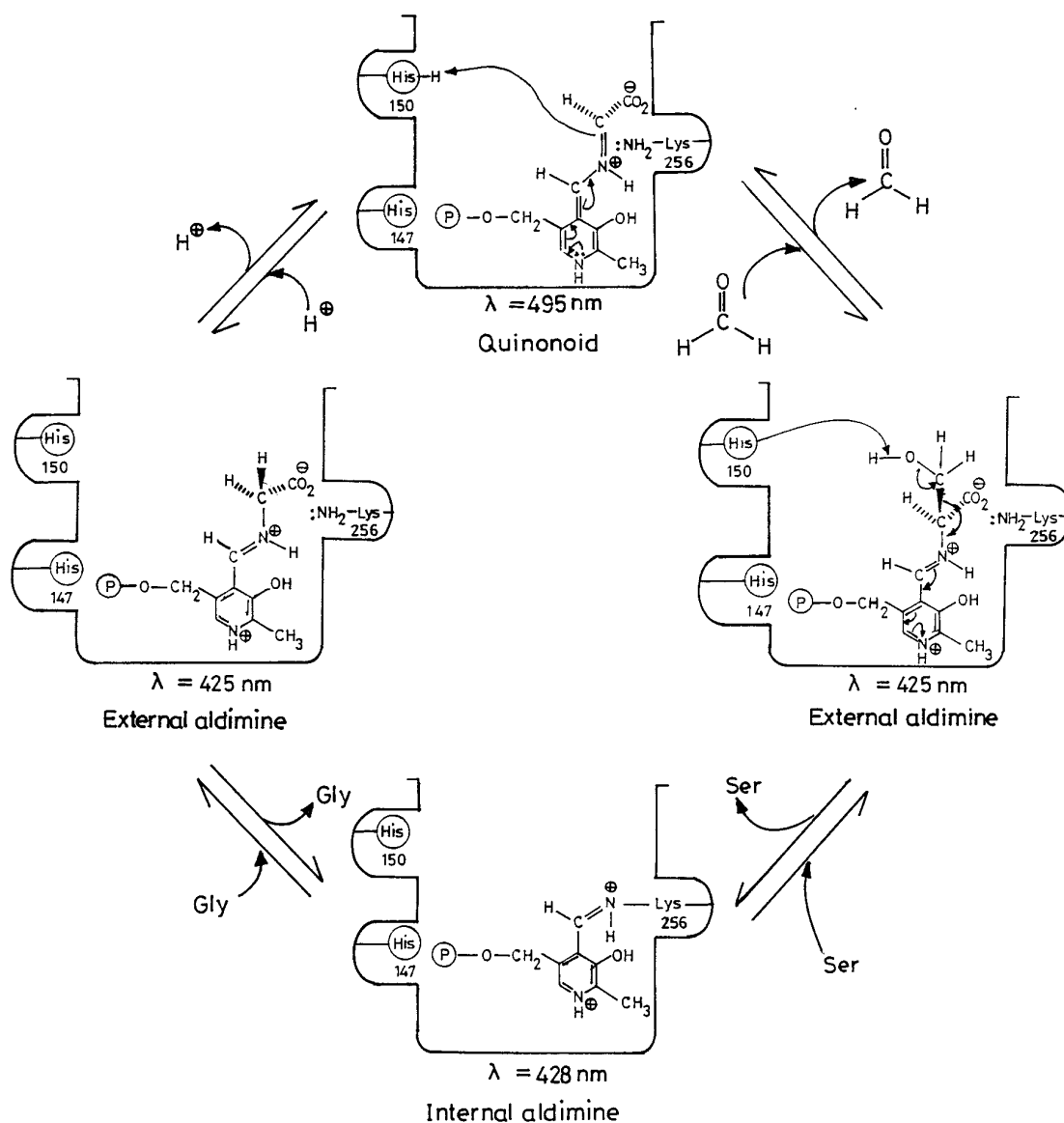
FIG. 6. **Role of His-150 in catalysis.**

substrates (serine or glycine) displaces the active site lysine and forms an external aldimine, resulting in a decrease of the enzyme's visible CD spectra. It can be seen from the Fig. 5 that the addition of serine or glycine resulted in the decrease of visible CD for rSHMT. Similar changes were observed in the case of H150N SHMT also, indicating the formation of an external aldimine.

DISCUSSION

The identification of the specific amino acid residues essential for the structure and function of enzymes is greatly facilitated by the availability of their three-dimensional structure. However, the unavailability of the crystal structure of SHMT has hampered the identification of possible residues at the active site. Chemical modification studies had indicated that Arg, Lys, Cys, and His residues are essential for activity of sheep liver cytosolic SHMT (17). A comparison of the sequences of SHMTs was carried out with the objective of identifying the conserved His residues. This comparison indicated that His-147, -150, -230, -255, -306, and -356 were conserved in all SHMTs, and His-134 and -304 were conserved among eukaryotic SHMTs. As a first step in identifying the His residue(s) involved in catalysis, His-134, -147, and -150 residues were

mutated to Asn. The consequences of such mutation on the structure and functional properties of the enzyme are discussed below.

The Role of His-134—His-134 is conserved in all the tetrameric eukaryotic SHMTs, whereas this residue is replaced by a glycine in prokaryotic SHMTs that exist as dimers (40, 41) (Table I), suggesting that this residue may have a role in the maintenance of the tetrameric structure. Mutation of this residue to Asn indeed resulted in the alteration of the quaternary structure of the enzyme, leading to the formation of dimers as shown in Fig. 2a with lowered enzyme activity and PLP binding. H134N SHMT follows the similar catalytic mechanism as the wild type enzyme without a change in the affinity for substrates. The results presented in this paper also show for the first time that the dimeric eukaryotic SHMT can be enzymatically active, albeit poorly, and are compatible with the explanation that the His-134 is probably interacting with an as yet unidentified negatively charged group on the neighboring subunit. Mutation of this His residue disrupts these interactions. The formation of the dimers rather than monomers would suggest that the enzyme is a dimer of dimers.

The Role of His-147—As shown in Table I, His-147 is con-

served among all SHMTs, and an equivalent residue in aspartate aminotransferase Trp-140 was shown to be involved in PLP binding (19). The results presented in this paper clearly demonstrate that His-147 plays a similar role in SHMT. Like H134N SHMT, this mutant enzyme had very little bound PLP (Fig. 1a), indicating that the efficiency of binding PLP with the mutant enzyme had decreased. Unlike the H134N SHMT, this mutant enzyme preparation prior to dialysis was present as a tetramer. The activity of the H147N SHMT (0.084 s^{-1}) (with serine as a substrate) was enhanced 9-fold (0.75 s^{-1}) upon the addition of PLP (500 μM), corresponding to 17% activity of the wild type enzyme. However, with L-*allo*-threonine as a substrate, the increase in activity was only 2.2-fold (Table II). This could be due to the interference of excess PLP (500 μM) with NADH absorption at 340 nm. It is possible that due to the decreased affinity for PLP, the H147N SHMT was less stable and it dissociated to the dimeric form rapidly (Fig. 2b). Similarly, the removal of PLP from rSHMT (Fig. 2b) or native sheep liver SHMT (42) resulted in the dissociation of tetramers to dimers, substantiating the role of PLP in maintenance of the oligomeric structure of the enzyme. The apparent K_a for PLP with the tetrameric mutant enzyme was approximately 18 times higher than rSHMT. The tetrameric nature of the mutant enzyme, considerable enzyme activity, unaltered K_m for serine, and decreased affinity for PLP indicated that His-147 might interact with PLP through ionic or hydrogen bonding interactions (Fig. 6).

The Role of His-150—His-150 is also highly conserved among all SHMTs (Table I), although it is not an invariant residue among other members of the fold type I group of PLP enzymes. This mutant enzyme was isolated predominantly as a tetramer with bound PLP, unlike the H134N and H147N SHMTs. Despite the presence of bound PLP, the mutant enzyme had very little enzyme activity (<2%). This loss in enzyme activity was reflected in the absence of quinonoid intermediate upon the addition of H$_4$-folate to the mutant enzyme in the presence of glycine or D-alanine (Figs. 3a and 4). The proton exchange studies using [2-^{3}H]glycine (Fig. 4b) showed that the step preceding the formation of quinonoid intermediate (*i.e.* proton abstraction) was affected by mutation of His-150 to Asn. The fluorescence quenching and visible CD studies (Fig. 5) with serine and glycine showed that the H150N SHMT was capable of forming the external aldimine, an early event in catalysis. However, when D-alanine was used as substrate, the K_m values for the H150N SHMT and rSHMT were similar (25 and 30 mM, respectively), and the k_{cat} decreased by 6-fold, suggesting that the mutant was capable, although less efficiently, of carrying out the transamination reaction. These results suggest that in the absence of His-150, some other proton acceptor might function, although less effectively. A similar observation was reported in the case of D-amino acid transaminase, where a second lysine residue could partially substitute for the active site lysine in the catalytic mechanism (10). In analogy with the scheme of reactions proposed earlier (7), the role of His-150 is shown in Fig. 6. Initially the bound PLP forms an internal aldimine with ϵ-amino group of Lys-256 (6). The addition of the substrate (serine and glycine) leads to the formation of the external aldimine via a geminal diamine. An abstraction of a proton stereospecifically from the 2-S position of the glycine-external aldimine complex leads to the formation of the quinonoid intermediate (39). This proton abstraction is facilitated by the presence of H$_4$-folate. The scheme visualizes the His-150 as the acceptor of this proton. In the presence of 5,10-CH$_2$-H$_4$-folate, this proton is probably added back to form serine and H$_4$-folate. Similarly, when serine or other β-hydroxyamino acids (L-*allo*-threonine and β-phenylserine) are used as substrates, His-150 could act as proton acceptor by interacting with the -CH$_2$OH group. It is worthwhile mentioning that the -CH$_2$OH group is located stereospecifically equivalent to the 2-S proton of glycine. This type of mechanism has been visualized in the reaction of hydroxyamino acids (43, 44). Thus, mutation of the crucial His-150 residue blocks the proton abstraction step, although it does not affect the formation of an external aldimine and leads to a marked decrease in enzyme activity. The sequence alignment of the fold type I group of PLP-dependent enzymes shows that, unlike SHMT His-147, His-150 is not a conserved residue and is equivalent to aspartate aminotransferase His-143. It was shown that mutation of aspartate aminotransferase His-143 did not affect the catalytic activity, and it was suggested that this residue may play an auxiliary role in the transaldimination reaction (45). It is pertinent to point out that the mechanism of the reactions catalyzed by aspartate aminotransferase and SHMT are different and that therefore the function of His-150 is probably unique to SHMT. The results presented in this paper clearly demonstrate the role of His-134 in subunit interactions, His-147 in cofactor binding, and His-150 in the proton abstraction step of catalysis.

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