Role of Tyrosine 65 in the Mechanism of Serine Hydroxymethyltransferase†

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ABSTRACT: Crystal structures of human and rabbit cytosolic serine hydroxymethyltransferase have shown that Tyr65 is likely to be a key residue in the mechanism of the enzyme. In the ternary complex of *Escherichia coli* serine hydroxymethyltransferase with glycine and 5-formyltetrahydrofolate, the hydroxyl of Tyr65 is one of four enzyme side chains within hydrogen-bonding distance of the carboxylate group of the substrate glycine. To probe the role of Tyr65 it was changed by site-directed mutagenesis to Phe65. The three-dimensional structure of the Y65F site mutant was determined and shown to be isomorphous with the wild-type enzyme except for the missing Tyr hydroxyl group. The kinetic properties of this mutant enzyme in catalyzing reactions with serine, glycine, *allo*threonine, D- and L-alanine, and 5,10-methenyltetrahydrofolate substrates were determined. The properties of the enzyme with D- and L-alanine, glycine in the absence of tetrahydrofolate, and 5,10-methenyltetrahydrofolate were not significantly changed. However, catalytic activity was greatly decreased for serine and *allo*threonine cleavage and for the solvent α-proton exchange of glycine in the presence of tetrahydrofolate. The decreased catalytic activity for these reactions could be explained by a greater than 2 orders of magnitude increase in affinity of Y65F mutant serine hydroxymethyltransferase for these amino acids bound as the external aldimine. These data are consistent with a role for the Tyr65 hydroxyl group in the conversion of a closed active site to an open structure.

Serine hydroxymethyltransferase[1] (SHMT) (EC 2.1.2.1) uses PLP and H_4PteGlu as coenzymes and catalyzes the reversible interconversion of serine and glycine (eq 1) and the irreversible hydrolysis of 5,10-CH^+-H_4PteGlu to 5-CHO-H_4PteGlu (eq 2) (*1, 2*). In addition to these physiological reactions, SHMT also catalyzes, in the absence of H_4PteGlu, the retroaldol cleavage of several 3-hydroxyamino acids, such as *allo*threonine (eq 3), the transamination of D- and L-alanine (eq 4) (*3, 4*), and the slow racemization of D- and L-alanine (*1*). Most of the reactions catalyzed by SHMT are the same as reactions catalyzed by other PLP enzymes, particularly those belonging to the α-class. Thus, elucidation of the mechanism of SHMT will contribute to our understanding of substrate and reaction specificity in PLP enzymes in general and the folate-specific reactions catalyzed by this enzyme.

L-serine + H_4PteGlu ⇔ glycine + CH_2-H_4PteGlu (1)

5,10-CH^+-H_4PteGlu ⟹ 5-CHO-H_4PteGlu (2)

*allo*threonine ⇔ glycine + acetaldehyde (3)

D- or L-alanine + PLP ⇔ pyruvate + PMP (4)

[2-^{3}H]glycine + H_2O ⇔ glycine + ^3H_2O (5)

The origins of substrate and reaction specificity of SHMT have been the subject of numerous mechanistic studies aided by characterization of site mutants designed without an experimental crystal structure of the enzyme (*3*−*9*). Pascarella et al. (*10, 11*) proposed that SHMT belongs to the α-class of PLP enzymes and modeled the active site of *E. coli* SHMT on the basis of the known structures of other members of this class. This provided a structural template for the design of site mutants. The recent solution of the crystal structures of both human and rabbit cytosolic SHMT confirmed that SHMT is a member of the α-class of PLP enzymes, thereby validating the use of the Pascarella homology model (*12, 13*). These structures have helped to retrospectively interpret many of the mechanistic studies of the mutant SHMTs. Lacking in these studies, however, was identification of a putative base that is required to remove the proton from the serine 3-hydroxyl group in the direction of serine cleavage, and to remove the *pro*-2*S* proton of glycine in the direction of serine synthesis. In other PLP enzymes, where a proton is removed from the α-carbon of the substrate, the active-site base is the ϵ-amino group of

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[1] Abbreviations: SHMT, serine hydroxymethyltransferase; H_4PteGlu, tetrahydrofolate; 5,10-CH^+-H_4PteGlu 5,10-methenyltetrahydrofolate; 5,10-CH_2-H_4PteGlu, 5,10-methylenetetrahydrofolate; 5-CHO-H_4PteGlu, 5-formyltetrahydrofolate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate.

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the Lys residue that forms the internal aldimine with PLP and removes the *R* proton on the α-carbon from the *si* face of C4′ (the carbon with the aldehyde group of PLP) (*14*). This is clearly not the case with SHMT, since the expelled amino group of the active-site Lys is on the *si* face of the α-carbon of the bound substrate and would remove the *pro-R* proton of glycine rather than the *pro-S* proton.

As a member of the α-group of PLP enzymes, SHMT differs in having evolved both a folate binding site and a base on the *re* face of the external aldimine. The structure of the folate site in *E. coli* SHMT clearly shows that the folate site was not formed by splicing with a folate binding domain but was created by several changes in amino acid sequence in the small domain of PLP enzymes (*15*). To understand the origin of the active-site base in SHMT we must first locate this base. The structures of the human and rabbit SHMTs suggest that either Tyr 65 or Glu 57 (numbering system based on *E. coli* SHMT) is this putative base, and this was further confirmed in the structure of *E. coli* SHMT in a ternary complex with glycine and 5-CHO-H_4PteGlu (*15*).

To investigate the putative role of Tyr 65 in the SHMT mechanism, we have prepared the Y65F mutant, characterized its properties in catalyzing reactions 1−5, and determined its crystal structure as the ternary complex with glycine and 5-CHO-H_4PteGlu. Both the activity of the Y65F mutant SHMT and its affinity for serine and glycine are greatly altered, but the crystal structure of its ternary complex is virtually identical to that of the wild-type enzyme. These observations permit us to conclude that Tyr65 is not the catalytic base but that it may play a critical role in the open to closed conformational change that takes place on substrate binding.

EXPERIMENTAL PROCEDURES

Materials. All buffers, amino acids, and coenzymes used in the purification and assay of enzymes were of the highest purity available. (6*S*)-H_4PteGlu was a gift from Eprova AG, Schaffhausen, Switzerland. Wild-type and Y65F SHMTs were expressed and purified as previously described (*16*). Methylene tetrahydrofolate dehydrogenase, used in the assay for serine, was purified from frozen rabbit liver obtained from Pel-Freeze Biologicals, Rogers, AR (*17*).

Site-Directed Mutagenesis. Mutations in SHMT were obtained by a PCR-based site-directed mutagenesis method as previously described (*16*). Enzyme expression was from a multicopy plasmid in a *rec*A$^-$ strain of *E. coli* that has been shown not to have any wild-type SHMT activity or undergo reversion by point mutations to form wild-type enzyme (*16*).

Kinetic Studies. All kinetic studies were done in either 20 mM potassium phosphate, pH 7.3, or 20 mM sodium *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonate (NaBes), pH 7.3, at 30 °C and containing 5 mM 2-mercaptoethanol and 0.2 mM ethylenediaminetetraacetic acid. These buffers at 20 mM concentration have no inhibitory effect on reaction rates or affinity of substrates. Kinetic assays for L-serine and H_4-PteGlu were performed by coupling the product CH_2-H_4-PteGlu to methylenetetrahydrofolate dehydrogenase with the concomitant reduction of NADP to NADPH (*18*). To determine the K_m for serine, H_4PteGlu was maintained at 0.23 mM and the serine concentration was varied between 0.06 mM and 6 mM, except for the mutant Y65F, where the serine concentration was varied between 3 and 100 μM. For K_m determinations of H_4PteGlu, L-serine concentrations were held constant at 30 mM and H_4PteGlu concentrations varied from 3 to 60 μM. For the Y65F SHMT the H_4PteGlu was varied between 8 and 160 μM. The concentration of Y65F SHMT in these assays was 9 μM. Values of k_{cat} for the wild-type enzyme were determined from the value of V_{max} determined from the *y*-axis intercept of double-reciprocal plots of velocity versus H_4PteGlu concentration. The value of k_{cat} for Y65F SHMT was determined by a computer fitting of the Scatchard equation for reactions where substrate and enzyme concentrations are similar. The K_m values for L-serine and H_4PteGlu correspond to the αK_m values previously reported for *E. coli* SHMT (*18*).

The rate of *allo*threonine cleavage was assayed by coupling the reduction of the product acetaldehyde with NADH and alcohol dehydrogenase (*19*). K_m and k_{cat} values were determined from double-reciprocal plots of the decrease in absorbance at 340 nm with *allo*threonine concentrations varied between 1.0 and 60 mM. For the Y65F mutant SHMT, *allo*threonine concentrations were varied from 15 μM to 30 mM.

The rates of transamination of D- and L-alanine were determined from first-order graphs of the disappearance of absorbance at 425 nm during the conversion of the enzyme-bound PLP to PMP (*8*, *9*). The K_m for D-alanine was determined by titrating enzyme with increasing concentrations of D-alanine (6−250 mM) and determining the maximum absorbance at 505 nm for the formation of intermediate III in Scheme 1. The K_m was determined from a best fit of the Michaelis−Menten equation for a graph of A_{505nm} versus D-alanine concentration.

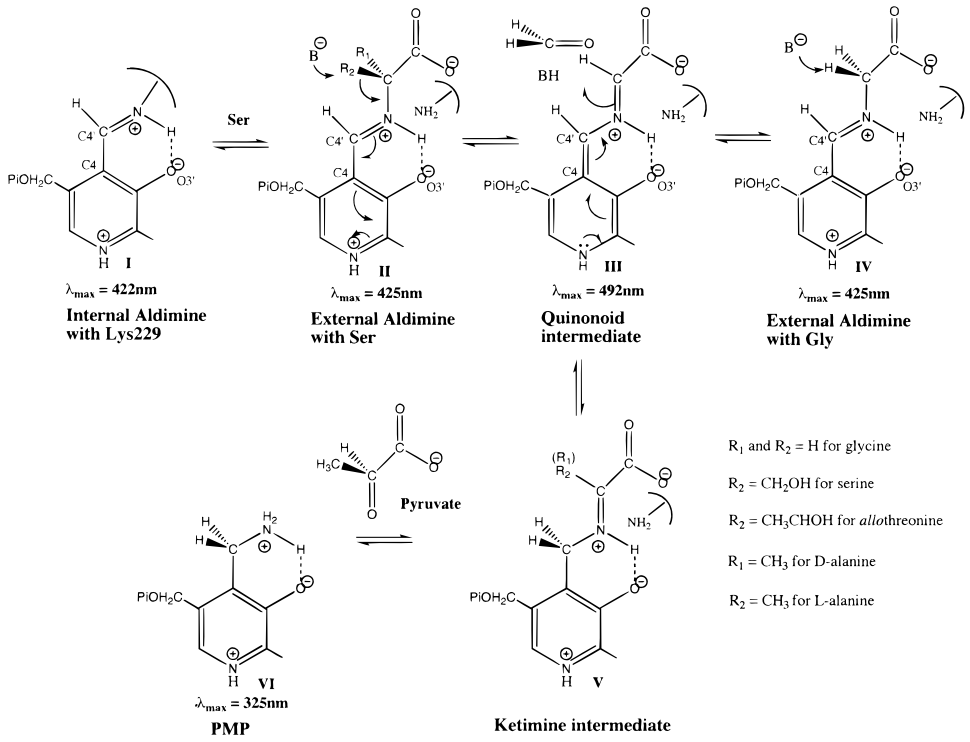
The rate of formation of the SHMT·D-alanine complex absorbing at 505 nm was determined by stopped-flow spectrophotometry in an Applied Biosystems spectrometer. SHMT, 100 μM, in one syringe was flowed against 400 mM D-alanine in the second syringe. The rate of formation of the complex absorbing near 505 nm was determined by fitting the absorbance versus time data to two parallel first-order reactions with the software provided by the instrument manufacturer.

The rate of exchange of the *pro*-2*S* proton of glycine (eq 5) with solvent protons was determined by incubating 0.4 mM [2-^{3}H]glycine (1.5×10^6 cpm) with SHMT and determining the amount of ^3H_2O formed in a 3 min incubation (*20*). The effect of H_4PteGlu on the rate of exchange was determined by adding 0.7 mM H_4PteGlu to the reaction solution.

The rate of hydrolysis of 5,10-CH^+-H_4PteGlu (15 μM) was determined by observing the rate of decrease in absorbance at 352 nm in 50 mM NaBES and 10 mM glycine, pH 7.0, at 30 °C (*2*). The rate of the nonenzymatic hydrolysis reaction in the absence of SHMT was subtracted from the rate of the SHMT-catalyzed reaction. The rate of the reaction was also verified by stopping the reaction with NaOH and determining the amount of 5-CHO-H_4PteGlu that had been formed by its ability to form the quinonoid complex with SHMT·Gly absorbing at 492 nm (*2*).

CD Spectra. Affinity of amino acid ligands for SHMT was determined from the decrease in optical activity of the

Scheme 1



PLP chromophore at 425 nm during titration of SHMT (44 μM) with successive increasing concentrations of the amino acid ligand. The serine concentrations were from 30 μM to 6 mM. Glycine concentrations were varied between 0.2 and 70 mM. L-Alanine was varied between 1 and 100 mM. Spectra were recorded between 500 and 350 nm at 30 °C on a Jasco 725 spectropolarimeter in the circular dichroism mode. Except for serine, K_d values for each amino acid were also determined in the presence of 0.23 mM H_4PteGlu. The addition of H_4PteGlu did not affect the CD spectrum at 425 nm. Values for optical activity were normalized to millidegrees per millimole of active site on SHMT.

The values of K_d for each amino acid ligand were determined by computer fitting to eq 6, where E_0 and S_0 were initial concentrations of SHMT and the amino acid ligand, respectively.

$$K_d = (E_0 - ES)(S_0 - ES)/ES \quad (6)$$

The value for *ES* was determined from the CD spectra by use of the relationship shown in eq 7, where ΔCD_{max} and ΔCD_{425} are the change in SHMT CD signal at 425 nm at saturating amino acid ligand and titration levels of amino acid ligand, respectively:

$$ES = (\Delta CD_{425}/\Delta CD_{max})E_0 \quad (7)$$

Determination of the Three-Dimensional Structure of Y65F. Monoclinic ($P2_1$, 4 molecules/asymmetric unit) crystals of the Y65F ternary complex with 5-CHO-H_4PteGlu and glycine were grown under the same conditions as the wild-type complex with which they are isomorphous (*21*). Intensity data were collected at room temperature on an R-axis II image plate detector with a rotating anode source run at 80 kV and 100 mA, as for the wild-type enzyme (*15*).

A difference Fourier synthesis between the Y65F mutant and wild-type ternary complexes showed a large negative peak at the position of the OH of Y65 but no other large-scale structural changes. The electron density was fit with the wild-type SHMT ternary complex model modified to phenylalanine at position 65, and solvent molecules were excluded from both the difference Fourier synthesis and the initial $2mF_o - F_c$ map calculation.

The model was iteratively refined via torsion angle dynamics with a maximum likelihood target function (*22*) by CNS 0.5b, with each refinement cycle followed by manual rebuilding in O with $2mF_o - dF_c$ cross-validated SIGMAA weighted maps (*23*). Noncrystallographic symmetry was enforced via positional NCS restraints between symmetry-related molecules with an initial weight of 300 kcal/(mol Å), which was reduced to 150 kcal/(mol Å) after the first cycle of refinement. All data between 20 and 2.7 Å with magnitudes $F > \sigma$ were included in the refinement. During iterative rebuilding, residue geometries were modeled with the program OOPS (*24*) and WHATCHECK (*25*). In the final stages of refinement, 318 solvent molecules were added on the basis of the presence of unused density in the SIGMAA-weighted $2mF_o - dF_c$ maps and proximal protein hydrogen-bond acceptors/donors within 3.3 Å of the water oxygen. The maximum estimated coordinate error in the refined structure is 0.18 Å and the optical resolution is 2.0 Å calculated from SFCHECK (*26*). Data collection and refinement statistics are shown in Table 1. Coordinates have been deposited in the RCSB Protein Data Bank, accession number 1EQB.

RESULTS

Comparison of Crystal Structures of Y65F and Wild-Type SHMT Ternary Complexes. Crystals of the Y65F mutant SHMT ternary complex are isomorphous with those of the

Table 1: Data Collection and Refinement Statistics for *E. coli* Y65F Ternary Complex

Data Collection	
resolution (Å)	2.7
R_{merge}[a]	0.28
$\langle I\rangle/\langle\sigma(I)\rangle$ overall/highest resolution shell	5.53/1.09
completeness overall[b]	0.95
completeness highest resolution shell (2.75−2.7 Å)[b]	0.86
N_{obs} (92−2.7 Å)	63328
% reflections with multiplicity > 3	73.6%
Refinement	
R_{work}[c] overall (20−2.7 Å)	0.205
R_{work}[c] highest resolution shell (2.83−2.70 Å)	0.298
R_{free}[c] (20−2.4 Å)	0.236
R_{free}[c] highest resolution shell (2.82−2.70 Å)	0.334
completeness, overall[c]	0.95
completeness, highest resolution shell[c]	0.86
$\langle B\rangle$ (Å^2)	36.8
N_{work}	56622
N_{free}	6000
N_{atoms}	13171
$N_{degrees\ of\ freedom\ torsion\ angle\ dynamics}$	8389
Rmsd from ideal:	
bond length (Å)	0.007
bond angles (deg)	1.2
dihedral angles (deg)	22.2
improper angles (deg)	2.09

[a] $R_{merge} = \sum\sum_i|I_h - I_{hi}|/\sum\sum_i I_h$ where I_h is the mean intensity of reflection *h*. All data with $I > -3\sigma$ are included. [b] For reflections with $I > -3\sigma$. [c] Calculated with reflections with $F > \sigma$.

wild-type ternary complex. A difference Fourier synthesis, using $F_{Y65F} - F_{wt}$ coefficients and phases from the refined structure of the wild-type ternary complex, clearly shows a hole at the position of the $O^{\zeta}H$ of Y65 with no major changes elsewhere in the structure, with the exception of some solvent molecules remote from the active site. Figure 1 shows the final refined electron density around the active site of the Y65F mutant with the wild-type structure superposed on it. The active sites of SHMT, like those of other α-type PLP enzymes, is constituted of residues from both subunits of the active dimer. In the SHMT ternary complex crystal structure, there are two of these tight dimers per asymmetric unit. Residues Y(orF)65 and E57 are on the B subunit heterologous to the A subunit containing the PLP they are closest to. The complete isomorphism of this mutant to the wild-type SHMT permits us to exclude effects from changes in enzyme structure in comparing their catalytic properties.

Proposed Mechanism. Scheme 1 shows our current understanding of the PLP-mediated steps of the SHMT mechanism. Serine reacts with the internal aldimine (structure I), forming the serine external aldimine (structure II), which is cleaved to form bound formaldehyde and the quinonoid complex (structure III) (*1*). Protonation of the quinonoid intermediate forms the external aldimine of glycine (structure IV). The mechanism by which formaldehyde reacts with H_4-PteGlu to form CH_2-H_4PteGlu is not shown. The proposed mechanism shown in Scheme 1 has been deduced from spectral studies of SHMT in the presence of different ligands (*1*). Wild-type SHMT is characterized by a single absorbance band at 422 nm (structure I) at wavelengths above 300 nm. Saturation with either serine or glycine results in a major band at 425 nm for the external aldimines (structures II and IV) and minor bands at 343 nm for the *gem*-diamine (not shown in Scheme 1; it occurs between the internal and external aldimines and has C4′ with one bond each to the ϵ-amino group of Lys229 and the amino group of serine). With glycine a small band at 492 nm can also be seen at pH 8, representing the quinonoid complex (structure III). The addition of H_4PteGlu to this SHMT·Gly complex forms an abortive ternary SHMT·Gly·H_4PteGlu complex, resulting in a greater than 50-fold increase in the absorbance at 492 nm, and a 3 orders of magnitude increase in the rate of exchange of the *pro*-2*S* proton of glycine with solvent protons (eq 5). The folate site positions N5 about 4 Å from the α-carbon of glycine (*15*).

The reactions with D- and L-alanine are shown as a side reaction from the quinonoid complex intermediate (structure III going to V and VI, Scheme 1) where a proton is added to C4′ of PLP in the quinonoid intermediate (structure III) to form the ketimine intermediate (structure V), which hydrolyzes to form pyruvate and PMP (structure VI). PMP has little affinity for the active site and dissociates, leaving apoSHMT (*8*). The two alanine stereoisomers are converted to the quinonoid complex by abstraction of either the 2*R* proton from L-alanine or the 2*S* proton from D-alanine. Previous studies have shown that different bases at the active site are involved in abstraction of the different α-protons from the alanine isomers (*8*, *9*). D-Alanine is an analogue of glycine, and the active-site base that removes the *pro*-2*S* proton of glycine is presumably the same base that removes the 2*S* proton of D-alanine. Likewise, the base that removes the 2*R* proton of L-alanine accounts for the very slow removal of the *pro*-2*R* proton of glycine observed by Malthouse (*27*).

The structure of the *E. coli* SHMT ternary complex shows that Tyr65 lies between the glycine product and N5 of H_4-PteGlu, suggesting it may be involved in mediating the effect of H_4PteGlu on the spectral changes at 492 nm and the rate of glycine proton exchange. When SHMT is saturated with either D- or L-alanine, intermediate III is populated, as evidenced by an absorption band at 505 nm. The appearance of this band is formed in the millisecond time range, but the half-life for transamination (III to VI) occurs with a $t_{1/2}$ in the minute time range. The rate of transamination of D-alanine is about 3-fold faster than the rate of transamination of L-alanine (Table 2).

Spectral Studies. Y65F SHMT exhibits a spectrum identical to that of the wild-type enzyme with a single absorption maximum at 422 nm for the internal aldimine of PLP. Saturation with either glycine or serine results in a small shift of the 422 nm band to 425 nm, characteristic of the external aldimine, as observed for the wild-type enzyme (data not shown). However, saturation of Y65F SHMT with glycine or serine results in no detectable increase in absorption at either 343 or 492 nm as observed with the wild-type enzyme. This suggests that, in the mutant enzyme, there is a decrease in the equilibrium population of the *gem*-diamine and quinonoid complexes compared to wild-type enzyme (Scheme 1). The addition of H_4PteGlu to form the Y65F SHMT·Gly·H_4PteGlu ternary complex does not result in an observable absorbance band at 492 nm, suggesting that Y65F SHMT may be blocked in conversion of the glycine external aldimine (structure IV) to the quinonoid complex (structure III).

Both L- and D-alanine form external aldimines absorbing at 425 nm with wild-type SHMT, and D-alanine forms an absorbance band at 505 nm that is characteristic of the quinonoid complex (*8*). Saturation of Y65F SHMT with

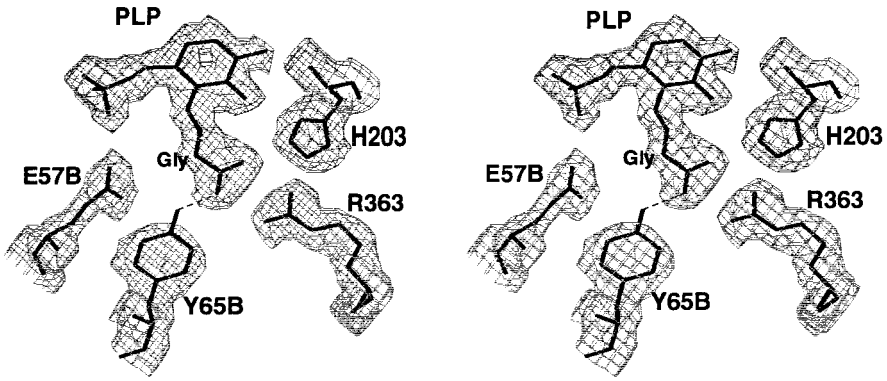


FIGURE 1: Stereoview of the final refined electron density ($2mF_o - dF_c$) for Y65F SHMT with the wild-type SHMT structure overlaid to show the absence of electron density for the $O^{\zeta}H$. The dotted line indicates a hydrogen bond (2.61 Å) to the glycine carboxylate, present in the wild-type ternary complex but missing in the Y65F mutant.

Table 2: Kinetic Constants for Wild-Type and Y65F SHMT Catalysis of Reactions Shown in Eqs 1−5

reaction	k_{cat} for wild-type (min^{-1})	k(wild type)/ k(Y65F)[a]
serine cleavage (eq 1)	640[b]	385
*allo*threonine cleavage (eq 3)	30[c]	70
[2-^{3}H]glycine exchange (eq 5)[d]		
−H_4PteGlu	0.9	1.9
+H_4PteGlu	1020	370
D-alanine transamination (eq 4)	0.033[e]	0.24
L-alanine transamination (eq 4)	0.012[e]	1.09
CH^+-H_4PteGlu hydrolysis (eq 2)	13[f]	0.93

[a] All kinetic constants are the average of at least three determinations. The range of values was always less than ±5%, except for serine cleavage with the Y65F mutant SHMT, where the range was ±10%. [b] Determined from steady-state initial velocity studies of eq 1. [c] Determined from steady-state initial velocity studies of eq 3. [d] Corrected for the difference in affinity for glycine between wild-type and Y65F eSHMTs. [e] Determined from rate of disappearance of absorbance at 425 nm. [f] Determined from rate of disappearance of absorbance at 358 nm.

D-alanine results in a peak at 425 nm and a 505 nm peak that is 2-fold higher than that observed in the wild-type enzyme (Figure 2). Saturation of this binary SHMT·D-Ala complex with H_4PteGlu results in about another 2-fold increase in the quinonoid complex for both wild-type and Y65F SHMTs (data not shown). Addition of H_4PteGlu to either the wild-type or Y65F SHMT·L-alanine complexes absorbing at 425 nm does not alter the spectrum for either complex (data not shown). The observed formation of the quinonoid complex of Y65F SHMT with D-alanine is in sharp contrast to the results with glycine, which does not show any evidence for the formation of a quinonoid complex with the mutant enzyme.

Catalytic Activity with Serine, Glycine, and Allothreonine. Catalysis of serine cleavage (eq 1) by Y65F SHMT is nearly 400-fold lower than by wild-type SHMT when saturated with both serine and H_4PteGlu (Table 2). To test the catalytic competency of Y65F SHMT with glycine, we looked at the rate of exchange of the *pro*-2*S* proton with solvent (eq 5). In the absence of H_4PteGlu this exchange reaction is very slow, but it is changed by no more than a factor of 2 between mutant and wild-type SHMTs. However, in the presence of tetrahydrofolate the rate of proton exchange with solvent is greatly increased for wild-type SHMT (1100-fold) but increased considerably less for Y65F SHMT (6-fold) (Table 2). This experiment was performed at a single concentration of glycine (0.4 mM), which is about the same as the K_d value for wild-type enzyme but 130-fold higher than the K_d value of glycine for Y65F SHMT (Table 3). Since glycine binds much more tightly to the mutant enzyme, we corrected the exchange rate for wild-type SHMT to saturating levels of glycine by doubling the value for the exchange rate. Previous studies have shown that K_m and K_d values for glycine and serine are the same for SHMT. With this correction there is a 370-fold decrease in the rate of solvent exchange of the α-H of glycine catalyzed by Y65F SHMT compared to wild-type SHMT when both are saturated with glycine and tetrahydrofolate (Table 2).

The value of k_{cat} for *allo*threonine cleavage (eq 3) by Y65F is 70-fold lower than observed in wild-type enzyme (Table 2). The rate of hydrolysis of 5,10-methenyltetrahydrofolate (eq 2) is essentially unchanged from the rate observed with wild-type enzyme (Table 2).

Catalytic Activity with D- and L-Alanine. Saturation of wild-type SHMT by D-alanine results in a biphasic increase in absorption at 505 nm that can be fit to two first-order reactions. The rapid phase exhibits a $k = 13\ s^{-1}$, with the slow phase being $0.2\ s^{-1}$ (data not shown), with the rapid phase exhibiting $^1/_3$ of the total amplitude. The two phases are most likely the result of two slightly different modes of binding or conformational states of the enzyme. With Y65F SHMT the formation of the 505-nm-absorbing complex with D-alanine is also biphasic with rate constants of $14.5\ s^{-1}$ and $0.4\ s^{-1}$, with the same relative amplitudes as observed for wild-type SHMT. The slightly faster rate of formation of the quinonoid complex with Y65F SHMT is most likely reflected in the 2-fold increase in its equilibrium concentration (Figure 2). The increase in concentration of the quinonoid complex may also explain why Y65F SHMT transaminates D-alanine 4-fold faster than wild-type SHMT, since the rate determining step occurs after quinonoid formation (Table 2).

Ligand Affinities. Determining the affinity of Y65F SHMT for glycine and serine proved to be difficult because of the very low catalytic activity of the mutant enzyme. Initial attempts to determine K_m values for glycine and serine quickly demonstrated that not only did the mutant enzyme have a very low activity but also it had a much higher affinity for its amino acid substrates (low micromolar range), increasing the difficulty in measuring K_m values. We were able to determine the K_m for serine when the enzyme was

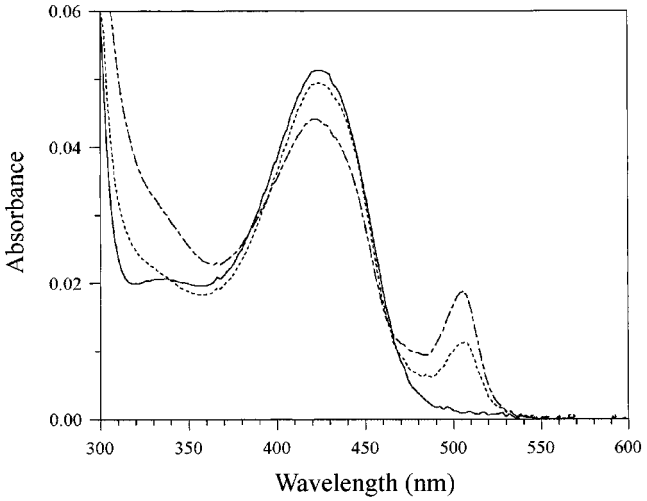


FIGURE 2: Spectra of SHMT saturated with D-alanine in 20 mM potassium phosphate, pH, 7.3. Spectra of 10 μM wild-type SHMT (—), with 0.2 M D-alanine (•••), and of 10 μM Y65F SHMT with 0.2 M D-alanine (—•—).

Table 3: Relative Affinities of Wild-Type and Y65F SHMTs for Substrates and Substrate Analogues

ligand	wild type K_m or K_d	wild type/Y65F K_m or K_d[a]
serine		
−H_4PteGlu	0.8[b]	37
+H_4PteGlu	0.3[b]	435
glycine		
−H_4PteGlu	6.7[c]	4.1
+H_4PteGlu	2.1[c]	300
*allo*threonine	1.5[d]	19
D-alanine	30[e]	3.7
L-alanine	28[c]	0.59
H_4PteGlu	0.025[b]	3.0

[a] The values of K_m or K_d are the average of at least three determinations with the range between values being less than ±5%, except for serine, where the range of values was ±10%. [b] Determined by steady-state initial velocity studies of eq 1. [c] Determined from changes in circular dichroism amplitude at 425 nm. [d] Determined by steady-state initial velocity studies of eq 3. [e] Determined from absorbance at 505 nm.

saturated with H_4PteGlu (Table 3). The value of 0.7 μM is 430-fold lower than the 0.3 mM value for wild-type SHMT. However, we could not determine K_m values for serine at lower H_4PteGlu concentrations, which is required for determining affinity for serine in the absence of H_4PteGlu. Likewise, we could determine the affinity of H_4PteGlu for Y65F SHMT only when the enzyme was saturated with serine (Table 3). Determining the affinity for glycine was complicated by the inability of the Y65F mutant enzyme to form a quinonoid complex absorbing at 492 nm, which had previously been used to determine the affinity of both glycine and H_4PteGlu for SHMT (*28*, *29*).

The problem of determining dissociation constants for Y65F SHMT was solved from CD spectra of the bound PLP during titration with amino acid ligands. Despite the small shift in wavelength absorbance maximum during the conversion of the internal aldimine to the external aldimine (structures I to II and VI), there is a significant change in the optical activity of the bound PLP as evidenced by the CD spectra at 425 nm during titration with amino acids (Figure 3). Y65F SHMT was titrated with glycine, L-serine,

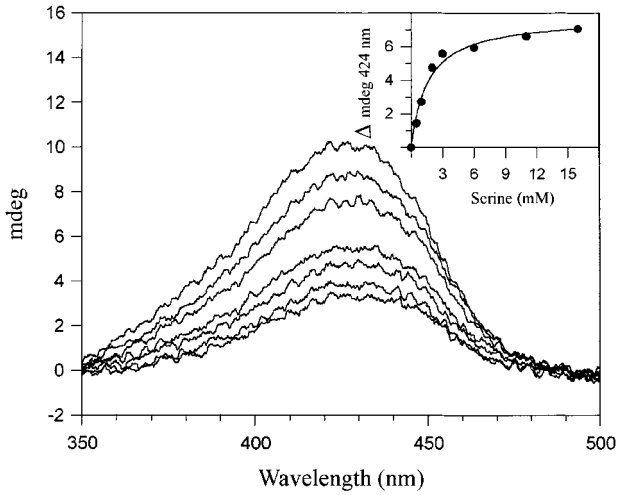


FIGURE 3: Circular dichroism spectra of wild-type SHMT (44 μM) in the presence of increasing concentrations of L-serine. The topmost curve is in the absence of serine and the bottom curve is in the presence of 15 mM serine. Inset: Plot of the change in millidegrees as a function of serine concentration. The solid line is a curve fit of the data as described under Experimental Procedures.

or L-alanine and the CD spectrum was recorded after each addition of the respective amino acid in the absence of H_4-PteGlu. The affinities of these amino acids were determined from saturation curve analyses of the change in optical activity as a function of ligand added. There was a 37-fold increase in affinity for serine but only a 4-fold increase in affinity for glycine. D-Alanine and L-alanine exhibit a 4-fold increase and a 2-fold decrease in affinity for the mutant enzyme, respectively (Table 3). These studies were repeated for glycine in the presence of saturating levels of H_4PteGlu. Under these conditions there is a 330-fold increase in affinity for glycine compared to the 4-fold increase in the absence of H_4PteGlu. Previous studies with wild-type SHMT have shown that there is about a 5-fold synergistic increase in affinity between the amino acid substrates and H_4PteGlu (*18*). With Y65F SHMT the increase in affinity of 80-fold for glycine in the presence of H_4PteGlu versus its absence is unique, and represents more than an order of magnitude increase in synergism that is not observed in any other SHMT.

DISCUSSION

Structural Properties of Y65F SHMT. Currently the 3-dimensional structures of human and rabbit cytosolic SHMT have been determined without bound ligands, and the 3-dimensional structure of *E. coli* SHMT has been solved as a ternary complex with glycine and 5-formyl-H_4PteGlu (*12*, *13*, *15*). In all of these structures the active-site residues and their orientations are highly conserved. Previous ligand binding studies suggested that binding of the substrate amino acids results in a closing of the active site. Three effects on protein properties associated with substrate binding led to this conclusion: (1) there is an increase in thermal stability, as evidenced by a large increase in the enthalpy of denaturation and up to a 12 °C increase in T_m for denaturation; (2) there are 17−19 fewer protons on the enzyme that rapidly exchange with solvent; and (3) there is a significant change in the optical activity of the bound PLP (9). These changes in properties are evoked when either serine is bound

or when glycine binds in the presence of a reduced folate, such as H_4PteGlu, 5-CHO-H_4PteGlu, or 5-CH_3-H_4PteGlu. *Allo*threonine binding also induces these changes, but neither D- nor L-alanine does, nor glycine in the absence of a reduced folate. It was concluded that closing of the active site requires a substrate carboxylate group and either a 3-hydroxy group or a reduced folate. The transamination and racemization of D- and L-alanine occur because they do not induce the closed active site, and in the open active site the enzyme loses control of proton placements, resulting in the nonphysiological side reaction shown in Scheme 1.

Comparison of the rabbit and *E. coli* structures supports the open−closed conformational change that takes place when substrate amino acids bind. The carboxyl group of substrate glycine and the Arg 363 guanidinium group form an ion pair with two H-bonds (2.7 and 2.8 Å, respectively) in the ternary complex of *E. coli* SHMT, consistent with a critical role for this interaction in inducing the conformational change (*15*). In the rabbit structure without bound ligands, glycine was modeled into the active site as an external aldimine with PLP (*12*). From this structure, Arg 363 can make only a single H-bond to the carboxylate group, and Tyr 65, which is on the other subunit, is about 1.5 Å further removed from the glycine carboxylate than in the *E. coli* SHMT ternary complex structure (*15*). We conclude that in the closing of the active site the carboxyl group of substrate glycine moves to make close contact with the side chains of Arg 363 and Tyr 65 (Figure 1). In addition to Tyr65 and Arg363, both Ser35 and His203 are also within H-bonding distance to the carboxyl group of glycine in the closed ternary complex.

As has been noted for both aspartate aminotransferase and thymidylate synthase, an extensive hydrogen-bonding network at the active site controls reaction specificity, and disruptions at any one of a number of sites can alter the catalytic efficiency and substrate specificity of these enzymes (*30*−*32*). In SHMT there is also an extensive H-bonding network (*15*). Previous studies on site mutants of SHMT have identified the function of some of these H-bond donors and acceptors. For example, changing R363 to Ala, and even to Lys, inactivates the enzyme and abolishes its ability to bind amino acid substrates having a carboxyl group. However, both serinamide and serine ethyl ester bind to the R363A mutant enzyme and are slowly turned over by the enzyme (binding and cleavage of these analogues are not observed with wild-type SHMT). Binding of these substrate analogues does not trigger the open-closed conformational change supporting the role of Arg363 in substrate affinity and stabilizing the anion on the α-carbon in the quinonoid complex.

The position of Y65 at the active site in both the human and rabbit cytosolic structure suggests it might be the elusive active-site base that is involved in proton abstraction from either the *pro*-2*S* position of glycine or the serine hydroxyl. However, in the *E. coli* ternary complex Y65 is more than 4 Å from the α-carbon of glycine and even farther from the putative site of the serine hydroxyl, making it unlikely that it is the active-site base (Figure 1). The complete isomorphism of this mutant to the wild-type ternary complex structure permits us to confidently assign or exclude mechanistic roles for this hydroxyl group without confounding effects due to positional changes in other residues.

Properties of Y65F SHMT. The 400-fold decrease in the rate of cleavage of serine (eq 1) and the 330-fold decrease in the rate of α-proton exchange of glycine (eq 5) in the Y65F mutant enzyme might suggest that Tyr65 is the active-site base. However, the determination of the affinity of both serine and glycine clearly shows that this decrease in catalytic activity is the result of tighter binding of these amino acid substrates. The values for k_{cat}/K_m for both serine and glycine have not changed, suggesting that in the Y65F mutant the transition-state energy for the rate-determining step(s) has not changed significantly. Thus, the decrease in rate is a consequence of the enzyme−substrate complex being in an energy well that is 3.6 kcal/mol deeper than in the wild-type enzyme. The bound serine and glycine substrates face an extra 3.6 kcal/mol energy barrier in conversion to their respective products.

This raises the question as to which enzyme−substrate complex on the reaction pathway has been stabilized. The spectral properties of Y65F SHMT, in the presence of substrates, show only absorption for the external aldimines for both serine and glycine (structures II and IV, Scheme 1), suggesting that the stabilization is unique to the external aldimine complexes. Further support for this is the absence in Y65F SHMT, saturated with both glycine and H_4PteGlu, of the 492 nm absorbance of the quinonoid complex, which is large in the wild-type enzyme where the equilibrium is shifted from the external aldimine (structure IV) to the quinonoid complex (structure III). Although there is no measurable increase in absorbance at 492 nm for the Y65F SHMT·Gly·H_4PteGlu ternary complex, a small amount of quinonoid complex must occur, because it undergoes exchange of the *pro* 2*S* proton of glycine with solvent, and this reaction requires that the intermediate quinonoid complex be formed.

The Y65F mutation has less effect on the rate of cleavage of *allo*threonine and the affinity for this substrate compared to serine (Tables 2 and 3). However, the decreases are still substantial, and as with glycine and serine, the value of k_{cat}/K_m for *allo*threonine has changed very little, suggesting that the mutant enzyme has stabilized the external aldimine with *allo*threonine without a significant change in the transition state for forming the quinonoid complex.

In contrast to glycine, serine, and *allo*threonine, the rate-determining steps for D- and L-alanine transamination and the rate at which they form the quinonoid complex are not significantly affected (Table 2). The rate-determining steps are clearly off the normal catalytic pathway for these reactions and involve the addition of a proton to C4′ of the PLP ring and hydrolysis of the ketimine intermediate (structure V). What is most interesting is that D-alanine, unlike glycine, gives the 505 nm absorbing peak that is characteristic of the quinonoid complex that is on the catalytic pathway for the physiological substrates. There is a small change in the properties of Y65F SHMT with D-alanine, since it transaminates a few times faster than wild-type enzyme, probably due to the increased concentration of the quinonoid complex (Figure 2). The affinities of D- and L-alanine for the mutant enzyme have changed by a factor of only a few in contrast to the other substrates.

The ability of Y65F SHMT to catalyze the hydrolysis of 5,10-CH^+H_4PteGlu (eq 2) has not been altered. The Tyr 65 hydroxyl does not make any hydrogen bonds to the folate

analogue 5-CHO-H_4PteGlu bound at the active site. In the mutant enzyme the K_m for H_4PteGlu has been increased by only 3-fold. These observations suggest that this mutation has not significantly altered the affinity or chemistry of the folate coenzyme.

Role of Tyr 65. The function of Tyr65 is mediated most likely through its interaction with the carboxylate group of either bound glycine or serine. Tyr65 is one of four residues that can form H-bonds to the substrate carboxyl group. Previous studies have suggested that the carboxyl group is an essential structural feature of substrate binding in triggering an open to closed conformational change in the enzyme (*9*). In the Y65F mutant, removal of one of these H-bonds to the carboxyl group results in a 400-fold increase in affinity of the substrate. This is counterintuitive, since removing a H-bond would be expected to weaken the interaction and result in lower affinity for the amino acid substrate. The key to understanding the role of Tyr65 may be the binding of L- and D-alanine and glycine in the absence of a reduced folate. Previous studies have shown that these three amino acids do not trigger changes in the properties of the enzyme associated with the switch to the closed structure. The affinity and kinetic constants for these three amino acids have changed little in Y65F SHMT. This is particularly important for the α-proton exchange and affinity of glycine in the absence of H_4PteGlu. Tyr65 does not involve the formation of the external aldimine since D- and L-alanine both form this complex and so does glycine in the absence of H_4PteGlu. It is the next step in the reaction pathway, where the external aldimine goes to the closed structure that seems to involve Tyr65. If the H-bond donated by Tyr65 to the carboxyl group of glycine is one of the determinants of the open−closed transition, its H-bond may not contribute to substrate binding, but rather it may weaken the interaction between the Arg363 guanidinium group and the substrate carboxylate. This may be required to permit the switch to the open form, which allows the amino acid to escape at the end of a catalytic cycle. Removal of this H-bond would thus result in a stabilization of the closed form.

We have noted previously that, in PLP enzymes, reaction specificity involves mostly the proper removal and placement of protons on the substrate (*8, 9*). We concluded that the lack of reaction specificity with D- and L-alanine was the result of these amino acids not being able to induce the closed structure but rather remaining in the open structure, where proper alignment for transfer of protons between intermediates is not possible. It is also clear that, by some mechanism, H_4PteGlu binding plays a role in the open−closed conformational change when glycine is the substrate. At this time we do not understand this connection, but we are currently changing other residues in the H-bonding network to identify the role of each in the mechanism of SHMT.

Nonproductive substrate binding is an alternative explanation for the 400-fold decrease in both k_{cat} and K_m values for serine and glycine with Y65F SHMT. If most molecules of serine or glycine bound tightly in a nonproductive mode in the mutant enzyme, and only a small fraction bound in the normal catalytically productive mode, one would observe the same kinetic properties as observed in this study. This mechanism seems less likely than the open−closed argument given above since there is no apparent reason for nonproductive binding of the amino acid substrate in Y65F SHMT. Furthermore, the absorbance and CD properties of the PLP ring would be expected to reflect nonproductive binding, but these two spectral properties are the same for serine and glycine binding in both wild-type and Y65F SHMTs. With all substrates we cannot detect any change between wild-type and Y65F SHMTs with respect to the position of the absorbance maxima or their optical properties. We see only changes in the equilibrium distribution of intermediates. Also, the structures of both Y65F and wild-type SHMTs were determined with glycine and 5-formyltetrahydrofolate bound and there is no detectable difference in the positions of any of the atoms of glycine or tetrahydrofolate. This suggests that both of these ligands bind to Y65F SHMT in the same manner as in the wild-type enzyme and that the amino acid substrate is not bound in some altered nonproductive complex.

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