Kinetic Mechanism and the Rate-Limiting Step of *Plasmodium vivax* Serine Hydroxymethyltransferase

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**Keywords:** Pyridoxal 5’-phosphate-dependent enzyme; *Plasmodium vivax* serine hydroxymethyltransferase (PvSHMT); random-order mechanism; deoxythymidylate (dTMP) cycle

**Background:** *Plasmodium vivax* serine hydroxymethyltransferase (PvSHMT) catalyzes formation of glycine from L-serine and tetrahydrofolate.

**Results:** Results indicate that PvSHMT can bind to either substrate first. The rate constant of glycine formation is similar to $k_{cat}$.

**Conclusion:** PvSHMT reaction occurs via a random-order mechanism and glycine formation is the rate-limiting step.

**Significance:** The data are useful for future investigation on inhibition of SHMT for antimalarial drug development.

**ABSTRACT**

Serine hydroxymethyltransferase (SHMT) is a pyridoxal 5’-phosphate (PLP)-dependent enzyme that catalyzes a hydroxymethyl group transfer from L-serine to tetrahydrofolate (H4folate) to yield glycine and 5,10-methylenetetrahydrofolate (CH2-H4folate). SHMT is crucial for dTMP biosynthesis and a target for antimalarial drug development. Our previous studies indicate that PvSHMT catalyzes the reaction via a ternary-complex mechanism. In order to define the kinetic mechanism of this catalysis, we explored the PvSHMT reaction by employing various methodologies including ligand binding, transient, and steady-state kinetics as well as product analysis by rapid-quench and HPLC/MS techniques. The results indicate that PvSHMT can bind first to either L-serine or H4folate. The dissociation constants for the enzyme:L-serine and enzyme:H4folate complexes were determined as 0.18 ± 0.08 mM and 0.35 ± 0.06 mM, respectively. The amounts of glycine formed after single turnovers of different preformed binary complexes were similar, indicating that the reaction proceeds via a random-order binding mechanism. In addition, the rate constant of glycine formation...
measured by rapid-quench and HPLC/MS analysis is similar to the $k_{cat}$ value ($1.09 \pm 0.05$ s$^{-1}$) obtained from the steady-state kinetics, indicating that glycine formation is the rate-limiting step of SHMT catalysis. This information will serve as a basis for future investigation on species-specific inhibition of SHMT for antimalarial drug development.

Serine hydroxymethyltransferase (SHMT) (L-serine:tetrahydrofolate 5,10-hydroxymethyl transferase; EC. 2.1.2.1) is a pyridoxal 5'-phosphate (PLP)-dependent enzyme that catalyzes the reversible transfer of a hydroxymethyl group from L-serine to tetrahydrofolate (H$_4$folate), yielding glycine and 5,10-methylenetetrahydrofolate (CH$_2$-H$_4$folate) as products (1,2). The enzyme is involved in the deoxythymidylate (dTMP) synthesis cycle, as CH$_2$-H$_4$folate is a substrate for thymidylate synthase, which synthesizes dTMP, a precursor for DNA biosynthesis (3). In addition to the physiological H$_4$folate-dependent SHMT reaction, SHMT from many organisms can also catalyze various other reactions such as H$_4$folate-independent retro-aldol cleavage of β-hydroxy amino acids including L-threonine, L-allo-threonine, (D,L)-β-phenylserine, and L-threo-phenylserine, transaminations, racemization, decarboxylation, and condensation (2, 4-7). Therefore, SHMT has been an enzyme of interest not only because of its important physiological function, but also for its reaction promiscuity (2, 8-10).

*Plasmodium* SHMT has been shown to be essential for parasite growth and development, making it a prime target for antimalarial drug chemotherapy development (11). The recombinant expression and purification of *Plasmodium falciparum* (PfSHMT) and *Plasmodium vivax* SHMT (PvSHMT) as well as their biochemical characterizations have been reported (5,12-14). In general, both of these enzymes are similar in their enzymatic properties. The crystal structure of PfSHMT has been recently solved, revealing the unique function of a cysteine pair at the folate binding pocket in regulating enzyme function through variation of the redox states of these cysteines. This regulation is different from the mammalian and bacterial SHMTs (15). Moreover, it has been shown that *Plasmodium* SHMTs display several properties that are distinct from the human enzyme, including the inactivation kinetics towards thiosemicarbazide (16). This implies that it is possible to design species-specific inhibitors for anti-malarial drug development. An in-depth understanding of *Plasmodium* SHMT kinetics and mechanism is therefore needed so that the differences in reaction details among SHMTs can be understood and used for the development of specific inhibitors.

Although the reaction of SHMT from various species has been investigated, many of these studies focused on the non-physiological reaction, or the reverse reaction of glycine and CH$_2$-H$_4$folate (2,7,8,17,18). The pre-steady state kinetics of the H$_4$folate-dependent SHMT reaction has never been investigated. Double-reciprocal plots of bi-substrate kinetics of SHMT from rabbit liver cytosol, *P. vivax*, and *P. falciparum* display intersecting lines, suggesting that the reaction occurs via a ternary-complex mechanism in which the formation of an enzyme:serine:H$_4$folate complex is required for catalysis (5,12,17). However, it was unclear whether the substrates bind in a compulsory or random order. While the binding of L-serine or other amino acids to SHMT can be clearly monitored by spectroscopic detection based on the formation of an external aldime, up to now, evidence supporting direct binding between SHMT and H$_4$folate has not been demonstrated.

In this study, we employed various methodologies including ligand binding measurements, as well as transient and steady-state kinetics to investigate the PvSHMT reaction. Upon anaerobic titration of H$_4$folate into the enzyme solution, a spectroscopic signal resulting from the direct binding of PvSHMT and H$_4$folate was detected. Results from rapid-quench and HPLC/MS analysis and steady-state kinetics have clearly established glycine formation as the rate-limiting step for the overall reaction of PvSHMT.

**EXPERIMENTAL PROCEDURES**

Reagents—All chemicals and reagents used were analytical grade and of the highest purity commercially available as described previously (5). An expression plasmid for hexa-histidine tagged FAD-dependent NAD(P)H:5,10-methylenetetrahydrofolate oxidoreductase (His$_6$-tagged MTHFR) was kindly provided by Dr.
Elizabeth E. Trimmer, Grinnell College (IA, USA). Concentrations of (6S)-tetrahydrofolate (H4folate) were determined based on its reaction with formic acid according to the protocol described previously (5,19,20). In brief, a solution of H4folate was added to formic acid (99%) containing 0.5 mM EDTA and 1 mM DTT and the reaction mixture was incubated in boiling water for 5 min. The solution was allowed to cool, and the absorption of the resulting stable compound, 5,10-methenyltetrahydrofolate (CH+-H4folate; \( \varepsilon_{350} = 26 \text{ mM}^{-1}\text{cm}^{-1} \)) was measured. Based on the assumption that all of the H4folate is converted to CH+-H4folate, the determined concentration of CH+-H4folate is equivalent to the concentration of H4folate. Concentrations of PvSHMT and NADH were determined using their molar absorption coefficients: NADH, \( \varepsilon_{340} = 6.22 \text{ mM}^{-1}\text{cm}^{-1} \); PvSHMT, \( \varepsilon_{422} = 6.37 \text{ mM}^{-1}\text{cm}^{-1} \) (5).

PvSHMT preparation and activity assay—The expression and purification of recombinant PvSHMT were performed as previously reported (5,14). In brief, the expression plasmid containing pET17b-pvshmt was transformed into E. coli BL21 (DE3) and induced by auto-induction method to express native PvSHMT without tag at 16 °C in ZYP-5052 rich medium (5 mM Na2SO4, 2 mM MgSO4, 1X NPS (25 mM Na2HPO4, 25 mM KH2PO4, 50 mM NH4Cl), 1X 5052 (0.5%(w/v) glycerol, 0.05%(w/v) D-glucose, and 0.2%(w/v) \( \alpha \)-lactose) containing 50 \( \mu \text{g/mL} \) of ampicillin for overnight (~16-18 h). Cells were disrupted by ultrasonication and then centrifuged to obtain crude extract. The enzyme was purified to homogeneity using polyethyleneimine precipitation DEAE- and SP-Sepharose chromatography. The activity of purified PvSHMT was assayed at 25 °C under anaerobic conditions by coupling its reaction with the reaction of His6-tagged MTHFR (5,21, 22). In brief, a mixture of enzyme solution containing PvSHMT (1 \( \mu \text{M} \)) and His6-tagged MTHFR (3 \( \mu \text{M} \)) in 50 mM HEPES, pH 7.0 containing 0.5 mM EDTA, and 1 mM DTT was mixed with a substrate solution containing NADH (100 \( \mu \text{M} \)), various concentrations of L-serine (200-6400 \( \mu \text{M} \)), and H4folate (25-400 \( \mu \text{M} \)) at 25 °C under anaerobic conditions by using a stopped flow-spectrophotometer. Initial rates were analyzed according to a rate equation (Equation 1) using the Enzfitter program (BIOSOFT, Cambridge, UK). Data are displayed according to Dalziel’s equation (double-reciprocal) (Equation 2) for a two-substrate reaction.

\[
\nu = \frac{\nu[A][B]}{K_A K_B + K_A[A] + K_B[B] + [A][B]} \tag{1}
\]

\[
\frac{1}{\nu} = \frac{\phi_A}{[A]} + \frac{\phi_B}{[B]} + \frac{\phi_{AB}}{[A][B]} \tag{2}
\]
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Binding of L-serine to PvSHMT at pH 8.0—The titration protocol of PvSHMT (∼31 µM; OD422 ~0.2) with L-serine (0.16-145 mM) in 50 mM HEPES, pH 8.0 containing 0.5 mM EDTA and 1 mM DTT and data analysis were carried out as described earlier (5,12).

Binding of H4folate to PvSHMT—In order to determine whether PvSHMT can directly bind H4folate without requiring prior binding of L-serine, we investigated the binding of PvSHMT and H4folate under anaerobic conditions to avoid interference signals from H4folate oxidation. An anaerobic solution (1 mL) of PvSHMT (∼47 µM; OD422 ~0.3) in 50 mM HEPES, pH 8.0 containing 0.5 mM EDTA and 1 mM DTT prepared inside the anaerobic glove box (<5 ppm oxygen; Belle Technology, UK) was first scanned to record an enzyme spectrum before adding various concentrations of H4folate (0.050 – 2.954 mM). After adding each concentration of H4folate, the mixture was thoroughly mixed and left for 5 min before recording a spectrum again. The spectrum of free enzyme was subtracted from all spectra obtained from each titration to determine the absorbance changes (ΔA). The ΔA values were plotted against the H4folate concentration and the Kd for the binding of H4folate to PvSHMT was determined according to Equation 3, where ΔA represents the absorbance change, ΔAmax is the maximum absorbance change, [L]free is a concentration of free ligand, and Kd is a dissociation constant of the enzyme-ligand complex. The analysis was done using Marquardt-Lavengren algorithms in the KaleidaGraph program version 4.0.

\[
\Delta A = \frac{[L]_{\text{free}}}{K_d + [L]_{\text{free}}} \tag{3}
\]

Kinetics for the binding of PvSHMT with L-serine—A solution of 31 µM PvSHMT was mixed with various concentrations of L-serine (0.4-12.8 mM) in 50 mM HEPES, pH 8.0 containing 0.5 mM EDTA and 1 mM DTT at 25 °C using a single-mixing mode stopped-flow spectrophotometer. The binding reaction was monitored at 435 nm where it gives a large absorbance change due to the formation of the external aldimine of the serine-PLP complex. All kinetic traces were analyzed using Program A. Observed rate constants (kobs) associated with each kinetic phase were plotted against the concentrations of L-serine and analyzed according to Equation 4 and 5. kf and kr are the forward and reverse rate constants for the free enzyme isomerization prior to the ligand binding according to the conformational selection model (23), kmax is the maximum observed rate constant, kintccept is the rate constant at an ordinate intercept, Ks represents a concentration of substrate that is a half value of kmax.

\[
k_{\text{obs}} = \frac{k_{\text{max}} \cdot [S]}{K_s + [S]} + k_{\text{intercept}} \tag{4}
\]

\[
k_{\text{obs}} = k_f + \left( \frac{K_s}{K_s + [S]} \right) k_r \tag{5}
\]

Single-turnover reactions of PvSHMT with L-serine and H4folate under different mixing conditions—Three different mixing set-ups were performed to identify the sequence of substrate binding as follows: (i) a solution of pre-equilibrated binary complex of PvSHMT (45 µM)-L-serine (30 µM) was mixed with various concentrations of H4folate (0.2-6.4 mM), (ii) a solution of pre-equilibrated binary complex of PvSHMT (45 µM) with either 0.4 or 3.2 mM H4folate was mixed with L-serine (30 µM), and (iii) a solution of PvSHMT (45 µM) was mixed with solution mixtures of L-serine (30 µM) and various concentrations of H4folate (0.2-6.4 mM). All reactions were carried out under anaerobic conditions at 25 °C in 50 mM HEPES, pH 8.0 containing 0.5 mM EDTA, and 1 mM DTT. Because the largest signal change was observed at 496 nm, the reaction progression was monitored at this wavelength using a stopped-flow spectrophotometer (single-mixing mode) to detect the formation of quinonoid species. Kinetic traces were analyzed by Program A. All concentrations specified are final mixing concentrations.

Detection of glycine product formation in single-turnover reactions of PvSHMT using rapid acid-quenched flow and HPLC/UV or HPLC/MS analysis—An anaerobic solution of pre-equilibrated binary complex of PvSHMT (45 µM):L-serine (30 µM) was mixed with 0.4 or 3.2 mM H4folate in 50 mM HEPES, pH 8.0 containing 0.5 mM EDTA, 1
mM DTT and 3 mM palatinose (as internal control) using a rapid-quenched flow (RQF) apparatus (TgK Scientific, UK), equipped inside an anaerobic glove box in which the oxygen concentration is <5 ppm oxygen (Belle Technology, UK). The reactions were quenched at various time points (0.03-6.30 s) by addition of a HCl solution (0.15 M), and the acid-quenched reaction mixtures were then collected for analysis.

Detection of glycine and serine was carried out either via phenylisothiocyanate (PITC) amino acid derivatization and monitored by HPLC/UV (Agilent 1100) or directly detected by HPLC/MS analysis (Agilent 1260 equipped with Agilent 6120 single quadrupole mass spectrometer). Samples from rapid quench flow (RQF) experiments were transferred into a Microcon ultrafiltration unit and then centrifuged at 10,000 rpm, 4 °C for 20 min to separate the small molecules from the macromolecules. The derivatization method and HPLC/UV analysis were performed according to the protocol described previously with slight modifications (24). For HPLC analysis, the dried samples were redissolved in 50 μL of solvent A (0.14 M sodium acetate, pH 6.4 containing 0.05% TEA) and centrifuged at 15,000 rpm at room temperature for 30 min to clarify the samples. A clear supernatant was injected onto a C18 column (Waters®; 3.9 x 150 mm) which was pre-equilibrated with 100% solvent A with a flow rate of 1 mL/min. The column was washed with 100% solvent A for 15 min, and then a gradient of 0-53% of solvent B (60% acetonitrile/H2O) was applied, in which B was gradually increased over the course of 10-min and maintained at the highest concentration for 5 more minutes. A gradient of 53-100% solvent B was applied over the next 5 min and held for another 5 min before re-equilibrating the column in 100% solvent A. Under these conditions, derivatized serine and glycine can be clearly separated and showed retention times of 7.9 and 9.1 min, respectively. Similar amino acid derivatization as described was applied to standard glycine and L-serine solutions (1-30 μM) and a standard curve was generated by plotting the averaged peak areas as a function of glycine or L-serine concentrations.

For the direct sample analysis using HPLC/MS, samples collected from RQF experiments were filtered through a Microcon ultrafiltration unit by centrifugation at 10,000 rpm for 20 min. The filtrate was injected onto a Zorbax-NH2 column (Agilent Technology; 4.6 x 250 mm, 5 μm) which was pre-equilibrated with 80% solvent C (acetonitrile + 0.1% (v/v) formic acid) with a flow rate of 0.5 mL/min for 60 min. The column was then washed with 80% solvent C for 2 min before the eluent was gradually changed to 60% solvent C over the course of 3 min and further held at this condition for 15 min. The percentage of solvent C was then gradually increased to 80% over 22 min and held at this condition for 10 more minutes to re-equilibrate the column for new injections. HPLC chromatograms of glycine and serine showed retention times of 14.2 and 15.2 min, respectively. The eluent from the HPLC column was continuously flowed into the mass spectrometer equipped with an API-electrospray ionizer (ESI). Conditions for mass spectrometric detection were as follows. Nebulizer pressure was set at 35 psi. The capillary source voltage was set at 3 kV. The drying gas temperature was maintained at 350 °C. Drying gas flow was 12 L·min⁻¹. Scan mode was from 50-150 m/z. The polarity was set in a positively selected ion mode (SIM) in which glycine and serine were detected separately at 76 m/z and 106 m/z, respectively. The peak area for glycine and serine was quantified based on glycine and serine standard curves, for which the amino acid was treated with the same condition as those from the rapid-quenched flow experiments. The amounts of glycine product formed at different time points were analyzed using a single exponential equation as shown in Equation 6, where A is the amount of glycine at any time, A₀ is the total amount of glycine that can be produced from the reaction, kₐₒₜ is the observed rate constant for the glycine formation and t is the reaction time.

\[ A = A_0(1 - e^{-k_{\text{obs}}t}) \]  

(6)

For analysis of the products resulting from the different types of mixing set-ups previously described, similar reactions as those performed in the stopped-flow experiments were carried out in a RQF machine, and the reactions were quenched at 10 s to measure glycine formation and to quantify the remaining L-serine. Measurement of L-serine and glycine was carried out as described above.
RESULTS

Bi-substrate steady-state kinetics of PvSHMT reaction at pH 8.0—As PvSHMT activity is the highest at pH 8.0 (5), we decided to re-investigate steady-state kinetics of PvSHMT at this pH. A solution of PvSHMT and His6-tagged MTHFR was mixed with various concentrations of L-serine and H4folate using the stopped-flow spectrophotometer under anaerobic conditions at pH 8.0 (Experimental Procedures). A double-reciprocal plot of v versus 1/[L-serine] shows a series of convergent lines at various concentrations of H4folate, indicating that the reaction proceeds via a ternary-complex mechanism as the reaction that takes place at pH 7.0 (Fig. 1). Steady-state kinetic parameters for the PvSHMT reaction at pH 8.0 are similar to those at pH 7.0 (5): Km of L-serine (pH 8.0 = 0.26 ± 0.04 mM, pH 7.0 = 0.18 ± 0.03 mM), Km of H4folate (pH 8.0 = 0.11 ± 0.01 mM, pH 7.0 = 0.14 ± 0.02 mM), and kcat (pH 8.0 = 1.09 ± 0.05 s−1, pH 7.0 = 0.98 ± 0.06 s−1).

Thermodynamics and kinetics of binding of PvSHMT with L-serine—Various concentrations of L-serine (0.16-145 mM) were added to a solution of PvSHMT (31 μM), pH 8.0 and the absorption changes were monitored. Results indicated that the enzyme binds L-serine and forms an external aldimine as an increase in absorbance at 435 nm could be observed (Fig. 2). The data analysis yielded a thermodynamic dissociation constant (Kd) of 2.2 ± 0.5 mM for the PvSHMT:L-serine complex (Inset of Fig. 2).

In order to gain insights into the binding mechanisms, stopped-flow spectrophotometry was used for investigating the kinetics of binding of L-serine to the enzyme. Kinetic traces of the absorption changes at 435 nm (Fig. 3) resulting from formation of the PvSHMT and L-serine complex showed three phases. The traces were analyzed to obtain the observed rate constants (kobs) of each phase. kobs of the first phase (0.001-10 s) was hyperbolically dependent on the L-serine concentration (Inset of Fig. 3), consistent with the maximum limiting rate constant of 0.38 ± 0.02 s−1 and L-serine concentration that gives a half-saturation value of 0.86 ± 0.16 mM (Inset of Fig. 3). kobs of the second phase (10-100 s) was independent of the L-serine concentration, with a rate constant of ~0.015 s−1. For the third phase (100-2500 s), kobs values hyperbolically decreased upon increasing the L-serine concentration, with the apparent lowest rate constant of 0.0020 ± 0.0001 s−1 and the L-serine concentration that gives a half-minimum value of 2 ± 1 mM (data not shown). Altogether, the data suggest that binding of PvSHMT and L-serine proceeds through three phases with the second and third phases not involved in the catalytic reaction because they are much slower than the kcat value (1.09 ± 0.05 s−1, Fig. 1). The first phase is likely the binding of L-serine to PvSHMT to form a PvSHMT:L-serine complex (ES) which then isomerizes to form the ES* complex with a rate constant of 0.30 ± 0.04 s−1 and an overall Kd value for the ES* complex formation was determined as 0.18 ± 0.08 mM (Fig. 8A). This Kd value is different from the value obtained from the plot in Inset of Fig. 2 (2.2 mM) which obtained from absorption signal changes after 15 min. The observed discrepancy between the Kd values obtained from the two techniques implies that the equilibrium Kd value (2.2 mM, Inset of Fig. 2) does not represent the binding event that is involved in the productive pathway. It is likely that in the absence of H4folate, after the first step of binary complex formation the PvSHMT:L-serine complex proceeds to the unproductive path or promiscuous activity to give a large absorbance signal change (second and third phases of Fig. 3).

Binding of PvSHMT and H4folate—While the binding of L-serine and SHMT to form an external aldime complex (as demonstrated in the previous section) is clearly evident, the interaction of SHMT with H4folate has never been documented. We therefore investigated the binding of PvSHMT and H4folate under anaerobic conditions by titrating PvSHMT (47 μM) with various concentrations of H4folate (0.050-2.954 mM) at pH 8.0. The results indicated that the enzyme can also bind to H4folate to form a binary complex of PvSHMT:H4folate because spectra (Fig. 4) of solutions of PvSHMT plus H4folate and free PvSHMT are clearly different, especially around 477 nm (Inset A of Fig. 4). The data were analyzed as described in Experimental Procedures to yield a Kd value of 0.35 ± 0.06 mM (Inset B of Fig. 4).

The binding experiment was also carried out using stopped-flow spectrophotometry under
anaerobic conditions to monitor the kinetics of PvSHMT and H4folate binding. Due to the very small change in signal, reliable kinetic data could not be obtained (data not shown). Nonetheless, the final spectral changes and the $K_d$ value of H4folate binding (0.25 ± 0.13 mM) obtained from the stopped-flow experiment are similar to that from the static titration experiments (Inset B of Fig. 4). These data clearly indicate that L-serine and H4folate can independently bind to PvSHMT and form a PvSHMT:L-serine or PvSHMT:H4folate binary complex, suggesting that the kinetic mechanism of PvSHMT is a random-order type.

**Single-turnover reaction of PvSHMT:L-serine binary complex and H4folate**—When a pre-equilibrated complex of PvSHMT (45 μM):L-serine (30 μM) was mixed with various concentrations of H4folate (0.2-6.4 mM) and the absorbance was monitored at 496 nm, the reactions showed five kinetic phases (Fig. 5A). The first phase (0.004-0.07 s) was the phase with a large increase in absorbance at 496 nm, while the second phase (0.07-0.2 s) showed a small absorbance decrease, indicating the formation and decay of a quinonoid species, respectively. The third phase (0.2-0.6 s) was a small lag phase that had no significant absorbance change. The fourth phase (0.6-4.0 s) was the phase where the reformation of a quinonoid species occurred. Interestingly, two different phenomena were observed for the fifth phase depending on the concentration of H4folate. For the reaction carried out in the presence of 0.2-0.8 mM H4folate, the fifth phase (4.0-300 s) was a slow decay of the quinonoid species, while for the reaction with 1.6-6.4 mM H4folate, the intermediate was stabilized until 1000 s. The final spectra for the various concentrations of H4folate were also recorded (data not shown).

All kinetic traces were analyzed as described in the Experimental Procedures and the observed rate constants ($k_{obs}$) of each phase were plotted against H4folate concentrations. The plot in Fig. 5B showed that the $k_{obs}$ of the first phase decreased upon increasing the concentration of H4folate. This result may be explained according to a model in which an isomerization of the enzyme-L-serine complex exists and only one form of the complex reacts with H4folate (23,25). This explanation agrees well with the results found in the previous section, showing that a binary complex of PvSHMT:L-serine can be isomerized into several forms of complexes. According to Equation 5, the forward and reverse rate constants for the enzyme isomerization were determined to be 17 ± 1 and 258 ± 180 s$^{-1}$ 275 ± 180 s$^{-1}$, respectively (Fig. 5B). A decreasing trend upon increasing the H4folate concentration gives a half-saturation value of 0.033 ± 0.025 mM (Fig. 5B). Observed rate constants of the second phase (~11 s$^{-1}$) and the third phase (~1 s$^{-1}$) were independent of H4folate concentration (Fig. 5A). The third phase was later assigned as the formation of glycine (a dotted line with filled circles of Fig. 5A). The last two phases showed a decrease in $k_{obs}$ upon increasing the H4folate concentration. Based on the currently available data, the identities of the intermediates formed during the fourth and fifth phases cannot be unequivocally assigned. Since their rate constants (<0.3 s$^{-1}$) are significantly less than the $k_{cat}$ value (1.09 ± 0.05 s$^{-1}$), these phases are not relevant to the catalytic turnovers. They may belong to the binding of H4folate to free PvSHMT or PvSHMT:glycine after product formation.

**Detection of glycine product formation using rapid acid-quenched flow/HPLC-UV or HPLC-MS techniques**—Although the stopped-flow experiments in the previous section showed the kinetics of quinonoid species formation, the data could not identify the step involved with glycine formation. Therefore, a rapid quench experiment was employed to elucidate the step that is involved in the C-C bond cleavage in L-serine to form glycine. A solution of PvSHMT (45 μM):L-serine (30 μM) binary complex was mixed with a low or high concentration of H4folate (0.4 or 3.2 mM) using rapid acid-quenched flow-HPLC/UV or HPLC/MS analysis as described in Experimental Procedures. Glycine (retention time of 14.2 min) and serine (retention time of 15.2 min) were clearly identified and well separated in the HPLC/MS chromatograms (Inset of Fig. 6). Glycine concentrations measured from reactions quenched at different time points were plotted against age times (Fig. 6) and analyzed according to a single-exponential equation (Equation 6) as described in Experimental Procedure. The observed rate constants ($k_{obs}$) for glycine formation derived from reactions carried out at different H4folate concentrations are similar, 1.02 ± 0.09 s$^{-1}$ and 1.24 ± 0.14 s$^{-1}$ for 0.4 and 3.2 mM H4folate,
respectively. It was noted that the total amount of glycine product formed in the reaction with 3.2 mM H4folate and detected by PITC derivatization was less than that produced from the reaction with 0.4 mM H4folate (data not shown). This might be due to low efficiency of glycine derivatization when H4folate is present in high amount.

In order to confirm if the rapid-quench experiment detected all glycine formed either in the free form or in the E:Gly:H4folate complex, we measured the amount of glycine detected under the rapid-quench condition in both forms (data not shown). The results indicate that H4folate does not have significant influence on the amount of glycine detected and that under the rapid-quench experiment detected all glycine formed.

When the rapid-quench results were compared to the kinetics of the stopped-flow experiments, the data indicated that the glycine formation phase can be correlated with a small lag period detected as the third phase (~0.2-0.6 s) in Fig. 5A. Furthermore, these kobs values are similar to the catalytic rate constant (kcat) of the PvSHMT reaction (1.09 ± 0.05 s−1) measured from steady-state kinetics (Fig. 1). These results have therefore, for the first time, unambiguously identified the glycine product formation as the rate-limiting step of the H4folate-dependent SHMT reaction.

Reaction of PvSHMT with L-serine and H4folate under different mixing conditions—The binding studies revealed that PvSHMT can independently bind L-serine or H4folate (Fig. 2 to Fig. 4), implying that the enzyme binds both substrates in random-order fashion. However, whether the random-order binding mechanism is relevant to the PvSHMT catalysis or whether the enzyme requires a specific pathway to form the product was not known. Three different types of stopped-flow mixing set-ups (see Experimental Procedures) were employed to pre-form different binary complexes and the absorbance was monitored at 496 nm to follow the formation of a quinonoid species. The appearance of quinonoid absorption was used as an indicative signal for detecting the ternary complex that was committed to catalysis (Fig. 5 and 7) because the quinonoid species has been proposed to be part of the SHMT catalytic cycle (1,2). The absorbance change at 435 nm was also monitored, but the signal change was too small to obtain reliable kinetic data.

Results from the first type of mixing set-up (mixing PvSHMT:L-serine with H4folate, solid lines with filled circles in Fig. 7A and 7B) were similar to that shown in Fig. 5A discussed earlier, but different from the other two experiments. For the second experiment in which a solution of PvSHMT:H4folate binary complex (0.4 and 3.2 mM, solid lines with filled rectangles in Fig. 7A and 7B, respectively) was mixed with L-serine, the reactions showed four phases in which all phases displayed an increase in absorbance. The first phase (0.005-0.2 s) of the reaction showed less amplitude change than that of the mixing of PvSHMT:L-serine with H4folate. kobs values of the reactions with 0.4 and 3.2 mM H4folate are 11 and 6 s−1, respectively. Although the kinetics of this phase is slower than the first kinetic phase from the mixing of PvSHMT:L-serine with H4folate, these values are still significantly faster than the rate-limiting step of the reaction (~1 s−1, Fig. 5A). The following kinetic phase (0.2–0.6 s) showed an increase in absorbance at 496 nm and is likely to be the glycine formation step that is the rate-limiting step in the overall reaction. The latter part of the reaction (2-1000 s) occurs much slower than the turnover number (~1 s−1), and thus is not relevant to catalysis. For the third experiment in which the free enzyme was mixed with a solution of L-serine and 0.4 and 3.2 mM H4folate (solid lines with filled triangles in Fig. 7A and 7B), kobs values for quinonoid formation in the first phase (0.005-0.2 s) in the reactions of 0.4 and 3.2 mM H4folate were 14.7 and 8 s−1, respectively. The second phase of the reaction (0.2-0.6 s) showed an increase of absorbance; this step likely corresponds to the glycine formation step. Other phases following this step (2-1000 s) are slower than the catalytic turnover.

Although kobs values for initial quinonoid formation in these three experiments are different, this step was faster than the glycine formation step (0.2-0.6 s) for all of the reaction setup conditions tested. Moreover, all reactions yielded similar amounts of product (see next section). Therefore, we conclude that all types of mixings can lead to a productive path for the SHMT reaction. As the mixing of free PvSHMT with a mixture of L-serine and H4folate resulted in kinetics and amplitude changes that are different from the reactions of either preformed complexes, the data imply that when providing the two substrates
simultaneously, the free enzyme binds randomly to both substrates, agreeing with the data shown in Fig. 3 and Fig. 4. Based on this study, the overall reaction catalyzed by PvSHMT can be described by the scheme displayed in Fig. 8B.

**Comparison of glycine generated from different reaction set ups** — The amount of glycine generated from the three mixing reactions (Fig. 7) was analyzed using rapid-quench and LC-MS techniques. Samples at the reaction times of 10 s were collected and analyzed. Similar amounts of glycine product (~22 µM) were obtained from all types of mixing reactions (Table 1). Since the conditions employed only allow for a single turnover of the reactions to proceed, these data indicated that all mixing set-ups resulted in similar amount of product after the glycine formation step, suggesting that all mixing set-ups led to a productive pathway and the PvSHMT reaction occurs via a random-order mechanism (Fig. 8B).

**Kinetic mechanism of PvSHMT** — Based on currently available data, the reaction of PvSHMT can be summarized as shown in Fig. 8. PvSHMT binds to either L-serine or H4folate as a first substrate. The binary complex then binds to another substrate and proceeds to form glycine. In principle, glycine or CH2-H4folate should also be released in random order. All data also imply that under steady-state enzyme turnovers in which L-serine is in excess, the glycine release is quick and the rate-limiting step is the glycine formation. However, in the presence of excess H4folate such as in Figs. 5 and 7, once glycine forms, it is trapped in the form of the enzyme:Gly:H4folate complex as noted by high absorbance at 496 nm (Fig. 8B).

**DISCUSSION**

Our investigation based on transient kinetics, thermodynamics and steady-state kinetics provides insight into the reaction mechanism of PvSHMT and has identified glycine formation as the rate-limiting step of this enzyme reaction. Direct binding experiments under anaerobic conditions has shown for the first time that the free enzyme can independently bind to H4folate and that the reaction of PvSHMT obeys a random-order mechanism in which the enzyme can bind either to L-serine or H4folate as the first substrate (Fig. 8B).

Using rapid-quench and HPLC/UV/MS experiments, the rate constant of glycine formation was measured as ~1 s⁻¹ which is similar to the $k_{cat}$ value of the PvSHMT reaction obtained from steady-state kinetics under the same condition. The data indicate that the C=C bond cleavage of L-serine to result in glycine formation is the rate-limiting step for the physiological H4folate-dependent reaction of PvSHMT. Recently, quantum mechanics calculation of H4folate-independent retro-aldol cleavage of β-hydroxyamino acids via the β-hydroxyl proton abstraction based on the structure of *Bacillus stearothermophilus* (bs) SHMT in complex with L-allo-threonine suggests that the rate-limiting step for the aldolase activity of bsSHMT reaction is also the C=C bond cleavage (26). Since the formation of E:L-serine:H4folate ternary complex (evidenced by formation of a quinonoid intermediate, Figs. 5 and 7) occurs before the glycine formation step (Fig. 7), it implies that the presence of H4folate is required for the C-C bond breakage. These results agree with the findings of our previous investigation that free formaldehyde could not be detected in the absence of H4folate (5). The results here also support the mechanism suggested (2,18), which proposes that the binding of H4folate may cause a conformational change that facilitates the glycine product formation.

Transient kinetics and direct binding experiments suggest that PvSHMT binds L-serine and H4folate via a random-order mechanism because the enzyme can bind to each ligand independently. The $K_d$ value for the H4folate binding is 0.35 ± 0.06 mM (Fig. 4), while a $K_d$ value for L-serine binding relevant to catalysis is 0.18 ± 0.08 mM (Fig. 8A). A key experimental setup that allowed us to directly observe the binary complex of PvSHMT:H4folate for the first time was conducting the reaction under anaerobic conditions. As H4folate can be readily oxidized, resulting in an absorbance increase at the 330-360 nm regions (5,27,28), it is impossible to aerobically monitor the interaction of H4folate and protein interaction without any background interference. Previously, most of the binding studies of SHMT and H4folate were only monitored in the presence of amino acid substrates, especially in the presence of glycine, for which an intense band of quinonoid signal could be observed (29-31). Different mixing set-ups—in which either PvSHMT:L-serine or
PvSHMT:H₄folate was preformed—resulted in the same amount of glycine product at the end of a single turnover, supporting the conclusion that both paths of ligand binding lead to the productive pathway (Fig. 8B). The previous study of the rabbit cytosolic SHMT using equilibrium isotope-exchange of L-serine and CH₂H₄folate also showed that the rate of isotope exchange under equilibrium conditions was not affected by increasing glycine concentrations, implying that the ordered preference for dissociation of both products, glycine and CH₂H₄folate, is not required (17). All results support that the kinetic mechanism of PvSHMT conforms to a random order model (Fig. 8B).

The binding of PvSHMT and L-serine is complicated and a multi-step process in which the PvSHMT:L-serine complex is in equilibrium of several forms (Fig. 8A). After the initial binding to form a Michaelis complex of PvSHMT:L-serine, the complex must proceed to form the gem-diamine and then the external aldimine complex. When the preformed PvSHMT-L-serine was mixed with various concentrations of H₄folate, the $k_{obs}$ was decreased. These data suggest that the isomerization step preceding the bi-molecular reaction with H₄folate exists. Based on our current data, we cannot distinguish if this isomerization step is caused by the enzyme conformational change or the equilibrium of gem-diamine and external aldimine complexes. In the presence of H₄folate, it is possible that a PvSHMT:H₄folate complex can readily bind and facilitate the formation of the external aldimine complex.

The random-order binding mechanism of PvSHMT implies that each ligand binds to the enzyme via separate binding pockets and that access to each pocket is independent. Currently, there are seven X-ray structures of SHMT ternary complexes with amino acid and folate analogues (PDB # 1EQB (32), (PDB # 1DFO) (33), (PDB # 1KL2) (34), (PDB # 2VGW) (35), (PDB # 2W7H and PDB # 2W7M) (36), (PDB # 2VMS) (37)) including the recently published structure of PvSHMT in complex with D-serine and H₄folate analogue (6R-5-CHO-H₄folate) at 2.4 Å resolution (PDB # 4OYT) (38) (Fig. 9). The crystal structure reveals defined binding pockets for amino acid and folate that could support the independent binding of each substrate via a random-order mechanism as proposed (Fig. 8B).

In conclusion, we have shown for the first time direct evidence supporting that the kinetic mechanism of PvSHMT occurs via a random-order model and that glycine formation is the rate-limiting step of SHMT. This information will serve as a basis for future investigation for other SHMTs. The comparative studies of SHMTs from parasite and host will pave the way for the design of species-specific inhibitors for antimalarial drug development.
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FOOTNOTES
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3The abbreviations used are: SHMT, serine hydroxymethyltransferase; H4folate, tetrahydrofolate; CH2-H4folate, methylenetetrahydrofolate; CH+-H4folate, methenyltetrahydrofolate; CHO-H4folate, formyltetrahydrofolate; PLP, pyridoxal 5'-phosphate; EQ, enzyme-quinonoid complex; RQF, rapid-quench flow; MTHFR, methylenetetrahydrofolate reductase

TABLE LEGENDS

TABLE 1. Amount of glycine resulting from three types of mixing experiments analyzed by rapid-quench and HPLC-MS techniques.

FIGURE LEGENDS

FIGURE 1. A double-reciprocal plot of bi-substrate kinetics of PvSHMT at pH 8.0. A solution of PvSHMT (1 µM) and His6-tagged MTHFR (3 µM) was mixed with solutions of NADH (100 µM), L-serine (0.2, 0.4, 0.8, 1.6 and 6.4 mM) and H4folate (0.025 (filled circles), 0.5 (filled squares), 0.1 (filled diamonds), 0.2 (empty circle), and 0.4 mM (empty squares)) at 25 °C and pH 8.0 under anaerobic conditions using a stopped-flow spectrophotometer. The plot of e/v and 1/L-serine shows a series of convergent lines indicating ternary complex kinetics. Symbols are represented for reciprocal initial velocities at given concentrations of both substrates. Insets are the secondary plots of slopes (A) and ordinate intercepts (B) of the primary plot as a function of 1/H4folate. The data analysis yielded the kinetic parameters as $K_m$ for L-serine = 0.26 ± 0.04 mM, $K_m$ for H4folate = 0.11 ± 0.01 mM, and the turnover number ($k_{cat}$) = 1.09 ± 0.05 s⁻¹.

FIGURE 2. Difference spectra for the binding of PvSHMT with various concentrations of L-serine at pH 8.0. The sample cuvette contained a solution of PvSHMT (~31 µM) in 50 mM HEPES, pH 8.0 containing 0.5 mM EDTA and 1 mM DTT and various concentrations of L-serine (0.16-145 mM, lower to upper spectra) while the reference cuvette contained only the enzyme solution and buffer. A difference spectrum was recorded after each addition. Inset shows the determination of $K_d$ for the binding of L-serine as 2.2 ± 0.5 mM.

FIGURE 3. Transient kinetics for the binding of PvSHMT with L-serine determined by stopped-flow spectrophotometry. A solution of PvSHMT (~31 µM) was mixed with various concentrations of L-serine (0.4-12.8 mM from lower to upper traces) and the change in absorbance was monitored at 435 nm. The results indicate the formation of an external aldimine complex with L-serine. The kinetic traces show three phases. Observed rate constants of the first phase were hyperbolically dependent on L-serine concentrations with the L-serine concentration that gives the half-saturation value ($K_S$) of 0.86 ± 0.16 mM.
and the maximum rate constant of 0.38 ± 0.02 s⁻¹ (Inset). While observed rate constants of the second and third phases are not involved with catalytic reaction because they are much lower than the $k_{cat}$ value.

**FIGURE 4.** Binding of PvSHMT with H₄folate. A solution of PvSHMT (~47 μM) was mixed with various concentrations of H₄folate (0.050-2.954 mM from lower to upper spectra) under anaerobic conditions. The free enzyme spectrum was subtracted from the spectra obtained after each titration to obtain difference spectra (Inset A). The increase of absorbance around 477 nm indicates the ability of PvSHMT to bind H₄folate. Based on the plot of absorbance changes at 477 nm versus H₄folate concentrations, the $K_d$ values obtained from both measurements using conventional and stopped-flow spectrophotometry were determined as 0.35 ± 0.06 (filled circles) and 0.25 ± 0.13 mM (filled rectangles), respectively (Inset B).

**FIGURE 5.** Single-turnover reaction of PvSHMT:L-serine binary complex with H₄folate. A solution of the PvSHMT-L-serine complex (45 μM PvSHMT and 30 μM L-serine) was mixed with various concentrations of H₄folate (0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 mM from lower to upper traces) under anaerobic conditions using a stopped-flow spectrophotometer. The kinetic traces monitored at 496 nm showed five phases (solid line) (A). The first phase (0.004-0.07 s) was a large increase of absorbance 496 nm and the $k_{obs}$ values were decreased upon the increasing concentration of H₄folate (B). The second and the third phases are independent of H₄folate concentrations. Based on rapid-quench data, the third phase (~1 s⁻¹) is the formation of glycine (dashed line with filled circles in (A)).

**FIGURE 6.** Detection and kinetic analysis of glycine product formation using rapid acid-quenched flow/HPLC-UV or HPLC-MS technique. A pre-mixed solution of PvSHMT (45 μM) and L-serine (30 μM) was mixed with H₄folate (0.4 mM) under anaerobic conditions using a rapid-quench flow apparatus in an anaerobic glove box and the glycine product formed was quantified by HPLC/UV or HPLC/MS. The plot shows the amount of glycine formed at various times. (Inset) HPLC/MS chromatograms of glycine (retention time of 14.2 min) and serine (retention time of 15.2 min), respectively.

**FIGURE 7.** Comparison of kinetic traces for the reaction of PvSHMT with L-serine and H₄folate under three different types of mixings. The first experiment (solid lines with filled circles) is a mixing of preformed binary complex of PvSHMT (45 μM):L-serine (30 μM) with 0.4 (A) and 3.2 (B) mM H₄folate. The second experiment (solid lines with filled rectangles) is a mixing of preformed binary complex of PvSHMT (45 μM) and 0.4 (A) or 3.2 (B) mM H₄folate with L-serine (30 μM). The third experiment (solid lines with filled triangles) is a mixing of free PvSHMT with mixtures of L-serine (30 μM) and 0.4 (A) or 3.2 (B) mM H₄folate. All reactions were monitored by the absorption changes at 496 nm using the stopped-flow spectrophotometer in single-mixing mode.

**FIGURE 8.** A proposed model for the PvSHMT reaction. (A) Binding of L-serine to PvSHMT is a multi-step process. An initial Michaelis complex of PvSHMT and L-serine (a) is isomerized and transformed to a gem-diamine intermediate and subsequently external aldimine complex (b-d). (B) A random-order model describing the overall reaction of PvSHMT mechanism. PvSHMT binds randomly either to L-serine or H₄folate to form an active E:Ser or E:H₄folate binary complex. The binding of enzyme and L-serine is a multi-step process as described in A. Only a relevant form of the external aldimine complex can bind to H₄folate to form the ternary complex of E:Ser:H₄folate. Glycine is generated with a rate constant of 1 s⁻¹. Both products are randomly released. The current data suggest that, in the presence of high concentration of H₄folate, the E:Gly complex can be trapped in the dead-end E:Gly:H₄folate complex.
FIGURE 9. Crystal structure of the homodimeric PvSHMT in complex with D-serine and formyltetrahydrofolate (6R-5-CHO-H₄folate) (PDB # 4OYT) shows two individual pockets on the surface representative for serine (yellow) and folate (white) (38).
Table 1

| Mixing conditions                     | Glycine (µM) |
|---------------------------------------|--------------|
| E + Ser vs. H₄folate (0.4 mM)         | 18 ± 3       |
| E + Ser vs. H₄folate (3.2 mM)         | 21 ± 3       |
| E + H₄folate (0.4 mM) vs. Ser         | 22 ± 4       |
| E + H₄folate (3.2 mM) vs. Ser         | 22 ± 2       |
| E vs. Ser + H₄folate (0.4 mM)         | 12 ± 3       |
| E vs. Ser + H₄folate (3.2 mM)         | 22 ± 8       |
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

![Graph showing the kinetic mechanism of Plasmodium vivax Serine Hydroxymethyltransferase](image-url)

- **Glycine (µM)** vs **Time (s)**
- **Abundance** vs **Retention Time (min)**

- **Inset**: Abundance profiles of Gly and Ser.
Figure 7
Figure 8

A

\[
\begin{align*}
E-\text{PLP} + \text{Ser} & \xrightarrow{\kappa_5 = 0.86 \text{ mM}} E-\text{PLP}:\text{Ser} \quad & (a) \quad k_2 = 0.30 \text{ s}^{-1} \\
E-\text{PLP}:\text{Ser} & \xrightarrow{\kappa_2 = 0.08 \text{ s}^{-1}} E-\text{PLP}:\text{Ser}^* \quad & (b) \quad k_3 \\
E-\text{PLP}:\text{Ser}^* & \xrightarrow{\kappa_4} E-\text{PLP}:\text{Ser}^{**} \quad & (c) \quad k_4 \\
E-\text{PLP}:\text{Ser}^{**} & \xrightarrow{\kappa_4} E-\text{PLP}:\text{Ser}^{***} \quad & (d)
\end{align*}
\]

\[\kappa_3 = 0.18 \text{ mM}\]

B

(Non-productive pathway)

(Productive pathway)

\[
\begin{align*}
\text{Ser} & \xrightarrow{\kappa_6 = 0.86 \text{ mM}} E:\text{Ser} \quad (\text{Non-productive pathway}) \\
E:\text{Ser} & \xrightarrow{\kappa_6} E:\text{Ser}:\text{H}_4\text{folate} \\
E:\text{Ser}:\text{H}_4\text{folate} & \xrightarrow{\kappa_6} E:Q:CH_2-\text{H}_4\text{folate} \quad k_{\text{cat}} = 1 \text{ s}^{-1} \\
E:Q:CH_2-\text{H}_4\text{folate} & \xrightarrow{\kappa_6} E:Gly:CH_2-\text{H}_4\text{folate} \\
E:Gly:CH_2-\text{H}_4\text{folate} & \xrightarrow{\kappa_6} CH_2-\text{H}_4\text{folate} \\
\text{CH}_2-\text{H}_4\text{folate} & \xrightarrow{\kappa_6} E \\
\text{E} & \xrightarrow{\kappa_6 = 0.86 \text{ mM}} E:\text{H}_4\text{folate} \quad (\text{Non-productive pathway}) \\
E:\text{H}_4\text{folate} & \xrightarrow{\kappa_6} E: \text{Ser}:\text{H}_4\text{folate} \\
E:\text{Ser}:\text{H}_4\text{folate} & \xrightarrow{\kappa_6} E:Q:CH_2-\text{H}_4\text{folate} \\
E:Q:CH_2-\text{H}_4\text{folate} & \xrightarrow{\kappa_6} E:Gly:CH_2-\text{H}_4\text{folate} \\
E:Gly:CH_2-\text{H}_4\text{folate} & \xrightarrow{\kappa_6} CH_2-\text{H}_4\text{folate} \\
\text{CH}_2-\text{H}_4\text{folate} & \xrightarrow{\kappa_6} E
\end{align*}
\]
Figure 9
Kinetic Mechanism and the Rate-Limiting Step of Plasmodium vivax Serine Hydroxymethyltransferase
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