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Enzymatic Mechanism for the Hydrolysis of 5,10-Methenyltetrahydropteroylglutamate to 5-Formyltetrahydropteroylglutamate by Serine Hydroxymethyltransferase†

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ABSTRACT: Serine hydroxymethyltransferase in the presence of glycine catalyzes the hydrolysis of (6*R*)-5,10-methenyltetrahydropteroylpolyglutamate to (6*S*)-5-formyltetrahydropteroylpolyglutamate. The enzyme also catalyzes the formation of (6*S*)-5-formyltetrahydropteroylpolyglutamate from a compound in equilibrium with (6*R*)-5,10-methenyltetrahydropteroylpolyglutamate believed to be (6*R*,11*R*)-5,10-hydroxymethylenetetrahydropteroylpolyglutamate, a putative intermediate in the nonenzymatic hydrolysis of 5,10-methenyltetrahydropteroylglutamate to 5-formyltetrahydropteroylglutamate [Stover, P., & Schirch, V. (1992) *Biochemistry* (preceding paper in this issue)]. The enzymatic mechanism for the formation of (6*S*)-5-formyltetrahydropteroylpolyglutamate from these substrates and the role of glycine in the reaction was addressed. Evidence suggests that (6*R*,11*R*)-5,10-hydroxymethylenetetrahydropteroyltetraglutamate is a catalytically competent intermediate in the enzyme-catalyzed hydrolysis of (6*R*)-5,10-methenyltetrahydropteroyltetraglutamate. The enzyme displays a high K_m of 40 μM for (6*R*)-5,10-methenyltetrahydropteroyltetraglutamate, while the K_m for (6*R*,11*R*)-5,10-hydroxymethylenetetrahydropteroyltetraglutamate is below 0.5 μM. The k_{cat} values for both reactions are identical and equal to the rate of formation of an enzyme ternary complex absorbing at 502 nm which is formed from glycine and (6*S*)-5-formyltetrahydropteroylpolyglutamate. The hydrolysis reaction proceeds with exchange of the C^{11} formyl proton of (6*R*)-5,10-methenyltetrahydropteroyltetraglutamate, suggesting that the enzyme-catalyzed reaction occurs by the same C^{11} carbanion inversion mechanism as the nonenzymatic reaction. Isotope exchange experiments using [2-^{3}H]glycine and differential scanning calorimetry data suggest both a catalytic and a conformational role for glycine in the enzymatic reaction. The results are discussed in terms of the similarity in mechanisms of the SHMT-catalyzed retroaldol cleavage of serine and hydrolysis of (6*R*)-5,10-methenyltetrahydropteroylpolyglutamates.

Serine hydroxymethyltransferase (SHMT)[1] catalyzes the reversible conversion of 5,10-CH_2-H_4PteGlu and glycine to form serine and tetrahydropteroylglutamate (H_4PteGlu) (reaction 1). Although several mechanisms have been proposed for this reaction, a favored mechanism involves the hydrolysis of 5,10-CH_2-H_4PteGlu to H_4PteGlu and enzyme-bound formaldehyde (Matthews & Drummond, 1991). The bound formaldehyde then adds to a glycine anion, which is present at the active site as a resonance-stabilized imine with pyridoxal-P. Recently, it was discovered that SHMT also catalyzes the hydrolysis of 5,10-CH^+-H_4PteGlu, forming 5-CHO-H_4PteGlu (reaction 2) (Stover & Schirch, 1990).

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[1] Abbreviations: cSHMT, cytosolic isoenzyme of serine hydroxymethyltransferase; C_1-THF synthase, C_1-tetrahydrofolate synthase; methenyl-THF synthetase, 5,10-methenyltetrahydrofolate synthetase; $H_4PteGlu_n$, tetrahydropteroylglutamate containing *n* glutamate residues; 5,10-CH^+-H_4PteGlu, 5,10-methenyltetrahydropteroylglutamate; 5-CHO-H_4PteGlu, 5-formyltetrahydropteroylglutamate; 5-CH_3-H_4PteGlu, 5-methyltetrahydropteroylglutamate; 10-CHO-H_4PteGlu, 10-formyltetrahydropteroylglutamate; 5,10-CHOH-H_4PteGlu, 5,10-hydroxymethylenetetrahydropteroylglutamate; KBES, potassium *N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonate; pyridoxal-P, pyridoxal phosphate; DMF, *N*,*N*-dimethylformamide.

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$$\text{glycine} + 5,10\text{-}CH_2\text{-}H_4PteGlu + H_2O \xrightarrow{\text{SHMT}} \text{serine} + H_4PteGlu \quad (1)$$

$$5,10\text{-}CH^+\text{-}H_4PteGlu + H_2O \xrightarrow[\text{Gly}]{\text{SHMT}} 5\text{-}CHO\text{-}H_4PteGlu \quad (2)$$

The one-carbon group in reaction 1 is at the oxidation level of formaldehyde, while in reaction 2 it is at the oxidation level of formate. This difference in oxidation levels suggests that the two reactions proceed by different mechanisms. However, analysis of the two mechanisms, as understood in terms of nonenzymatic studies, suggests that the two reactions are related. In reaction 1, an initial step in the transfer of the methylene group is believed to be the opening of the five-membered imidazolium ring by breaking of the C^{11}–N^{10} bond to form the N^5 iminium cation. The iminium cation then reacts with water to form N^5-hydroxymethyl-H_4PteGlu. In reaction 2, the proposed nonenzymatic mechanism suggests that the initial step is the addition of water to 5,10-CH^+-H_4PteGlu to form 5,10-CHOH-H_4PteGlu, followed by breaking of the C^{11}–N^{10} bond to form the product 5-CHO-H_4PteGlu. Each mechanism involves the breaking of the C^{11}–N^{10} bond and the addition of water to a carbon–nitrogen double bond. Both would require similar acid–base-catalyzed steps to perform these reactions (Stover & Schirch, 1990).

SHMT catalyzes the hydrolysis of (6*R*)-5,10-CH^+-H_4PteGlu$_n$ with biphasic kinetics, with the product of each phase being 5-CHO-H_4PteGlu$_n$ (Stover & Schirch, 1990). Previous evidence suggests that the rapid phase of the reaction is the result of catalysis of a C^{11} hydrated compound, (11*R*)-5,10-CHOH-H_4PteGlu$_n$, and the slow phase is the hydrolysis of 5,10-CH^+H_4PteGlu (Stover & Schirch, 1992). The rabbit liver cSHMT-catalyzed reaction has an absolute requirement for either glycine or D-alanine. Both of these amino acids form a quinonoid complex with the enzyme-bound pyridoxal-P in which the 2*S* proton of the amino acid has been transferred to a base on the enzyme (Besmer & Arigoni, 1968; Jordan & Akhtar, 1970). Previous studies showed that the polyglutamate forms of 5,10-CH^+-H_4PteGlu are much better substrates for reaction 2 compared to the monoglutamate form of the coenzyme (Stover & Schirch, 1990). Reaction 2 is catalyzed by rabbit liver cytosolic and mitochondrial isoenzymes of SHMT as well as by *Escherichia coli* SHMT, all of which display these same catalytic requirements.

5-CHO-H_4PteGlu$_n$ is found to exist as a part of the folate pool in virtually all cells (Cossins, 1984). Our previous studies suggest that, in *E. coli*, the SHMT catalysis of reaction 2 is the source of the cellular 5-CHO-H_4PteGlu$_n$ (Stover & Schirch, 1990). The observation that 5-CHO-H_4PteGlu inhibits many of the enzymes in one-carbon metabolism suggests that it may play a role as a regulator of one-carbon metabolism. This cofactor has been demonstrated to tightly bind or inhibit AICAR transformylase (Bertrand & Jolivet, 1989), sarcosine dehydrogenase and dimethylglycine dehydrogenase (Witter & Wagner, 1981), sarcosine oxidase (Kvalnes-Krick & Jorns, 1987), methionyl-tRNA formyltransferase (Lucchini & Bianchetti, 1980), prokaryotic thymidylate synthase (North & Matthews, 1977), CH^+-H_4PteGlu cyclohydrolase, 5,10-CH_2-H_4PteGlu dehydrogenase,[2] and dihydrofolate reductase in the absence of NADPH (Birdsall et al., 1981). Also, 5-CHO-H_4PteGlu$_n$ is a slow tight-binding inhibitor of SHMT (Stover & Schirch, 1991).

The questions addressed in this paper center on understanding the mechanism of SHMT catalysis of reaction 2. Of particular interest is determining the role of glycine in the reaction, determining why the polyglutamate forms of the coenzyme are much better substrates than the monoglutamate forms, and comparing the enzymatic mechanism with the nonenzymatic mechanism discussed in the previous paper (Stover and Schirch, 1992).

[2] Stover and Schirch, unpublished results.

Experimental Procedures

Materials. Glycine, L-serine, MgATP, $NADP^+$, NADPH, $NaCNBH_3$, deuterium oxide, H_4PteGlu, and 2-mercaptoethanol were purchased from Sigma. [2-^{3}H]Glycine was purchased from Amersham and purified as described previously (Schirch & Chen, 1973). Sodium [^{3}H]formate was purchased from Moravek Biochemicals Inc. and used without further purification. [^{2}H]Glycine was purchased from MSD Isotopes. 5-CHO-H_4PteGlu was purchased from Fluka, and pteroylpolyglutamates were purchased from Dr. B. Schirck's laboratory and reduced to the tetrahydro form as described previously (Stover & Schirch, 1990). 5-CHO-H_4PteGlu and 5-CH_3-H_4PteGlu polyglutamates were prepared as described previously (Stover & Schirch, 1991). Formic acid, ammonium sulfate, and all buffers were reagent grade and used without further purification. The cytosolic isoenzyme of SHMT, methenyl-THF synthetase, and C_1-THF synthase were purified from fresh-frozen rabbit livers, and the cSHMT-bound pyridoxal-P was reduced with $NaCNBH_3$ (Schirch & Peterson, 1980; Schirch et al., 1985; Schirch & Mason, 1963, Stover & Schirch, 1990).

Preparation of Folate Compounds. The concentration of 5,10-CH^+-H_4PteGlu$_n$ was determined from its absorbance at 360 nm using an extinction coefficient of 25 100 M^{-1} cm^{-1} (Temple & Montgomery, 1984). 5-CHO-H_4PteGlu$_n$ concentration was determined by its ATP-dependent methenyl-THF synthetase catalyzed conversion to 5,10-CH^+-H_4PteGlu, measuring the increase in absorbance of the product at 360 nm (Stover & Schirch, 1991). The concentration of the compound believed to be (6*R*,11*R*)-5,10-CHOH-H_4PteGlu$_n$ (Stover & Schirch, 1992), present in 5,10-CH^+-H_4PteGlu$_n$ solutions, was determined by preincubating the solutions with 1.0 mM ATP, methenyl-THF synthetase (14 μg), and C_1-THF synthase (220 μg) in a buffer of 50 mM glycine and 50 mM KBES, pH 7.5. These enzymes convert any 5-CHO-H_4PteGln$_n$ and 5,10-CH^+-H_4PteGlu$_n$ in the reaction solutions to 10-CHO-H_4PteGlu$_n$ (Stover & Schirch, 1992). After a several-minute incubation, 20 μM cSHMT was added and the increase in absorbance at 502 nm was immediately recorded. The increase in absorbance at 502 nm is due to the rapid formation of the SHMT·Gly·5-CHO-H_4PteGlu$_n$ complex. The concentration of total 5-CHO-H_4PteGlu$_n$ formed from (6*R*,11*R*)-5,10-CHOH-H_4PteGlu$_n$ was determined using previously reported K_d values (Stover & Schirch, 1991) of 5-CHO-H_4PteGlu$_n$ for the cSHMT·Gly binary complex and an ϵ_{502} of 40 000 M^{-1} cm^{-1}.

(6*R*,*S*)-5,10-CH^+-H_4PteGlu$_n$ was prepared from (6*R*,*S*)-5-CHO-H_4PteGlu$_n$ by adjusting 1 mM solutions to pH 1.5 with HCl and incubating overnight at 4 °C. The resulting yellow crystals were washed with 10 mM HCl and stored at 0 °C as a crystalline suspension (Stover & Schirch, 1991). (6*R*,*S*)-5,10-C[^{2}H]$^+$-H_4PteGlu$_n$ was prepared by lyophilizing a crystalline suspension of 5,10-CH^+-H_4PteGlu and dissolving 10 mg in 0.5 mL of D_2O, adjusting the pH to 3.5 with NaOH, and incubating the solution at 50 °C for 5 h. The solution was then adjusted to pH 1.5 with DCl. [^{1}H]NMR spectra of the reaction showed complete exchange of the C^{11} proton with D_2O as determined by the absence of a resonance at 9.89 ppm.

(6*R*)-5,10-C[^{3}H]$^+$-H_4PteGlu$_4$ was prepared by incubating 10 nmol of (6*S*)-H_4PteGlu$_4$, 10 nmol of sodium [^{3}H]formate, 20 nmol of MgATP, and 500 μg of C_1-THF synthase in 300 μL of 20 mM KBES and 5 mM 2-mercaptoethanol buffer, pH 7.2, for 10 min at 35 °C. The reaction was terminated by the addition of 300 μL of 1 N HCl and the precipitated protein removed by centrifugation. After adjusting the pH of the solution to 1.5 by the addition of HCl, the solution was incubated anaerobically at room temperature for 1 h to effect conversion of 10-C[^{3}H]O-H_4PteGlu$_4$ to 5,10-C[^{3}H]$^+$-H_4PteGlu$_4$. The synthesis of (6*R*)-5,10-C[^{3}H]$^+$-H_4PteGlu$_4$ from (6*S*)-H_4PteGlu$_4$ was greater than 90% as determined from its absorbance at 360 nm. The solution was lyophilized, and the remaining solid washed with 2 mL of 100 mM HCl and lyophilized again to remove any unreacted formic acid or exchanged tritium. The washing procedure was repeated two additional times.

Incorporation of tritium into the C^{11} position of (6*R*)-5,10-C[^{3}H]$^+$-H_4PteGlu$_4$ was verified by its ability to exchange with solvent protons. Five-microliter aliquots, containing 5 nmol of (6*R*)-5,10-C[^{3}H]$^+$-H_4PteGlu$_4$ (23 000 cpm/nmol), were incubated in 1 mL of 50 mM KBES, pH 7.0, at 55 °C. Aliquots of 20 μL were removed with time, lyophilized to remove exchanged counts, redissolved in 50 μL of H_2O and prepared for scintillation counting. The (6*R*)-5,10-C[^{3}H]$^+$-H_4PteGlu$_4$, incubated in KBES at pH 7.0, displayed a first-order C^{11} proton exchange with a $t_{1/2}$ of approximately 425 min.

Assay for Conversion of 5,10-CH$^+$-H_4PteGlu$_n$ to 5-CHO-H_4PteGlu$_n$. The rate of 5,10-CH$^+$-H_4PteGlu hydrolysis by cSHMT was determined using the method of initial rates by measuring the rate of increase in absorbance at 502 nm following the addition of 5–200 μM 5,10-CH$^+$-H_4PteGlu$_4$ to a solution of 4 μM cSHMT, 5 mM glycine, 50 mM KBES, pH 7.0, and 5 mM 2-mercaptoethanol at 30 °C. Concentrated (6*R*)-5,10-CH$^+$-H_4PteGlu$_n$ solutions were made at pH 1.5 and solubilized in 10% DMF/H_2O, pH 2.0, in order to minimize formation of (6*R*,11*R*)-5,10-CHOH-H_4PteGlu$_n$. These precautions were necessary to minimize the burst phase of this reaction, which results in the rapid formation of the stable dead-end cSHMT·Gly·5-CHO-H_4PteGlu$_n$ complex (Stover & Schirch, 1991). Initial velocity measurements were determined from the first 20 s of the reaction in order to minimize the nonenzymatic hydrolysis of 5,10-CH$^+$-H_4PteGlu$_n$ to 10-CHO-H_4PteGlu$_n$, which was insignificant over this time period. K_m and k_{cat} values were determined from Lineweaver–Burk plots using initial velocity measurements.

Assay for Conversion of 5,10-CHOH-H_4PteGlu$_n$ to 5-CHO-H_4PteGlu$_n$. Determination of kinetic constants for the conversion of the hydrated adduct of CH$^+$-H_4PteGlu$_n$ to the quinonoid complex, absorbing at 502 nm, posed difficult problems. First, the compound cannot be made in pure form. In this study, we used solutions enriched in the compound and free of any 5-CHO-H_4PteGlu$_n$, which were prepared by the methods described in the previous paper (Stover & Schirch, 1992). Second, the rate of reaction is rapid and requires the use of a stopped-flow spectrophotometer. Third, the hydrated adduct has a high affinity for the enzyme and its product dissociates very slowly. Using the same reaction solutions described for assays with 5,10-CH$^+$-H_4PteGlu$_n$, stopped-flow determinations were performed by flowing increasing concentrations of 5,10-CHOH-H_4PteGlu$_n$ (5–40 μM) against solutions of cSHMT (5 μM) and glycine (20 mM) and measuring the rate of increase in absorbance at 502 nm. K_m and k_{cat} values were determined for the monoglutamate form of the substrate from double-reciprocal plots of initial velocity versus substrate concentration. However, with the polyglutamate forms of the substrate, the initial velocity did not vary with substrate concentration even when less than a 2-fold excess of substrate over enzyme was used, suggesting that the enzyme had a high affinity for the substrate. A value of k_{cat} was calculated from the invariant value of either the initial velocity or k_{obs}, and a limiting estimate of K_m was determined from the ability of the method to detect changes in values of the initial velocity.

Tritium Exchange of the Glycine pro-2S Proton. Determination of the relative rates of the SHMT-catalyzed exchange of the *pro*-2*S* proton of glycine in the absence and presence of folate cofactors was performed essentially as described previously (Schirch & Chen, 1973). [2-^{3}H]Glycine was added to a solution of 50 mM KBES, pH 7.2, 10 mM glycine, and 10 mM 2-mercaptoethanol such that the solution contained 2500 cpm/μL. Aliquots of 0.5 mL were incubated at 37 °C with 10 × K_m concentrations of the folate derivatives (Stover & Schirch, 1991), and the reaction was initiated with the addition of 1 nmol of cSHMT. Aliquots (50 μL) were removed with time, diluted with 200 μL of 1 N HCl, and placed on ice to terminate the reaction. The diluted reaction mixture was applied to a 0.5- × 4-cm Dowex-50-W-X12 column equilibrated with 10 mM HCl. The column was washed with 2 mL of 10 mM HCl, and the flowthrough was collected. The amount of exchanged tritium was determined by aliquoting 50 μL of the eluate into 1 mL of scintillation fluid and measuring counts per minute. The value of k_{cat} was determined from initial rates of counts released into the solvent.

Stopped-Quench Experiments. A comparison of the rates of 5-CHO-H_4PteGlu$_4$ and quinonoid complex formation from (6*R*)-5,10-CH$^+$-H_4PteGlu$_4$ was determined by stopped-quench experiments at 30 °C. The rate of 5,10-CH$^+$-H_4PteGlu$_4$ hydrolysis to the quinonoid complex was determined by measuring the increase in absorbance at 502 nm with a 500-μL solution containing 10 nmol of cSHMT, 15 nmol of 5,10-CH$^+$-H_4PteGlu$_4$, 1 mM glycine, and 1 mM 2-mercaptoethanol in 50 mM KBES, pH 7.0. This rate was compared to the rate of 5,10-CH$^+$-H_4PteGlu$_4$ hydrolysis to 5-CHO-H_4PteGlu$_4$ by adding 15 nmol of (6*R*)-5,10-CH$^+$-H_4PteGlu$_4$ to a series of 500-μL solutions containing 10 nmol of cSHMT in the same buffer as just described. These reactions were quenched at various time intervals by the addition of 1 mL of 15 N NH_4OH and incubated at 5 °C for 24 h to destroy the unreacted folate derivatives. The solvent was removed by lyophilization, and total 5-CHO-H_4PteGlu$_4$ was measured by either adding ATP and methenyl-THF synthetase and determining the increase in absorbance at 360 nm (Stover & Schirch, 1990) or adding cSHMT and glycine and determining the increase in absorbance at 502 nm.

Exchange of the C^{11} Proton of 5,10-CH$^+$-H_4PteGlu$_4$ during Its cSHMT-Catalyzed Hydrolysis to 5-CHO-H_4PteGlu$_4$. A 5-nmol (5-μL) quantity of 5,10-C[^{3}H]$^+$-H_4PteGlu$_4$ (23 500 cpm/nmol) was added to 450 μL of a buffer containing 100 mM KBES, pH 7.2, 5 mM 2-mercaptoethanol, 2 mM glycine, and 10 nmol of cSHMT subunits. The solution was incubated for 4 min at 35 °C to effect complete conversion of the substrate to 5-CHO-H_4PteGlu$_4$. The resulting cSHMT·Gly·5-CHO-H_4PteGlu$_4$ ternary complex is stable and dissociates very slowly (Stover & Schirch, 1991). This permitted the ternary complex to be rapidly isolated by gel filtration at 5 °C. One hundred microliters of reaction solution was added to a 1-mL syringe filled with Sephadex G-25 at 0 °C which had been equilibrated with cold 20 mM KBES buffer, pH 7.3. The

reaction solution was washed into the column with an additional 300 μL of cold buffer and centrifuged at 2000 rpm in a glass tube. The enzyme, with bound 5-CHO-H_4PteGlu$_4$, was collected in the eluate in greater than 85% yield (Schirch et al., 1991). The concentration of 5-CHO-H_4PteGlu$_4$ formed in the reaction was determined by adding 50 μL of the eluant to a 950-μL solution containing 10 mM glycine and 20 μM cSHMT and measuring the absorbance at 502 nm. Alternatively, 5-CHO-H_4PteGlu$_4$ was determined by adding a 100-μL aliquot of the reaction solution to a solution of ATP and methenyl-THF synthetase, at pH 6.0, and determining the increase in absorbance at 360 nm.

The amount of ^{3}H remaining in the isolated 5-CHO-H_4PteGlu$_4$ was determined from a 50-μL aliquot of the eluted cSHMT·Gly·5-CHO-H_4PteGlu$_4$ ternary complex using a scintillation counter. Less than 1.5% of the original [^{3}H] was present in the 5-CHO-H_4PteGlu$_4$. To determine if the measured ^{3}H was associated with 5-CHO-H_4PteGlu$_4$, the remaining eluted cSHMT·Gly·5-CHO-H_4PteGlu$_4$ ternary complex was placed in a boiling water bath for 10 min, which precipitates the enzyme and liberates free 5-CHO-H_4PteGlu$_4$. After centrifugation to remove the precipitated protein, the solution was lyophilized to remove any exchanged cSHMT-bound tritium. Following lyophilization, the powder was redissolved in 200 μL of H_2O and tritium was determined in a scintillation counter. This reduced the total measured ^{3}H to 0.5% of the original 5,10-C[^{3}H]$^+$-H_4PteGlu$_4$. This procedure was repeated with reaction vials having either reduced cSHMT or no enzyme as controls.

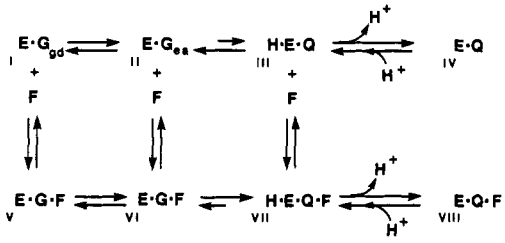
Differential Scanning Calorimetry Determinations. The denaturation of cSHMT solutions was performed with an MC-2 differential scanning microcalorimeter equipped with data acquisition software from Microcal Inc., Amherst, MA. cSHMT was dialyzed for 24 h against 100 volumes of 50 mM KBES, pH 7.3, and 10 mM 2-mercaptoethanol prior to each experiment. The enzyme was degassed and diluted to 3.2 mg/mL, and microliter volumes of concentrated solutions of the appropriate ligands were added immediately prior to use. All ligands were present at at least 10 times their K_m values to insure enzyme saturation. All thermodynamic values were made from triplicate determinations. The scanning rate was 30 °C/h. Enthalpy of denaturation (ΔH_d) values were determined as described previously (Schirch et al., 1991).

Rapid Reaction Kinetic Studies. Rapid reaction studies were performed on a Kinetic Instruments stopped-flow spectrophotometer, and absorbance versus time data were curve-fitted by single- or double-exponential algorithms supplied by Kinetic Instruments. All experiments were done in 50 mM KBES buffer, pH 7.0, and all data are the average of at least 5 measurements. The variance among measurements was less than 5%. For determination of the rate of formation of the enzyme–glycine binary quinonoid complex, either 20 mM glycine or [2-^{2}H]glycine was flowed against 60 μM cSHMT and the increase in absorbance at 492 nm was recorded. For experiments involving H_4PteGlu$_n$ and formyl- or methyl-substituted folates, one syringe contained 5 μM cSHMT and 10 mM glycine or [2-^{2}H]glycine and the second syringe contained 10 mM glycine with a 10 times K_m concentration of the folate ligand. When H_4PteGlu$_n$ was the ligand, the absorbance was followed at 495 nm, but when the folate was N^5-substituted the reactions were followed at 502 nm.

Results

Kinetic Constants for Formation of the Quinonoid Complex. We have previously shown that the cSHMT catalysis of 5,10-CH$^+$-H_4PteGlu to 5-CHO-H_4PteGlu requires either

Scheme I[a]



[a] E, SHMT; G, glycine; F, folate derivative including H_4PteGlu$_n$, 5-CHO-H_4PteGlu$_n$, or 5-CH_3-H_4PteGlu$_n$; *gd*, SHMT with glycine bound as a geminal diamine; ea, SHMT with glycine bound as an external aldimine; Q, SHMT with glycine bound as a resonance-stabilized quinonoid after removal of the *pro*-2*S* proton of glycine.

glycine or D-alanine. The evidence suggested that a quinonoid complex (E·Q) of these amino acids with the enzyme-bound pyridoxal-P was a requirement for catalysis. In this quinonoid complex the *pro*-2*S* proton of glycine is transferred to a base at the active site of cSHMT (H·E·Q) (Besmer & Arigoni, 1968; Jordan & Akhtar, 1970). Scheme I shows a minimal diagram of the relationship of the quinonoid complex, H·E·Q, with other complexes known to exist with glycine, e.g., *gem*-diamine (gd), external aldimine (ea), and quinonoid complexes shown as I, II, and III, respectively. The quinonoid complex absorbs near 500 nm and has a large apparent molar extinction coefficient of 40 000 M^{-1} cm^{-1} (Schirch & Ropp, 1967). The complex H·E·Q can exchange its proton with solvent protons through the putative intermediate, E·Q, shown as complex IV in Scheme I. The equilibrium distribution of the complexes of cSHMT and glycine favors the external aldimine and geminal diamine, with less than 1% of SHMT·Gly complexes existing as H·E·Q.

The addition of H_4PteGlu to the enzyme–glycine complex results in a shift in the equilibrium toward the ternary complex shown as structure VII in Scheme I. The increase in absorbance at 500 nm is about 50-fold, and the resulting distribution of complexes suggests that 50% exists as structure VII with the other 50% being divided between structures V and VI (Stover & Schirch, 1991). The *pro*-2*S* proton of glycine equilibrates rapidly with solvent protons, presumably through the complex shown as structure VIII in Scheme I. Considerable information on the kinetics of the formation of the ternary complex of glycine and H_4PteGlu, and the exchange of the *pro*-2*S* proton of glycine with solvent protons has been published (Schirch & Chen, 1973; Schirch, 1975; Hopkins & Schirch, 1986). However, it has also been shown that both 5-CH_3-H_4PteGlu and 5-CHO-H_4PteGlu can form ternary complexes with SHMT and glycine which have almost identical absorption properties as the ternary complex with H_4PteGlu (Schirch & Ropp, 1967; Stover & Schirch, 1991). The effects of 5-CH_3-H_4PteGlu and 5-CHO-H_4PteGlu on the exchange of the *pro*-2*S* proton of glycine have not been previously reported. The effect of glutamate chain length on both the rate of formation of ternary complex VII and the exchange of the *pro*-2*S* proton of glycine has also not been reported.

Because the formation of the ternary quinonoid complex appears to be crucial to the SHMT-catalyzed formation of 5-CHO-H_4PteGlu, we have extended previous studies on the effects of both N^5-substitution and glutamate chain length on this reaction. Table I records previous and current results for the rate of formation of the quinonoid ternary complex H·E·Q and H·E·Q·F with glycine and [2-^{2}H]glycine, the rate of proton exchange with [2-^{3}H]glycine, and the effect of glutamate chain length on these two properties. In the absence of any folate compound, the rate of formation of complex III is biphasic

Table I: Comparison of the Rate of SHMT-Catalyzed *pro*-2*S* Proton Exchange of Glycine and Rate of E·Q_{502} Formation[a]

ligand	formation of E·Q_{502} (s^{-1}) [^{1}H]Gly	[^{2}H]Gly	2*S*-H exchange (s^{-1}) [^{3}H]Gly
Gly	85, 0.1[b]	0.06, 0.004	0.02[c]
Gly + H_4PteGlu	875[c]	>500	2.0[c]
Gly + H_4PteGlu$_3$	>500	>500	0.75 ± 0.02
Gly + 5-CH_3-H_4PteGlu	4.8[d]	1.09	0.022 ± 0.004
Gly + 5-CH_3-H_4PteGlu$_3$	2.0, 6.7[d]	1.13	0.018 ± 0.004
Gly + 5-CHO-H_4PteGlu	0.5[d,e]	0.58	<0.0008 ± 0.00005
Gly + 5-CHO-H_4PteGlu$_3$	0.19, 0.05[d]	0.30, 0.06	<0.0008 ● 0.00005

[a] pH 7.0, 30 °C [b] Rate is biphasic; associated amplitudes are the same. [c] Schirch (1975). [d] Stover and Schirch (1991). [e] Rate is biphasic; the amplitude of the slow reaction is 9 times the amplitude of the fast reaction.

with a rapid phase and a much slower phase. At pH 7.3 the two phases are equal in amplitude. The use of [2-^{2}H]glycine in the reaction results in the disappearance of the observed rapid phase with only two very slow phases remaining. The rate of exchange of the *pro*-2*S* proton of [2-^{3}H]glycine is slow, being only about 0.02 s^{-1}.

The addition of either H_4PteGlu or H_4PteGlu$_3$ to the E·Gly complexes results in a large increase in absorbance at 500 nm (structure VII in Scheme I) and an order of magnitude increase in its rate of formation. No significant isotope effect with [2-^{2}H]glycine could be observed in the formation of structure VII. However, the reactions were too fast to determine an isotope effect of less than 2-fold. The addition of both H_4PteGlu and H_4PteGlu$_3$ causes about a 100-fold increase in the rate of exchange of the *pro*-2*S* proton of [2-^{3}H]glycine with solvent protons (Table I).

When the studies on the rate of formation of complex VII (E·Q·F) were repeated with 5-CH_3-H_4PteGlu and 5-CH_3-H_4PteGlu$_3$, some differences were observed compared to the results found with H_4PteGlu$_n$. First, the rate of formation of the ternary quinonoid complex is much slower, with the triglutamate showing two slow phases. The reaction shows a deuterium isotope effect of about 4 for both 5-CH_3-H_4PteGlu and 5-CH_3-H_4PteGlu$_3$. The addition of neither the monoglutamate nor triglutamate forms of 5-CH_3PteGlu$_n$ results in an increased rate of solvent exchange of the α-proton of glycine (Table I).

The experiments with 5-CHO-H_4PteGlu$_n$ revealed very different results compared to H_4PteGlu$_n$ and 5-CH_3-H_4PteGlu$_n$. The rate of formation of the ternary quinonoid complex is very slow and biphasic with the triglutamate form of the folate (Stover & Schirch, 1991). Neither the monoglutamate nor triglutamate compound results in deuterium isotope effects with [2-^{2}H]glycine. Also, there is no measurable exchange of the α-proton of glycine with solvent protons (Table I). Even though the effects of 5-CH_3-, 5-CHO-, and H_4PteGlu$_n$ on the spectral properties of the enzyme–glycine complexes are essentially identical, the rates of formation of complex VII (Scheme I) and the rate of exchange of the α-proton of glycine vary considerably with the nature of the substituent on N^5 and the number of glutamate residues. The results show that the base on the enzyme which accepts the *pro*-2*S* proton of glycine is exposed to solvent when H_4PteGlu$_n$ is bound. The lack of an isotope effect with [2-^{2}H]glycine suggests that a conformational change is the rate-limiting step as opposed to breaking the *pro*-2*S* H–C bond of glycine in the presence of either H_4PteGlu$_n$ or 5-CHO-H_4PteGlu$_n$.

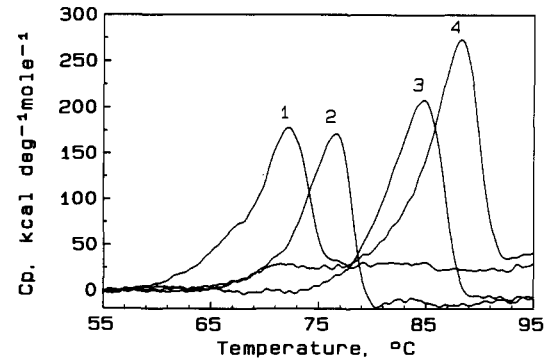


FIGURE 1: Differential scanning calorimeter thermograms of cSHMT with four ligand combinations. Curve 1, cSHMT·Gly; curve 2, cSHMT·Gly·H_4PteGlu$_3$; curve 3, cSHMT·Gly·5-CH_3-H_4PteGlu$_3$; curve 4, cSHMT·Gly·5-CHO-H_4PteGlu$_3$. The buffer used in all experiments was 50 mM KBES, pH 7.3, and 20 mM glycine. The concentration of cSHMT was 3.2 mg/mL and the folate concentration was 5 μM for each experiment.

Effect of N^5-Substituted Folates on the Stability of cSHMT. Previous studies measuring amide proton exchange, optical activity of pyridoxal-P, and thermal stability have shown that the formation of the ternary complexes E·Gly·F (Scheme I) results in conformational changes when the folate monoglutamates, H_4PteGlu, 5-CH_3-H_4PteGlu, or 5-CHO-H_4PteGlu, are bound (Schirch et al., 1991). One of the most sensitive means of detecting conformational changes in proteins is by measuring their T_m and enthalpy of denaturation (ΔH_d) by differential scanning calorimetry (Relimpio et al., 1981; Farach & Martinez-Carrion, 1983; Strong et al., 1987; Shriver & Kamath, 1990). Previous studies with cSHMT have shown that the addition of the monoglutamate form of the three folate compounds to E·Gly resulted in about a 4–6 °C increase in T_m. Values for ΔH_d varied with the nature of the folate with increases of 0, 120, and 160 kcal/mol for H_4PteGlu, 5-CH_3-H_4PteGlu, and 5-CHO-H_4PteGlu, respectively (Schirch et al., 1991). The effect of glutamate chain length on the stability of the E·Gly·F ternary complexes has not been determined previously by this technique. Figure 1 shows the thermal denaturation curves for E·Gly·F, with F being either H_4PteGlu$_3$, 5-CH_3-H_4PteGlu$_3$, or 5-CHO-H_4PteGlu$_3$, compared to the thermal curve for E·Gly. Increasing the glutamate chain length from 1 to 3 had no effect on the thermal denaturation curve for H_4PteGlu$_n$, which has the same T_m and ΔH_d values found with the monoglutamate form of the coenzyme. However, significant differences were found with 5-CH_3H_4PteGlu$_3$ and 5-CHO-H_4PteGlu$_3$. The addition of the two glutamate residues increased T_m to 84 and 87 °C for the methyl and formyl derivatives, which are 4 and 8 °C higher than their monoglutamate forms, respectively. The additional glutamate residues had no effect on ΔH_d.

An important difference in the effect of 5-CHO-H_4PteGlu$_3$ with respect to the other folate compounds used in these studies was observed. With all previous thermal denaturation studies done with cSHMT, heating to 95 °C results in irreversible denaturation with the enzyme being completely precipitated. With 5-CHO-H_4PteGlu$_3$ as the ligand and heating to 95 °C, the denaturation was not totally irreversible as it did not lead to complete precipitation of the enzyme. Cooling the enzyme solution showed that the quinonoid complex was still present and a second scan showed a thermal denaturation curve with as much as 30% of the peak height as observed in the first scan. The ΔH_d value in this study was 160 kcal/mol higher than observed for the E·Gly complex and about 220 kcal/mol higher than the enzyme alone, but these values cannot be directly

Table II: Kinetic Constants for 5,10-CH^+-$H_4PteGlu_n$ and 5,10-CHOH-$H_4PteGlu_n$ Conversion to 5-CHO-$H_4PteGlu_n$ (Reactions 3 and 4)

	5,10-CH^+-$H_4PteGlu_n$ (reaction 3)		5,10-CHOH-$H_4PteGlu_n$ (reaction 4)	
n	K_m (μM)	k_{cat} (s^{-1})	K_m(μM)	k_{cat} (s^{-1})
1	140 ± 30	0.0001 ± 0.00005	8.0 ± 1	0.500
4	40 ± 5	0.055 ± 0.01	<0.5	0.058

compared because the final states are different in the two studies. These studies further confirm that the binding of 5-CHO-$H_4PteGlu_n$ results in conformational changes not present when $H_4PteGlu_n$ is the ligand. In addition, the results suggest that the addition of glutamate residues to 5-CHO-$H_4PteGlu$ forms a unique conformational state of the ternary complex H·E·Gly·F not observed with either $H_4PteGlu_3$ or 5-CHO-$H_4PteGlu$.

Kinetic Constants for Formation of E·Q·5-CHO-$H_4PteGlu_n$ from 5,10-CH^+-$H_4PteGlu_n$ and 5,10-CHOH-$H_4PteGlu_n$. The rate of increase in absorbance at 502 nm upon the addition of solutions of 5,10-CH^+-$H_4PteGlu_n$ to cSHMT is biphasic, having a rapid phase which is complete in a few seconds followed by a slow phase which is complete in several minutes. As has been previously demonstrated, the rapid phase is attributable to the conversion of a compound in the solutions of 5,10-$CH^+H_4PteGlu_n$ [proposed to be (6*R*,11*R*)-5,10-CHOH-$H_4PteGlu_n$] to 5-CHO-$H_4PteGlu_n$ (reaction 4). The slow phase is the result of conversion of 5,10-CH^+-$H_4PteGlu_n$ to 5-CHO-$H_4PteGlu_n$ (reaction 3). As noted in a previous study, solutions of 5,10-CH^+-$H_4PteGlu_n$ can be prepared either enriched in the hydrated intermediate (incubated at 50 °C, pH 4.0) or containing undetectable amounts of this compound (incubated at 4 °C, pH 1.5) (Stover & Schirch, 1992). The initial rates of increase in absorbance at 502 nm with solutions of the mono- and tetraglutamate forms of 5,10-CH^+-$H_4PteGlu$ were determined. In these studies, saturation kinetics were observed with increasing substrate concentrations (reaction 3). K_m and k_{cat} values were determined from double-reciprocal plots for both substrates (Table II). We have previously shown that the effect of the number of glutamate residues on the properties of cSHMT does not vary significantly from the triglutamate to the hexaglutamate (Strong & Schirch, 1989). The triglutamate has been used in a few of these studies, and the results were the same as observed for the more extensive studies with the tetraglutamate. Repeating these studies with 5,10-C[^{2}H]$^+$-$H_4PteGlu_n$ as a substrate did not result in an observed isotope effect.

$$\text{5,10-CH}^+\text{-H}_4\text{PteGlu}_n + \text{E·Gly} \rightarrow \text{E·Gly·5-CHO-H}_4\text{PteGlu}_n \quad (3)$$

$$\text{5,10-CHOH-H}_4\text{PteGlu}_n + \text{E·Gly} \rightarrow \text{E·Gly·5-CHO-HPteGlu}_n \quad (4)$$

Using stopped-flow techniques, similar studies were done with solutions enriched in the hydrated adduct 5,10-CHOH-$H_4PteGlu_n$ (reaction 4). With the tetraglutamate form, no variation in initial rate was observed with increasing concentration of this substrate. The value of k_{cat} was taken from k_{obs} for the formation of the quinonoid complex, and limits of K_m were estimated from the concentration range of substrate used in the study (Table II).

The results recorded in Table II show that the addition of three glutamate residues lowers the K_m values for both 5,10-CH^+-$H_4PteGlu_n$ and 5,10-CHOH-$H_4PteGlu_n$ by more than an order of magnitude. The K_m for 5,10-CHOH-$H_4PteGlu_4$ was too low to measure. It may be comparable to the K_m

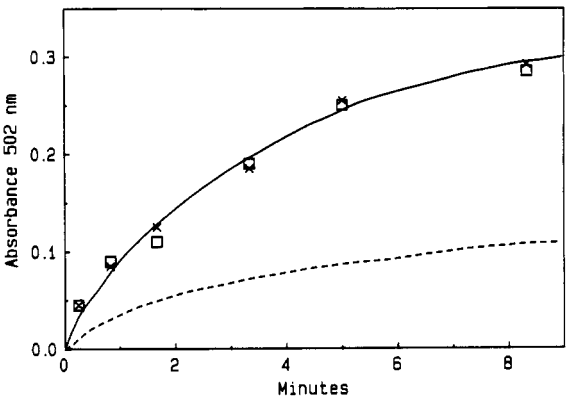


FIGURE 2: Comparison of the rate of the conversion of 5,10-CH^+-$H_4PteGlu_4$ to a quinonoid complex absorbing at 502 nm and 5-CHO-$H_4PteGlu_4$. A 0.5-mL solution containing 10 μM cSHMT and 15 μM 5,10-CH^+-$H_4PteGlu_4$, pH 7.0, was monitored with time at 502 nm in the presence of 4 mM [2-^{1}H]glycine (—) or [2-^{2}H]glycine (---) at 30 °C. Stopped-quench experiments under identical conditions show the amount of 5-CHO-$H_4PteGlu_4$ formed at designated time intervals in the presence of [2-^{1}H]glycine (□) or [2-^{2}H]glycine (×). The 5-CHO-$H_4PteGlu_4$ concentrations were determined as described in Experimental Procedures.

found for $H_4PteGlu_3$ or the K_d of 5-CHO-$H_4PteGlu_3$, which are both about 0.2 μM (Stover & Schirch, 1991). Increasing the number of glutamate residues has the opposite effect on k_{cat} for the two substrates. With 5,10-CH^+-$H_4PteGlu_n$, increasing the number of glutamate residues from 1 to 4 results in a 500-fold increase in k_{cat}. However, with 5,10-CHOH-$H_4PteGlu_n$, the values of k_{cat} for the tetraglutamate is 10-fold slower than with the monoglutamate (Table II). Comparison of the tetraglutamate form of the two substrates shows that the k_{cat} values are very similar and about equal to the rate observed for the reaction of 5-CHO-$H_4PteGlu_3$ with cSHMT·Gly to form H·E·Q·5-CHO-$H_4PteGlu_3$ (Table I). This raises the question of whether both substrates, 5,10-CH^+-$H_4PteGlu_4$ and 5,10-CHOH-$H_4PteGlu_4$, may not be converted more rapidly to 5-CHO-$H_4PteGlu_4$ by the enzyme than is suggested by observing the increase in absorbance at 502 nm and whether the rate-determining step is the removal of the *pro*-2*S* proton of glycine to form H·E·Q·5-CHO-$H_4PteGlu_4$.

The rate of formation of 5-CHO-$H_4PteGlu_4$ from 5,10-CH^+-$H_4PteGlu_4$ was addressed by doing a stopped-quench experiment. To a solution of 10 μM cSHMT and 1 mM glycine was added 15 μM 5,10-CH^+-$H_4PteGlu_4$. Since this substrate concentration is well below its K_m value of 40 μM, the rate of formation of H·E·Q·5-CHO-$H_4PteGlu_4$, as determined by the increase in absorbance at 502 nm, requires about 10 min to reach completion. At several intervals during this period, different vials of reaction solution were stopped by the addition of concentrated NH_4OH, which denatures the enzyme and converts any remaining 5,10-CH^+-$H_4PteGlu_4$ to 10-CHO-$H_4PteGlu_4$ in a few minutes. Under these conditions the 5-CHO-H_4-$PteGlu_4$, which had been formed in the reaction, would remain stable. After removal of the denatured enzyme, the amount of 5-CHO-$H_4PteGlu_4$ in the stopped reaction was determined either by lowering the pH to 7.3, adding fresh cSHMT and glycine, and determining the amplitude of the quinonoid complex from its absorbance at 502 nm or adding ATP and methenyl-THF synthetase and measuring the increase in absorbance at 360 nm. Both methods gave the same amount of 5-CHO-$H_4PteGlu_4$.

The solid line in Figure 2 shows the rate of increase in absorbance at 502 nm of the initial reaction solution (formation of H·E·Q·5-CHO-$H_4PteGlu_4$). The open boxes show the

Table III: C^{11} Proton Exchange during the cSHMT-Catalyzed Hydrolysis of 5,10-C[^{3}H]$^+$-H_4PteGlu$_4$ to 5-CHO-H_4PteGlu$_4$

enzyme[a]	substrate[b] (cpm)	product[c] (cpm)	product[c] (nmol)
cSHMT·Gly	118 000	630 ± 100	5.0
red cSHMT·Gly	118 000	620 ± 100	<0.25
glycine	118 000	670 ± 100	<0.25

[a] 10 nmol of cSHMT subunits. [b] CH^+-PteGlu$_4$, 5 nmol, 23 500 cpm/nmol [c] 5-CHO-PteGlu$_4$.

amount of quinonoid complex (H·E·Q·5-CHO-H_4PteGlu$_4$) formed from quenched reactions of identical reaction solutions. It is clear that 5-CHO-H_4PteGlu$_4$ is not formed faster than the rate of appearance of absorbance at 502 nm. Therefore, the enzyme-catalyzed rate of formation of 5-CHO-H_4PteGlu$_n$ from 5,10-CH^+-H_4PteGlu$_4$ is the same as the rate of formation of H·E·Q·5-CHO-H_4PteGlu$_4$.

When the experiment was repeated with [2-^{2}H]glycine, an interesting observation was made. With this substrate, the amount of H·E·Q·5-CHO-H_4PteGlu$_4$ formed is decreased by 70% (dashed line in Figure 2). However, the rate of approach to equilibrium shows no isotope effect. The results in Table I show that the *pro*-2*S* proton of glycine does not equilibrate with solvent protons with enzyme-bound 5-CHO-H_4PteGlu$_4$, suggesting that the decreased amount of quinonoid complex formed with deuterated glycine is the result of a change in the equilibrium between the glycine anion (Q) and the base on the enzyme which accepts the proton. In stopped-quench experiments, both the rate of formation and the amount of 5-CHO-H_4PteGlu$_4$ formed are not affected by substitution of a deuterium atom on glycine for a proton (Figure 2). This suggests that it is the rate of formation of H·E·Q which is important and not its equilibrium concentration in the cSHMT catalysis of reaction 3. These results further suggest that the rate of H·E·Q formation in the presence of 5,10-CH^+-H_4PteGlu$_4$ is not determined by the breaking of the *pro*-2*S* C–H bond of glycine. The results are consistent with the rate of H·E·Q formation being determined by a conformational change.

The value for k_{cat} reported in Table II is about an order of magnitude greater than the previously reported rate for reaction 3 (Stover & Schirch, 1990). This is the result of anion inhibition of this reaction. In the previous studies, the reaction solution contained 30 mM ammonium sulfate and 10 mM ammonium formate. Forty millimolar sulfate causes about a 90% decrease in the rate of reaction 3.

Exchange of the C^{11} Proton during the Hydrolysis of 5,10-CH^+-H_4PteGlu$_4$ to 5-CHO-H_4PteGlu$_4$. In the study of the mechanism of the nonenzymatic conversion of 5,10-CH^+-H_4PteGlu to 5-CHO-H_4PteGlu, the C^{11} proton was exchanged with solvent (Stover & Schirch, 1992). We investigated whether this was also observed in the enzymatic reaction. In this study, 5,10-C[^{3}H]$^+$-H_4PteGlu$_4$ was incubated with cSHMT, the product 5-CHO-H_4PteGlu$_4$ was isolated, and the amount of tritium remaining in the formyl group was determined. Control reactions included a no-enzyme control and cSHMT in which the enzyme-bound pyridoxal-P had been reduced with $NaCNBH_3$. The reduced enzyme cannot form a quinonoid complex with glycine. The results are reported in Table III. Five nanomoles of 5,10-CH^+-H_4PteGlu$_4$ was completely converted to 5-CHO-H_4-PteGlu$_4$ in the 4-min incubation. No radioactive tritium above control levels was detected in the product 5-CHO-H_4PteGlu$_4$.

We have previously shown that the exchange of the C^{11} proton of 5,10-CH^+-H_4PteGlu is very slow at 30 °C and pH 7.5. The rate of exchange of the C^{11} proton, at this pH and 40 °C, exhibits a $t_{1/2}$ of about 60 min (Stover & Schirch, 1992). To determine if cSHMT catalyzes the exchange of the C^{11} proton in the absence of glycine, the 5,10-C[^{3}H]$^+$-H_4PteGlu$_4$ solutions described above were incubated at pH 7.0 with either cSHMT, cSHMT with 10 mM aminomethylphosphonate, or a no-enzyme control. At 10-min time intervals, 100-μL aliquots were removed and lyophilized to dryness to remove any exchanged protons. The residue was redissolved in scintillation fluid and counted. More than 90% of the original counts were in the residue, showing that cSHMT, either alone or with aminomethylphosphonate, did not catalyze the exchange of the C^{11} proton in a 20-min incubation. Aminomethylphosphonate is a glycine analogue that forms an external aldimine with the enzyme corresponding to structure II in Scheme I, but it does not form a quinonoid complex. We have previously shown that this glycine analogue does not result in conversion of CH^+-H_4PteGlu$_n$ to 5-CHO-H_4PteGlu$_n$.

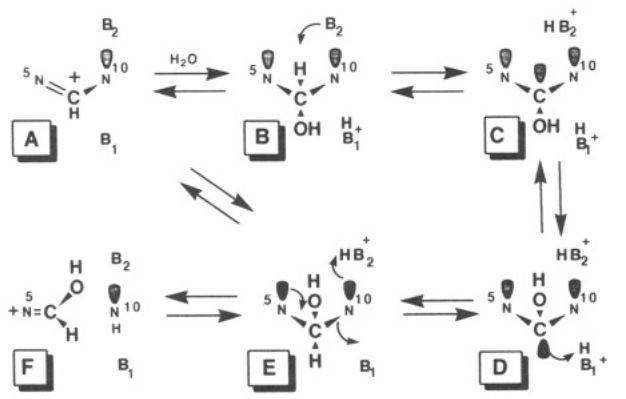
In summary, cSHMT catalyzes the exchange of the C^{11} proton of 5,10-CH^+-H_4PteGlu$_n$ with solvent protons in its conversion to 5-CHO-H_4PteGlu$_n$, but it does not catalyze the exchange in the absence of glycine.

Role of Glycine in the Conversion of 5,10-CHOH-H_4PteGlu$_3$ to 5-CHO-H_4PteGlu$_3$. Glycine has been shown previously to be required for the cSHMT catalysis of reaction 3 (Stover & Schirch, 1990). However, glycine has not previously been tested as a requirement for the cSHMT catalysis of reaction 4. Attempts were made to determine if cSHMT alone could catalyze the breakdown of the putative (6*R*,11*R*)-5,10-CHOH-H_4PteGlu$_n$ to 5-CHO-H_4PteGlu$_n$ so that the role of glycine could be more narrowly defined. Solutions of 5,10-CH^+-H_4PteGlu$_4$, enriched in (6*R*,11*R*)-5,10-CHOH-H_4PteGlu$_4$ (20 μM), were incubated with C_1-THF synthase to convert any remaining 5,10-CH^+-H_4PteGlu$_4$ to 10-H_4PteGlu$_4$. To this solution was added either cSHMT (5 μM) or cSHMT and aminomethylphosphonate as a control. After a 15-min incubation at 30 °C the two solutions were placed in a boiling water bath for 3 min to precipitate the enzyme and free any 5-CHO-H_4PteGlu$_4$ which had been formed. After centrifugation to remove the precipitated protein, MgATP and methenyl synthetase were added to determine the concentration of 5-CHO-H_4PteGlu$_4$. Less than the detection limit of 1 μM 5-CHO-H_4PteGlu$_4$ was found in either of the two solutions. In the presence of glycine, the enzyme forms 5 μM 5-CHO-H_4PteGlu$_4$ in less than 1 min. Therefore, glycine appears to be required for both reactions 3 and 4.

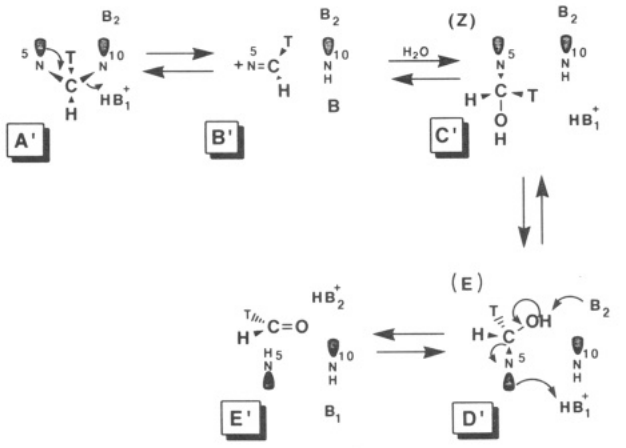
Discussion

The research reported in this paper addresses three questions. One, does the SHMT catalysis of reaction 2 occur by the same mechanism as proposed for the nonenzymatic reaction? Two, what is the role of glycine in the enzyme-catalyzed reaction? Three, why does increasing the number of glutamate residues on 5,10-$CH^+$$H_4$PteGlu$_n$ increase the enzyme-catalyzed rate of hydrolysis? We have assumed that the mechanism of reaction 2 is closely related to the mechanism of the SHMT catalysis of reaction 1, i.e., the conversion of 5,10-CH_2-H_4PteGlu$_n$ and glycine to H_4PteGlu$_n$ and serine. Although the exact mechanism of reaction 1 is not known, there are several experiments which define the stereochemical relationship of the transfer of the one-carbon group from 5,10-CH_2-H_4PteGlu$_n$ to glycine. These requirements restrict the number of possible mechanisms which are consistent with current results. A discussion of these stereochemical requirements and possible mechanisms has been recently re-

Scheme II



Scheme III



viewed by Matthews and Drummond (1991). Much of the mechanism we propose for the SHMT catalysis of reaction 2 is based on the mechanism proposed by Matthews and Drummond for reaction 1, in which enzyme-bound formaldehyde is an intermediate in the reaction. Although other mechanisms in which formaldehyde is not an intermediate are discussed in this review, we will restrict our discussion to only the one mechanism.

Evidence for Similar Enzymatic and Nonenzymatic Mechanisms in the Conversion of $CH^+H_4PteGlu_n$ to 5-CHO-$H_4PteGlu_n$. Scheme II shows a proposed mechanism for the SHMT catalysis of 5,10-CH^+-$H_4PteGlu_n$ to 5-CHO-$H_4PteGlu_n$, and Scheme III shows the proposed mechanism for the principal physiological reaction (reaction 1). The structures of $H_4PteGlu_n$ in Schemes II and III are drawn with C^6 of folate in the same horizontal plane as N^5 and N^{10}. The proton on C^6 is above the plane of C^6, N^5, and N^{10} in each structure. Both N^5 and N^{10} are in the plane of the paper and C^{11} is in front of this plane.

The reactions in Scheme II propose a cSHMT-catalyzed mechanism for conversion of 5,10-CH^+-$H_4PteGlu_n$ to 5-CHO-$H_4PteGlu_n$. The mechanism is essentially the same as that proposed for the nonenzymatic mechanism in terms of intermediates and stereochemical considerations (Stover & Schirch, 1992). An additional constraint on the stereochemistry of the reaction, not present in the nonenzymatic studies or the enzyme mechanism considered by Matthews and Drummond (1991), is that cSHMT binds only one of two rotamers of 5-CHO-$H_4PteGlu_n$ (Stover & Schirch, 1991). The rotamer that is bound is shown in the enol form as structure F in Scheme II. The proposed mechanism accounts for the correct isomer of 5-CHO-$H_4PteGlu_n$ (structure F) being formed at the active site of cSHMT. Looking at the mechanism in the reverse direction, structure F would be formed by the antiperiplanar elimination of N^{10} which must occur from the (11*R*) isomer of 5,10-CHOH-$H_4PteGlu_n$ (structure E). This would account for structure E being an intermediate in the reaction and could account for its more rapid conversion to 5-CHO-$H_4PteGlu$ (in the case of the monoglutamate substrate) if the rate-limiting step involves the conversion of A to E. There are at least two possible paths for the conversion of 5,10-CH^+-$H_4PteGlu_n$ to structure E. First, there could be the syn addition of water at C^{11}, which would form structure E directly. Second, water could make an antiperiplanar addition to form structure B. If structure B undergoes the antiperiplanar elimination of N^{10}, it would result in the rotamer of 5-CHO-$H_4PteGlu_n$ which does not bind to cSHMT (Stover & Schirch, 1991). Arriving at the correct isomer (structure E) requires the inversion at C^{11} through the putative carbanions shown as structures C and D. The involvement of these two carbanions is supported by the data of the nonenzymatic mechanism. The involvement of the two carbanions also explains the enzymatic results reported in Table III, which show that in the conversion of structure A to structure F the C^{11} proton is not retained. Direct conversion of A to E would not explain the loss of the C^{11} proton observed in our experiments.

Additional evidence in support of the mechanism shown in Scheme II is its consistency with the stereochemical requirements of the principal physiological mechanism shown in Scheme III. We assume that the location of the oxygen atom, derived from either serine in Scheme III or 5-CHO-$H_4PteGlu_n$ in Scheme II, must occupy similar space(s) at the active sites in the two mechanisms. Using the arguments put forth by Matthews and Drummond (1991), Scheme III shows the conversion of 5,10-CH_2-$H_4PteGlu_n$ to $H_4PteGlu_n$ and enzyme-bound formaldehyde, which accounts for the presently known stereochemistry of this reaction. Previous studies show that, starting with (3*R*)-[3-^{3}H]serine, the result is (11*R*)-[11-^{3}H]CH_2-$H_4PteGlu_n$ (Vanoni et al., 1990). If free formaldehyde is an intermediate, then it cannot rotate at the active site, the glycine anion must attack the *si* face of the bound formaldehyde, and N^5 must attack the *re* face. The intermediate N^5 iminium cation (structure B′) must be attacked either from the *si* face to form (11*R*)-5,10-CH_2-$H_4PteGlu_n$ or the *si* face by water to form the N^5 carbinolamine.

In Scheme III, (11*R*)-[11-^{3}H]-CH_2-$H_4PteGlu_n$ undergoes elimination of N^{10} to form the N^5 iminium cation (A′ → B′). Water attacks the *si* face of the iminium cation (B′) to form the (*Z*) conformer of the N^5 carbinolamine (B′ → C′), in which the hydroxymethyl group on N^5 and the alkyl group on C^6 are on the same side of the pteridine ring. Positioning formaldehyde at the active site on the less hindered side of $H^4PteGlu_n$, so that the glycine anion (Q in Scheme I) can attack the *si* face, requires an inversion of the N^5 carbinolamine to form the (*E*) conformer (C′ → D′). Elimination of N^5 results in bound $H_4PteGlu_n$ and formaldehyde (D′ → E′). The glycine anion would be above the plane of formaldehyde and would attack the *si* face to form (3*R*)-[3-^{3}H]serine (Matthews & Drummond, 1991).

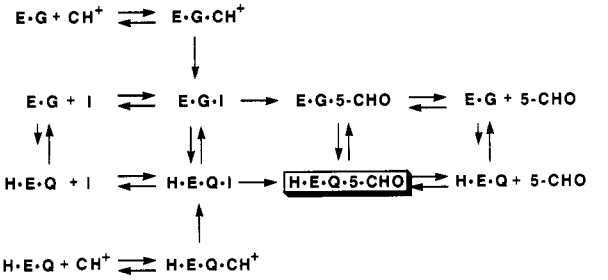
In Scheme II the sequence of events is a hydration at C^{11} of 5,10-CH^+-$H_4PteGlu_n$, inversion at C^{11}, followed by the elimination of the N^{10} nitrogen. The mechanism shown in Scheme III displays three similar steps: elimination of the N^{10} nitrogen, hydration at C^{11}, and inversion at N^5. The inversion step in each mechanism requires the movement of the oxygen atom from below to above the plane of the pteridine ring. The structures in the two mechanisms suggest a similar path for

the oxygen atom. Structure B is analogous to structure C′, structure E is analogous to D′, and structure F is analogous to B′. Currently there is no experimental evidence that an inversion of the carbinolamine at N^5 is required in the mechanism of the physiological reaction; it has been suggested in order to satisfy stereochemical constraints (Matthews & Drummond, 1991). However, previous evidence has shown that the N^5 carbinolamine is not a substrate for cSHMT (Schirch & Chen, 1973). This could be reconciled with the proposed mechanism if it is assumed that the location of the oxygen atom in the free N^5 carbinolamine is hydrogen-bonded to the 4-keto group. In either the (*Z*) or (*E*) conformations, a rotation around the N^5–C^{11} bond would place the hydroxyl group in a position to hydrogen-bond to the carbonyl group at C^4. This would place the oxygen atom in a different position than in either structure C′ and D′. This could prevent binding to cSHMT just as the rotamer of 5-CHO-H_4PteGlu that has the formyl oxygen pointed toward the C^4 keto group does not bind (Stover & Schirch, 1991).

Role of Glycine in the Enzyme Mechanism. The results presented in Figure 1 and Table II show that glycine is required to convert 5,10-CH^+-H_4PteGlu$_n$ to compound F (Scheme II). Glycine could be required for catalysis for at least three different reasons. First, when glycine binds in the presence of folates there is a conformational change (Schirch et al., 1991). It may be that this conformational change is required for proper alignment at the active site. However, the glycine analogue aminomethylphosphonate also causes a conformational change when it binds at the active site, and it does not support catalysis at a measurable rate (Schirch et al., 1991; Stover & Schirch, 1990). We conclude that the conformational change is not the critical role played by glycine, but probably is involved in the effects observed with the polyglutamate forms as discussed below. Second, the glycine anion could be directly involved in the mechanism. Matthews and Drummond (1991) have proposed a mechanism for the major physiological reaction that involves an attack of the glycine anion (Q, Scheme I) on the N^5 iminium cation (B′, Scheme III), forming a covalent attachment of glycine and folate. A similar mechanism could be proposed for the mechanism of reaction 3, where the glycine anion (Q) attacks C^{11} in structure A. However, this mechanism would not readily explain compound E as a substrate for the reaction and would require the folate-independent cleavage of allothreonine and β-phenylserine to occur by a mechanism different from that for serine. At this time there is little evidence for a direct attack of the glycine anion on the N^5 iminium cation. Third, the *pro*-2*S* proton of glycine which is transferred to a base on the enzyme may be required in one of the catalytic steps. The evidence supports this third possibility.

There are several steps in the mechanisms shown in Schemes II and III that require proton transfers involving groups at the active site. Glycine is required for the exchange of the C^{11} proton of 5,10-CH^+-H_4PteGlu$_n$, suggesting that its role comes early in the mechanism. However, glycine also appears to be required for the conversion of compound E to F, suggesting that it also is required for the last step. As shown in Scheme II, at least two bases at the active site are required to account for the proton transfers. More bases may be involved. It is possible that the proton shown on B_1 (Scheme II) comes from glycine and that the conformational change induced by glycine aligns these bases with respect to the proton transfer steps. Aminomethylphosphonate may help align the bases, but without the 2*S* proton being on B_1 it cannot support the catalytic cycle.

Scheme IV



Effect of the Polyglutamate Chain on the Enzymatic Mechanism. The addition of two or more glutamate residues to 5-CHO-H_4PteGlu has a profound effect on the rate of formation of enzyme-bound 5-CHO-H_4PteGlu$_n$ (H·E·Q·5-CHO-H_4-PteGlu$_n$), which absorbs at 502 nm. It results in a 500-fold increase in the rate starting from 5,10-CH^+-H_4PteGlu$_4$ and a 10-fold decrease in rate starting from both 5-CHO-H_4PteGlu$_4$ and 5,10-CHOH-H_4PteGlu$_4$ (Table II). The glutamate residues are not directly involved in the chemistry at N^5 and N^{10}, suggesting that the glutamate residues exert their effect by causing a conformational change in the enzyme. Support for this interpretation is the effect of the two glutamate residues on the thermal denaturation properties of the cSHMT·Gly complex in the presence of 5-CHO or 5-CH_3-H_4PteGlu$_3$ and the lack of deuterium isotope effects with [2-^{2}H]glycine on any of the reactions involved in the formation of H·E·Q·5-CHO-H_4PteGlu$_3$ (Figure 1).

Scheme IV shows a series of enzyme complexes that can lead to the formation of the ternary complex H·E·Q·5-CHO-H_4PteGlu$_n$ absorbing at 502 nm (boxed structure in Scheme IV). In this scheme, Q is the glycine anion H is the *pro*-2*S* proton of glycine on a putative base at the active site, 5-CHO is 5-CHO-H_4PteGlu$_n$, CH^+ is 5,10-CH^+-H_4PteGlu$_n$, and I is (11*R*)-5,10-CHOH-H_4PteGlu$_n$. With the monoglutamate form of the coenzyme, 5-CHO-H_4PteGlu$_n$ and 5-CHOH-H_4PteGlu (I) are converted to H·E·Q·5-CHO-H_4PteGlu at the same rate (0.5 s^{-1}) (Tables I and II). With the tetraglutamate form of the coenzyme, all three compounds (CH^+, I, and 5-CHO) are converted to the complex absorbing at 502 nm at the same rate (0.06 s^{-1}) (Tables I and II). This suggests that with the polyglutamate forms of the coenzyme there is a common intermediate in all three pathways which is converted to H·E·Q·5-CHO in a rate-determining step. This common intermediate does not appear to be E·G·5-CHO, since the stopped-quench experiments show that, starting with either I or CH^+, the rate of formation of 5-CHO-H_4PteGlu$_4$ is the same as the appearance of the complex absorbing at 502 nm. The observation that [2-^{2}H]glycine shows no isotope effect is also consistent with the rate-determining step not being the transfer of the *pro*-2*S* proton of glycine to a base on the enzyme. Previous studies on the binding of 5-CHO-H_4PteGlu$_n$ to cSHMT suggested that the rate-determining step in forming H·E·Q·5-CHO-H_4PteGlu$_n$ involved a conformational change (Stover & Schirch, 1991). If the similar conversion of E·G·I to H·E·Q·I was controlled by the same conformational change and H·E·Q·I was converted rapidly to H·E·Q·5-CHO, the common rate for formation of the complex absorbing at 502 nm could be explained for each of the three substrates.

Concluding Remarks. The long-term goal of this research is to determine if the SHMT-catalyzed formation of 5-CHO-H_4PteGlu$_n$ is the in vivo source of this derivative of the coenzyme and if 5-CHO-H_4PteGlu$_n$ has a physiological role. The results reported in Tables I and II suggest that, in the cell, even the polyglutamate form of 5,10-CH^+-H_4PteGlu$_n$ could

not serve as a substrate for SHMT. At pH 7.3, the concentration of free 5,10-CH^+-H_4PteGlu$_n$ (assuming it is in equilibrium with 10 μM 10-CHO-H_4PteGlu$_n$) would be about 0.5 μM, which is well below the K_m of 40 μM found for cSHMT. Either direct channeling of this putative substrate to SHMT from other folate enzymes occurs or the hydrated compound (6*R*,11*R*)-5,10-CHOH-H_4PteGlu$_n$ is the true substrate for this reaction. It is conceivable that another folate enzyme catalyzes the formation of the hydrated compound.

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