5-Formyltetrahydrofolate Polyglutamates Are Slow Tight Binding Inhibitors of Serine Hydroxymethyltransferase*

(Received for publication, June 27, 1990)

Patrick Stover and Verne Schirch
From the Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University.
Medical College of Virginia, Richmond, Virginia 23298

The interaction of the mono- and triglutamate forms of 5-methyltetrahydrofolate and 5-formyltetrahydrofolate with serine hydroxymethyltransferase were determined by several methods. These methods included: determining dissociation constants by observing the absorbance at 502 nm of a ternary complex of the enzyme, glycine, and the folate compounds; determining inhibition constants from steady-state reactions; and determining the rate of formation and breakdown of the enzyme inhibitor complex by rapid reaction kinetics. Studies of the dissociation and inhibitor constants showed that both 5-methyltetrahydrofolate and 5-formyltetrahydrofolate have essentially the same affinity for the enzyme-glycine binary complex. However, rapid reaction and steady-state kinetic studies showed that the triglutamate form of 5-formyltetrahydrofolate both binds and is released much more slowly from the enzyme-glycine binary complex, compared with the triglutamate form of 5-methyltetrahydrofolate. The results also showed that only one rotamer of 5-formyltetrahydrofolate binds at the active site of serine hydroxymethyltransferase. The results are discussed in terms of the possible role of 5-formyltetrahydrofolate polyglutamates in regulation of one-carbon metabolism.

The conversion of serine and H4PteGlu1 to glycine and 5,10-CH2-H4PteGlu by serine hydroxymethyltransferase (SHMT) provides the cell with approximately 70% of the C1 units required for the synthesis of thymidine, purines, choline, and methionine. SHMT, which exists at the branch point of one carbon metabolism, binds all reduced folate coenzymes in the cell with high affinity, with the exception of 10-CHO-H4PteGlu. The determination of intracellular folate pools by Horne et al. (1989) revealed that over 50% of folate derivatives in rat liver are N5-substituted in the form of 5-CH3-H4PteGlu.

Both the high affinity of 5-CHO-H4PteGlu and 5-CHO-H4PteGlu for SHMT and their high concentrations in the cell suggest that these cofactors are capable of regulating the activity of this enzyme in vivo. In this paper, we investigate the interaction of the monoglutamate and triglutamate forms of the cofactors (5-CH3-H4PteGlu, 5-CHO-H4PteGlu) with SHMT. These studies show that there are significant differences in the mechanism of binding of the triglutamate forms of these two N5-reduced folates to SHMT.

EXPERIMENTAL PROCEDURES

Materials—Glycine, aminomethylphosphonate, DL-allothreonine, MgATP, NADPH, folic acid, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, alcohol dehydrogenase, and 2-mercaptoethanol were purchased from Sigma. (6R,S)-H4PteGlu and (6R,S)-5-CHO-H4PteGlu were purchased from Fluka and used without further purification. Pteroylpolyglutamates were purchased from Dr. B. Schirch's laboratory in Switzerland and reduced to the tetrahydro
form as described by Strong et al. (1987). The reduced pterinopropylglutamates were purified on DEAE-Sephadex according to the method described by Strong and Schirch (1989). (6S)-5-CHO-H$_4$PteGlu were prepared as described previously (Stover and Schirch, 1990). (6S)-5-CHO-H$_4$PteGlu was prepared by incubating a 1.0 mM solution of (6S)-H$_4$PteGlu with a 1.5 M excess of formaldehyde for 5 min at pH 7.0, followed by the addition of 0.05 mM excess of sodium borohydride. The solution was concentrated and purified as described previously (Strong and Schirch, 1990). All other chemicals were purchased from Fisher.

Rabbit liver C$_7$THF synthase, CH$_4$THF synthetase, and the mitochondrial and cytosolic isozymes of SHMT, and Escherichia coli THF synthase, were purified as described previously (Schirch and Peterson, 1980; Villar et al., 1985; Hopkins and Schirch, 1984; Schirch et al., 1985).

Determination of Enzyme and Folate Derivative Concentrations—The concentration of all enzymes was determined by their absorbance at 280 nm (Gavilanes et al., 1982; Schirch et al., 1985; Villar et al., 1985; Hopkins and Schirch, 1984).

Determination of Dissociation Constants for N$_5$-H$_4$PteGlu$_3$—Dissociation constants for 5-CHO-H$_4$PteGlu, and 5-CHO-H$_4$PteGlu$_3$, were calculated from Scatchard plots by titrating a cSHMT-glycine solution with N$_5$-H$_4$PteGlu (Scatchard, 1949). A solution of 50 mM KMES, 0.4 mM cSHMT, and 50 mM glycine was titrated with increasing concentrations of the coenzyme in a 10-cm pathlength cell. The increase in absorbance at 360 nm, resulting from the reduction of NADP$^+$ and the decrease in absorbance at 280 nm, resulting from the reduction of NADPH, were determined by the method of Dixon (Segal, 1975) using the apparent extinction coefficient of 40,000 M$^{-1}$ cm$^{-1}$ at pH 7.3 (Schirch, 1978). The concentration of 5-CHO-H$_4$PteGlu stock solutions was determined by incubating the cofactor with 5,10-CHO$^+$-THF synthetase in 1 mM MgATP, 50 mM KMES, pH 6.0, and measuring the increase in absorbance at 360 nm. The extinction coefficient of the product CH$_4$THF at 360 nm is 26,000 M$^{-1}$ cm$^{-1}$ (Blakeley and Benkovic, 1986). The concentrations of free and bound N$_5$-H$_4$PteGlu$_3$ were determined using the extinction coefficient of 32,000 M$^{-1}$ cm$^{-1}$ at 290 nm, pH 7.0 (Blakeley and Benkovic, 1986). All concentrations of folate coenzymes are recorded as the concentration of the physiological stereoisomer.

Inhibition of cSHMT with N$_5$-H$_4$PteGlu$_3$—Inhibition constants of allostereonine cleavage for the inhibitors 5-CHO-H$_4$PteGlu, and 5-CHO-H$_4$PteGlu$_3$, were determined by initial velocity measurements using a coupled assay system with alcohol dehydrogenase as described previously (Schirch et al., 1977). Assays were performed by determining the rate of decrease in absorbance at 340 nm after addition of 170 pmol of cSHMT to a 1-ml cuvette containing 150 mM L-allothreonine, 0.15 mM NADPH, 0.05 mg of alcohol dehydrogenase, and between 0 and 200 mM N$_5$-H$_4$PteGlu in 50 mM KMES, pH 7.0. The concentration of the inhibitor was increased until the rate of decrease in absorbance at 340 nm became independent of inhibitor concentration.

Inhibition constants for the SHMT catalyzed aldol cleavage of serine by the inhibitors 5-CHO-H$_4$PteGlu, and 5-CHO-H$_4$PteGlu$_3$, were determined by the method of Dixon (Segal, 1975) using a coupled assay system (Schirch and Quashnock, 1981). Reactions were initiated by the addition of 25 pmol of cSHMT to a solution containing 40 mM 1-serine, 0.5 mM NADP$^+$, H$_4$PteGlu$_3$ (5-40 mM), 0.5 mM C$^+$-THF synthase, and 5 mM 2-mercaptoethanol in 50 mM KMES, pH 7.0, and the rate of increase in absorbance at 340 nm determined.

Determination of initial rates of serine aldol cleavage by the cSHMT-Gly-5-CHO-H$_4$PteGlu$_3$ ternary complex were performed by preparing stock solutions of the cSHMT-Gly binary complex (100 pmol cSHMT, 500 mM glycine, and the cSHMT-Gly-5-CHO-H$_4$PteGlu$_3$ ternary complex (100 pmol cSHMT, 500 mM glycine, 150 mM H$_4$PteGlu$_3$) and diluting the enzyme complexes 4000-fold into a serum assay solution. Absorbance measurements at 502 nm showed that the stock solution was over 98% in the ternary complex. After dilution in the assay solution the rate of increase in absorbance at 502 nm was determined. The initial rate assay contained 10 mM 1-serine, 0.5 mM C$^+$-THF synthase, 0.5 mM NADP$^+$, 40 mM H$_4$PteGlu$_3$, and 5 mM 2-mercaptoethanol in 50 mM KMES, pH 7.0. Recovery of activity experiments were performed as described above with the exception that the ternary complex reaction was diluted 4000-fold into a serum assay, minus the substrate H$_4$PteGlu$_3$. After incubation of the diluted ternary complex in the serum assay solution for various time periods, the reaction was started by the addition of H$_4$PteGlu$_3$, and the rate determined as described above.

Rapid Reaction Kinetics—First order rate constants describing the rate of formation and breakdown of the cSHMT·Gly·N$_5$-H$_4$PteGlu$_3$ quinonoid complex at 502 nm were obtained using a stopped-flow spectrophotometer from Kinetic Instruments Inc. Rate constants for quinonoid formation, as shown in Table II, were obtained from measurements of the increase in absorbance at 502 nm with time after flowing a solution containing 10 pm H$_4$PteGlu$_3$, 50 mM glycine in 50 mM KMES against a solution of 200 pm (6R,S)-N$_5$-H$_4$PteGlu$_3$, 50 mM glycine in 50 mM KMES. Alternatively, cSHMT·N$_5$-H$_4$PteGlu$_3$ solutions were flowed against N$_5$-H$_4$PteGlu$_3$-Gly solutions. Absorbance versus time data were curve-fitted by single or double exponential algorithms as described previously (Shostak and Schirch, 1988). For the determination of rate of rotamer interconversion, a solution of 50 pm H$_4$PteGlu$_3$ and 50 mM glycine in 50 mM KMES, pH 7.0, was flowed against a solution of 4 pm (6R,S)-5-CHO-H$_4$PteGlu. The concentration of 50 mM glycine in the KMES buffer. All reactions performed in the stopped-flow instrument were done at 30°C.

The rate of dissociation of glycine from the ternary complex was determined by flowing a solution of 50 mM KMES, pH 7.0, 5 mM glycine, 50 pm N$_5$-H$_4$PteGlu$_3$, and 10 pm cSHMT against a solution containing 50 mM KMES, pH 7.0, 100 mM aminomethylphosphonate and 50 pm N$_5$-H$_4$PteGlu$_3$. The decrease in absorbance at 502 nm was recorded as a function of time. N$_5$-H$_4$PteGlu$_3$ dissociation rates were obtained in a similar manner using a 20-fold molar excess of folic acid as the competitive binding ligand.

RESULTS

Spectra of the cSHMT-Gly·N$_5$-H$_4$PteGlu$_3$, Ternary Complexes—SHMT contains pyridoxal-P bound as an internal aldimine at the active site (Scheme I, structure I). The absorption properties of this coenzyme have enabled it to be used as a reporter group in the determination of the mechanism of this enzyme (Schirch, 1982). Scheme I shows the current accepted mechanism for the observable intermediates. SHMT catalyzes an exchange reaction of groups located on the 2S-position of serine and glycine, which are shown as R and R' in Scheme I. When the enzyme is saturated with glycine, three complexes can be observed by their absorption spectra at 343, 425, and 495 nm. These correspond to structures III, IV, and V in Scheme I, respectively. The addition of H$_4$PteGlu$_3$ to the enzyme-glycine binary complex has been shown to shift the equilibrium toward structure V, which is referred to as the quinonoid complex (Schirch and Ropp, 1967). The ternary SHMT-Gly·H$_4$PteGlu$_3$ complex has an apparent extinction coefficient of 40,000 M$^{-1}$ cm$^{-1}$ (Strong and Schirch, 1989). However, because of the impurities present in H$_4$PteGlu$_3$ solutions, and their intense absorption below 400 nm, the effect of H$_4$PteGlu$_3$ on the distribution of the complexes between structures III, IV, and V is unknown.

Fig. 1A (curve 2) shows the absorption spectra of the SHMT-Gly complex at pH 7.0. Curve 3 shows the effect of adding a 1.5 M excess of 5-CHO-H$_4$PteGlu, with respect to the enzyme, which results in the formation of the ternary SHMT-Gly·5-CHO-H$_4$PteGlu$_3$ complex in greater than a 98% yield. Because of the purity, stability, and tight binding of the triglutamate, you can determine from the spectrum if the ternary complex is essentially all structure V (Scheme I). The retention of considerable absorbance at both 343 and 425 nm suggests that structures III and IV are present in significant concentrations in the ternary complex or whether the ternary complex is essentially all structure V.
the binding of glycine and the reduced folate compounds.

H₄PteGlu₃ ternary complex (curve 3). CD spectra show less interference from 5-CHO-H₄PteGlu₃, because it selects only for chromophores bound to the enzyme that exhibit optical activity. The addition of saturating amounts of 5-CHO-H₄PteGlu₃ to the cSHMT activity. The addition of saturating amounts of 5-CHO-H₄PteGlu₃ displays a 200-fold increase in affinity for glycine compared with the affinity with the monoglutamate SHMT-complex (Matthews et al., 1982). The effect of increasing glutamate chain length on the affinity of the SHMT-5-CHO-H₄PteGlu₃ complex for glycine has not been reported previously.

The effects of polyglutamate chain length on the affinity of both N⁵-H₄PteGlu₃₋₋ derivatives and glycine for rabbit cSHMT were determined by observing the increase in absorbance at 502 nm as a function of increasing ligand concentration (Table I). The dissociation constants for the triglutamate derivatives of 5-CHO-H₄PteGlu₃ and 5-CH₃-H₄PteGlu₃ are similar, with their dissociation constants being decreased about 30-fold compared with the monoglutamate derivatives. The dissociation constants for glycine are reduced 50- and 15-fold, respectively, for SHMT-5-CHO-H₄PteGlu₃ and 5-CH₃-H₄PteGlu₃. The similarities in the binding constants and effects of the polyglutamate chain structure for 5-CHO-H₄PteGlu₃ and 5-CH₃-H₄PteGlu₃, suggest that these compounds interact with the cSHMT-Gly complex by the same mechanism.

The effectiveness of 5-CHO-H₄PteGlu₃ and 5-CH₃-H₄PteGlu₃ in inhibiting the cleavage of serine with H₄PteGlu₃ as the variable substrate was also determined. Both compounds were found to be effective competitive inhibitors of H₄PteGlu₃ in the reaction. The Kᵢ values for the two triglutamate forms were found to be essentially identical to their Kᵢ values (Table I). The initial velocity patterns showed that the inhibition was immediate for both the 5-CH₃-H₄PteGlu₃ and 5-CHO-H₄PteGlu₃ derivatives. However, the 5-CHO-H₄PteGlu₃ appeared to display a slow time dependent increase in inhibition, a property which was not displayed by the 5-CH₃-H₄PteGlu₃ derivative. This slow increase in inhibition could not be quantitated for two reasons. First, to show competitive inhibition, less than saturating levels of H₄PteGlu₃ were used as substrate in the assay. The concentration of the coenzyme decreased during the assay resulting

**Table I**

<table>
<thead>
<tr>
<th>Ternary complex</th>
<th>Glycine</th>
<th>N⁵-Folate* (Kᵢ)'</th>
<th>μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>cSHMT-Gly-5-CHO-H₄PteGlu₃</td>
<td>1600 ± 1.0</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>cSHMT-Gly-5-CHO-H₄PteGlu₃</td>
<td>32 ± 5</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>cSHMT-Gly-5-CH₃-H₄PteGlu₃</td>
<td>520</td>
<td>11'</td>
<td>1.0</td>
</tr>
<tr>
<td>cSHMT-Gly-5-CH₃-H₄PteGlu₃</td>
<td>34 ± 5</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

* Determined from the absorbance of the ternary complex of 502 nm.
* Determined from initial velocity studies using H₄PteGlu₃ and serine as substrates.
* Values from Schirch and Ropp (1966).

To unsubstituted H₄PteGlu₃ does not significantly alter the affinity of the rabbit liver cSHMT-H₄PteGlu₃ binary complex for glycine (Strong and Schirch, 1989). However, as shown with pig liver SHMT, the 5-CH₃-H₄PteGlu₃ binary complex displays a 200-fold increase in affinity for glycine compared with the affinity with the monoglutamate SHMT-complex (Matthews et al., 1982). The dissociation constants for glycine are reduced 50- and 15-fold, respectively, for SHMT-5-CHO-H₄PteGlu₃ and 5-CH₃-H₄PteGlu₃. The similarities in the binding constants and effects of the polyglutamate chain for 5-CHO-H₄PteGlu₃, and 5-CH₃-H₄PteGlu₃, suggests that these compounds interact with the cSHMT-Gly complex by the same mechanism.

Dissociation Constants of N⁵-H₄PteGlu₃—Previously, the intense absorption of the quinonoid structure of the SHMT ternary complexes has been used to determine the Kᵢ values of glycine and unsubstituted and N⁵-substituted H₄PteGlu₃ (Schirch and Ropp, 1966; Matthews et al., 1982). These studies have shown that substitution at the N⁵ position with either a methyl or formyl group has little effect on the affinity of the reduced folate for the SHMT-Gly complex (Kᵢ = 10-15 μM). There was, however, a 5-fold synergism detected for the binding of glycine and the reduced folate compounds (Schirch and Ropp, 1966). The addition of glutamate residues...
in curves which stayed linear for only about 20 s. Second, 
H₄PteGlu undergoes oxidation nonenzymatically in the assay 
solution giving rise to oxidation products which absorb at 340 
nm. This results in a nonlinear base line which is difficult to 
accurately subtract from the reaction curve over a period of 1 
to 2 min.

In order to better quantitate this apparent slow time-
dependent inhibition, a series of enzyme activity recovery 
experiments were performed (Fig. 2). A concentrated solution 
of cSHMT (100 μM), glycine (500 μM), and 5-CHO-H₄PteGlu₃ 
(150 μM) was prepared and calculated to be greater than 98% 
as the ternary complex as determined from its absorption at 
502 nm. This solution was diluted 4000-fold into a reaction 
mixture containing saturating concentrations of serine and 
H₄PteGlu. The addition and mixing took 4 s. The increase 
in absorbance was linear, but exhibited only 52% of the 
activity of a control in which 5-CHO-H₄PteGlu₃ had been 
ominixed from the concentrated SHMT-Gly solution (Fig. 2A). 
In a second experiment, the concentrated solution of the 
SHMT-Gly-5-CHO-H₄PteGlu₃ complex was diluted in an 
assay solution which did not contain H₄PteGlu as a substrate. 

The control was added to initiate the catalytic reaction 
after several seconds. The results of this experiment are 
shown in Fig. 1B. If the folate substrate was added 8 s after 
the dilution of the ternary complex the rate was 66% of the 
control, respectively. When these experiments were repeated 
with either 5-CHO-PteGlu₃ or 5-CH₃-H₄PteGlu, no inhibition 
of activity was observed even in the absence of any 
incubation time after the dilution of the ternary complexes.

The results in the previous paragraph suggest that both 5-
CHO-H₄PteGlu₃ and 5-CH₃-H₄PteGlu,₃ are classical competitive inhibitors of cSHMT as shown by reaction (1).

However, the results suggest that 5-CHO-H₄PteGlu₃ inhibition 
may fit a two step model with an initial rapid onset of 
hibition, followed by a slower time-dependent increase in 
hibition as shown by reaction (2). The data shown in Fig. 2 
suggest that the equilibrium between E-I and E-1* is about 1. 
This would explain why only 52% of the activity of the 
ternary complex is recovered rapidly after dilution and the 
remaining 48% activity is recovered only after a several minute 
incubation.

\[
E + I \overset{\text{fast}}{\longrightarrow} E \cdot I \\
E + I \overset{\text{slow}}{\longrightarrow} E \cdot I^* \quad (1)
\]

The competitive inhibition of serine cleavage by both 5-
CHO- and 5-CH₃-H₄PteGlu₃ shows that the inhibitors can 
bind to the free enzyme. Previously, inhibition studies of 
allothreonine cleavage by 5-CH₃-H₄PteGlu, showed that 5-
CH₃-H₄PteGlu, could also bind to the enzyme-Gly complex (structure V, Scheme 1) (Schirch et al., 1977). These 
inhibition studies of allothreonine cleavage, which do not require a 
folate substrate, were repeated with N⁵-H₄PteGlu, and N⁵-
H₄PteGlu,₃. As shown in Fig. 3, increasing concentrations of 
5-CHO-H₄PteGlu, and 5-CH₃-H₄PteGlu, result in inhibition 
with a limiting value for \( k_{\text{on}} \) of 0.4 s⁻¹. This value was also 
found previously for the limiting rate of allothreonine cleavage 
in the presence of 5-CH₃-H₄PteGlu, (Schirch et al., 1977). In 
these previous studies the limiting rate at a saturating 
concentration of 5-CH₃-H₄PteGlu, was shown to be the rate at 
which glycine dissociates from the ternary complex. Matthews 
et al. (1982) found similar results using pig liver SHMT and 
went on to show that the limiting rate of allothreonine cleavage 
in the presence of high concentrations of all polyglutamate 
forms of 5-CH₃-H₄PteGlu, was independent of the number of 
glutamate residues. The conclusion drawn from these results 
was that the increased affinity of the polyglutamate forms 
must be a function of the \( k_{\text{on}} \) rate for 5-CH₃-H₄PteGlu, (Matthews et al., 1982).

When 5-CHO-H₄PteGlu₃ was used as an inhibitor of allo-
theornine, a different result was observed as shown in Fig. 3. 
The final \( k_{\text{off}} \) at saturating inhibitor concentration was 0.05 
\( s^{-1} \) as opposed to the value of 0.4 \( s^{-1} \) observed with 5-CHO-
H₄PteGlu,₃. This shows that the number of glutamate residues 
on this form of folate does affect the dissociation rate of 
glycine from the ternary complex.

Rapid Reaction Kinetics Associated with Quinonoid Forma-
tion and Breakdown—A more direct approach to defining the 
factors affecting the binding of inhibitors to an enzyme is to 
examine the kinetic constants associated with formation and 
breakdown of the E-I complex using rapid reaction tech-
The amplitudes of the two phases were similar.

Partial double bond character of the C"-N5 formamide bond, the
experiments measuring the apparent rate of quinonoid forma-
tion were performed with at least a 10-fold excess N5-H,PteGlu, over SHMT. With each form of the enzyme studied, the apparent rate of quinonoid formation and breakdown was about an order of magnitude slower with 5-CHO-H,PteGlu, than with 5-CH3-H,PteGlu, (Table II). The observation was not pursued further in this study. These results suggest that the limiting k values for glycine and N5-H,PteGlu, are the result of interconversion of enzyme complexes rather than dissociation of the ligand from the enzyme.

There were two notable features associated with quinonoid formation upon the addition of the two additional glutamate residues of 5-CH3-H,PteGlu, and 5-CHO-H,PteGlu, First, the absorbances at 502 nm versus time traces for both cofactors were biphasic, with both phases being of approximately equal amplitude (Table II). The biphasic character remained even in the presence of excess cofactor with respect to enzyme. Second, both the rate of quinonoid formation and breakdown was decreased by less than 2-fold when 5-CH3-
H,PteGlu, was replaced with the triglutamate, the rate of quinonoid formation and breakdown with 5-CHO-H,PteGlu, was almost an order of magnitude slower compared with the monoglutamate derivative. The rates of formation and dissociation of the SHMT-Gly-N5-H,PteGlu, quinonoid complex were also determined for rabbit liver mitochondrial SHMT, E. coli SHMT, and a mutant form of the E. coli enzyme H228D.

Evidence That Only One Rotamer of 5-CHO-H,PteGlu, Binds to the SHMT-Gly Complex—The solution structure of 5-CHO-H,PteGlu, has been determined by both 13C and 1H NMR spectroscopy and found to be an equilibrium mixture of two slowly interconverting forms (Feeney et al., 1980). These two forms were attributed to the partial double bond character of the C15-N3 formamide bond, creating two rotamers present at a ratio of 2.35 to 1.0 at 25 °C. The N5 formyl group is bent out of the plane of the pteridine ring and has been demonstrated to lie on the same side of the ring as the C6 hydrogen (Poe and Benkovic, 1980). The more abundant rotamer has the formyl proton oriented in the same plane as the keto group of C4, which is deshielded in the 1H NMR spectra. These spectra also display deshielding of the C2 proton due to its proximity to the formyl carbonyl group. The tetrahydropyrazine ring exists in a half chair conformation with the C6 proton in the equatorial position (Poe and Benkovic, 1980).

The relatively slow rate of formation and high apparent extinction coefficient of the quinonoid species permits sensitive and accurate determinations of the rate of formation and breakdown of this complex using stopped-flow spectroscopy. During these experiments, it was observed that quinonoid formation was biphasic when 5-CHO-H,PteGlu, was flowed against an excess of the SHMT-Gly binary complex. The slow phase of quinonoid formation with 5-CHO-H,PteGlu, had a half-life of approximately 2 s⁻¹ at 40 °C, which is similar to the rate of rotamer interconversion demonstrated by Feeney et al. (1980).

Performing the identical experiment, described in the previous paragraph, but with a 10-fold excess of 5-CHO-
H,PteGlu, over the SHMT-Gly binary complex results in a single exponential reaction with a first order rate constant equal to that of the previously described rapid phase. This suggests that the slow phase is not a property of the enzyme. If the rate of the slow phase represents rotamer interconversion, then this rate should be independent of the source of SHMT. Unfortunately, mSHMT and E. coli SHMT could not be used in this study, since the rate of quinonoid formation is less than the rate of putative rotamer interconversion (Table II). We were, however, able to use a mutant form of the E. coli enzyme, H228D SHMT, which does display a rapid formation of the quinonoid complex with excess 5-CHO-
H,PteGlu, (Table II). When quinonoid formation was determined with excess SHMT-Gly complex, a biphasic reaction was observed with a slow phase having the same rate as observed with cSHMT.

Fig. 4 shows the rate of SHMT-Gly-5-CHO-H,PteGlu, quinonoid complex formation at 502 nm with a 10-fold excess of E. coli H228D SHMT-Gly binary complex over 5-CHO-
H,PteGlu, using a stopped-flow apparatus. The slower rate in this biphasic curve is identical for both cytosolic and H228D E. coli SHMT over the temperature range 25–40 °C, suggesting that it represents the first order rate of rotamer interconversion. Under the same conditions of excess enzyme, neither 5-CH3-H,PteGlu, nor H,PteGlu, exhibit biphasic kinetics upon forming the quinonoid complex with either the cytosolic or H228D SHMT. These results suggest that SHMT is capable of forming the quinonoid complex with only a single

<table>
<thead>
<tr>
<th>Enzyme-Gly complex</th>
<th>No. of Glu residues</th>
<th>Formation of ternary complex</th>
<th>Breakdown of ternary complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5-CHO-H,PteGlu (kₚ)</td>
<td>5-CHO-H,PteGlu (kᵣ)</td>
</tr>
<tr>
<td>cSHMT-Gly</td>
<td>1</td>
<td>0.50</td>
<td>4.80</td>
</tr>
<tr>
<td>cSHMT-Gly</td>
<td>3</td>
<td>0.05; 0.19</td>
<td>2.6; 6.70</td>
</tr>
<tr>
<td>mSHMT-Gly</td>
<td>1</td>
<td>0.15</td>
<td>3.50</td>
</tr>
<tr>
<td>E. coli SHMT</td>
<td>1</td>
<td>0.05</td>
<td>3.10</td>
</tr>
<tr>
<td>H228D-Gly</td>
<td>1</td>
<td>2.20; 12.70</td>
<td>0.36</td>
</tr>
</tbody>
</table>

* Determined from the breakdown of the SHMT-Gly-N5-H,PteGlu, quinonoid complex with folic acid as the competing ligand.
* The rate of formation of the quinonoid complex was biphasic. The amplitudes of the two phases were similar.

**FIG. 4. Rate of formation of E. coli H228D SHMT-Gly-5-CHO-H,PteGlu, quinonoid complex with limiting 5-CHO-H,PteGlu, by stopped-flow spectrophotometry.** A solution of 50 μM H228D SHMT in 50 mM glycine was flowed against 4 μM 5-CHO-
H,PteGlu, and monitored at 502 nm. The solid line is a curve fit for a two exponential reaction. The inset shows the change in the slower rate as a function of temperature, plotted by the method of Arrhenius.
rotamer of 5-CHO-H4PteGlu. The relative amplitude of the fast and slow phases for both cSHMT and H228D SHMT are 2.5 to 1 at 30 °C, consistent with SHMT binding the more populated rotamer where the C1 and C4 carbonyl oxygens are maximally separated.

Previous 'H NMR studies of 5-CHO-H4PteGlu demonstrated that the relative distribution of the two rotamer populations was temperature dependent and the associated equilibrium thermodynamic parameters were determined (Feeney et al., 1980). The changes we observed in relative amplitude of the fast and slow phases with both enzymes as a function of temperature (2.5:1 at 30 °C and 3.3:1 at 40 °C) is consistent with the change in the relative populations of the two rotamers (2.35:1 at 25 °C and 4.66:1 at 77 °C) determined by NMR (Feeney et al., 1980).

The rate of rotamer interconversion as a function of temperature was determined and plotted by the method of Arrhenius (Fig. 4, inset). The data show the slow phase of quinonoid ternary complex formation, associated with rotamer interconversion, over the temperature range 10-40 °C for the H228D enzyme. These values were identical for the cytosolic enzyme over the temperature range 25-40 °C. Below 25 °C, quinonoid formation became rate limiting for cSHMT. From this plot, the Ea for rotamer conversion from the less populated to the more populated state was determined to be 24 kcal/mol.

**DISCUSSION**

The conversion of 5,10-CH2-H4PteGlu4 to 5-CHO-H4PteGlu4 by SHMT is an unusual example of enzymatic regulation whereby an enzyme, displaying a second unrelated enzymatic activity, produces an effective inhibitor of its principal physiological reaction (Stover and Schirch, 1990). The high concentrations of 5-CHO-H4PteGlu4 found in certain cells, particularly in seedlings, suggests that this compound may play a major role in regulating SHMT activity. In order to better define the role of this compound in vivo, we have further investigated the properties of the SHMT:Gly-5-CHO-H4PteGlu4 ternary complex and the related SHMT:Gly-5-CH3-H4PteGlu4 ternary complex. The results reported in this study show that the triglutamate forms of 5-CHO- and 5-CH3-H4PteGlu4 exhibit similar Ks and K values for cSHMT. The similarity of the constants suggests that these two inhibitors are of the classical competitive type and inhibit SHMT by rapidly forming an E-I complex as shown in Reaction 1. However, closer inspection of the kinetics of inhibitor binding and dissociation clearly show that the polyglutamate forms of 5-CHO-H4PteGlu4 inhibit by a mechanism that is different than the inhibition by 5-CHO- and 5-CH3-H4PteGlu4.

**Scheme 2**

FAST

\[
\text{cSHMT} \cdot \text{Gly} + 5\text{-CHO-H}_4\text{PteGlu}_n \xrightarrow{k_3} \text{cSHMT} \cdot 5\text{-CHO-H}_4\text{PteGlu}_n
\]

(Geminal diamine - External aldimine)

SLOW

\[
\text{cSHMT} \cdot \Theta + 5\text{-CHO-H}_4\text{PteGlu}_n \xrightarrow{k_5} \text{cSHMT} \cdot \Theta + \text{5-CHO-H}_4\text{PteGlu}_n
\]

(Quinonoid)

Reaction 1 cannot explain the results on the rate of formation and breakdown of the quinonoid complex and the inhibition of serine catalysis by 5-CHO-H4PteGlu4.

We conclude that forming the SHMT:Gly-5-CHO-H4PteGlu4 ternary complex occurs by a different mechanism than forming the ternary complex with 5-CH3-H4PteGlu4. We also conclude that the polyglutamate chain of 5-CHO-H4PteGlu4 plays some role in the binding process, which does not occur with the 5-CH3-H4PteGlu4 cofactor. One model, which explains most of the results for forming the SHMT:Gly-5-CHO-H4PteGlu4 is shown in Scheme 2. In this mechanism, 5-CHO-H4PteGlu4 binds relatively rapid to the cSHMT:Gly complex forming the geminal diamine and external aldimine complexes (structures III and IV in Scheme 1). This is followed by a much slower conversion to the quinonoid complex absorbing at 502 nm (structure V in Scheme 1). Furthermore, considering the slower rate of formation, the ratio of k6/k5 appears to be near 1. This would mean that in the ternary complex, about half of the complex resides in the quinonoid form which is in slow equilibrium with the other half of the complex. This model is consistent with several observations. First, the spectral properties of the ternary complex recorded in Fig. 1 suggest that significant concentrations of the geminal diamine and external aldimine complexes are present. Although we cannot determine quantitatively the exact relative concentrations of structures III, IV, and V, it is possible to estimate that only about 50% of the ternary complex is present as structure V. Second, the model in Scheme 2 explains why when the ternary complex is diluted in an assay solution, only 52% of the activity is immediately recovered and the remaining 48% is recovered in a time frame which is consistent with the value of k6 (Fig. 2 and Table II). Third, the rate of formation of the quinonoid complex as determined by the increase in absorbance at 502 nm is slow (and biphasic) with a half-life for the slow-phase of 14/s (k6 + k5). However, the rate of inhibition of serine cleavage by 5-CHO-H4PteGlu4 appears to show no lag phase. This is explained by the fact that most of the inhibition occurs by the rapid formation of the ternary complex in the geminal diamine and external aldimine complexes and the rapid phase of forming the quinonoid complex (t5 = 3 s) (Table II). Since the initiation of the inhibition assay takes about 4 s, this means that about 70% of the ternary complex has already been formed when absorbance versus time data is recorded. The serine assay does not stay linear long enough to clearly determine that the remaining 30% inhibition occurs with a t5 of 14 s. Fourth, the model in Scheme 2 predicts that the value of Ks should be similar to the value of K. This is because the values of k6 and k5 are nearly equal. We cannot accurately determine the value of k5 because the rate of formation of the
quinoioid complex is biphasic. The value of $K^{*}$ should be similar to the value of $K_{d}$ determined by titrating the SHMT-Gly complex with 5-CHO-H$_4$PteGlu. The value of $K_{a}$ is the inhibition constant determined from the initial velocity studies in the cleavage of serine. The values for both $K_{d}$ and $K^{*}$ were determined to be 0.2 μM (Table I). However, they both have errors of ±0.1 μM.

The model does not explain why the rate of formation of the quinoioid complex is biphasic with 5-CHO-H$_4$PteGlu. Nor is it clear why in the recovery of activity when the ternary complex is diluted into an assay mixture that the rate remains linear with time for at least 15 s (Fig. 3). One would predict that the rate should increase due to the conversion of the quinoioid complex to free enzyme ($t_{1/2}$ = 17 s). One factor which may contribute to an explanation of why the rate does not increase is that in the assay one of the intermediates is the SHMT-Gly complex which has a high affinity for the diluted 5-CHO-H$_4$PteGlu (Fig. 2).

Typical slow binding inhibitors are substrate intermediate analogs which have a higher affinity for a catalytic intermediate state of the enzyme than the ground state enzyme. H$_4$PteGlu, 5-CH$_3$-H$_4$PteGlu, and 5-CHO-H$_4$PteGlu all exhibit rapid binding to the SHMT-Gly complex. This is a property consistent with competitive ground state inhibitors. However, the three coenzymes differ in their rates in forming quinonoid complexes (Scheme 3). Compared to $k_{cat}$ in the conversion of serine to glycine (8.4 s$^{-1}$), formation of the H$_4$PteGlu quinoioid complex is rapid (875 s$^{-1}$) (Schirch, 1975), whereas formation of the 5-CH$_3$-H$_4$PteGlu quinoioid complex is the same order of magnitude (4.8 s$^{-1}$). Only formation of the 5-CHO-H$_4$PteGlu quinoioid complex exhibits slow formation (0.05 s$^{-1}$). The slow apparent rate of inhibitor binding defines 5-CHO-H$_4$PteGlu as an intermediate state inhibitor (Frieden et al., 1980). Based on these criteria, the 5-CHO-H$_4$PteGlu quinoioid complex best mimics the catalytic intermediate, suggesting that the cSHMT-Gly-5-CHO-H$_4$PteGlu quinoioid complex is (structure B, Scheme 4) a true intermediate-state analog of the catalytically competent cSHMT-Ser-H$_4$PteGlu, ternary complex (structure A, Scheme 4). The slow apparent rate of binding of intermediate-state inhibitors is typically understood in terms of considerable conformational changes required to adjust the enzyme from the ground state to the intermediate state (Morrison and Walsh, 1987). On the other hand, the rate at which the folate dissociates from the quinoioid complex is reflective of the degree at which the intermediate state is stabilized. Only the 5-CHO-H$_4$PteGlu quinoioid exhibited $k_{i}$ values considerably less than $k_{cat}$ values.

In addition, there appears to be communication between the one-carbon and polyglutamate binding sites on the enzyme. This communication is suggested by the dramatic changes in the rate of quinoioid formation and breakdown between ternary complexes formed with 5-CHO-H$_4$PteGlu, and 5-CHO-H$_4$PteGlu (Table II). By comparison, rate constants associated in quinoioid formation and breakdown with 5-CH$_3$-H$_4$PteGlu and 5-CH$_3$-H$_4$PteGlu are very similar. The differences in the rates of quinoioid formation and breakdown for 5-CHO-H$_4$PteGlu and 5-CHO-H$_4$PteGlu complexes suggest that the formyl group binding at the one-carbon site on the enzyme confers changes in the conformation of the polyglutamate binding site. Therefore, the structural relationship between the enzyme one-carbon site and the polyglutamate binding site may be different between the ground-state enzyme and the intermediate-state enzyme.

Finally, as would be expected for an intermediate-state-like complex, the cSHMT-Gly-5-CHO-H$_4$PteGlu quinoioid complex shows specificity for a particular rotamer of 5-CHO-H$_4$PteGlu. The high energy of activation (24 kcal/mol) determined for rotamer interconversion is due to the partial double bond character of the N“-C“ formyl bond, and this value is similar to that found for other compounds exhibiting rotamers (Lee and Querijero, 1985). If the 5-CHO-H$_4$PteGlu quinoioid species does closely mimic the SHMT-Ser-H$_4$PteGlu, catalytic complex, then we can make predictions about the relative displacement of the serine hydroxyl group relative to the C“ carbonyl oxygen of H$_4$PteGlu (Scheme 4). A single rotamer state of 5-CHO-H$_4$PteGlu has also been observed for the cofactor when bound to dihydrofolate reductase (Birdsall et al., 1981). Like SHMT, this enzyme is specific for the rotamer with the C“ and C“ carbonyl oxygens maximally separated.

One of the unexplained observations about the mechanism
Serine Hydroxymethyltransferase

of cSHMT is that N\textsuperscript{5}-hydroxymethyl-H\textsubscript{4}PteGlu is not a substrate for the enzyme (Schirch and Chen, 1973). The observation that cSHMT binds only one of the rotamers of 5-CHO-H\textsubscript{4}PteGlu may have the hydroxyl group pointing toward the C\textsuperscript{4} carbonyl where it can form a hydrogen bond. If this stabilizes this structure, then it would correspond to the rotamer of 5-CHO-H\textsubscript{4}PteGlu which does not bind to the active site of cSHMT.

The product of serine catabolism, 5,10-CH\textsubscript{2}-H\textsubscript{4}PteGlu, is the principal entry point of one-carbon units into the one-carbon pool. In vivo, there are four enzymes capable of converting an amino acid substrate and tetrahydrofolate to 5,10-CH\textsubscript{2}-H\textsubscript{4}PteGlu, all of which bind 5-CHO-H\textsubscript{4}PteGlu with high affinity. In the mitochondria, dimethylglycine dehydrogenase and sarcosine dehydrogenase have been demonstrated to bind both 5-CH\textsubscript{3}-H\textsubscript{4}PteGlu and 5-CHO-H\textsubscript{4}PteGlu with \(K_d\) values in the 1.5 \(\mu\)M range (Wittwer and Wagner, 1980). Bacterial sarcosine oxidase also displays high affinity for 5-CHO-H\textsubscript{4}PteGlu (Kvalnes-Krick and Jorns, 1987). The high concentration of these \(N^5\)-substituted folates in the one-carbon pool suggests that these compounds are capable of regulating the entry of one-carbon units into the folate pool. Therefore, regulation of the supply of these folate derivatives may be critical in maintaining cellular homeostasis.

REFERENCES


