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In Vitro Conversion of Formate to Serine: Effect of Tetrahydropteroylpolyglutamates and Serine Hydroxymethyltransferase on the Rate of 10-Formyltetrahydrofolate Synthetase†

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ABSTRACT: Serine hydroxymethyltransferase and C_1-tetrahydrofolate synthase catalyze four reactions which convert formate and glycine to serine. The one-carbon carrier in these reactions if tetrahydropteroylglutamate which is regenerated in the coupled reaction and thus can be used in catalytic concentrations with respect to serine synthesis. The rate of serine synthesis is followed by the oxidation of NADPH during reduction of the intermediate 5,10-methenyltetrahydropteroylglutamate. K_m values for the substrates of cytosolic serine hydroxymethyltransferase and the 10-formyltetrahydrofolate synthetase activity of the trifunctional enzyme C_1-tetrahydrofolate synthase were determined. This included the values for the polyglutamate forms of tetrahydropteroylglutamate containing from one to six glutamate residues. The results suggest that the synthetase active site binds the polyglutamate forms of the coenzyme synergistically with respect to formate and ATP. Using saturating levels of all substrates, the k_{cat} values for the serine hydroxymethyltransferase and 10-formyltetrahydrofolate synthetase activities were also determined. The synthetase reaction is the rate-determining step in the conversion of formate to serine. The effect of glutamate chain length and the concentration of serine hydroxymethyltransferase were studied with respect to the rate of serine formation. Tetrahydropteroylmonoglutamate gave slower than expected rates which is attributed to its inhibition of the reduction of the intermediate 5,10-methenyltetrahydropteroylglutamate. This inhibition was not a factor with the di- through hexaglutamate forms of the coenzyme. The addition of an excess of serine hydroxymethyltransferase was predicted to lower the rate of the formation of serine by lowering the concentration of free coenzyme in the assay. However, activation of the rate was observed which was at least 2-fold greater than the predicted rate. This increase in predicted rate appears to result from an interaction between C_1-tetrahydrofolate synthase and serine hydroxymethyltransferase. The in vivo concentrations of serine hydroxymethyltransferase and C_1-tetrahydrofolate synthase in rabbit liver were determined.

The stereospecific incorporation of formate into the C_3 carbon of L-serine has been demonstrated to occur under physiological conditions (Biellmann & Schuber, 1970). The enzymes involved in this conversion are the multifunctional enzyme C_1-THF synthase[1] and SHMT (EC 2.1.2.1). C_1-THF synthase possesses 10-CHO-THF synthetase (EC 6.3.4.3), 5,10-CH^+-THF cyclohydrolase (EC 3.5.4.9), and 5,10-CH_2-THF dehydrogenase (EC 1.5.1.5) activities. These reactions are shown in Figure 1. During the conversion of formate and glycine to serine, the coenzyme $H_4PteGlu_n$, which mediates

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[1] Abbreviations: C_1-THF synthase, C_1-tetrahydrofolate synthase; 10-CHO-THF synthetase, 10-formyltetrahydrofolate synthetase; 5,10-CH^+-THF cyclohydrolase, 5,10-methenyltetrahydrofolate cyclohydrolase; 5,10-CH_2-THF dehydrogenase, 5,10-methylenetetrahydrofolate dehydrogenase; $H_4PteGlu_n$, tetrahydropteroylglutamate containing *n* glutamyl residues; SHMT, serine hydroxymethyltransferase; c or m preceding SHMT, either the cytosolic or mitochondrial isoenzyme forms; red-cSHMT, cSHMT in which the internal ε-lysine–pyridoxal phosphate Schiff's base has been reduced with sodium cyanoborohydride; AMPPNP, 5′-adenylyl imidodiphosphate; AMPPCP, 5′-adenylyl methylene diphosphate.

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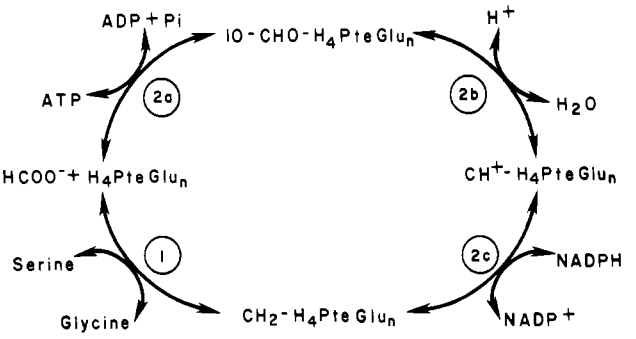

FIGURE 1: Reactions involved in the coupled cyclic system which converts formate to L-serine. The enzymes involved are (1) serine hydroxymethyltransferase, (2a) 10-formyltetrahydrolate synthetase, (2b) 5,10-methenyltetrahydrofolate cyclohydrolase, and (2c) 5,10-methylenetetrahydrofolate dehydrogenase. Reactions 2a, 2b, and 2c are catalyzed by the enzyme C_1-tetrahydrofolate synthase.

the transfer of the one-carbon group, is regenerated by the action of SHMT for use by 10-CHO-THF synthetase in subsequent catalytic cycles.

The ability of $H_4PteGlu_n$ to function in the cycle at catalytic levels permits us to determine the kinetic properties of enzymes involved in one-carbon metabolism under conditions which more closely reflect the in vivo environment. In the cell, $H_4PteGlu_n$ exists as polyglutamate forms, where Glu_n represents four to eight glutamate residues linked by amide bonds through the γ-carboxyl group (McGuire & Coward, 1984). These polyglutamate derivatives have a high affinity for most folate-requiring enzymes, exhibiting K_m values in the 0.1–1 μM range (MacKenzie & Baugh, 1980; Matthews et al., 1982; Rabinowitz 1983; Ross et al., 1984; Lu et al., 1984; Pacquin et al., 1985; Strong et al., 1989; McGuire & Coward, 1984). Increasing evidence also suggests that the concentration of enzymes which utilize $H_4PteGlu_n$ in the cell is similar to the concentration of this coenzyme (Schirch & Strong, 1989). These two observations, the high affinity of the coenzyme for folate-dependent enzymes and the near-equimolar coenzyme–enzyme concentrations, suggest that in vivo most of the intracellular $H_4PteGlu_n$ is enzyme bound and that the concentration of free coenzyme is very low. Virtually all in vitro studies with enzymes in one-carbon metabolism have been performed with the concentration of $H_4PteGlu_n$ in large excess over the concentration of the enzyme. The K_m and k_{cat} values from these studies are being used to make predictions about the control and flux of one-carbon groups through metabolic pathways in vivo (Wasserman et al., 1983; Rebandel et al., 1986; Kalman, 1986; Green et al., 1988). It is important that these predicted rates of flux in one-carbon metabolic pathways be determined under conditions where the free coenzyme concentration more closely reflects in vivo conditions.

Using the reactions in the metabolic cycle shown in Figure 1, we have determined kinetic constants for the SHMT and 10-CHO-THF synthetase activities of the cycle for polyglutamate chain lengths from one to six. As previously shown, the rate-determining step in the conversion of formate to serine is the 10-CHO-THF synthetase reaction. Using the kinetic parameters determined for the 10-CHO-THF synthetase, we compare the predicted and observed properties of the cycle under conditions where $H_4PteGlu_n$ is similar to the concentration of total enzyme active sites present in the cycle.

Experimental Procedures

Materials

The cytosolic and mitochondrial isoenzymes of SHMT and C_1-THF synthase were purified from fresh frozen rabbit livers as previously described (Schirch & Peterson, 1980; Villar et al., 1985). *Escherichia coli* SHMT was purified as described by Schirch et al. (1985). SHMT preparations, at enzyme concentrations up to 50 μM, showed no 10-CHO-THF synthetase activity. The reduced form of cSHMT (red-cSHMT) was formed by reacting a solution of the enzyme in 10 mM potassium phosphate, pH 7.0, with a 1.2-fold molar excess of sodium cyanoborohydride, followed by Sephadex G-50 chromatography with 50 mM potassium *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonate, pH 7.0, containing 30 mM ammonium sulfate and 10 mM 2-mercaptoethanol as the eluting solvent. The dehydrogenase–cyclohydrolase fragment of C_1-THF synthase was purified from a tryptic digest of the enzyme in the presence of $NADP^+$ (Villar et al., 1985) and had a specific activity of 14.8 μmol min^{-1} (mg of dehydrogenase monomer)$^{-1}$.

Glycine, MgATP, Na_2ADP, $MgCl_2$, $NADP^+$, NADPH, L-serine, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma and used without further purification. The tetralithium salts of 5′-adenylyl imidodiphosphate (AMPPNP) and 5′-adenylyl methylenediphosphate (AMPPCP) were purchased from Boehringer Mannheim Biochemicals. Stock ammonium formate solution was prepared by neutralizing formic acid with the addition of a concentrated solution of ammonium hydroxide. Pteroylpolyglutamates were purchased from Dr. B. Schircks Labs in Switzerland and reduced to the tetrahydro form as described by Strong et al. (1987). The reduced pteroylpolyglutamates were purified on DEAE-Sephadex according to the method described by Matthews et al. (1982), except that a linear gradient of 0–0.8 M potassium chloride containing 0.2 M 2-mercaptoethanol was used to elute the reduced coenzyme. $H_4PteGlu_n$ eluted at a salt concentration between 0.3 M and 0.4 M. At the concentrations of the coenzyme used in these studies, the concentration of added potassium chloride in a typical assay amounted to less than 5 mM.

All spectrophotometric titrations and enzymatic assays were performed on a Cary 210 spectrophotometer at a temperature of 24 °C.

Methods

Determination of Enzyme and $H_4PteGlu_n$ Concentrations. The concentrations of all forms of SHMT, except red-cSHMT, were determined from the amount of bound pyridoxal phosphate released from the enzyme upon the addition of 0.1 N NaOH. An extinction coefficient of 6550 M^{-1} cm^{-1} at 388 nm was used to quantitate the released pyridoxal phosphate (Harruff & Jenkins, 1976). The concentration of red-cSHMT (mg/mL) was determined by dividing the absorbance at 280 nm of a 1-mL solution of the enzyme by 0.72 (Gavilanes et al., 1982). The concentration of C_1-THF synthase (mg/mL) was determined by dividing the absorbance at 280 nm of a 1-mL solution of the enzyme by 0.62 (Villar et al., 1985).

The concentrations of stock solutions of $H_4PteGlu_n$ were determined by a coupled enzymatic assay using SHMT and 5,10-CH_2-THF dehydrogenase and measuring the increase in the absorbance at 340 nm, at pH 7.3, attributed to the reduction of $NADP^+$. The combined extinction coefficient for NADPH and 5,10-CH^+-THF produced in this assay at 340 nm is 7200 M^{-1} cm^{-1} at pH 7.3 (Schirch, 1978).

Determination of C_1-THF Synthase and SHMT Concentrations in Fresh Rabbit Liver. A 20-g portion of rabbit liver from two newly sacrificed New Zealand adult white rabbits was homogenized in a Waring blender in 100 mL of 30 mM dibasic potassium phosphate buffer containing 10 mM ammonium sulfate, 10 mM 2-mercaptoethanol, 0.1 mM pyridoxal

phosphate, 1 mM EDTA, 1 mM PMSF, and 25% glycerol. The homogenate was centrifuged at 24460*g* for 30 min at 4 °C, and the supernatant was placed on ice. The pellet was reextracted with 40 mL of homogenizing buffer and centrifuged as described above. The two supernatants were combined. The pellet from the second centrifugation was once again resuspended in 40 mL of homogenation buffer and then heated in a water bath to 61 °C for 30 s. Upon completion of this heat step, the homogenate was immediately placed on ice, cooled to 25 °C, and centrifuged as described earlier. The supernatant was then transferred to a new vessel and placed on ice. A portion of these two supernatants was assayed for SHMT and 5,10-CH_2-THF dehydrogenase activities, using the standard assays described previously (Schirch et al., 1977; Schirch, 1978). The concentrations of SHMT and C_1-THF synthase (reported as the micromolar concentration of H_4PteGlu binding sites) were calculated from the observed activities and the known turnover numbers for the purified proteins. The intracellular volume per gram of rabbit liver was assumed to be 0.7 mL/g of tissue.

Kinetic Constants. Kinetic parameters for cSHMT were determined by using the coupled enzyme cycle essentially as described by Strong et al. (1987), except for the following changes. The standard buffer used in these studies was 50 mM potassium *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonate, pH 7.0, containing 30 mM ammonium sulfate and 10 mM 2-mercaptoethanol.

For the determination of K_m values for glycine, a typical assay contained the following in 1 mL: 0.5 mM MgATP, 25 mM ammonium formate, 0.2 mM NADPH, (10–50)K_m amounts of $H_4PteGlu_n$, 3.1×10^{-7} M C_1-THF synthase, and 1.06×10^{-8} M cSHMT. Mixing of the above assay components results in the rapid formation of 5,10-CH_2-$H_4PteGlu_n$. The assay was then initiated by the addition of increasing concentrations of glycine (0.5–50 mM), and the decrease in the absorbance at 340 nm was measured. K_m and apparent k_{cat} values were determined from the abscissa and ordinate intercepts of double-reciprocal plots of the data.

For the determination of K_m values for L-serine, a typical assay contained the following in 1 mL of standard buffer: 0.2 mM Na_2ADP, 0.3 mM $MgCl_2$, 50 mM potassium phosphate, 0.3 mM $NADP^+$, (10–50)K_d amounts of $H_4PteGlu_n$, 3.1×10^{-7} M C_1-THF synthase, and 4×10^{-9} M cSHMT. The assays were initiated by the addition of 0.5–10 mM L-serine, and the increase in the absorbance at 340 nm was monitored. K_m and apparent k_{cat} values were determined from the abscissa and ordinate intercepts of double-reciprocal plots of the data.

K_m values for $H_4PteGlu_n$ for 10-CHO-THF synthetase were determined by using the coupled cyclic assay described by Strong et al. (1987), except in the case of $H_4PteGlu_1$, which was measured by the standard acid quench method (Strong et al., 1987). In the case of $H_4PteGlu_2$, the assays were performed in 1 mL of standard buffer containing 0.4 mM MgATP, 25 mM ammonium formate, 0.2 mM NADPH, 25 mM glycine, 1×10^{-8} to 1×10^{-7} M cSHMT, and 1×10^{-6} to 1.5×10^{-4} M coenzyme. The assays were initiated by the addition of 3×10^{-8} to 3×10^{-7} M C_1-THF synthase active sites. Initial rates for 10-CHO-THF synthetase were determined from the decrease in the absorbance at 340 nm on a full scale absorbance of either 0.1 or 0.2. K_m values for $H_4PteGlu_3$ through $H_4PteGlu_6$ were determined as described above, except that the assays were performed in a cuvette with a 10-cm path length and in a total volume of 25 mL. Because of the increased path length, the NADPH concentration was lowered to 0.02 mM. $H_4PteGlu_n$ concentrations used in these reactions ranged from 5×10^{-8} to 2×10^{-6} M, and C_1-THF synthase concentrations from 3×10^{-9} to 3×10^{-8} M.

Apparent k_{cat} values for 10-CHO-THF synthetase as a function of the glutamate chain length of $H_4PteGlu_n$ were determined under the conditions described for the coupled system with all three substrates for the enzyme at saturating concentrations. Apparent k_{cat} values were also determined, using an assay which directly measured the disappearance of $H_4PteGlu_n$ at 298 nm. An extinction coefficient of 19700 M^{-1} cm^{-1} at 298 nm and pH 7.0, which accounts for absorbance of the product 10-CHO-$H_4PteGlu_n$ at this wavelength, was used to calculate the rate of 10-CHO-THF synthetase (Black et al., 1978). In both assays, maximal rates for 10-CHO-THF synthetase varied less than 5% over a 2-fold range of $H_4PteGlu_n$ concentrations.

Determination of Dissociation Constants for $H_4PteGlu_n$ for cSHMT and 10-CHO-THF Synthetase. Dissociation constants for $H_4PteGlu_{1-6}$ for cSHMT were determined as described by Strong et al. (1989) for the mitochondrial isoenzyme, except that the buffer used was 50 mM potassium *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonate, pH 7.0, containing 50 mM glycine, 30 mM ammonium sulfate, and 10 mM 2-mercaptoethanol. Under these conditions, an apparent extinction coefficient assuming the fully formed ternary complex was determined to be 40000 ± 2400 M^{-1} cm^{-1} at 492 nm.

The equilibrium binding constants for $H_4PteGlu_4$ for C_1-THF synthase in the absence and presence of varying concentrations of the cosubstrate, formate, and the substrate analogues MgAMPPNP and MgAMPPCP were determined by the competitive binding method described by Strong et al. (1989). Briefly, this method is based on the ability of a second enzyme to decrease the concentration of SHMT–glycine–$H_4PteGlu_n$ ternary complex (quinonoid intermediate) which absorbs intensely at 492 nm. The extent of the decrease in the quinonoid complex in the presence of the second THF-binding enzyme is a measure of the equilibrium binding constant for the coenzyme.

A typical experiment included 4–5 μM cSHMT, 50 mM glycine, 1 μM–2 mM ammonium formate, 0.5 mM $Li_4AMPPNP$ or 0.5 mM $Li_4AMPPCP$, 1 mM $MgCl_2$, and 4–5 μM C_1-THF synthase subunits in a final volume of 1 mL of standard buffer. Spectra associated with the quinonoid complex were recorded as cSHMT was titrated with $H_4PteGlu_4$.

Analysis of the C_1-THF Synthase–SHMT Coupled Enzyme System. All studies of the coupled enzyme system, which looked either at the utilization of $H_4PteGlu_1$ or at the effect of excess SHMT, were performed in 1 mL of standard buffer containing 0.2 mM MgATP, 10 mM ammonium formate, 0.2 mM NADPH, and 50 mM glycine. The concentrations of other components of the cycle, C_1-THF synthase, SHMT (cytosolic, mitochondrial, *E. coli*, or reduced cytosolic), $H_4PteGlu_1$, and $H_4PteGlu_5$, were varied depending on the experiment and are described in more detail in the text.

Equation 1 describes a simple equilibrium process for the binding of $H_4PteGlu_n$ to a protein, in this case cSHMT.

$$[F]_f = K_d[E{\cdot}F]/[E]_f \quad (1)$$

Substitution of the equality described by eq 2 and, subsequent to this, that of eq 3 for $[E]_f$ and $[E{\cdot}F]$, respectively, results in eq 4. In these equations, $[F]_f$ refers to the concentration

$$[E]_f = [E]_T - [E{\cdot}F] \quad (2)$$

$$[E{\cdot}F] = [F]_T - [F]_f \quad (3)$$

$$[F]_f = K_d([F]_T - [F]_f)/\{[E]_T - ([F]_T - [F]_f)\} \quad (4)$$

Table I: Parameters Associated with the Binding of Tetrahydropteroylpolyglutamates with the 10-Formyltetrahydrofolate Synthetase Activity of C_1-Tetrahydrofolate Synthase and Cytosolic Serine Hydroxymethyltransferase[a]

	10-formyltetrahydrofolate synthetase			serine hydroxymethyltransferase				
coenzyme deriv	K_m (μM)	$k_{cat,app}$ uncoupled (min^{-1})	$k_{cat,app}$ cycle (min^{-1})	K_d (μM)	K_m serine (mM)	$k_{cat,app}$ Ser→Gly (min^{-1})	K_m Gly (mM)	$k_{cat,app}$ Gly→Ser (min^{-1})
$H_4PteGlu_1$	15 ± 1.0	1200	170	15 ± 0.3	1.2	1500	ND[b]	ND
$H_4PteGlu_2$	5.5 ± 0.6	260	260	2.6 ± 0.6	ND	1100	4.5	350
$H_4PteGlu_3$	0.3 ± 0.2	78	75	0.4 ± 0.2	1.0	1200	4.4	710
$H_4PteGlu_4$	0.1 ● 0.1	82	80	0.4 ± 0.2	ND	1200	5.0	750
$H_4PteGlu_5$	0.1 ± 0.1	82	81	0.2 ± 0.2	ND	1400	5.0	980
$H_4PteGlu_6$	0.3 ± 0.1	85	83	0.3 ± 0.2	1.0	1200	5.0	980

[a] Conditions are as described under Experimental Procedures. [b] Not determined.

of free $H_4PteGlu_n$, $[F]_T$ to the total concentration of $H_4PteGlu_n$, $[E]_T$ to the total concentration of cSHMT active sites, $[E]_f$ to the concentration of free cSHMT, [E·F] to the concentration of cSHMT–$H_4PteGlu_n$ bound complex, and K_d to the dissociation constant for $H_4PteGlu_n$ binding to cSHMT. In all cases, values for these parameters are entered as molar quantities.

Cross multiplication followed by rearrangement of terms results in eq 5. The final equation, eq 6, is the positive root

$$[F]_f^2 + ([E]_T - [F]_T + K_d)[F]_f + -K_d[F]_T = 0 \quad (5)$$

solution of this quadratic equation.

$$[F]_f = \{-([E]_T - [F]_T + K_d) + (([E]_T - [F]_T + K_d)^2 + 4K_d[F]_T)^{1/2}\}/2 \quad (6)$$

In most experiments which compare observed and predicted rates for the C_1-THF synthase–SHMT coupled system, the theoretical concentration of free $H_4PteGlu_n$ was calculated by using eq 6. The predicted rates for the cycle (M/min) were calculated by substituting the concentration of free $H_4PteGlu_n$ and the kinetic parameters for 10-CHO-THF synthetase (Table I) into the standard Michaelis–Menten velocity equation:

$$v = k_{cat}[C_1\text{-THFS}][H_4PteGlu_n]_f/(K_m + [H_4PteGlu_n]_f) \quad (7)$$

For some experiments, data were available from the direct measurement of the concentration of the quinonoid species (SHMT–glycine$^-$–$H_4PteGlu_n$; $\epsilon_{492} = 40\,000 \pm 2400\ M^{-1}\ cm^{-1}$) and by using the dissociation constants for the coenzyme listed in Table I for cSHMT to quantitate the free coenzyme. This concentration of free coenzyme was then used to calculate the predicted rates for 10-CHO-THF synthetase.

Results

Determination of C_1-THF Synthase and cSHMT Enzyme Levels in Rabbit Liver. The concentrations of two tetrahydrofolate-dependent enzymes, C_1-THF synthase and SHMT, were measured by determining the total enzyme units in a 20-g portion of liver obtained from two fresh rabbit livers. A total of 110 SHMT units (μmol of product/min) was measured in the liver sample. This same liver portion also contained 61 units (μmol of product/min) of the enzyme 5,10-CH_2-THF dehydrogenase, which is one activity of the trifunctional enzyme C_1-THF synthase. By using a k_{cat} value of 500 min^{-1} for the reaction catalyzed by SHMT (Schirch et al., 1985) and assuming a specific volume of 0.7 mL/g of tissue, the concentration of SHMT tetrahydrofolate binding sites was calculated to be 18 μM. In the case of C_1-THF synthase, a k_{cat} value of 1600 min^{-1}, determined under the assay conditions employed here, was used for the turnover number for the dehydrogenase activity of this trifunctional enzyme. Each enzyme subunit was assumed to contain two tetrahydrofolate binding sites, i.e., four sites per C_1-THF synthase enzyme molecule. By using these values, the concentration of C_1-THF synthase tetrahydrofolate binding sites in rabbit liver was determined to be 6 μM. The levels of enzyme activity in this 20-g portion of rabbit liver were characteristic of the levels found by us in our many preparations of these enzymes from frozen liver homogenates.

Dissociation Constants and Kinetic Constants Associated with the Binding of $H_4PteGlu_n$ to cSHMT. Kinetic constants and equilibrium binding constants of $H_4PteGlu_n$ for porcine liver cytosolic SHMT have been previously determined by Matthews et al. (1982). Here, we have repeated and extended these earlier determinations with rabbit liver cytosolic SHMT. Titrations of cSHMT, at saturating glycine concentrations, with each $H_4PteGlu_n$ were used to quantitate the amount of bound coenzyme. Scatchard plots of this binding data were linear and gave a K_d value of 15 μM for the monoglutamate, which decreased to ca. 0.2 μM with the pentaglutamate derivative (Table I).

In a previous publication we determined K_m values for serine and glycine and apparent k_{cat} values for the forward and reverse reactions of mSHMT as a function of the number of glutamate residues on $H_4PteGlu_n$ (Strong et al., 1989). We repeated these same studies with the cytosolic isoenzyme and found that like the mitochondrial isoenzyme no change was observed in either the K_m values of L-serine (1 mM) or the apparent k_{cat} values (1200 min^{-1}) with increasing chain length of $H_4PteGlu_n$ in the serine to glycine direction (Table I). However, these apparent k_{cat} values of 1200 min^{-1} were about 1.5 times greater than those seen for mSHMT. The K_m values for glycine (ca. 5 mM) also did not change with increasing glutamate chain length, using the cytosolic enzyme, in contrast to the mitochondrial isoenzyme, which exhibited a decrease in the K_m value for glycine from 8 mM with $H_4PteGlu_1$ to 3 mM with $H_4PteGlu_5$. The apparent k_{cat} values for the glycine to serine reaction increased 3-fold as the number of glutamate residues was increased from two to five, to a value of 980 min^{-1} (Table I). We were unable to determine the K_m or apparent k_{cat} value with $H_4PteGlu_1$ in the cyclic assay, due to the nonlinearity of the reaction with respect to the concentration of cSHMT. Therefore, it is possible this change in the value of the apparent k_{cat} may be somewhat larger when these values are compared, using $H_4PteGlu_1$ and $H_4PteGlu_5$. Again, the maximum apparent k_{cat} value (980 min^{-1}) was about 1.5 times greater than that observed with the mitochondrial isoenzyme.

Determination of K_m and k_{cat} Values for 10-CHO-THF Synthetase with $H_4PteGlu_n$. Previously we have determined the K_m values for both MgATP and formate at saturating concentrations of $H_4PteGlu_n$ for the 10-CHO-THF synthetase activity of C_1-THF synthase using the coupled assay with

Table II: Effect of Substrates and Substrate Analogues on the Affinity of 10-Formyltetrahydrofolate Synthetase for $H_4PteGlu_4$[a]

added ligands	K_d $H_4PteGlu_4$ (μM)
none	4.4
formate (2 mM)	1.9
AMPPNP (0.5 mM)	1.2
AMPPNP (0.5 mM), formate (1 μM)	0.8
AMPPNP (0.5 mM), formate (5 μM)	0.7
AMPPNP (0.5 mM), formate (10 μM)	0.6
AMPPNP (0.5 mM), formate (2 mM)	0.4
AMPPCP (0.5 mM), formate (2 mM)	1.9

[a] Conditions are as described under Experimental Procedures.

cSHMT (Strong et al., 1987). By using a cuvette with a 10-cm path length, we have now determined the K_m values for $H_4PteGlu_n$ having from one to six glutamyl residues. As shown in Table I, K_m values were found to decrease from 15 μM for the monoglutamate to 0.3 μM for the triglutamate, with the maximum decrease in the K_m value seen with the tetra- and pentaglutamyl derivatives, having a value of 0.1 μM. Apparent k_{cat} values for the synthetase in the coupled system, under conditions where all three substrates were at saturating levels, were observed to decrease about 3.5-fold as the number of glutamate residues of $H_4PteGlu_n$ was increased from one to six. All of this decrease in k_{cat} occurred upon binding the triglutamyl form of the coenzyme. Apparent k_{cat} values for the synthetase were also measured in an assay which followed the direct disappearance of the $H_4PteGlu_n$. Apparent k_{cat} values determined by this method were nearly identical with those found by using the coupled system for $H_4PteGlu_2$ through $H_4PteGlu_6$; however, the maximal rate of the synthetase reaction in the presence of the monoglutamate was 7 times greater in the direct assay than was seen in the coupled system (Table I). We address this discrepency in rates in a subsequent section.

Synergistic Binding of $H_4PteGlu_4$ and Formate with 10-CHO-THF Synthetase in the Presence of MgAMPPNP. We have previously shown that the K_m value of formate for 10-CHO-THF synthetase decreases over 500-fold when $H_4PteGlu_1$ is replaced with $H_4PteGlu_{3-5}$ (Strong et al., 1987). The effect of increasing glutamate chain length had little effect on the K_m value of MgATP. The large decrease in K_m for formate may reflect synergistic binding between this substrate and the polyglutamate forms of $H_4PteGlu_n$. In order to answer this question of coenzyme–formate synergism, we used the competitive binding method described previously (Strong et al., 1989), which relies on the ability of a second tetrahydrofolate-binding enzyme to decrease the quinonoid intermediate associated with cSHMT–glycine–$H_4PteGlu_n$ ternary complexes. We determined the equilibrium binding constants for $H_4PteGlu_4$ for C_1-THF synthase in the absence and presence of formate and the ATP analogues AMPPNP and AMPPCP. The results indicated that there was over a 10-fold enhancement in the binding affinity of the trifunctional enzyme for $H_4PteGlu_4$ in the presence of all substrates as compared to that of the unliganded enzyme. About one-third of this enhanced affinity for $H_4PteGlu_4$ could be attributed to a formate concentration-dependent effect (Table II).

Effect of $H_4PteGlu_n$ and SHMT on the Properties of the Coupled Enzyme System. Previously, we reported (Strong et al., 1987) two unusual properties of the C_1-THF synthase–cSHMT coupled enzyme system (Figure 1). First, it was noted that the rate of the cycle in the presence of $H_4PteGlu_1$ exhibited an initial lag, showing an increase in the absorbance at 340 nm before approaching a linear steady-state rate of decrease in the absorbance at this wavelength (i.e., disappearance of NADPH). With $H_4PteGlu_n$ (n = 2–6) no such lag was observed. Second, it was observed that a large excess of cSHMT over the concentration of $H_4PteGlu_5$ in the coupled reaction did not, as would be expected, inhibit the rate of the cycle. In the following sections, we present our initial attempts at characterizing this coupled enzyme cycle with regard to these two unusual properties.

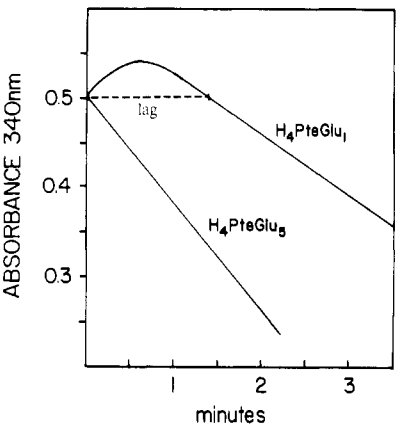

FIGURE 2: Effect of glutamate chain length on the coupled spectrophotometric assay which converts formate to L-serine. The rate of cycle, in the presence of $H_4PteGlu_1$ or $H_4PteGlu_5$, is determined from the decrease in the absorbance at 340 nm due to the conversion of NADPH to $NADP^+$ by the 5,10-methylenetetrahydrofolate dehydrogenase activity of C_1-tetrahydrofolate synthase. The concentration of $H_4PteGlu_1$ is 75 μM, and the concentration of $H_4PteGlu_5$ is 5 μM. The concentration of C_1-tetrahydrofolate synthase used in these two reactions was 8×10^{-8} M and 2.4×10^{-7} M, respectively. The dashed line refers to the lag time in the formation of product discussed in the text.

Figure 2 shows the rate of a typical assay using the coupled system in the presence of either 75 μM $H_4PteGlu_1$ ($5K_m$) or 5 μM $H_4PteGlu_5$ ($50K_m$) versus time. As seen in the figure, with $H_4PteGlu_1$ as the coenzyme substrate, there is not only a significant lag period prior to attaining a linear steady-state rate but also an observed increase in the absorbance at 340 nm. Assays containing $H_4PteGlu_{2-6}$ remain linear for longer than 5 min if the cosubstrates for the cycle are not significantly depleted during this time and the reaction cuvette is maintained anaerobic. During these 5 min, the total pool of $H_4PteGlu_5$ would turn over in the cycle about 20 times.

In order to explain the increase in the absorbance at 340 nm using $H_4PteGlu_1$, we have varied individual components of the coupled enzyme cycle and observed the effect on both the lag and steady-state portions of the time course of the coupled assay. When 75 μM $H_4PteGlu_1$, 8×10^{-8} M trifunctional enzyme, and 2.8 μM cSHMT were used in the cycle, with all other substrates at saturating levels, the length of the lag phase was about 80 s and the steady-state rate (linear portion of activity profile) 11.5 μM $NADP^+$ min^{-1} (Figure 2). Increasing cSHMT concentrations to 15 μM had no effect on either of these parameters. Increasing the concentration of C_1-THF synthase in the assay by 10-fold resulted in a decreased lag period, to about 5 s, and a proportional 10-fold increase in the steady-state rate. Although difficult to assess, the increase in the trifunctional enzyme concentration appeared to have little effect on the extent of the observed increase in the absorbance at 340 nm. When 80 μg of purified cyclohydrolase–dehydrogenase fragment of C_1-THF synthase was included in the assay, the lag was decreased to about 6 s and no increase in the absorbance at 340 nm was observed. An approximately 50% enhancement in the steady-state rate of the cycle was also seen. Finally when the concentration of $H_4PteGlu_1$ was varied in the assay over a 10-fold range, from

7.5 μM to 75 μM, a progressive increase in the lag portion of the assay was observed, as well as the expected increase in the steady-state rate.

As both the cyclohydrolase–dehydrogenase enzyme fragment and the lower $H_4PteGlu_1$ concentrations diminish the lag in the disappearance of NADPH, the data suggest that, at the high $H_4PteGlu_1$ concentrations required to saturate 10-CHO-THF synthetase, we are inhibiting the reduction of 5,10-CH^+-$H_4PteGlu_1$ by the dehydrogenase activity of C_1-T-HF synthase. This conclusion is supported by the 4-fold higher affinity of the dehydrogenase for $H_4PteGlu_1$ (K_I = 3.7 μM) (Schirch, 1978) in comparison to the affinity of the synthetase active site for this same coenzyme form (K_m = 15 μM, this paper). At the $H_4PteGlu_1$ concentration used above (75 μM), less than 5% of the dehydrogenase sites would be expected to be initially free of coenzyme. In contrast to this observation, when $H_4PteGlu_5$ is used as the substrate, the synthetase active site has nearly 40-fold greater affinity for this coenzyme form than does the dehydrogenase site. Therefore, significantly less $H_4PteGlu_5$ is required to saturate the synthetase enzyme than is required to saturate this site with $H_4PteGlu_1$. At the 5 μM $H_4PteGlu_5$ concentration used in the cycle, 35% of the dehydrogenase sites would be free.

If we are in fact inhibiting the dehydrogenase activity of the trifunctional enzyme, the increase in the absorbance at 340 nm could be attributed to a buildup of 5,10-CH^+-$H_4PteGlu_1$. We have investigated this possibility by measuring both the formation of the 5,10-CH^+-$H_4PteGlu_1$ and the 5,10-CH^+-$H_4PteGlu_1$ plus 10-CHO-$H_4PteGlu_1$ coenzyme pools during the cycle. To determine only the increase in the concentration of the methenyl derivative during the progress of the assay, we included in the normal assay an NADPH-regenerating system consisting of excess glucose 6-phosphate and glucose-6-phosphate dehydrogenase. Recording spectra at 10-s intervals after initiating the assay with the addition of MgATP, we observed the appearance of an absorbance peak at 350 nm which matched the spectra of 5,10-CH^+-$H_4PteGlu_1$ at pH 7.0. In a second experiment, aliquots of a running assay were removed at various times and quenched with concentrated HCl, lowering the pH to about 3. After 15 min at 50 °C in a nitrogen atmosphere, and following centrifugation in a microfuge, a spectrum was recorded, and the absorbance at 360 nm was used to calculate the concentration of 5,10-CH^+-$H_4PteGlu_1$ (ϵ_{360} = 25 100 M^{-1} cm^{-1}). These values represent the total concentration of the combined 5,10-CH^+- and 10-CHO-$H_4PteGlu_1$ pools. The linear steady-state rate of the cycle using $H_4PteGlu_1$ was achieved when the concentration of 5,10-CH^+-$H_4PteGlu_1$ reached its maximum value. During the linear part of the assay, the combined concentrations of 10-CHO- and 5,10-CH^+-$H_4PteGlu_1$ were about 50% of the total added coenzyme (136 μM), with about 5% of this combined pool being the methenyl form. Assuming an extinction coefficient of 19000 M^{-1} cm^{-1} (estimated from spectral data at pH 7.0) at 340 nm for 5,10-CH^+-$H_4PteGlu_1$, this concentration of methenyl coenzyme (about 3 μM) would be sufficient to produce the observed increase in absorbance at 340 nm in the coupled assay system.

As stated above, another unusual property of the coupled enzyme cycle is the failure of high concentrations of the coupling enzyme, SHMT, to inhibit the rate of the 10-CHO-THF synthetase reaction. This reaction has previously been shown to be the rate-determining step in the cycle (Strong et al., 1987). High concentrations of SHMT should lower the level of free $H_4PteGlu_n$ available to 10-CHO-THF synthetase, which would then decrease the rate of this reaction in the cycle.

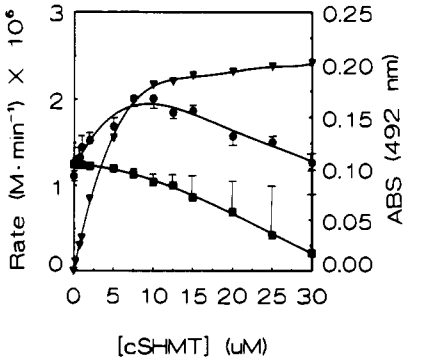

FIGURE 3: Observed (●) and predicted (■) rates for the cyclic assay shown in Figure 1 in the presence of 5 μM $H_4PteGlu_5$ and 1.6 × 10^{-8} M C_1-tetrahydrofolate synthase at increasing concentrations of cytosolic serine hydroxymethyltransferase. All other components of the cycle were present at saturating levels. Under these conditions the 10-formyltetrahydrofolate synthetase enzyme is the rate-determining step in the cycle. The concentration of SHMT-bound $H_4PteGlu_5$ at each concentration of serine hydroxymethyltransferase was determined from the absorbance at 492 nm (▼) (right ordinate). From this value, the concentration of free coenzyme at each point in the titration was calculated. The predicted rate of the cycle was determined by using the concentration of $(H_4PteGlu_5)_{free}$ and the K_m and k_{cat} values for $H_4PteGlu_5$ for 10-formyltetrahydrofolate synthetase listed in Table I. Error bars refer to standard deviations (95% confidence). In the case of the predicted curve, these marks account for the standard deviation in the extinction coefficient for the cSHMT–glycine–$H_4PteGlu_5$ ternary complex, as well as the standard error in the kinetic constants for 10-CHO-THF synthetase.

The method for predicting the rate of the cycle is described in detail under Methods.

The rate of the cycle with increasing concentrations of cSHMT is recorded in Figure 3, using 5 μM $H_4PteGlu_5$ and 1.6 × 10^{-8} M C_1-THF synthase (Figure 3, circles). As the concentration of cSHMT is raised, an increase in the absorbance at 492 nm (cSHMT–glycine$^-$–$H_4PteGlu_5$) is observed (Figure 3, triangles). As described above, this absorbance value reflects the amount of $H_4PteGlu_5$ bound to cSHMT. The concentration of free $H_4PteGlu_5$ was then determined from the difference between the total and the cSHMT-bound coenzyme. These measured concentrations of free $H_4PteGlu_5$ and the K_m and k_{cat} values reported for 10-CHO-THF synthetase in Table I were used to calculate the predicted rates for the cycle (Figure 3, squares).

Calculating the concentration of free $H_4PteGlu_5$ as the difference between the total $H_4PteGlu_5$ and the cSHMT-bound $H_4PteGlu_5$ does not take into account that steady-state levels of 10-CHO-$H_4PteGlu_5$, 5,10-CH^+-$H_4PteGlu_5$, and 5,10-CH_2-$H_4PteGlu_5$ may be significant. Acid quenching of the solutions during the cycle indicated that the combined pools of 5,10-CH^+-$H_4PteGlu_5$ and 10-CHO-$H_4PteGlu_5$ account for less than 7% of the total $H_4PteGlu_5$ added to initiate the cycle. This represents the limit in our detection of these products and represents at most 0.3 μM coenzyme in these two pools. In fact, we could detect no difference in the concentration of 5,10-CH^+-$H_4PteGlu_5$ plus 10-CHO-$H_4PteGlu_5$ in the presence and absence of the trifunctional enzyme. We assumed that the concentration of 5,10-CH_2-$H_4PteGlu_5$ was negligible due to the high concentration of glycine and SHMT in the assay. On the basis of these studies, we have assumed the concentration of free $H_4PteGlu_5$ to be the difference between the total coenzyme added to the assay and the coenzyme bound to SHMT.

The data in Figure 3 show that as the cSHMT concentration is increased to a level about 2 times that of $H_4PteGlu_5$, there is about a 2-fold enhancement in the rate of the synthetase

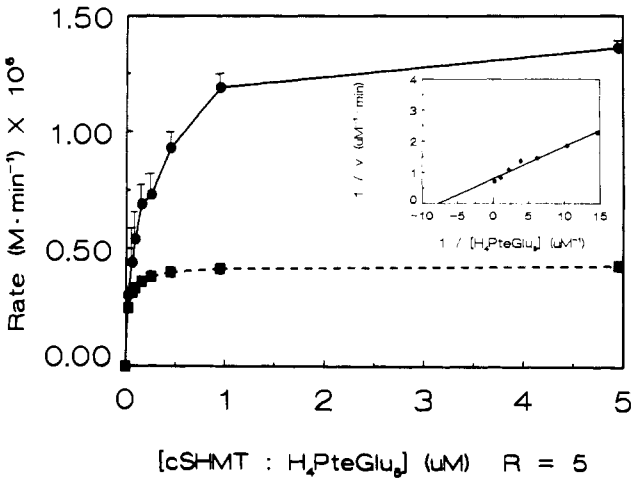

FIGURE 4: Observed (●) and predicted (■) rates for the cycle shown in Figure 1 with 1.6×10^{-8} M C_1-tetrahydrofolate synthase at increasing amounts of cytosolic serine hydroxymethyltransferase and $H_4PteGlu_5$, which were maintained at a fixed ratio of 5:1 (cSHMT:$PteGlu_5$). The observed initial rates are plotted versus the concentration of cSHMT-bound $H_4PteGlu_5$ in the assay. On the basis of the K_d value for $H_4PteGlu_5$ (Table I) for cytosolic serine hydroxymethyltransferase, the concentration of free $H_4PteGlu_5$ at each point in the titration was calculated. The predicted rate of the cycle was determined by using this free concentration of $H_4PteGlu_5$ and the K_m and k_{cat} values for $H_4PteGlu_5$ for 10-formyltetrahydrofolate synthetase also listed in Table I. The inset shows the double-reciprocal plot of the initial velocity data. The apparent K_m and k_{cat} values for the cSHMT–glycine–$H_4PteGlu_5$ complex calculated from this plot were 0.1 μM and 80 min^{-1}, respectively.

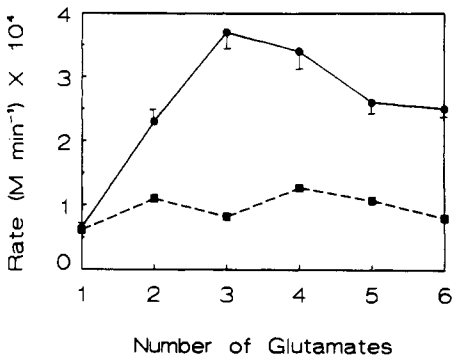

FIGURE 5: Observed (●) and predicted (■) rates for the cycle shown in Figure 1 with 5 μM $H_4PteGlu_n$ (n = 1–6) and substrate amounts of cytosolic serine hydroxymethyltransferase (10 μM) and C_1-tetrahydrofolate synthase (2.0 μM). The predicted rate of the cycle for each $H_4PteGlu_n$ was calculated from the concentration of free coenzyme (prior to initiating the assay with C_1-tetrahydrofolate synthase) and the kinetic parameters for 10-formyltetrahydrofolate synthetase (Table I).

enzyme as compared to the enzyme rate in the absence of cSHMT. The predicted rate suggests that this level of cSHMT should lower the concentration of free $H_4PteGlu_5$ to a level which would decrease the rate of 10-CHO-THF synthetase by 20%. Further increases in the concentration of cSHMT result in a decrease in the rate of the cycle which parallels, but remains much faster than, the predicted rate. At 30 μM cSHMT, the rate of the cycle is still slightly faster than the rate observed in the absence of cSHMT and is more than 5-fold higher than the mean value for the predicted rate. At high concentrations of cSHMT the predicted rate becomes very sensitive to small changes in both the extinction coefficient of the quinonoid complex and the K_m of $H_4PteGlu_5$ for the synthetase site. When the standard deviations in the values for these constants are considered, the significant difference between the observed and the predicted rates for the cycle is much less pronounced. However, the faster than predicted rate of the coupled enzyme cycle cannot be explained simply by errors in determining these constants and suggests that the SHMT–glycine–$H_4PteGlu_5$ ternary complex serves as a competent substrate for 10-CHO-THF synthetase.

Substitution of mSHMT for cSHMT in the coupled assay results in a similar activation profile, but the maximum increase in the rate of the cycle is only 1.4 times the rate of 10-CHO-THF synthetase in the absence of any SHMT. At 30 μM mSHMT, the rate is 3-fold greater than the mean predicted rate (data not shown). When *E. coli* SHMT serves as the coupling enzyme, no significant increase in the rate of the synthetase is observed at concentrations of SHMT up to 30 μM. At 30 μM *E. coli* SHMT, the concentration of bound coenzyme would be similar to that seen with about 7 μM cSHMT (K_d, $H_4PteGlu_5$ for *E. coli* SHMT = 2 μM; Strong et al., 1989). We also examined the effect of substituting cSHMT which had been reduced with sodium cyanoborohydride (red-cSHMT) on the rate of the cycle. This catalytically inactive cSHMT also showed no significant effect on the rate of the cycle at concentrations as high as 30 μM (data not shown).

If the cSHMT–glycine–$H_4PteGlu_5$ ternary complex is serving as a competent substrate for 10-CHO-THF synthetase, increasing concentrations of cSHMT and $H_4PteGlu_5$ held at a fixed ratio should exhibit saturation-type kinetics and allow for the determination of an apparent K_m and V_{max} value for this complex. Figure 4 shows the rate of the cycle with increasing concentrations of $H_4PteGlu_5$ which is held at the fixed cSHMT:$H_4PteGlu_5$ ratio of 5:1 in the presence of saturating concentrations of all other cyclic assay components. Assays were initiated by the addition of C_1-THF synthase. These data gave an apparent K_m and k_{cat} for the cSHMT–glycine–$H_4PteGlu_5$ complex of about 0.1 μM and 80 min^{-1}, respectively (inset to Figure 4). By using the K_d value for the dissociation of $H_4PteGlu_5$ from cSHMT–glycine–$H_4PteGlu_5$ ternary complex reported in Table I, the concentration of free $H_4PteGlu_5$ in these studies were calculated and a predicted rate for the cycle was determined as before (Figure 4, dashed line). Above 0.1 μM coenzyme, the predicted rate indicates only a small increase in the rate of the cycle at increasing concentrations of the cSHMT–glycine–$H_4PteGlu_5$ complex.

Effect of the Number of Glutamyl Residues of $H_4PteGlu_n$ on the Rate of the Cycle. Using concentrations of $H_4PteGlu_n$, cSHMT, and C_1-THF synthase which more closely reflect in vivo levels, we have determined the rate of conversion of formate to L-serine (Figure 1) as a function of glutamate chain length for $H_4PteGlu_n$. Figure 5 shows the observed and predicted initial rates for such a study, using 5 μM $H_4PteGlu_n$ (each one), 10 μM cSHMT, and 2.0 μM C_1-THF synthase. The concentration of $H_4PteGlu_n$ and the ratio of the concentration of cSHMT to that of C_1-THF synthase may approximate the in vivo situation. The assays were performed by incubating the coenzyme with cSHMT prior to initiating the reaction by the rapid addition of C_1-THF synthase. The predicted rates are based on the theoretical free concentration of each $H_4PteGlu_n$ in the reaction prior to the addition of C_1-THF synthase (i.e., $H_4PteGlu_n$ not bound to cSHMT). The predicted rates (Figure 5, squares) show essentially no change in the rate of the cycle as a function of glutamate chain length. This reflects the offsetting values for the decrease in K_m and k_{cat} with increasing glutamate chain length. The observed data, however, show that with the penta- and hexaglutamate forms of the coenzyme, which are the major physiological forms, there is about a 3-fold increase in the rate of the cycle as compared with the rate in the presence of $H_4PteGlu_1$.

Discussion

Determination of the control of enzymes in one-carbon metabolism represents a very complex problem. For any single metabolic pathway involving $H_4PteGlu_n$ coenzymes the following factors have to be considered. There are at least six different forms of this coenzyme in the cell with respect to the pteridine portion of the coenzyme: i.e., $H_2PteGlu$, $H_4PteGlu$, CH_2-PteGlu, 10-CHO-PteGlu, 5-CHO-PteGlu, 5-CH_3-PteGlu. Each enzyme in a metabolic pathway involving this coenzyme will use one of these forms as a substrate, but may also be inhibited by one or more of the other forms. In addition to the variations in the pteridine portion of the molecule, the coenzyme also is divided into forms with different numbers of glutamate residues. For most eukaryotic cells, the number of glutamate residues varies from four to eight ($H_4PteGlu_{4-8}$). There appear to be two different classes of enzymes with respect to affinity for the polyglutamate portion of the coenzyme (Green et al., 1988). One class is represented by an increasing affinity for the coenzyme with an increasing number of glutamate residues. The second class has a similar affinity for all polyglutamate forms of the coenzyme. In general those enzymes which have a polyglutamate binding site have K_m values in the 0.1 μM range while those enzymes which appear to lack a polyglutamate binding site have K_m values in the 1–5 μM range. Evidence also suggests that the concentration of all forms of the coenzyme in the cell is similar to the concentration of coenzyme binding sites, suggesting that most of the coenzyme in the cell is bound and the concentration of free coenzyme is very small.

To add to the difficulty in elucidating the flux of one-carbon groups through the different one-carbon metabolic pathways are the occurrence of several multifunctional enzymes, channeling of the coenzyme between some enzymes, and evidence for enzyme–enzyme interactions which affect activity [reviewed by Schirch and Strong (1989)]. Most past studies have focused on the kinetic properties of single-purified enzymes. It is clear that to fully understand the interaction of all the above-named factors more complex systems need to be studied.

The conversion of formate and glycine to serine offers a system which involves many of the complicating factors involved in understanding one-carbon metabolism (Figure 1). The combined reactions of SHMT and C_1-THF synthase utilize $H_4PteGlu_n$ in a catalytic cycle, permitting the study of the cycle at low concentrations of the coenzyme and under conditions where most of the coenzyme is bound. The cycle contains a multifunctional enzyme (C_1-THF synthase), which catalyzes three reactions and contains at least two separate catalytic sites (Villar et al., 1985). The enzymes in this cycle contain sites which interact with the polyglutamate portion of the coenzyme with K_m values in the 0.1–0.2 μM range (cSHMT and 10-CHO-THF synthetase) and at least one active site which appears not to have a polyglutamate site having a K_m value in the micromolar range (5,10-CH_2-THF dehydrogenase and 5,10-CH^+-THF cyclohydrolase). Both cSHMT and C_1-THF synthetase are present in high concentration in rabbit liver with a combined active site concentration of about 25 μM, which may represent as much as two-thirds of the concentration of all forms of folate in the cell. Both enzymes can be purified in high yield and have been studied in some detail with respect to their interaction with reduced folates; i.e., cSHMT is known to be inhibited by both 5-CH_3-H_4PteGlu and 5-CHO-H_4PteGlu (Schirch & Ropp, 1967; Matthews et al., 1984). However, the system is not so complicated that it should be impossible to determine all of the individual kinetic constants for analysis by computer simulation. The results reported in this paper are an initial attempt to determine some of the kinetic properties of the individual reactions in the cycle. We also compare some of the properties of the cycle with those based on predictions of the formation of 10-CHO-$H_4PteGlu_n$ being the rate-determining step.

We have determined the K_m or K_d values for $H_4PteGlu_n$, with n = 1–6, for the cytosolic isoenzyme of SHMT from rabbit liver. Many of these values have been previously determined for the porcine enzyme and the rabbit mitochondrial enzyme (Matthews et al., 1982; Strong et al., 1989). In general the values we determined for the cytosolic enzyme are very similar to the results previously reported in these other studies. The tri-through hexaglutamate forms of the coenzyme have a 50-fold higher affinity for cSHMT than the monoglutamate form. The addition of glutamate residues to the coenzyme has no effect on the K_m values for serine and glycine or the k_{cat} value for conversion of serine to glycine. The addition of glutamate residues does result in a 3-fold increase in the k_{cat} value for the conversion of glycine to serine (Table I). These values are needed to understand the role of this enzyme in the cycle we are studying.

We have also determined for the first time the K_m values of $H_4PteGlu_{1-6}$ for 10-CHO-THF synthetase from a mammalian source. A detailed study of the kinetic constants for $H_4PteGlu_n$ with 10-CHO-THF synthetase from yeast has previously been reported (Rabinowitz, 1983). This study indicated a nearly 1000-fold decrease in the K_m value for the coenzyme as the glutamate chain length was increased from one to four glutamyl residues, 265 μM down to 0.27 μM, respectively. Wasserman et al. (1983) found the K_m values for $H_4PteGlu_1$ and $H_4PteGlu_3$ with 10-CHO-THF synthetase from chicken liver to be 67 μM and 4.1 μM, respectively. In comparison to both of these previous studies, our results indicate that rabbit liver 10-CHO-THF synthetase has about a 1 order of magnitude higher affinity for the triglutamyl derivative of the coenzyme than the proteins purified from yeast and chicken liver.

In our previous studies with this enzyme, we also showed that in the presence of ATP and NH_4^+ ion the binding of $H_4PteGlu_{3-6}$ resulted in a conformational change in the synthetase domain of this enzyme (Strong et al., 1987). Concomitant with this structural change was a nearly 3 order of magnitude decrease in the K_m for formate. This decrease in K_m value with respect to polyglutamate chain length suggests that formate will only serve as a substrate for this reaction if the coenzyme has at least three glutamate residues. The large decrease in K_m for formate also suggested that binding synergism was occurring and that there may be a comparable decrease in the K_m for $H_4PteGlu_{3-6}$ between the enzyme and the enzyme–formate–ATP complex. In this study we have tried to confirm this synergistic binding between formate and the folate substrate. By using a 10-cm path length cell, we have been able to determine the K_m values for glutamate chain lengths of one to six. The decrease in K_m from 15 μM for the monoglutamate to 0.3 μM for the triglutamate confirms our earlier study which shows that the binding of the third glutamate to this enzyme is the critical residue and that the enzyme has a polyglutamate binding site. These studies were done at saturating levels of ATP and formate. Unfortunately, even with a 10-cm cell it is not possible to determine the K_m values of $H_4PteGlu_n$ at formate levels near its K_m value to determine if these two ligands bind synergistically. Our ability to accurately assess initial velocities measured at nonsaturating formate and low coenzyme concentrations limits the use of this

approach to determine these parameters.

To determine the effect of formate and ATP levels on the affinity of the synthetase active site for the folylpolyglutamates, we used a spectrophotometric competitive binding assay to determine the K_d value for $H_4PteGlu_4$ in the absence of both ATP and formate (Strong et al., 1989). The observed value of 4.4 μM is 44-fold higher than the observed K_m value in the presence of ATP and formate, suggesting that synergistic binding is occurring. However, C_1-THF synthase is a multifunctional enzyme and contains another folate binding site which catalyzes the cyclohydrolase and dehydrogenase reactions. We have previously shown that the K_m for $H_4PteGlu_n$ for this site is 4 μM and is insensitive to the number of glutamate residues (Villar et al., 1985). This suggests that the K_d value of 4.4 μM we found for $H_4PteGlu_4$ in our competitive binding assay is measuring the affinity of this coenzyme for the dehydrogenase–cyclohydrolase site and that the K_d value for the synthetase site may be even larger than 4.4 μM.

We could not add both ATP and formate in these competitive binding assays to determine the K_d for the synthetase since the enzyme would rapidly convert all the substrates to products. To avoid this problem, we tried to use ADP to form a dead-end complex with formate and $H_4PteGlu_4$. We found that the ADP was being converted in the competitive binding assay to ATP, which resulted in the conversion of $H_4PteGlu_4$ to 10-CHO-$H_4PteGlu_4$. We suspected that the formation of ATP from ADP in this system was the result of contaminating myokinase activity. Several attempts were made to further purify our apparent homogeneous enzyme preparation and to inhibit the myokinase activity by including magnesium diadenosine pentaphosphate, a potent inhibitor of this enzyme (Schoff et al., 1989). However, neither of the procedures decreased the rate of ADP conversion to ATP. We cannot rule out the possibility that C_1-THF synthase itself may be able to catalyze a formate-dependent conversion of ADP to ATP, especially since formyl phosphate has been indicated as an intermediate in the formation of 10-CHO-THF by this enzyme (Smithers et al., 1987).

To circumvent the ADP conversion to ATP problem, we substituted AMPPNP and AMPPCP at saturating concentrations as two nonhydrolyzable ATP analogues in our competitive binding assay. Unfortunately, neither of these analogues when bound to the synthetase domain results in the conformational change in this domain observed with either ATP or ADP. However, we were able to demonstrate a 10-fold decrease in the K_d for $H_4PteGlu_4$ to 0.4 μM, which is about 4-fold higher than the K_m for $H_4PteGlu_4$ determined in the presence of ATP and formate (Table II). About equal amounts of this decrease in K_d could be attributed to synergistic binding with formate and the ATP analogue. However, this effect may have been much greater since the K_m for $H_4PteGlu_4$ in the absence of ATP and formate may be much larger than 4.4 μM as noted in the previous paragraph.

These results strongly suggest that $H_4PteGlu_{3-6}$ binds synergistically with formate and ATP and that most of the 500-fold decrease previously observed in the K_m for formate upon binding of the folylpolyglutamates is also reflected in a decrease in the K_m values for the coenzyme. In a cell with a limited supply of free $H_4PteGlu_n$, the formation of an unproductive ternary synthetase–$H_4PteGlu_n$–ATP complex in the absence of formate would decrease the availability of the coenzyme for other pathways in one-carbon metabolism. Our data suggest that $H_4PteGlu_{3-6}$ would bind to the synthetase domain only when both formate and ATP were available to form the catalytically productive complex. Previously, evidence has shown that all three substrates are required for the formation of a catalytically competent active site in 10-CHO-THF synthetase from *Clostridium cylindrosporum* (Buttlaire et al., 1975a,b; Wendland et al., 1983). Formate was shown in these studies to be released from the enzyme at a reduced rate in these quaternary complexes in comparison to its presence in binary and ternary complexes.

The rate-determining step in the cycle is the conversion of formate to 10-CHO-$H_4PteGlu_n$ (Strong et al., 1987). The dehydrogenase and cyclohydrolase reactions have k_{cat} values that are at least an order of magnitude larger than the synthetase reaction (Schirch, 1978). However, when $H_4PteGlu_1$ is used as the folate substrate the rate-determining step appears to become the conversion of 5,10-CH^+-$H_4PteGlu_1$ to 5,10-CH_2-$H_4PteGlu_1$ with the concomitant accumulation of 5,10-CH^+-$H_4PteGlu_1$. This appears to be the result of the combined effect of the higher affinity of the dehydrogenase site for $H_4PteGlu_1$ compared to the synthetase site and the inhibition of the dehydrogenase reaction by $H_4PteGlu_1$. This observation points out the importance of knowing not only the K_m values for substrates for each activity in the cycle but also the K_i values for other folate forms present in the reaction solution.

To further probe the role of the synthetase reaction to control the rate of the cycle, we investigated the effect of increasing concentrations of SHMT. As this enzyme concentration is increased, it will sequester the $H_4PteGlu_n$ by forming a nonproductive ternary enzyme–glycine–$H_4PteGlu_n$ complex, depressing the concentration of free coenzyme. This ternary complex absorbs at 500 nm with an apparent extinction coefficient of 40 000 ± 2400 M^{-1} cm^{-1}. This permits confirmation by spectrophotometric measurements that most of the $H_4PteGlu_n$ is bound to cSHMT during the catalytic cycle. On the basis of the available free $H_4PteGlu_n$ and the known k_{cat} and K_m values for the synthetase reaction, one can calculate the effect of increasing concentrations of cSHMT on the rate of conversion of formate to serine. As shown in Figure 3, the observed rate of the cycle is faster than the predicted rate at all concentrations of cSHMT. At 30 μM cSHMT the actual rate is at a minimum 2-fold higher than the predicted rate and as much as 5-fold larger. The observed rate would require that all of the folate be free and available to the synthetase reaction under these conditions. However, we can directly observe that nearly all of the $H_4PteGlu_n$ is bound to cSHMT.

There are several possible explanations which could account for the difference between the observed and predicted rates of the cycle as a function of increasing cSHMT concentration. First, the cSHMT–$H_4PteGlu_n$ complex could be a substrate for the synthetase by channeling the coenzyme between the active sites as has been proposed for the transfer of NADH between several dehydrogenases (Srivastava & Bernhard, 1984, 1985, 1987). In support of this interpretation is the observation that increasing the concentration of cSHMT–$H_4PteGlu_n$ shows saturation kinetics on the rate of the cycle (Figure 4). The calculated K_m and k_{cat} values for the enzyme–enzyme complex, 0.1 μM and 80 min^{-1}, respectively, are similar to the values determined under conditions where the concentration of $H_4PteGlu_n$ is greater than the concentration of C_1-THF synthase. The significance of this finding is uncertain at present. Also, mSHMT and *E. coli* SHMT gave reduced and no activation of the rate of the cycle, respectively, suggesting that there is some structural specificity to the effect of cSHMT activation. A second possible explanation for the activation by cSHMT is that $H_4PteGlu_n$ is an inhibitor of some other step in the cycle and that as you

lower the concentration of free $H_4PteGlu_n$ you reduce this inhibition and thus increase the rate of the cycle. This role for cSHMT in the stimulation of the rate of the cycle could also apply to any other assay component as well. We attempted to test this possibility by including in the assay the reduced form of cSHMT, which binds $H_4PteGlu_n$ but has no catalytic activity. However, the red-cSHMT did not produce the rate enhancement, suggesting that inhibition of some step in the cycle by $H_4PteGlu_n$ or another assay factor is not responsible for the activation by cSHMT. The study with red-cSHMT also suggests that glycine (which cannot form the external aldimine with pyridoxal phosphate) may play a role in this stimulatory process. A third possible explanation is that cSHMT and C_1-THF synthase form a complex that results in lower K_m and larger k_{cat} values of the synthetase reaction. Under these conditions there would be no need to invoke channeling of the coenzyme. It is also possible that some as yet unknown property of the cyclohydrolase and dehydrogenase reactions account for the discrepancy between the observed and predicted rates. We have not yet determined the effect of polyglutamate chain length on the conversion of 10-CHO-$H_4PteGlu_n$ to 5,10-CH_2-$H_4PteGlu_n$ as catalyzed by the cyclohydrolase and dehydrogenase reactions. This aspect of the problem is currently being investigated.

We also looked at the effect of glutamate chain length on the rate of conversion of formate to serine under conditions which reflect in vivo concentrations of the enzymes and folate substrates. Again, using the synthetase as the rate-determining step, we calculated predicted rates as a function of glutamate chain length and compared them to the observed initial rates (Figure 5). The predicted rates suggest no change in rate with increasing glutamate chain length, due to compensating decreases in K_m and k_{cat} values. The observed rate of the cycle, however, increased with glutamate chain length, reaching a maximum with chain lengths of three to six. Even if the total $H_4PteGlu_n$ added were free to react with 10-CHO-THF synthetase, the predicted rate would be less than one-half the observed value. The observed 3-fold higher than predicted rates probably are a reflection of the same observations discussed in the previous paragraph.

In conclusion, of the four reactions involved in the conversion of formate to serine we have determined K_m values for all substrates for two of the reactions, including the polyglutamate forms of $H_4PteGlu_n$. On the basis of the observation that the synthetase reaction has the smallest k_{cat} value of the four reactions and is the rate-determining step, we performed several experiments to test whether the observed rate of conversion of formate to serine was the same as the predicted rate. The observed rate was, under some conditions, 5-fold higher than the predicted rate. It is our intention to fully characterize this cycle of four reactions to elucidate all of the factors involved so that predicted rates match those that are observed.

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