Serine Hydroxymethyltransferase Catalyzes the Hydrolysis of 5,10-Methenyltetrahydrofolate to 5-Formyltetrahydrofolate*

Patrick Stover and Verne Schirch†

From the Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University, Medical College of Virginia, Richmond, Virginia 23298

The combined activities of rabbit liver cytosolic serine hydroxymethyltransferase and C₅-tetrahydrofolate synthase convert tetrahydrofolate and formate to 5-formyltetrahydrofolate. In this reaction C₅-tetrahydrofolate synthase converts tetrahydrofolate and formate to 5,10-methenyltetrahydrofolate, which is hydrolyzed to 5-formyltetrahydrofolate by a serine hydroxymethyltransferase-glycine complex. Serine hydroxymethyltransferase, in the presence of glycine, catalyzes the conversion of chemically synthesized 5,10-methenyltetrahydrofolate to 5-formyltetrahydrofolate with biphasic kinetics. There is a rapid burst of product that has a half-life of formation of 0.4 s followed by a slower phase with a completion time of about 1 h. The substrate for the burst phase of the reaction was shown not to be 5,10-methenyltetrahydrofolate but rather a one-carbon derivative of tetrahydrofolate which exists in the presence of 5,10-methenyltetrahydrofolate. This derivative is stable at pH 7 and is not an intermediate in the hydrolysis of 5,10-methenyltetrahydrofolate to 10-formyltetrahydrofolate by C₅-tetrahydrofolate synthase. Cytosolic serine hydroxymethyltransferase catalyzes the hydrolysis of 5,10-methenyltetrahydrofolate pentaglutamate to 5-formyltetrahydrofolate pentaglutamate 15-fold faster than the hydrolysis of the monoglutationate derivative. The pentaglutamate derivative of 5-formyltetrahydrofolate binds tightly to serine hydroxymethyltransferase and dissociates slowly with a half-life of 16 s. Both rabbit liver mitochondrial and Escherichia coli serine hydroxymethyltransferase catalyze the conversion of 5,10-methenyltetrahydrofolate to 5-formyltetrahydrofolate at rates similar to those observed for the cytosolic enzyme. Evidence that this reaction accounts for the in vitro presence of 5-formyltetrahydrofolate is suggested by the observation that mutant strains of E. coli, which lack serine hydroxymethyltransferase activity, do not contain 5-formyltetrahydrofolate, but both these cells, containing an overproducing plasmid of serine hydroxymethyltransferase, and wild type cells do have measurable amounts of this form of the coenzyme.

Intracellular folate pools contain three known derivatives of tetrahydrofolate at the oxidation level of formate; CH⁺.

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† To whom correspondence should be addressed.

H₄PteGlu, 10-010-H₄PteGlu, and 5 CHOH-H₄PteGlu. Enzymatic reactions involving the first two of these derivatives have been characterized extensively whereas little information exists elucidating the metabolic role of 5-CHO-H₄PteGlu. 5-CHO-H₄PteGlu comprises approximately 10% of the total folate pool in most organisms. However, in some organisms the levels are much higher; these include 35% in Neurospora crassa (Cossins et al., 1976), 70% in soybeans (Shin et al., 1975), 25% in mouse L1210 leukemia cells (Moran et al., 1976; Fujii et al., 1982), and 14% in human fibroblasts (Rosenblatt et al., 1979). Many studies have suggested that this compound is merely an artifact of isolation. Clinically, the D.L-mixture of 5 CHOH-H₄PteGlu is known as leucovorin, a therapeutic drug used to rescue patients from methotrexate toxicity. The only proposed in vivo source of 5-CHO-H₄PteGlu is the nonenzymatic hydrolysis of 5,10-CH⁻H₄PteGlu. 5,10-CH⁻H₄PteGlu undergoes nonenzymatic hydrolysis at neutral or basic pH, yielding a mixture containing predominately 10-CHO-H₄PteGlu and trace amounts of 5-CHO-H₄PteGlu (May et al., 1951). In addition, it has also been shown that 10-CHO-H₄PteGlu is capable of slowly isomerizing to 5-CHO-H₄PteGlu, the more thermodynamically stable compound (May et al., 1951). The first extensive study elucidating the mechanism of 5,10-CH⁻H₄PteGlu hydrolysis proposed the existence and accumulation of a tetrahedral intermediate whose breakdown was kinetically driven to form the product 10-CHO-H₄PteGlu while thermodynamically driven to produce 5-CHO-H₄PteGlu. Numerous subsequent studies have verified and further elucidated this mechanism (Robinson and Jencks, 1967; Benkovic, 1980 and references therein). Although these studies implicate this nonenzymatic hydrolysis to be the source of intracellular 5-CHO-H₄PteGlu, the slow rate at which this occurs in vitro is not likely to account for the substantial levels of this cofactor found in vivo.

Previously, this laboratory has purified and characterized 5,10-CH⁻THF synthetase from rabbit liver (Hopkins and Schirch, 1984). This is the only known mammalian enzyme that utilizes 5-CHO-H₄PteGlu as a substrate, converting it to CH⁻H₄PteGlu in an irreversible ATP-dependent reaction.

1 The abbreviations used are: CH⁻H₄PteGlu, methenyltetrahydrofolate; C₅-THF synthase, C₅-tetrahydrofolate synthase; CH⁻THF synthetase, methenyltetrahydrofolate synthetase; THF, tetrahydrofolate; CH⁻H₄PteGlu, 5,10-methenyltetrahydropteroylglutamate containing n glutamate residues; 5-CHO-H₄PteGlu, 5-formyltetrahydropteroylglutamate containing n glutamate residues; 10-CHO-H₄PteGlu, 10-formyltetrahydropteroylglutamate containing n glutamate residues; SHMT, serine hydroxymethyltransferase; CSMT, cytosolic serine hydroxymethyltransferase; mSHMT, mitochondrial serine hydroxymethyltransferase; redSHMT, cytosolic serine hydroxymethyltransferase in which the pyridoxal phosphate aldimine has been reduced with sodium borohydride; KMES, potassium 2-(N-morpholino)ethane-sulfonate; HPLC, high performance liquid chromatography.
The discovery of this enzyme not only verified the in vivo existence of 5-CHO-H₄PteGlu but also indicated that 5,10-CH⁺-H₄PteGlu was not only an intermediate in the conversion of 5,10-CH₂-H₄PteGlu to 10-CHO-H₄PteGlu by the dehydrogenase and cyclohydrolase activities of C₇-THF synthase but rather an autonomous member of the one-carbon pool. More recently, CH⁺-H₄PteGlu has been discovered to be a cofactor of the flavin enzyme DNA photolyase (Johnson et al., 1988; Jorns et al., 1987).

Serine hydroxymethyltransferase (SHMT) catalyzes the reversible interconversion of serine and glycine with H₄PteGlu, as the one-carbon carrier. This reaction is the major source of one-carbon units in the cell in the form of 5,10-CH₂-H₄PteGlu (Schirch, 1982). Recently, this laboratory has reported the conversion of formate to serine by the coupled enzyme system of cSHMT and C₁-tetrahydrofolate synthase mediated by the coenzyme H₄PteGlu (Strong and Schirch, 1989). Further investigation of this cycle has identified a new reaction that generates 5-CHO-H₄PteGlu. In this paper, we characterize this reaction and provide evidence that it is the in vivo source of 5-CHO-H₄PteGlu.

EXPERIMENTAL PROCEDURES

Materials—Glycine, L-serine, MgATP, NADP⁺, NADPH, Na CNBH₃, glucose 6-phosphate, phosphoenolpyruvate, pyruvate kinase, glucose-6-phosphate dehydrogenase, and 2-mercaptoethanol were purchased from Sigma. H₄PteGlu and 5-CHO-H₄PteGlu were purchased from Dr. B. Schirck's laboratory in Switzerland and reduced to the tetrahydro form as described by Strong et al. (1987). The reduced pteroylglutamates were purified by DEAE-Sephadex according to the method described by Strong and Schirch (1989). All other chemicals were purchased from Fisher Scientific.

C₇-THF synthase, CH⁺THF synthetase, Escherichia coli SHMT, and the mitochondrial and cytosolic isozymes of SHMT were purified to homogeneity from fresh frozen rabbit livers as described previously (Schirch and Peterson, 1980; Villar et al., 1985; Hopkins and Schirch, 1984, 1986; Schirch et al., 1985). RedSHMT was prepared by reduction of the pyridoxal-P aldimine with NaCNBH₃ (Schirch and Mason, 1983). ApoSHMT was prepared by treatment of the holoenzyme with L-cysteine (Schirch et al., 1973).

Determination of Intracellular Concentration of 5-CHO-H₄PteGlu— Cultures, 500 ml, of E. coli strains JM105, GS245, and (GS)-5,10-CH⁺-H₄PteGlu, in 50 mM KMES, pH 7.0, for 1 h at 30 °C. The solution was acidified to pH 1.5, and the precipitated protein was removed by centrifugation. The solution was allowed to incubate at room temperature for either 2 or 3 h until the absorbance at 360 nm reached its maximum. The solution was applied to a 3 x 5-cm Florosil column that had been equilibrated with a degassed 1% 2-mercaptoethanol, 5 mM HCl solution. The column was washed with 5 column volumes of the mercaptoethanol/HCI solution, and the (GS)-5,10-CH⁺-H₄PteGlu and 5,10-CH⁺-H₄PteGlu was eluted with 40% ethanol containing 5 mM HCl.

Determination of Enzyme and H₄PteGlu Concentrations—The concentrations of all enzymes were determined by their absorbance at 280 nm (Gavilanes et al., 1982; Schirch et al., 1985). The concentrations of stock H₄PteGlu solutions were determined by a coupled enzymatic assay using cSHMT and 5,10-CH⁺-H₄PteGlu. The increase in absorbance at 340 nm, resulting from the reduction of NADP⁺ and the formation of 5,10-CH⁺-H₄PteGlu, has an extinction coefficient of 7200 M⁻¹ cm⁻¹ (Schirch, 1978).

Determination of the Concentration of Free and SHMT-bound 5-CHO-H₄PteGlu—Dissociation constants for 5-CHO-H₄PteGlu were determined by titrating a solution of SHMT and 50 mM glycine with increasing concentrations of the coenzyme and determining the increase in absorbance at 502 nm of the SHMT-Gly-5-CHO-H₄PteGlu ternary complex (Schirch and Ropp, 1966). Using an extinction coefficient of 40,000 M⁻¹ cm⁻¹ for the ternary complex the concentrations of free and bound 5-CHO-H₄PteGlu were determined from the expression for the dissociation constant as described previously (Strong and Schirch, 1989).

Enzyme Assays—Experiments performed with the coupled enzyme system of SHMT and C₇-THF synthase were performed in 50 mM KMES, pH 7.0, 10 mM 2-mercaptoethanol, 10 mM ammonium formate, and 30 mM ammonium sulfate in a 1-ml cuvette. ATP, NADPH, H₄PteGlu, and SHMT were varied according to the desired experimental conditions. The reaction was monitored by taking spectra at several time intervals between 530 and 460 nm and determining the rate of formation of the SHMT-Gly-5-CHO-H₄PteGlu complex absorbing at 502 nm. The rate of conversion of 5,10-CH⁺-H₄PteGlu to 5-CHO-H₄PteGlu by either cytosolic, mitochondrial, or E. coli SHMT was determined by following the increase in absorbance at 502 nm after the addition of ATP, NADPH, H₄PteGlu, and SHMT at several concentrations until the absorbance at 502 nm was determined with a stopped-flow absorbance spectrometer from Kinetic Instruments, Inc. Absorbance versus time data were stored on an IBM XT computer and curve fitted as described by Shostak and Schirch (1988).

HPLC Analysis—The product of 5,10-CH⁺-H₄PteGlu, hydrolysis in the presence and absence of SHMT was analyzed by C-18 reverse phase chromatography. Reaction solutions containing SHMT and coenzyme were placed in Centricon 30 filtration tubes and centrifuged at 4000 rpm for 10 min. The filtrate was injected onto a C-18 column equilibrated with 18% methanol-water containing 5 mM tetrabutylammonium phosphate as an ion pair reagent (McMartin et al., 1981). The eluates were eluted isocratically and detected by absorbance at 280 nm. The product of the reaction was compared with authentic 5-CHO-H₄PteGlu, 10-CHO-H₄PteGlu, and 5,10-CH⁺-H₄PteGlu standards.

RESULTS

Spectral Changes Occuring during the Interconversion of Formate and Serine—Recently, we have published preliminary characterization of the reactions and associated kinetic parameters involved in the reversible interconversion of for-
mate and serine (Strong and Schirch, 1989). SHMT and the
trifunctional enzyme, C1-THF synthase, catalyze this cyclic
reaction in which H4PteGlu is required at only catalytic
concentrations (Fig. 1). When SHMT is present in excess
over the trifunctional enzyme and at concentrations equal
to or greater than the concentration of the folate coenzyme,
most of the folate is present as H4PteGlu, and bound as the
SHMT-Gly-H4PteGlu, ternary complex. This complex ex-
hibits an intense absorption band at 499 nm with a molar
absorbivity coefficient of 40,000 cm⁻¹ M⁻¹ (Strong and
Schirch, 1989). The rate of the cycle can be followed by
observing the decrease in absorption of NADPH at 340 nm
because of the reduction of 5,10-CH3-H4PteGlu, to 5,10-CH2-
H4PteGlu, by the dehydrogenase activity of the trifunc-
tional enzyme. When the dehydrogenase reaction reaches equilib-
rium as the result of the depletion of NADPH, the H4PteGlu,
pool decreases with a concomitant increase in 10-CHO-
H4PteGlu, concentration. The conversion of H4PteGlu to 10-
CH10-11PteGlu, results in the disappearance of the SHMT-
Gly-H4PteGlu, ternary complex absorbing at 492 nm. Also,
because of the cytidolylactase activity of the trifunc-
tional enzyme, 10-CHO-H4PteGlu, is in equilibrium with a small
amount of 5,10-CH3-H4PteGlu.
When the dehydrogenase reaction reaches equilibrium the
decrease in absorbance at 492 nm, to nearly base-line levels,
occur in a few min. However, during the next 70 min a new
absorption band at 502 nm appears which exceeds the inten-
sity of the original 492 nm band (Fig. 2). Previously, we
have shown that cSHMT forms E-Gly ternary complexes with
both 6-CH3-H4PteGlu and 5 CHO-H4PteGlu, which exhibit
absorption maxima at 502 nm (Schirch and Ropp, 1966). As
shown in the next section, we have identified the folate species
in the 502 nm absorbing complex as 5-CHO-H4PteGlu,. The
spectral results shown in Fig. 2 suggest that in the cycle,
shown in Fig. 1, H4PteGlu, is being converted to stoichiomet-
ric amounts to 5-CHO-H4PteGlu,. The observation that the
absorbance at 502 nm is greater than the original absorbance
at 492 nm is the result of a lower Kd (10 μM) of 5-CHO-
H4PteGlu, for the ternary complex compared with the Kd of
H4PteGlu, (15 μM).
If the metabolic reactions shown in Fig. 1 are the in vivo
source of 5 CHO-H4PteGlu, then these reactions must also
occur with the polymerglutamate forms of the coenzyme at sig-
nificant rates in the presence of physiological concentrations
of cSHMT (18 μM) and C1-THF synthase (6 μM) (Strong and
Schirch, 1989). Fig. 3 compares the rate of increase in absorb-
bance at 502 nm for 10 μM solutions of the monogluta-
mate and the pentaglutamate forms of H4PteGlu, with 10 μM cSHMT
and 5 μM C1-THF synthase using the conditions of the met-
abolic cycle described under "Experimental Procedures," ex-
cept the NADPH was omitted (Strong and Schirch, 1989).
The pentaglutamate form of the coenzyme is converted to the
species resulting in the absorbance of 502 nm 15-fold more
rapidly than the monoglutamate. The inset of Fig. 3 shows
that the rate of formation of the complex absorbing at 502
nm is linearly dependent upon the cSHMT concentration,
but the rate does not change with increasing C1-THF synthase
concentration.
Identification of the Folate Compound in the Complex
Absorbing at 502 nm—To identify the folate species formed in
the cyclic system shown in Fig. 1, 25 nmol of H4PteGlu, was
incubated with cSHMT, formate, glycine, and C1-THF syn-
thase with limiting NADPH and an ATP-regenerating system
for 2 h. After removing the proteins by filtration, the folate
species was purified on a Florosil column that removed
NADP⁺ and ATP. This partially purified folate compound
was shown to form a complex with cSHMT and glycine which
absorbed at 502 nm. A spectrum of the folate compound was
shown to be identical to the spectrum of 5-CHO-H4PteGlu, (maximum
absorption at 287 nm). Upon acidification to pH 2 with HCl,
the folate compound was converted to a species exhibiting an absorption maximum at 360 nm, which is characteristic of 5,10-CH\(^+\)-H\(_2\)PteGlu. The unknown folate compound eluted from an HPLC C-18 column at the position and with the peak shape of authentic 5-CHO-H\(_2\)PteGlu. In this chromatographic system, 5-CHO-H\(_2\)PteGlu is resolved from other folate compounds (McMartin et al., 1981). The unknown folate also served as a substrate for pure CH\(^+\)THF synthetase in the presence of ATP (Hopkins and Schirch, 1984). These results confirm that in the cyclic system the spectral changes observed and recorded in Fig. 2 are the result of the conversion of H\(_2\)PteGlu to 5-CHO-H\(_2\)PteGlu.

The conversion of 5-CHO-H\(_2\)PteGlu to CH\(^+\)-H\(_2\)PteGlu, by CH\(^+\)THF synthetase provides a rapid and convenient method for determining the concentration of 5-CHO-H\(_2\)PteGlu. In the reaction described in the previous paragraph we obtained 17 nmol of 5-CHO-H\(_2\)PteGlu or a 70% yield. The intermediate 10-CHO-H\(_2\)PteGlu, is very unstable, and degradation probably accounts for the less than quantitative conversion.

Components of the Cycle Required for Formation of 5-CHO-H\(_2\)PteGlu,——In order to determine if all components of the metabolic cycle (Fig. 1) are required for the conversion of H\(_2\)PteGlu, to 5-CHO-H\(_2\)PteGlu, each component of the cycle was removed systematically. All components were required except NADPH. This suggested that the sequence of reactions was the conversion of H\(_2\)PteGlu, and fromate to 10-CHO-H\(_2\)PteGlu, and CH\(^+\)-H\(_2\)PteGlu, by the synthetase and cyclohydrolase activities of CY-THF synthase followed by conversion of one or both of these products to 5-CHO-H\(_2\)PteGlu, by cSHMT. Using each of these potential substrates in the assay system showed that cSHMT catalyzed the formation of 5-CHO-H\(_2\)PteGlu, only from CH\(^+\)-H\(_2\)PteGlu.

Previously, cSHMT has been shown to exhibit broad substrate and reaction specificity, catalyzing not only the aldol cleavage of many 3-hydroxyamino acids, but also decarboxylation, transamination, and racemization reactions (Schirch, 1982; Shostak and Schirch, 1988). All of these reactions involve the amino acid substrate. The hydrolysis of CH\(^+\)-H\(_2\)PteGlu is the first example of cSHMT catalyzing an alternate reaction involving the folate coenzyme. Since the amino acid substrates glycine and serine and the bound pyridoxal-P are not directly involved in the folate binding site, we expected that these ligands would not be required for the hydrolysis of CH\(^+\)-H\(_2\)PteGlu. However, cSHMT in the absence of glycine did not catalyze the hydrolysis of CH\(^+\)-H\(_2\)PteGlu. This prompted a more detailed study of the ligand requirements for the reaction. Table I lists the components that were added to cSHMT, with their ability to form a complex absorbing near 500 nm and their ability to support the formation of 5-CHO-H\(_2\)PteGlu, from CH\(^+\)-H\(_2\)PteGlu. The results show that in addition to cSHMT, either glycine, D-alanine, or L-alanine was required as ligand. D-Alanine is a glycine analog and is a product of the cleavage of α-methylerynine. D-Alanine forms a complex absorbing at 505 nm and undergoes a slow transamination reaction with the enzyme (Schirch and Jenkins, 1964). E. coli SHMT also forms a quinonoid complex with L-alanine which undergoes transamination (Shostak and Schirch, 1988). This complex also supports the hydrolysis of CH\(^+\)-H\(_2\)PteGlu, to 5-CHO-H\(_2\)PteGlu.

Pyridoxal-P is required for the hydrolytic reaction since neither apoSHMT nor enzyme in which the coenzyme had been reduced with NaCNBH\(_3\) was capable of catalyzing the reaction. L-Serine could not replace glycine in this system. The results shown in Table I suggest that the ability to form a quinonoid ternary complex absorbing near 500 nm is required for SHMT catalysis of this reaction.

Purified rabbit liver mitochondrial SHMT and E. coli SHMT also catalyze the hydrolysis of CH\(^+\)-H\(_2\)PteGlu, to 5-CHO-H\(_2\)PteGlu, and have the same ligand requirements as the cytosolic enzyme.

Kinetic Properties of the Enzyme-catalyzed Reaction——Since the cSHMT-Gly, 5-CHO-H\(_2\)PteGlu, ternary complex absorbs at 502 nm, this property was used to determine directly the rate of conversion of CH\(^+\)-H\(_2\)PteGlu, to 5-CHO-H\(_2\)PteGlu, by solutions of cSHMT and glycine. The results, as recorded in Fig. 4, show the spectra of a mixture of cSHMT, glycine, and CH\(^+\)-H\(_2\)PteGlu, determined with a diode array spectrophotometer. Aliquots of less than 10 µl of chemically synthesized CH\(^+\)-H\(_2\)PteGlu stock solutions at pH 2.0, 40 °C, were rapidly added to the enzyme-glycine buffered solution. The first recorded spectra taken 10 s after mixing show a large absorption peak at 502 nm. Later spectra show a slower increase in this absorption band. The inset to Fig. 4 shows how the absorbance at 502 nm varies with time after mixing of all reagents. The kinetic curve is clearly biphasic with a rapid phase that is over in less than 30 s followed by a slow phase that takes up to 1 h to reach completion. The burst phase exhibits first order kinetics with a half-life of 0.4 s at 30 °C.

The burst in formation of the complex absorbing at 502 nm (Fig. 4, inset) could be the result of some unconverted 5-CHO-H\(_2\)PteGlu, in the CH\(^+\)-H\(_2\)PteGlu, solutions. This is a possibility since the CH\(^+\)-H\(_2\)PteGlu, was made from acidifying solutions of 5-CHO-H\(_2\)PteGlu. However, the amplitude of the burst was the same with CH\(^+\)-H\(_2\)PteGlu, made by acidifying.

<table>
<thead>
<tr>
<th>Enzyme and substrate</th>
<th>Observed quinonoid complex (A(_{max}))</th>
<th>5-CHO-H(_2)PteGlu formed as a product</th>
</tr>
</thead>
<tbody>
<tr>
<td>cSHMT</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>cSHMT-Gly</td>
<td>Yes (492 nm)</td>
<td>Yes</td>
</tr>
<tr>
<td>cSHMT-Ser</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>cSHMT-D-Ala</td>
<td>Yes (505 nm)</td>
<td>Yes</td>
</tr>
<tr>
<td>cSHMT-L-Ala</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>E. coli SHMT-D-Ala</td>
<td>Yes (505 nm)</td>
<td>Yes</td>
</tr>
<tr>
<td>cSHMT-aminomethyl-phosphonate</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>RedSHMT-Gly</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>apoSHMT-Gly</td>
<td>No</td>
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</tr>
<tr>
<td>Cy-THF synthase-Gly</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>mSHMT-Gly</td>
<td>Yes (492 nm)</td>
<td>Yes</td>
</tr>
<tr>
<td>E. coli SHMT-Gly</td>
<td>Yes (495 nm)</td>
<td>Yes</td>
</tr>
</tbody>
</table>
fying solutions of 10-CHO-H,PteGlu. Also, preincubating neutral solutions of CH'-H,PteGlul with ATP and CH'THF synthetase did not decrease the size of the burst upon addition of SHMT and glycine. These results suggest strongly that the initial burst of absorbance at 502 nm is not due to residual 5-CHO-H,PteGlul, but rather to another folate compound in these solutions which can serve as a substrate in this reaction.

It was next determined whether the initial burst of absorbance at 502 nm was the result of either the formation of an E-Gly-CH'-H,PteGlul complex or represented an E-Gly-5-CHO-H,PteGlul complex. This was accomplished by stopping the reaction after 30 s by adding cold ethanol and removing the denatured enzyme by centrifugation. After removal of the ethanol the resulting solution was assayed for 5-CHO-H,PteGlul by adding ATP and CH'THF synthetase and observing the increase in absorbance at 360 nm. The results showed that 5-CHO-H,PteGlul had been formed in an amount predicted from the amplitude of the burst. Under the same experimental conditions, CH'-H,PteGlul, in the absence of enzyme gave no detectable 5-CHO-H,PteGlul. We conclude that the burst in absorbance at 502 nm is the result of formation of either 5-CHO-H,PteGlul or an intermediate that forms this compound nonenzymatically when removed from the enzyme. The burst is not the result of simply binding the CH'-H,PteGlul.

To confirm that CH'-H,PteGlul was a substrate for the SHMT-catalyzed reaction, samples of CH'-H,PteGlul in a solution of 50 mM glycine were first converted to 10-CHO-H,PteGlul by adding the cyclohydrase activity of Cs-THF synthase at pH 7.5. The hydrolysis of CH'-H,PteGlul, to 10-CHO-H,PteGlul, was followed by observing the decrease in absorbance at 360 nm. When the absorbance at 360 nm had reached a minimum value, cSHMT was added and the reaction monitored at 502 nm. A burst of absorbance was observed which had the same amplitude as an experiment in which the CH'-H,PteGlul, had not been preincubated with the CH'THF synthase. However, there was no slow phase increase in absorbance. These results suggest that the formation of 5-CHO-H,PteGlul, in the slow phase is the result of the hydrolysis of the CH'-H,PteGlul, and that the burst is the result of some other folate structure being converted to 5-CHO-H,PteGlul. This was confirmed further by titrating neutral solutions of CH'-H,PteGlul, (50 nmol of a (6R, 6S)-mixture) with increasing amounts of cSHMT-Gly (up to 80 nmol) (Fig. 5). The amount of 5-CHO-H,PteGlul formed in the burst was 5 nmol or about 20% of the enzymatically active isomer. The addition of C1-THF synthase to the neutral solutions of CH'-H,PteGlul did not reduce the amount of 5-CHO-H,PteGlul, in the burst phase of the reaction (Fig. 5). The unknown folate compound that gives rise to the burst phase of the reaction is stable at pH 7 for at least several days at 4°C.

The rate of formation of the complex absorbing at 500 nm upon mixing solutions of CH'-H,PteGlul with either mitochondrial or E. coli SHMT was repeated as described in the previous paragraph for cSHMT. Both mSHMT and E. coli SHMT showed biphasic kinetics; however, there were differences in the relative rates of the fast and slow phases compared with the cytosolic enzyme. Preincubation of the CH'-H,PteGlul, used in these assays with C1-THF synthase blocked the slow phase of the reaction but not the fast phase for each enzyme. Therefore, both the mitochondrial and E. coli enzymes seem to catalyze the hydrolysis of CH'-H,PteGlul, by the same mechanism as the cytosolic enzyme.

Role of SHMT in Forming 5-CHO-H,PteGlul, in Vivo—To determine if the SHMT-catalyzed hydrolysis of CH'-H,PteGlul, to 5 CHO-H,PteGlul, is the physiological source of this form of the coenzyme, the levels of 5-CHO-H,PteGlul, in three strains of E. coli were determined. One strain, GS245, is glyA negative and lacks any functional SHMT due to a deletion in the glyA gene (Stauffer et al., 1981). This strain, transformed with a plasmid carrying the glyA gene, results in a 25-fold increase in the expression of SHMT over wild-type strains of E. coli (Schirch et al., 1983). The third strain used was wild-type JM105, which does not contain the overproducing plasmid and produces normal levels of functional SHMT. Each strain was grown under identical conditions and the cell contents analyzed for the presence of 5-CHO-H,PteGlul, using the enzyme 5,10 CH'THF synthetase. The GS245 cells, which lack a functional SHMT, contained less than 0.2 nmol of 5-CHO-H,PteGlul, per g of cells, wet weight, which was the detection limit for 5-CHO-H,PteGlul (Table II). Wild-type JM105 E. coli cells were found to contain 0.9 nmol of 5-CHO-H,PteGlul, per g of cells, wet weight. The GS245 bacteria containing the overproducing plasmid were found to contain 22 nmol of 5-CHO-H,PteGlul, or more than 20 times the level found in the JM105 cells.

The presence of 5-CHO-H,PteGlul, occurring only in the E. coli cells that contain a functional SHMT is consistent with the SHMT-catalyzed hydrolysis being the in vivo source of this form of the coenzyme. However, bacteria also contain the enzyme CH'THF synthetase, which catalyzes the irreversible conversion 5-CHO-H,PteGlul, to CH'-H,PteGlul, (Grimshaw et al., 1984). This raises the question of why a pool of 5-CHO-H,PteGlul, exists in cells containing the synthetase. One possible explanation is that 5-CHO-H,PteGlul, bound in the E. coli SHMT-Gly-5-CHO-H,PteGlul, ternary complex is not available for the synthetase because of its slow rate of dissociation from SHMT. To test this hypothesis the off rate of 5-CHO-H,PteGlul, from the E. coli SHMT-Gly-5-CHO-H,PteGlul, ternary complex was determined by flowing the ternary complex in a stopped-flow spectrophotometer.

### Table II

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>5-CHO-H,PteGlul, nmol/g cells</th>
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<tbody>
<tr>
<td>JM101</td>
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<td>GS245</td>
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against excess PteGlu. The rate of decrease in absorbance at 502 nm was first order, with a $t_\text{1/2}$ of 14 s. When this experiment was repeated with cSHMT, the off rate of 5-CHO-H$_4$PteGlu had a $t_\text{1/2}$ of 1.5 s. However, when the experiment was repeated with cSHMT-Gly-H$_2$PteGlu, the $t_\text{1/2}$ was 16 s. This slow rate of dissociation of 5-CHO-H$_4$PteGlu from the enzyme-glycine ternary complex would protect this form of the coenzyme from rapid cleavage by CH$^+$THF synthetase.

**DISCUSSION**

The in vivo interrelationships among the many derivatives of tetrahydrofolate, and the enzymes associated with their interconversion, are still poorly understood. It seems unlikely, however, that a significant concentration of the folate pool would be in a form that was metabolically unimportant and would play a passive role in one-carbon homeostasis. Yet, this has been proposed to be the case for 5-CHO-H$_4$PteGlu. This is the most stable form of reduced folates and has been found in significant concentrations in nearly all cells that have been studied. Identification of the physiological source of 5-CHO-H$_4$PteGlu may begin to provide some insight into the role this cofactor plays in the regulation of one-carbon metabolism in the cell. The results in this paper show that the combined catalytic properties of C$_i$-THF synthase and SHMT can convert H$_2$PteGlu and formate to 5-CHO-H$_4$PteGlu.

Of primary importance in this study is to determine if the hydrolysis of CH$^+$-H$_4$PteGlu, by SHMT is the physiological source of 5-CHO-H$_4$PteGlu. The most important evidence in support of this possibility is that E. coli cells, which do not contain a functional SHMT, have little or no 5-CHO-H$_4$PteGlu, but wild-type cells and cells with an increased level of SHMT do have 5-CHO-H$_4$PteGlu. (Table II). In a similar study Cossins and co-workers (1976) have described two mutants of N. crassa which have low levels of SHMT. In these mutants there is a 75% decrease in the cellular concentration of 5-CHO-H$_4$PteGlu, (Cossins et al., 1976). Additional evidence supporting the SHMT reaction as the physiological source of 5-CHO-H$_4$PteGlu, is that the reaction occurs fast enough with the polyglutamate forms of the coenzyme to account for the intracellular concentrations. Also, the observation that the SHMT-bound 5-CHO-H$_4$PteGlu, dissociates very slowly accounts for why CH$^+$THF synthetase does not deplete the intracellular pool of this form of the coenzyme. The data suggest that the 5-CHO-H$_4$PteGlu, pools in the cell are bound to SHMT. There is enough SHMT in rabbit liver (19 pm), L1210 cells (9 pm), and E. coli cells in vivo to support this argument. Also, E. coli cells which overproduce SHMT by 25-fold have more than a 20-fold increase in their cellular pools of 5-CHO-H$_4$PteGlu, (Table II).

Rabbit liver cytosolic SHMT catalyzes the hydrolysis of CH$^+$-H$_4$PteGlu, only in the presence of glycine or D-alanine (Table I). These two amino acids are unique in that they form an amino acid anionic complex with the bound pyridoxal-P which absorbs near 500 nm (Schirch, 1982). In this intermediate both glycine and D-alanine have lost their 2 S proton, which resides on a putative active site base. Either this proton or the resonance-stabilized amino acid anion must play an important role in the mechanism of the hydrolysis of CH$^+$-H$_4$PteGlu. This is supported by the observation that glycine cannot be replaced by aminomethylphosphonate, an analog of glycine that can form an external aldime with pyridoxal-P but does not lose its pro-2 S proton to form the complex absorbing near 500 nm. The data also explain the requirement for pyridoxal-P since this coenzyme is essential both in form-

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ing an external aldime with glycine and in stabilizing the glycine anion after loss of its pro-2 S proton. Of interest was the observation that L-alanine also forms a ternary quinonoid complex with E. coli SHMT and 5-CHO-H$_4$PteGlu. The E. coli enzyme also catalyzes the conversion of CH$^+$-H$_4$PteGlu, to 5-CHO-H$_4$PteGlu, in the presence of L-alanine, confirming the need of the quinonoid complex in the mechanism of this reaction.

When chemically synthesized CH$^+$-H$_4$PteGlu, is used as substrate for SHMT there is a burst in appearance of either 5-CHO-H$_4$PteGlu, or a compound that is rapidly hydrolyzed nonenzymatically to 5-CHO-H$_4$PteGlu, (Fig. 4). This compound is neither CH$^+$-H$_4$PteGlu, nor an intermediate in its hydrolysis to 10-CHO-H$_4$PteGlu, by the cyclohydrolase activity of C$_i$-THF synthase. However, it accounts for 20% of the available folate in the solution. Much work has been done on the nonenzymatic hydrolysis of CH$^+$-H$_4$PteGlu, to 10-CHO-H$_4$PteGlu, by several investigators (Benkovic, 1980). These studies have shown that a hydrated intermediate is formed at C-11. What is not discussed in these papers is that this hydrated intermediate forms a new chiral center, and therefore, two possible hydrated intermediates exist. Probably only one of these hydrated intermediates is on the pathway for hydrolysis to 10-CHO-H$_4$PteGlu, by C$_i$-THF synthase. It may be that the compound that gives a burst is the wrong isomer. However, it is not readily evident why this intermediate would be stable at pH 7. We are currently pursuing the isolation of the compound that gives rise to the burst phase of the reaction. In the experiments by Cossins and co-workers (1976) on the N. crassa mutants depleted in SHMT an unknown folate compound was found to exist in these cells. It may be that this is the same compound that gives rise to the burst phase of our reaction with SHMT.

Scheme 1 shows a set of reactions that explain our observations on the relationship of SHMT and CH$^+$-THF synthetase in the formation and utilization of 5-CHO-H$_4$PteGlu, in vivo. In this scheme 5-CHO-H$_4$PteGlu, (structure I) binds to

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CH\(^+\)-THF synthetase and is converted to bound CH\(^-\)H\(_2\)PteGlu (structure II), which dissociates from the enzyme. The free CH\(^-\)H\(_2\)PteGlu, then reacts with water to form a hydrated intermediate (structure IV) and another stable structure, which we show here as a second hydrated isomer (structure III). One of these (structure IV) is converted to 10-CHO-H\(_4\)PteGlu, either concomitantly or by the cyclohydrolase activity of C\(_7\)-THF synthase. The other isomer (structure III) is relatively stable and binds to SHMT where it is converted rapidly to 5-CHO-H\(_2\)PteGlu in what is the burst phase of the reaction. The compound represented by structure III represents about 20% of the total reduced folate in pH 2.0 solutions of CH\(^-\)H\(_2\)PteGlu. SHMT can also bind CH\(^-\)H\(_2\)PteGlu, and convert it in a slow step to the putative hydrated intermediate (structure III).

There are several possibilities why a hydrated intermediate such as structure III might be stable at pH 7. Microscopic reversibility would require that the stable intermediate III be formed from either structure IV or CH\(^+\)H\(^-\)PteGlu. Factors that might contribute to the formation of a stable intermediate are either the inversion of the lone electron pair on N\(_6\) or the formation of an ylide intermediate (Poe and Benkovic, 1980; Burdick et al., 1977). However, the stable intermediate may also be a tautomer of structure II in which the double bond has migrated to the 5-6 position giving a methylendioxyfolate derivative.

Although little work has been done on the mechanism of H\(_2\)PteGlu, in the SHMT reaction some studies have been done on model systems and suggest what some of the intermediates must be in the transfer of the one-carbon group from CH\(_2\)H\(_2\)PteGlu, (structure VI in Scheme 2) to glycine via a free formaldehyde (Kallen and Jencks, 1966). A critical step in this mechanism is the acid-catalyzed conversion of CH\(_2\)H\(_2\)PteGlu, to the 5-iminium cation (structure VII in Scheme 2). This step requires the transfer of a proton to N\(_6\)

The iminium cation reacts with water to form the \(\text{CHO-H}_2\text{PteGlu}\), although not shown, the breakdown of \(\text{N}^0\)-hydroxymethyl \(\text{H}_2\text{PteGlu}\) to form formaldehyde (structure VIII) would require a base-catalyzed removal of a proton (B), which could be the same as the base required in the breakdown of the hydrated intermediate to form 5-CHO-H\(_2\)PteGlu. The requirement for the same acid-base groups at the same positions in the active site suggests that SHMT may catalyze the hydrolysis of CH\(^-\)H\(_2\)PteGlu, by essentially the same mechanism as it would catalyze the conversion of CH\(_2\)H\(_2\)PteGlu, to H\(_2\)PteGlu, and formaldehyde. This mechanism could explain the need for the quinone-oxido complex if, for example, the acid group that protonates \(\text{N}^0\) gets its proton from glycine. The model assumes that free formaldehyde is an intermediate in the SHMT-catalyzed reaction. However, there is little direct evidence for this mechanism, and other mechanisms not involving a free formaldehyde intermediate are possible.

REFERENCES