Evidence for the Accumulation of a Stable Intermediate in the Nonenzymatic Hydrolysis of 5,10-Methenyltetrahydropteroylglutamate to 5-Formyltetrahydropteroylglutamate†

Patrick Stover and Verne Schirch*

Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University, Richmond, Virginia 23298

Received August 9, 1991; Revised Manuscript Received October 18, 1991

ABSTRACT: Solutions of 5,10-methenyltetrahydropteroylglutamate can be converted to a stable hydrated adduct by heating solutions at 50 °C at pH values of 3–5 for several hours. The adduct is stable at pH values from 4 to 9 for hours, but at pH values below 2 it is converted to 5,10-methenyltetrahydropteroylglutamate and at pH values above 8 it is converted to 5-formyltetrahydropteroylglutamate. Arguments are presented that the adduct is (11*R*)-5,10-hydroxymethylenetetrahydropteroylglutamate formed from (11*S*)-5,10-hydroxymethylenetetrahydropteroylglutamate by formation of an ylide at C-11 which undergoes inversion of the electron pair to form the (11*R*) isomer. The (11*R*) hydrated adduct is believed to be the isomer of 5,10-methenyltetrahydropteroylglutamate referred to as anhydroleucovorin B by Cosulich et al. [Cosulich, D. C., Roth, B., Smith, J. M., Hultquist, M. E., & Parker, R. P. (1952) *J. Am. Chem. Soc. 74*, 3252–3263]. In addition, a new mechanism for the formation of 5-formyltetrahydropteroylglutamate from either 5,10-methenyltetrahydropteroylglutamate or 10-formyltetrahydropteroylglutamate via (11*R*)-5,10-hydroxymethylenetetrahydropteroylglutamate is proposed. A requirement for this pathway is that the formyl proton of 10-formyltetrahydropteroylglutamate exchange with solvent protons. The exchange of this formyl proton was observed at all pH values from 5.5 to 11.5 at a rate which exceeded by more than an order of magnitude the rate of formation of 5-formyltetrahydropteroylglutamate.

Tetrahydropteroylglutamate (H_4PteGlu)[1] is the principal physiological carrier of one-carbon groups, being capable of carrying one-carbon units at the oxidation levels of methanol, formaldehyde, and formate. There are three one-carbon derivatives of H_4PteGlu at the oxidation level of formate that exist in chemical equilibrium: 10-CHO-H_4PteGlu, 5,10-CH^+-H_4PteGlu, and 5-CHO-H_4PteGlu. While all three of these compounds are members of the cellular folate pool, only 10-CHO-H_4PteGlu, which supplies C^2 and C^8 in purine ring biosynthesis, serves in vivo as a one-carbon donor (Rowe, 1984).

The nonenzymatic reversible interconversion of the three principal formyl derivatives of H_4PteGlu, first identified by May et al. (1951), has received extensive study and is the prototype of the amidine hydrolysis reaction (for review see Benkovic, 1978). Previous studies have suggested that 5,10-CH^+-H_4PteGlu is hydrolyzed at C^{11}, producing an equilibrium mixture of 5-CHO-H_4PteGlu and 10-CHO-H_4PteGlu through a common hydrated intermediate (Robinson & Jencks, 1967; Benkovic et al., 1972). Breakdown of this intermediate requires protonation of either N^{10} or N^5 and subsequent formation of a free amine. In this reaction, formation of the thermodynamically favored product, 5-CHO-H_4PteGlu, is compromised in favor of the more kinetically accessible product, 10-CHO-H_4PteGlu. This has been attributed to the relative macroscopic pK_a difference of 6.1 between N^5 (4.8) and N^{10} (−1.3) (Benkovic et al., 1972).

While the mechanistic pathway of 5,10-CH^+-H_4PteGlu hydrolysis to 10-CHO-H_4PteGlu, as described by Robinson and Jencks (1967), is widely accepted, there remains some controversy as to the optimal conditions, and therefore mechanism, resulting in the formation of 5-CHO-H_4PteGlu. Initial studies of this reaction reported that optimal accumulation of 5-CHO-H_4PteGlu from 5,10-CH^+-H_4PteGlu occurs under conditions of extreme heat at neutral or highly alkaline pH (May et al., 1951; Cosulich et al., 1952). More recently, studies by Temple et al. (1979) suggest that formation of 5-CHO-H_4PteGlu occurs optimally under slightly acidic conditions during prolonged reflux. In this latter study, the reaction was performed in the absence of buffers. In addition, apparently forgotten in the literature was the discovery of anhydroleucovorin B, an isoform of 5,10-CH^+-H_4PteGlu identified by Cosulich et al. (1952) that has not been addressed in any subsequent literature pertaining to formyltetrahydrofolate chemistry.

Recently we have demonstrated the catalytic hydrolysis of 5,10-CH^+-H_4PteGlu to 5-CHO-H_4PteGlu by serine hydroxymethyltransferase (SHMT). The reaction exhibits biphasic kinetics with an initial rapid burst of product formation, followed by a slow linear steady-state rate (Stover & Schirch, 1990). The amplitude of the burst phase was demonstrated to be saturable by titration with increasing amounts of SHMT, excluding the possibility that the burst in formation of 5-CHO-H_4PteGlu was a property of the enzyme. This suggested

† This work was supported by Grant GM 28143 from the National Institutes of Health.

* To whom correspondence should be addressed.

[1] Abbreviations: SHMT, serine hydroxymethyltransferase; H_4PteGlu, tetrahydropteroylglutamate; 5,10-CH^+-H_4PteGlu, 5,10-methenyltetrahydropteroylglutamate; 5-CHO-H_4PteGlu, 5-formyltetrahydropteroylglutamate; 10-CHO-H_4PteGlu, 10-formyltetrahydropteroylglutamate; 5,10-CHOH-H_4PteGlu, 5,10-hydroxymethylenetetrahydropteroylglutamate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; MES, 2-[*N*-morpholino]ethanesulfonic acid; CHES, 2-[*N*-cyclohexylamino]ethanesulfonic acid; CAPS, (cyclohexylamino)propanesulfonic acid; KBES, potassium *N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonate.

0006-2960/92/0431-2148$03.00/0

that the observed biphasic kinetics were the result of an unknown compound in equilibrium with the parent substrate 5,10-CH^+-H_4PteGlu that was preferentially converted to 5-CHO-H_4PteGlu. In our efforts to understand the SHMT-catalyzed mechanism of formation of 5-CHO-H_4PteGlu, we have reinvestigated the nonenzymatic reaction. In this paper, we propose a mechanism for the nonenzymatic formation of 5-CHO-H_4PteGlu from either 10-CHO-H_4PteGlu or 5,10-CH^+-H_4PteGlu through a common tetrahedral intermediate distinct from the one described by Robinson and Jencks (1967). In addition, we provide evidence that this intermediate is the stable derivative of CH^+-H_4PteGlu (anhydroleucovorin B) previously identified by Cosulich et al. (1952).

Experimental Procedures

Materials. Glycine, MgATP, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, and 2-mercaptoethanol were purchased from Sigma. (6*R*,*S*)-H_4PteGlu and (6*R*,*S*)-5-CHO-H_4PteGlu were purchased from Fluka and used without further purification. [^{13}C]Formate and H_2[^{18}O] were obtained from MSD Isotopes, and D_2O, 99.8%, was purchased from Aldrich. Isolation of cSHMT, C_1-tetrahydrofolate synthase, methenyltetrahydrofolate synthetase, and preparation of (6*S*)-5-CHO-H_4PteGlu and (6*R*)-5,10-CH^+-H_4PteGlu were performed as previously described (Stover & Schirch, 1990).

Folate Assays. The concentration of 5,10-CH^+-H_4PteGlu was determined from its absorbance at 360 nm using an extinction coefficient of 25 100 M^{-1} cm^{-1} (Temple & Montgomery, 1984). 5-CHO-H_4PteGlu concentration was determined by its ATP-dependent enzymatic conversion to 5,10-CH^+-H_4PteGlu using the methenyl synthetase assay as described previously (Stover & Schirch, 1991).

Determination of the concentration of (6*R*,11*R*)-5,10-CHOH-H_4PteGlu, the putative compound that gives rise to the burst phase of the SHMT-catalyzed hydrolysis of 5,10-CH^+-H_4PteGlu to 5-CHO-H_4PteGlu, was performed by incubating aliquots of folate solutions to be tested in 1 mL of 50 mM KBES, pH 7.5, containing 20 mM glycine, 10 mM 2-mercaptoethanol, 2 μM (220 μg) C_1-tetrahydrofolate synthase, 0.5 μM (14 μg) methenyl synthetase, and 1 mM MgATP for several minutes. The synthase converts any remaining 5,10-CH^+-H_4PteGlu to 10-CHO-H_4PteGlu, and the methenyl synthetase converts any 5-CHO-H_4PteGlu to 5,10-CH^+-H_4PteGlu. The absorbance was zeroed at 502 nm, and 2 mg of SHMT was added. The burst in absorbance, due to the formation of the SHMT·Gly^-·5-CHO-H_4PteGlu ternary complex, was determined during the first 30 s. The amplitude of the A_{502} burst is a measure of (6*S*)-5-CHO-H_4PteGlu formed from the putative (6*R*,11*R*)-5,10-CHOH-H_4PteGlu. A K_d of 10 μM and an extinction coefficient of 40 000 M^{-1} cm^{-1} was used to calculate total 5-CHO-H_4PteGlu concentration formed from the burst in absorbance at 502 nm (Stover & Schirch, 1991).

Preparation of 5-[^{13}C]HO-H_4PteGlu. (6*R*,*S*)-5-[^{13}C]HO-H_4PteGlu was prepared by reacting a 10-fold molar excess of [^{13}C]formate with 25 mg of (6*R*,*S*)-H_4PteGlu using a carbodiimide coupling reagent as described by Moran and Coleman (1982). Upon completion of the reaction, the solution was neutralized with 6 N ammonium hydroxide and diluted with three volumes of *N*,*N*-dimethylformamide. The diluted solution was applied to a 1- × 5-cm neutral alumina column that was previously equilibrated with *N*,*N*-dimethylformamide, and the column was washed with three column volumes of 90% ethanol followed by three column volumes of water. The pure 5-^{13}CHO-H_4PteGlu was eluted from the column using a linear gradient consisting of 50 mL of water and 50 mL of 1 N NH_4OH as the eluting solvent. The fractions containing the purified compound, identified by absorbance spectra, were pooled and lyophilized.

Preparation of 10-CH[^{18}O]-H_4PteGlu. (6*R*,*S*)-5,10-CH^+-H_4PteGlu was made by incubating (6*R*,*S*)-5-CHO-H_4PteGlu at pH 1.5 and 4 °C for 48 h. The crystalline CH^+-H_4PteGlu was collected by centrifugation and the crystals dissolved in a minimal volume of warm, deoxygenated H_2O. The CH^+-H_4PteGlu was recrystallized 2 times at 0 °C. The crystals were dissolved in dimethyl sulfoxide and two aliquots (0.25 μmol each) of 5,10-CH^+-H_4PteGlu were placed in a 1-mL vial and dried under vacuum. The samples were redissolved with 200 μL of H_2[^{18}O] and 10 μL of 2-mercaptoethanol and made pH 10.0 with powdered NaOH. The solutions were converted to 10-CHO-H_4PteGlu by incubation at 4 °C for 30 min. The samples were frozen and lyophilized.

[^{18}O] *Exchange Experiments.* 10-CH[^{18}O]-H_4PteGlu samples were dissolved in 200 μL of degassed 100 mM K_2HPO_4 or 100 mM KH_2HPO_4 in 10 mM 2-mercaptoethanol and immediately placed in a water bath at 60 °C under anaerobic conditions for several hours to effect conversion to 5-CHO-H_4PteGlu. The product, 5-CHO-H_4PteGlu, was purified on a 5- × 25-cm Bio-Gel P-2 column equilibrated with H_2O. Purified 5-CHO-H_4PteGlu was the first UV-absorbing material to elute from the column. The overall yields of 5-CHO-H_4PteGlu from 5,10-CH^+-H_4PteGlu were 42% and 35% for the K_2HPO_4- and KH_2PO_4-treated samples, respectively.

Mass spectra were acquired on a dual quadrupole mass spectrometer constructed from components from Extrel Corporation (Pittsburgh, PA) that has been described elsewhere (Wysocki et al., 1991). The samples were added to the matrix (glycerol) on the probe tip and were bombarded with 6 keV of Cs^+ ions (Antek).

Determination of Rate Constants for 5-CHO-H_4PteGlu Formation. The rate of formation of (6*S*)-5-CHO-H_4PteGlu from solutions containing equilibrium mixtures of (6*R*)-10-CHO-H_4PteGlu and (6*R*)-5,10-CH^+-H_4PteGlu were determined over the pH range 5.5–12 in solutions containing 10 mM 2-mercaptoethanol and 100 mM sulfonate buffer (MES, pH 5.5; HEPES, pH 7–9; CHES, pH 9–10; CAPS, pH 10–11). Experiments were performed by dissolving 2 μmol of 5,10-CH^+-H_4PteGlu, pH 1.2, in 1 mL of the desired argon-purged buffer, adjusting the pH as necessary with 6 N KOH, sealing the vessel under argon, and allowing the formyl tetrahydrofolates to equilibrate on ice for 60 min. The vessel was then placed in a water bath at 75 °C, and 10-μL aliquots were removed with time and assayed for 5-CHO-H_4PteGlu. The value of k_{obs} was determined from the initial velocity assuming the reaction was first order in 10-CHO-H_4PteGlu and 5,10-CH^+-H_4PteGlu.

The stability of the compound in equilibrium with CH^+H_4PteGlu which is rapidly converted to 5-CHO-H_4PteGlu by SHMT was determined by incubating solutions enriched in the compound under various conditions. Two milliliters of a 1 mM solution of 5,10-CH^+-H_4PteGlu was incubated at pH 4.5 in 20 mM sodium citrate for 3 h. During this period about 20% of 5,10-CH^+-H_4PteGlu was converted to the compound with only a small percentage of 5-CHO-H_4PteGlu being formed. The solution was adjusted to pH 8.0 with NaOH and lyophilized. The lyophilized powder was redissolved in 200 μL of H_2O, and the pH was adjusted to the desired value. From these solutions, 20-μL aliquots were removed with time and assayed for the concentrations of both 5-CHO-H_4PteGlu and the intermediate compound.

C^{11} Proton Exchange Experiments. Rates of proton exchange of the C^{11} hydrogen of formyl tetrahydrofolates were determined by incubating 6 mM solutions of (6*R*,*S*)-5-CHO-H_4PteGlu, 10-CHO-H_4PteGlu, or 5,10-CH^+-H_4PteGlu in various buffers containing 10 mM 2-mercaptoethanol, and allowing the solutions to reach equilibrium on ice for 40 min. The solutions were lyophilized overnight and dissolved in deoxygenated D_2O at 5 °C the following morning. Proton spectra were immediately recorded at given time intervals on a Bruker 270-MHz AF NMR spectrometer equipped with a variable temperature probe. Percent deuterium incorporation into the C^{11} position was determined by integration of the spectra, and peak assignments were made on the basis of previously published chemical shift determinations (Temple & Montgomery, 1984). 10-CHO-H_4PteGlu was prepared from 5,10-CH^+-H_4PteGlu at pH 10.0 by the addition of 1-μL aliquots of a 1 M NaOH solution which had been vacuum-deoxygenated (60 min) and argon-purged (15 min).

NMR Analysis. [^{1}H] and [^{13}C] NMR spectra were recorded on an IBM/Bruker Model AF270 instrument equipped with a carbon-tuned broad-band probe. All [^{13}C] spectra were recorded as proton-decoupled spectra and contained [^{13}C]-formate as an internal spectral reference for all peak assignments. Each spectrum represents the average of approximately 200 transients. 5,10-[^{13}C]H^+-H_4PteGlu spectra were recorded in HCl/H_2O, pH 1.0, 20% D_2O. All other spectra were recorded in 20% D_2O/H_2O, pH 7.0, unless otherwise indicated.

Results

Chemical Properties of 5,10-CH^+-H_4PteGlu in Acidic Solutions. We have previously suggested that the burst phase of the SHMT-catalyzed hydrolysis of 5,10-CH^+-H_4PteGlu to 5-CHO-H_4PteGlu was the result of a compound which exists in equilibrium with 5,10-CH^+-H_4PteGlu in acidic solutions. This compound was hypothesized to be a hydrated adduct of 5,10-CH^+-H_4PteGlu that was stable over the physiological pH range. We now believe this compound to be (6*R*,11*R*)-5,10-CHOH-H_4PteGlu (Stover & Schirch, 1990). Identification of this compound, which will be referred to as the "intermediate", is an important step in the determination of the overall mechanism of CH^+-H_4PteGlu hydrolysis by SHMT. Historically, 5,10-CH^+-H_4PteGlu (anhydroleucovorin A) was identified as the acid degradation product of 5-CHO-H_4PteGlu (Cosulich et al., 1952). Solutions of 5,10-CH^+-H_4PteGlu at pH 2.0 were believed to contain a mixture of several substances, while incubation at pH 1.3 or below yielded a homogeneous crystalline material. Heating or prolonged incubation of 5,10-CH^+-H_4PteGlu at pH 4.0 resulted in the transformation of 5,10-CH^+-H_4PteGlu to anhydroleucovorin B, a compound with unique spectral and melting point properties.

This led us to believe that anhydroleucovorin B and our intermediate were possibly the same compound. Therefore, optimal conditions for formation of the unknown compound were determined. A pH and temperature study showed that the intermediate was formed in solutions between pH 2 and 5.5, with optimal concentrations being formed at pH values of 4–4.5 and optimal temperatures being 40–50 °C. The concentrations achieved for the intermediate never exceeded 40% of the original concentration of 5,10-CH^+-H_4PteGlu. Incubation of 5,10-CH^+-H_4PteGlu in 100 mM potassium phosphate, pH 4.5 and 40 °C, results in a decrease in absorbance at 360 nm with concomitant increases in the intermediate (determined enzymatically) and a species absorbing at 278 nm. This correlation between the decrease in absorbance at 360 nm and the appearance of the intermediate lasts for about 40–50 min and then deviates as 5-CHO-H_4PteGlu and degradation products accumulate in the solution.

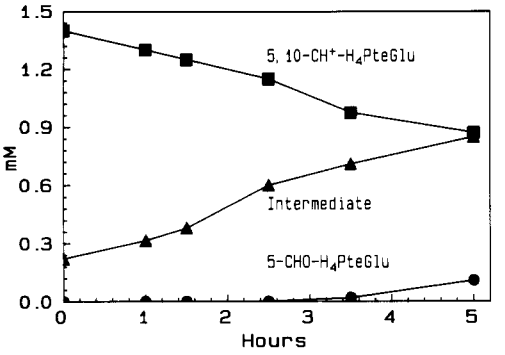


FIGURE 1: Rate of conversion of (6*R*)-5,10-CH^+PteGlu, at pH 4.5 and 50 °C, to the intermediate and 5-CHO-H_4PteGlu. Concentrations of 5,10-CH^+-H_4PteGlu (■), intermediate (▲), and 5-CHO-H_4PteGlu (●) are shown.

The formation of the intermediate is severalfold slower in the absence of any phosphate buffer, but more of the intermediate accumulates before 5-CHO-H_4PteGlu begins to appear in the reaction solutions. In the absence of buffer the pH drops during the incubation. Addition of standard NaOH solutions showed that between 0.8 and 1.2 equiv of hydroxide ion are consumed for each equivalent of CH^+-H_4PteGlu converted to the intermediate. The predicted stiochiometry for the hydration reaction would be a maximum of 1 equiv of hydroxide/equiv of intermediate formed. When (6*R*,*S*)-5,10-CH^+-H_4PteGlu was used in these studies in place of (6*R*)-5,10-CH^+-H_4PteGlu, the rate and accumulation of the intermediate, as determined by its reaction with SHMT, were the same, suggesting that SHMT recognizes only one of the C-6 isomers of the intermediate. Most of the experiments reported in this study used the (6*R*,*S*) mixture of the H_4PteGlu compounds.

Figure 1 shows the analysis of solutions of 5,10-CH^+H_4PteGlu for individual folate derivatives during incubation at a high temperature. In this experiment, a solution of CH^+-H_4PteGlu, 1.5 mM, in H_2O/HCl, pH 4.0, was incubated at 50 °C and 10-μL aliquots were removed for determination of the concentrations of both the intermediate and 5-CHO-H_4PteGlu. As shown in Figure 1, there is a good correlation between the decrease in concentration of 5,10-CH^+H_4PteGlu and increase in the intermediate. No detectable 5-CHO-H_4PteGlu was found in these solutions in the first 2.5 h of reaction. However, if the solution was incubated for 24 h, about 70% of the original CH^+-H_4PteGlu was converted to 5-CHO-H_4PteGlu.

Stability of the Hydrated Intermediate of 5,10-CH^+H_4PteGlu. In order to elucidate the structure of the putative hydrated intermediate, attempts were made to purify the compound. We first investigated the stability of the compound as a function of pH. A solution of (6*R*)-5,10-CH^+H_4PteGlu was incubated at pH 4.5 until the concentration of the intermediate was about 20% of the total folate. The solution was adjusted to pH 8.0 and lyophilized. During this procedure all of the remaining 5,10-CH^+-H_4PteGlu in the solution was converted to 10-CHO-H_4PteGlu and degradation products. The lyophilized powder was redissolved in H_2O and assayed for the concentration of the intermediate. Adjustment to pH 8.0 and lyophilization had not decreased the amount of the intermediate. Two aliquots of the solution were then incubated at either 30 or 75 °C and the concentrations of the intermediate and 5-CHO-H_4PteGlu determined. No loss in the concentration of the intermediate was observed at 30 °C

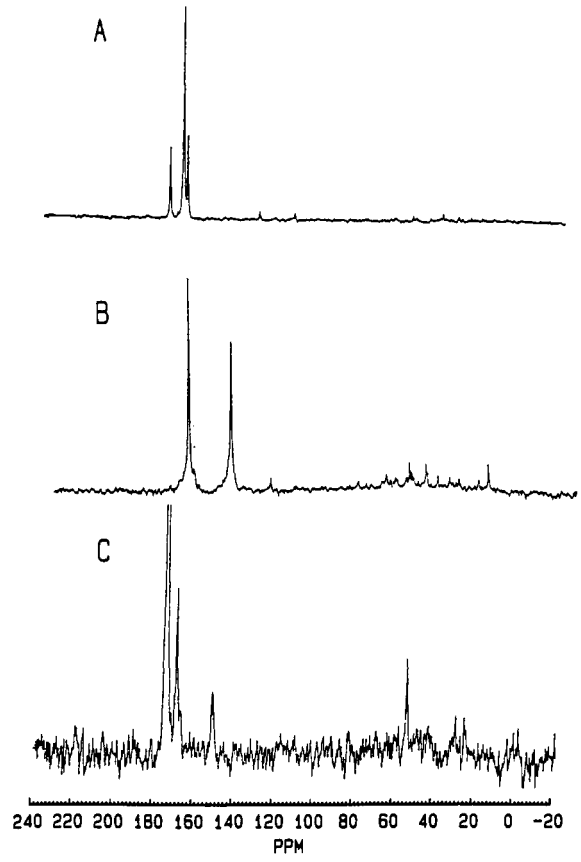


FIGURE 2: [^{13}C] NMR spectra of H_4PteGlu compounds substituted with [^{13}C]formyl groups at N^5 and N^{10}. Each solution contained [^{13}C]formate as an internal standard. Panel A: [^{13}C] NMR spectrum of a 10 mM solution of 5-[^{13}C]HO-H_4PteGlu at pH 7.0. Panel B: Spectrum of the solution recorded in panel A after the pH was lowered to 1 and the solution was incubated at 4 °C for 24 h. Panel C: Spectrum of 5,10-[^{13}C]H^+-H_4PteGlu at pH 4.0 after incubation for 1 h at 55 °C. The pH was maintained at 4.0 by the addition of 0.6 equiv of KOH.

in 8 h. The solution incubated at 75 °C for 1 h showed a 15% decrease in the amount of the intermediate with an equal increase in the amount of 5-CHO-H_4PteGlu.

At pH 12, solutions containing the intermediate were incubated at both 30 and 75 °C. At 75 °C the compound had a $t_{1/2}$ of 5 min with 5-CHO-H_4PteGlu being formed in about 95% yield. At 30 °C the $t_{1/2}$ was 40 min and the yield of 5-CHO-H_4PteGlu was greater than 90%.

A variety of chromatographic supports were used in an attempt to purify the adduct. These supports included Florosil, silica, alumina, and DEAE-Sephadex. All of these columns resulted in the loss of the intermediate, with only folate degradation products or 5-CHO-H_4PteGlu being detected in the eluant. In some cases, there was evidence that the column matrices catalyzed the formation of 5-CHO-H_4PteGlu from the intermediate.

NMR Evidence for the Stable Hydrated Intermediate of 5,10-CH$^+$-H_4PteGlu. In an attempt to determine if the intermediate was a hydrated adduct of 5,10-CH$^+$-H_4PteGlu, [^{13}C] NMR spectra of solutions of 5,10-[^{13}C]H^+-H_4PteGlu and 5-[^{13}C]HO-H_4PteGlu were recorded, and the spectra were analyzed for evidence of a tetrahedral adduct at C^{11} (Figure 2). The expected chemical shift difference between a C^{11} sp^2 carbon (as present in 5,10-CH$^+$-H_4PteGlu and 5-CHO-H_4PteGlu) and a C^{11} sp^3 carbon (which the putative tetrahedral intermediate would contain) would be in the range of 100 ppm, with the tetrahedral carbon appearing upfield. Panel A of Figure 2 shows the [^{13}C] NMR spectrum of 5-[^{13}C]-HO-H_4PteGlu with the resonances of the two rotamer forms at 165 and 163 ppm at pH 7.0 (Feeney et al., 1980). [^{13}C]-Formate is present as an internal standard and exhibits a resonance at 171 ppm. Panel B shows the effect of incubating 10 mM (6*R*,*S*)-5-[^{13}C]HO-H_4PteGlu in 0.1 N HCl for 24 h at 5 °C. The [^{13}C]^{11} resonance of 5,10-[^{13}C]H^+-H_4PteGlu is seen at 148 ppm (spectrum B) with [^{13}C]formic acid as the internal standard (164 ppm). Spectrum C shows the results after incubating 10 mM (6*R*,*S*)-5,10-[^{13}C]H^+-H_4PteGlu at pH 4.0 and 55 °C for 1 h. The pH was maintained at pH 4.0 by the addition of KOH. The reaction was stopped after the addition of 0.6 equiv of KOH. In this spectrum, at least three different formyl resonances are visible. The resonance at 150 ppm is unreacted 5,10-[^{13}C]H^+-H_4PteGlu. The resonance at 170 ppm is characteristic of either 5-CHO-H_4PteGlu or 10-CHO-H_4PteGlu or a mixture of the two. The resonance at 58 ppm is characteristic of an sp^3 carbon, suggesting that this is the [^{13}C]^{11} resonance of the putative tetrahedral intermediate. When the pH of the solution used to obtain spectrum C was lowered to pH 1.5, the spectrum reverted to the one shown in Figure 2B. The resonance at 58 ppm is also observed in spectra of 5,10-CH$^+$-H_4PteGlu solutions incubated at elevated temperatures over the pH range 2–5. The appearance of the resonance at 58 ppm was always observed under conditions that were known to result in the formation of the compound that is converted to 5-CHO-H_4PteGlu by SHMT.

NMR and Mass Spectral Analysis of 5-CHO-H_4PteGlu Formed from 10-CHO-H_4PteGlu. Previous studies of the hydrolysis of formamidinium salts suggested that a hydrated intermediate exists only fleetingly and breaks down rapidly to the two formyl isomers (Robinson & Jencks, 1967; Benkovic et al., 1972). The proposed mechanism suggested that in the conversion of 10-CHO-H_4PteGlu to 5-CHO-H_4PteGlu one need not go through CH$^+$-H_4PteGlu as an intermediate. This would suggest that the formyl oxygen would be retained in this conversion of the two formyl isomers. To determine the fate of the formyl oxygen, 10-CH[^{18}O]-H_4PteGlu was synthesized and converted to 5-CHO-H_4PteGlu at pH values of about 10 and 5 as described in Experimental Procedures. 5-CHO-H_4PteGlu was purified to homogeneity, and retention of the [^{18}O] was determined by mass spectral analysis. No [^{18}O] was found in the 5-CHO-H_4PteGlu, which exhibited a mass ion identical to samples of 5-CHO-H_4PteGlu containing only [^{16}O]. However, a control sample of 5-CH[^{18}O]-H_4PteGlu, incubated under the same reaction conditions, lost the [^{18}O] from the formyl oxygen faster than the rate of formation of 5-CHO-H_4PteGlu from 10-CHO-H_4PteGlu. Therefore, it is impossible to determine if the conversion of 10-CHO-H_4PteGlu to 5-CHO-H_4PteGlu proceeds through an intermediate without a C^{11} oxygen by this method.

C^{11} Proton Exchange Studies. The C^{11} proton of 5,10-CH$^+$-H_4PteGlu has been known to exchange with solvent, and the associated rate constants for this exchange have been determined for model compounds (Hafferl et al., 1963; Poe & Benkovic, 1980). The mechanism of this exchange was postulated to involve a C^{11} ylide intermediate in which C^{11} exists as a carbanion stabilized by a protonated N^5 existing as a bridgehead quaternary amine. The C^{11} proton exchange pathway was proposed to compete with a hydrolytic pathway resulting in 10-CHO-H_4PteGlu formation (Robinson, 1970). Study of the pH-dependent exchange rate is limited due to the conversion of 5,10-CH$^+$-H_4PteGlu to 10-CHO-H_4PteGlu above pH 5.0. However, the exchange of the C^{11} proton from the other two equilibrium derivatives of formyl folates has never been addressed. Therefore, the rates of C^{11} proton

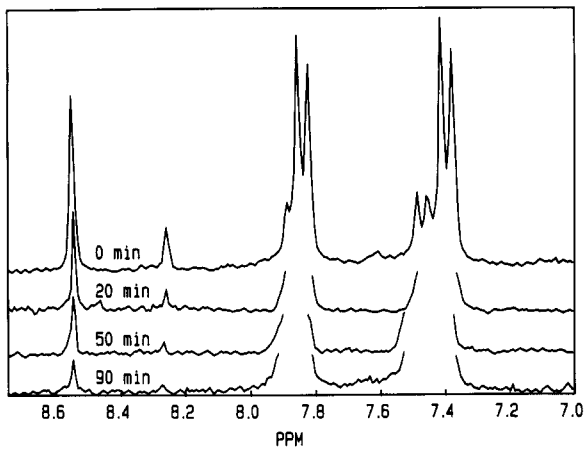


FIGURE 3: [^{1}H] NMR spectra of (6*R*,*S*)-10-CHO-H_4PteGlu incubated at 40 °C in 100 mM potassium phosphate, pH 9.2, in 99% D_2O. The disappearance of the resonances at 8.5 and 8.25 ppm represent the exchange of the C^{11} proton of 10-CHO-H_4PteGlu.

exchange of 5-CHO-H_4PteGlu, 10-CHO-H_4PteGlu, and equilibrium mixtures of 5,10-CH^+-H_4PteGlu and 10-CHO-H_4PteGlu were determined as a function of pH.

Figure 3 shows the [^{1}H] NMR spectra of 10-CHO-H_4PteGlu in deuterated 100 mM potassium phosphate, pH 9.2, and 10 mM 2-mercaptoethanol buffer, taken over a 90-min period at 40 °C. The major doublets at 7.4 and 7.9 ppm represent the aromatic C′3–C′5 and C′2–C′6 protons, and the singlet at 8.55 ppm represents the C^{11} proton as determined by Poe and Benkovic (1980). What was not reported by these authors was the time-dependent exchange of the C^{11} proton at alkaline pH. As seen in Figure 3, the C^{11} proton exchange has a $t_{1/2}$ of approximately 20 min at pH 9.2 and 40 °C. The spectra also display a minor doublet at 7.5 ppm, what appears to be a second minor doublet at 7.9 ppm, and a singlet at 8.3 ppm. The proton giving rise to the singlet at 8.3 ppm exchanges at the same rate as the proton exhibiting the singlet at 8.5 ppm. Previously, ^{1}H NMR spectra of 5-CHO-H_4PteGlu in this region displayed two minor doublets slightly offset from the principal aromatic doublets (Poe & Benkovic, 1980) and a second minor singlet. This was attributed to an equilibrium mixture of two rotamer forms of 5-CHO-H_4PteGlu (Poe & Benkovic, 1980; Feeney et al., 1980). This suggests that the unidentified peaks in the [^{1}H] NMR spectrum of 10-CHO-H_4PteGlu (Figure 3) may also be attributable to a rotamer of 10-CHO-H_4PteGlu. This putative second rotamer was probably not observed by Poe and Benkovic (1980) in the initial NMR structural studies of 10-CHO-H_4PteGlu since the C^{11} proton was greater than 50% exchanged in their studies. Additionally, reducing the temperature of the 10-CHO-H_4PteGlu to between 5 and 10 °C shifted the apparent equilibrium toward the less abundant rotamer.

These C^{11} proton exchange studies were repeated with 5-CHO-H_4PteGlu. No C^{11} proton exchange was observed for this compound under the conditions used for 10-CHO-H_4PteGlu.

It seems unlikely that the C^{11} proton of 10-CHO-H_4PteGlu exchanges by conversion to 5,10-CH^+H_4PteGlu at pH 9.2. To further understand the mechanism for the exchange of the C^{11} proton of 10-CHO-H_4PteGlu, exchange studies were investigated over an extended pH range at 40 °C in sulfonate buffers, to limit buffer catalysis, by the procedure used to record the data shown in Figure 3. Below pH 7, the 10-CHO-H_4PteGlu will be in equilibrium with 5,10-CH^+H_4PteGlu. Figure 4 shows the results of the C^{11} proton exchange rate at four pH values between pH 5.5 and 11.0.

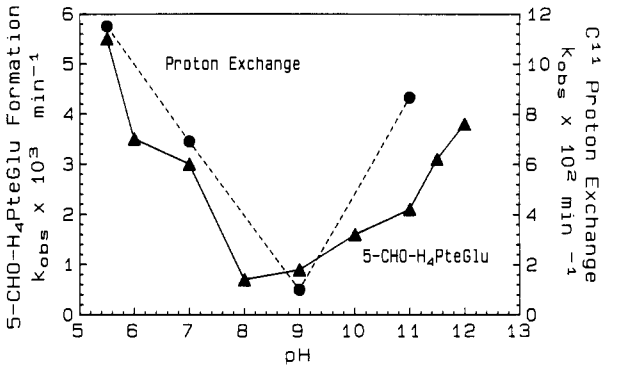


FIGURE 4: First-order rate constants for 5-CHO-H_4PteGlu formation (▲) and C^{11} proton exchange (●) from equilibrium solutions of (6*R*,*S*)-5,10-CH^+-H_4PteGlu and (6*R*,*S*)-10-CHO-H_4PteGlu in sulfonate buffers as a function of pH. [^{1}H] exchange studies were performed at 40 °C and 5-CHO-H_4PteGlu formation studies were performed at 75 °C.

During the NMR studies, no degradation products were observed either spectroscopically or by assay for 10-CHO-H_4PteGlu below pH 11.0. Above pH 11.0, some degradation products were observed by both methods. The rate constant was therefore determined from initial rates assuming first-order kinetics. The increasing rate of exchange of the C^{11} proton above pH values of 9, as recorded in Figure 4, suggests that the C^{11} proton exchanges from 10-CHO-H_4PteGlu without the proton-dependent conversion to 5,10-CH^+-H_4PteGlu.

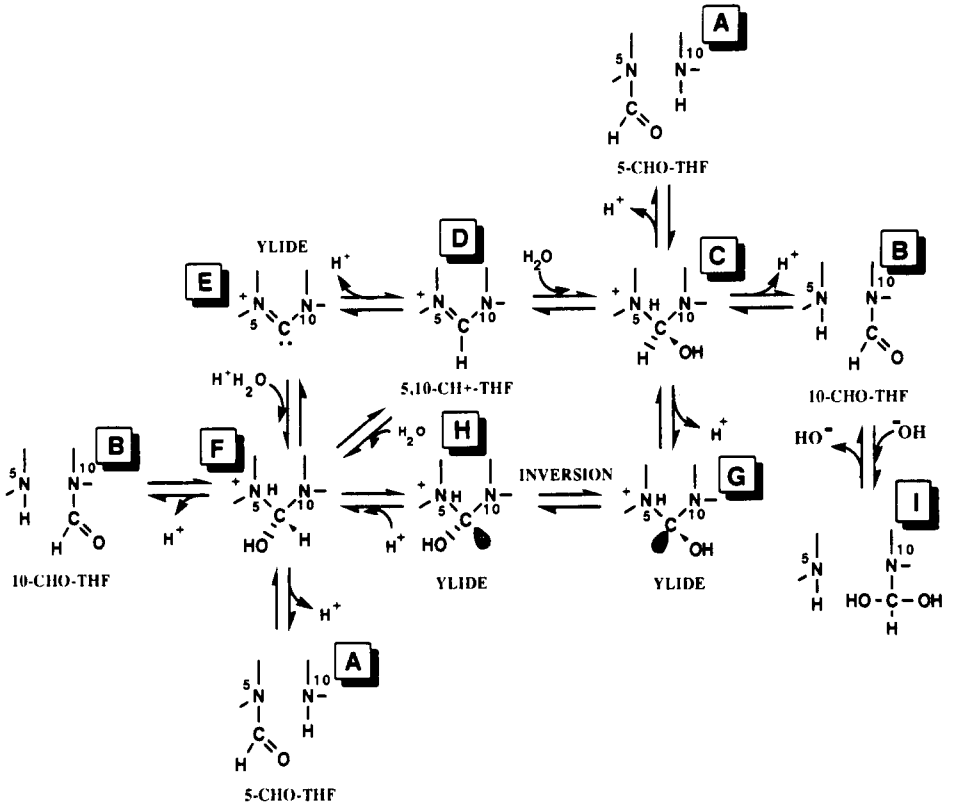
Rate of Formation of 5-CHO-H_4PteGlu. The rate of formation of 5-CHO-H_4PteGlu was determined with the same solutions of 10-CHO-H_4PteGlu used in the C^{11} proton exchange studies as recorded in Figure 4. The rate of formation of the 5-CHO derivative was much slower than proton exchange; therefore, the temperature was increased from 40 °C to 75°C for these studies. Figure 4 shows the comparison of the rates of C^{11} proton exchange and 5-CHO-H_4PteGlu formation over the pH range 5.5–12. These data show that the pathway(s) for C^{11} proton exchange and the pathway(s) for 5-CHO-H_4PteGlu formation display similar pH dependence with a minimum rate at about pH 8–9 and increasing rates in both the acid and alkaline pH ranges. Although these results are consistent with the C^{11} proton exchange being required in the mechanism of formation of 5-CHO-H_4PteGlu, we cannot rule out the possibility that proton exchange and formation of 5-CHO-H_4PteGlu are competing pathways.

Discussion

In this study, we present evidence that there is a stable compound which exists in equilibrium with 5,10-CH^+-H_4PteGlu in acidic solutions. Formation of this compound occurs optimally at about pH 4.0 at an elevated temperature. Conversion of 5,10-CH^+-H_4PteGlu to this compound requires approximately 1 equiv of hydroxide ion, decreases the 360-nm absorbance of the formamidinium salt, displays a [^{13}C] chemical shift consistent with an sp^3-hybridized hydroxyl carbon, breaks down to form 5-CHO-H_4PteGlu at pH 12, and is rapidly converted to 5-CHO-H_4PteGlu by SHMT. All of these properties are consistent with the intermediate being a hydrated adduct of 5,10-CH^+-H_4PteGlu.

The hydrated adduct, which accumulates at pH 4.0 in both buffered and unbuffered solutions, is unlikely to be the hydrated intermediate which occurs in the formation of 10-CHO-H_4PteGlu proposed by Robinson and Jencks (1967), Robinson (1970), and Benkovic et al. (1972). Unlike their proposed intermediate, the intermediate identified in this study was relatively stable in neutral to slightly acidic solutions. It

Scheme I



was also not a substrate for either methenyltetrahydrofolate cyclohydrolase, which converts 5,10-CH^+-H_4PteGlu to 10-CHO-H_4PteGlu, or 5,10-methenyltetrahydrofolate synthetase, which converts 5-CHO-H_4PteGlu and ATP to 5,10-CH^+-H_4PteGlu$_n$, ADP, and Pi (Stover & Schirch, 1990). The first evidence for a stable derivative of 5,10-CH^+-H_4PteGlu was reported by Cosulich et al. (1952). These authors described the formation of a B form (anhydroleucovorin B) of 5,10-CH^+-H_4PteGlu obtained by either boiling or prolonged incubation of 5,10-CH^+-H_4PteGlu in water or buffer at pH 4.0. Anhydroleucovorin B precipitated from the boiling solution and had the empirical formula $C_{20}H_{21}N_7O_6 \cdot {}^1/_2H_2O$. Since this empirical formula differed from the A form of 5,10-CH^+-H_4PteGlu ($C_{20}H_{21}N_7O_6 \cdot 4H_2O$) only in the degree of hydration, the authors concluded that anhydroleucovorin B was an isomer of 5,10-CH^+-H_4PteGlu. In addition, the absorbance spectrum of anhydroleucovorin B was distinct from the A form. While 5,10-CH^+-H_4PteGlu displayed a single absorption maximum at 360 nm, anhydroleucovorin B exhibited two absorption maxima, the principal maximum being around 280 nm with a weaker absorption band around 350 nm. Anhydroleucovorin B was converted to 5,10-CH^+-H_4PteGlu only upon acidification with hot 0.1 N HCl. The melting point of the B form was greater than 350 °C, compared to the melting point of 258 °C for 5,10-CH^+-H_4PteGlu. However, the purity of anhydroleucovorin B as isolated was never reported.

We were never able to get greater than a 40% conversion of 5,10-CH^+-H_4PteGlu to our intermediate without accumulating 5-CHO-H_4PteGlu and several degradation products. Therefore, we could not determine the spectral properties, elemental analysis, and melting point of our intermediate. However, the initial rate of formation of our intermediate at pH 4.5 was correlated with a loss of the 360-nm absorbance of 5,10-CH^+-H_4PteGlu and an increase of absorbance at 278 nm, in agreement with the spectral properties of anhydroleucovorin B.

The currently accepted mechanism for the hydrolysis of 5,10-CH^+-H_4PteGlu to 10-CHO-H_4PteGlu requires nucleophilic attack at C^{11} by H_2O, resulting in the formation of a hydrated intermediate. To conform to stereoelectronic rules, H_2O would add antiperiplanar to the two lone pairs on N^5 and N^{10}. This would result in the formation of (11*S*)-5,10-CHOH-H_4PteGlu (structure C in Scheme I), in which the C^{11} hydroxyl group is on the opposite site of the five-membered imidazolinium ring from the C^6 proton. The breakdown of this hydrated intermediate to 5-CHO-H_4PteGlu and 10-CHO-H_4PteGlu has been interpreted in terms of the macroscopic differences in the pK_a values of N^5 and N^{10} (C → A or C → B in Scheme I). This sequence of reactions does not explain the observed stable intermediate described in this paper. We suggest that the stable hydrated intermediate, in equilibrium with 5,10-CH^+-H_4PteGlu, is (11*R*)-5,10-CHOH-H_4PteGlu (structure F, Scheme I). In this intermediate, the hydroxyl group is on the same side of the imidazolinium ring as the lone pairs of electrons on N^5 and N^{10} and the C^6-H. Arguments to support that the intermediate is (11*R*)-5,10-CHOH-H_4PteGlu must explain how it is formed, why it is stable at pH values from 2 to 12, and why it breaks down in neutral and alkaline solutions exclusively to 5-CHO-H_4PteGlu.

The formation of (11R)-5,10-CHOH-H_4PteGlu (structure F in Scheme I) could occur by several different pathways. First, it could be directly formed from 10-CHO-H_4PteGlu either by attack of N^5 on the *si* face of the formyl group (B → F) or by attack of N^{10} on the *re* face of 5-CHO-H_4PteGlu (A → F). Formation of the hydrated adduct C would require attack by N^5 and N^{10} on the opposite faces of the respective formyl groups. We assayed for the presence of the intermediate (structure F) after incubating solutions of 5-CHO-H_4PteGlu and 10-CHO-H_4PteGlu at 50 °C and pH 4.0 for 10 min. The results suggested that direct ring closure was not a preferred pathway for formation of the stable intermediate,

as little or no intermediate was detected. Second, compound F could be formed by syn addition of hydroxide to 5,10-CH^+-H_4PteGlu (D → F). A similar pathway would be the addition of H_2O to the ylide E (E → F). The syn addition of water or hydroxide to the imidazolinium ring is not consistent with stereoelectronic predictions since the electron density of the nitrogen lone pairs and the oxygen heteroatom are localized on the same side of the ring. Since the electron pair on the ylide can probably invert, addition could be anti for this pathway. A third possible pathway for formation of compound F would be for intermediate C to lose its C^{11} proton to form the ylide G. This would be predicted to be unstable because it would have three lone electron pairs on the same side of the imidazolinium ring. If the carbanion (compound G) inverted its electron pair, it would form the ylide H. Reprotonation of compound H would give (11*R*)-CHOH-H_4PteGlu (compound F).

If ylides G and H are intermediates in the conversion of 10-CHO-H_4PteGlu to 5-CHO-H_4PteGlu, then the C^{11} proton of 10-CHO-H_4PteGlu must be lost in its conversion 5-CHO-H_4PteGlu. Previously, it has been demonstrated that 5,10-CH^+-H_4PteGlu (structure D) undergoes reversible exchange of the C^{11} proton (Poe & Benkovic, 1980). We have again confirmed this observation. We have also shown that the C^{11} proton of 10-CHO-H_4PteGlu rapidly exchanges with solvent protons. This could occur by either the pathway B → E via CH^+H_4PteGlu or by B → C → G (Scheme I). The conversion of intermediate C → D is an acid-catalyzed step and would be expected to be slow at pH 11.0. However, we observed that proton exchange increases with pH in the alkaline range. At pH 12 both exchange of the C^{11}-H of 10-CHO-H_4PteGlu and breakdown of the intermediate to 5-CHO-H_4PteGlu occur at least an order of magnitude more rapidly than the formation of 5-CHO-H_4PteGlu from 10-CHO-H_4PteGlu. This suggests that the rate-determining step in the formation of structure F is the inversion of the ylide G to the ylide H. The rate of formation of compound F would increase with pH because the conversion of C → G would be proportional to OH^- concentration. Compound F does not accumulate at high pH because the rate of its dehydration to 5-CHO-H_4PteGlu is faster than the rate of its formation. Since N^5 is not protonated above pH 7 there would appear to be no positive charge available to stabilize the carbanion on C^{11} in compound G. However, the rapid exchange of the 10-formyl C^{11} proton at high pH suggests that N^{10} retains considerable positive charge as a result of resonance of its lone electron pair with the benzamide moiety.

The increase in both the exchange rate of the C^{11} proton and the formation of 5-CHO-H_4PteGlu with decreasing pH, below pH 7, is probably the result of protonation of N^5. This would increase the acidity of the C^{11} proton on compound C, thus increasing the concentration of compound G, which would be converted to compound H in a pH-independent step. Decreases in pH below 4.0 would be expected to decrease the rate of formation of ylide G and therefore the formation of compound F. This predicted decrease in the formation of compound F below pH 4 may account for the observation that at pH 1.5 no hydrated intermediate nor C^{11} proton exchange can be detected.

The pathway for conversion of 10-CHO-H_4PteGlu to 5-CHO-H_4PteGlu, via the ylides G and H, would predict that the formyl oxygen would be retained. We found that the formyl oxygen is not retained, but this is probably the result of faster exchange reactions taking place through other pathways such as the reaction of B → I (Scheme I). The conversion of B → I probably also accounts for the known deformylation of 10-CHO-H_4PteGlu at alkaline pH.

The most notable feature of the hydrated intermediate F, which distinguishes it from hydrated intermediate C, is its stability. The structure of compound F could be stable for at least two reasons. First, the compound contains both the N^5 and N^{10} electron pairs and the hydroxyl heteroatom in the same plane. In this position, the C^{11}-OH could form a hydrogen bond with the C^4 carbonyl oxygen, forming a seven-membered ring and thus stabilizing this structure. Previous evidence that the C^4 carbonyl is in a position to interact with the substituents on C^{11} was demonstrated by NMR, where the resonance of the C^{11} formyl proton of 5-CHO-H_4PteGlu was found to be deshielded by the C^4 carbonyl oxygen (Poe & Benkovic, 1980). Second, the pathway for elimination of either N^{10} or N^5 from structure F would require a syn elimination mechanism.

A remaining question is why compound F breaks down only to 5-CHO-H_4PteGlu and not to 10-CHO-H_4PteGlu, as opposed to compound C, which greatly favors the anti elimination of N^5 to form 10-CHO-H_4PteGlu. Elimination of either N^5 or N^{10} from compound F would require a syn elimination. However, models of compound F suggest that inversion of the lone pair of electrons on N^{10} would convert compound F into a form where elimination of N^{10} can occur by the more favorable anti elimination mechanism. Since N^5 is a bridgehead atom, it cannot invert and thus would require a syn elimination of N^5 to form 10-CHO-H_4PteGlu.

Definitive evidence for the structure of the compound in equilibrium with 5,10-CH^+-H_4PteGlu in slightly acidic solution, which breaks down to form 5-CHO-H_4PteGlu, is not yet available. However, the properties of this compound can be rationalized in favor of the hydrated adduct (11*R*)-5,10-CHOH-H_4PteGlu (compound F in Scheme I). Pathways for the formation of this compound in both acidic and alkaline pH ranges appear to be available. In the alkaline range, evidence suggests that it occurs by the inversion of ylides G and H (Scheme I) and that formation of 5-CHO-H_4PteGlu from 10-CHO-H_4PteGlu occurs by elimination of N^{10} from (11*R*)-5,10-CHOH-H_4PteGlu. It cannot be ruled out that even in acidic solutions the preferred pathway for formation of 5-CHO-H_4PteGlu is via intermediate F and that the direct breakdown of intermediate C to 5-CHO-H_4PteGlu is not operative. Support of the role of intermediate F in the formation of 5-CHO-H_4PteGlu is presented in the accompanying paper on the mechanism of the SHMT-catalyzed conversion of 5,10-CH^+-H_4PteGlu to 5-CHO-H_4PteGlu (Stover & Schirch, 1992).

Acknowledgments

We are grateful to Dr. Wyoski for performing mass spectral analysis.

References

Benkovic, S. J. (1978) *Acc. Chem. Res. 11*, 314–320.
Benkovic, S. (1980) *Annu. Rev. Biochem. 49*, 227–251.
Benkovic, S. J., Bullard, W. P., & Benkovic, P. A. (1972) *J. Am. Chem. Soc. 94*, 7542–7549.
Cosulich, D. C., Roth, B., Smith, J. M., Hultquist, M. E., & Parker, R. P. (1952) *J. Am. Chem. Soc. 74*, 3252–3263.
Feeney, J., Albrand, J. P., Boicelli, C. A., Charlton, P. A., & Young, D. W. (1980) *J. Chem. Soc., Perkin Trans. 2*, 176–180.
Hafferl, W., Lundin, R., & Ingraham, L. (1963) *Biochemistry 2*, 1298–1305.

May, M., Bardos, T. J., Barger, F. L., Lansford, M., Ravel, J. M., Sutherland, G., & Shive, W. (1951) *J. Am. Chem. Soc. 73*, 3067–3075.
Moran, R. G., & Colman, P. D. (1982) *Anal. Biochem. 122*, 10–78.
Poe, M., & Benkovic, S. J. (1980) *Biochemistry 19*, 4576–4583.
Robinson, D. R. (1970) *J. Am. Chem. Soc. 92*, 3138–3146.
Robinson, D. R., & Jencks, W. P. (1967) *J. Am. Chem. Soc. 89*, 7098–7103.
Rowe, P. B. (1984) in *Chemistry and Biochemistry of Folates* (Blakely, R. L., & Benkovic, S. J., Eds.) Vol. 1, pp 329–344, John Wiley and Sons, New York.
Stover, P., & Schirch, V. (1990) *J. Biol. Chem. 265*, 14227–14233.
Stover, P., & Schirch, V. (1991) *J. Biol. Chem. 266*, 1543–1550.
Stover, P., & Schirch, V. (1992) *Biochemistry* (following paper in this issue).
Temple, C., Elliott, R. D., Rose, J. D., & Mongomery, J. A. (1979) *J. Med. Chem. 22*, 731–734.
Temple, C., Jr., & Montgomery, J. A. (1984) in *Chemistry and Biochemistry of Folates* (Blakely, R. L., & Benkovic, S. J., Eds.) Vol. 1, p 80, John Wiley and Sons, New York.
Wysocki, V. H., Ding, J. M., Jones, J. L., Callahan, F. L., & King, F. L. (1991) *J. Am. Chem. Soc.* (in press).

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

Patrick Stover and Verne Schirch*
Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University, Richmond, Virginia 23298
Received August 9, 1991; Revised Manuscript Received October 18, 1991

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

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

† This work was supported by Grant GM 28143 from the National Institutes of Health.
* To whom correspondence should be addressed.

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

0006-2960/92/0431-2155$03.00/0