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Primary Structure and Tetrahydropteroylglutamate Binding Site of Rabbit Liver Cytosolic 5,10-Methenyltetrahydrofolate Synthetase*

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Bruno Maras‡, Patrick Stover§, Sofia Valiante‡¶, Donatella Barra‡, and Verne Schirch§‖

From the §Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University, Richmond, Virginia 23298 and the ‡Dipartimento di Scienze Biochimiche and Centro di Biologia Molecolare del Consigilio Nazionale delle Ricerche, Università La Sapienza, 00185 Rome, Italy

The primary sequence of 5,10-methenyltetrahydrofolate synthetase from rabbit liver was determined by amino acid sequencing of the purified enzyme. The enzyme contains 201 amino acid residues with a predicted mass of 22,779 Da. The enzyme is located in the cytosolic fraction of liver homogenates. Carbodiimide-activated 5-formyltetrahydropteroylmonoglutamate and the pentaglutamate form of the substrate both irreversibly inactivate the enzyme by forming a covalent bond to Lys-18. Non-activated 5-formyltetrahydropteroylpentaglutamate protected against this inactivation. Substrate specificity studies showed that increasing the number of glutamate residues from zero to five on 5-formyltetrahydropteroate results in a 2 order of magnitude increase in the affinity of the substrate for the enzyme but only a 3-fold increase in the value of V_{max}.

5-Formyltetrahydropteroylglutamate (5-CHO-H_4PteGlu$_n$)[1] (clinically known as leucovorin) is used in cancer chemotherapy to either rescue patients from methotrexate toxicity or to enhance the effectiveness of 5-fluorouracil (1–5). 5-CHO-H_4PteGlu$_n$ exerts its effects by first being converted to 5,10-CH^+H_4PteGlu$_n$ by an ATP-dependent reaction (Equation 1). The product 5,10-CH^+-H_4PteGlu$_n$ is converted to other reduced folate intermediates involved in 1-carbon metabolism by the trifunctional enzyme C_1-tetrahydrofolate synthase, serine hydroxymethyltransferase, and methylenetetrahydrofolate reductase (6–8).

$$\text{5-CHO-}H_4\text{PteGlu}_n + \text{MgATP} \rightarrow \text{5,10-}CH^+\text{-}H_4\text{PteGlu}_n + \text{MgADP} + P_i \quad \text{(Eq. 1)}$$

Reaction 1 is catalyzed by 5,10-methenyltetrahydrofolate synthetase (methenyl-THF synthetase), which has been purified from both procaryotic and eucaryotic sources (9–11). At the time of the purification of the rabbit and bacterial enzymes, the source of intracellular 5-CHO-H_4PteGlu$_n$ was not known but was suspected of being the result of the nonenzymatic hydrolysis of 5,10-CH^+-H_4PteGlu$_n$ (10). It was proposed that the function of methenyl-THF synthetase was to serve as a salvage pathway for reincorporating 5-CHO-H_4PteGlu$_n$ back into the one-carbon pool.

Recently, serine hydroxymethyltransferase was shown to catalyze the hydrolysis of 5,10-CH^+-H_4PteGlu$_n$ to 5-CHO-H_4PteGlu$_n$, suggesting that the source of intracellular 5-CHO-H_4PteGlu$_n$ was enzymatic (12). The combined activities of methenyl-THF synthetase and serine hydroxymethyltransferase result in a futile cycle that is characteristic of other metabolic regulatory systems, suggesting that 5-CHO-H_4PteGlu$_n$ may have a regulatory function. This was supported by the observation that 5-CHO-H_4PteGlu$_n$ polyglutamates are slow tight-binding inhibitors of serine hydroxymethyltransferase and that some dormant cells, such as seeds and spores, appear to have high concentrations of 5-CHO-H_4PteGlu$_n$ (13, 14). To better understand the structure and function of methenyl-THF synthetase and its putative role in regulation, we have purified it from rabbit liver and determined its primary structure by amino acid sequencing. We have also located the polyglutamate binding site of the coenzyme and have shown that the enzyme is located in the cytosol. The sequence of methenyl-THF synthetase shows no homology to other folate-requiring enzymes.

EXPERIMENTAL PROCEDURES

Materials—Fresh frozen rabbit livers were obtained from Pel-Freez Biologicals (Rogers, AR) and stored at −70 °C. Enzyme markers, pepsin, EDC, and amino acid substrates were obtained from Sigma. Trypsin (code TRTPCK) and chymotrypsin were from Worthington, and cyanogen bromide was from Fluka Chemie AG. Iodo[2-^{14}C]acetate was from Radiochemical Centre, Amersham, and guanidinium chloride (recrystallized from methanol) was from Merck. All chromatography solvents were HPLC grade and obtained from Farmitalia Carlo Erba, and sequence grade chemicals were purchased from Applied Biosystems. (6*S*)-5-[^{14}C]CHO-H_4PteGlu$_n$ mono- and pentaglutamate were prepared from either (6*S*)-H_4PteGlu using L-[U-^{14}C]serine (120 mCi/mmol, Moravek Biochemicals) or 5-CHO-H_4PteGlu$_5$ using sodium [^{14}C]formate (50 mCi/mmol, DuPont NEN) (14). The enzyme C_1-tetrahydrofolate synthase was purified from fresh frozen rabbit liver according to the method of Stover and Schirch (14). Samples of 5-CHO-H_4PteHis and 5-CHO-H_4Pte were a gift from P. Stover.

Purification of Methenyltetrahydrofolate Synthetase—In the early stages of this study, methenyl-THF synthetase was purified by the procedure of Hopkins and Schirch (10). In the later stages of the study, an entirely new purification procedure was developed, which resulted in about three times as much enzyme in a purification period of 2 days (15). This new procedure was important in isolating enzyme for the determination of the first few residues at the NH_2 terminus of the protein.

Enzymatic and Chemical Cleavages of Methenyl-THF Synthetase—The purified protein (10 mg) was carboxymethylated with iodo[2-^{14}C]acetate as described (16). An aliquot (2.5 mg) of the carboxymethylated protein was suspended in 0.5 ml of 0.1 M ammonium bicarbonate and

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This work is dedicated to the memory of Gian Paolo Nitti.

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‖ To whom correspondence should be addressed. Tel.: 804-828-9482; Fax: 804-828-1473.

[1] The abbreviations used are: 5-CHO-H_4PteGlu$_n$, 5-formyltetrahydropteroylglutamate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; methenyl-THF synthetase, 5,10-methenyltetrahydrofolate synthetase; H_4PteGlu$_n$, tetrahydropteroylglutamate with *n* glutamate residues; KMES, potassium 2-(*N*-morpholino)ethanesulfonic acid; KBES, potassium *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; 5-CHO-H_4PteHis, 5-formyltetrahydropteroylhistidine; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; 5-CHO-H_4Pte, 5-formyltetrahydropteroic acid; 5,10-CH^+-H_4PteGlu, 5,10-methenyltetrahydropteroylglutamate.

incubated at 37 °C for 3 h after addition of 0.2 mg of trypsin. A second sample of protein (2.5 mg) was dissolved in 0.5 ml of 70% (v/v) formic acid and incubated with 20 mg of CNBr for 20 h at room temperature in the dark. A third aliquot of protein (2.5 mg) was digested with 0.2 mg of chymotrypsin in 0.1 M ammonium bicarbonate for 3 h at 37 °C. A fourth sample (2.5 mg) was digested with pepsin in 5% formic acid for 5 min at room temperature at an enzyme/substrate ratio of 1/50 (w/w).

Samples of native protein from different preparations (see above) were utilized to determine the NH_2-terminal sequence.

Peptide Purification—The tryptic peptides were purified using a Beckman System Gold chromatography apparatus with a reverse-phase column (Aquapore RP-300, 4.6 × 250 mm, 7 μm, Applied Biosystems). Peptides were eluted with a linear gradient from 0 to 35% acetonitrile in 0.2% (v/v) trifluoroacetic acid at a flow rate of 1.0 ml/min. Elution of the peptides was monitored using a diode array detector (Beckman model 168) at 220 and 280 nm.

Peptides obtained after CNBr cleavage were purified on a reverse-phase column (Vydac C4, 4.6 × 250 mm, 5 μm), using the same solvent system as above, at a flow rate of 1.0 ml/min. Peptide mixtures obtained after chymotryptic and peptic digestions were purified under the same conditions as described for the tryptic peptides.

Analytical Techniques—Quantitative amino acid analyses were carried out on 0.5–1 nmol of purified peptides hydrolyzed in vapor phase with 6 N HCl containing 0.1% phenol at 110 °C for 24 h. The amino acid composition of hydrolyzed peptides was determined using a Pharmacia 4151 Alpha Plus instrument.

The amino acid sequence of peptides was determined by automated Edman degradation using Applied Biosystems models 475A or 476A sequenators. Samples (0.2–1 nmol) were loaded onto a polyvinylidene difluoride membrane (Problott®) previously activated with methanol. The presence of carboxymethylcysteine in the sequence was confirmed by measuring the radioactivity of the aliquot delivered in the fraction collector after each cycle of degradation.

Mass spectral analyses were performed at the Dana Farber Mass Spectrometry Core Facility (Boston, MA) and by Professor Piero Pucci, Servizio di Spettrometria di Massa del Consiglio Nazionale delle Ricerche, Università di Napoli.

Peptide Nomenclature—The peptides were retrospectively numbered according to their location in the sequence, starting from the N terminus. Tryptic peptides are designated with T, CNBr peptides with B, peptic peptides with P, and chymotryptic peptides with C.

Location of Polyglutamate Binding Site—The glutamate residues on (6*S*)-5-CHO-H_4PteGlu$_n$ were activated by incubation with EDC (17). This complex was observed to inhibit methenyl-THF synthetase activity irreversibly. A 10-fold molar excess of EDC was added to a 10 mM solution of (6*S*)-5-CHO-H_4PteGlu$_n$ in 10 mM potassium phosphate, pH 6.0, for 5 min at 0 °C prior to inactivation studies. Inactivation of methenyl-THF synthetase was determined by incubating 200-μl aliquots of methenyl-THF synthetase (0.1 μM) in pH 6.0 potassium phosphate buffer with increasing concentrations of the EDC·(6*S*)-5-CHO-H_4PteGlu$_n$ solution. After a 10-min incubation, a 50-μl aliquot of the enzyme solution was added to a cuvette containing 950 μl of reaction buffer that included 50 mM KMES, pH 6.0, 2.0 mM MgATP, and 100 μM (6*S*)-5-CHO-H_4PteGlu$_n$. The rate of the enzymatic reaction was monitored at 360 nm (10). This procedure was repeated until the activity of methenyl-THF synthetase had decreased to less then 20% of a control methenyl-THF synthetase solution that did not contain the added EDC-activated (6*S*)-5-CHO-H_4PteGlu$_n$. Protection experiments were performed by adding to the 0.1 μM methenyl-THF synthetase solution 10 μM (6*S*)-5-CHO-H_4PteGlu$_5$ prior to the addition of the EDC-activated substrate.

Identification of the amino acid residue modified with the irreversibly bound 5-CHO-H_4PteGlu$_n$ was accomplished as follows. A 10-fold excess of solid EDC was added to a 0.5 mM solution of (6*S*)-5-[^{14}C]CHO-H_4PteGlu$_n$ (2000 cpm/pmol) as described above. A 50-μl aliquot of this solution was added to the purified methenyl-THF synthetase (10 mg/ml), which had been dialyzed for 5 h against 10 mM potassium phosphate, pH 6.0. After a 15-min incubation at 0 °C, an additional 50-μl aliquot of nonlabeled EDC-activated 5-CHO-H_4PteGlu$_n$ was added to the enzyme solution and incubated an additional 15 min at 0 °C. The reaction was terminated by the addition of 200 μl of 2-mercaptoethanol, and the enzyme was separated from excess EDC and 5-CHO-H_4PteGlu$_n$ by passage through a Sephadex G-50 column (1 × 20 cm). The labeled enzyme was concentrated by lyophilization and dissolved in 0.1 M NH_4HCO_3 with a final enzyme concentration of about 5 mg/ml. The ratio of covalently attached (6*S*)-5-[^{14}C]CHO-H_4PteGlu$_n$/mol of methenyl-THF synthetase was 0.9 for both the mono- and pentaglutamate derivatives of 5-CHO-H_4PteGlu$_n$.

The labeled methenyl-THF synthetase was dialyzed against 8 M urea to denature the protein. Cysteine residues were blocked with iodoacetate as previously described, and the enzyme was dialyzed against 0.1 M NH_4HCO_3, pH 8.0 (16). The precipitated enzyme was digested with a 1% solution of trypsin for 8 h at 25 °C. The tryptic peptides were purified on a C-8 HPLC column with a linear gradient from 0 to 60% solvent B in 60 min (buffer A was 0.3% triethylamine acetate, pH 6.8, in water, and buffer B was 0.3% triethylamine acetate, pH 6.8, in 60% acetonitrile). Peptides were monitored at both 220 (peptides) and 280 nm (bound 5-CHO-H_4PteGlu$_n$). Aliquots of each fraction were also analyzed for radioactivity in a liquid scintillation counter. Radioactive peptides were analyzed by amino acid sequencing (16).

TABLE I
Cellular location of methenyl-THF synthetase

Cell fraction	Lactate dehydrogenase	Succinate-cytochrome *c* reductase	Methenyl-THF synthetase
	% total activity	*% total activity*	*% total activity*
Homogenate	100	100	100
Cytosol	106	20	92
Mitochondria	3	75	2

Cellular Location—An excised rabbit liver was rapidly divided into two 10-g portions. All further procedures were performed at 5 °C, and all buffers were kept on ice. The liver portions were washed with the homogenizing buffer consisting of 10 mM potassium phosphate, pH 7.5, 1 mM EDTA, and 0.3 M sucrose. Mitochondria were isolated from one of the 10-g portions. The other portion was chopped into fine pieces and placed in 50 ml of homogenizing buffer containing 0.2% Triton X-100. The tissue was homogenized in a blender and centrifuged at 7500 × *g* for 10 min, and the supernatant was placed on ice. This fraction contained both mitochondrial and cytosolic enzyme activities and will be referred to as the crude homogenate.

Mitochondria were isolated from the other 10-g portion of liver after diluting 1/10 (w/v) in homogenizing buffer. The liver tissue was finely chopped and homogenized in a rotary tissue disrupter. Tissue was further disrupted by 30 strokes in a Dounce homogenizer. The homogenate was centrifuged at 500 × *g* for 10 min, and the pellet was discarded. The supernatant was centrifuged at 7500 × *g* for 10 min to pellet the mitochondria, and the supernatant, which contained cytosolic proteins, was placed on ice. The mitochondria were washed by gently resuspending the pelleted mitochondria in 50 ml of homogenization buffer and centrifuged at 500 × *g* for 10 min. The pellet was discarded and the supernatant centrifuged at 7500 × *g* to again pellet the mitochondria. The supernatant was pooled with the cytosolic fraction. The pelleted mitochondria were resuspended in 20 ml of homogenization buffer, and the washing procedure was repeated. The pellet was resuspended in 10 ml of homogenization buffer, and the mitochondria was lysed by making the buffer 0.2% in Triton X-100.

The mitochondrial, cytosolic, and crude homogenate fractions were assayed for lactate dehydrogenase (cytosolic marker), succinate-cytochrome *c* reductase (mitochondria marker), and methenyl-THF synthetase activities. Lactate dehydrogenase activity was determined by measuring the increase in absorbance at 340 nm during the oxidation of lactate and NAD^+ to pyruvate and NADH. The assay was performed in 1 ml of KBES, pH 7.0, 1 mM lactate, 100 μM NAD^+, and 5–25-μl additions of the three fractions. Succinate-cytochrome *c* reductase activity was determined at 550 nm as previously described (18). The reaction mixture contained 700 μl of 0.1 M potassium phosphate, pH 7.4, 1 mM KCN, 100 μl of 0.5 mM cytochrome *c*, 100 μl of 200 μM succinate, and 5–25-μl additions of the three fractions.

Methenyl-THF Synthetase Assay—Activity was determined at 30 °C by measuring the increase in absorbance at 360 nm during the ATP-dependent conversion of 5-CHO-H_4PteGlu$_n$ to 5,10-CH$^+$-H_4PteGlu$_n$ (10). Standard assay conditions contained 100 mM KMES, pH 6.0, 1 mM MgATP, 25 μM (6*R*,6*S*)-5-CHO-H_4PteGlu$_n$, and 2–20-μg additions of methenyl-THF synthetase solutions. For the determination of kinetic constants for the analogs and polyglutamate forms of 5-CHO-H_4PteGlu$_n$, the MgATP concentration was 3 mM (10-fold K_m), and the substrate concentration was varied over a 10-fold range that bracketed the K_m concentration. Values for K_m and k_{cat} were determined from double-reciprocal plots of initial velocity *versus* substrate concentration. Initial velocity values were determined from the slope of the first 10% of the reaction.

RESULTS

Cell Location of Methenyl-THF Synthetase—Table I shows the activities of lactate dehydrogenase, succinate-cytochrome *c*

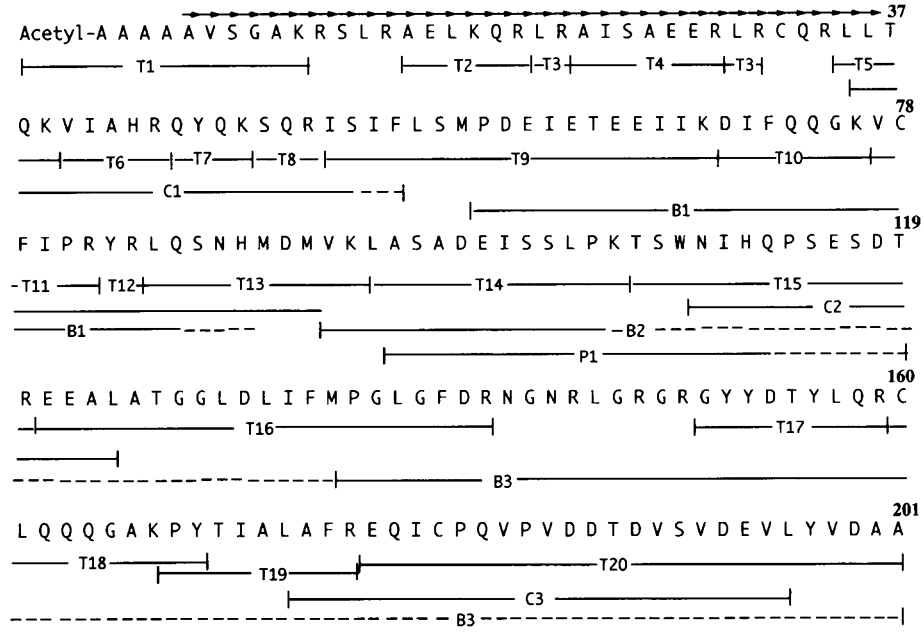

FIG. 1. **Primary sequence of rabbit liver methenyl-THF synthetase.** The *arrows* above the sequence show the residues determined from sequencing of a truncated form of the enzyme. The *lines* under the sequence represent the peptides determined from tryptic (*T*), CNBr (*B*), peptic (*P*), and chymotryptic (*C*) digestions. The *dashed lines* represent residues of peptides that were not sequenced to the end but inferred from amino acid composition and the sequence of the first residues. The sequence of peptide T1 was mute to sequencing but was determined from its amino acid composition with a mass of 858 Da, as determined by fast atom mass spectrometry.

reductase, and methenyl-THF synthetase in the total cell homogenate and the cytosolic and mitochondrial fractions. The activities in the crude homogenate were assigned the value of 100%. Lactate dehydrogenase activity was essentially confined to only the cytosol (106%) with only 3% being found in the mitochondrial fraction. This gives an indication of the degree of contamination of the mitochondrial fraction by cytosolic proteins. Succinate-cytochrome *c* reductase activity was primarily found in the mitochondrial fraction (75%) with 20% being in the cytosolic fraction. This suggests that there was some mitochondrial breakage during the isolation procedure. Methenyl-THF synthetase was primarily found in the cytosolic fraction (92%) with only 2% being in the mitochondrial fraction (less than the amount of lactate dehydrogenase). These results confirm that the methenyl-THF synthetase purified and sequenced in this study is a cytosolic enzyme.

Primary Structure—The proposed amino acid sequence of rabbit liver methenyl-THF synthetase is reported in Fig. 1. The enzyme subunit has 201 amino acid residues yielding a molecular mass of 22,779 Da, which is 5,000 Da less than the mass predicted from SDS-PAGE (10). The size of the protein was verified by mass spectrometric analysis of a carboxymethylated sample that gave an *m/z* of 23,007. This size is exactly the molecular mass calculated from sequence data considering the alkylation of the 4 cysteine residues and including an acetylated amino terminus. The native enzyme was also analyzed by mass spectrometric analysis. The protein sample, which contained high levels of Triton X-100, gave a broad peak with an *m/z* in the range of 22,770–22,790, which is close to the predicted mass of 22,779 Da for the protein with an acetylated NH_2 terminus.

The sequence was deduced following the isolation and identification of an almost complete set of tryptic peptides that were ordered with the help of overlapping peptides produced by cyanogen bromide and chymotryptic and peptic cleavages. Fig. 1 reports only the peptic and chymotryptic peptides that were useful in constructing the sequence.

Attempts to determine the NH_2-terminal sequence by direct automated Edman analysis of a first preparation of the enzyme, either native or reduced and carboxymethylated, were unsuccessful, suggesting the presence of a blocked NH_2 terminus. However, truncated forms at the NH_2 terminus of the enzyme, probably due to proteolysis during the purification procedures, were observed in samples obtained from different preparations. Reverse-phase HPLC purification of these samples yielded different peaks of protein that eluted very close in the chromatogram. Two of these peaks gave clear results when analyzed by automated Edman degradation in accord with the occurrence of truncated molecular forms of the enzyme, starting with NH_2-terminal Ala-23 and Leu-35, respectively (Fig. 1). Finally, a sample of enzyme prepared according to the rapid procedure described by Stover *et al.* (15) gave two very close bands of almost equal concentration when analyzed by SDS-PAGE. These two forms of the enzyme could not be resolved by reverse-phase HPLC. Sequence analysis of the two SDS-PAGE bands, after blotting onto the sequencing membrane, revealed that one corresponded to the mute NH_2-terminal-blocked protein and the other to a more extended truncated form starting from Ala-5 (Fig. 1). This was sequenced until Thr-37.

A tryptic peptide (*T1* in Fig. 1) that was mute to automated Edman degradation was suspected of being the blocked NH_2 terminus of the protein. The amino acid composition of this peptide was Ser (0.9 mol/mol of peptide), Gly (1.1), Ala (5.8), Val (0.9), and Lys (1.0). Considering the sequence AVSGAK present at the NH_2 terminus of the longest truncated form of the protein, we suspected that the 4 remaining alanines expected from the amino acid composition of the peptide are placed in a row at the NH_2 terminus of the protein. Fast atom bombardment mass spectral analyses of this peptide gave an *m/z* of 858, which is the predicted size of the T1 peptide *N*-acetyl-AAAAAVSGAK as shown in Fig. 1. The COOH-terminal sequence was identified after covalent attachment to a membrane (Sequelon AA™, Millipore) of the tryptic peptide T20 that had neither lysine nor arginine residues in the amino acid composition (Fig. 1).

The properties of the protein were analyzed by several computer models. A hydropathy profile (19) shows that the protein is very hydrophilic with only residues 1–6 and 125–145 having clusters of hydrophobic amino acids. Charge distribution is not uniform throughout the molecule, in particular the fragment comprising residues 6–41, which has 32% positively charged amino acid residues. The predicted pI for the protein is between 8 and 8.5. Secondary structure prediction (20) shows an extended α-helix in the NH_2-terminal region (residues 1–41).

TABLE II
Kinetic constants for substrate analogs of methenyl-THF synthetase

Substrate	K_m	k_{cat}
	μM	s^{-1}
5-CHO-H_4PteGlu	8	5
5-CHO-H_4PteGlu$_5$	0.2	5
5-CHO-H_4PteHis	11	3
5-CHO-H_4Pte	21	2

Substrate Specificity—The specificity of methenyl-THF synthetase for nucleotide triphosphates and divalent metal ions has been previously determined (10). In this study, we have investigated the role of the polyglutamate chain on K_m and V_{max}. Table II shows that both 5-CHO-H_4PteHis and 5-CHO-H_4Pte are substrates but with K_m values that are 1.5–2-fold higher than the K_m for 5-CHO-H_4PteGlu. This suggests that the γ-carboxyl group of the glutamate of 5-CHO-H_4PteGlu$_1$ does not significantly increase the affinity of the substrate for methenyl-THF synthetase. Also, the k_{cat} values for these two substrates differ by less than 2-fold. The 40-fold decrease in K_m value for 5-CHO-H_4PteGlu$_5$ suggests that methenyl-THF synthetase has a polyglutamate binding site (Table II).

Location of Polyglutamate Binding Site—Previously, dimethylglycine dehydrogenase and thymidylate synthase have been covalently inhibited with EDC-activated folate (17, 21). The EDC activates the carboxyl group of the glutamate side chain of H_4PteGlu to nucleophilic attack. Amino groups rapidly react to form stable amide bonds. We have observed that methenyl-THF synthetase loses activity when incubated with an EDC-activated solution of either 5-CHO-H_4PteGlu or 5-CHO-H_4PteGlu$_5$. Activity was not recovered upon prolonged dialysis at pH 7.0, suggesting that an amide bond had been formed with one of the glutamate carboxyl groups. Solutions of 0.1 μM methenyl-THF synthetase were titrated with increasing additions of EDC-activated 5-CHO-H_4PteGlu for 5 min at 0 °C. 50% inhibition was observed at 1.5 μM inhibitor. Incubation with 25 μM inhibitor for 5 min resulted in an 80% loss of activity. However, there was only a 5% loss of activity when 30 μM 5-CHO-H_4PteGlu$_5$ was added to the reaction solution prior to the addition of 25 μM EDC-activated 5-CHO-H_4PteGlu. This protection by the pentaglutamate is consistent with the inactivation occurring at the folate binding site. Similar inhibition and protection results were observed with EDC-activated 5-CHO-H_4PteGlu$_5$.

EDC-activated 5-CHO-H_4PteHis also resulted in inactivation of methenyl-THF synthetase. Again, the substrate 5-CHO-H_4PteGlu$_5$ protected against inactivation. However, EDC-activated 5-CHO-H_4Pte resulted in a 3-fold decreased level of inactivation of methenyl-THF synthetase even under saturating conditions of this substrate. Apparently, a free nucleophile is not close enough to the activated carboxyl group of the benzoic acid moiety to rapidly form a stable amide adduct.

Using EDC-activated [^{14}C]CHO-H_4PteGlu$_{1+5}$ substrates, 0.9 eq of 5-CHO-H_4PteGlu or 5-CHO-H_4PteGlu$_5$ were covalently attached to the enzyme. After denaturation and blocking of the sulfhydryl groups, the labeled enzyme was digested with trypsin. Peptides were purified using a triethylamine acetate-acetonitrile gradient on a C-8 reverse-phase HPLC column. For both the mono- and pentaglutamate substrates, a single radioactive peptide was isolated in greater than 70% yield. Amino acid sequencing showed that both the mono- and pentaglutamate EDC-activated substrates were bound to a peptide with the sequence Ala-Glu-Leu-*X*-Gln-Arg. The labeled peptide matches the sequence Ala^{15}-Glu-Leu-Lys-Gln-Arg^{20}. In the amino acid sequence analysis, the residue in the fourth cycle was not a known amino acid, but it was the first cycle to produce radioactivity. However, the radioactivity was very low, and examination of the filter at the end of sequencing showed that most of the counts remained on the filter. This was also found to be true when radiolabeled 5-CHO-H_4PteGlu was added to the sequencing filter. Under the acidic conditions used in sequencing, the formyl group of 5-CHO-H_4PteGlu will form 5,10-methenyl-H_4PteGlu, whose formyl group will react with any available nucleophile. Apparently, during sequencing this results in the transfer of the formyl group to the membrane.

DISCUSSION

Leucovorin is widely used in chemotherapy, but little is known about its metabolism. An enzyme activity for the conversion of 5-CHO-H_4PteGlu to other folate metabolites was first described by Greenberg in 1954 (22). A homogenous preparation of the enzyme was not obtained until 1984 when both the *Lactobacillus casei* and rabbit liver methenyl-THF synthetases were purified (9, 10). More recently, the enzyme has also been purified from human liver (11). All three enzymes share common kinetic and structural properties. Each enzyme is a monomeric 25–30-kDa protein that can use a variety of nucleotide triphosphates as an energy source.

Both the *in vivo* existence and function of 5-CHO-H_4PteGlu have been in question (23). Its existence has been attributable to the nonenzymatic hydrolysis of 5,10-CH$^+$-H_4PteGlu during the extraction of folates from biological tissue. This nonenzymatic source of 5-CHO-H_4PteGlu suggested that it did not exist in cells and, therefore, had no function. Evidence that 5-CHO-H_4PteGlu may have a physiological function was suggested by the studies of Bertrand *et al.* (11) who found that the *in vivo* inhibition of methenyl-THF synthetase in MCF-7 cells resulted in a 2-fold increase in cellular 5-CHO-H_4PteGlu levels with a concomitant decrease in both cell growth and *de novo* purine biosynthesis. A possible enzymatic source for 5-CHO-H_4PteGlu was discovered by Stover and Schirch (12). Serine hydroxymethyltransferase from both procaryotic and eucaryotic cells was found to catalyze the hydrolysis of 5,10-CH$^+$-H_4PteGlu to 5-CHO-H_4PteGlu. The combined activities of serine hydroxymethyltransferase and methenyl-THF synthetase catalyze a futile cycle that consumes ATP. The possible regulatory role of this futile cycle in controlling the *in vivo* levels of 5-CHO-H_4PteGlu has prompted us to investigate the structure and function of methenyl-THF synthetase.

Our first goal was to determine the primary structure of methenyl-THF synthetase to determine if it was related to other folate-requiring enzymes. We found that the enzyme is smaller by several kilodaltons than indicated from mobility on SDS-PAGE. Both mass spectral analysis and amino acid sequence are in agreement that the protein is 23 kDa and not 28 kDa as previously suggested (10). Evidence that the sequence reported in Fig. 1 is correct is supported by the perfect agreement between the mass of the reduced and carboxymethylated enzyme (as determined by mass spectrometry), the agreement between the mass and amino acid sequence of the native enzyme, and the agreement between the mass and amino acid sequence of the amino-terminal tryptic peptide T1.

The rabbit enzyme is stabilized by non-ionic detergents (15). Similar observations have been made for the *L. casei* enzyme (9). A hydrophobicity profile shows that the enzyme is very hydrophilic with only two short hydrophobic sequences. Searching for sequence identity of other proteins in the Swiss Prot and EMBL Data Banks resulted in only one match, an *Escherichia coli* 21-kDa protein that shares 20.8% identity with rabbit liver methenyl-THF synthetase (24). This percentage rises to 50% when only the central region of the two proteins is considered. This protein has been expressed, but its physiological role is still unknown. It may be either *E. coli* methenyl-THF synthetase or another folate-dependent protein. Studies are in

progress to assess the function of this bacterial protein. However, none of the sequences of known folate-binding proteins were found to show homology with methenyl-THF synthetase.

Methenyl-THF synthetase is inactivated by carbodiimide-activated 5-CHO-H_4PteGlu$_{1+5}$ and 5-CHO-H_4PteHis. 5-CHO-H_4Pte only partially inactivated the enzyme. All of these compounds serve as substrates for the enzyme. However, removal of glutamate residues results in some lowering of affinity for the enzyme (Table II). Both the carbodiimide-activated mono- and pentaglutamate of 5-CHO-H_4PteGlu appear to bind covalently to Lys-18. The observation that carbodiimide-activated 5-CHO-H_4PteHis, which lacks a γ-carboxyl group, also inactivates the enzyme suggests that Lys-18 forms a salt bridge with the α-carboxyl of the first glutamate residue. This region of the protein is predicted to be an α-helix with repeating Lys or Arg residues at positions 10, 11, 14, 18, 20, and 22. Model building suggests that the positive charges of these Lys and Arg residues are spaced so that they could interact with the negative charges of the polyglutamate portion of 5-CHO-H_4PteGlu$_n$. Not finding other labeled peptides with the carbodiimide-activated pentaglutamate may be due to the fact that residues 11, 14, 20, and 22 are Arg residues and would not react with the carbodiimide-activated polyglutamate. Evidence that this region may serve as a polyglutamate binding site is its similarity to the polyglutamate binding site of *E. coli* thymidylate synthase (25). An analysis of the crystal structure of the enzyme-ternary complex with deoxyuridine monophosphate and the polyglutamate analog CB3717 shows that the α-carboxyl group of Glu-1 forms an ionic bond with Lys-50. Additional positive charged residues are at positions 51, 53, 55, and 59. It appears that the free carboxyl of Glu-3 forms a bond with Arg-55. His-53 may play some role in binding of the polyglutamate chain, but we conclude that it does not have a positive charge. Unfortunately, Glu-4 and Glu-5 of CB3717 were linked through the γ-carboxyl groups rather than the α-carboxyl groups found in the natural substrates. Probably for this reason, the binding of Glu-4 and Glu-5 to specific residues could not be determined.

Usha *et al.* (26) have determined that Arg-269 and Arg-462 in cytosolic serine hydroxymethyltransferase were protected from phenylglyoxal modification by H_4PteGlu. Arg-462 is part of a sequence of predicted α-helix that contains 8 Lys or Arg residues between residues 442 and 465 (27). A similar sequence of Lys and Arg residues is present in the mitochondrial serine hydroxymethyltransferase (16). Future studies may show that in many folate-requiring enzymes, an α-helical segment containing multiple Lys and Arg residues serves as a polyglutamate binding site.

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