

5-Formyltetrahydropteroylpolyglutamates Are the Major Folate Derivatives in *Neurospora crassa* Conidiospores*

(Received for publication, May 23, 1994, and in revised form, August 19, 1994)

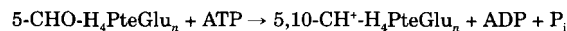
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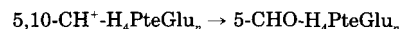
5-Formyltetrahydropteroylpolyglutamate (5-CHO-H₄PteGlu_n) is the only reduced folate derivative that is stable to oxidation and alkaline pH. However, no metabolic role has been assigned to this folate derivative, and evidence for its existence in cells has been questioned. Recently, serine hydroxymethyltransferase was shown to catalyze the formation of 5-CHO-H₄PteGlu_n from 5,10-methenyl-H₄PteGlu_n (Stover, P., and Schirch, V. (1990) *J. Biol. Chem.* 265, 14227–14233). We have proposed that 5-CHO-H₄PteGlu_n may serve as a storage form of reduced folates and one-carbon groups in cells that are in a dormant stage. This hypothesis was tested by determining the levels of H₄PteGlu_n derivatives in the mycelia and conidiospores of *Neurospora crassa* and a mutant strain that lacks cytosolic serine hydroxymethyltransferase. *N. crassa* serine hydroxymethyltransferase was purified to homogeneity and characterized with respect to kinetic constants, quaternary structure, stability, and reaction specificity. A new assay for determining the concentration of the polyglutamate forms of H₄PteGlu_n derivatives was also developed. Using this assay, it was shown that 85% of the tetrahydropteroylpolyglutamates in conidiospores was 5-CHO-H₄PteGlu_n. After adding the spores to growth media, the 5-CHO-H₄PteGlu_n was reduced to less than 10% of the folate pool in 20 min. Mycelia had no detectable 5-CHO-H₄PteGlu_n. Only 10–20% of the folate pool in conidiospores from the mutant strain lacking cytosolic serine hydroxymethyltransferase was in the form of 5-CHO-H₄PteGlu_n.

One-carbon metabolism is essential in providing activated one-carbon groups for the biosynthesis of purines, thymidylate, methionine, choline and many other methylated compounds. These one-carbon groups are carried and donated by tetrahydropteroylglutamate (H₄PteGlu_n)¹ (1, 2). Most one-carbon H₄PteGlu_n derivatives are subject to degradative oxidation with the exception of 5-CHO-H₄PteGlu_n, which is stable to ox-

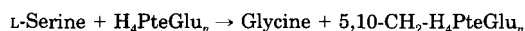
ygen and alkaline pH. The metabolic function of 5-CHO-H₄PteGlu_n is not clear, as this derivative does not donate its one-carbon group in any known biosynthetic reaction. However, there are two known enzymes that may be involved in the metabolism of this derivative. 5,10-Methenyltetrahydrofolate synthetase catalyzes the irreversible ATP-dependent conversion of 5-CHO-H₄PteGlu_n to 5,10-CH⁺-H₄PteGlu_n (Reaction 1) (3, 4). This reaction is responsible for the *in vivo* conversion of leucovorin (the clinical name for 5-CHO-H₄PteGlu) to forms of H₄PteGlu_n that can serve as one-carbon donors and thus relieve the toxic effects of the chemotherapeutic drug methotrexate. In the presence of glycine, SHMT catalyzes the hydrolysis of 5,10-CH⁺-H₄PteGlu_n to 5-CHO-H₄PteGlu_n (Reaction 2) (5). The physiological reaction catalyzed by SHMT is the conversion of serine to glycine and the generation of 5,10-CH₂-H₄PteGlu_n, which is the major source of one-carbon groups in the cell (Reaction 3) (1). Whether the SHMT catalysis of Reaction 2 is of physiological importance is yet to be proven. However, the combined activities of SHMT and 5,10-methenyltetrahydrofolate synthetase (Reactions 1 and 2) result in a futile cycle involving 5-CHO-H₄PteGlu_n. Often futile cycles are involved in metabolic regulation.



REACTION 1



REACTION 2



REACTION 3

No metabolic role has previously been assigned to 5-CHO-H₄PteGlu_n, even though the intracellular folate pools of most organisms have been found to contain about 10% of this H₄PteGlu_n derivative. The amount of 5-CHO-H₄PteGlu_n varies dramatically in different cell types, but it has been argued that it is formed during the cell extraction procedure and, therefore, is an artifact of isolation. This is unlikely since significant concentrations of 5-CHO-H₄PteGlu_n have been found in some organisms. These include *Neurospora crassa* mycelia (35% of total folates), mouse L1210 cells (25%), human fibroblasts (14%), and soybeans (70%) (6–10). However, even in these examples, the length and pH of the extraction process cast doubt on the true *in vivo* levels of 5-CHO-H₄PteGlu_n. Because of the stability of 5-CHO-H₄PteGlu_n, we have proposed that it may function as a storage form of folate in cells that have a dormant stage, such as spores or seeds. Another possibility is that this derivative plays a regulatory role in one-carbon metabolism. Studies have shown that 5-CHO-H₄PteGlu_n is an inhibitor of many of the enzymes involved in one-carbon metabolism (11–15). Therefore, the accumulation of this derivative might result in a broad inhibition of one-carbon metabolism resulting in the inhibition of many biosynthetic pathways.

To determine whether 5-CHO-H₄PteGlu_n serves as a storage

* This work was supported by Grant GM 28143 from the National Institutes of Health (to V. S.) and Grant A-1747 from the Natural Sciences and Engineering Research Council of Canada (to E. A. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: H₄PteGlu_n, tetrahydropteroylglutamate; 5-CHO-H₄PteGlu_n, 5-formyltetrahydropteroylglutamate containing *n* glutamate residues; 10-CHO-H₄PteGlu_n, 10-formyltetrahydropteroylglutamate; 5,10-CH₂-H₄PteGlu_n, 5,10-methylenetetrahydropteroylglutamate; 5,10-CH⁺-H₄PteGlu_n, 5,10-methenyltetrahydropteroylglutamate; 5,10-CHOH-H₄PteGlu_n, 5,10-hydroxymethylenetetrahydropteroylglutamate; SHMT, serine hydroxymethyltransferase; PAGE, polyacrylamide gel electrophoresis; BES, *N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonate; HPLC, high performance liquid chromatography.

form of folates and one-carbon groups, we have investigated the distribution of $H_4PteGlu_n$ pools during the asexual life cycle of *N. crassa*. If 5-CHO- $H_4PteGlu_n$ serves as a storage form of folate, then the conidiospores, formed during the life cycle of this organism, would be expected to have higher concentrations of this $H_4PteGlu_n$ derivative than the mycelial stage of the life cycle where cell growth and replication are taking place. We have purified and characterized the catalytic properties of *N. crassa* SHMT to determine if it is involved in the formation of 5-CHO- $H_4PteGlu_n$ in this organism. The results strongly suggest that 5-CHO- $H_4PteGlu_n$ serves as a storage form of folates and one-carbon groups in the conidiospores of this organism and that *N. crassa* SHMT plays an important role in its formation.

EXPERIMENTAL PROCEDURES

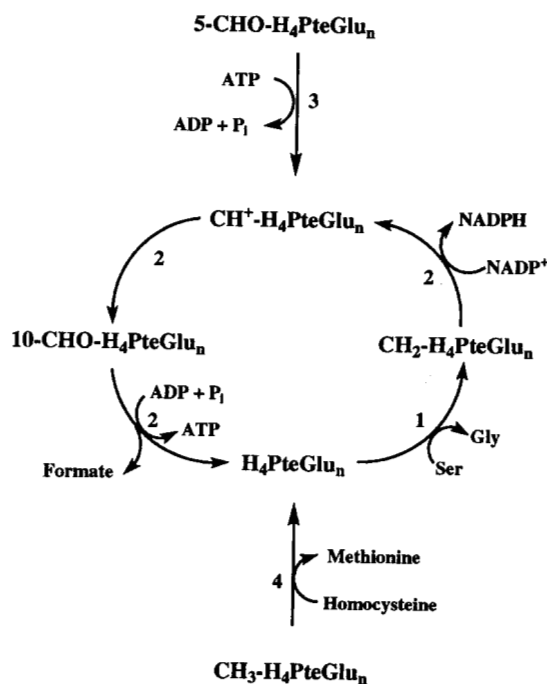
Materials—Glycine, serine, NADH, NADP⁺, $H_4PteGlu$, alcohol dehydrogenase, L-threonine, D-alanine, 2-mercaptoethanol, EDTA, methionine, NaADP, MgATP, MgCl₂, and peroxidase-conjugated goat anti-rabbit IgG were purchased from Sigma. Allothreonine was obtained from ICN Pharmaceuticals. C₁-tetrahydrofolate synthase, rabbit liver cSHMT, and 5,10-methenyltetrahydrofolate synthetase were purified as described previously (16, 17). Pteroylpolyglutamates were obtained from Dr. B. Schirck's Laboratories, Jona, Switzerland. Cultures of *N. crassa* lindegren A wild-type (Fungal Genetics Stock Center (FGSC) no. 853), *met-6* (FGSC no. 1330), and *for* (FGSC no. 9) were obtained from the Fungal Genetics Stock Center, Kansas City, KN. SHMT was purified from the *met-6* mutant by a recently published procedure (18). *Lactobacillus casei* dihydrofolate reductase and cobalamin-independent *Escherichia coli* methionine synthase were generous gifts from Dr. Roy Kisliuk and Dr. Rowena Matthews, respectively. Samples of (6S)-5-CHO- $H_4PteGlu_n$ derivatives were generously provided by Dr. Anthony Fitzhugh.

Preparation of Folate Compounds—(6S)- $H_4PteGlu_n$ and (6S)-5-CHO- $H_4PteGlu_n$ were prepared as described previously (16). 5,10-CH⁺- $H_4PteGlu_n$ was synthesized by incubating solutions of 5-CHO- $H_4PteGlu_n$ at pH 2 overnight at 4 °C. 10-CHO- $H_4PteGlu_n$, 5,10-CH₂- $H_4PteGlu_n$, and 5-CH₃- $H_4PteGlu_n$ were synthesized and quantified as described previously (16). The concentrations of stock 5-CHO- $H_4PteGlu_n$ solutions were determined at pH 7 using an extinction coefficient of 31,500 M⁻¹ cm⁻¹ at 286 nm. Concentrations of 5,10-CH⁺- $H_4PteGlu_n$ solutions were determined at pH 2 using an extinction coefficient of 25,100 M⁻¹ cm⁻¹ at 360 nm (19). A coupled enzymatic assay involving SHMT and 5,10-CH₂-tetrahydrofolate dehydrogenase was used to measure the concentrations of stock $H_4PteGlu_n$ solutions (20).

Enzyme Assays—The aldol cleavage of serine, allothreonine, threonine, and 3-phenylserine; the transamination of L-alanine; and the rate of 5-CHO- $H_4PteGlu_n$ synthesis by SHMT were measured by published methods (5, 20–23).

Enzyme activity of conidiospores and mycelial extracts was determined by a sensitive assay based on the ability of SHMT to catalyze the rapid exchange of the *pro* 2 S proton of glycine with solvent protons in the presence of $H_4PteGlu_n$ (24). To 50 µl of potassium BES, pH 7.3, was added 2-[³H]glycine (800,000 cpm) and 10 µmol of $H_4PteGlu$. The reaction was initiated by the addition of 1–10-µl aliquots of either mycelial or spore extracts of the respective acetone powders. After a 5-min incubation at 30 °C, 5 µl of 20% trichloroacetic acid was added and the precipitated protein removed by centrifugation. The supernatant was then added to a 1-cm high column of Dowex 50 in a 1-ml plastic syringe that had been equilibrated with 10 mM HCl. The eluate from this column was collected directly in a scintillation counting vial containing 6 ml of scintillation fluid. The column was washed with another 600 µl of 10 mM HCl. The eluate was collected in the vial and counted. The substrate glycine binds to the column and only the exchanged protons appear in the eluate.

Extraction of *N. crassa* Mycelial and Conidiospore $H_4PteGlu_n$ Derivatives—The extraction of $H_4PteGlu_n$ cofactors from conidia was performed by transferring a known amount, usually 5 mg dry weight, into a 1.5-ml Eppendorf tube, adding 0.5 ml of hot extraction buffer (50 mM potassium HEPES, pH 7.6, with 0.2 mM EDTA, 10 mM (NH₄)₂SO₄, 50 mM 2-mercaptoethanol, and 2% sodium ascorbate) and immediately boiling for 5 min. Extracts were then cooled on ice, sonicated for approximately 30 s, and centrifuged. The supernatant was diluted with 0.5 ml of 20 mM potassium phosphate, pH 6.8, containing 1 mM MgCl₂ and 10 mM (NH₄)₂SO₄ before determining the concentrations of



SCHEME 1. Cyclic assay for measuring folylpolyglutamates. The enzymes involved are the following: 1, serine hydroxymethyltransferase; 2, C₁-tetrahydrofolate synthase; 3, 5,10-methenyltetrahydrofolate synthetase; 4, methionine synthase.

$H_4PteGlu_n$ derivatives. Mycelial extracts were obtained using the same procedure, except that 13 mg (dry weight) of mycelia were used for each assay. All buffers were made with HPLC grade water and extensively purged with argon before use.

Determination of $H_4PteGlu_n$ Pools—The coupled enzyme activities of SHMT and C₁-tetrahydrofolate synthase catalyze a metabolic cycle with the conversion of serine to formate and glycine with substrate amounts of NADP⁺ and ADP and catalytic concentrations of either $H_4PteGlu_n$, 10-CHO- $H_4PteGlu_n$, 5,10-CH⁺- $H_4PteGlu_n$, or 5,10-CH₂- $H_4PteGlu_n$ (Scheme 1). These folates will be referred to as the "combined folate pool." The $H_4PteGlu_n$ compounds are regenerated with each catalytic cycle with concomitant reduction of NADP⁺ to NADPH. Using excess SHMT and C₁-tetrahydrofolate synthase, the rate of reduction of NADP⁺ to NADPH, as determined by the increase in absorbance at 340 nm, was linear with 10–200 pmol $H_4PteGlu_n$ /1-ml assay. The cycle did not generate NADPH when the monoglutamate forms of the coenzyme were used.

A typical assay contained 800 µl of *N. crassa* extract, 1 mM MgADP, 0.2 mM NADP⁺, 5 mM L-serine, and 150 µg of SHMT at 30 °C. The reaction mixture was placed in an HP 8452A spectrophotometer, and a background rate was obtained by determining the change in absorbance of a 335–345-nm wavelength window with time. The instrument subtracted from this absorbance window the absorbance of a 440–450-nm window. NADP⁺ does not absorb at these longer wavelengths, but this corrects for random fluctuations in absorbance. The reaction was started by the addition of 100 µg of C₁-tetrahydrofolate synthase, and the increase in absorbance followed for about 1 min. The background rate was then subtracted from this rate to obtain the folate-dependent rate.

The concentrations of 5-CHO- $H_4PteGlu_n$ and 5-CH₃- $H_4PteGlu_n$ were determined by converting these two folate derivatives into one of the combined folate forms prior to determining the folate concentrations as described above. An aliquot of *N. crassa* extract was incubated for either 1 min with 1 mM MgATP and 10 µg of purified 5,10-methenyltetrahydrofolate synthetase or for 15 min at 37 °C with 0.2 mM DL-homocysteine and 10 µg of methionine synthase. After preincubation, the other reagents were added as described above for folate determinations. Any increase in rate over the combined folate pool is due to either 5-CHO- $H_4PteGlu_n$ or 5-CH₃- $H_4PteGlu_n$.

Standard curves for $H_4PteGlu_n$ derivatives were determined by adding known concentrations to the assay buffer in the absence of extract. Otherwise, the assay was performed as described above. Control experiments were performed to determine the yield of each folate form by adding a known amount of each derivative of $H_4PteGlu_n$ to a spore

extract and determining the increase in rate over the rate without the addition of the $H_4PteGlu_3$ derivative. Each folate determination was performed at least in triplicate.

***Neurospora crassa* Cultures**—Lindegren A wild-type was maintained on agar slants of Vogel's media (25) containing 2% (w/v) sucrose and 2% (w/v) agar. The methionine auxotroph, *met-6*, and the formate auxotroph, *for*, were maintained on the same media supplemented with 1 mM DL-methionine and 10 mM formate, respectively. For large scale production of conidiospores, 2.5-liter Fernbach flasks containing 500 ml of the above media were inoculated from a fresh slant culture using a sterile loop and incubated at 30 °C in the dark for 2–4 days. After this period, they were inverted and allowed to grow at room temperature in constant light with aeration for 8–10 more days. Wet-harvested conidiospores were prepared by washing cultures with 0.2% Tween 80, filtering through cheesecloth, and pelleting by centrifugation. The conidiospores were then washed with distilled water and lyophilized. Dry-harvested conidiospores were obtained by inverting 10-ml slant cultures grown in 18 × 150-mm test tubes and tapping the sides as described previously (26). The conidiospores were collected in sterile tubes.

Physical and Kinetic Properties of *N. crassa* SHMT—The molecular weight of purified *N. crassa* SHMT was determined using molecular sieve chromatography. The enzyme and standards were prepared in 100 mM potassium phosphate buffer, pH 7.0, and filtered before being analyzed on a TSK-G4000 HPLC column. The equilibrating buffer was 100 mM potassium phosphate, pH 7.0. The molecular weight standards were apoferritin, rabbit liver cytosolic SHMT, bovine serum albumin, alcohol dehydrogenase, hexokinase, and ovalbumin. The subunit molecular weight was determined by SDS-PAGE analysis (27).

The extinction coefficient at 278 nm for *N. crassa* cytosolic SHMT was calculated from the number of tryptophan and tyrosine residues in the protein (28). This was determined based on the predicted amino acid sequence derived from the cloned gene (29). The calculated extinction coefficient is 37,550 $M^{-1} cm^{-1}$ at 278 nm or an absorbance of 0.68 for a 1 mg/ml solution.

The K_m and V_{max} values for serine and $H_4PteGlu_n$ were determined using double-reciprocal plots of initial velocity versus substrate concentration. Varying $H_4PteGlu$ concentrations of 50, 20, 10, and 7 μM were used with fixed L-serine concentrations of 1.0, 0.5, 0.25, and 0.167 mM. The K_m and V_{max} values for allothreonine were also determined by double-reciprocal plots of initial velocity versus allothreonine concentration.

Dissociation constants for $H_4PteGlu_3$ and $H_4PteGlu_5$ were determined by titrating solutions containing enzyme and glycine with $H_4PteGlu_n$ and measuring the formation of the stable SHMT-Gly- $H_4PteGlu_n$ quinonoid complex at 492 nm as described previously (30). An extinction coefficient of 19,000 $M^{-1} cm^{-1}$, determined using saturating $H_4PteGlu_n$, was used to determine the concentration of quinonoid at each $H_4PteGlu_n$ concentration. Data were plotted according to the method of Scatchard (31).

The thermal denaturation temperature of SHMT was determined by a previously published method (32).

First-order rate constants for the formation and breakdown of the 5-CHO- $H_4PteGlu_n$ -SHMT-Gly ternary complexes were determined by measuring the absorbance at 502 nm using a stopped-flow spectrophotometer from Kinetic Instruments Inc. as described previously (33).

Determination of Intracellular SHMT Concentrations—The amount of SHMT in conidiospores was determined by Western blot analysis. Wet-harvested conidiospores were resuspended in 20 mM potassium phosphate buffer and immediately lysed in a French pressure cell at 20,000 psi. The extracts were centrifuged, and SDS-PAGE of both the soluble and insoluble fractions was performed using a 12.5% slab gel according to the method of O'Farrell (27). Western blots were performed on protein that had been separated by SDS-PAGE and transferred to nitrocellulose according to the method of Burnette (34). The blotted nitrocellulose was incubated with a 1:2000 dilution of rabbit anti-*N. crassa* cSHMT serum overnight at 27 °C. After washing, the nitrocellulose was incubated with a 1:2000 dilution of peroxidase-conjugated goat anti-rabbit IgG for 1 h at room temperature. After extensive washing, the membrane was incubated with substrate for 10 min, and scanned in a Shimadzu CS-9000 scanning densitometer. A standard curve was constructed by loading known quantities of purified *N. crassa* cSHMT on the same SDS-PAGE gels.

Isolation of Soluble Proteins from Conidiospores and Mycelia—Dry-harvested spores, 50 mg, were placed in a polypropylene centrifuge tube and cooled to -60 °C. The spores were then extracted with 10 ml of acetone, also at -60 °C, and stirred for 5 min. The extract was centrifuged for 5 min at -15 °C and 13,000 × *g*. The centrifuge tube was placed

in an ice-ethanol bath, and the acetone was removed with a pipette. The centrifuge tube containing the pelleted spore proteins was quickly placed under vacuum to remove the excess acetone. The dry acetone powder was then extracted at 0 °C with 5 ml of 50 mM potassium BES buffer, pH 7.3, with stirring for 30 min. The extract was centrifuged at 13,000 × *g* for 15 min, and the supernatant was assayed for SHMT activity. Mycelial extracts were obtained by grinding 1 g of mycelia with 2 g of acid-washed sand and 5 ml of 20 mM potassium phosphate buffer in a mortar and pestle for several minutes. The suspension was transferred to a polypropylene centrifuge tube at -60 °C, and 40 ml of acetone at -60 °C was added. These suspensions were centrifuged and dried as before. Extracts of these acetone powders were obtained by resuspending the pellet in 20 ml of buffer and stirring at 0 °C for 30 min. The solution was then centrifuged at 13,000 × *g* for 20 min, and the supernatant was used to assay for enzyme activities.

The similarity between mycelial and conidiospore SHMT was determined by isoelectric focusing. The soluble proteins were separated by isoelectric focusing in 6 M urea as described previously (35) and analyzed by Western blotting with anti-*N. crassa* cSHMT antibodies as described (36).

RESULTS AND DISCUSSION

Method for Determining $H_4PteGlu_n$ Pools in *N. crassa*—The nonenzymatic conversion of 10-CHO- $H_4PteGlu_n$ to 5-CHO- $H_4PteGlu_n$ is well documented (37). Many of the previous methods for determining folate levels in biological tissues have not rigorously demonstrated that the 5-CHO- $H_4PteGlu_n$, observed in tissue extracts, was not generated by this nonenzymatic reaction during the extraction and analysis of the folate pools. We have devised a rapid and sensitive assay for 5-CHO- $H_4PteGlu_n$, which can be easily tested for artifactual formation during the extraction process.

The combined concentrations of $H_4PteGlu_n$, 10-CHO- $H_4PteGlu_n$, 5,10-CH₂- $H_4PteGlu_n$, and 5,10-CH⁺- $H_4PteGlu_n$ can be determined by measuring the rate of NADPH formation at 340 nm in the metabolic cycle shown in Scheme 1. In this cycle, the $H_4PteGlu_n$ derivatives are regenerated during each catalytic cycle, and the rate of NADPH formation suggests there are about 100 cycles of $H_4PteGlu_n$ /min. The same rate was obtained starting with equal concentrations of any of the four derivatives of $H_4PteGlu_n$ that are a part of the cycle. However, no rate of NADPH formation was observed when either 5-CHO- $H_4PteGlu_n$ or 5-CH₃- $H_4PteGlu_n$ were used to initiate the cycle. This assay does not measure the monoglutamate derivatives of $H_4PteGlu_n$, but previous studies have shown that in *N. crassa* the folates are present almost exclusively as the hexaglutamate forms (38). Varying the number of glutamate residues between 3 and 6 did not change the rate of the cycle.

Although 5-CHO- $H_4PteGlu_n$ is not a substrate for the cycle, it can be converted into a form that supports the cycle by preincubation with ATP and 5,10-methylenetetrahydrofolate synthetase for 2 min (Reaction 1 and Scheme 1) (39). Using this method, a standard curve for 5-CHO- $H_4PteGlu_n$ versus $\Delta A_{340}/min$ was constructed (Fig. 1). There was some variation in the slope of the standard curve with different preparations of C₁-tetrahydrofolate synthase. Therefore, the standard curve was reconstructed whenever C₁-tetrahydrofolate synthase from a new preparation was used. Experiments performed with varying concentrations of 5-CHO- $H_4PteGlu_3$ added to a mycelial extract showed that the slope of the standard curve did not change when mycelial extract was present but was higher by the amount of folates in the extract (data not shown).

The concentration of 5-CH₃- $H_4PteGlu_n$ can be determined in tissue extracts by a method similar to the one described for 5-CHO- $H_4PteGlu_n$. This method involves preincubation of cell extracts with homocysteine and methionine synthase to convert 5-CH₃- $H_4PteGlu_n$ to $H_4PteGlu_n$ (Scheme 1). When 20 pmol of 5-CH₃- $H_4PteGlu_n$ and 0.2 mM DL-homocysteine are added to a mycelial extract and the rate of the cycle was determined in the absence of methionine synthase, a 10% reduction in rate was

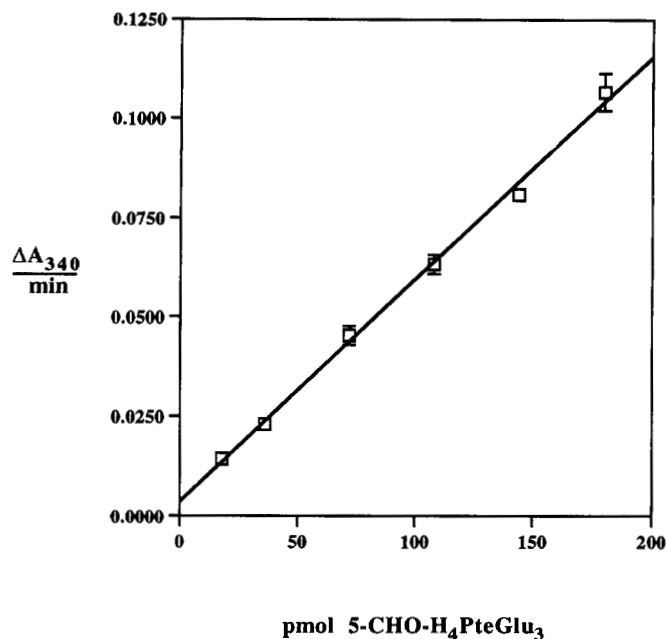


FIG. 1. Cyclic assay standard curve constructed with (6S)-5-CHO- H_4 PteGlu $_3$. Known concentrations of 5-CHO- H_4 PteGlu $_3$ were added to the assay buffer in the absence of extract. After a 1-min preincubation with 5,10-methylenetetrahydrofolate synthetase and ATP, the other reaction components were added and the reaction rate measured at 340 nm. Error bars represent one S.D. ($n = 3-5$).

observed. This was due to homocysteine inhibiting the SHMT activity in the cycle, but the results confirm that 5-CH $_3$ - H_4 PteGlu $_n$ is not a substrate for the cyclic assay for folates.

The recovery of each H_4 PteGlu $_n$ derivative during the extraction process was determined by comparing the rates of the cycle for each H_4 PteGlu $_n$ derivative by adding it either directly to the assay mixture or by first adding it to 3–5 mg of spores prior to the extraction process. The decrease in rate of the cycle after the extraction process was used to determine the yield of each H_4 PteGlu $_n$ derivative. The recovery for each folate was between 70 and 80%, except for 5-CH $_3$ - H_4 PteGlu $_3$, which was only 58%. We assumed that the recovery of the spore folates was also about 75%, so each experimentally determined concentration was corrected by multiplying by 1.35. However, since the recovery of 5-CH $_3$ - H_4 PteGlu $_3$ was significantly lower, the experimentally determined concentration was corrected by multiplying by 1.7. The validity of our extraction method was confirmed by comparing the levels of H_4 PteGlu $_n$ derivatives in spores determined by our method and by a previously used microbiological method. Our method showed higher levels of folates by 1.7-fold (6). These higher levels may be either the result of some degradation of reduced folates during the much longer period required for the microbiological assay or the failure to correct for the efficiency of the extraction of folates as we have done.

H_4 PteGlu $_n$ Pools in Conidiospores—The levels of H_4 PteGlu $_n$ compounds in dry-harvested conidiospores were determined by extracting H_4 PteGlu $_n$ derivatives as described under "Experimental Procedures." Analysis of dry-harvested spore extracts with and without preincubation with ATP and 5,10-methylenetetrahydrofolate synthetase showed that 85% of the folate pool was 5-CHO- H_4 PteGlu $_n$ (Table I). The concentration of 5-CHO- H_4 PteGlu $_n$ was 22 nmol/g of spores. When spores were harvested in water, little or no 5-CHO- H_4 PteGlu $_n$ was found in the boiled spore extracts. The lack of 5-CHO- H_4 PteGlu $_n$ in wet-harvested spores suggested that the brief 10–15-min exposure to water during harvesting resulted in all of the 5-CHO- H_4 PteGlu $_n$ being converted to other H_4 PteGlu $_n$ compounds,

TABLE I
Concentrations of H_4 PteGlu $_n$ derivatives and SHMT in conidiospores and mycelia of wild-type and the for mutant of *N. crassa*

Compound	Wild-type <i>N. crassa</i>		For mutant <i>N. crassa</i>	
	spores	mycelia	spores	mycelia
	nmol/g		nmol/g	
Combined ^a	3.8 ± 0.9	76 ± 1.4	18 ± 1.1	213 ± 6.6
5-CHO- H_4 PteGlu $_n$	23 ± 1.1	<12	5.6 ± 1.2	<24
5-CH $_3$ - H_4 PteGlu $_n$	<2.8	47 ± 11	3.4 ± .4	26 ± 5.2
cSHMT (Western blot)	1.0			
SHMT (activity)	0.6	2.7	0.06	0.5

^a Total concentration of H_4 PteGlu $_n$, 10-CHO- H_4 PteGlu $_n$, 5,10-CH $_2$ - H_4 PteGlu $_n$, and 5,10-CH- H_4 PteGlu $_n$.

which are involved in the cyclic assay. This prompted a study of the rate at which 5-CHO- H_4 PteGlu $_n$ in dry-harvested spores is converted into other H_4 PteGlu $_n$ compounds after the addition of spores to aqueous conditions. Dry-harvested spores were incubated in Vogel's media for varying lengths of time before H_4 PteGlu $_n$ compounds were extracted. Fig. 2 shows that 5-CHO- H_4 PteGlu $_n$ is mostly converted into the active folate pool during the first 10 min, decreasing from 85% of the folate pool to 25%. However, with different spore preparations, the 5-CHO- H_4 PteGlu $_n$ often decreased to less than 10% of the folate pool during a 10-min incubation. Because of this rapid conversion and the time it takes to increase the temperature to high levels during spore extraction, it is possible that all of the folates in spores are stored as 5-CHO- H_4 PteGlu $_n$. After 20 min of incubation in Vogel's media, all spore extracts gave undetectable levels of 5-CHO- H_4 PteGlu $_n$.

No 5-CH $_3$ - H_4 PteGlu $_n$ was found in any of our spore extracts. After a 10-min incubation of spores in Vogel's media, the 5-CH $_3$ - H_4 PteGlu $_n$ increased to 16% of the folate pool (Fig. 2). However, in mycelia, 5-CH $_3$ - H_4 PteGlu $_n$ represents about 38% of the folate pool, and 5-CHO- H_4 PteGlu $_n$ is less than 10% (Table I). Mycelia contain about 5 times the level of folates/g of dry weight than spores.

During the first 10 min of incubation of conidiospores in Vogel's media, there is a 1.7-fold increase in the concentration of total H_4 PteGlu $_n$ (Table I). It is not clear if this increase represents more efficient extraction of H_4 PteGlu $_n$ compounds, conversion of monoglutamate forms to polyglutamate forms, or *de novo* synthesis.

The results presented in Table I unequivocally show that during the asexual life cycle of *N. crassa*, the levels of 5-CHO- H_4 PteGlu $_n$ go from undetectable levels in the mycelia to greater than 80% of the folate pool in the conidiospores. During conditions for germination of the spores, the 5-CHO- H_4 PteGlu $_n$ is rapidly converted back into forms that support one-carbon metabolism. Previous studies have shown that both rabbit liver and *E. coli* SHMTs slowly catalyze the conversion of 5,10-CH- H_4 PteGlu $_n$ to 5-CHO- H_4 PteGlu $_n$ (5). Correlation of 5-CHO- H_4 PteGlu $_n$ and SHMT levels in *E. coli* supported the interpretation that in this organism, the source of the 5-CHO- H_4 PteGlu $_n$ was the SHMT catalysis of Reaction 2 (5). The life cycle of *N. crassa* offers an excellent model for studying the role of SHMT in regulating the *in vivo* levels of 5-CHO- H_4 PteGlu $_n$. However, SHMT has not previously been purified or characterized from any fungal source so it is not clear whether any analogy can be drawn between its role in 5-CHO- H_4 PteGlu $_n$ metabolism in *N. crassa* and other organisms. To correct for this lack of information, we have purified *N. crassa* cSHMT and characterized it with respect to properties important for understanding its role in 5-CHO- H_4 PteGlu $_n$ metabolism.

Purification and Properties of *N. crassa* SHMT—SHMT was purified from an overproducing mutant that requires methio-

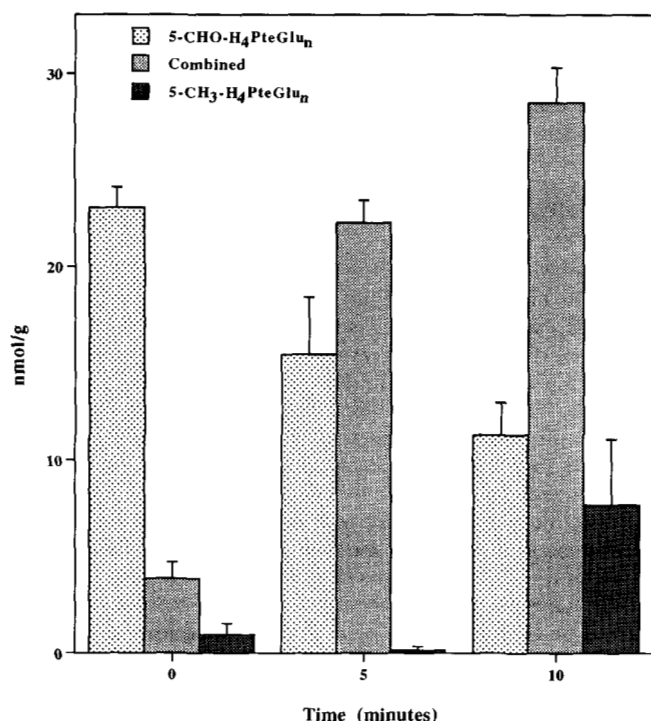


FIG. 2. Distribution of folylpolyglutamate derivatives during the initial stages of germination. Dry-harvested conidiospores were incubated in minimal media for the indicated time periods and immediately boiled in extraction buffer. Extracts were then assayed as described under "Experimental Procedures." Error bars represent one S.D. ($n = 3-5$).

nine for growth. The enzyme was purified by standard methods and was found to be greater than 95% homogeneous based on SDS-PAGE analysis (18). The overall purification was 746-fold with a 30% yield. Approximately 70 mg of pure enzyme were obtained from 250 g (wet weight) of mycelia. After obtaining about one-third of the amino acid sequence the gene for *N. crassa*, cytosolic SHMT was cloned, and the predicted amino acid sequence was determined from the cDNA (29). The predicted sequence was found to be 60% identical to rabbit cytosolic, 56% to rabbit mitochondrial, and 47% to *E. coli* SHMTs (29). Our partial sequence was identical to the cDNA predicted sequence, confirming that we had purified cytosolic SHMT.

The subunit molecular weight was determined using SDS-PAGE analysis in which a single band with a molecular weight of 54 kDa was obtained. This is in agreement with the predicted amino acid sequence obtained from the cloned cytosolic SHMT gene, which would result in a protein with a molecular weight of 53 kDa (29). The native molecular weight, determined by molecular sieve chromatography, was 230 kDa, indicating a tetramer of identical subunits. These results are similar to those obtained for the rabbit isoenzymes that are also tetramers of identical 54 kDa subunits but differs from the *E. coli* enzyme, which is a dimer (1, 40).

Enzyme Characterization—Like the other SHMT enzymes, purified *N. crassa* SHMT is pale yellow due to the absorption of the covalently bound internal aldimine of pyridoxal phosphate at 428 nm. The 280/428 ratio for the pure enzyme, which varies in different species from 6.0 to 8.5, was 6.8 for the *N. crassa* enzyme. Saturation of the rabbit and *E. coli* enzymes with glycine and H₄PteGlu_n results in an absorption peak at 495 nm due to the formation of a resonance stabilized quinonoid complex of the bound pyridoxal phosphate (1). Addition of 5-CHO-H₄PteGlu_n or 5-CH₃-H₄PteGlu_n to the SHMT·Gly complex also results in the formation of a quinonoid species, but the wavelength is shifted to 502 nm (5). The *N. crassa* enzyme displays

the same characteristic absorption maxima.

Kinetic constants for serine and H₄PteGlu were determined using double-reciprocal plots of initial velocity versus substrate concentration. These plots gave converging lines, indicating that the mechanism involves sequential addition of substrates. The K_m values obtained were 0.5 mM for serine and 30 μ M for H₄PteGlu, while the αK_m values obtained were 0.2 mM for serine and 18 μ M for H₄PteGlu. The decrease observed between the K_m and αK_m values indicates that the two substrates display synergism in binding. Both rabbit isoenzymes and the *E. coli* enzyme display similar characteristics (40, 41).

The ability of *N. crassa* SHMT to cleave several 3-hydroxy amino acids in the absence of H₄PteGlu_n was also studied. Like the rabbit and *E. coli* enzymes, the *N. crassa* enzyme cleaves allothreonine to glycine and acetaldehyde with a K_m for L-allothreonine of 1.7 mM and a k_{cat} of 14 min⁻¹, which is approximately one-tenth of the rate of the rabbit enzyme. The enzyme also catalyzes the transamination of D-alanine with a first-order rate constant of 0.08 min⁻¹, which is 16 times the rate of the *E. coli* enzyme (42).

Studies with rabbit liver cSHMT have shown that the polyglutamate derivatives of H₄PteGlu have much higher affinities for the enzyme than the monoglutamate forms (30). As with the rabbit cytosolic enzyme, *N. crassa* cSHMT also displayed much higher affinity for the polyglutamate derivatives of H₄PteGlu. While the K_m for H₄PteGlu was 30 μ M, the K_d values for H₄PteGlu₃ and H₄PteGlu₅ were 0.2 μ M and 0.1 μ M, respectively.

The ability of *N. crassa* SHMT to catalyze Reaction 2 was determined by observing the rate of formation of the SHMT·Gly·5-CHO-H₄PteGlu_n ternary complex absorbing at 502 nm. Like the rabbit and *E. coli* enzymes, the synthesis of 5-CHO-H₄PteGlu_n by *N. crassa* SHMT displays biphasic kinetics, having a rapid phase that is over in approximately 30 s and a slow phase that lasts for several minutes. Previous studies with the rabbit enzyme have shown that the two rates can be attributed to the presence of two different folate derivatives in the 5,10-CH⁺-H₄PteGlu_n solutions (33). These studies suggested that the rapid rate is due to the formation of 5-CHO-H₄PteGlu_n from a hydrated intermediate, 5,10-CHOH-H₄PteGlu_n, while the slow rate is the result of hydrolysis of 5,10-CH⁺-H₄PteGlu_n to 5-CHO-H₄PteGlu_n. Thus the *N. crassa* SHMT appears to catalyze Reaction 2 with the same characteristics as the rabbit and *E. coli* SHMTs.

Previous studies with rabbit liver cSHMT have shown that the enzyme undergoes conformational changes when various ligands are bound. One of the most sensitive methods for measuring conformational changes in proteins is by determining their thermal denaturation temperature (T_m) and enthalpy of denaturation (ΔH_d) by differential scanning calorimetry. In order to determine the stability of *N. crassa* SHMT, the thermal denaturation temperature was determined in the presence and absence of various ligands. Like the rabbit enzyme, *N. crassa* SHMT shows a transition peak with variable T_m and ΔH_d in the presence of different ligands. The T_m for the free enzyme is 57 °C with a ΔH_d of 800 kcal/mol. With saturating serine, the T_m increased to 68 °C with a ΔH_d of 1240 kcal/mol, indicating that the SHMT·Ser binary complex is more stable. Differential scanning calorimetry thermograms of the 5-CHO-H₄PteGlu·SHMT·Gly ternary complex also showed a further increase in stability, with a T_m of 72 °C and ΔH_d of 900 kcal/mol. The polyglutamate chain length of 5-CHO-H₄PteGlu_n also appears to play a role in stabilizing the ternary complex of the *N. crassa* enzyme. The 5-CHO-H₄PteGlu₃·SHMT·Gly and 5-CHO-H₄PteGlu₅ ternary complexes had T_m values of 78 and 80 °C, respectively. The ΔH_d values were 1670 and 1640 kcal/mol. The effects observed with the *N. crassa* SHMT are similar to those

reported previously for the rabbit and *E. coli* enzymes (32, 33).

Previous studies have shown that the polyglutamate forms of 5-CHO-H₄PteGlu_n are slow tight-binding inhibitors of rabbit cSHMT (14). These studies indicated that 5-CHO-H₄PteGlu_n initially binds rapidly to the SHMT-Gly complex with a subsequent slow step which significantly lowers the *K_d* value. The rate of formation and breakdown of the quinonoid complex of the rabbit cSHMT with 5-CHO-H₄PteGlu₃ was slower by a factor of 10 compared with the rates with 5-CHO-H₄PteGlu₁. In order to determine if *N. crassa* SHMT displays the same slow-binding characteristics, rapid reaction techniques were used to determine the rates of formation and breakdown of the 5-CHO-H₄PteGlu₁·SHMT·Gly and 5-CHO-H₄PteGlu₃·SHMT·Gly quinonoid complexes. Unlike the rabbit enzyme, the rates of formation and breakdown for the monoglutamate form of the *N. crassa* 5-CHO-H₄PteGlu₁·SHMT·Gly quinonoid complex were slow, with half-lives of 27 and 21 s, respectively. The rates obtained with 5-CHO-H₄PteGlu₃ were slower by a factor of three, with half-lives for formation and breakdown of 83 and 67 s, respectively. The results show that the physiological polyglutamate forms of 5-CHO-H₄PteGlu_n are slow binding inhibitors of *N. crassa* SHMT.

These limited studies on the characterization of *N. crassa* cSHMT show that the properties are qualitatively the same as both the rabbit and *E. coli* enzymes. The conservation of properties of all SHMTs, especially with respect to 5-CHO-H₄PteGlu_n, suggests that the role of cSHMT in regulating one-carbon metabolism in *N. crassa* will also be applicable to rabbit and *E. coli* cells.

Concentration and Activity of SHMT in Conidiospores—To determine if SHMT in the conidiospores differs from SHMT in the mycelia, antibodies were raised to the pure enzyme and used in Western blots of spore and mycelial acetone powder extracts separated by isoelectric focusing in 6 M urea. These experiments showed that there are three major forms of subunits of SHMT in both mycelia and spores. The origin of the three bands on isoelectric focusing is unknown but may indicate either some posttranslational modification of the homotetrameric SHMT or cross-reactivity of the antibodies to mitochondrial SHMT subunits. There were no reproducible differences in the Western blot isoelectric focusing patterns obtained from spore and mycelial extracts.

Western blot analysis of SDS-PAGE gels of spore extracts was used to determine the concentration of SHMT in spores. Using a standard curve developed from the purified enzyme, the amount of SHMT was determined to be about 1.0 nmol of SHMT subunits/gram of conidiospores (Table I).

Determination of the activity of SHMT in conidiospore acetone powder extracts was determined by using the ability of the enzyme to catalyze the H₄PteGlu_n-dependent exchange of the *pro* 2 S proton of glycine with solvent protons (24). Again, the amount of activity was compared with a standard curve developed from assays with the purified enzyme. The results showed that the acetone powder extracts of conidiospores have an active SHMT with an estimated amount of 0.6 nmol of SHMT subunits/g of spore. The similarity between the Western blot studies (1.0 nmol/g) and activity measurements suggests that the SHMT in the spores probably has the same activity and structure as SHMT in mycelia.

The concentration of 5-CHO-H₄PteGlu_n in spores is about 25-fold higher than the concentration of cSHMT subunits. Even though SHMT catalyzes slowly the formation of 5-CHO-H₄PteGlu_n (Reaction 2) it would take only 25 catalytic turnovers to account for the observed levels of 5-CHO-H₄PteGlu_n in spores. The 25-fold excess of 5-CHO-H₄PteGlu_n with respect to cSHMT subunits and the *K_d* of 0.1 μM for the polyglutamate

forms of 5-CHO-H₄PteGlu_n suggests that in spores the enzyme is stored as the cSHMT·Gly·5-CHO-H₄PteGlu_n ternary complex. This form of the enzyme has a thermal denaturation temperature that is 25 °C higher than the enzyme alone. This increased stability of cSHMT would be important for spore viability.

Levels of SHMT and H₄PteGlu_n Compounds in the *for* Mutant—A mutant strain of *N. crassa*, which is auxotrophic for formate has previously been shown to lack an active cSHMT (6). To answer the question of whether the SHMT catalyzed hydrolysis of 5,10-CH⁺-H₄PteGlu_n (Reaction 2) is the source of 5-CHO-H₄PteGlu_n in spores, we have determined the levels of SHMT and H₄PteGlu_n derivatives in the spores of this *for* mutant strain. The *for* strain was grown as described under "Experimental Procedures." Both mycelia and spores were harvested, and acetone powders were extracted with buffer as described for the wild-type strain. Western blot analysis of these extracts by isoelectric focusing in 6 M urea showed cross-reactivity between a protein and the antibodies raised to the purified cytosolic enzyme. Western blot analysis suggests that the subunits of SHMT in mycelial and spore extracts of the *for* mutant have slightly different pI values compared with the enzyme in wild-type mycelia and spores, suggesting that the lack of SHMT activity in this mutant strain is the result of a change of a single amino acid residue.

The SHMT catalytic activity in mycelia and spore extracts was determined using the glycine tritium exchange assay. The levels in the *for* mutant extracts were found to be about 10% of the activity determined for wild-type extracts (Table I). This assay will also reflect the presence of mitochondrial SHMT, which has previously been estimated to be about 10% of the activity of the cytosolic isoenzyme (6).

The distribution of folates in spores and mycelia was also determined for the *for* mutant. In several different conidiospore preparations, 5-CHO-H₄PteGlu_n represented 10–20% of the total folate pool. In mycelia, there was no detectable 5-CHO-H₄PteGlu_n and 5-CH₃-H₄PteGlu_n was 11% (Table I). There was about a 2-fold elevated level of total folates in the mycelia of the *for* strain compared with the wild-type strain. The low levels of 5-CHO-H₄PteGlu_n in this strain support the interpretation that the origin of this form of the coenzyme is the SHMT-catalyzed hydrolysis of 5,10-CH⁺-H₄PteGlu_n. The small amount of 5-CHO-H₄PteGlu_n found in the spores of this mutant strain can be accounted for by the mitochondrial isoenzyme of SHMT.

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