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Methenyltetrahydrofolate Cyclohydrolase Catalyzes the Synthesis of (6*S*)-5-Formyltetrahydrofolate

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The methenyltetrahydrofolate cyclohydrolase activity of the bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase domain catalyzes the conversion of 5,10-methenyltetrahydrofolate to 10-formyltetrahydrofolate. We have observed that in the presence of this domain, 10-formyltetrahydrofolate is converted to 5-formyltetrahydrofolate. The effects of nucleotide analogs on the initial rates of this reaction were found to be similar to their effects on the cyclohydrolase activity, establishing that 5-formyltetrahydrofolate production is dependent on the cyclohydrolase. The specific activity of 5-formyltetrahydrofolate production is approximately 7×10^4-fold lower than that of the cyclohydrolase activity but can be used to obtain quantitative conversion of 10-formylH_4folate to 5-formyltetrahydrofolate, in mg amounts. This ''side-reaction'' may contribute to the *in vivo* production of (6*S*)-5-formyltetrahydrofolate.

INTRODUCTION

The function of tetrahydrofolate (H_4folate)[1] is to transfer one-carbon units at the oxidation states of methanol, formaldehyde, and formate to acceptor molecules for use in biosynthetic reactions. The one-carbon unit is covalently bound at N_5 of the pteridine ring, N_{10} of the *p*-aminobenzoyl moiety, or to both forming a N_5–N_{10} bridge. Figure 1 illustrates the enzymatic addition of formate to H_4folate **[1]** in the ATP-dependent 10-formylH_4folate synthetase reaction, forming 10-formylH_4folate **[2]** (reviewed in *1*). This product can be used in the biosynthesis of purines or can be reversibly converted to 5,10-methenylH_4folate **[3]** by the action of methenylH_4folate cyclohydrolase without the need of a cofactor or second substrate. 5,10-MethenylH_4-folate can be converted to 5,10-methyleneH_4folate by NAD(P)-dependent methyleneH_4folate dehydrogenase, for use in biosynthesis of thymidylate, serine, or methionine. 5,10-MethenylH_4folate **[3]** is also converted chemically to 5-formylH_4folate **[4],** with K_{eq} **4/3** $= 1.5 \times 10^4$ (*2*). This chemical conversion has been proposed to contribute to the *in vivo* occurrence of (6*S*)-5-formylH_4folate (*3*). However, the

[1] Abbreviations used: H_4folate, tetrahydrofolate; D, methyleneH_4folate dehydrogenase; C, methenyl-H_4folate cyclohydrolase; S, 10-formylH_4folate synthetase; SHMT, serine hydroxymethyl transferase; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; FPLC, fast protein liquid chromatography; EDTA, ethylenediaminetetraacetic acid; AADP, 3-aminopyridine adenine dinucleotide phosphate; NADP, nicotinamide adenine dinucleotide 2′-phosphate; 2′,5′-ADP, 2′-phosphoadenosine 5′-phosphate.

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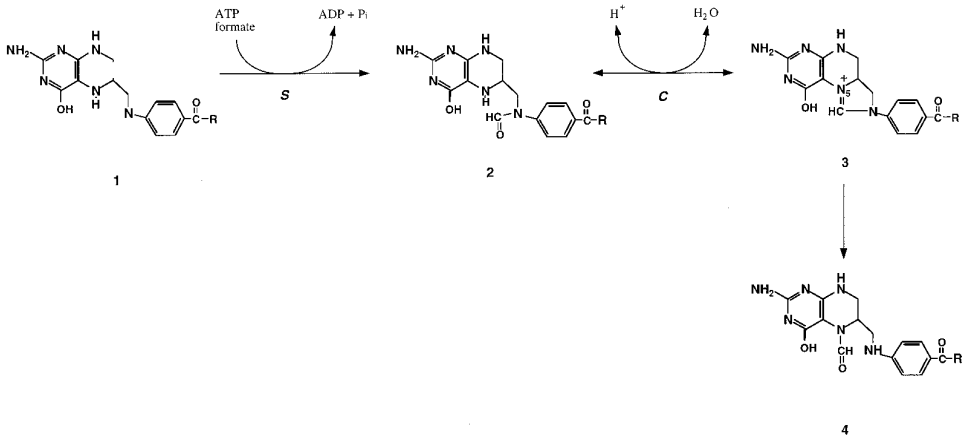

FIG. 1. **1,** H_4folate; **2,** 10-formylH_4folate; **3,** 5,10-methenylH_4folate; **4,** 5-formylH_4folate; S, 10-formyl-H_4folate synthetase activity; C, 5,10-methenylH_4folate cyclohydrolase activity. It should be noted that one-carbon substitution at N_{10} changes the (R,S) designation at position 6.

rate appears to be too slow to account for the concentrations detected (*4*), which vary from about 5% of the folate pool in rat liver (*5*) up to 85% of that in *Neurospora crassa* conidiospores (*6*). Serine hydroxymethyltransferase (SHMT) produces 5-formylH_4folate as a side-reaction (*4*) and this reaction was shown to be the main source in *N. crassa* and *Escherichia coli* (*6*, *7*)

5-FormylH_4folate **[4]** is of considerable interest as its metabolic role has long been a source of debate. Unlike other forms of H_4folate, the 1-carbon unit carried by 5-formylH_4folate does not participate in any known biosynthetic reaction. This prompted the proposal that it serves as a stable storage form of H_4folate (*8*). The enzyme methenylH_4folate synthetase is the only enzyme known to utilize 5-formylH_4folate as a substrate. It converts 5-formylH_4folate **[4]** to 5,10-methenylH_4-folate **[3]** using ATP to overcome the thermodynamically unfavorable reaction; this results in cycling the stored H_4folate back into the pool of metabolically useful H_4folate forms. This reaction forms the basis for the use of 5-formylH_4folate as an agent to rescue normal cells from methotrexate toxicity during cancer treatment (for a review, see *9*). Currently, the racemic mixture of 5-formylH_4folate, leucovorin, is clinically administered. Although the unnatural isomer seems to have no impact on rescue from methotrexate (*9*), it is a weak inhibitor of cellular uptake of the natural isomer, (6*S*)-5-formylH_4folate (*9*, *10*), and it accumulates in plasma relative to the natural isomer (*11*). Therefore, it may be an advantage to administer only the natural isomer (*12, 13*).

We have found that methenylH_4folate cyclohydrolase (C) catalyzes the stereospecific synthesis of (6*S*)-5-formylH_4folate. In many organisms, the C and methyleneH_4folate dehydrogenase (D) activities belong to a single polypeptide (D/C) and appear to share a common active site (*14*). Furthermore, the D/C domain in eukaryotes is covalently associated with a 10-formylH_4folate synthetase (S) domain,

forming a trifunctional D/C/S enzyme. The human D/C/S has been cloned and overexpressed in *E. coli* in this laboratory and the recombinant D/C domain has been separately expressed and retains the kinetic characteristics of the D/C/S (*15–17*). In this study, we examine the synthesis of (6*S*)-5-formylH_4folate by the C activity of the human D/C domain in order to assess its putative role in contributing to *in vivo* 5-formylH_4folate levels, and report an alternative method to the stereospecific synthesis of (6*S*)-5-formylH_4folate **[4].**

MATERIALS AND METHODS

All the reagents used were of analytical grade. DEAE-cellulose DE-23 was purchased from Whatman. Centriprep-10 concentrators are Amicon products. Sodium formate was from J.T. Baker Chemical Co., β-mercaptoethanol from Kodak, and ATP from Sigma. All other reagents were BDH products. Recombinant human D/C/S enzyme and its bifunctional D/C domain (D/C301) were purified from *E. coli* crude lysate in a 1-day purification as described previously (*16, 17*). Enzyme was made NADP-free by desalting and was concentrated by centrifugation using Centriprep-10. Typical purifications of D/C/S enzyme and of D/C domain yield approximately 10 and 60 mg of enzyme, respectively (10 and 35 U/mg dehydrogenase activity, respectively) from 10 g of cells.

Synthesis of (*6R*)*-10-formylH_4folate* **[*2*].** The enzymatic synthesis is similar to that reported by Ho and Jones (*18*). (6*R,S*)-H_4folate **[1]** is the starting material and in this protocol, the purified recombinant human D/C/S enzyme was used as the source of 10-formylH_4folate synthetase (S) activity. Formate is the source of the one-carbon unit and ATP is the cofactor for the reaction, which is illustrated in Fig. 1. A solution of (6*R,S*)-H_4folate **[1]** (0.125 mmol in 12.5 ml) containing 5 mM Tris buffer, pH 7.5, 0.36 M β-mercaptoethanol, and 250 mM NaCl was made to 67 mM sodium formate, 5 mM $MgCl_2$, 33 mM KCl, 33 mM triethanolamine · HCl, pH 8.0, and 1.5 mM ATP, in a final volume of 48 ml. The solution was incubated at 37°C and 15 U synthetase activity (in 2 ml of 30% glycerol, 50 mM potassium phosphate, pH 7.3, and 35 mM β-mercaptoethanol) was added. The reaction was placed under vacuum at 37°C. Conversion to **2** was monitored by periodic acidification of aliquots and spectroscopic quantitation of **3** (described below). The reaction was complete after 40 min. The reaction mixture was diluted with 167 ml of 0.1 M β-mercaptoethanol at 4°C and applied to a 75-ml DEAE-cellulose column. The product was purified and eluted in NH_4OAc as previously outlined (*18*).

Conversion of (*6R*)*-10-formylH_4folate* **[*2*]** *to* (*6R*)*-methenylH_4folate* **[*3*].** For stable storage, **2** was converted to **3** by acidification essentially as described in (*18*). The purified **2,** in approximately 0.175 M NH_4OAc and 0.1 M β-mercaptoethanol, was lyophilized. The resulting yellow powder was redissolved in 5.0 ml 0.1 N HCl with heating to 55°C. The solution, now at pH 1.7, was placed under vacuum and precipitated at 4°C over 48 h. The yellow crystals were washed with ice-cold EtOH and dried. A 64% yield of **3** was obtained from the biologically active isomer of **1.**

Conversion of (*6R*)*-methenylH_4folate* **[*3*]** *to* (*6R*)*-10-formylH_4folate* **[*2*].** Immediately before use **3** was dissolved in a solution of 0.1 M potassium phosphate, pH

7.3, and 0.1 M β-mercaptoethanol and was stirred gently at 23°C under vacuum, for 1 h, for conversion to **2.**

Synthesis of (*6S*)*-5-formylH$_4$folate* **[*4*]** *using cyclohydrolase activity.* The source of cyclohydrolase activity used in the synthesis was purified D/C domain as it is obtained in higher yields than the D/C/S. **2** (7.5 μmol) was combined with 1500 U of cyclohydrolase activity (500 U dehydrogenase activity) in 100 mM β-mercaptoethanol, 25 mM potassium phosphate, pH 7.3, and 0.1% ascorbate in a final volume of 20 ml. The concentrations of substrate and enzyme were 375 and 20 μM, respectively, or a 19 : 1 ratio. The extent of the reaction was followed by differential acidification (described below). When the reaction was complete, the mixture was made enzyme-free by filtration to dryness in a Centriprep-10 microfiltration device. The filtrate was diluted 1 : 1 with water and purified according to the parameters defined by Moran *et al.* (*19*) adapted to purification by FPLC at 4°C.

Differential acidification. An aliquot of reaction mix was made to 3.5% TCA and placed in boiling water for 55 s. Under these conditions, **2** and **4** are both rapidly converted to **3,** which is quantified by spectrophotometry (E_{350} = 24.9 mM^{-1} cm^{-1}). A second aliquot of reaction mix was made to 0.5 M sodium acetate, pH 3.3, at 23°C. **2** is rapidly converted to 99.7% **3,** according to the following equilibrium: **[3]**/**[2]** $\times$ [H$^+$] = 9 $\times$ 10^5 M^{-1} (*2*). However, at pH 3, the half-time of cyclization of **4** to **3** is 270 min (*20*); thus its conversion is negligeable. The difference between the concentration of **3** determined in TCA and in acetate provides the concentration of **4.**

Characterization of [*6S*]*-5-formylH$_4$folate synthetic activity.* Initial rates were measured using 200 μM (*R,S*)-10-formylH$_4$folate **[2]** and 5 μM D/C301 in the absence of nucleotide or in the presence of saturating nucleotide (40 μM AADP, 200 μM NADP, or 200 μM 2′,5′-ADP). When maximum conversion of **2** to 5-formylH$_4$folate **[4]** was sought, equivalent concentrations of substrate and enzyme were used. The amount of product formed was determined by differential acidification. It was not possible to study classical initial rate kinetics of this activity as its turnover is very low compared to that of the interconversion of **2** and **3** by the cyclohydrolase activity.

RESULTS

Enzymatic Synthesis of (*6S*)*-5-formylH$_4$folate*

We have observed that 10-formylH$_4$folate **[2]** is converted to 5-formylH$_4$folate **[4]** in the presence of the human D/C domain. In order to demonstrate the enzymatic nature of the synthesis, we measured the reaction rate in the presence of varying D/C domain concentrations. Figure 2 illustrates that the initial rate of the reaction is directly proportional to the enzyme concentration, at saturating substrate concentrations. Further evidence of the enzymatic nature of the reaction is provided by demonstrating its stereospecificity, illustrated in Fig. 3. The natural isomer, (6*R*)-10-formylH$_4$folate, is entirely converted to product under conditions where only 50% of the racemate is converted. The chemical rate of interconversion is negligible compared to that of the enzymatic reaction (Fig. 3).

The very slow rate observed for the previously unrecognized activity prompted