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Isolation and characterization of a novel dihydrofolate formylating enzyme from human MCF-7 breast cancer cells

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Methotrexate (MTX*) is a clinically important drug for the treatment of a variety of neoplasms [1]. MTX is felt to produce its cytotoxic effects through a potent direct inhibition of the enzyme dihydrofolate reductase (DHFR) [2]. Recent investigations using MCF-7 breast cancer cells and normal human myeloid precursor cells have shown that following exposure to MTX, the intracellular levels of dihydrofolate (H_2folate) increase rapidly [3]. Over time, a novel intracellular folate identified as formyl-H_2 folate appears and reaches concentrations equal to that of H_2 folate within 12 hr [3]. The effect of this new folate on several folate-requiring enzymes, including thymidylate synthase [TS] and the *de novo* purine enzymes AICAR and GAR transformylase, was examined [4]. These studies revealed that formyl-H_2folate pentaglutamate was an inhibitor of both GAR transformylase and TS, and an alternate formyl donor for AICAR transformylase. Enzymatic conversion of tetrahydrofolate (H_4folate) to 10-formyl-H_4folate (the required cofactor for both GAR and AICAR transformylase) has been demonstrated using cytosolic extracts of porcine liver [5], but the possible role of H_2folate as a substrate for this reaction has not been examined. In this report, we describe the enzymatic conversion of H_2folate to 10-formyl-H_2folate by a cytoplasmic activity that appears to be distinct from the enzyme responsible for formylation of H_4folate.

Materials and methods

MCF-7 cells were maintained in RPMI-1640 medium (Biofluids, Rockville, MD) enriched with 10% dialyzed fetal calf serum (Gibco, Grand Island, NY). Cells were harvested at 80% confluency and stored at −70° until used. Cytosolic preparations were made by lysing cells with three 5-sec bursts from a sonicator (Branson model 350) in hypotonic buffer (0.05 M Tris buffer, pH 8.5) followed by centrifugation at 100,000 *g* for 30 min. An extract of *Lactobacillus casei* bacterial cytosol was purchased from the New England Enzyme Center (Boston, MA).

H_2folate and H_4folate were purchased from the Sigma Chemical Co. (St Louis, MO). [3′,5′,7,9-^{3}H]Folic acid (20 Ci/mmol; 95–98% pure by HPLC) was supplied by Moravek Biochemicals (Brea, CA).

* Abbreviations: MTX, methotrexate; H_2folate, dihydrofolate; H_4folate, tetrahydrofolate; TS, thymidylate synthase; GAR, glycinamide ribotide; AICAR, aminoimidazolecarboxamide ribonucleotide; and DHFR, dihydrofolate reductase.

[^{3}H]H_2folate was prepared by dithionite reduction of [^{3}H]folic acid using a modification of the method of Blakley [6]. The final product was determined by HPLC analysis to be 80–85% H_2folate with 15–20% residual folic acid. A second crystallization was not performed in the preparation of the radiolabeled H_2folate; thus, the preparation used contained residual folic acid. Further purification was not performed as the yield of H_2folate after a second crystallization was poor, and the residual folic acid would not interfere with these experiments. While folic acid may be formylated during these experiments, formyl-folic acid is easily separable from formyl-dihydrofolate by HPLC. The residual folic acid in the commercial preparations and the radiolabeled preparation is evident in the HPLC separations shown in Fig. 1. Standard formyl-H_2folate was prepared by first formylating folic acid using formic acid according to the method of Blakley [7]. Formylfolic acid was then reduced to formyl-H_2folate using the dithionite reaction followed by alcohol precipitation of the product as previously described [8]. The purity of the formyl-H_2folate was greater than 95% as determined by HPLC [4].

All other chemicals were the highest grade obtainable and were purchased from the Sigma Chemical Co.

Measurement of H_2folate formylating activity. Dialyzed cytosolic preparations of either MCF-7 cells or *L. casei* were incubated in a shaking water bath at 37° with H_2folate (0 to 0.4 mM), [^{3}H]H_2folate (0.5 μCi), 100 mM sodium formate, 20 mM $MgCl_2$, 100 mM potassium chloride, 50 mM ATP, and 250 mM 2-mercaptoethanol in 0.05 M Tris–HCl buffer, pH 8. MTX (10^{-5} M) was included in each assay to prevent the reduction of H_2folate or of the formyl-H_2folate product by DHFR present in the enzyme preparations. Reactions were terminated by immersing reaction tubes in boiling water for 60 sec in preparation for analysis by HPLC (*vide infra*). [^{3}H]H_2folate incubated at 37° with 0.5 mM 2-mercaptoethanol for 4 hr was found to be 97% preserved and had a half-life of 26 hr under the assay conditions.

Folate extraction and identification. Separation, identification, and quantitation of formyl-H_2folate, formyl-H_4folate, H_2folate, H_4folate, and folic acid were accomplished by using a reverse-phase HPLC assay as previously described [3] (see Fig. 1). After termination of the enzymatic reaction by immersion of assay tubes into a boiling water bath for 1 min, the folates in the reaction mixture were concentrated using a Waters C_{18} SepPak as previously described [3]. Standards and samples were separated on a Waters high-pessure liquid chromatograph using a C_8 radial

pac cartridge. Elution was carried out at 2 mL/min under isocratic conditions using 84% PicA (pH 7.6) and 16% methanol. Separations of folates were monitored by UV detection at 280 nm. Samples were collected at 1-min intervals and dissolved in 10 mL of liquid scintillation fluid (3a70B, RPI, Mt. Prospect, IL); tritiated counts were quantitated in a Searle Mark III liquid scintillation counter (Searle, Chicago, IL). Elution times for folate standards separated under these conditions were as follows: 10-formyl-H_4folate, 4.2 min; 10-formyl-H_2folate, 4.8 min; H_4folate, 6.8 min; 10-formyl-folic acid, 9.8 min; H_2folate, 12.0 min; and folic acid, 16.3 min. As further proof of the formation of formyl-H_2folate, spectral analysis of the reaction product was carried out using a Hewlett–Packard model 8452A diode array spectrophotometer equipped with a flow cell in series with the effluent from the HPLC. Full spectra (190–800 nm) were acquired and stored by the spectrophotometer every 20 sec and the spectra corresponding to the HPLC peaks of interest were analyzed by comparison to the spectra and retention times of known standards (see Fig. 2).

Measurement of formyl-H_4folate synthase. Formyl-H_4folate synthase was measured in the cytosol of both *L. casei* and MCF-7 using the method of Tan *et al.* [5]. An incubation mixture containing cytosol, triethanolamine hydrochloride, pH 8, 20 mM sodium formate, 1 mM ATP, 1 mM $MgCl_2$, 50 mM potassium chloride, 0.63 mM H_4folate and 200 mM 2-mercaptoethanol in a volume of 1 mL was incubated for 10 min at 37°. The reaction was terminated by the addition of 1 mL of 0.36 M HCl (to stop the reaction and convert the 10-formyl-H_4folate produced to 5,10-methenyl-H_4folate). After standing at room temperature for 10 min, the absorbance at 350 nm was measured and the production of 5,10-methenyl-H_4folate calculated using ε_{350} = 24,900 m^{-1} cm^{-1}.

Calculations. Kinetic constants (K_m) were first calculated using standard double-reciprocal plots (Lineweaver–Burk). These were then used as initial estimates for a nonlinear, least-squares computer program capable of fitting the kinetic data without linearization [9].

Results

We first assessed the ability of the cytosolic preparations from MCF-7 cells to catalyze the formation of formyl-H_2folate from H_2folate. Using conditions identical to those described by Tan *et al.* [5] for the formylation of H_4folate, we found that FH_2 was formylated at a rate of 0.8 pmol/min per mg of cytosolic protein, and that the reaction was specifically dependent on the presence of formate and a cytosolic protein (Fig. 1). Further, we found that an extract of *L. casei* could also catalyze the formylation of H_2folate (Table 1). The specific activity of this preparation was 1.7 pmol/min/mg. The activity of either the human or bacterial preparation was abolished completely by denaturation of the cytosolic proteins using either immersion in boiling water for 60 sec or by denaturation with 5% perchloric acid. Catalytic activity was found to be dependent on the presence of ATP, formate, and Mg^{2+} (Table 1). Activity was enhanced by 50% with the addition of K^+. For the MCF-7 preparation, activity was found to be linear with respect to time for periods up to 4 hr of incubation under standard reaction conditions using cytosol containing 0.2 mg total protein. Production of formyl-H_2folate using these same reaction conditions (4-hr incubation) was also linear with respect to cytosolic protein using a range of 0.1 to 0.4 mg per reaction. The reaction was pH dependent, displaying an optimum pH 8.5 when incubated under the above conditions. The HPLC peak coeluting with standard formyl-H_2folate was analyzed by UV spectroscopy. The putative formyl-H_2folate peak had a retention time and spectrum that was found to be identical to that of standard formyl-H_2folate and the published spectrum of formyl-H_2folate [4] (Fig. 2).

The Michaelis–Menten constant (K_m) was measured for

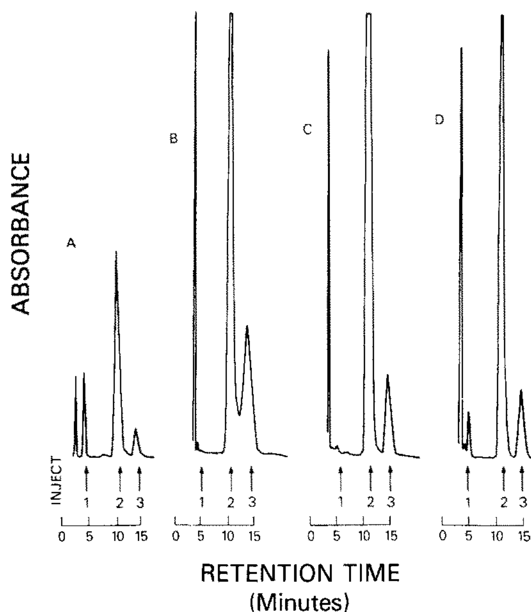

Fig. 1. Chromatographic separation:
(A) Standards

	Compound	RT (min)
↑ 1	Formyl-H_2folate	4.8
↑ 2	H_2folate	12.0
↑ 3	Folic acid	16.3

(B) Standard reaction mixture containing 0.04 mM H_2folate, 100 mM sodium formate, 20 mM $MgCl_2$, 100 mM KCl, 50 mM ATP, 10^{-5} M MTX and 0.05 M Tris buffer, pH 8.5, incubated without MCF cytosol for 4 hr at 37°.
(C) Standard reaction mixture incubated without sodium formate with dialyzed MCF cytosol (0.2 mg protein).
(D) Standard reaction mixture incubated with dialyzed MCF cytosol (0.2 mg protein) with the addition of sodium formate.

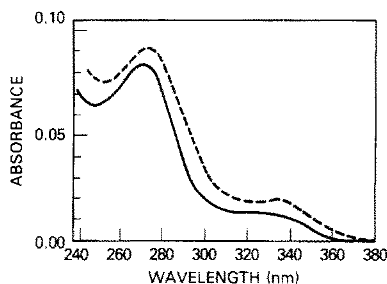

Fig. 2. Spectral analysis. Spectral analysis of HPLC peak coeluting with formyl-H_2folate (———). Published ultraviolet spectrum for formyl-H_2folate [4] is also illustrated for comparison (---).

the reaction with respect to H_2folate and was found to be 97 ± 9 μM for *L. casei* and 911 ± 97 μM for the MCF-7 preparation.

In an effort to determine whether the enzyme responsible for formylating H_4folate is the same as, or different from,

Table 1. Cofactors required for enzyme activity

	Formyl-H_2 folate produced (nmol)
Enzyme + standard reaction mixture*	18.0
Enzyme + standard mixture minus ATP	0
Enzyme + standard mixture minus formate	0
Enzyme + standard mixture minus Mg^{2+}	1.9
Enzyme + standard mixture minus K^+	11.4

* H_2folate 0.04 mM, sodium formate 100 mM, $MgCl_2$ 20 mM, KCl 100 mM, ATP 50 mM, MTX 10^{-5} M, and Tris–HCl buffer, 0.05 M, pH 8.5, incubated at 37° with 0.6 mg *L. casei* enzyme for 4 hr.

the enzyme that formylates H_2folate, substrate competition experiments were performed. When formyl-H_4folate synthase was measured using 16 μM H_4folate in the presence of a 36-fold higher concentration of H_2folate (567 μM), no inhibition of product formation was observed. Conversely, H_2folate formylation using 87 μM H_2folate was measured in the presence of 36-fold excess of H_4folate (3.1 mM), with no apparent decrease in the amount of formyl-H_2folate produced.

Discussion

This report suggests that the formylation of H_2folate is an enzyme-mediated reaction and that the enzyme is found in the cytosol of human breast cancer cells and in an extract of *L. casei*. The reaction depended on ATP, a metal cofactor (Mg^{2+}), and the presence of formate to act as a substrate. These requirements along with the lability of activity with heating and protein precipitation support the enzymatic nature of this reaction. Furthermore, since the formylation of H_2folate or H_4folate was unaffected by the inclusion of the alternate potential substrate in the incubation mixture in concentrations up to 36-fold excess, it may be suggested that the formylating activities for these two folates reside with independent enzymes. Although the K_m of the reaction indicated a low affinity for dihydrofolte as a substrate, formyl-H_4folate synthase has a similar K_m for H_4folate (230 μM) [5], indicating that either the reaction conditions were not optimal or that this reaction may not be the primary function of the protein. Another explanation for the seemingly low affinity of the enzyme for H_2folate could be the monoglutamated state of the folate used in this study. Studies using the more physiologic polyglutamated H_2folate were hampered by the unavailability of radiolabeled polyglutamated folate compounds that would be essential for these experiments.

Based on this study and previous work that demonstrated the presence of 10-formyl-H_2folate only after MTX treatment of normal or malignant cells, we propose the following scheme for formation of this novel physiologic folate. In the presence of a functioning DHFR, H_2folate is quickly converted to H_4folate and H_2folate levels are inadequate to catalyze significant or measurable concentrations of formyl-H_2folate. Any formyl-H_2folate that is formed would be quickly consumed in either the AICAR transformylase reaction or by DHFR. In the presence of MTX, DHFR is inhibited, intracellular H_2folate accumulates to high levels, and the slow conversion of H_2folate to formyl-H_2folate by the observed cytosolic enzyme activity occurs. The product, formyl-H_2folate, is not consumed by AICAR transformylase, since the folate-dependent *de novo* purine enzymes appear to be directly inhibited by H_2folate and MTX polyglutamates. An alternate explanation for the source of intracellular formyl-H_2folate may be the direct oxidation of formyl-H_4folate, which would allow the accumulation of formyl-H_2folate in the presence of DHFR inhibition by MTX. This scenerio seems unlikely, given the high degree of preservation of the intracellular formyl-H_4folate pools [3, 4, 10].

In summary, recent reports have described the presence of a new oxidized folate, 10-formyl-dihydrofolate, following exposure of human MCF-7 breast cancer cells of human myeloid precursor cells to MTX. The mechanism of generation of this novel folate has not been elucidated. In the present work, we found that cytosolic preparations of human MCF-7 cells, as well as an extract of *L. casei*, were able to catalyze the formation of formyl dihydrofolate from dihydrofolate. This catalytic activity was destroyed by protein denaturation using either acid precipitation or heating. The enzymatic activity was found to be formate-, ATP-, and Mg^{2+}-dependent. The Michaelis–Menten constant (K_m) of dihydrofolate was 911 μM for the human enzyme and 97 μM for *L. casei*. Enzyme activity was unaffected by addition of a 36-fold higher concentration of H_4folate. These data suggest that the dihydrofolate formylating activity is enzymatic and distinct from that responsible for the formylation of H_4folate.

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The effect of 90 days treatment with Omeprazole on 24 hour plasma gastrin profiles in female Wistar rats

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Omeprazole (OM) is a novel irreversible proton pump inhibitor which can virtually abolish acid secretion in the stomach and has great utility in the treatment of acid related disorders [1]. In a 2-year carcinogenicity study conducted as part of the safety evaluation of OM, the contract laboratory performing the study reported the occurrence of carcinoids and enterochromaffin-like (ECL) cell hyperplasia in the oxyntic mucosa of Sprague–Dawley rats [2]. Following the discovery of carcinoids, the clinical programme was suspended and research to clarify the mechanism of carcinoid development was initiated. A further carcinogenicity study was conducted at lower doses in female Sprague–Dawley rats only, in an attempt to produce a "no-effect" dose. This was not successful and resulted in a low incidence of carcinoids even at the lowest dose of 5 μmol/kg (1.73 mg/kg) [3]. Since then, much interest and research has identified and begun to define a clear association between ECL cell hyperplasia, carcinoids and the trophic hormone gastrin [4–6]. It is now widely thought that gastrin, which is secreted in response to high pH and the presence of food in the stomach, is hypersecreted during periods of profound inhibition of acid secretion and during this time high concentrations of plasma gastrin may be sustained [5]. Gastrin has a general trophic effect on the oxyntic mucosa and stimulates ECL cells to divide and proliferate [4, 7, 8]. Therefore, prolonged administration of high doses of the proton pump inhibitor OM will result in profound long term inhibition of acid secretion and sustained hypergastrinaemia, leading to ECL cell hyperplasia and because of the sustained proliferative stimulus, eventually the appearance of carcinoids. If this hypothesis is correct, other potent long acting inhibitors of acid secretion should also induce carcinoids. This is indeed the case and carcinoids have been found in rat carcinogenicity studies of three long-acting H_2-receptor antagonists, namely SK&F 93479 in Wistar rats [9], loxitdine and tiotidine in Sprague–Dawley rats [10, 11], whereas shorter-acting agents such as cimetidine, ranitidine, famotidine and nizatidine were not carcinoid-inducing in carcinogenicity studies.

The case for the causative role of gastrin in the induction of gastric carcinoids is quite strong, but there has been little attempt to define a quantitative relationship between gastrin, the hyperplastic response and the induction of carcinoids. This area of knowledge is a vital component in understanding the precise role of gastrin in gastric mucosal growth and may be of great value if potent inhibitors of acid secretion are to be developed as therapeutic agents and if toxicologists are to continue to evaluate their carcinogenic potential in rats. The present studies were designed to investigate the effect of OM on 24 hr gastrin profiles in female rats taken at intervals over a 13-week dosing period to allow a detailed examination of the total exposure of animals to the gastrin stimulus.

Materials and methods

Female Wistar (Charles River) rats (250–280 g) were group housed in plastic cages on soft Greenwood granules. Food (PRD pellets, Labsure Ltd) and water were provided *ad lib.* The air temperature was maintained at 21 ± 2°, humidity was maintained at 40–60% and fluorescent lighting provided between 0600 and 1800 hr GMT. Each animal was dosed daily by oral gavage with either vehicle, 1, 5, 15 or 100 mg/kg Omeprazole (99.4% pure) suspended in 0.5% gum tragacanth for 90 days. Dosing was carried out between 0800 and 1000 hr each day. Timed blood samples were taken from a lateral tail vein on days 1, 15, 30, 61, and 90. The samples were taken at 3, 6, 9, 12, 16, 20 and 24 hr post-dose on each bleed day. Plasma samples were assayed in duplicate for gastrin by radioimmunoassay using a commercially available kit (Becton Dickinson). Duplicate results did not vary by more than 10%. Gastrin values were calculated as mean ± SE and statistical analysis was performed using Wilcoxon's rank sum test.

Results

Clinical observations and necropsy. There were no clinically observable treatment related effects and at necropsy there were no macroscopic abnormalities in any tissue.

Plasma gastrin. Plasma gastrin concentrations in the control group were fairly constant over 24 hr even at peak feeding times. The mean values were between approximately 70 and 125 pg/mL. Control values were distributed normally and did not change significantly during the 90 day experiment (Table 1).

Mean plasma gastrin concentrations in rats dosed at 1 mg/kg were not significantly elevated at any time during the experiment (Fig. 1A).

In rats dosed with 5 mg/kg OM plasma gastrin concentrations were not significantly elevated above control values on day 1. On day 15, mean peak plasma gastrin concentrations were elevated to 184 ± 72 and 186 ± 46 pg/mL at 3 and 6 hr post-dose, respectively, although there is no statistical difference from control mean values. By 9 hr, mean gastrin concentrations had returned to control levels. Essentially the same picture was presented on day 30 for these animals, except that the values from the treated animals at 3 and 6 hr were statistically different ($P < 0.05$) from concurrent controls. On day 61, plasma gastrin had