DIHYDROFOLATE REDUCTASE FROM A METHOTREXATE-RESISTANT STRAIN OF *ESCHERICHIA COLI:* DIHYDROFOLATE MONOOXYGENASE ACTIVITY.

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SUMMARY: Dihydrofolate reductase from strain MB 1428 of *Escherichia coli* was shown to catalyze the oxidative cleavage of dihydrofolate at the C(9)-N(10) bond. One of the products of the reaction was identified as 7,8-dihydropterin-6-carboxaldehyde through its proton magnetic resonance spectrum. The maximal enzymatic rate was 0.05 moles dihydrofolate cleaved per minute per mole enzyme at 25° and pH 7.2, and the K_M for dihydrofolate was 17.5 ± 2.5 μM. The enzymatic reaction was fully inhibitable with methotrexate. The mechanism of enzyme action was proposed to be an apparent "acidification" of dihydrofolate upon binding to the enzyme. Folate underwent an analogous oxidative cleavage by enzyme with a turnover number of 0.0014, which produced pterin-6-carboxaldehyde. Methotrexate was also slowly degraded by the enzyme.

Aerobic solutions of dihydrofolate at neutral pH are unstable (*1-3*). A yellow-green fluorescing compound with absorbancy maxima at 270 and 420 nm and an aromatic amine, presumably p-aminobenzoylglutamate, are produced (*1*). Authentic synthesis of the yellow-green fluorescing compound identified it as 2-amino-4-hydroxy-6-formyl-7,8-dihydropteridine, or 7,8-H_2-pterin-6-CHO (*4*). It was discovered that dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase E.C.1.5.1.3) from *E. coli* significantly increased the rate of decomposition of dihydrofolate. The kinetic parameters of the enzyme-catalyzed reaction were measured, and the identity of the pterin degradation product established through proton magnetic resonance studies. From these studies it appears that the decomposition reaction catalyzed by the enzyme is an monooxygenase reaction with the following formal stoichiometry:

$$7,8\text{-}H_2\text{-folate} + \tfrac{1}{2}O_2 \rightarrow 7,8\text{-}H_2\text{-pterin-6-CHO} + \text{p-aminobenzoylglutamate} \quad (1)$$

An analogous reaction with folate was also studied.

MATERIALS AND METHODS

Dihydrofolate reductase was purified from a methotrexate-resistant strain

of *Escherichia coli* (strain MB 1428) as described previously (*5,6*). Folic acid, dihydrate, was purchased from Cyclo, methotrexate (4-amino-4-deoxy-N(10)-methyl-folate) from Nutritional Biochemicals, pterin-6-carboxylate from Aldrich. Dihydrofolate was prepared from folate according to the method of Futterman (*7*) as modified by Blakley (*8*). The numbering system for folates of the IUPAC-IUB is given in Figure 1.

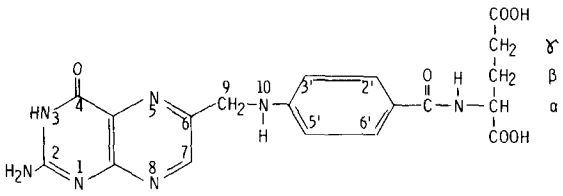

FIGURE 1: Numbering system for folate.

Dihydrofolate, folate and methotrexate solutions were standardized spectrophotometrically as described before (*9*). Dihydrofolate reductase was standardized by methotrexate titration (*6*). Pterin-6-carboxaldehyde was quantitated spectrophotometrically (*2*). The change in molar absorbance at 420 nm for the reaction of equation 1 was assumed to be +7,000 cm^{-1} M^{-1}; this value is uncertain to ±25% due to lability of the pterin product. The change in molar absorbance at 370 nm for the conversion of folate to p-amino-benzoylglutamate and pterin-6-carboxaldehyde was measured to be +3100 cm^{-1} M^{-1} at pH 7.2.

The rate of oxidative cleavage of folate and dihydrofolate was determined spectrophotometrically at 25° using the above molar absorbance changes. When folate was stored with the enzyme and then chromatographed on DEAE-Sephadex A-25 in 0.55 M NaCl 0.05 M Tris-HCl pH 7.2, the only detectable pterins were folate and pterin-6-carboxaldehyde. A similar experiment with dihydrofolate demonstrated that equation (1) described the principal route for dihydrofolate degradation. The rate of enzyme-catalyzed destruction of methotrexate was followed by storing a solution of 500 μM methotrexate at pH 7.2 in 0.05 M Tris-

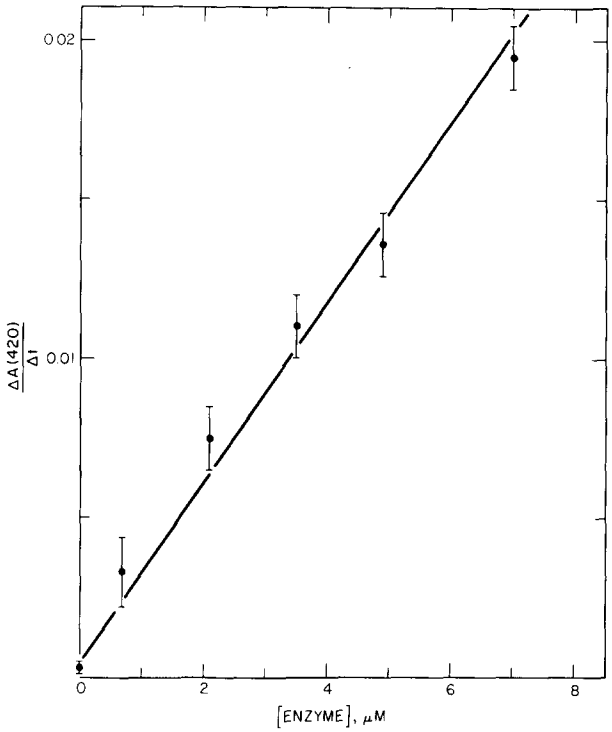

FIGURE 2: Dependence of rate of dihydrofolate monooxidase upon enzyme concentration. $\Delta A(420)/\Delta t$ is increase in A(420) in 500 sec. Dihydrofolate, 314 μM. T, 25°. The rate in the absence is indicated at zero enzyme concentration.

HCl 0.30 M NaCl with 12 μM enzyme in foil-wrapped vials at 25° in a constant temperature oven. Aliquots of the solution were layered on a 1.5 x 36 cm column packed with Sephadex (R) G-25 (fine) at 0, 30, 47, 119 and 126 hrs after mixing and eluted with H_2O; the fractions containing methotrexate were combined and standardized. No appreciable loss of methotrexate was noted in solutions incubated under the same storage conditions with buffer alone or with heat-denatured enzyme.

The proton magnetic resonance spectrum of 7,8-H_2-pterin-6-CHO was taken on a Varian HA-100 with Fourier Transform accessory. Spectra were taken of a roughly 0.002 M solution in d_5-pyridine (Merck Sharp & Dohme, Canada) at 34° and were internally referenced to $(CH_3)_4Si$ with downfield shifts assigned positive values and expressed in parts-per-million (ppm) of the polarizing

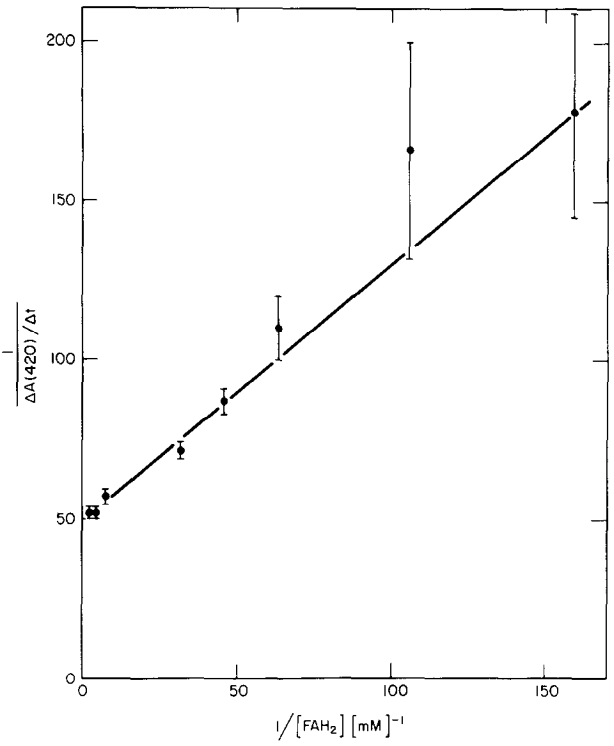

FIGURE 3: Dependence of rate of dihydrofolate monooxidase upon dihydrofolate concentration. Rates corrected for nonezymatic activity.

field. The pterin was purified from enzyme-treated dihydrofolate by chromatography in a darkened room on a 2 x 12.5 cm DEAE-Sephadex A-25 column in 0.55 M NaCl 0.05 M Tris-HCl pH 7.2, followed immediately by chromatography on a 2 x 36 cm Sephadex G-25 column in H_2O, and then by lyophilization. The lyophilization was followed by dissolution in d_5-pyridine and then by spectral accumulation. From the beginning of lyophilization to the end of spectral accumulation took about 3 hrs; in this time, an appreciable amount of the pterin (roughly 30%) had degraded to 7,8-H_2-xanthopterin, and xanthopterin.

RESULTS AND DISCUSSION

When the absorbance of a solution of dihydrofolate at neutral pH is monitored at 420 nm, there is a slow steady increase in absorbance with time. Addition of *E. coli* MB 1428 dihydrofolate reductase appreciably increases this

rate. Upon addition of the reductase inhibitor methotrexate, the rate of absorbance increase returns to its slow nonenzymatic value. The amount of rate increase is linearly dependent upon the concentration of enzyme present, as is shown in the data replotted for Figure 2. The rate at zero enzyme concentration is the nonenzymatic rate. The nonenzymatic rate is much lower in dihydrofolate solutions exhaustively bubbled with nitrogen.

The dependence of the initial rate of the enzyme-catalyzed absorbance increase at 420 nm upon dihydrofolate concentration is portrayed in Figure 3. From analysis of the slope and intercept of this plot, the K_M or Michaelis constant for dihydrofolate is 17.5 ± 2.5 μM. This K_M is much larger than the dissociation constant of about 1 μM (*9*,*10*), which suggests that the rate constant for product formation is appreciably larger than the rate constant for dihydrofolate dissociation from the enzyme. The maximal rate of reaction at infinite dihydrofolate concentration was 0.050 moles of dihydrofolate cleaved per minute per mole enzyme; this turnover number is much smaller than the turnover number for dihydrofolate reduction, which is 1050 moles reduced per minute per mole enzyme under comparable condition (*5*,*6*).

The primary products of the enzymatic reaction are the same as those of the nonenzymatic reaction, namely p-aminobenzoylglutamate and a labile, yellow-green fluorescing compound proposed (*1*) and shown (*4*) to be 2-amino-7, 8-dihydro-4-hydroxypteridine-6-carboxaldehyde. This latter compound at roughly 0.002 M in d_5-pyridine had a proton magnetic resonance spectrum consisting of a resonance of unit intensity at 11.01 ppm and two resonances each with twice this intensity at 6.68 and 4.07 ppm. The resonance at 11.01 ppm corresponds to the aldehyde proton. Aldehyde protons generally exhibit chemical shifts in chloroform between 9.8 and 10.5 ppm (*11*), but solvent interactions with pyridine can give rise to downfield shifts (*12*). In trifluoroacetic acid, the aldehyde resonance is at 9.63 ppm (*4*). This resonance of the enzymatic product could possibly be a carboxyl proton resonance; but: 1) carboxyl groups α to rings generally have chemical shifts at lower field (*11*), 2) there are

TABLE I

Turnover numbers for folate and dihydrofolate monooxygenase and methotrexate destruction by *E. coli* MB 1428 dihydrofolate reductase. All at 25° and pH 7.2.

Substrate	Turnover Number moles/min/mole
Folate	0.0014
Dihydrofolate	0.050
Methotrexate	0.00004

no ultraviolet bands to indicate appreciable formation of the appropriate carboxylates, and 3) pterin-6-COOH in the same solvent has an unobservable carboxyl resonance possibly due to exchange with the water impurity. The resonance at 6.68 ppm probably corresponds to the two protons on the 2-amino group (see reference *13* for examples) and the resonance at 4.07 ppm to the two protons at C-7 (*14*). The resonances of the 4-hydroxyl protons and the proton on N(8) were not observed, possibly due to exchange with the water impurity or obscuration by the solvent and water resonances. Thus, the proton magnetic resonance spectrum is consistent with the proposal of Hillcoat *et al* (*1*) and Whitely *et al* (*4*) that the yellow-green fluorescing compound is 7,8-H_2-pterin-6-CHO.

The dihydrofolate reductase from *E. coli* MB 1428 will also oxidatively cleave folate at the C(9)-N(10) bond, producing p-aminobenzoylglutamate and pterin-6-carboxaldehyde, which are also produced by photolytic cleavage of folate (*15*). Methotrexate was found to be slowly degraded by the enzyme, although the products could not be identified. The turnover numbers for the three enzyme-catalyzed degradation reactions are listed in Table I.

Despite initial results to the contrary (*2*), dihydrofolate has been found to be more labile at acid pH values than at neutral pH (*1*,*16*). For a 353 μM solution of the dihydrofolate preparation described herein, the initial non-

enzymatic rate of absorbance increase at 420 nm was 42.6-fold more rapid at pH 4.00 than at pH 7.00 at 25°. When dihydrofolate is bound to *E. coli* MB 1428 dihydrofolate reductase, charged groups on the enzyme raise the pK values for ionizable groups on dihydrofolate to make the dihydrofolate ultraviolet absorption bands correspond to dihydrofolate at acid pH (*8*). A plausible mechanism for the dihydrofolate monooxygenase activity of the reductase is that upon binding to enzyme dihydrofolate changes to an ionic form of greater lability, this form being the ionic form found at acid pH values.

ACKNOWLEDGEMENT

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