Dismutation of Dihydrofolate by Dihydrofolate Reductase†

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ABSTRACT: Degradation of 7,8-dihydrofolate (H_2folate) in the presence of dihydrofolate reductase (DHFR) has been shown due not to an oxygenase activity of the reductase as previously reported but to dismutation of H_2folate to folate and 5,6,7,8-tetrahydrofolate (H_4folate). The reaction can be followed spectrophotometrically or by analysis of the reaction mixture by high-performance liquid chromatography (HPLC). The products have also been isolated and characterized. Oxygen uptake during the reaction is much less than stoichiometric with H_2folate disappearance and is attributed to autoxidation of the H_4folate formed. The dismutation activity is a property of highly purified *Streptococcus faecium* DHFR isoenzyme 2 (but not isoenzyme 1) and of *Lactobacillus casei* DHFR, but not of bovine liver DHFR. The activity is dependent on tightly bound $NADP^+$ and/or NADPH. Removal of the nucleotide results in loss of dismutation activity, which is restored by adding $NADP^+$ or NADPH. Maximum activity is obtained when approximately 1 mol equiv of nucleotide is added per mol of DHFR. It is proposed that in the dismutation reaction bound NADP(H) is alternately reduced and oxidized by incoming molecules of H_2folate with release of folate and H_4folate, respectively. The relatively slow rate of folate formation presumably limits the rate of the overall reaction. The equilibrium constant for the dismutation reaction is 19.4 ± 7.4 at 22 °C and pH 7.0. Calculation of standard oxidation–reduction potentials at pH 7 gave values of –0.230 V for the H_2folate/H_4 folate pair and –0.268 V for the folate/H_2folate pair. The mechanism by which $NADP^+$ is retained by the enzyme from some sources during purification procedures is unclear. With one exception, fluorescence titration gave dissociation constants in the normal range (0.08–24 μM) for complexes of $NADP^+$ and NADPH with the enzymes either as isolated or after removal of bound nucleotide.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3; DHFR) normally catalyzes the reduction of 7,8-dihydrofolate (H_2folate) to tetrahydrofolate (H_4folate) by NADPH. However, Poe (1973) reported that DHFR from *Escherichia coli* MB 1428 also catalyzes the oxidative cleavage of H_2folate at the C9–N10 bond and tentatively identified one of the products by proton magnetic resonance as 7,8-dihydropterin-6-carboxaldehyde. The reaction proposed was

$$H_2\text{folate} + {}^1/_2O_2 \rightarrow \text{7,8-dihydropterin-6-carboxaldehyde} + (p\text{-aminobenzoyl})\text{glutamate} \quad (1)$$

In the course of ^{13}C nuclear magnetic resonance (NMR) studies on the binding of ligands to DHFR (Cocco et al., 1981a,b, 1983) [2-^{13}C]H_2folate was incubated with an approximately equimolar concentration of highly purified DHFR (isoenzyme 2 from *Streptococcus faecium*). When the ^{13}C NMR spectrum obtained subsequently from this solution was examined, two resonances were observed, neither of which corresponded to [2-^{13}C]H_2folate at any pH between 6 and 10 (L. Cocco, J. A., Montgomery, C. Temple, Jr., and R. L. Blakley, unpublished results). One of these resonances had chemical shift values at all pH values similar to those of folate, but the chemical shift of the other did not correspond to any folate known to us.

This interaction between H_2folate and high concentrations of DHFR is a hindrance to studies on the binding of this substrate, and it was of some practical importance to determine whether it could be avoided. Although we at first assumed that we were dealing with the reaction described by Poe (1973), this turned out not to be the case, but instead the reaction proved to be a dismutation to folate and H_4folate, as the results presented below indicate.

Experimental Procedures

Materials

DHFR isoenzyme 2 was isolated and purified from *S. faecium* var. *durans* strain A as described previously (Blakley et al., 1978). *S. faecium* DHFR isoenzyme 1 was separated from isoenzyme 2 during purification of the latter by ion-exchange chromatography on DEAE-Sephadex (Nixon & Blakley, 1968) and was used without further purification. DHFR from *L. casei* was prepared as described by Cocco et al. (1981a) and from bovine liver as described by Peterson et al. (1975). Folate was removed from the bovine liver DHFR by hydroxylapatite chromatography (Kaufman & Kemmerer, 1976), as follows. Five grams of Bio-Gel HTP hydroxylapatite was suspended in 200 mL of 10 mM potassium phosphate buffer, pH 6.8, and allowed to settle for 10 min, and the buffer was decanted. This procedure was repeated once. The hydroxyapaptite was poured into a 1 × 12 cm column and washed with 50 mL of the same buffer. About 6 mg of enzyme was applied to the column, which was then washed with 15 mL of the same buffer. A linear gradient (50 mL of 10 mM potassium phosphate buffer, pH 6.8, in the stirred compartment and 50 mL of 200 mM potassium phosphate buffer, pH 6.8, in the reservoir) was used to elute the enzyme. Two peaks of activity were obtained, the first having bound folate and the second being folate free. By this procedure about 50% of the applied enzyme could be obtained folate free.

Crystalline H_2folic acid was prepared according to Blakley (1960). NADP, NADPH, superoxide dismutase, and alkaline phosphatase from bovine intestinal mucosa were from Sigma. Bound nucleotides were removed from *S. faecium* DHFR (isoenzyme 2) by stirring a solution of the enzyme in 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 7.3, with charcoal (0.1–1 mg added per mg of protein, depending on the enzyme preparation) for 30 min at 5 °C. Charcoal was removed by centrifugation, and in some

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cases the solution was also passed through a Millipore filter (0.2 μm). The final solution had only a slightly decreased protein concentration, and the specific activity was unchanged.

Methods

Ultraviolet Spectrophotometry. Spectral changes and reaction rate were determined by the use of Cary 219 and Cary 14 recording instruments. In measurements of the rate of the dismutation reaction from the absorbance change at 340 nm, the reaction mixture contained 0.9 mM H_2folate, 50 mM potassium phosphate buffer, pH 7, and 0.2–2.0 mg of DHFR in a volume of 1 mL at 25 °C. A standard quartz curvette (1-cm path length) was used with a quartz insert that gave an effective path length of 1 mm. The molar absorbance change at 340 nm for the dismutation reaction was measured by determining the absorbance change that had occurred when equilibrium was reached. Initial concentrations of H_2folate were in the range 75–300 μM, and the reaction mixtures contained 2 mg of *S. faecium* DHFR isoenzyme 2. The total absorbance change was plotted vs. the initial H_2folate concentration, and the slopes of the least-squares line was taken as the apparent molar absorbance change. This value was corrected for residual H_2folate at equilibrium as found in the determination of the equilibrium constant for the dismutation reaction. The corrected absorbance change found is 3.65×10^3 M^{-1}.

In the determination of the apparent K_m for H_2folate, concentrations of the latter ranged from 150 to 2100 μM, and 103 μM enzyme was used. The apparent K_m was calculated from the data by fitting to the appropriate equation by the use of Cleland's HYPER program (Cleland, 1979).

Fluorescence Titrations. Determination of dissociation constants for complexes of $NADP^+$ and NADPH with DHFR was determined by titration of the protein fluorescence measured with a Perkin-Elmer MPF-44B spectrofluorometer at 20 °C. Excitation was at 290 nm (8 nm slit), and emission was measured at 340 nm (12 nm slit). To DHFR (2 μM) in 3.0 mL of 50 mM potassium phosphate buffer, pH 7.3, was added successive small volumes of approximately 400 μM NADPH or $NADP^+$, with measurement of fluorescence before additions and after each addition. Fluorescence intensity data corrected for volume change and internal filter effects were computer fitted to the equation described by Torikata et al. (1979), in order to calculate the dissociation constant, K_D.

High-performance liquid chromatography (HPLC) was performed on an Altex-Beckman system consisting of two Model 110A pumps, Model 420 microprocessor, Model 153 UV–visible detector with analytical flow cell, Model C-RIA integrator–recorder, and Model 210 injector. Separation of folate, H_2folate, H_4folate, NADPH, and NADP was achieved on an Altex 5-μm ultrasphere–ODS column (4.6 × 250 mm) with the use of a solvent gradient formed from the following components: solvent A, 5 mM *tert*-butylammonium phosphate in water; solvent B, 5 mM *tert*-butylammonium phosphate in 80% methanol. The column was equilibrated with a solvent mixture containing 18% B, and at time zero the gradient was developed as follows: 18–40% B linear gradient over 35 min; 40–60% B linear gradient over 15 min; 60% B for 10 min. In order to observe H_4folate, it was found necessary to rigorously degas both solvents with helium (3–4 h) and to add sodium ascorbate to samples (20 mg of solid sodium ascorbate/mL). Under these conditions, the following retention times (min) were found: (*p*-aminobenzoyl)glutamate, 36.2; H_4folate, 41.0; H_2folate, 45.9; $NADP^+$, 47.3; folate, 52.1; NADPH, 54.4. In a typical enzymatic reaction 8 mg of solid sodium ascorbate was weighed into a small test tube (7 × 50 mm) and was dissolved in 300 μL of 0.05 M Tris-HCl buffer, pH 7.3. To this solution was added 10 μL of 3 mM H_2folate in the same buffer, and the reaction was started by the further addition of 5–150 μL of a solution of DHFR (1–5 mg, 23–55 IU). The tube was incubated in a bath at 22 °C for a designated period, then heated in a block for 1 min at 100 °C, and then chilled in ice. The suspension was transferred to a small centrifuge tube and centrifuged at 7000*g* for 2 min, and 10 μL of the supernatant was injected for HPLC with recording at 280 nm and the recorder set at 0.08 absorbance unit full scale. When the analysis of nucleotides was to be performed on boiled enzyme supertant solution, the procedure was similar, but detection was at 254 nm.

Oxygen Uptake Studies. These were made with a Clark-type oxygen electrode (Fisher Scientific Co., Pittsburgh, PA). A solution (2.0 mL) of *S. faecium* DHFR isoenzyme 2 (0.21 mM, specific activity 23 IU/mg) in 0.05 M Tris-HCl buffer, pH 7.3, was placed in the oxygen electrode cell and allowed to equilibrate at 25 °C under air. Then 1.0 mL of H_2folate in the same buffer was added to give a final concentration of 0.92 mM, the electrode was inserted to close the cell, and recording was commenced. In some experiments, oxygen was readmitted after the oxygen concentration reached a very low value and recording resumed. In other cases after a period of recording oxygen consumption, samples were removed from the cell and analyzed by HPLC as described above to determine folates present.

Isolation of Reaction Products. Products were isolated from a reaction mixture containing 7.2 mM potassium phosphate buffer, pH 7.3, 0.36 M KCl, 0.72 mM ethylenediaminetetraacetic acid (EDTA), 0.014% sodium azide, 4.3 mM H_2folate, and 0.43 mM *S. faecium* DHFR isoenzyme 2. The reaction mixture (total volume 13.8 mL) was incubated at 37 °C for 110 min. The pH was then adjusted to 9.5 with 2 M Tris and 10 N NaOH, the mixture centrifuged to remove some precipitated protein, and the supernatant applied to a Sephadex G-50 column (4.5 × 95 cm) which had been equilibrated with 10 mM Tris-HCl buffer, pH 9.5. The column was eluted with the same buffer, and fractions of about 15 mL were collected. Two peaks having absorption at 280 nm emerged, one in fractions 23–32 and a second larger peak in fractions 69–82. The first of these contained protein. Fractions in the second peak were combined and freeze-dried, the residue was dissolved in a small volume of water, and the solution was applied to a Bio-Gel P2 column (4.7 × 174 cm) which had been equilibrated with 0.05 M ammonia. The column was eluted with 0.05 M ammonia, and fractions of 15 mL were collected. Two peaks with yellow color and absorbance at 280 nm emerged. The first, larger peak was found in fractions 44–51 and the second in fractions 60–61. Fractions within each of these peaks were combined, and the two solutions so obtained were each concentrated to dryness by rotary evaporation at ≈1 mmHg pressure. Residues were dissolved in a small volume of water by addition of a drop of concentrated ammonia.

The composition of these fractions were investigated by HPLC. A Waters μBondapak C_{18} column (4.7 × 250 mm) was used with a solvent gradient formed from the following components: solvent A, 0.1 M sodium phosphate, pH 5.5; solvent B, acetonitrile. The column was equilibrated with solvent A, and after sample injection the gradient was developed as follows: 0–2 min, 0% solvent B; after 2 min, a linear gradient from 0 to 10% B over 8 min; constant at 10% B for 5 min; 10–11% B over 1 min; constant at 11% B for 4 min; 11–0% over 5 min. Detection was performed at 254 nm. The yellow material from frations 60–61 exhibited two major and

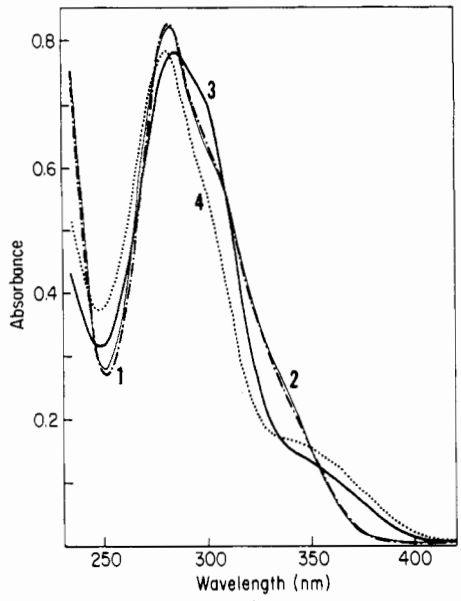

FIGURE 1: Changes in the ultraviolet spectrum of H_2folate during degradation by DHFR. Solutions in both reference and sample cuvettes contained 10 mM potassium phosphate buffer, pH 7.3, 0.5 M KCl, 1 mM EDTA, and 0.02% sodium azide. The sample cuvette also contained 0.29 mM H_2folate. Each standard cuvette contained 1.0 mL of reaction mixture and was fitted with a quartz insert that reduced the optical path to 1 mm. Spectrum 1, no other additions, 0 min; spectrum 2, no other additions, after 30 min; spectrum 3, both cuvettes also contained 0.13 mM *S. faecium* DHFR isoenzyme 2 and recording was commenced immediately after addition of H_2folate to the sample cuvette, mixing, and positioning of the insert; spectrum 4, as for spectrum 3 but recording commenced after 30 min.

some partially resolved minor peaks in this HPLC system. These components did not correspond in regard to either elution time or spectrum to any folates that were examined and are considered to have been unconjugated pterins. They were not examined further. The major fraction from the Bio-Gel P2 column (tubes 44–51) showed two components by HPLC which corresponded in their elution times to folate (15.5 min) and (*p*-aminobenzoyl)glutamate (5.3 min). Separation of these components by preparative HPLC in the same system yielded 18.6 μmol of folate and 15 μmol of (*p*-aminobenzoyl)glutamate. Their identities were confirmed by spectra at pH 1, 7.3, and 13.

Results

Spectrophotometric Observations on the Transformation of H_2folate by DHFR. When H_2folate was incubated at 20 °C with a relatively large amount of *S. faecium* DHFR isoenzyme 2 (0.01–0.4 molar equivalents) at pH 7.3, the spectrum quickly changed with increases in absorbance in the 350–400-nm region, accompanied by changes throughout the rest of the ultraviolet (Figure 1). The spectra of unchanged H_2folate and of the complete mixture at 0 min and at 30 min did not show isosbestic points, an observation suggesting that more than one transformation reaction was occurring under these conditions. Reductase was absolutely required for the reaction.

Reaction rates can be estimated by recording at any one of a number of wavelengths with essentially identical results, but for most experiments the decrease in absorbance at 340 nm was used. Rates of absorbance change at this wavelength under the usual reaction conditions ranged from 1×10^{-3} to 35×10^{-3} min^{-1}. The reaction rate was proportional to enzyme concentration up to at least 100 μM DHFR (2 mg/mL). When the effect of H_2folate concentration on reaction velocity was determined, the process was found to be saturable (linear double-reciprocal plot) with an apparent Michaelis constant of 180 ± 8 μM.

Isolation of Products of Dihydrofolate Transformation. To elucidate the nature of the process, H_2folate (60 μmol) was incubated with *S. faecium* DHFR under air, and the products of the reaction were separated by gel filtration and HPLC (see Methods). The major products isolated were folate (18.6 μmol) and (*p*-aminobenzoyl)glutamate (15 μmol). Another fraction contained a mixture of unconjugated pterins. This result is contrary to the predictions for the oxygenase reaction (eq 1) proposed by Poe (1973) and suggests that a completely different reaction mechanism is responsible.

Oxygen Uptake Measurements during Dihydrofolate Transformation. As a further test of whether transformation was due to an oxygenase reaction, oxygen consumption was determined by the use of an oxygen electrode. In a typical experiment the initial H_2folate concentration was 0.92 mM, and the decrease in oxygen concentration over a period of 18 min was 0.079 mM. When a sample was removed at this point, and the enzyme inactivated by heating, analysis by HPLC indicated that 81% of the dihydrofolate had reacted, corresponding to a 0.73 mM decrease in concentration. The utilization of oxygen was therefore not stoichiometric with H_2folate transformation.

A further indication that oxygen was not directly involved in dihydrofolate removal was obtained by examining the rate of oxygen consumption at different intervals during H_2folate reaction. In the experiment referred to above the rate of oxygen consumption remained constant during the 18-min period despite the drop in H_2folate concentration to 0.19 mM, which is near the apparent K_m value. In other experiments it was found that during longer incubations in which the H_2folate concentration was depleted to an even lower level, oxygen consumption continued at an almost constant rate (after reequilibration of the system with air to raise the oxygen concentration). These results indicate that oxygen is not directly involved in the enzymatic reaction involving H_2folate but reacts with a labile product of the primary reaction, namely, H_4folate. The (*p*-aminobenzoyl)glutamate and unconjugated pterins isolated from the large-scale reaction therefore arise from oxidative degradation of H_4folate. It is well established that solutions of H_4folate are unstable, undergoing degradation by oxygen with cleavage at the 9,10 bond to give (*p*-aminobenzoyl)glutamate and a multitude of dihydropterin products that are further oxidized to pterins (Chippel & Scimgeour, 1970; Kallen, 1971; Pearson, 1974; Reed & Archer, 1980). In confirmation of this interpretation it was found that the introduction of superoxide dismutase (100 units/mL) virtually abolished oxygen consumption (decreased by >90%). This is in agreement with previously published evidence that superoxide dismutase substantially inhibits the autoxidation of H_4pterins like H_4folate (Nishikimi, 1975).

Use of HPLC To Analyze Products of H_2folate Transformation. In preliminary experiments with no precautions to exclude oxygen, the disappearance of H_2folate and the appearance of folate were found to be first order with identical rate constants. In a reaction allowed to proceed to completion, the folate formed corresponded to about half the amount of H_2folate originally present. The other product or products from the reaction were not readily identified in this system. A (*p*-aminobenzoyl)glutamate peak was identified, this product arising from oxidative degradation of H_4folate.

When degradation and HPLC were performed anaerobically (ascorbate in the reaction mixture and prolonged deoxygenation of chromatography solvents), a peak due to H_4folate was

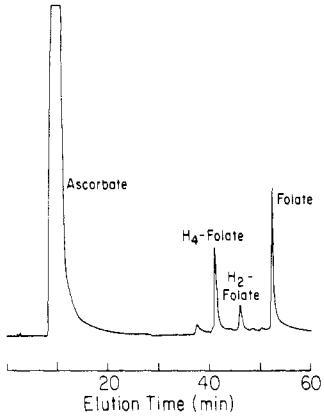

FIGURE 2: HPLC of the products from incubation of H_2folate with *S. faecium* dihydrofolate reductase. The incubation period at 20 °C was 20 min. For other details see Methods.

Table I: Dismutation Rates for DHFR from Various Sources[a]

enzyme source	specific activity (IU/mg)		
		H_2folate dismutation	
	H_2folate reduction	without added NADP$^+$	with added NADP$^+$
S. faecium isoenzyme 1	23.8	0	0.002[b]
S. faecium isoenzyme 2	34.9	0.031	0.09[b]
L. casei	21.9	0.038	0.121[b]
bovine liver	55.6	0	0.0030[c]
			0.0071[d]

[a] Rates of H_2folate dismutation or reduction were measured as described under Methods. [b] 1.0 mol of NADP$^+$/mol of DHFR. [c] 0.5 mol of NADP$^+$/mol of DHFR. [d] 5.0 mol of NADP$^+$/mol of DHFR.

clearly visible in the elution profile of a sample of the degradation products (Figure 2), and no additional major peaks other than those for folate and residual H_2folate appeared. The reaction catalyzed by *S. faecium* DHFR is therefore a dismutation according to the equation

$$2H_2\text{folate} \rightleftharpoons \text{folate} + H_4\text{folate} \quad (2)$$

H_2folate Dismutation by DHFR from Other Sources. H_2folate was incubated for 30 min with the *L. casei* enzyme under conditions similar to those used for the isolation of products formed by the *S. faecium* enzyme, and the products were subsequently separated by HPLC. Three major peaks were recovered, and these were identified by their elution times and by the ultraviolet spectra at pH 1, 7.3, and 13. In order of elution they were (*p*-aminobenzoyl)glutamate, H_2folate, and folate. On the basis of absorbance the yield of the latter was 43%.

The dismutation of H_2folate catalyzed by the *L. casei* enzyme could also be assayed spectrophotometrically and corresponded to a specific activity quite similar to that of the *S. faecium* DHFR isoenzyme 2 (Table I). However, neither DHFR isoenzyme 1 as isolated from *S. faecium* nor bovine liver DHFR had measurable dismutation activity (Table I).

Presence of Bound Nucleotide in DHFR Preparations. Although there are several possible explanations for the occurrence of the dismutation reaction, one is that the isolated enzyme retains bound NADP$^+$ or NADPH. The dismutation reaction 2 would then be the result of coupling reactions 3 and 4.

$$H_2\text{folate} + \text{NADPH} + H^+ \rightleftharpoons H_4\text{folate} + \text{NADP}^+ \quad (3)$$

$$H_2\text{folate} + \text{NADP}^+ \rightleftharpoons \text{folate} + \text{NADPH} + H^+ \quad (4)$$

Table II: Nucleotide Bound to Bacterial DHFR[a]

enzyme preparation	nucleotide bound (mol/mol of DHFR)		
	NADPH	NADP$^+$	total
S. faecium, batch 7/82	0	0.093	0.093
S. faecium, batch 5/83	0.044	0.065	0.109
S. faecium, batch 6/83	0.019	0.151	0.17
S. faecium, batch 7/83	0.062	0	0.062
L. casei	0	0.189	0.189

[a] The amount of bound nucleotide was determined in the HPLC system described under Methods. Quantities were determined by comparing integrated areas with those from NADPH and NADP$^+$ standards run immediately before the enzyme samples.

In order to examine this possibility, concentrated enzyme (20–30 mg/mL) was heated for 1 min at 100 °C, chilled in ice, and centrifuged. A sample of the supernatant (40 μL) was used for HPLC analysis of released nucleotide as described under Methods. Results obtained with preparations of DHFR from several sources are shown in Table II. The preparations of bacterial DHFR contained material that eluted with the same elution times as NADPH and NADP$^+$. To confirm the identity of these materials, samples containing about 12 nmol of nucleotide were treated with alkaline phosphatase (69 units). After incubation for 30 min at 20 °C the mixture was heated 1 min at 100 °C, chilled in ice, and centrifuged. A 10-μL sample of the supernatant was used in the HPLC analysis. Phosphatase treatment of authentic NADPH or NADP$^+$ changed the elution times from 54.74 and 48.5 min to 13.45 and 13.79 min, respectively. Phosphatase treatment produced a similar effect on the elution times of the material present in the boiled DHFR preparations, and on mixing, this material coeluted with the phosphatase products from NADPH and NADP$^+$.

Removal of Bound Nucleotide from DHFR. To further establish the presence of bound nucleotide and its role in the dismutation reaction, DHFR preparations from *S. faecium* were treated with activated charcoal. This treatment abolished the dismutation reaction as demonstrated both by spectrophotometry and also by HPLC. HPLC analysis of the supernatant solution from a heated, centrifuged sample of the charcoal-treated reductase confirmed that bound nucleotide has been removed.

It was also found that nucleotide was lost when *S. faecium* DHFR passed through a methotrexate–Sepharose column (Dann et al., 1976), followed by removal of folate (used to elute the enzyme from the affinity column) by gel filtration on Sephadex G-50 at pH 9.0. Bovine liver DHFR preparations that had been purified through these steps also had no dismutase activity (Table I), and no bound nucleotide was detected.

When *S. faecium* DHFR was treated with sufficient charcoal to decrease the dismutation rate to a very low level, the dismutation activity was restored by subsequent addition of NADPH or NADP$^+$ (Figure 3). The rate of the dismutation reaction increased with the amount of nucleotide added until 1 mol equiv of nucleotide had been added per mol of DHFR. Further additions did not increase the rate. The specific activity of the nucleotide–enzyme complex was 0.09 IU/mg which corresponds to a turnover number of 2 min^{-1}, whereas the specific activity of this preparation for the normal reductase reaction was approximately 30 IU/mg. Addition of a molar equivalent of NADP$^+$ to an untreated DHFR preparation gave the same specific activity (Table I). Addition of much larger amounts of nucleotide, approximately equiv-

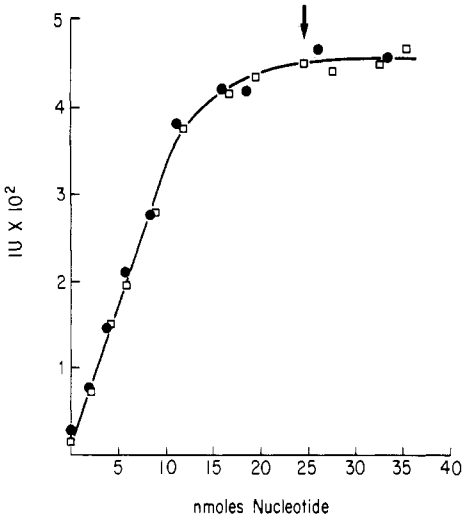

FIGURE 3: Dependence of the rate of H_2folate dismutation by charcoal-treated DHFR on the amount of coenzyme added. The reaction mixtures contained *S. faecium* DHFR (0.5 mg of protein; previously treated with 0.1 mg of charcoal/mg of protein), 0.9 mM H_2folate (total 324 nmol), phosphate buffer, and the amounts of nucleotide indicated. Other details are given under Methods. The arrow indicates an amount of nucleotide stoichiometric with the DHFR present. (□) NADPH; (●) $NADP^+$.

alent to H_2folate, gave predominantly oxidation of H_2folate according to reaction 4 when a high concentration of $NADP^+$ was added or reduction of H_2folate according to reaction 3 when a high concentration of NADPH was added.

When *L. casei* DHFR was stirred with charcoal (0.5 mg/mg of protein) for 24 h, there was no loss in dismutation activity. Nucleotide was stripped from the enzyme, however, by adjustment to pH 9.5 and passage through a Sephadex G-50 column (3.5 × 110 cm) equilibrated at this pH. Enzyme treated thus and subsequently concentrated by ultrafiltration at neutral pH was unable to catalyze dismutation of H_2folate, and analysis by HPLC indicated that no detectable amount of nucleotide was now present. On addition of $NADP^+$ in slight molar excess of dismutation rate became 0.08 IU/mg. This was 67% of the rate under these conditions for the original preparation (Table I), and the specific activity for the normal catalytic reaction (H_2folate reduction to H_4folate) had decreased by a similar factor.

As indicated above, purified bovine liver DHFR did not catalyze dismutation of H_2folate, but this might be due simply to absence of bound nucleotide. However, in the presence of 1 equiv of added $NADP^+$ one preparation gave an apparent dismutation rate 2700 times slower than the rate of the normal reaction. For a second preparation the dismutation rate was 19 000 times slower than the normal catalytic reaction in the presence of 0.5 equiv of added $NADP^+$/mol of DHFR (Table I), and in the presence of 5 equiv it was still 7800 times slower. In contrast to bacterial DHFR, bovine enzyme therefore has little capacity for catalyzing dismutation, even when supplied with $NADP^+$.

S. faecium DHFR isoenzyme 1 appeared to have a small amount of dismutase activity in the presence of 1 equiv of $NADP^+$ (Table I), but this was probably attributable to slight contamination with isoenzyme 2.

Role of DHFR in H_2folate Dismutation. Since the DHFR catalyzing the dismutation reaction carries bound $NADP^+$ or NADPH, it is theoretically possible that in the dismutation reaction the enzyme is serving solely to provide nucleotide and does not catalyze the reaction. This possibility was eliminated by the demonstration that there was no reaction during incubation of 300 μM H_2folate with 300 μM NADPH or $NADP^+$, under the usual assay conditions but in the absence of DHFR. Furthermore, methotrexate and trimethoprim, known inhibitors of DHFR that bind at the catalytic site, inhibited the dismutation reaction. It was not possible to determine a K_i because of the high concentration of DHFR relative to inhibitor concentration that must be used in the assay. However, in a standard assay reaction mixture containing 43 μM DHFR, the dismutation reaction was abolished by the presence of 45 μM methotrexate or 44 μM trimethoprim.

Table III: Dissociation Constants for Complexes of $NADP^+$ or NADPH with DHFR from Various Sources

source of DHFR	K_D (μM) NADPH complex	K_D (μM) $NADP^+$ complex
bovine liver	0.077 ± 0.021	0.784 ± 0.028
L. casei	*a*	6.39 ± 0.19
S. faecium (isoenzyme 2)[b]	2.53 ± 0.10	13.2 ± 1.7
S. faecium (isoenzyme 2)[c]	2.27 ± 0.17	24.3 ± 2.8

[a] Too low for estimation. [b] After treatment with charcoal to remove nucleotide. [c] Without charcoal treatment.

Dissociation Constants for DHFR–Coenzyme Complexes. The finding that highly purified DHFR retains bound nucleotide was unexpected because published values for dissociation constants of coenzyme complexes of bacterial DHFR are not unusually low (D'Souza & Freischeim, 1972; Williams et al., 1973; Erickson & Mathews, 1973; Gleisner & Blakley, 1975; Dunn et al., 1978; Reddy et al., 1978; Williams et al., 1979; Cayley et al., 1981). The lowest values reported for an NADPH complex is 0.01 μM (Dunn et al., 1978), but most are of the order of 1 μM, and $NADP^+$ complexes have even higher constants.

To verify that nucleotide complexes of DHFR from the sources used in these experiments have similar dissociation constants, these were determined by fluorescence titration with the results shown in Table III. With the possible exception of the complex of NADPH with *L. casei* DHFR, the values are in the same range as those previously reported, and the $NADP^+$ complexes all have quite high values.

Equilibrium Constant for Dismutation and Standard Oxidation–Reduction Potentials. The equilibrium constant for the dismutation reaction was estimation by determining residual H_2folate remaining at equilibrium. In two experiments approximately 80 μM H_2folate was incubated with 137 μM *L. casei* DHFR in a solution (360 μL) containing 8 mg of sodium ascorbate and 50 mM Tris-HCl buffer, pH 7.3. Two experiments in which the amount of DHFR was decreased 10-fold gave similar values. Attempts at achieving greater precision by also measuring folate present at equilibrium were complicated by the observation that a more than stoichiometric amount of folate was formed, presumably due to some decomposition of the H_4folate. Estimates of the equilibrium concentrations of H_2folate were performed by HPLC, with comparison of the integrated peak area with that for the peak for the initial H_2folate concentration. The latter concentration was determined spectrophotometrically. The mean value from four estimations was 19.36 ± 7.36 (SD), at 22 °C.

For the reduction of H_2folate by NADPH according to reaction 1, we have previously determined an equilibrium constant of 8.4×10^{10} at 37 °C and pH 8.0 (Nixon & Blakley, 1968). This corresponds to a value for the ratio

$$\frac{[H_4\text{folate}][NADP^+]}{[H_2\text{folate}][NADPH]}$$

of 840. This leads to a value of −0.230 V for E_0', the standard

oxidation–reduction potential at pH 7.0 for the H_2folate/H_4folate pair, based on a value of −0.320 V for the $NADP^+$/NADPH pair (Rodkey & Donovan, 1959). This value of E_0' at 37 °C may be compared with a value of −0.18 V reported by Mathews & Huennekens (1963) presumably for reaction at 25 °C. The value of −0.230 V for E_0' together with the equilibrium constant for the dismutation reaction at 22 °C leads to a value for E_0' for the folate/H_2folate pair of −0.268 V.

Discussion

The data presented indicate that, in the absence of added nucleotide coenzyme, DHFR from two bacterial sources catalyzes a dismutation of H_2folate to folate and H_4folate and that $NADP^+$ or NADPH bound to the enzyme is essential for this reaction. The enzyme–nucleotide complex evidently plays a catalytic role in this process, and any nucleotide added in excess of the amount required to saturate the catalytic site does not cause an increase in the rate of dismutation. It is proposed that in the dismutation reaction bound NADP(H) is alternatively reduced and oxidized by incoming molecules of H_2-folate, with formation and release of folate and H_4folate, respectively. The unfavorable equilibrium constant for H_2-folate oxidation to folate by bound $NADP^+$ suggests that the rate of this step is probably quite slow, and this presumably is what limits the rate of the overall dismutation process.

No measurements have been made of the rate constants for NADP(H) binding to enzyme or for NADP(H) release from complexes with the enzyme. Although the rate of release is probably very low, it is presently impossible to judge whether it is very much slower than the rate of $NADP^+$ reduction by H_2folate. In other words, it is not clear whether or not nucleotide remains bound during many cycles of the coupled oxidation–reduction responsible for dismutation. Regardless of this, it is apparent that enzyme-bound nucleotide shuttles electron between molecules of H_2folate that bind successively to the catalytic site of the reductase.

The dismutation activity of DHFR is analogous to its transhydrogenase activity reported some time ago by Huennekens et al. (1971). In the latter case the enzyme was reported to catalyze the reduction of the acetylpyridine analogue of $NADP^+$ by NADPH. This reaction required the presence of a small amount of folate, H_2folate, or H_4folate, and the transhydrogenation rate was about 5% that of the normal reaction. In this process, therefore, the folate–reductase complex played a catalytic role in shuttling electrons between the nucleotides. As would be expected, the transhydrogenation reaction was also inhibited by methotrexate.

The discovery that the destruction of H_2folate by DHFR in the absence of added $NADP^+$ or NADPH is due to an oxygenase-type activity (Poe, 1973) but to a dismutation reaction raises the question of whether such dismutation has any physiological significance. Conceivably the conversion of H_2folate to folate and H_4folate might replenish intracellular stores of the latter under conditions in which reduction of H_2folate to H_4folate is impaired. One such condition would be in the presence of antifolates that inhibit DHFR. However, in these circumstances binding of antifolate to the catalytic site would prevent dismutation of H_2folate as well as its catalytic reduction by NADPH. Even when accumulation of H_2folate results in displacement of antifolate (White, 1979), catalytic reduction would predominate over dismutation. No other circumstances have been reported in which significant amounts of H_2folate accumulate, and in particular no circumstances in which accumulation is due to depletion of NADPH.

It must also be recognized that the rate of dismutation is very slow even for the bacterial enzymes, being several 100-fold slower than the rate of H_2folate reduction to H_4folate. The overall rate of dismutation is undoubtedly limited by the rate of folate formation (reaction 3), since it is well-known that folate reduction (reverse of reaction 3) is much slower than the rate of H_2folate reduction.

A further reason that a physiological role for the dismutation reaction is doubtful is that it is presently uncertain whether dismutation is catalyzed by vertebrate DHFR's. Although bovine liver DHFR is the only DHFR from a vertebrate source that we have examined, it catalyzed dismutation of H_2folate only at a very low rate even when adequate amounts of nucleotide were supplied. The reason for this is obscure. Clearly the thermodynamics for dismutation are independent of the enzyme source, and binding of $NADP^+$ and NADPH was even more favorable for DHFR from bovine liver than for the *S. faecium* enzyme isoenzyme 2 (Table III). One possibility is that folate is released at an insignificant rate from the catalytic site of the bovine reductase. Consistent with this is our observation that after elution of the enzyme from the methotrexate–Sepharose column with folate, folate bound to the reductase cannot be removed by treatment with charcoal for 24 h, by passage through Dowex-1 Cl^{-1}, or by gel filtration on Sephadex G-50 at pH 9. Even chromatography on hydroxylapatite, recommended by Kaufman & Kemerer (1976), resulted in only partial removal of folate. Consequently, it seems very likely that folate dissociation is extremely slow and effectively limits the dismutation rate. Whether this is so for DHFR from other vertebrate sources is uncertain at this point but seems quite possible.

The presence of bound nucleotide in preparations of purified DHFR that was disclosed when the dismutation reaction was investigated is surprising and indeed paradoxical in view of the relatively high dissociation constants determined by fluorescence titration for complexes of NADPH and especially for $NADP^+$ (Table III). However, isolation of such relatively stable complexes has been reported in the past for DHFR from *L. casei* (Huennekens et al., 1971; Otting & Huennekens, 1972; Gundersen et al., 1972), from chicken liver (Huennekens et a., 1970, 1971; Mell et al., 1968), and from methotrexate-resistant L1210 murine lymphoma cells (Huennekens et al., 1971; Harding et al., 1970; Neef & Huennekens, 1975). How the relative stability of these complexes, as exemplified by their survival during enzyme purification and during procedures such as gel electrophoresis, can be reconciled with the measured dissociation constants is still unclear.

One possible explanation of the paradox is that more than one DHFR isoenzyme is present in the preparations and that a minor isoenzyme has a much higher affinity for the nucleotide cofactors than the predominant species. However, no evidence for such heterogeneity has been seen in sequencing studies, in electrophoretic data, or in fluorescence titration data. Another possibility is that although the dissociation constants are not particularly low, the rate constant for dissociation may be very low. A detailed study of the binding of $NADP^+$, NADPH, and analogues was performed by Dunn et al. (1978), by the use of a stopped-flow fluorospectrometric method. For $NADP^+$ the association rate constant at pH 6.0 and 25 °C was found to be 8×10^6 M^{-1} s^{-1} and the dissociation rate constant 133 s^{-1}. Although binding was complicated by the enzyme undergoing a conformational transition, there is certainly no indication from their results that bound $NADP^+$ would require long periods of time to dissociate. It is unclear, therefore, why $NADP^+$ remained bound to the enzyme during

purification and even during stirring with charcoal for 24 h. The way in which this paradox can be resolved is unclear, and further data are necessary before resolution is possible.

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Registry No. DHFR, 9002-03-3; NADP, 53-59-8; NADPH, 53-57-6; H_2folate, 4033-27-6; H_4folate, 135-16-0; folate, 59-30-3.

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