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Purification and Properties of *Escherichia coli* Dihydrofolate Reductase†

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ABSTRACT: Dihydrofolate reductase has been purified 40-fold to apparent homogeneity from a trimethoprim-resistant strain of *Escherichia coli* (RT 500) using a procedure that includes methotrexate affinity column chromatography. Determinations of the molecular weight of the enzyme based on its amino acid composition, sedimentation velocity, and sodium dodecyl sulfate gel electrophoresis gave values of 17680, 17470, and 18300, respectively. An aggregated form of the enzyme with a low specific activity can be separated from the monomer by gel filtration; treatment of the aggregate with mercaptoethanol or dithiothreitol results in an increase in enzymic activity and a regeneration of the monomer. Also, multiple molecular forms of the monomer have been detected by polyacrylamide gel electrophoresis. The unresolved enzyme exhibits two pH optima (pH 4.5 and pH 7.0) with dihydrofolate as a substrate. Highest activities are observed in buffers containing large organic cations. In 100 m*M* imidazolium chloride (pH 7), the specific activity is 47 μmol of dihydrofolate reduced per min per mg at 30°. Folic acid also serves as a substrate with a single pH optimum of pH 4.5. At this pH the K_m for folate is 16 μM, and the V_{max} is 1/1000 of the rate observed with dihydrofolate as the substrate. Monovalent cations (Na^+, K^+, Rb^+, and Cs^+) inhibit dihydrofolate reductase; at a given ionic strength the degree of inhibition is a function of the ionic radius of the cation. Divalent cations are more potent inhibitors; the I_{50} of $BaCl_2$ is 250 μM, as compared to 125 m*M* for KCl. Anions neither inhibit nor activate the enzyme.

Dihydrofolate reductase [5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase (EC 1.5.1.3)] is the target enzyme of several important drugs. We have previously shown that dihydrofolate reductases from bacterial and mammalian sources can be distinguished from each other on the basis of their relative ability to bind a series of small molecule analogues of dihydrofolate. These differences in binding explain how the drugs can function as potent and nontoxic antibacterials even though their target is common to both host and parasite (Burchall and Hitchings, 1965).

Dihydrofolate reductase has been purified to homogeneity from a variety of sources, including *Lactobacillus casei* (Pastore et al., 1974; Gundersen et al., 1972), *Streptococcus faecium* (Nixon and Blakley, 1968; D'Souza et al., 1972), T4 bacteriophage (Erickson and Mathews, 1973), and a methotrexate-resistant strain of *Escherichia coli* (Poe et al., 1972). Previously, the enzyme was partially purified in our laboratories from a strain of *Escherichia coli* resistant to 128 μg/ml of trimethoprim (Burchall, 1970). However, larger quantities of pure enzyme were needed to physically characterize the protein and its ligand complexes. In this paper, we describe the properties of dihydrofolate reductase purified to homogeneity from a strain of *E. coli* B (RT 500) resistant to 500 μg/ml of trimethoprim. The RT 500 *E. coli* produces at least 300-fold more enzyme than the wild type and tenfold more than the strain resistant to 128 μg/ml of trimethoprim. Sufficient dihydrofolate reductase was isolated for structural studies (Stone et al., 1975) and investigations of enzyme–ligand interactions (Pattishall et al., 1975).

Experimental Procedure

Materials. Folic acid was purchased from Calbiochem. Dihydrofolate was prepared by the method of Futterman (1957), as modified by Blakley (1960), and stored as a suspension in 5 m*M* HCl at −70°. NADPH was from P.L. Biochemicals; methotrexate was supplied by Nutritional Biochemicals; and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was purchased from Pierce Chemical Company. AH-Sepharose 4B, Sephadex G-50, and Sephadex G-100

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were from Pharmacia. Polyoxyethylene 20 cetyl ether (Brij 58), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, and proteins used as molecular weight markers were bought from Sigma. All other chemicals were reagent grade.

Dihydrofolate Reductase Assay. The standard enzyme assay mixture contained 50 μM dihydrofolate, 60 μM NADPH, 12 mM 2-mercaptoethanol, 50 mM potassium phosphate buffer (pH 7) or 100 mM imidazole chloride buffer (pH 7), and enzyme in a final volume of 1.0 ml at 30°. One enzyme unit is defined as the amount of enzyme required to reduce 1 μmol of dihydrofolate/min, based on a molar extinction coefficient of 12.3×10^3 (Hillcoat et al., 1967). When determining the apparent K_m of NADPH, the dihydrofolate and 2-mercaptoethanol concentrations in the assay were increased to 100 μM and 112 mM, respectively. The apparent K_m of dihydrofolate was determined in 120 μM NADPH and 112 mM 2-mercaptoethanol.

Folate Reductase Assay. The standard enzyme assay mixture contained 320 μM folic acid, 60 μM NADPH, 12 mM 2-mercaptoethanol, 100 mM succinic acid (adjusted to pH 4.5 with Tris), and enzyme in a final volume of 1.0 ml at 30°. One enzyme unit is defined as the amount of enzyme required to reduce 1 μmol of folate/min, based on the molar extinction coefficient of 18.4×10^3 (Hillcoat and Blakley, 1966). When determining the apparent K_m of folic acid, the folate concentration was varied between 2.5 and 50 μM.

Bacterial Growth. A mutant strain of *E. coli* B, originally selected for its ability to grow in 500 μg/ml of trimethoprim, was used as a source of enzyme. It is an overproducer of dihydrofolate reductase. Cells were grown in 10-l. cultures containing 150 g of Tryptic Soy Broth (Difco) supplemented with 40 g of glucose in the absence of trimethoprim. Optimal harvest time was determined by measuring the specific activity of the enzyme from cells collected at various times during the growth of the culture. Highest specific activities and total enzyme were obtained when the cells were grown to a final optical density of 2.0 at 600 nm. Usually 90 l. of culture was grown and harvested by centrifugation at 45000 rpm in a Sharpless supercentrifuge (Pennsalt Corp.) The cells were suspended in 40 mM Tris-Cl (pH 8.1), centrifuged at 16000g for 30 min at 5°, and then frozen.

Cell Lysis. A modification of the lysozyme-nonionic detergent method of Godson and Sinsheimer (1967) was used for cell lysis. All steps were conducted at 5°. The cells (650 g wet weight) obtained from a total of 380 l. of culture were pooled and suspended in 40 mM Tris-Cl (pH 8) (1.4 l. total volume). The following additions were made to the stirred suspension at 30-min intervals: (1) Na_4EDTA (260 ml, 20 mg/ml) and lysozyme (100 ml, 17 mg/ml); (2) Brij 58 (100 ml, 50 mg/ml); (3) $MgCl_2$ (36 ml, 1 M) and DNase (10 ml, 13.2 mg/ml). The suspension was centrifuged at 16000g for 30 min, and the precipitate was resuspended in 40 mM Tris-Cl (pH 8) (1.4 l. total volume). Steps 1–3 were then repeated. After the second treatment, the cells were washed with 600 ml of 40 mM Tris-Cl (pH 8) and centrifuged. All three supernatants were pooled and frozen; these constitute the crude extract.

Methotrexate Affinity Column Chromatography. Methotrexate was coupled to AH-Sepharose 4B (250 mg of methotrexate/15 g of resin) using the method supplied by Pharmacia. It was calculated that the Sepharose bound 8.5 μmol of methotrexate/ml of resin. Before applying the enzyme, the affinity column (1.5 × 30 cm) was equilibrated with 10 mM potassium phosphate buffer (pH 6). Enzyme from the ammonium sulfate step (Table I) was diluted to a protein concentration of 19 mg/ml then applied to the column at a flow rate of 8 ml/hr. The column was washed with 1.2 l. of 0.01 M potassium phosphate buffer (pH 6) then 10 l. of 0.2 M potassium phosphate buffer–1 M KCl (pH 6) at a flow rate of 15 ml/hr. When the eluent no longer contained material which absorbed at 280 nm, the dihydrofolate reductase was eluted with 0.2 M potassium phosphate, 1 M KCl, and 3 mM folic acid (pH 8). Fractions containing enzyme were pooled and concentrated by ultrafiltration (PM-10 membrane, Amicon Corp.).

Protein Determinations. Protein was measured by the method of Lowry et al. (1951) using, as a standard, crystalline bovine serum albumin dried to constant weight in a vacuum oven at 45°. The $E_{280}(1\%)$ of dihydrofolate reductase was calculated from the Lowry protein value.

Polyacrylamide Gel Electrophoresis. The method of Ornstein and Davis (1964) was used for discontinuous electrophoresis. The cylindrical separating gels (0.5 cm × 6 cm) contained acrylamide and bisacrylamide at 7.5% T and 2.5% C, using the notation of Hjertèn (1968). The stacking gel varied between 1 and 3 cm in length, depending upon volume of sample, and was made up of 3% T and 20% C. Electrophoresis was conducted at 5° with a constant power of 0.5 W/gel until the bromophenol blue marker migrated to the bottom of the gel. Enzyme bands were located either by the protein staining method of Fairbanks et al. (1971) or the enzyme activity stain described by Hiebert et al. (1972). Gels were scanned at 560 nm using the Gilford linear transport accessory.

Amino Acid Analysis. A dihydrofolate reductase sample (0.5 mg) was hydrolyzed in 6 N HCl for 21 hr at 105° in an evacuated, sealed tube, and the hydrolysate was analyzed on a Beckman Spinco Model 120B amino acid analyzer. Cysteine was determined as cysteic acid on hydrolysates of the enzyme after oxidation with performic acid for 4 hr at 0° (Hirs, 1956). The colorimetric method of Spies and Chambers (1949) employing *p*-dimethylaminobenzaldehyde was used for the determination of tryptophan. Before reaction with the reagent, the enzyme was digested by Pronase for 22 hr at 40°.

Identification of Amino Terminus. The terminal amino acid was identified by dansylation and hydrolysis of 75 nmol of the enzyme according to the method of Gray (1967). The dansylated protein was isolated by precipitation with trichloroacetic acid (250 mg), washed twice with 1 N HCl (1 ml for each wash), then suspended in 6 N HCl and hydrolyzed for 8 hr at 110°. The hydrolysate was evaporated to dryness in vacuo, and the dansylated end amino acid was extracted three times from the dry residue with water-saturated ethyl acetate (1.5 ml). After evaporating the ethyl acetate nearly to dryness, any dansyl amino acids present were identified by two-dimensional chromatography on plastic silica gel coated thin-layer sheets (Merck, Darmstadt) using the solvent systems of Spivak et al. (1971).

Ultracentrifugal Analysis. Sedimentation velocity measurements were made in a Beckman Spinco Model E analytical centrifuge using schlieren optics. Sedimentation coefficients were determined at rotor speeds of 59780 and 50740 rpm. Diffusion coefficients were calculated by height–area analysis of schlieren optic patterns obtained at a rotor speed of 10589 rpm (Schachman, 1957). All experi-

Table I: Purification of Dihydrofolate Reductase from *E. coli* RT 500.

Step	Volume (ml)	Total Protein (mg)	Total Enzyme (units)[a]	Specific Activity (units/mg)	Purification (−fold)	Yield (%)
1. Crude extract	4000	92000	49000	0.53	1	100
2. Ammonium sulfate	1250	51250	40400	0.79	1.5	82
3. Affinity chromatography	600	618	12670	20.5	38.7	(52)[b]
4. Sephadex G-100	181	381	7280	19.1	36.1	(30)[b]

[a] Enzymic activity was measured in 50 m*M* potassium phosphate buffer (pH 7). [b] Half of the enzyme from step 2 was applied to the affinity column. The volume, total protein, and total enzyme in steps 3 and 4 are those of single affinity and Sephadex G-100 columns, however, the yields have been doubled to reflect the actual recoveries observed when the second half of the enzyme is processed through these steps.

ments were carried out at 20°. Molecular weights were calculated by making use of the Svedberg equation. Data were corrected for solvent density. The partial specific volume was calculated from the amino acid composition and the partial specific volumes of the constituent amino acids.

Results

Enzyme Purification. The crude extract was fractionated at 0° between 45 and 90% saturated ammonium sulfate, and the precipitate was dissolved in 900 ml of 10 m*M* potassium phosphate (pH 6.0) then dialyzed overnight against 8 l. of the same buffer (Table I). This step resulted in a minimal loss of enzymic activity (18%) yet removed sufficient protein (40 g) to allow direct application to the affinity column. Although the methotrexate-Sepharose in the column had a calculated capacity of 7.6 g of dihydrofolate reductase (based on 1:1 stoichiometry), in practice its capacity was less, and only half of the ammonium sulfate treated enzyme was used in this preparation. After the affinity column chromatography step folate was removed from the enzyme by overnight dialysis against 0.1 *M* potassium phosphate–1 *M* KCl (pH 8) (2 l.), then the remaining folate was stripped from the enzyme by subjecting the preparation to Sephadex G-100 column chromatography (2.6 × 100 cm) at a flow rate of 12 ml/hr. In addition to removing folate, the Sephadex G-100 step also separated the approximately 18000 mol wt dihydrofolate reductase from a group of higher molecular weight proteins which had also bound to the methotrexate affinity column. The pooled enzyme was dialyzed against 10 m*M* potassium phosphate buffer (pH 7.2) and stored at −10°.

The ultraviolet absorption spectrum of the purified enzyme has a maximum at 282 nm and a sharp shoulder at 290 nm. The $E_{280}(1\%)$ of the dihydrofolate reductase is 24.6. Also, the low absorbance of the enzyme between 320 and 360 nm indicates the preparation is relatively free of ligands absorbing in this region (NADPH, dihydrofolate, or folate). The concentration of active dihydrofolate reductase was also determined by fluorescent methotrexate titrations (Perkins and Bertino, 1966). Assuming a 1:1 stoichiometry between the enzyme and methotrexate and an enzyme molecular weight of 18000, the results agree within 90% of the Lowry protein measured with bovine serum albumin as a standard.

At protein concentrations of 1–2 mg/ml in potassium phosphate buffer, the enzyme is stable to repeated freezing and thawing, but freezing in the presence of sulfhydryl reagents (e.g., 0.1 m*M* dithiothreitol) results in protein precipitation. The enzyme can also be stored refrigerated in the presence or absence of sulfhydryl reagents for several weeks without significant loss of activity. During the purification,

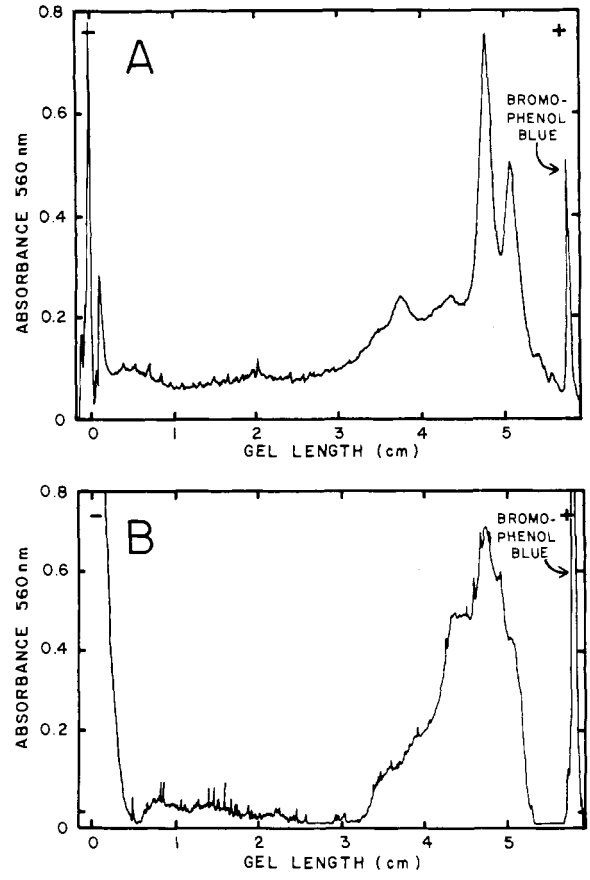

FIGURE 1: Polyacrylamide gel electrophoresis. (A) The absorption scan of an analytical gel run with 10 μg of dihydrofolate reductase then stained for protein. (B) The absorption scan of an analytical gel run with 0.4 μg of protein then stained for enzymic activity. In both cases, the origin is on the left and the position of the bromophenol blue marker is indicated near the anode.

enzymic activity was routinely measured in 50 m*M* potassium phosphate (pH 7.0); however, highest reaction rates were found in 100 m*M* imidazolium chloride buffer (pH 7.0) (specific activity, 47 μmol of dihydrofolate reduced per min per mg). This corresponds to a turnover number of 850 mol of dihydrofolate reduced per min per mol of enzyme.

Multiple Forms of the Enzyme. Electrophoresis of the purified dihydrofolate reductase on polyacrylamide gels resulted in multiple protein staining bands (Figure 1A). Since each protein band corresponds to an area of enzymic staining (Figure 1B), all are dihydrofolate reductase. This conclusion is supported by the results of preparative gel electrophoresis. As the enzyme migrated off the end of the preparative gel, fractions were collected and monitored for both

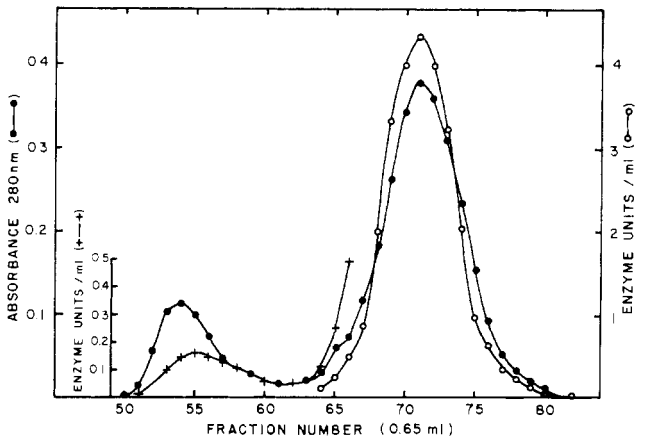

FIGURE 2: Sephadex G-50 gel filtration of dihydrofolate reductase. Enzyme (2.5 mg in 1 ml) from step 4 of the purification scheme was applied to a 1 × 100 cm column of Sephadex G-50 equilibrated with 20 m*M* potassium phosphate buffer (pH 7) and eluated with the same buffer at a flow rate of 6 ml/hr. Fractions containing 0.65 ml were collected and assayed for both enzymic activity and protein. Note that the activity of fractions 50–66 is plotted on an expanded scale.

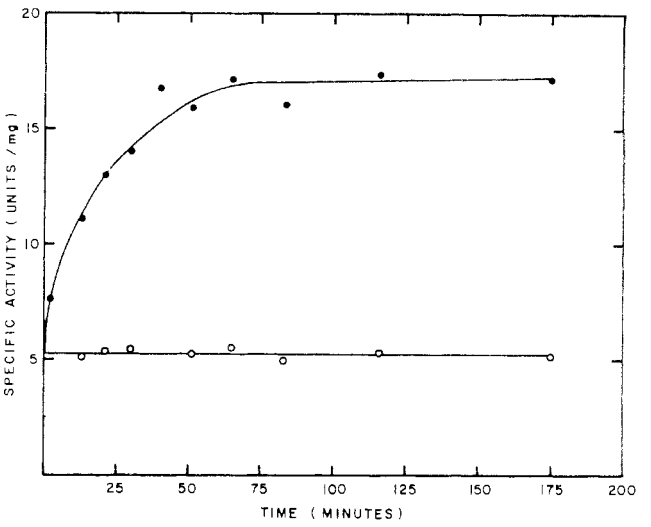

FIGURE 3: Activation of the void volume enzyme by dithiothreitol. Purified dihydrofolate reductase was filtered through a Sephadex G-50 column as in Figure 5, and the void volume enzyme was pooled and divided into two portions. Either 0.075 ml of 100 m*M* dithiothreitol in 20 m*M* potassium phosphate buffer (pH 7) (●—●) or 20 m*M* potassium phosphate buffer (pH 7) (○—○) was added to 0.675 ml of enzyme (87 μg/ml). Aliquots were removed at the indicated time intervals and assayed for enzymic activity in 100 m*M* imidazolium chloride (pH 7.0).

protein and enzymic activity. Every protein containing fraction also demonstrated dihydrofolate reductase activity (D. Baccanari and D. Averett, unpublished). In preparing these large quantities of reductase, extensive washing of the affinity column was needed to remove nonspecifically bound proteins, and it is possible that the multiple forms were generated during this step. However, when smaller amounts of the enzyme were purified much more rapidly (7 days vs. more than 30 days) multiplicity was still evident upon electrophoresis and is therefore not an artifact of the longer purification procedure. Experiments in which the enzyme was preincubated with either folate, dihydrofolate, $NADP^+$, or NADPH before electrophoresis indicate that the multiplicity is not due to enzyme–ligand complexes. However, preincubation of the enzyme with NADPH did result in the appearance of new rapidly migrating protein bands similar to that previously described for the reductase–NADPH binary complex (Gundersen et al., 1972).

Multiple molecular weight forms of dihydrofolate reductase are also observed. Dodecyl sulfate gel electrophoresis of the purified enzyme using the system described by Smith and Bernstein (1973), results in two protein staining bands. The major species (approximately 90% of the total protein) has a molecular weight of 18300, as determined by comparing the relative mobility to that of standard protein markers; the minor species has a molecular weight of 16500. Both of these enzyme forms are observed with several preparations of the enzyme; they have not been resolved by Sephadex gel filtration or sedimentation velocity studies.

Aggregated Form of the Enzyme. When the purified enzyme was rechromatographed on Sephadex (G-50), a high molecular weight form, which comprised about 20% of the total protein recovered, was eluted in the void volume of the column (Figure 2). The aggregate has a low specific activity (5 μmol of dihydrofolate reduced per mg per min) and is inhibited by methotrexate. Since the enzyme had been previously fractionated by Sephadex column chromatography during purification, the aggregate was formed from the monomer sometime during the storage. An indication of the time course of aggregation is given by rechromatography of the peak tubes of the monomeric species (fractions 71 and 72, Figure 2). Within 17 hr at 5°, approximately 5–10% of the monomer was converted into the aggregate (data not shown). The degree of enzyme aggregation is not a function of buffer ionic strength, since patterns similar to Figure 2 are observed with columns equilibrated and eluted with 0.1 *M* potassium phosphate–1 *M* KCl (pH 7). However, since the aggregate is not detected by dodecyl sulfate gel electrophoresis, after the enzyme is incubated with mercaptoethanol, it appeared that sulfhydryl groups may be involved in the aggregation process. When the enzyme preparation which contains aggregates was incubated with 10 m*M* dithiothreitol for 2 hr at room temperature and filtered through a Sephadex G-50 column equilibrated with 1 m*M* dithiothreitol (20 m*M* potassium phosphate (pH 7)) a single peak of 280-nm absorption and enzymic activity corresponding to the monomeric species was observed. Since the specific activity of the aggregate is only about $1/10$ that of the monomers, it was of interest to determine whether or not incubation of the aggregate with dithiothreitol resulted in an increase in specific activity as well as the formation of the monomeric species. Figure 3 shows that incubation of the isolated aggregate at 23° in 10 m*M* dithiothreitol resulted in a maximal 3.5-fold activation within 2 hr. The activity of the enzyme without dithiothreitol remained constant. Therefore, as the aggregate is converted to the monomer, there is a concomitant increase in enzymic activity, though not to the level usually observed with the monomeric species (i.e., 47 μmol per min per mg).

Sedimentation Velocity. In the sedimentation velocity experiments, only a single symmetrical band of protein was observed. The sedimentation coefficient ($s_{20,w}$) was calculated to be 2.14 S with a diffusion coefficient ($D_{20,w}$) of 11.05. Concentration dependence was not detected when the protein concentration was varied between 2.5 and 7.5 mg/ml. The partial specific volume of the enzyme was calculated to be 0.73 ml/g. By substituting these values into the Svedberg equation, an apparent molecular weight of 17470 was found. Under the conditions employed, the 16500 mol wt species (observed on dodecyl sulfate gels) could not be detected, and no attempts were made to ana-

Table II: A Comparison of the Amino Acid Composition of RT 500 with MB 1428 Dihydrofolate Reductase.

Residue	RT 500 Suggested Content	MB 1428 Content[a]
Lys	6	7
His	5	5
Arg	9	9
Asp	19	18
Thr	6	6
Ser	8	8
Glu	19	15
Pro	10	10
Gly	11	10
Ala	13	13
½-Cys	2	2
Val	11	11
Met	5	5
Ile	11	11
Leu	11	11
Tyr	3	4
Phe	6	6
Try	5	5
	Total 160	156

[a] Taken from Bennett (1974).

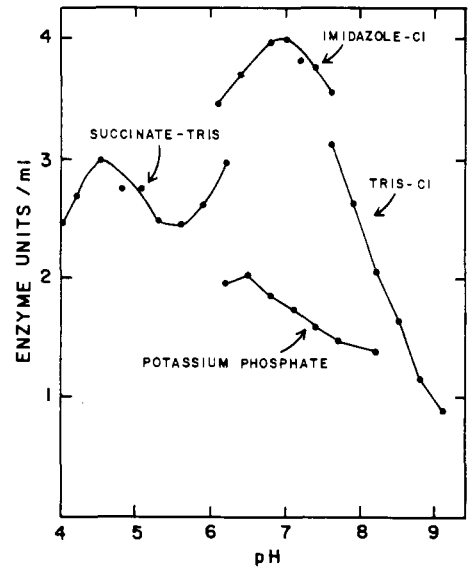

FIGURE 4: The pH optimum of dihydrofolate reductase. The purified enzyme was assayed at 30° in 100 m*M* succinate (titrated with Tris base), 100 m*M* imidazole chloride, 100 m*M* Tris-Cl, or 50 m*M* potassium phosphate buffers at various pH values. Each assay contained 50 μM dihydrofolate, 12 m*M* 2-mercaptoethanol, and 60 μM NADPH in a final volume of 1.0 ml. Corrections were made for nonenzymatic changes in absorbance at 340 nm at acidic pH.

lyze the aggregated form of the enzyme by sedimentation velocity studies.

Amino Acid Content. The amino acid composition of *E. coli* RT 500 dihydrofolate reductase is shown in Table II. The enzyme has a total of 38 aspartate and glutamate residues per mole, and the acidic isoelectric point of the protein, p*I* = 4.6 (D. Baccanari, unpublished), indicates that the majority of these residues are in the form of free acids. The molecular weight of the enzyme based on its amino acid composition is 17680.

Table II also shows a comparison of the *E. coli* RT 500 enzyme with the amino acid composition of dihydrofolate reductase from the methotrexate resistant strain of *E. coli* (MB 1428) isolated by Poe et al. (1972). The similar amino acid contents of the *E. coli* RT 500 enzyme and the *E. coli* MB 1428 enzyme is interesting considering that the amino acid compositions of dihydrofolate reductase from two methotrexate-resistant strains of *S. faecalis* differ, most noticeably in cysteine content (D'Souza et al., 1972; Gleisner et al., 1974). One might anticipate that the homology between the enzymes from two strains of *S. faecalis* both resistant to the same inhibitor, methotrexate, would be greater than the homology between two strains of *E. coli* resistant to different inhibitors, either methotrexate or trimethoprim.

The N-terminal amino acid analysis of the enzyme from *E. coli* RT 500 dihydrofolate reductase gave rise to dansyl methionine. No other dansyl amino acids were detected.

pH Optimum. The activity of dihydrofolate reductase depends upon the buffer used in the enzyme assay. At a given pH, assays in buffers containing large organic cations, such as Tris-Cl, show higher rates than those in buffers containing smaller inorganic cations, such as potassium phosphate (e.g., Figure 4). This effect is an inhibition by potassium phosphate rather than an activation by Tris-Cl since, at a fixed pH, enzymic activity decreases as the concentration of potassium phosphate buffer increases yet remains relatively constant as the concentration of Tris-Cl increases (data not shown). In Figure 4, the pH–activity profile of the enzyme

Table III: K_m Values for Dihydrofolate Reductase Substrates in 100 m*M* Imidazolium Chloride (pH 7.0).

Substrate	Apparent K_m (μM) Without $BaCl_2$	Apparent K_m (μM) 300 μM $BaCl_2$	Relative V_{max} Without $BaCl_2$	Relative V_{max} 300 μM $BaCl_2$
Dihydrofolate	3.2 ± 0.3	2.1 ± 0.15	1	0.67
NADPH	6.8 ± 0.4	6.2 ± 0.6	1	0.64
NADH	268 ± 22		0.21	
Folate	16		0.001	

is shown for a variety of buffers. The pH optimum is pH 6.5 in 50 m*M* potassium phosphate buffer. In 100 m*M* imidazolium chloride, the pH optimum is about pH 7.0, and the enzyme activity is 2.3-fold higher than in potassium phosphate at the same pH. Another minor pH optimum is observed at pH 4.5 in succinate-Tris buffer; this second pH optimum is also seen with citrate-Tris, sodium citrate, and sodium acetate buffers.

The *E. coli* dihydrofolate reductase can also use folate as a substrate with a single pH optimum at about pH 4.5. However, in the folate assay, 750-fold more enzyme is needed to obtain a reaction rate (change in absorbance at 340 nm per min) equal to that with dihydrofolate as a substrate. Since the apparent K_m for folic acid was found to be 16 μM (Table III), this low reaction rate with folate represents a slow catalysis rather than a poor affinity of the enzyme for folate.

Cation Inhibition. Since potassium phosphate inhibits the enzyme assay, the effects of the group IA and IIA cations on dihydrofolate reductase activity were studied. Figure 5 shows that the inhibition of the enzyme assay by KCl or NaCl is nonlinear and the effect becomes saturating. Also, at any given concentration, KCl is a better inhibitor than NaCl. Both of these observations indicate that the cation inhibition is not due to some general phenomenon such as protein denaturation at high ionic strength. In support of

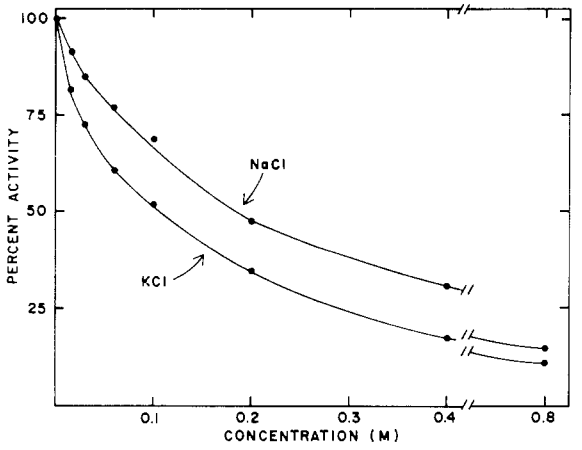

FIGURE 5: Inhibition of dihydrofolate reductase by NaCl and KCl. The enzyme was assayed in 100 m*M* imidazole chloride (pH 7) containing the indicated concentrations of NaCl or KCl. The buffer, salt, and NADPH were equilibrated at 30°, then enzyme was added and the reaction was started with dihydrofolate.

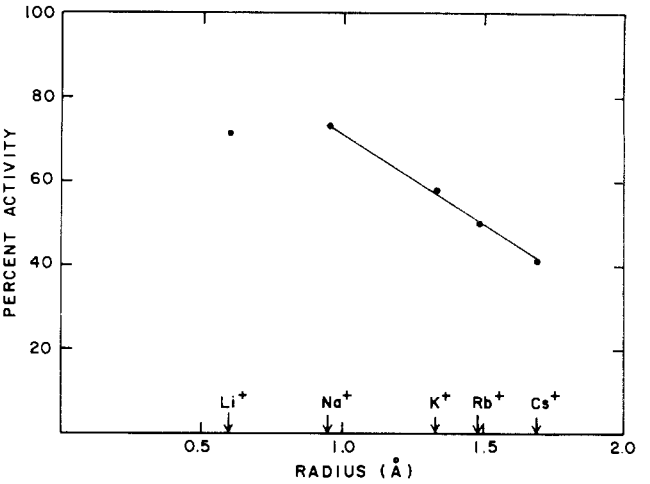

FIGURE 6: Effect of monovalent cations on dihydrofolate reductase activity. The enzyme was assayed in 100 m*M* imidazole chloride (pH 7) containing 100 m*M* LiCl, NaCl, KCl, RbCl, or CsCl. The buffer, salt, and NADPH were equilibrated at 30°, then enzyme was added, and the reaction was started with dihydrofolate. The reaction velocity in the presence of salt was compared to the control, and percent activity was plotted as a function of the crystalline ionic radius of the cation.

this, it was found that the inhibition by KCl occurred rapidly and did not change if the enzyme was preincubated with the cation for various lengths of time. Also the KCl effect was immediately reversed upon dilution.

When the effects of other monovalent cations were examined, the correlations illustrated in Figure 6 were observed. The enzyme was assayed in 50 m*M* imidazolium chloride (pH 7) containing 100 m*M* NaCl, KCl, RbCl, or CsCl, and the percent activity of each assay system (with the assay in the absence of added salt being 100%) was plotted against the crystalline atomic radius of the cation. For Na^+, K^+, Rb^+, and Cs^+, the inhibition increased as a linear function of the cation radius.

The cation survey was extended to some of the divalent ions. The effects with $MgCl_2$, $CaCl_2$, or $BaCl_2$ were also saturating, but the inhibitions occurred at lower concentrations than the monovalent cation effects. About 30 m*M* $MgCl_2$ or 15 m*M* $CaCl_2$ was needed to inhibit the reaction 50%. $BaCl_2$ was the most effective inhibitor of all the cations tested. In the enzyme assay, only 250 μM $BaCl_2$ was needed for 50% inhibition, making it 800-fold more potent than NaCl. As with KCl, the inhibition by $BaCl_2$ occurred instantaneously and was immediately reversed upon dilution. The addition of a twofold excess of EDTA to an enzyme assay already inhibited by $BaCl_2$ results in an immediate reversal of the inhibition.

Since the effect of $BaCl_2$ is not due to denaturation of the enzyme, the cation must alter V_{max}, K_m, or both. The apparent K_m values of NADPH and dihydrofolate were measured with and without added 300 μM $BaCl_2$ (Table III). It can be seen that this level of $BaCl_2$ does not increase the K_m value for either substrate yet decreases the calculated V_{max} about 35%. Table III also shows that NADH is a substrate for *E. coli* dihydrofolate reductase with a 40-fold higher K_m and a fivefold lower V_{max} than the reaction with NADPH.

Anions neither inhibit nor activate the enzyme. Activities were essentially identical in Tris buffer (pH 7.3) with phosphate, fluoride, chloride, bromide, iodide, or acetate (at a concentration of 0.1 *M*) as the anion. Urea, up to 1.5 *M*, had no effect on the enzyme; assays performed in 3 *M* urea showed a 15% loss of activity. At concentrations of 12 m*M* in the enzyme assay, the organic diamino cations spermine, spermidine, ethylenediamine, diaminobutane, and diaminopentane inhibited 60, 54, 48, 28, and 28%, respectively.

Discussion

E. coli RT 500 is a mutant selected for its ability to grow in the presence of 500 μg/ml of trimethoprim. Its presumed mechanism of resistance is the synthesis of large amounts of dihydrofolate reductase. Whenever such a mutant is isolated, the question arises as to whether or not the enzyme from the mutant is identical with the wild type. Erickson and Mathews (1973) have purified small amounts of the wild type *E. coli* enzyme to homogeneity and reported a molecular weight and amino acid content different than the enzyme from *E. coli* RT 500, though similar salt inhibitions were observed with both enzymes. An additional criterion for comparing the two enzymes is a study of their enzyme inhibitor profiles. When the small molecule inhibitors used with the wild type enzyme (Burchall and Hitchings, 1965) were tested with the enzyme from *E. coli* RT 500 identical inhibitor profiles were observed (J. Burchall, unpublished), indicating the functional similarities of the two enzymes.

A comparison of the *E. coli* RT 500 enzyme with that obtained from a methotrexate resistant *E. coli* MB 1428 (Poe et al., 1972; Greenfield, 1974) shows both enzymes have similar molecular weights, K_m values for NADPH, NADH, and dihydrofolate, and can reduce folate to the same extent at pH 4.5. A comparison of their pH–activity profile, amino acid content, and sequence (Stone et al., 1975) indicates that both are similar but not identical.

The effects of cations on *E. coli* RT 500 dihydrofolate reductase and those reported for several mammalian enzymes (Greenberg et al., 1966; Reyes and Huennekens, 1967) differ both qualitatively (in the direction of the response) and quantitatively (in the concentration needed to elicit a response). Divalent cations inhibit the *E. coli* RT 500 enzyme at low concentrations (200–300 μM for $BaCl_2$) as opposed to the 200 m*M* $BaCl_2$ needed for maximal activation of the L1210 enzyme (Reyes and Huennekens, 1967). Also the inhibition of the *E. coli* RT 500 dihydrofolate reductase is a function of the radius of monovalent cations; a simple correlation between size of the cation and response is not evident for the mammalian enzyme (Greenberg et al., 1966; Reyes

and Huennekens, 1967). The *E. coli* RT 500 enzyme is not activated by urea, or anions. The effect of $BaCl_2$ on the *E. coli* dihydrofolate reductase is interesting in another respect. The radius of Ba^{2+} (1.35 Å) is intermediate between K^+ (1.33 Å) and Rb^+ (1.48 Å), yet Ba^{2+} inhibits at about a 500-fold lower concentration than either. Thus, charge of the cation must also play a role in the mechanism of inhibition. Inhibitory concentrations of $BaCl_2$ do not change the fluorescence or ultraviolet absorption spectra of the enzyme and only had a slight effect on the circular dichroism (B. Kitchell, personal communication). This, combined with the low concentration of cation needed and the immediate reversal by dilution or EDTA, indicates the conformation of the enzyme is not greatly altered and denaturation is not the mechanism of inhibition. It is possible that Ba^{2+} (and to a lesser extent the other cations) is of the proper size and charge to bind to the enzyme in such a manner that the transfer of hydride from NADPH to dihydrofolate is partially blocked (lowering V_{max}) without grossly altering the binding (K_m) of either ligand.

Several different types of multiplicity have been observed with the *E. coli* RT 500 enzyme: multiple forms on polyacrylamide gel electrophoresis; an aggregated enzyme; and a 16500 mol wt species. Although this latter form has been separated from the 18300 mol wt enzyme only under the denaturing conditions of dodecyl sulfate and urea, all available evidence indicates that it is not a contaminating nonreductase protein. It is possible that this low molecular weight form may have been generated from the 18300 mol wt species by limited proteolysis, either intracellularly or during the initial stages of purification, yet has retained sufficient substrate and/or inhibitor affinity to bind to the methotrexate-Sepharose column.

High molecular weight forms of dihydrofolate reductases from several species can occur either alone (Ferone et al., 1969) or in combination with lower molecular weight enzymes (Gutteridge et al., 1970; Sirotnak, 1973). However, a process similar to the conversion of the *E. coli* RT 500 aggregate to the monomeric species and its concominent activation by disulfide reducing agents has not been observed with other dihydrofolate reductases. The implication that enzyme sulfhydryl groups are involved in the aggregation is not without its drawbacks. Upon Sephadex G-200 gel filtration of the isolated aggregate, the major protein peak elutes in the void volume of the column (D. Baccanari, unpublished). Assuming the elution volume of the aggregate is a function of its true molecular weight rather than an anomalous shape and if disulfide bonds formed the only cross-links between molecules, then the aggregate would have to be composed of at least 28 monomers.

The major forms of *E. coli* RT 500 dihydrofolate reductase have recently been isolated by preparative gel electrophoresis and work is now in progress on further characterizing all of the various multiple forms of the enzyme.

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