T^4 BACTERIOPHAGE-SPECIFIC DIHYDROFOLATE REDUCTASE: PURIFICATION TO HOMOGENEITY BY AFFINITY CHROMATOGRAPHY[1]

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SUMMARY

Dihydrofolate reductase specified by bacteriophage T^4 has been purified 6,000-fold by a simple affinity chromatographic method employing N^{10}-formylaminopterin (4-amino-10-formylpteroylglutamate), bound by peptide linkage to the solid support aminoethyl Bio-Gel P-150. About 4.5 mg of enzyme (80% recovery) can be obtained from 500 g of infected cells. The product is homogeneous in two different electrophoresis systems and shows a molecular weight of 29,000 as determined by sodium dodecyl sulfate gel electrophoresis. Inhibitor studies show a single binding site per enzyme molecule for folic acid analogues.

Dihydrofolate reductase activity is abundant in cultures of Escherichia coli infected by T-even bacteriophages or T5 (1,2,3), due to synthesis of a virus-coded enzyme in infected cells. We have been interested in the molecular properties of this enzyme for several years, since it provides a route to understanding the interaction between chemotherapeutically active folate analogs and their biochemical site of action, namely, dihydrofolate reductase. An additional reason for our interest in phage-coded dihydrofolate reductase relates to its role as a structural element in the tail plates of T-even phages (4). Productive research on both of the above problems demands a convenient method for preparation of the virus-specific enzyme in homogeneous form. This paper describes just such a method.

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MATERIALS AND METHODS DEAE-cellulose and Bio-Gel P-150 were obtained from BioRad. Methotrexate (4-amino-10-methylpteroylglutamate) was the gift of Dr. J. M. Smith, Jr., of Lederle. Aminopterin (4-aminopteroylglutamate) was purchased from Nutritional Biochemical Co. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride was purchased from Ott Chemical Co., Muskegon, Michigan. NADPH, streptomycin sulfate, sodium dodecyl sulfate, folic acid and standard proteins were obtained from Sigma. Dihydrofolate was prepared by the method of Futterman as modified by Friedkin et al. (5) and stored in sealed ampoules as a dry powder under nitrogen. The aminoethyl derivative of Bio-Gel P-150 was prepared as described by Inman and Dintzis (6), by the direct reaction between Bio-Gel P-150 and ethylenediamine. The heating period was 3.5 hours. After the final wash the material was stored in distilled water at 4°. The preparation of N^{10}-formylaminopterin and its linkage to the aminoethyl Bio-Gel P-150 are described in Fig. 1. Electrophoresis chemicals were from Canalco.

The assay for dihydrofolate reductase has been described (10). A unit is defined as that amount of protein which will reduce one μmole of dihydrofolate per minute under the conditions of the assay. A molar absorbancy change of 12,000 $M^{-1}cm^{-1}$ at 340 mμ was used for calculation of units (8). Protein concentrations were determined by the biuret reaction for impure fractions or by the microbiuret reaction of Goa (11) for pure fractions.

The production of large amounts of T-even phage-infected E. coli cells has been described (12). In the present case T^4amN82 was grown productively on E. coli CR63. Infected cells of E. coli B were used as an enzyme source. The purification procedure through the streptomycin sulfate step was as previously described (3) except that a 20% cell suspension was prepared and the crude extract was treated with an 8% streptomycin sulfate solution instead of 5%. The details of the present purification are presented under Table I. The data is for 500 g of infected cells. Operations were performed at 0-4° except as noted. The affinity material has been reused several times without change in its performance.

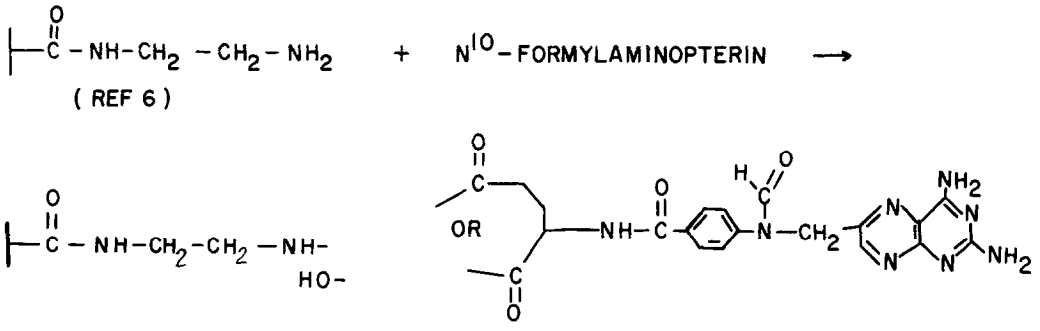


FIGURE 1. N^{10}-formylaminopterin was synthesized by a method analogous to the synthesis of N^{10}-formylfolic acid (7). Two hundred mg of aminopterin, purified as described by Mathews and Huennekens (8), was dissolved in a reagent composed of 20 ml of 98% formic acid and 5 ml of acetic anhydride and heated at 100° for one hour. Volatile reactants were removed in vacuo and the product was dissolved in 10 ml of 50% aqueous dimethylformamide. One to two ml of 3% $NaHCO_3$ may be required to complete the solution process. This solution was used directly in the coupling reaction. The conditions used in the coupling reaction are essentially those described by Cuatrecasas for coupling 3-O-succinylestradiol to aminoethyl Sepharose (9). Excess water was drained from the aminoethyl Bio-Gel P-150 by means of a filter funnel. About 50 ml of this slurry was transferred to a 250 ml TPX beaker (Nalge Co.). 30 ml of dimethylformamide was added, followed by the addition of about 50 mg of N^{10}-formylaminopterin, (2.5 to 3 ml). The pH was lowered to 4.7 with concentrated HCl while the material was stirred gently. Five hundred mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride in 5 ml of water was added over a 5-minute period. The reaction was allowed to proceed for about 18-24 hours at room temperature with gentle stirring. The material was then transferred to a column, 6 x 15 cm, and washed with 100 ml of 50% aqueous dimethylformamide, followed by about 5 liters of 0.5 M NaCl dissolved in 1% $NaHCO_3$ over a period of 3 days. It is important that all glass coming in contact with the material be previously treated with Siliclad (6).

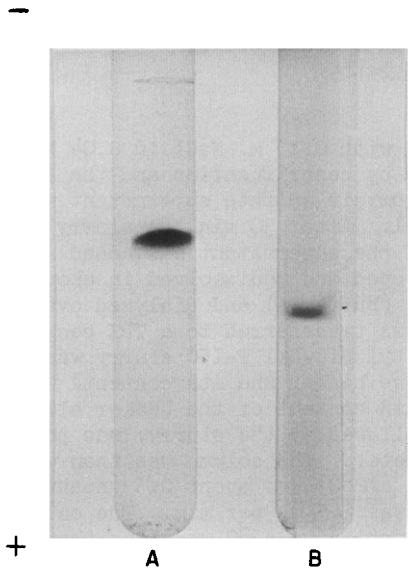
RESULTS AND DISCUSSION The DEAE-cellulose extraction removes substances which can seriously interfere with the binding of enzyme to the affinity material; therefore, the step cannot be bypassed. During the washing of the column no detectable activity is found until the pH is shifted to 8.0. The amount of enzyme lost in the pH 8.0 wash is less than 10% of the activity applied, however. The overall recovery of enzyme is about 80%; 500 g of infected cells yield about 4.5 mg of dihydrofolate reductase. As is shown in Table I, the overall purification is about 6,000-fold, of which 3,000-fold is accomplished by affinity chromatography. The purity of the preparation has been examined by electrophoresis of the native enzyme on the polyacrylamide system of Davis (13)

TABLE I
Purification of T4 Dihydrofolate Reductase by
Affinity Chromatography

70 g of DEAE-cellulose was equilibrated with 0.15 M. NaCl in 0.04 M Tris-HCl, pH 7.0. The slurry was sedimented by centrifugation and the DEAE-cellulose pellet was stirred into the streptomycin sulfate supernatant which had previously been brought to 0.15 M in NaCl. After 30 min the slurry was again removed by centrifugation. To each liter of the supernatant was added 270 g of ammonium sulfate. The precipitate was collected and redissolved in about 300 ml of 0.04 M potassium phosphate buffer, pH 7.0 (buffer A) and dialyzed overnight against 10 liters of buffer A. The sample was transferred to a TPX beaker, about 40 ml of N^{10}-formylaminopterin aminoethyl Bio-Gel P-150 slurry was added and the mixture stirred gently overnight. The beaker and its contents were then brought to room temperature and the solid content of the beaker allowed to settle. After settling, the solution, followed by the slurry, was poured into a column (stopcock open) 2.8 cm in diameter. The column was then washed with buffer A until the absorbance at 280 mμ ($\underline{A280}$) was about 0.5 (about 300 ml). The flow rate of this and subsequent washes was 2.5 ml per min. The column was then washed with 0.4 M NaCl in buffer A until the A280 was less than 0.05 (about 100 ml). Then 20 ml of 0.4 M NaCl in 0.4 M potassium phosphate, pH 8.0, was applied to the column. This was followed by 0.20 M NaCl in 0.04 M potassium phosphate, pH 8.0, until the A280 was essentially zero (about 60 ml). The enzyme was eluted from the column by applying 0.2 mM dihydrofolate in 0.2 M NaCl, 0.04 M potassium phosphate, pH 8.0, 1% 2-mercaptoethanol. The appearance of the enzyme in the effluent was signaled by a steep rise in the A280. The enzyme can be conveniently collected in a single 125 ml flask by collecting about 40 ml of effluent immediately upon the rise in A280 and then assaying the effluent and collecting it until the activity has dropped to a low level. The sample was concentrated to a few ml by means of dialysis against solid sucrose and then passed through a Sephadex G-25 column, 2.2 x 30 cm., equilibrated with buffer to remove dihydrofolate. After use, the affinity material was washed overnight with five liters of 1.0 M NaCl in buffer A and brought to pH 9.0 with NaOH. The material was then washed with buffer A containing 0.02% sodium azide and transferred to a cold room for storage at 0-4°.

Purification step	Volume	Total Activity	Total Protein	Specific Activity
	ml	units	mg	units/mg
Crude extract	1,900	835	39,000	.024
Streptomycin	2,200	835	39,000	.024
DEAE-cellulose	2,100	815	31,000	.026
0-43% $(NH_4)_2SO_4$	280	750	15,000	.050
Affinity Chromatography	50	660	4.5	145

and of enzyme treated with sodium dodecyl sulfate (SDS), by the method of Weber and Osborn (14). As is shown in Fig. 2, only a single band is observed. The electrophoretic mobility of the SDS-treated enzyme in the presence of the marker proteins bovine serum albumin, ovalbumin, carboxypeptidase A, and α-chymotrypsinogen gives a molecular weight of 29,000, ± 1500. Since this value is in good agreement with previous results obtained with the native



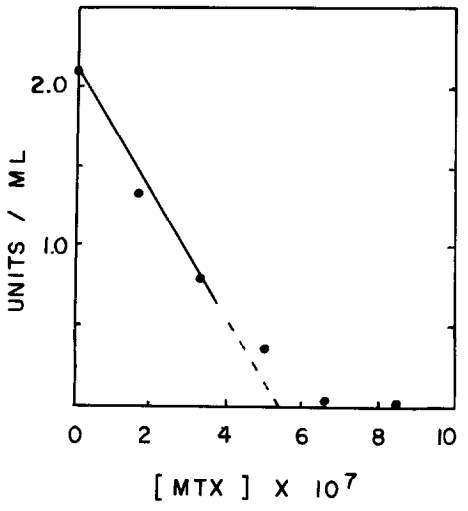


FIGURE 2. (Left) Analytical gel electrophoresis of T4 dihydrofolate reductase purified by affinity chromatography. Gel A (20 μg of protein) was electrophoresed on gels and buffers after Davis (13) except that both separating and stacking gels contained riboflavin and were photopolymerized, and the separating gel contained 0.005% 2-mercaptoethanol. Electrophoresis was at 3 ma per gel and was performed in the cold. The sample contained 20% sucrose and was layered onto the surface of the stacking gel. Gel B, (20 μg) was the same as for gel A except that the sample was pretreated with SDS and electrophoresed as described by Weber and Osborn (14).

FIGURE 3. (Right) Titration of the activity of dihydrofolate reductase with methotrexate (MTX). 45μg of homogeneous T4 dihydrofolate reductase in 3 ml of 0.04 M potassium phosphate buffer, pH 7.0 (corresponding to a molar concentration of 5.17×10^{-7} M) was titrated with a solution of MTX, 5×10^{-5} M. At the indicated points 5 μl of enzyme was removed and assayed for activity. The data are not corrected for the small volume changes due to additions and withdrawals. This error is less than 3%. An extrapolation of the linear portion of the curve intersects the abscissa at an MTX concentration of 5.2×10^{-7} M, indicating the presence of only one binding site per molecule for this inhibitor.

enzyme (10) the presence of subunits appears to be excluded.

The specific activity of 145 units per mg of homogeneous enzyme can readily be translated into a turnover number of about 4,350. Since this is one of the highest turnover numbers reported for dihydrofolate reductase (15), it seemed possible that the enzyme possessed more than one catalytic site per molecule. This would imply the existence of more than one binding site for dihydrofolate per molecule. In order to determine the number of binding sites per

molecule, the enzymatic activity was titrated with the stoichiometric inhibitor (16) methotrexate. As can be ascertained from Fig. 3, the data indicate the presence of but one binding site per molecule.

Attempts to purify T4 dihydrofolate reductase on columns of methotrexate covalently linked to Bio-Gel P-150 were unsuccessful because the enzyme, once bound, could not be eluted[3]. Apparently, the linkage of methotrexate to the insoluble polyacrylamide through a carboxyl group on the glutamate moiety does not interfere with the stoichiometric and irreversible characteristics of this inhibition. N^{10}-formylaminopterin, while still a potent inhibitor (Ki - 10^{-7} M), is readily dissociable upon treatment with dihydrofolate. In the absence of dihydrofolate the enzyme can still be eluted from the affinity material described here but the elution is very inefficient. It is this property that permits the washing away of inactive protein which binds nonspecifically to the affinity material.

The method described here can potentially be extended in two interesting directions: (1) since it takes advantage of a universal property of dihydrofolate reductases, namely the ability to bind 4-amino folate analogs, it is, in principle, capable of application to the purification of this enzyme from many biological sources; (2) the method can, in principle, be used for purifying mutationally altered, catalytically inactive dihydrofolate reductase molecules, so long as they retain the ability to bind pteridines. Since such mutants can be easily prepared (10,17), a powerful approach is at hand for probing the relationship between protein structure and biological activity in this important enzyme.

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