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Purification and Properties of Dihydrofolate Reductase from an Amethopterin-Resistant Strain of *Streptococcus faecium*†

L. D'Souza, P. E. Warwick,‡ and J. H. Freisheim*

ABSTRACT: Dihydrofolate reductase from an amethopterin-resistant strain of *Streptococcus faecium* has been isolated and purified (*ca.* 360-fold) employing a procedure that involves sonication of intact cells, filtration through Bio-Gel P-150, and chromatography on hydroxylapatite and DEAE-Sephadex. Two forms of the enzyme (I and II) were separated following hydroxylapatite chromatography. Rechromatography of form II enzyme on hydroxylapatite resulted in the conversion of II to I as judged by electrophoretic analysis. Form I enzyme was isolated in *ca.* a 40% overall yield following DEAE-Sephadex chromatography. At pH 6.5, the turnover number was 6000 moles of dihydrofolate reduced min^{-1} $mole^{-1}$ of enzyme at 25°. The enzyme showed a single protein band following polyacrylamide gel electrophoresis and was monodisperse in the ultracentrifuge ($s_{20,w}$ = 2.10 S). The reductase had a molecular weight of *ca.* 20,000 as judged by sedimentation equilibrium analysis, gel filtration, and amethopterin titration. Amino acid analyses indicated the presence of one cysteine residue and nine tryptophan residues per mole of protein. The circular dichroic absorption spectrum of the enzyme revealed the presence of an aromatic side-chain Cotton effect consisting of three distinct ellipticity bands in the 250- to 310-nm region.

Dihydrofolate reductase (EC 1.5.1.3), which catalyzes the TPNH-dependent reduction of dihydrofolate to tetrahydrofolate, has been obtained in essentially pure form from chicken liver (Kaufman and Gardiner, 1966; Kaufman and Pierce, 1971; Huennekens *et al.*, 1970), L-1210 lymphoma (Perkins *et al*,. 1967), *Streptococcus faecium* (Nixon and Blakley, 1968), and *Lactobacillus casei* (Gundersen *et al.*, 1972) among others. This enzyme appears to be the main, if not the only, target site for the 4-amino-4-deoxy analogs of folic acid.

Elevated levels of dihydrofolate reductase have been reported for certain cell types exhibiting amethopterin resistance as compared to that observed in the corresponding sensitive parent strain. This phenomenon has been observed in amethopterin-resistant strains of various bacteria (Freisheim *et al.*, 1972; Crusberg *et al.*, 1970; Dunlap *et al.*, 1971; Nixon and Blakley, 1968; Sirotnak and Salser, 1971) as well as in certain mammalian (Hakala, 1965) and tumor cells (Sartorelli *et al.*, 1964; Friedkin *et al.*, 1962).

The results of this study describe the purification and certain molecular properties of dihydrofolate reductase from an amethopterin-resistant strain of *S. faecium*. A comparison of certain properties of the enzyme isolated in the present study to those of the dihydrofolate reductases isolated by Nixon and Blakley (1968) suggest that different strains of amethopterin-resistant *S. faecium* were employed.

Materials and Methods

Materials. The amethopterin-resistant strain of *S. faecium* (ATCC 8043) was a gift from Dr. Carl Smith, University of Cincinnati. The characterization of this strain in terms of specific growth requirements is being done by Dr. Smith and coworkers. Dihydrofolate was prepared from folic acid (Calbiochem) by the dithionite method of Futterman (1957) as modified by Blakley (1960). TPNH was obtained from P-L Biochemicals. DEAE-Sephadex A-50 was obtained from Pharmacia Fine Chemicals. Hydroxylapatite (Bio-Gel HT) and Bio-Gel P-150 were purchased from Bio-Rad Laboratories. The hydroxylapatite was mixed with an equal weight of Celite (Baker) as a support to obtain better chromatographic flow rates. Dialysis tubing (Union Carbide) was treated as described by Kaufman and Gardiner (1966). All other chemicals were of reagent or analytical grades.

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Enzyme Assay. The assay of dihydrofolate reductase was performed at 25° by a spectrophotometric method which utilizes the decrease in absorbance at 340 nm when TPNH and dihydrofolate are converted to TPN and tetrahydrofolate, respectively (Mathews and Huennekens, 1963). The standard assay mixture contained 33 μM dihydrofolate, 50 μM TPNH, and 50 mM KPO_4 buffer (pH 6.5) in a total volume of 1.0 ml. The value of 12,300 (Hillcoat *et al.*, 1967) was taken as the molar extinction change at 340 nm for the reaction. One unit of enzyme activity is defined as the amount which catalyzes the reduction of 1 μmole of dihydrofolate to tetrahydrofolate per min at 25°.

Protein concentration was determined in impure samples according to the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. In purified samples, the protein concentration was determined from absorbance measurements at 215 and 225 nm (Waddell, 1956) or at 280 nm (*vide infra*).

Polyacrylamide gel electrophoresis was performed essentially as described by Ornstein (1964) and Davis (1964). The tubes (0.5 × 5 cm) contained 10% w/v acrylamide and were run at 4° and at 2.5 mA/gel until the Bromophenol Blue marker had migrated approximately 4.5 cm. Protein on the gels was detected by staining with Amido-Schwarz (Davis, 1964). Enzyme activity was detected directly on the gels by incubation of TPNH and dihydrofolate in the presence of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide as described by Dunlap *et al.* (1971). The tetrazolium dye is reduced to an insoluble blue formazan by tetrahydrofolate, but not by TPNH or dihydrofolate.

Sedimentation velocity analysis was carried out at 20° in a Beckman Model E analytical ultracentrifuge equipped with phase-plate schlieren optics. The analyses were performed in a double-sector cell at 63,650 rpm. Plate negatives of the observed schlieren patterns were taken at regular time intervals during the run and the radial boundary position of the sedimenting protein was measured with a microcomparator at each time interval. The sedimentation constants ($s_{20,w}$) were calculated from the least-squares slopes of the log of the radial boundary position *vs.* time (Schachman, 1957).

Molecular Weight. Sedimentation equilibrium measurements were performed in the ultracentrifuge employing Rayleigh interference optics according to the method of Yphantis (1964) at a rotor speed of 44,800 rpm. A double-sector synthetic boundary cell was employed. The partial specific volume was assumed to be 0.73 ml/g. Additional molecular weight determinations were also carried out by the Sephadex gel filtration method of Whitaker (1963) and by amethopterin titration of the enzyme. In the latter determination, 1 mole of inhibitor is presumed to bind 1 mole of enzyme in a "stoichiometric" manner (Bertino *et al.*, 1964; Werkheiser, 1961).

Amino Acid Analysis. Amino acid analyses were performed according to Spackman *et al.* (1958) on the Beckman amino acid analyzer, Model 120C. Acid hydrolyses (24, 48, and 72 hr) containing 2 mg of protein/tube were carried out in duplicate with 6 N HCl *in vacuo* at 110°. Cysteine and methionine were analyzed as cysteic acid and methionine sulfone, respectively, after oxidation with performic acid (Moore, 1963). Serine and threonine values were obtained from extrapolated values to zero time of hydrolysis according to Moore and Stein (1963). Tryptophan determinations were carried out by titration of the enzyme with *N*-bromosuccinimide in 0.15 M acetate–formate (pH 4.0) containing 6 M urea (Patchornik *et al.*, 1958; Freisheim and Huennekens, 1969) and by the spectrophotometric method of Edelhoch (1967).

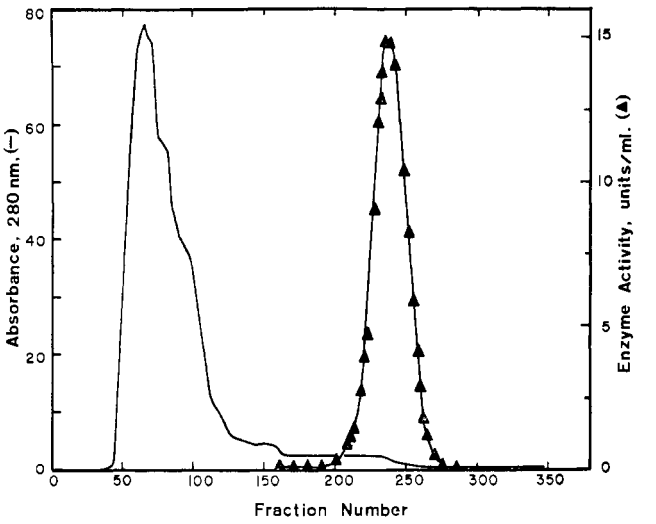

FIGURE 1: Bio-Gel P-150 effluent profile of dihydrofolate reductase. The details are given in the text.

Bacterial Growth. The amethopterin-resistant strain of *S. faecium* used in these studies was grown in a medium as previously described by Freisheim *et al.* (1972). Approximately 1.5 l. of the stock culture (in log phase) was used to inoculate 50 l. of sterilized medium. Growth (turbidity) at 37° was followed using a Klett photometer equipped with a red 66 filter. Freisheim *et al.* (1972) have shown that maximum dihydrofolate reductase production occurs in this strain during the late logarithmic growth stage. The late exponential growth phase was reached in approximately 16–18 hr; the cells were then cooled in ice to a temperature of approximately 8–10° and harvested at 50,000*g* using a Sharples centrifuge. The wet weight of the bacteria obtained was *ca.* 220 g from about 50 l. of medium.

Purification of Dihydrofolate Reductase. All operations were carried out at 0–4° and centrifugations were done in a Sorvall Model RC-2B refrigerated centrifuge. Concentration of dilute enzyme solutions was accomplished by passage through an Amicon diaflow ultrafiltration cell, employing UM-10 membranes. Sephadex gels and ion exchangers were swollen with water as recommended by the manufacturer, washed at room temperature with the initial buffer to be used in the chromatographic procedure and then stored at 4°. Columns were packed at 4° in solutions of the starting buffer prior to sample application.

STEP 1. CELL-FREE EXTRACT. Frozen, packed cells (*ca.* 220 g) were suspended in six volumes of 0.05 M KPO_4–0.5 mM EDTA (pH 6.5), sonicated, and centrifuged as described previously (Freisheim *et al.*, 1972). The supernatant fraction (*ca.* 1300 ml) was then concentrated by the addition of ammonium sulfate to 90% of saturation. After centrifugation at 20,000 *g* the ammonium sulfate precipitate was dissolved in 150 ml of 0.05 M KPO_4–0.5 mM EDTA (pH 6.5) and dialyzed against two changes of 8 l. each of the same buffer for *ca.* 20 hr.

STEP 2. FILTRATION THROUGH BIO-GEL P-150. The crude enzyme solution (*ca.* 200 ml) was then applied to a 10 × 100 cm Bio-Gel P-150 column which had been previously equilibrated with 0.05 M KPO_4–0.5 mM EDTA (pH 6.5). Following elution with the same buffer, the effluent fractions (18

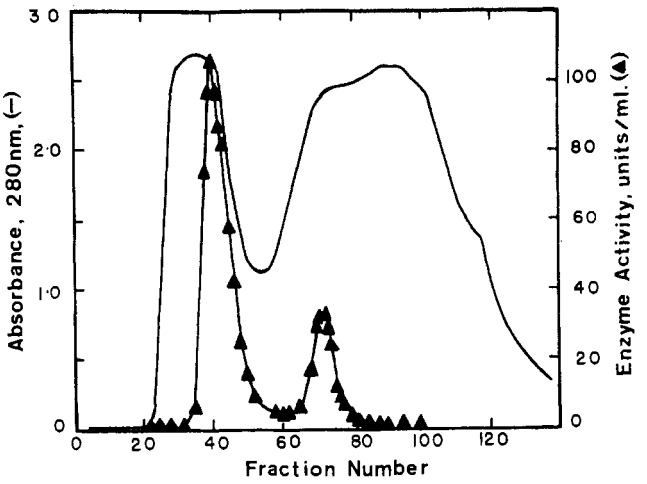

FIGURE 2: Hydroxylapatite chromatography of dihydrofolate reductase. The details are described in the text.

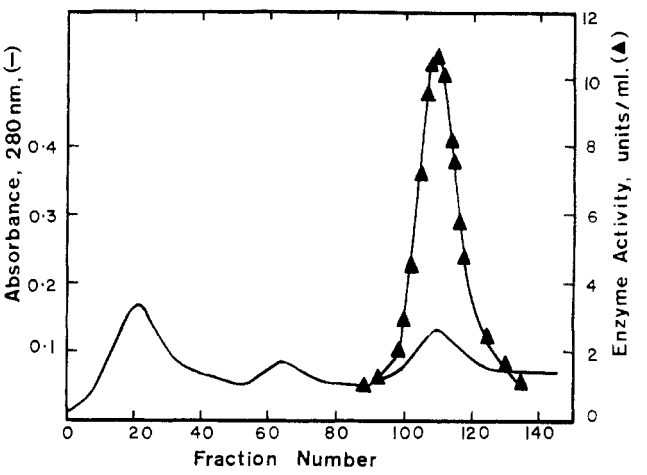

FIGURE 3: DEAE-Sephadex A-50 chromatography of dihydrofolate reductase. Other details are given in the text.

ml each) emerging from the column were collected automatically. The effluent profile is indicated in Figure 1. Those fractions containing enzyme (205–270) were pooled and concentrated using an Amicon diaflow apparatus fitted with a UM-10 membrane. As shown in Figure 1, dihydrofolate reductase was well separated from the bulk of the protein following filtration through Bio-Gel P-150.

STEP 3. CHROMATOGRAPHY ON HYDROXYLAPATITE. Following dialysis *vs.* 6 l. of 0.05 M KPO_4–0.5 mM EDTA (pH 6.5) for 24 hr, the enzyme solution (125 ml; 135 units/ml) was added to a 5 × 25 cm hydroxylapatite column. Elution was carried out with a linear gradient consisting of 2 l. each of 0.05 M KPO_4 (pH 6.5) *vs.* 0.30 M KPO_4 (pH 7.5) each containing 0.5 mM EDTA and 15-ml fractions were collected. As indicated in Figure 2 chromatography on hydroxylapatite resulted in the separation of two distinct dihydrofolate reductases. The HA-2 enzyme (fractions 65–80) accounted for approximately 20–25% of the total dihydrofolate reductase applied to the column. The HA-1 enzyme (fractions 35–55) constituted *ca.* 75–80% of the total enzyme applied. Since both enzyme fractions showed a much higher absorbance at 260 nm than at 280 nm at this stage of purification, the latter wavelength cannot be taken as a measure of protein content alone.

Approximately 0.3 unit each of HA-1 and HA-2 enzyme was applied to 10% polyacrylamide gels. Following electrophoresis, the location of the enzyme was detected directly on the gels using the enzymatic staining technique (*vide supra*). The gels containing the HA-2 samples showed a single enzyme band (form I). The HA-1 samples, however, contained two well-resolved enzyme bands. The slower migrating enzyme band was identical with that of the HA-2 band (form I); the second band (form II) had migrated nearer to the anode.

STEP 4. RECHROMATOGRAPHY OF HA-1 ENZYME ON HYDROXYLAPATITE. The HA-1 enzyme (*ca.* 300 ml), following dialysis *vs.* 6 l. of 0.05 M KPO_4–0.5 mM EDTA (pH 6.5) for 24 hr, was rechromatographed on a hydroxylapatite column under the same conditions as described in step 3 of the purification procedure. All of the enzyme emerging from the column appeared in fractions 65–80 (the position of HA-2 enzyme in step 3) rather than in earlier fractions, suggesting a conversion of HA-1 to HA-2 enzyme. This was confirmed using the enzymatic staining technique following polyacrylamide gel electrophoresis of the pooled enzyme fractions. A single band was observed which corresponded to the form I enzyme previously shown for the HA-2 material (step 3). Rechromatography on hydroxylapatite results in the conversion of form II to form I enzyme. The form I enzyme, however, cannot be converted to the form II enzyme by chromatography on hydroxylapatite.

STEP 5. CHROMATOGRAPHY ON DEAE-SEPHADEX. The fractions containing form I (HA-2) enzyme from steps 3 and 4 were pooled and dialyzed *vs.* 0.05 M KPO_4 (pH 7.2). The dialyzed sample was then chromatographed on a DEAE-Sephadex A-50 column (2.5 × 15 cm). Elution was performed using a gradient consisting of 1000 ml each of 0.05 M KPO_4 (pH 7.2) *vs.* 0.30 M KPO_4 (pH 8.0) and 10-ml fractions were collected. As shown in Figure 3, dihydrofolate reductase emerged as a single peak. Fractions containing enzyme (100–120) were pooled (200 ml). The purified enzyme contained 0.021 mg of protein/ml and 6.4 units/ml (specific activity 305 units/mg). It was subsequently shown that five to six times the amount of HA-2 enzyme could be applied to the DEAE-Sephadex column without altering the conditions of column chromatography (*cf.* Table I).

Results

Enzyme Purification. The results of the purification procedure described in the Methods section are summarized for a typical preparation in Table I. Following chromatography on DEAE-Sephadex (step 5), the enzyme has a specific activity of *ca.* 300 units/mg. The enzyme was purified *ca.* 360-fold relative to the cell-free extract and was recovered in an overall yield of 40%.

All subsequent studies were performed with enzyme that had been purified through the DEAE-Sephadex step.

Gel Electrophoresis. The enzyme showed a single protein band following polyacrylamide gel electrophoresis as indicated in Figure 4. A coincidence of enzyme activity with the single protein band was demonstrated by use of the enzymatic staining technique directly on the gel (see Methods section).

Sedimentation Analysis. The enzyme sedimented as a single symmetrical peak with evidence of boundary spreading. The spreading of the boundary was probably the result of the diffusion of this relatively low molecular weight enzyme. A study of sedimentation velocity in 0.10 ionic strength KPO_4 buffer (pH 6.5) at 20° indicated a decrease of $s_{20,w}$ with

TABLE I: Purification of Dihydrofolate Reductase from Amethopterin-Resistant *S. faecium*.

Step	Fraction	Vol (ml)	Protein (mg/ml)	Act. (Units/ml)	Sp Act. (Units/mg)	Total Act. (Units)	Recov (%)
1	Cell-free extract[a]	1270	11.2	9.4	0.84	11,600	100
2	Bio-Gel P-150	1170	0.141	8.4	60.0	9,860	85
	Combined fractions[b]	125	2.20	135.0	61.3	16,900	85
3	Hydroxylapatite						
	HA-1	300	0.29	38.0	131	11,400	57
	HA-2	225	0.062	12.6	203	2,350	12
4	Hydroxylapatite on HA-1	240	0.189	37.0	196	8,890	45
5	DEAE-Sephadex on combined HA-2 fractions	210	0.121	36.7	302	7,680	39

[a] From 230 g of cells. [b] Two preparations from step 2 were combined for subsequent steps. The combined fractions were from 394 g of cells.

increasing protein concentration. Extrapolation to zero protein concentration gave a value of $s_{20,w} = 2.10$ S.

Molecular Weight. The molecular weight of dihydrofolate reductase was determined by three independent methods. Sedimentation equilibrium analysis over a protein concentration range of 0.2–2.5 mg/ml at 7.5° gave an apparent weight-average molecular weight in the range of 19,400–20,200 in four separate experiments. The buffer was the same as that used in the sedimentation velocity experiments. The partial specific volume used in the molecular weight calculations was determined to be 0.73 ml/g from the amino acid composition data (*vide infra*).

Filtration of the enzyme through a 1.5 × 84 cm Sephadex G-100 column calibrated with proteins of known molecular weight (Whitaker, 1963) gave a value of 19,200. Titration of dihydrofolate reductase with amethopterin, a "stoichiometric" inhibitor (Bertino *et al.*, 1964; Werkheiser, 1961), gave a molecular weight of 20,200 (Figure 5). This titration assumes that the inhibitor interacts on an equimolar basis with the enzyme at low pH values.

Amino Acid Composition. The results are summarized in Table II. The enzyme is quite rich in acidic amino acid resi-

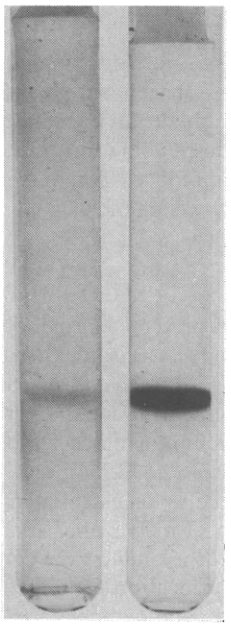

FIGURE 4: Polyacrylamide gel electrophoresis of dihydrofolate reductase. Approximately 35 μg of protein was applied to each gel. The gel on the right was stained for protein and that on the left for enzyme as described in the Methods section. Single protein and enzyme bands were similarly observed at protein concentrations up to 150 μg/gel.

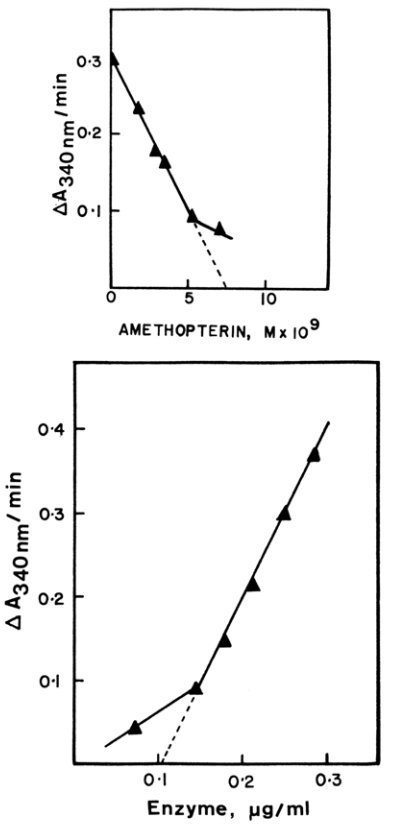

FIGURE 5: (A, left) Inhibition of dihydrofolate reductase as a function of increasing concentrations of amethopterin. The enzyme (0.142 μg/ml) was titrated with inhibitor at the indicated concentrations in 0.05 M KPO_4–0.001 M EDTA (pH 5.9). (B, right) The effect of amethopterin (5.25×10^{-9} M) on the activity of dihydrofolate reductase. The intersection with the abscissa corresponds to an enzyme concentration of 1.06×10^{-4} mg of protein ml^{-1}. Other conditions are described in part A.

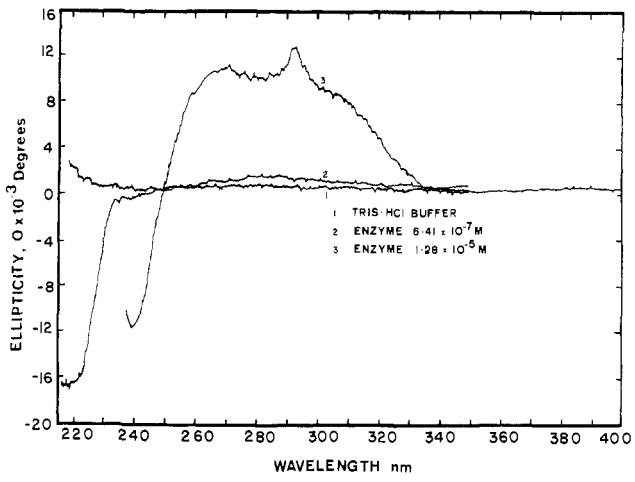

FIGURE 6: The circular dichroic absorption spectrum of dihydrofolate reductase. The spectra were taken using 10-mm cells containing 3.0 ml of the indicated solutions.

dues explaining its rapid electrophoretic mobility on polyacrylamide gels. The enzyme contains only one half-cystine residue, presumably as cysteine, and an unusually high tryptophan content. The value of 8.9 residues of tryptophan (based on a molecular weight of 20,000) was determined according to the spectrophotometric methods of Patchornik *et al.* (1958) and Edelhoch (1967). The molecular weight, calculated from the data in Table II, is 20,300 which compares well to the values obtained by physical means and by amethopterin titration.

Absorption Spectrum. The enzyme exhibited a single absorption maximum at 278 nm and a distinct shoulder at 290 nm. The shoulder at 290 nm is undoubtedly due to the relatively high tryptophan content of this enzyme. An absorbancy index of 22.0 for a 1% solution of protein at 280 nm was determined by relating absorbance to protein concentration as measured in the ultracentrifuge by refractometric means. The refractive index increment was assumed to be 0.00185 dl g^{-1} at 5500 Å (Doty and Edsall, 1951).

Circular Dichroic Absorption Spectrum. The near ultraviolet circular dichroic (CD) spectrum of the enzyme shows a positive band system as indicated in curve 3 of Figure 6. The side chain Cotton effect in the 260- to 310-nm region shows three distinct ellipticity bands centered around 305, 295, and 270 nm, respectively. The observed fine structure in this region is most probably determined by the aromatic side-chain bands; the enzyme shows a negative CD band centered at *ca.* 220 nm, presumably due mainly to the intrinsic polypeptide backbone absorption (curve 2, Figure 6). Another negative band centered at *ca.* 240 nm has also been observed at relatively high enzyme concentrations. The transition involved at 240 nm is unknown, although phenylalanyl residues might be considered (Beychok, 1968). Titration of the reductase with TPNH to a 1:1 stoichiometry results in the generation of an extrinsic Cotton effect at *ca.* 340 nm with a concomitant decrease in the magnitude of the side-chain Cotton effect (Freisheim and D'Souza, 1971).

Kinetic Properties. The reductase from this particular amethopterin-resistant strain of *S. faecium* shows a single pH optimum centered at *ca.* 6.5. The enzyme activity at the pH optimum was virtually identical in either Tris-HCl of KPO_4 buffers (0.05 M). Apparent K_m values of 7×10^{-6} M for dihydrofolate and 2×10^{-5} M for TPNH were determined for the purified reductase at pH 6.5. These values are essentially identical with those reported for the partially purified enzyme; the assays were performed as described by Freisheim *et al.* (1972).

TABLE II: Amino Acid Composition of Dihydrofolate Reductase from Amethopterin-Resistant *S. faecium*.

	Residues/ 20,000 g of Protein	Nearest Integer/ 20,000 g	Integral No. × Mol Wt of Residue
Lysine	10.8	11	1,410
Histidine	4.7	5	686
Arginine	9.6	10	1,562
Aspartic acid	19.5	20	2,302
Threonine[a]	7.8	8	809
Serine[a]	5.9	6	523
Glutamic acid	25.4	25	3,228
Proline	6.5	6	583
Glycine	10.9	11	628
Alanine	9.7	10	711
Valine	9.4	9	892
Isoleucine	8.6	9	1,019
Leucine	10.8	11	1,245
Tyrosine	5.7	6	979
Phenylalanine	8.1	8	1,178
Half-cystine[b]	1.1	1	103
Methionine[b]	6.0	6	787
Tryptophan[c]	(8.9)	9	1,676
	(20,091)[d]	171	20,321

[a] Values extrapolated to zero time of hydrolysis. [b] Measured as cysteic acid and methionine sulfone, respectively, after performic acid oxidation. [c] Measured by spectrophotometric methods as described in the text. [d] Summation of residues × residue mol wt.

Using the standard spectrophotometric assay system (see Methods section), the specific activity of the purified enzyme is *ca.* 300 μmoles of dihydrofolate reduced min^{-1} mg^{-1} of protein at 25°. The corresponding turnover number for dihydrofolate is 6000 moles reduced per min per mole of enzyme, assuming a molecular weight of 20,000.

Discussion

The physical data presented provide strong evidence that the dihydrofolate reductase isolated from an amethopterin-resistant strain of *S. faecium* is essentially homogeneous. Titration of the enzyme with amethopterin (Figure 5), assuming that 1 mole of inhibitor binds 1 mole of enzyme (Werkheiser, 1961; Bertino *et al.*, 1964), gives a molecular weight of 20,200. This value is in essential agreement with those determined by physical means and is virtually identical with that calculated from the amino acid composition (Table II).

In step 4 of the purification procedure the form II enzyme is converted to form I following hydroxylapatite chromatography. Thus, the basis for the difference of these chromatographic forms would not be expected to reside in significant structural variations in acidic or basic amino acid residues. In addition, none of the physical studies performed on the purified enzyme have indicated that a subunit ⇄ aggregate

TABLE III: Comparison of Certain Properties of the Form I Dihydrofolate Reductase to the Enzymes Isolated by Nixon and Blakley.

	Form I	Mutant[a]	Wild Type[a]
Molecular weight	20,000	20,000	28,000
Sedimentation constant	2.10	2.04	2.58
pH optimum	6.5	5.8	5.8
Turnover number[b]	6000 (25°)	900 (37°)	8000 (37°)

[a] Data from Nixon and Blakley (1968). [b] In moles of substrate transformed per min per mole of enzyme. The numbers in parentheses indicate the temperature at which the enzyme assays were performed.

system is operative; this is not surprising in view of the low molecular weight of the reductase.

Gunderson *et al.* (1972) have resolved dihydrofolate reductase from amethopterin-resistant *L. casei* into two separate forms by chromatography on hydroxylapatite. Dunlap *et al.* (1971) have shown that these two enzyme forms can also be distinguished on the basis of their electrophoretic migration on polyacrylamide gels. The latter authors have shown that form II differs from form I by the binding of 1 mole of TPNH/mole of protein. Removal of TPNH from form II results in the generation of the form I enzyme. Similarly, Niethammer and Huennekens (1971) have shown that the molecular basis for the two chromatographic and electrophoretic forms of human erythrocyte methemoglobin reductase resides in the binding of TPN to one of the forms. In the present study it is quite possible that the resolution of two enzyme forms (step 3) resulted from the removal of a cofactor, perhaps TPNH or TPN, previously bound to the enzyme. In step 4 the remaining protein-bound cofactor may have been stripped from the enzyme. A possible explanation for the fact that these multiple forms of dihydrofolate reductase survive chromatography and gel electrophoresis may be related to the relatively low dissociation constant of 4×10^{-7} M for the TPNH·enzyme complex (Freisheim and D'Souza, 1971).

Nixon and Blakley (1968), however, indicate that two dihydrofolate reductases differing in molecular weight, turnover number and other properties are synthesized by the particular amethopterin-resistant strain (SF/A) of *S. faecium* which they employ. Properties of the "mutant" and "wild-type" enzymes isolated by Nixon and Blakley (1968) are compared with the form I enzyme isolated in these studies in Table III. The enzyme isolated in the present studies (form I) resembles the mutant enzyme in molecular weight and sedimentation constant, but the respective pH optima and turnover numbers are quite different. The form I enzyme resembles the wild-type enzyme somewhat in turnover number, although at 37° the value for the form I enzyme would be considerably increased. Based on the marked differences in properties between the form I enzyme and those isolated by Nixon and Blakley, the most probable explanation is that different strains of amethopterin-resistant *S. faecium* are being employed as a source of dihydrofolate reductase in the two laboratories.

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Exchange of Methyl Protons of Acetyl Coenzyme A Catalyzed by Adenosine Triphosphate Citrate Lyase†

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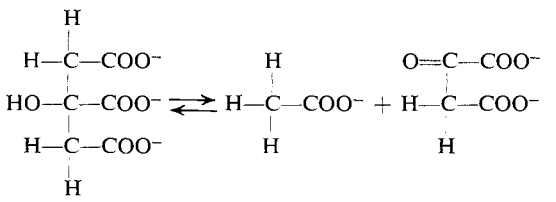
ABSTRACT: ATP citrate lyase requires the presence of oxalacetate in order to catalyze the exchange of the methyl protons of acetyl-CoA. This activity was measured by nuclear magnetic resonance spectroscopy by following exchange of deuterons of D_2O into acetyl-CoA, and by following the exchange of tritium from [^{3}H]acetyl-CoA into H_2O. The reaction requires sulfhydryl groups on the enzyme. Oxalacetate cannot be replaced by L-malate or α-ketoglutarate, substances which induce an enolase in citrate synthase.

There are three enzymes known to catalyze the same bond-making and -breaking reaction on citrate. These three



are citrate lyase (EC 4.1.3.6), citrate synthase (citrate lyase (CoA acetylating) EC 4.1.3.7), and ATP citrate lyase (citrate lyase (CoA acetylating and ATP dephosphorylating) EC 4.1.3.8). In order for citrate to be formed, a proton must first be removed from an acetyl unit in each case. Eggerer (1965) first demonstrated that such an enolization could be measured in citrate synthase only if the inducer substance L-malate was added. Srere (1967) was also able to demonstrate this activity of citrate synthase and showed α-ketoglutarate, too, could serve as an inducer for this enolization.

We have examined the ATP citrate lyase for the similar reaction measuring the exchange of deuterons from D_2O into acetyl-CoA by nuclear magnetic resonance spectroscopy and also by measuring the exchange of tritium from [^{3}H]acetyl-CoA into the water of the medium. The results of these experiments are presented in this paper.

Materials and Methods

Rat liver ATP citrate lyase (mol wt 500,000) was purified using the procedure of Srere (1959) for the chicken liver enzyme followed by chromatography on Bio-Gel A 1.5 m. Such preparations show single bands by disc gel electrophoresis and have specific activities of 3.5–4.0 μmoles of citrate cleaved per min per mg of protein. This preparation contains 2 munits of citrate synthase/mg and 3 of malate dehydrogenase/mg. The protein was estimated by the method of Lowry *et al.* (1951). Acetyl-CoA and deuterated acetyl-CoA were prepared using the method of Simon and Shemin (1953) and both preparations contained some free acetate. Control experiments showed that the presence of acetate did not effect the results. Nmr spectra were recorded with a Varian A-60-A nmr spectrometer and were performed at room temperature (27°).

[^{3}H]Acetyl-CoA was prepared by reacting with [^{3}H]acetic anhydride and CoASH in 0.1 M Tris base. [^{3}H]Acetyl-CoA was subsequently isolated by ion-exchange chromatography on a DEAE-cellulose column using the method of Moffatt and Khorana (1961). Tritium-exchange experiments were performed in 0.1 M Tris-Cl (pH 8.1) with enzyme, [^{3}H]acetyl-CoA and other substrates. The tritiated water was collected by a lyophilization technique described by Bloom (1958). The collected H_2O (10 μl) was placed in 10 ml of Bray's solution and the radioactivity determined in a Packard Tri-Carb scintillation counter.

Rates were calculated as suggested by Rose (1970) using the equation $v = 2.3nt^{-1}$[AcCoA] log (1-fraction reacted), where $n = 3$ since 3 equiv of protons are involved.

Results

Exchange of Protons of Acetyl-CoA with D_2O Nuclear Magnetic Resonance. The nmr spectrum of acetyl-CoA is shown in Figure 1. The line at −2.35 ppm (sodium 3-(trimethylsilyl)-1-propanesulfonate = 0) is due to the protons of the acetyl group of acetyl-CoA. The peaks at 0.72 and −0.85 ppm are due to the methyl groups of the pantothenic acid portion of the acetyl-CoA. The separation in resonances of the two methyl groups is due to the neighboring asymmetric carbon atom. Since the protons of methyl groups of the pantothenic acid portion of the molecule do not exchange, the amount of exchange of protons of the acetyl group with deuterons of the medium is estimated from the decrease in ratio of peak heights at −2.35 ppm to that of −0.85 ppm.

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