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Purification and Characterization of the Bifunctional Thymidylate Synthetase-Dihydrofolate Reductase from Methotrexate-Resistant *Leishmania tropica*†

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ABSTRACT: Thymidylate synthetase (TS) and dihydrofolate reductase (DHFR) in *Leishmania tropica* exist as a bifunctional protein. By use of a methotrexate-resistant strain, which overproduces the bifunctional enzyme, the protein was purified 80-fold to apparent homogeneity in two steps. The native protein has an apparent molecular weight of 110000 and consists of two subunits with identical size and charge. Available data indicate that each of the subunits possesses TS and DHFR. The TS of the bifunctional protein forms a covalent 5-fluoro-2′-deoxyuridylate (FdUMP)-(±)-5,10-methylenetetrahydrofolate–enzyme complex in which 2 mol of FdUMP is bound per mole of enzyme. In contrast, titration of DHFR with methotrexate indicated that only 1 mol of the inhibitor is bound per mole of dimeric enzyme. Both TS and DHFR activities of the bifunctional enzyme were inactivated by the sulfhydryl reagent *N*-ethylmaleimide. Substrates of the individual enzymes afforded protection against inactivation, indicating that each enzyme requires at least one cysteine for catalytic activity. Kinetic evidence indicates that most, if not all, of the 7,8-dihydrofolate produced by TS is channeled to DHFR faster than it is released into the medium. Although the mechanism of channeling is unknown, the possibility that the two enzymes share a common folate binding site has been ruled out.

Thymidylate synthetase (TS;[1] EC 2.1.1.45) and dihydrofolate reductase (DHFR; EC 1.5.1.3) catalyze sequential reactions in the de novo synthesis of dTMP. TS catalyzes the conversion of dUMP and CH_2-H_4folate to dTMP and H_2folate; it is unique among enzymes that utilize folate cofactors in that H_4folate is oxidized in the course of the one-carbon transfer reaction. DHFR catalyzes the subsequent NADPH-dependent reduction of the H_2folate produced by TS to regenerate H_4-folate, which serves as a carrier of one-carbon units in a number of metabolic processes. Because blocking either TS or DHFR results in depletion of dTMP and subsequent cessation of DNA synthesis, these enzymes have been studied extensively and exploited as targets for chemotherapeutic agents for a number of diseases.

TS and DHFR are distinct and readily separable in sources as varied as bacteria, bacteriophage, yeast, and vertebrates. Usually, TS is a dimer of identical subunits with a native molecular weight of about 70000 and DHFR is a monomer with a molecular weight of about 20000 [for reviews see Blakley (1984) and Santi & Danenberg (1984)]. In contrast, TS and DHFR have recently been reported to exist as a bi-

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[1] Abbreviations: TS, thymidylate synthetase; DHFR, dihydrofolate reductase; CH_2-H_4folate, (±)-5,10-methylenetetrahydrofolate; H_4folate, (±)-tetrahydrofolate; H_2folate, 7,8-dihydrofolate; MTX, methotrexate; FdUMP, 5-fluoro-2′-deoxyuridylate; NEM, *N*-ethylmaleimide; BSA, bovine serum albumin; Tes, 2-[[tris(hydroxymethyl)methyl]amino]-ethanesulfonic acid; DTT, dithiothreitol; $NaDodSO_4$-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. All other abbreviations are those recommended by IUPAC.

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functional protein in a number of protozoa that span a diverse group of the subkingdom (Ferone & Roland, 1980; Garrett et al., 1984). Depending upon the species, the bifunctional TS–DHFRs have apparent native molecular weights ranging from 100000 to 240000 as determined by gel filtration chromatography.

Because of their unique structures, bifunctional TS–DHFRs in protozoa represent interesting proteins for kinetic and structural characterization. Further, many parasitic protozoa are major health problems, and the bifunctional protein is a promising target for selective chemotherapeutic agents. In this regard, pyrimethamine, a species-specific inhibitor of DHFR, has been extensively used to treat malaria (Rollo, 1980).

Thus far, the only protozoan TS–DHFR that has been purified to homogeneity and partially characterized is that from *Crithidia fasciculata* (Ferone & Roland, 1980), a nonpathogenic trypanosomatid that can be cultivated in large quantities. In general, purifying sufficient amounts of TS–DHFR from pathogenic protozoa has been impractical because of source limitations. We recently reported the development of a MTX-resistant strain of the parasitic protozoa *Leishmania tropica* (Coderre et al., 1983); this strain overproduces TS–DHFR to the extent that milligram amounts of the bifunctional protein are readily obtainable. In this paper we describe the purification and characterization of TS–DHFR from MTX-resistant *L. tropica*.

Materials and Methods

Folic acid, H_4folate, dUMP, FdUMP, pyridoxal phosphate, protease inhibitors, and all buffers were obtained from Sigma. H_2folate (Friedkin, 1959), CH_2-H_4folate, and (6*R*)-L-CH_2-H_4folate (Bruice & Santi, 1982) were prepared as described and stored under argon at −80 °C. Concentrations of H_2folate and CH_2-H_4folate were determined enzymatically with DHFR and TS, respectively. MTX was obtained from the Lederle Parenterals, Inc. [6-^{3}H]FdUMP (20 Ci/mmol) and [3′,5′,7-(N)-^{3}H]MTX (28 Ci/mmol) were purchased from Moravek Biochemicals. [6-^{3}H]-L-Serine (28 Ci/mmol) was an Amersham product. Sephadex G-15, Sephadex G-25, DEAE-Sepharose CL-6B, and Sepharose CL-6B were from Pharmacia. Cibacron Blue F3GA–agarose was obtained from Pierce Chemical Corp. PteGlu_4-lysine–Sepharose was a gift from Dr. Roy L. Kisliuk. MTX–Sepharose was prepared by coupling MTX to aminohexyl-Sepharose CL-6B (Dann et al., 1976), which was prepared according to the method of Bethell (1979); spectrophotometric assay of the digested resin (Failla & Santi, 1973) indicated that the preparation contained 0.6 μmol of MTX/mL of wet gel. Reagents and protein standards for $NaDodSO_4$–PAGE and Bio-Gel P-300 were from Bio-Rad. Ampholines were LKB products. *L. casei* TS and DHFR were prepared from a methotrexate-resistant strain (Crushberg et al., 1970) as described (Wataya & Santi, 1977; Dann et al., 1976). The following buffers were used: buffer A, 50 mM Tes (pH specified in the text), 75 mM 2-mercaptoethanol, 1 mM EDTA; buffer B, 50 mM Tes (pH 7.4), 5 mM DTT, 1 mM EDTA; buffer C, 10 mM potassium phosphate (pH 7.0).

Growth of Organisms. *L. tropica* promastigotes (strain 252, Iran), obtained from S. Meshnik (Cornell University Medical College), were cultured in M199 medium (Gibco) containing Earl's buffered saline salts, 20% fetal calf serum, 25 mM Hepes (pH 7.4), and 50 μg/mL gentamicin. The MTX-resistant strain of *L. tropica* (Coderre et al., 1983) was maintained in the same media containing 1 mM MTX. For large-scale preparations of the latter, cells were grown to stationary phase (approximately 2×10^7 cells/mL) and harvested by centrifugation (1500*g*, 5 min); the medium containing MTX was removed, and the cells were seeded at about 10^6 cells/mL into larger volumes (1–10 L) of medium not containing MTX and incubated by stirring in Bellco spinner flasks at 26 °C. Organisms were harvested at late logartihmic phase [$(1.0–1.5) \times 10^7$ cells/mL] by centrifugation (1500*g*, 5 min) and washed twice with ice-cold phosphate-buffered saline. If cell pellets were not used immediately, they were stored at −80 °C, although freezing resulted in some loss of enzyme activity.

Purification of TS–DHFR from L. tropica. The following protocol was used to purify the enzyme from 1–10-L cultures of wild-type or MTX-resistant *L. tropica*. All steps were performed at 4 °C, and the entire procedure was performed without interruption.

Step I: Preparation of Cell Extracts. Packed cell pellets of *L. tropica* were suspended at a density of approximately 3×10^9 cells/mL in buffer B, which contained 10% glycerol and the following protease inhibitors: 1 mM 1,10-phenanthroline, 1 mM benzamidine, 50 μM phenylmethanesulfonyl fluoride, 20 μg/mL leupeptin, 50 μg/mL crude soybean trypsin inhibitor, and 50 μg/mL aprotinin. The cells were disrupted by sonication (Bronwill Biosonic IV; 5-s bursts at 100 W with intermittent 30-s cooling periods) and centrifuged at 48000*g* for 70 min.

Step II: DEAE-Sepharose Column Chromatography. The supernatant was applied to a column of DEAE-Sepharose CL-6B (2.5 × 8.6 cm) previously equilibrated with buffer B. The column was washed with the equilibration buffer (approximately 25 mL) until protein was undetectable in the effluent, and the enzyme was eluted with 30 mL of 0.15 M KCl in buffer B. Alternatively, a 200-mL linear gradient of 0–0.2 M KCl in buffer B was applied to the equilibrated column, and the enzyme was eluted at 75 mM salt.

Step III: MTX–Sepharose Affinity Chromatography. The fractions containing TS–DHFR from Step II were pooled and applied to a column of MTX–Sepharose (1.0 × 2.5 cm) that had been equilibrated with buffer C. The enzyme was applied by circulating the protein solution through the column with a peristaltic pump (flow rate = 0.3 mL/min) for 4–6 h or until less than 5% of the activity remained in the reservoir. The column was washed with buffer C, containing 1 M KCl, until protein was undetectable in the effluent, and then washed with 10 mL of buffer C. One column volume of buffer B, containing 1 mM H_2folate, was applied to the column, allowed to equilibrate for 20 min, and then eluted with the same buffer. Fractions of 1.0 mL were collected, and the enzyme eluted in the first 4 mL.

Fractions containing the enzyme were pooled; H_2folate was removed by filtration through a column of Sephadex G-15 (120 mesh; 1 × 10 cm), equilibrated, and eluted with buffer B. TS–DHFR eluted in 5–7 mL and H_2folate eluted in 15–21 mL. Alternatively, pooled fractions were applied to a small DEAE-Sepharose column (0.4 × 8 cm) previously equilibrated with buffer B. After the column was washed with about 20 mL of the equilibration buffer, TS–DHFR was eluted in about 2 mL of buffer B containing 0.15 M KCl.

Enzyme Assays. DHFR activity was determined spectrophotometrically at 25 °C (Hillcoat et al., 1967). The standard assay mixture (1.0 mL) contained buffer A (pH 7.0), 1 mg/mL BSA, 0.1 mM NADPH, 0.1 mM H_2folate, and limiting enzyme; controls included 2 μM MTX. TS activity was determined spectrophotometrically (Wahba & Friedkin, 1961) at 25 °C. The standard assay mixture (1.0 mL) contained buffer A (pH 7.8), 5 mM H_2CO, 1 mg/mL BSA, 0.2 mM

CH_2-H_4folate, 0.1 mM dUMP, and limiting enzyme; controls included 2 μM FdUMP. Serine transhydroxymethylase activity was determined at 37 °C as reported by Taylor & Weissbach (1965), except the reaction mixture (0.1 mL) contained buffer A (pH 8.0), 1 mg/mL BSA, 0.2 mM pyridoxal phosphate, 1.7 mM H_4folate, 1.8 μM [^{3}H]-L-serine (28 Ci/mmol), and appropriate amounts of enzyme; controls omitted enzyme. One unit of enzyme activity is defined as that amount of enzyme that produces 1 nmol of product/min.

DHFR and TS were also quantitated by binding assays in which we used the active-site titrants MTX and FdUMP, respectively. For DHFR, 0.1-mL solutions containing 0.25–4 μg of protein in buffer B were incubated with 0.4 μM [^{3}H]-MTX (28 Ci/mmol) and 0.5 mM NADPH for 20 min. Macromolecular-bound radioactivity was separated from free [^{3}H]MTX by gel filtration on small columns of Sephadex G-15 (Garrett et al., 1984). For TS, the [6-^{3}H]FdUMP–CH_2-H_4folate–enzyme complex was formed in buffer B and was quantitated by the nitrocellulose filter binding assay as previously reported (Santi et al., 1974) or isolated by gel filtration through Sephadex G-25 (Washtien & Santi, 1979).

The TS and DHFR coupled system was assayed by monitoring the decrease in NADPH absorbance at 340 nm in the absence of added H_2folate. The assay mixture (1.0 mL), containing buffer A (pH 7.8), 5 mM H_2CO, 1 mg/mL BSA, 30 μM NADPH, 28 μM CH_2-H_4folate, and specified amounts of both enzymes, was incubated at 25 °C to enzymatically reduce traces of endogenous H_2folate. When the absorbance at 340 nm was stable, the reaction was initiated by addition of 0.02 mL of 5 mM dUMP to give a final concentration at 0.1 mM. The TS and DHFR rates required to analyze the coupled system were obtained by initial velocity determinations under conditions of the coupled assay, with the following modifications: for DHFR, CH_2-H_4folate and dUMP were omitted and 100 μM H_2folate was included; for TS, NADPH was omitted and the formation of H_2folate was monitored spectrophotometrically.

Electrophoretic Methods. NaDodSO$_4$–PAGE was performed on slab gels 0.15 cm thick and 14.5 cm long; stacking gels (3.5 cm) were 4.5% polyacrylamide, and separating gels (11 cm) were 8.5–12.5% polyacrylamide. Procedures used were those described by Laemmli (1970). Gels containing the [6-^{3}H]FdUMP–CH_2-H_4folate–TS complex were cut in 1-mm strips, treated with 1 mL of Protosol at 60 °C for 20 min, neutralized with 0.1 mL of HOAc, and counted.

Nondenaturing isoelectric focusing was performed in cylindrical polyacrylamide gels (0.25 cm × 10 cm) prepared with 4.7% acrylamide, 0.15% *N*,*N*′-methylenebis(acrylamide), 10% glycerol, 2% ampholines (pH 5–8, 1.5%; pH 3–10, 0.5%), 0.02% ammonium persulfate, and 0.001% TEMED. The pH gradient was determined by electrophoresis of colored protein standards with known isoelectric points (p*I* = 4.7–10.6) (BDH Chemicals, Poole, England). Electrophoresis was performed at 400 V for 7000–8000 V h, and gels were subsequently fixed and stained with Coomassie blue as described by Winter et al. (1977). Denaturing isoelectric focusing gels were prepared and conducted according to the method of O'Farrell (1975). Isoelectric focusing gels that contained urea were fixed for 6–8 h in 10% trichloroacetic acid and then washed for 12 h in 25% 2-propanol–10% HOAc and for 2 h in H_2O. The gels were stained for 10 min at 60 °C in a solution containing 0.12% Coomassie Blue R-250 in 25% EtOH–10% HOAc and destained overnight with a solution of 25% EtOH–10% HOAc.

Analytical Gel Filtration. Gel filtration of *L. tropica* TS–DHFR was performed on a 2 × 56 cm column of Bio-Gel P-300 (100–200 mesh) at a flow rate of 3 mL/h. The column was calibrated by separate determinations with the following protein standards: catalase (Stokes radius, a = 5.2 nm), aldolase (a = 4.8 nm), bovine serum albumin (a = 3.6 nm), and ovalbumin (a = 3.1 nm).

Density Gradient Centrifugation. Glycerol density gradients (6.7–20% v/v) were prepared in 4.5 mL of 50 mM Tes (pH 7.2) and 5 mM DTT in cellulose nitrate tubes. A solution (0.10 mL) containing 8 μg of yeast alcohol dehydrogenase, 5 μg of beef catalase, 6 μg of *Escherichia coli* alkaline phosphatase (s = 7.4, 11.3, and 6.4 S, respectively), and 1.5 μg of TS–DHFR was added to two of the above tubes, which were then centrifuged at 38 000 rpm for 18 h with an SW 50.1 rotor in a Beckman Model L2-65B ultracentrifuge. Samples were collected and the protein standards were assayed as described by Martin & Ames (1961); the TS and DHFR activities were assayed as described above.

Protein Analysis. Protein concentrations were determined by the method of Read & Northcote (1981) using BSA as a standard. Purified TS–DHFR was submitted to high-performance liquid chromatography on a C_4 reverse-phase column (Vydac Model 214TP54; 0.4 × 25 cm) prior to amino acid analysis. A 40-min linear gradient was run at 0.5 mL/min from 24% to 95% acetonitrile in aqueous 0.1% trifluoroacetic acid, and protein was detected by its absorbance at 220 nm. TS–DHFR eluted in a single peak with a retention volume of 12.0 mL (70% acetonitrile). Protein samples were hydrolyzed for 24, 48, and 72 h in 6 M HCl at 110 °C; amino acid compositions were determined in duplicate on a Beckman 6300 amino acid analyzer. Tryptophan content was determined by hydrolyzing TS–DHFR in 4 M methanesulfonic acid (Simpson et al., 1978); the hydrolysate was analyzed on a Beckman 121 amino acid analyzer.

RESULTS

Purification of TS–DHFR from MTX-Resistant L. tropica. Most freshly prepared crude extracts from MTX-resistant *L. tropica* possess 20–40-fold higher levels of TS–DHFR than extracts of wild-type organisms. Early in this work we found that storage of either frozen cells or crude extracts resulted in loss of TS and DHFR activities and that during purification the TS activity decreased disproportionately to that of DHFR, presumably because of proteolysis. Ultimately, we obtained optimal yields when harvested cells were immediately lysed in the presence of a mixture of protease inhibitors and when purification steps were performed without interruption.

Preliminary studies were performed to evaluate a number of affinity chromatography matrices that have previously been used to purify TS or DHFR from other sources. In these experiments, crude extracts were applied to small columns containing the affinity matrix. After the column was washed with salt to remove nonspecifically bound protein, the enzyme was eluted biospecifically. First, we examined MTX–Sepharose, an affinity matrix that has been used extensively to purify a number of DHFRs (Kaufman, 1974). As shown in Figure 1A, both activities were retained on this column and coeluted when treated with 1.0 mM H_2folate; the TS–DHFR was obtained in about 75% yield and was about 100-fold purified. Second, we examined Cibacron Blue–agarose, a matrix that has affinity for some enzymes that bind to nicotinamide cofactors and that has also been used to purify DHFR (Johnson et al., 1980). In our initial experiments, the *Leishmania* TS–DHFR did not elute with buffers containing 1 M KCl, 0.1 mM dUMP, or 0.5 mM NADPH but did elute with buffer containing 0.5 mM H_2folate. In the final protocol, we included 0.5 mM NADPH in the loading buffer in an

Table I: Purification of TS–DHFR from *L. tropica* (R1000)[a]

purification step	total protein (mg)	DHFR sp act. (units/mg)	DHFR total units	TS sp act. (units/mg)	TS total units	purification (*x*-fold)[b]	yield (%)
I, crude extract	48.7	179	8700	40.6	1980	1.0	100
II, DEAE-Sepharose chromatography	10.9	631	6880	192	2091	3.5	80
III, MTX–Sepharose chromatography	0.41	14620	6000	3620	1480	82	69

[a] Prepared from 4×10^{10} cells. [b] Based on DHFR activity.

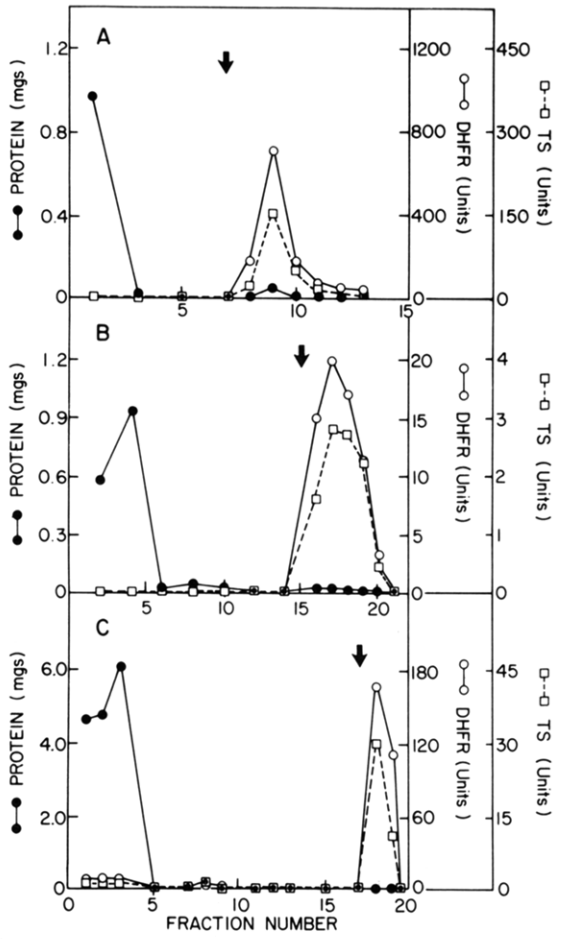

FIGURE 1: Affinity chromatography of TS–DHFR. (A) The extract was applied to a column containing MTX–Sepharose (0.1 × 2.5 cm) equilibrated with buffer C. The column was washed with buffer C containing 1.0 M KCl, and TS–DHFR was eluted with buffer B containing 1.0 mM H_2folate (arrow). (B) The extract in buffer B containing 0.5 M NaCl and 0.5 mM NADPH was applied to a column containing Cibacron Blue–agarose (0.5 × 4 cm) equilibrated with the same buffer. The column was washed with 10 mL of loading buffer and 5 mL of buffer B containing 0.5 M NaCl; TS–DHFR was eluted with buffer B containing 0.5 M NaCl and 0.5 mM H_2folate (arrow). (C) The extract was applied to a column of $PteGlu_4$-lysine–Sepharose (0.5 × 3 cm) equilibrated with buffer B. The column was washed with 15 mL of buffer B containing 0.5 M NaCl, and TS–DHFR was eluted with buffer B containing 1 M NaCl and 0.5 mM H_2folate (arrow).

attempt to prevent binding of other enzymes that use NADPH. After the column was washed with salt, TS and DHFR activities were eluted with H_2folate in 50% overall yield with a 20-fold purification (Figure 1B). The third affinity column was $PteGlu_4$-lysine–Sepharose, an affinity resin that has been used to purify TS (Rao & Kisliuk, 1983). However, unlike previously reported uses of this matrix, the *Leishmania* TS–DHFR bound tightly to the resin in the absence of dUMP and was eluted with 0.5 mM H_2folate (Figure 1C). The enzyme was obtained in 65% yield and was about 100-fold purified

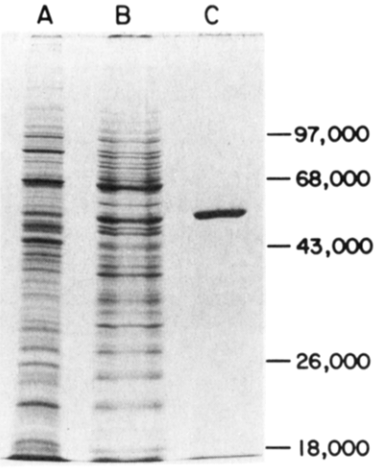

FIGURE 2: $NaDodSO_4$–PAGE (25.5% polyacrylamide) of *L. tropica* TS–DHFR following each step of protein purification: (A) crude extract (30 μg); (B) after DEAE-Sepharose column chromatography (30 μg); (C) after MTX–Sepharose column chromatography (3 μg). The molecular weight standards were phosphorylase *b* (97 000), bovine serum albumin (68 000), ovalbumin (43 000), α-chymotrypsinogen (26 000), and β-lactoglobulin (18 000).

compared with the crude extract. Of the affinity columns described, MTX–Sepharose and $PteGlu_4$-lysine–Sepharose were clearly the most effective; because of its availability, MTX–Sepharose was chosen for further study.

The TS–DHFR from MTX-resistant *L. tropica* was purified by sequential DEAE-Sepharose chromatography and MTX–Sepharose affinity chromatography (Table I). Early in this work DEAE-Sepharose chromatography was performed with a linear KCl gradient; both enzyme activities coeluted at about 0.07 M salt, and pooled fractions were about 8-fold purified. Subsequently, we found it more convenient to elute the enzyme activities with a single salt concentration of 0.15 M KCl. This provided a 3–4-fold purification, which was sufficient to provide homogeneous enzyme after affinity chromatography on MTX–Sepharose. The highly purified TS–DHFR exhibited a single band on $NaDodSO_4$–PAGE (Figure 2) and a single component upon isoelectric focusing under both denaturing and nondenaturing conditions. The isoelectric point of the native protein was 6.4 ± 0.1 (three determinations).

At concentrations of at least 0.2 mg/mL, purified preparations of TS–DHFR could be stored in buffer B containing 40% glycerol at −20 °C for at least 1 month without loss of either activity; with lower concentrations of enzyme, BSA (0.5 mg/mL) was added to maintain full activity. When the purified protein was stored at 4 °C, there was a 40–50% loss of DHFR activity and a 70–80% loss of TS activity after 1 month; BSA (0.5 mg/mL) afforded some protection, especially for dilute solutions of protein.

Serine Transhydroxymethylase. Crude extracts of wild-type and MTX-resistant *L. tropica* possessed 0.003 and 0.001 unit/mL serine transhydroxymethylase, respectively. The enzyme coeluted with TS–DHFR from DEAE-Sepharose upon

Table II: Amino Acid Composition of *L. tropica* TS-DHFR

amino acid	residues/dimer	amino acid	residues/dimer
Asx	89.1	Met	29.6
Thr	55.1	Ile[b]	38.2
Ser	58.1	Leu[b]	105.1
Glx	117.8	Tyr	24.3
Pro	52.9	Phe	37.9
Gly	79.6	His	16.0
Ala	104.4	Lys	53.5
Cys[a,b]	6.1	Arg	60.7
Val[b]	49.8	Trp[c]	9.6

[a] Determined as cysteic acid. [b] Data obtained from 72-h hydrolysates. [c] Obtained from protein hydrolysis in 4 M methanesulfonic acid.

elution with 0.15 M salt but was separated from TS-DHFR during MTX-Sepharose chromatography.

Native and Subunit Molecular Weights of L. tropica TS-DHFR. NaDodSO$_4$-PAGE of the purified TS-DHFR showed a single protein band (over 99% of total protein by densitometric scanning) with a molecular weight of 56 200 ± 200 (eight determinations with 8.5%, 9%, 10%, and 12.5% polyacrylamide gels). A single radioactive peak corresponding to a molecular weight of 56 400 was observed upon NaDodSO$_4$-PAGE of the complex formed with [^{3}H]FdUMP and CH_2-H$_4$folate. Gel filtration of the purified protein on Bio-Gel P-300 resulted in coelution of TS and DHFR activities in a single peak that corresponded to an apparent molecular weight of 150 000 and a Stokes radius of 4.4 nm. Glycerol density gradient centrifugation of the purified TS-DHFR indicated a sedimentation coefficient of 6.0 S. Using the data and a partial specific volume of 0.734 cm^3/g determined as described below, we calculated a molecular weight of 110 000 and a frictional coefficient of 1.40 (Siegel & Monty, 1966).

Amino Acid Analysis. The amino acid composition of *L. tropica* TS-DHFR is given in Table II. Using the nearest integer of the experimentally determined values, we calculated that the native protein has 988 amino acids and a molecular weight of about 108 800. The extinction coefficient calculated from the tyrosine and tryptophan content (Edelhoch, 1967) was ϵ_{280} = 87 600 M^{-1} cm^{-1}, which agreed with the value of 83 600 M^{-1} cm^{-1} determined by the method of Scopes (1974). From the amino acid composition the partial specific volume for TS-DHFR was calculated to be 0.734 cm^3/g (Cohn & Edsall, 1943).

Interaction of TS-DHFR with MTX and FdUMP. MTX was a potent inhibitor of DHFR activity and FdUMP was a potent inhibitor of TS activity in the bifunctional protein; both compounds exhibited patterns characteristic of stoichiometric inhibition (Ackerman & Potter, 1949). However, MTX did not inhibit TS and FdUMP did not inhibit DHFR. When 1.0 μM MTX was added to on-going reactions, DHFR was rapidly inhibited, but the rate of dTMP formation was unchanged. Similarly, in the presence of 25 μM CH_2-H$_4$folate, adding 2.0 μM FdUMP rapidly inhibited TS but did not affect the rate of DHFR-catalyzed reduction of H$_2$folate.

The inhibitory properties of MTX and FdUMP were analyzed by the method reported by Cha (1975). This method requires determining the concentration of inhibitor necessary for 50% inhibition (I_{50}) at several concentrations of enzyme (E_t) and, from eq 1, permits approximations of both the K_i

$$I_{50} = 0.5E_t + K_i \quad (1)$$

of the inhibitor and the stoichiometry of binding. The I_{50} values for MTX inhibition of DHFR in the presence of 0.10 mM NADPH were determined under standard assay conditions with 0.32–1.6 nM TS-DHFR; similarly, I_{50} values for FdUMP inhibition of TS in the presence of 0.24 mM CH_2-H$_4$folate were determined with 2.0–6.0 nM bifunctional protein. Plots of I_{50} values (n = 5) of the inhibitors vs. the concentration of TS-DHFR gave K_i values of 0.13 ± 0.04 nM for MTX and 0.3 ± 0.1 nM for FdUMP; the stoichiometry determined from the slopes of such plots indicated that 0.98 mol of MTX and 1.44 mol of FdUMP were bound per mole of dimeric enzyme. The stoichiometry of binding was also directly determined by forming the [^{3}H]MTX-enzyme and [^{3}H]FdUMP-Ch-H$_4$folate-enzyme complexes, as described under Materials and Methods, and then isolating the complexes by Sephadex G-25 chromatography at 4 °C. With a crude extract, 316 pmol of [^{3}H]FdUMP and 163 pmol of [^{3}H]MTX were bound per milligram of protein. After the protein was purified to homogeneity, 2.20 mol of [^{3}H]FdUMP and 1.05 mol of [^{3}H]MTX were bound per mole of enzyme.

The rates of dissociation of MTX and FdUMP from purified TS-DHFR were determined by a previously described method (Santi et al., 1974). In separate solutions, [^{3}H]MTX-enzyme and [^{3}H]FdUMP-CH_2-H$_4$folate-enzyme complexes were formed as described under Materials and Methods. A 500-fold excess of unlabeled inhibitor was added to each solution and the solutions were incubated at 25 °C. At intervals, aliquots were removed and assayed for enzyme-bound [^{3}H]FdUMP by the nitrocellulose-binding assay or enzyme-bound [^{3}H]-MTX by gel filtration. In each case, loss of bound radioactivity was first order for at least 3 half-lives; FdUMP dissociated with $k \simeq 0.0056$ min^{-1} and MTX dissociated with k = 0.033 min^{-1}.

Inactivation of TS-DHFR by NEM and Protection by Substrates. Treatment of TS-DHFR with NEM resulted in a time-dependent loss of both activities. In a typical experiment, the enzyme was incubated with the specified amount of NEM in 50 mM Tes (pH 7.4), 1 mM EDTA, and 0.5 mM DTT; at various time intervals, 50-μL aliquots were removed and added to 0.95 mL of the standard assay mixture, and the initial velocity was determined. With 15 units/mL TS and 0.2 mM NEM, there was a first-order loss of enzyme activity with $t_{1/2}$ = 9 min. When 1.0 μM dUMP was included in the incubation mixture there was no loss in activity for as long as 15 min. Similarly, treatment of 60 units/mL DHFR with 0.01 mM NEM resulted in a first-order loss of DHFR activity with $t_{1/2}$ = 17 min. The presence of 1.0 μM NADPH afforded complete protection for up to 30 min; with 1.0 μM H$_2$folate, 85% of the activity remained after 30 min. Treatment of the FdUMP-CH_2-H$_4$folate-enzyme complex with 0.01 mM NEM for 30 min resulted in a 90% loss in DHFR activity, which was identical with a control in which the enzyme was not bound to FdUMP.

Kinetic Properties. Kinetic constants of the *L. tropica* TS-DHFR were determined from double-reciprocal plots in experiments in which the nonvaried substrate was kept at a fixed, saturating concentration. For DHFR, apparent K_m values for H$_2$folate and NADPH were 1.5 ± 0.1 μM and 2.7 ± 0.1 μM, respectively, at pH 7.0 and 25 °C in the presence of 100 μM fixed substrate. For TS at pH 7.8 and 25 °C, the apparent K_m for dUMP was 4.7 ± 0.9 μM with 1.7 mM CH_2-H$_4$folate and the K_m for (6R)-L-CH_2H$_4$folate was 35 ± 4 μM with 0.1 mM dUMP. The K_m for the diastereomeric mixture of CH_2-H$_4$folate was 65 ± 8 μM, twice that of the natural isomer, indicating that the inactive 6S diastereomer does not significantly inhibit TS. Our most active preparations of the bifunctional enzyme gave V_{max} values of 19 ± 2 μmol min^{-1} mg^{-1} for DHFR and 4.5 ± 0.4 μmol min^{-1} mg^{-1} for TS.

To obtain kinetic parameters of DHFR required for the coupled TS-DHFR assay enzyme (see below), initial velocities

Scheme I

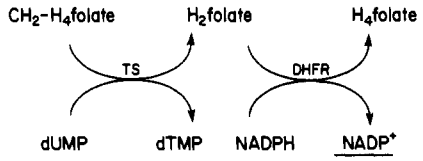

Table III: Coupled TS–DHFR Assays Using Mixtures of TS and DHFR from *L. casei* and the Bifunctional TS–DHFR from *L. tropica*

source	rate (μM min^{-1})		lag time (s)		steady-state H_2folate (μM)	
	TS	DHFR	calcd	exptl	calcd	exptl
L. casei	1.0	9.0	10	12	0.17	0.10
	0.30	3.0	30	35	0.15	0.16
	0.30	1.5	60	65	0.30	0.24
L. tropica	0.11	0.57	63	*a*	0.12	*a*
	0.22	1.1	33	*a*	0.12	*a*
	0.33	1.7	21	*a*	0.12	*a*

[a] Not detectable.

were determined in buffer A (pH 7.8), which contained 100 μM NADPH, 5 mM formaldehyde, and limiting *L. tropica* TS–DHFR or *L. casei* DHFR. For the bifunctional protein, the apparent K_m for H_2folate was 0.6 ± 0.1 μM and V_{max} was 18 ± 1 μmol min^{-1} mg^{-1}. The *L. casei* DHFR showed an apparent K_m for H_2folate of 1.5 ± 0.2 μM and V_{max} = 17 ± 1 μmol min^{-1} mg^{-1}.

Coupled Assay for TS and DHFR. The coupled assay depicted in Scheme I was used to monitor the DHFR-catalyzed reduction of H_2folate formed by TS. With certain assumptions (Easterby, 1973; Rudolph et al., 1979), eq 2 de-

$$[\text{NADP}] = v_1 t + (v_1/v_2)K_m(e^{-v_2 t/K_m} - 1) \qquad (2)$$

scribes the time course of NADP formation. Here, K_m is the Michaelis constant for H_2folate, v_1 is the rate (μM min^{-1}) of TS under conditions of the coupled assay, and v_2 is the rate of DHFR using near-saturating concentrations of substrates. In the experiments described below, extrapolation of the linear portion of plots of NADP to the horizontal axis (K_m/v_2) provided the lag time that precedes attainment of a steady-state concentration of H_2folate, and extrapolation to the vertical axis (v_1K_m/v_2) provided the steady-state concentration of H_2folate.

Early in these studies, we found that CH_2-H_4folate inhibited DHFR of the bifunctional enzyme (K_i = 150 μM) and caused concentration-dependent increases in the lag time of NADP production. To minimize this effect, we used a low concentration of CH_2-H_4folate (30 μM) in the coupled assays. Although this amount was insufficient to saturate TS, the necessary data could be obtained over a period of time where only a small fraction of the cofactor was consumed (<10%), so v_1 closely approximates a zero-order reaction.

The validity of eq 2 under the coupled assay conditions of TS and DHFR was confirmed by using mixtures of the individual enzymes from *L. casei*. In contrast to the bifunctional enzyme, CH_2-H_4folate showed no inhibition of *L. casei* DHFR at concentrations 80-fold higher than that of the substrate H_2folate. With limiting TS, the production of NADP shows a lag as expected of two noninteracting enzymes in a coupled assay system; this closely corresponds to the curve simulated from eq 2 (Figure 3A). As shown in Table III, both the lag times of NADP formation and steady-state concentrations of H_2folate agree with those calculated from eq 2 by using K_m = 1.5 μM for H_2folate and specified values for v_1 and v_2. The

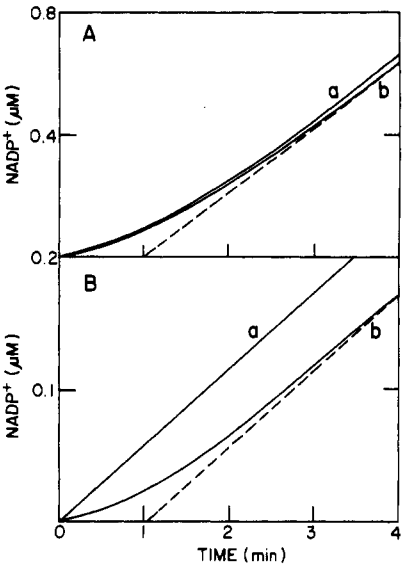

FIGURE 3: Experimental and simulated time courses for NADP formation in the TS–DHFR coupled assay using the enzymes from *L. casei* and *L. tropica*. Reactions were performed as described under Materials and Methods, and simulations were computer generated from eq 2. (A) Curve a is the observed time course using 0.30 μM min^{-1} TS and 1.5 μM min^{-1} DHFR from *L. casei*. Curve b is the time course simulated from the aforementioned values, and K_m = 1.5 μM. (B) Curve a is the observed time course using the bifunctional enzyme from *L. tropica*, which had 0.11 μM min^{-1} TS and 0.57 μM min^{-1} DHFR; curve b is the time course simulated from these values and K_m = 0.6 μM.

results also show that we were able to detect lag times as low as 12 s and steady-state concentrations of H_2folate as low as 0.10 μM.

Figure 3B shows the experimental and simulated time course of NADP production in the coupled assay using a preparation of the *L. tropica* bifunctional protein that had a TS rate (v_1) of 0.11 μM min^{-1} and a DHFR rate (v_2) of 0.57 μM min^{-1}. When K_m = 0.6 μM for H_2folate was used and inhibition of DHFR by CH_2-H_4folate was ignored, eq 2 predicts a 63-s lag time and 0.12 μM steady-state concentration of H_2folate. However, no lag in NADP production was observed. Since we could experimentally detect lag times as low as 12 s, this result indicates that at least 80% of the H_2folate produced by TS in the bifunctional protein is channeled to DHFR faster than it is released into the medium. Similar experiments that used higher concentrations of the bifunctional TS–DHFR resulted in expected increases in the steady-state rate of NADP production, but lag periods and H_2folate could not be detected (Table III). If inhibition of *L. tropica* DHFR by CH_2-H_4folate is taken into consideration, the simulated lag periods and steady-state concentrations of H_2folate would be even greater than the values used here.

TS–DHFR from Wild-Type L. tropica. It has been previously reported that TS–DHFR from wild-type and MTX-resistant *L. tropica* is inhibited to the same extent by MTX (Coderre et al., 1984). For additional characterization, TS–DHFR from wild-type *L. tropica* was purified as described for the MTX-resistant organisms except that a linear gradient of 0–0.2 M KCl was used in the DEAE-Sepharose step. From 4 × 10^{10} cells possessing 8.9 units of DHFR and 4.0 units of TS per milligram of protein, the enzyme was purified 1500-fold and obtained in 46% yield. The purified protein had specific activities similar to those of purified TS–DHFR from MTX-resistant organisms, eluted from gel filtration chromatography with an apparent molecular weight of 150 000, and showed a single band on NaDodSO$_4$–PAGE corresponding to a mo-

lecular weight of 56000. The K_m (1.0 ± 0.2 μM) and V_{max} (23 μmol min^{-1} mg^{-1}) of H_2folate at pH 7.0, as well as the rate of dissociation of the [^{3}H]MTX–enzyme complex (k = 0.038 min^{-1}), were similar to the values obtained for TS–DHFR from MTX-resistant cells.

Discussion

TS and DHFR have been shown to exist as a bifunctional protein in diverse species of protozoa (Ferone & Rowland, 1980; Garrett et al., 1984). We have initiated investigations of this unusual protein in protozoa that are pathogenic to man. However, in most cases, studies of TS–DHFR from such organisms are limited by the amount of protein that can be obtained; the TS–DHFRs are present in relatively low abundance, and it is impractical to obtain large quantities of pathogenic protozoa. Recently, we developed a MTX-resistant strain of *L. tropica* promastigotes that, by DNA amplification, overproduces TS–DHFR (Coderre et al., 1983). The bifunctional protein represents about 1% of the soluble protein in these organisms, and milligram amounts of purified protein can be obtained from a few liters of organisms grown in tissue culture. A comparison of kinetic parameters of DHFR, MTX binding, and native and subunit molecular weights of TS–DHFR from resistant and parent cell lines revealed no differences; unless otherwise specified, we used the protein from MTX-resistant *L. tropica*.

The bifunctional TS–DHFR from MTX-resistant *L. tropica* has been purified to homogeneity by ion-exchange chromatography on DEAE-Sepharose and affinity chromatography on MTX–Sepharose. As indicated in Table I, only an 80-fold purification was required, and the homogeneous protein was obtained in about 70% yield. By use of a similar procedure, TS–DHFR from wild-type organisms was purified 1500-fold and obtained in 46% yield. Two additional affinity chromatography systems were effective in purifying TS–DHFR: the bifunctional protein bound avidly to $PteGlu_4$-lysine–Sepharose and Cibacron Blue–agarose and, after elution of nonspecifically bound proteins, was obtained by biospecific elution with H_2-folate. The major difficulty encountered in purifying TS–DHFR was loss of enzyme activities, particularly TS, which occurred upon storage of cells or crude extracts and during purification. We attributed this problem in part to proteolysis and were able to circumvent it to a great extent by using freshly harvested cells, including protease inhibitors during cell lysis, and performing the purification without interruption. When the purified protein was used, it was subsequently found that TS but not DHFR activity was extremely sensitive to a variety of endopeptidases as well as carboxypeptidase A (unpublished results).

The purified protein migrated as a single band on native isoelectric focusing gels with pI = 6.4 and on $NaDodSO_4$–PAGE with an apparent molecular weight of 56200. The molecular weight of the native enzyme appeared to be 150000 as determined by gel filtration chromatography. However, using the Stokes radius, sedimentation coefficient, and partial specific volume, we calculated a molecular weight of 110000, which agrees with the value of 108800 calculated from the amino acid composition. These parameters closely resemble those reported for the bifunctional protein from *C. fasciculata* (Ferone & Rowland, 1980) and demonstrate that the *L. tropica* TS–DHFR is a dimer of subunits with identical size. In addition, the homogeneous protein showed a single band upon isoelectric focusing under denaturing conditions, demonstrating that the subunits also have identical charge. The probability is very low that two different subunits of the protein would have both identical molecular weights and charge (O'Farrell, 1975), and it is reasonable to conclude that the subunits are identical, each possessing TS and DHFR. Although not proven, a similar arrangement of subunits is probably common to the bifunctional TS–DHFR in other protozoa. Preliminary attempts to sequence the N-terminal region of *L. tropica* TS-DHFR have not yielded derivatized amino acids (unpublished results), suggesting that N-terminal amino acids of the subunits may be blocked.

It has been suggested that additional enzymes might be associated with the TS–DHFR of protozoa (Ferone & Rowland, 1980). In particular, serine transhydroxymethylase would be an attractive candidate because it catalyzes the conversion of H_4folate to CH_2-H_4folate and completes the cycle necessary for continuing dTMP synthesis. However, *L. tropica* TS–DHFR and serine transhydroxymethylase are readily separable upon MTX–Sepharose chromatography, and if an association does exist, it must be weak. Further, serine transhydroxymethylase levels are not increased in MTX-resistant cells, indicating that the gene that codes for this protein is not present in the amplified region of DNA that possesses the TS–DHFR gene.

FdUMP was a potent inhibitor of TS activity in the bifunctional protein, showing an apparent K_i of 0.3 nM. As with TS from other sources, covalent [^{3}H]FdUMP–CH_2-H_4-folate–enzyme complexes were formed that could be isolated and quantitated and were stable upon $NaDodSO_4$–PAGE. Similarly, MTX was a potent inhibitor of DHFR activity with an apparent K_i of 0.1 nM, and [^{3}H]MTX–enzyme complexes were formed that were sufficiently stable for isolation and quantitation. Kinetic analysis indicated that about 1.5 mol of FdUMP was bound per mole of enzyme, but direct binding experiments that used [^{3}H]FdUMP as an active-site titrant for TS clearly demonstrated that 2 mol of inhibitor was bound per mole of TS–DHFR. In contrast, kinetic analysis and binding experiments demonstrated that only 1 mol of MTX was bound per mole of dimeric enzyme. We interpret this result to indicate that two DHFR active sites interact in such a manner that when one is bound to MTX, the other is both catalytically incompetent and unable to bind to MTX.[2] Whether an interaction between the DHFR sites is important in the catalytic reaction was not revealed by the kinetic experiments performed in the present study.

Studies of TS from a variety of sources revealed that a cysteine residue is essential in catalysis; an early event in the reaction involves attack of a sulfhydryl group of the enzyme at the 6-position of dUMP, which activates the 5-position for reaction with the cofactor, CH_2-H_4folate [for a review, see Santi & Danenberg (1984)]. This aspect of the mechanism has been verified by studies of the interaction of TS with the mechanism-based inhibitor, FdUMP, which forms a covalent FdUMP–CH_2-H_4folate–TS complex in which the 6-position of the inhibitor is covalently attached to the catalytic cysteine residue [for a review, see Maley et al. (1984)]. As shown here, treatment of the bifunctional protein with the sulfhydryl reagent NEM resulted in rapid inactivation of TS activity that was prevented by the substrate dUMP. By analogy to what is known of the enzyme from other sources, it is reasonable to assume that a cysteine residue of the bifunctional enzyme serves as a nucleophilic catalyst in the TS reaction and is directly involved in forming the covalent FdUMP–CH_2-H_4-

[2] A reviewer has pointed out the formal possibility that the native protein could possess a single DHFR site formed at the junction of the two subunits; this would require a heterologous interaction of the subunits in a manner that would preclude further polymerization (Monod et al., 1965; Steitz et al., 1973).

folate–enzyme complex. Cysteine residues are also found in a number of DHFRs, but available evidence indicates that they do not play a direct role in catalysis (Williams & Bennett, 1977; Blakley, 1984). The DHFR activity of the bifunctional protein was also inactivated by NEM. This inactivation also occurred with the FdUMP–CH_2-H_4folate–enzyme complex, demonstrating that the susceptible sulfhydryl group in DHFR is distinct from that in TS. Although NADPH and, to a lesser extent, H_2folate protected DHFR from inactivation, it is not known whether the modified cysteine is within the active center and is directly protected by substrate binding or whether substrate binding causes a conformational change that results in protection of a thiol remote from the active center. Nevertheless, both DHFR and TS domains of the bifunctional protein appear to possess at least one cysteine residue that is essential for catalytic activity.

One of the possible biological advantages of the bifunctional TS–DHFR is channeling of the H_2folate produced in the TS reaction to the substrate binding site of DHFR. To test this, we used a coupled assay system containing all substrates except H_2folate and monitored the formation of NADP that occurred upon DHFR-catalyzed reduction of the H_2folate generated in situ. Equations that describe the kinetics of a coupled system containing two enzymes predict that channeling of H_2folate would be manifested by a decrease in its expected steady-state concentration and a resultant decrease in the lag period that precedes linearity of the rate of the second enzyme, DHFR. As a model system, mixtures of the individual TS and DHFR from *L. casei* were used to verify that these enzymes followed the expected kinetic behavior of two separate sequential enzymes under the conditions of the coupled assay; both lag times for NADP production and steady-state concentrations of H_2folate closely matched predicted values for two noninteracting enzymes in a coupled assay. In contrast, under similar conditions with the *L. tropica* bifunctional TS–DHFR there was no observable lag time preceding NADP production and H_2folate was undetectable. Since we could experimentally detect lag times as low as 12 s and predicted lag times were as high as 63 s for the bifunctional assay, we conclude that at least 80% of the H_2folate produced by TS was channeled to DHFR faster than it was released into bulk solvent.

Although unlikely, TS and DHFR might share a common folate binding site. However, when TS activity was completely inhibited by formation of the FdUMP–CH_2-H_4folate–enzyme complex, DHFR remained active. Conversely, complete inhibition of DHFR by MTX did not significantly affect TS activity. Together with the aforementioned disproportionate sensitivity of TS toward proteolysis, these results clearly demonstrate that TS and DHFR have autonomous binding sites.

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Registry No. H_2folate, 4033-27-6; NADPH, 53-57-6; dUMP, 964-26-1; (6*R*)-L-CH_2-H_4folate, 31690-11-6; MTX, 59-05-2; FdUMP, 134-46-3.

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Purification and Properties of a Novel Nucleolar Exoribonuclease Capable of Degrading both Single-Stranded and Double-Stranded RNA†

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ABSTRACT: A ribonuclease that hydrolyzes either linear duplex or single-stranded RNA in an exonucleolytic manner has been partially purified from Ehrlich ascites tumor cell nucleoli and is free from other ribonucleases. The enzyme will also degrade the RNA complement of an RNA·DNA duplex; however, no nuclease activity is observed on linear duplex or single-stranded DNA. The exonuclease acts on RNA nonprocessively from the 3′ end releasing 5′-mononucleotides. The enzyme has a broad pH optimum around pH 8.0, requires Mg^{2+} or Mn^{2+} (0.06 mM) for optimum activity, and is sensitive to ethylenediaminetetraacetic acid and *N*-ethylmaleimide inhibition. Monovalent cations including K^+, Na^+, and NH_4^+ are inhibitory. Gel filtration studies of this enzyme gave a Stokes radius of 40 Å. Sedimentation velocity measurements in glycerol gradients yield a $s_{20,w}$ of 6.0 S. From these values a native molecular weight of 100 000 was calculated. Copurification of the single- and double-stranded activities, identical reaction requirements, and identical heat-inactivation curves strongly suggest that both activities reside with the same enzyme.

The mouse ribosomal RNA system has proven extremely useful for studies of ribosomal RNA transcription and processing both in vivo and in vitro. These studies have included specific transcription of cloned mouse rRNA genes (Miller & Sollner-Webb, 1981; Grummt, 1981; Bach et al., 1981; Grummt et al., 1982; Mishima et al., 1982; Cizewski & Sollner-Webb, 1983), identification of control regions important for rRNA synthesis, (Miller & Sollner-Webb, 1981; Grummt, 1981; Bach et al., 1981; Grummt et al., 1982; Mishima et al., 1982; Cizewski & Sollner-Webb, 1983), definition of the type and order of processing events occurring during maturation of rRNA precursors (Hamada et al., 1980; Michot et al., 1982, 1983; Walker et al., 1982; Bachellerie et al., 1983; Bowman et al., 1983; Goldman et al., 1983), and elucidation of the various steps on the route to synthesis of a functional rRNA (Bowman et al., 1983; Goldman et al., 1983; Eichler & Eales, 1983; Lasater & Eichler, 1984). However, despite the extensive effort that has gone into the study of rRNA metabolism, relatively little is yet known about the enzymes actually involved in the various processing steps or in the turnover of discarded RNA sequences.

In our initial studies of ribonucleases associated with mouse nucleoli, we have used a variety of substrates in an attempt to define the multiplicity of nucleases present. Thus far we have identified four distinct nucleolar ribonucleases. Two of these enzymes were single-strand specific endonucleases which we have designated nucleolar RNase I and II, respectively (Eichler & Tatar, 1980; Eichler & Eales, 1982; Eichler & Eales, 1983). A third single-strand specific activity, nucleolar RNase III, was recently shown to be a 5′ → 3′ exoribonuclease (Lasater & Eichler, 1984). In this paper, we report on a nucleolar exoribonuclease that acts on both single-stranded and double-stranded RNA and describe its purification as well as some of its properties which differentiate and distinguish this novel ribonuclease.

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Materials and Methods

Chemicals. Labeled [^{3}H]CDP (16 Ci/mmol) and [^{3}H]ADP (16 Ci/mmol) were obtained from Amersham. Hydroxylapatite was purchased from Bio-Rad Laboratories, DEAE-cellulose 52 was from Whatman, and Sephacryl S-200 and DEAE Sephadex A-25 were from Pharmacia.

Enzymes. Polynucleotide phosphorylase was purchased from P-L Biochemicals. T_4 RNA ligase, T_4 terminal transferase, and bacterial alkaline phosphatase were purchased from Bethesda Research Laboratories.

RNAs. ^{32}P-Labeled f_2RNA was prepared essentially according to the procedure of Glitz (1971) for MS2 RNA. The specific radioactivity was 3×10^4 cpm/nmol. [^{3}H]Poly(C) (specific activity 2×10^3 cpm/nmol) and [^{3}H]poly(A) (specific activity 9×10^3 cpm/nmol) were synthesized with polynucleotide phosphorylase according to the procedure of Moses & Singer (1970). [^{3}H]Poly(U) (specific activity 2×10^3 cpm/nmol) was purchased from Miles Chemical Corp. [^{3}H]Poly(C)·poly(I) and [^{3}H]poly(A)·poly(U) were prepared as described by Eichler & Tater (1980). Double-labeled poly(C) was prepared as follows: [^{3}H]poly(C) (14×10^3 cpm/nmol) was treated with bacterial alkaline phosphatase to remove terminal phosphate residues and then purified by phenol extraction and concentrated by ethanol precipitation. The 3′-^{32}P labeling was accomplished following the procedure of Peattie (1979) using T_4RNA ligase and [5′-^{32}P]pCp (2.5×10^8 cpm/nmol). The double-labeled [3′-^{32}P,^{3}H]poly(C) was purified by phenol extraction, ethanol precipitation, and spun-column chromatography (Maniatis et al., 1982). [3′-

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