Isolation and Characterization of a Mutant Dihydrofolate Reductase-Thymidylate Synthase from Methotrexate-resistant *Leishmania* Cells*

Rosalia Arrebola‡§, Asuncion Olmo‡§, Pedro Reche‡¶, Edward P. Garvey‖, Daniel V. Santi**, Luis M. Ruiz-Perez‡ ‡‡, and Dolores Gonzalez-Pacanowska‡

*From the ‡Instituto de Parasitología y Biomedicina "Lopez-Neyra," Consejo Superior de Investigaciones Científicas, 18001-Granada, Spain, the ‖Wellcome Research Laboratories, RTP, North Carolina 27709, and the **Departments of Biochemistry and Biophysics and Pharmaceutical Chemistry, University of California, San Francisco, California 94143*

The MTX-resistant *Leishmania major* promastigote cell line D7BR1000 displays extrachromosomal amplified R-region DNA, which contains the gene for dihydrofolate reductase-thymidylate synthase (DHFR-TS) (Garvey, E. P., and Santi, D. V. (1986) *Science* 233, 535–540). Now we report that these methotrexate (MTX)-resistant cells also possessed a structurally altered DHFR-TS. We have performed the cloning, expression, and characterization of the altered DHFR-TS gene. The DNA sequence of the altered DHFR-TS gene revealed a single base change in position 158 which resulted in the substitution of a methionine in position 53 of DHFR for an arginine. Steady-state measurements of the purified recombinant enzyme indicated that the mutation did not cause significant modifications in the K_m for DHFR or TS substrates but lowered the k_{cat} by 4-fold. Of greater interest, there was a modification in the effect on MTX inhibition of DHFR. The initial inhibition complex appeared to have been unaffected by the alteration, but the subsequent slow-binding step of inhibition in the wild-type enzyme is absent in the altered enzyme. Consequently, the overall K_i for MTX was 30-fold greater for the mutant than for the wild-type enzyme. Transfection of *L. major* with the mutant DHFR-TS gene gives parasites that are capable of growing in medium containing 10 mM methotrexate, showing that the altered DHFR gene is in itself capable of conferring MTX resistance in *Leishmania*.

Dihydrofolate reductase (DHFR)[1] (EC 1.5.1.3) and thymidylate synthase (TS) (EC 2.1.1.45) act sequentially in the *novo* synthesis of pyrimidines. In parasitic protozoa, both DHFR and TS exist as a bifunctional protein ranging in size from 110 to 140 kDa, with subunit sizes of 55–70 kDa (1–3). The importance of DHFR to the biochemistry of the cell and in the treatment of a variety of diseases has made this enzyme the focus of numerous studies on its structure and function. In recent years, the crystal structures of DHFR from several prokaryotic and eukaryotic organisms have been determined (4–8). The large data base of structural information on DHFR coupled with the technique of site-directed mutagenesis has allowed researchers to investigate how the structure of the enzyme is related to its function and inhibition by anti-folates. In the case of *Leishmania major* or any other protozoal parasite, the crystal structure of DHFR-TS is yet to be determined, so an understanding of the structure-function relationships in *L. major* DHFR can only be achieved through the analysis of mutants with altered catalytic and inhibition properties and homology comparisons with other DHFRs. Such mutants may be isolated from MTX-resistant *L. major* cells or may be engineered via site-directed mutagenesis.

Methotrexate (MTX) is an extremely potent DHFR inhibitor which is often used as an antiproliferative agent. The binding of MTX to DHFR is characteristic of a class of inhibitors that form an initial complex which isomerizes slowly to a tighter complex and are referred to as "slow, tight-binding" inhibitors (9–11).

Considerable effort has been dedicated to the understanding of the biochemical basis for the selectivity of MTX and for the development of cellular resistance to the drug (12). It has been well established that several individual or concurrent mechanisms can be responsible for resistance: amplification of the DHFR gene, alteration in the transport of the drug into the cell, and expression of an altered DHFR protein (for reviews, see Refs. 13 and 14). Such studies have engendered insight into the relationship between DHFR structure and catalytic function and provided tools for molecular genetic studies. In the particular case of *Leishmania*, resistance to methotrexate can be attained by amplification of the gene for DHFR-TS contained in an extrachromosomal DNA called R-region DNA (15), reduction in the permeability of cells to the drug (16, 17), or by amplification of the chromosomal H region as extrachromosomal circles (17–21).

We have explored further the question of MTX resistance in *Leishmania* and report here that the previously characterized

[1] The abbreviations used are: DHFR, dihydrofolate reductase; TS, thymidylate synthase; WT rLMDT, wild-type recombinant *L. major* dihydrofolate reductase-thymidylate synthase; M53R rLMDT, Met-53 to Arg mutant recombinant *L. major* dihydrofolate reductase-thymidylate synthase; MTX, methotrexate (4-amino-4-deoxy-10-methylfolic acid); pE1, expression plasmid for WT rLMDT; pD7BE1–20, expression plasmids for M53R rLMDT; H_2folate, dihydrofolate; CH_2-H_4folate, methylene tetrahydrofolate; FdUMP, 5-fluoro-2'-deoxyuridine monophosphate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-ethanesulfonic acid.

resistant cell line D7BR1000[2] contains an altered DHFR-TS. This finding represents the first description of MTX resistance in *Leishmania* mediated by a mutation in the target enzyme. We describe the cloning, sequencing, and expression of the DHFR-TS gene from this cell line and have identified the mutation Met-53 to Arg as being responsible for MTX resistance. An expression system for the altered protein has been developed and studies on some structural and kinetic properties of the protein have been performed and compared with those of the wild-type recombinant enzyme. Experiments are also presented showing that transfection of *L. major* with the mutant gene confers high levels of methotrexate resistance to cells.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, Taq Polymerase, T4 DNA ligase, and Random Priming kit were purchased from Boehringer Mannheim Biochemicals. Methotrexate (4-amino-4-deoxy-10-methylfolic acid) was obtained from the Lederle Parentals, Inc. Folic acid, H_4folate, dUMP, FdUMP, protease inhibitors, β-NADPH, and all buffers were from Sigma. Dihydrofolate was prepared from folic acid by the method of Blakley (22). DNA sequencing was carried out using the Sequenase Version 2.0 from U. S. Biochemical Corp. Reagents and protein standards for SDS-PAGE and IEF were from Bio-Rad and Serva Feinbiochemical. [6-^{3}H]FdUMP (23 Ci/mmol) and Hybond-N membranes for Southern blotting were from Amersham Corp. [3',5',7-^{3}H]MTX, sodium salt (32.7 Ci/mmol), was purchased from Du Pont-NEN® Research Products. MTX-Sepharose was prepared by coupling MTX to aminohexyl-Sepharose CL-6B according to the method of Bethell (23). Expression vector pKK223-3 and Ampholineγ PAG plates pH 5.5–8.5 for IEF were purchased from Pharmacia LKB Biotechnology Inc.

Selection and Cloning of MTX-resistant L. major and Cell Transfection—L. major promastigotes were derived from a clone (D7B) isolated from strain 252, Iran (S. Meshnik). Organisms were grown at 26 °C in M199 medium (Life Technologies Inc.) supplemented with 20% fetal calf serum, 25 mM Hepes, pH 7.4, and, when specified, MTX. MTX-resistant strains of *L. major* promastigotes were obtained by stepwise selection as described for the original MTX-resistant *L. major* (15). Clones of the D7BR1000 line were prepared by agar plating (24) in collaboration with Dr. Buddy Ullman at the Oregon Health Sciences University. For transfection experiments, the wild-type *L. major* 252 and *L. major* WR454 (Walter Reed Army Institute of Research) (25) strains were used. Parasites were transfected by electroporation and selected in M199 medium containing 16 μg/ml G418 (geneticin; Life Technologies Inc.) as described (26). Electroporation was performed with an ECM 600® Electroporation System from BTX Inc. at 1300 microfarads, 2,250 V/cm. Transfected cells were selected for growth in 480 μg/ml G418 and tested for resistance to methotrexate. The EC_{50} is the concentration of MTX (in μM) which decreases cell density by 50% (17). The presence of the plasmid DNA as a circular extrachromosomal element in the transfected cell line was determined by Southern analysis of chromosome gels.

DNA Manipulation Procedures—Total *L. major* DNA was prepared as described (15). Oligonucleotide synthesis was performed at the UCSF Biomolecular Resource Center. PCR was performed in a Perkin-Elmer thermocycler using Taq polymerase. Amplification was performed in reactions containing 0.5 μg of genomic DNA, 25 pmol of each primer, 100 μM of each dNTP, 100 mM Tris-HCl, pH 8.4, 60 mM $MgCl_2$, 500 mM KCl, 200 μg/ml gelatin, and 5 units of enzyme. PCR products were separated by electrophoresis and Southern blot hybridization using the DHFR-TS *L. major* gene as probe confirmed the identity of the correct band. Bands were excised, electroeluted, and cloned in M13 mp18. DHFR sequences were determined using the dideoxy chain termination technique (27); a series of complementary 17- and 19-mer oligonucleotides derived from the DHFR and TS coding regions were used as primers for sequencing single stranded template.

Parasites were embedded in agarose blocks for pulsed field gradient electrophoresis as described (28). Chromosomes from *L. major* were fractionated in a contour-clamped homogeneous electric field apparatus by pulsed-field gradient electrophoresis (29). Running conditions were:

1-s pulses for 30 min followed by a 36-h run with 75-s pulses at 8.5 V/cm and a temperature of 12–15 °C (30). Chromosomes were then transferred to Hybond-N™ membranes and hybridized with a *L. major* DHFR-TS probe as described in standard protocols. The expression plasmids (pD7BE1 to pD7BE20) were constructed by cloning the *EcoRI-Hind*III restriction fragment from different M13 mp18-DHFR-TS clones into the expression vector pKK223-3 (Pharmacia). *Escherichia coli* χ2913 (Thy$^-$) and PA414 (Thy$^-$,DHFR$^-$) (31) cells transformed with the expression plasmids were grown in LB medium containing 50 μg/ml ampicillin. All general DNA manipulations not mentioned were as described (32).

Protein Analysis—*Leishmania* crude extracts, either for determination of DHFR-TS levels or for characterization of the bifunctional protein, were prepared as reported (33). Electrophoretic procedures were performed as previously described (34). Two-dimensional electrophoresis was performed by Protein Database Inc., modifying the procedure of O'Farrell (35). Recombinant M53R and wild-type *L. major* DHFR-TS were isolated and purified as previously described (33, 36). For TS quantifications, covalent TS-[^{3}H]FdUMP·CH_2-H_4 folate complexes were formed as indicated (37). Nondenaturing isoelectric focusing was performed in the precasting polyacrylamide gels above indicated. The pH gradient was determined by electrophoresis of colored protein standards with known isoelectric points (pI = 4.7–10.6 and 3.5–10.5). Electrophoresis was performed in a LKB 2117 Multiphor electrophoresis system at 8 watts for 5000 V-h. Gels were previously focused at 6 watts for 600 V-h. 25 μg of purified protein in a final volume of 5 μl were charged at 2.5 cm from negative electrode to avoid precipitation. After electrophoresis, gels were subsequently fixed and stained as described (33).

The rate of [^{3}H]MTX dissociation from the MTX·NADPH·enzyme complex was determined by: 1) incubating 6 μg (54.5 pmol) of M53R rLMDT or 3.6 μg (32.7 pmol) of WT rLMDT with 100 μM NADPH and 0.1 μM [^{3}H]MTX in 0.6 ml of 50 mM TES, pH 7.4, 2 mM dithiothreitol, and 1 mM EDTA, at 25 °C, for 45 min; 2) initiating dissociation by addition of 50 μM cold MTX; and 3) separating the macromolecular-bound from free [^{3}H]MTX by filtering 100-μl aliquots of the reaction mixture on small columns of Sephadex G-15 by a previously described method (2); the chromatographic separation was performed at 4 °C. To quantitate the complex formed, it was assumed that DHFR-TS binds 1 mol of MTX/mol of dimer (33).

Steady-state kinetic data were obtained with a Hewlett-Packard 8452A Diode Array Spectrophotometer interfaced with a Compaq PC. Data were translated and the computer program KaleidaGraph™ 2.0 was used to analyze data. DHFR and TS specific activities were monitored at 25 °C at 340 nm. The DHFR and TS standard assays were as described (15). To determine the character and extent of MTX inhibition, 0–1000 nM MTX was included, the reaction was then initiated with 4.2 nM M53R rLMDT. The K_i for wild-type enzyme was determined by including 0–30 nM MTX, and initiating the reaction with 0.8 nM wild-type enzyme. H_2folate concentration was kept fixed at 0.1 μM. K_m values for NADPH, H_2folate, CH_2-H_4folate, and dUMP were obtained by varying the substrate at subsaturating concentrations while keeping the other at constant saturating concentrations. Nonlinear regression analysis using Enzkinetic was used to determine both k_{cat} and K_m values.

Concentrations were determined spectrophotometrically using molar extinction coefficients of 28,000 M^{-1} cm^{-1} at 282 nm for H_2folate (22, 38), 6,220 M^{-1} cm^{-1} at 340 nm for NADPH (39) and 22,100 M^{-1} cm^{-1} at 302 nm, pH 13, for MTX (38). The molar extinction coefficients for H_2folate and CH_2-H_4folate utilization by DHFR and TS were 12,300 and 6,400 M^{-1} cm^{-1} at 340 nm, respectively (40). Protein concentrations were obtained either by Bradford determinations or for purified enzyme, using a molar extinction coefficient for DHFR-TS of 87,600 M^{-1} cm^{-1} at 280 nm. All other protein techniques not mentioned were as described (33).

RESULTS AND DISCUSSION

DHFR-TS in D7B Crude Extracts—It has been reported that the properties of DHFR-TS from the *L. major* cell lines R1000 (resistant to MTX) or CB50 (resistant to CB3717) (41) were indistinguishable from those of the enzyme isolated from wild-type cells and that the major mechanism of drug resistance in these cells was gene amplification (18, 41). When compared with levels of DHFR-TS in wild-type cells, *Leishmania* D7B cells resistant to 10, 50, 100, and 1000 μM MTX showed increased levels of the bifunctional protein (Table I). However, the levels of TS, and especially DHFR, did not directly correlate with the concentration of drug to which the cells were resistant

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[2] Resistant cell lines are designated as R followed by the micromolar concentration of drug to which they were resistant, *e.g.* R1000 refers to cells that were resistant to 1000 μM MTX. In addition, the clone name of D7B is used as a prefix to differentiate between the resistant cells described here and the original MTX-resistant cells (15).

TABLE I
DHFR and TS levels in crude extracts from D7B cells resistant to MTX and from wild-type cells

Each value is an average from at least four different preparations of crude extract from the given cell line; each preparation taken from cells at least 10 generations apart. Each determination consists of assaying a crude extract for DHFR activity, TS binding, and protein; and each assay is done at least twice, with the average taken.

Cell type	DHFR	TS	Ratio DHFR/TS
	units/mg	*pmol/mg*	*units/pmol*
Wild-type	8 ± 2	4 ± 2	1.5
R10	40 ± 20	30 ± 10	1.3
R50	35 ± 9	90 ± 20	0.4
R100	10 ± 3	50 ± 30	0.2
R1000	15 ± 5	70 ± 20	0.2

FIG. 1. **Two-dimensional gel electrophoresis of wild-type (A) and D7BR1000 (B) crude extracts and immunoprecipitate of D7BR1000 crude extract (C).** Figures show the upper right-hand corner of the two-dimensional maps, so that the pH gradient runs from approximately 6 to 8, and the molecular mass decreases from the origin to approximately 25 kDa. The *large arrows* point to the spots which represent wild-type and the mutant DHFR-TS. The spot described in the text that is present in the D7BR1000 extract and absent in wild-type extract is directly to the basic side of the wild-type DHFR-TS, at the same molecular weight. The *small arrows* point to the spots which represent other proteins that are increased in D7BR1000.

and the amount of DHFR-TS overproduction. The *L. major* D7BR1000 line overproduced the bifunctional protein DHFR-TS by amplification of a region of DNA called R-region DNA that contains the gene for DHFR-TS (28). D7B R-region DNA was significantly larger than the other DNAs (42 *versus* 30 kilobases) and occurred in a different pattern from that observed in previous resistant cell lines. We have determined by contour-clamped homogeneous electric field electrophoresis and Southern hybridization that the D7B cells resistant to 10, 100, and 1000 μM MTX possessed, respectively, 25-, 15-, and 30-fold increases of the R-region DNA when compared to the wild-type copy number (results not shown). Other MTX-resistant cells (15) and the cells resistant to high levels of CB3717 (41) had shown at least an 85-fold increase in copy number. In addition, when copy number was determined during the selection of CB3717-resistant cells, the level increased proportionately to the concentration of drug to which cells were resistant. Therefore, although DNA amplification had occurred in the MTX-resistant D7B cells, the amount of amplification in cells resistant to 1 mM MTX was significantly less than in resistant cells previously described.

The ratio of DHFR activity to amount of TS (unit:pmol) is a fairly constant number in crude extracts of wild-type, the original MTX-resistant cells, and CB3717-resistant cells (Table I). This ratio is usually between 1.5 and 2.0, but it can be higher due to the lability of TS (33). When we examined D7B cells resistant to 50 μM or higher concentrations of MTX, we found that the ratio of activity of DHFR to amount of TS was significantly lower than the ratio in either D7BR10 or wild-type cells (Table I). Considering these and the DNA results previously mentioned, we were curious to see if DHFR-TS had been altered during the selection process. We analyzed the crude extracts from wild-type and D7BR1000 cells by two-dimensional electrophoresis (Fig. 1). Initially, we located DHFR-TS on the two-dimensional map by immunoprecipitating DHFR-TS from crude extracts of D7BR1000 cells, and then examined wild-type and D7BR1000 whole crude extracts. Extracts from D7BR1000 cells showed a new spot which had a slightly more basic pI relative to the wild-type DHFR-TS (Fig. 1B). The relative intensity of the new spot was approximately 5 times greater than the spot that corresponded to wild-type DHFR-TS (as shown by densitometry). At least two other major new spots also appeared in the D7BR1000 map, relative to the wild-type map: an acidic protein, with an approximate molecular mass of 40 kDa, and a protein with an approximate molecular mass of 45 kDa and pI of about 6.8. These proteins might result from the co-amplification of other genes during the drug selection process but are probably irrelevant to drug resistance since, as shown below, transfection experiments of the DHFR-TS gene into *Leishmania* demonstrate that the enzyme is in itself sufficient to confer resistance of cells to MTX. To summarize, when extracts from MTX-resistant cells were analyzed either for
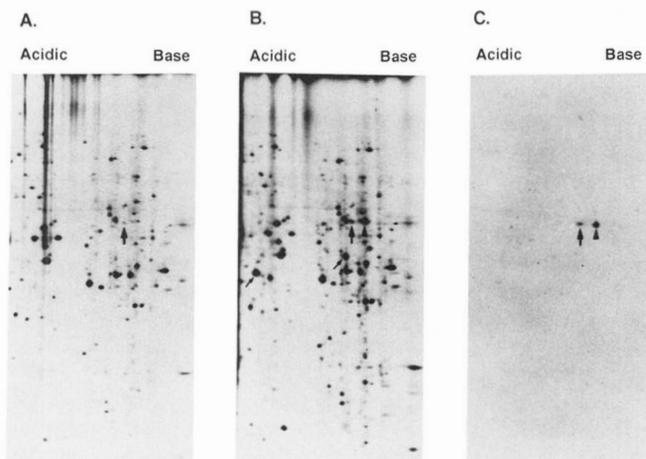
amount of DHFR-TS or for possible structural changes in DHFR-TS, data was consistent with the existence of an altered bifunctional protein.

Cloning and Sequencing of the MTX-resistant DHFR-TS Gene—Several clones of *L. major* D7BR1000 cells were obtained by a previously described procedure (24) and grown in media with 1 mM MTX. DHFR and TS specific activities were measured in crude extracts of these cells and compared with the corresponding values from nonresistant and classical resistant *L. major* (R1000–11) cells (18) (Table II). All of the clones examined presented a decreased DHFR/TS ratio compared to wild-type cells suggesting that an altered DHFR-TS was present in all cases. One clone (C6) presented the most different value of DHFR/TS (0.56) compared to that from wild-type cells (4.4) so we selected this clone for further studies assuming that the probability of isolating an altered DHFR sequence was higher in this case.

Total DNA from the 1 mM MTX-resistant *L. major* D7BR1000-C6 was isolated and used as template in the polymerase chain reaction to amplify DHFR-TS sequences and facilitate isolation of the DHFR-TS gene. Primers complementary to the *L. major* DHFR-TS gene were designed introducing an *Eco*RI and a *Hin*dIII site at the 5' and 3' ends, respectively, for convenient cloning of the PCR products in M13. The correct identity of the 1583-base pair PCR product was verified by hybridization with the DHFR-TS gene from wild-type *L. major*. The PCR product was digested with *Eco*RI and *Hin*dIII, then directionally cloned between *Eco*RI and *Hin*dIII sites in M13 mp18 and transformed in *E. coli* XL1-Blue cells; 20 positive clones were randomly selected for further study. Single and double strand DHFR-TS M13 DNA from each of these clones was isolated, checked by restriction analysis, and the entire DHFR domain insert sequences of the 20 M13 clones were determined. The sequences in 19 clones were identical to the *L. major* DHFR-TS DNA sequence reported by Beverley *et al.* (42), except at position 2 of codon 53 (ATG to AGG) which would cause a methionine to arginine substitution. In one of the clones a second mutation (T instead of C at nucleotide position 607) giving rise to a Thr-202 to Met change in the DHFR domain was also detected. We do not know if this change is due to

TABLE II

DHFR and TS levels in crude extracts from R1000–11, D7B clones resistant to MTX and wild-type cells

L. major strain 252	Clone[a]	Protein concentration	DHFR	TS	DHFR/TS
		mg/ml	nmol/min/mg[b]		
POJ1[c]		7.4	6	1	4.4
R1000–11[d]		9.7	28	13	2.1
D7BR1000[e]	Uncloned	6.0	19	22	0.8
D7BR1000[e]	1	7.0	20	25	0.8
D7BR1000[e]	2	8.4	14	18	0.8
D7BR1000[e]	3	6.8	16	21	0.8
D7BR1000[e]	5	3.7	25	25	1.0
D7BR1000[e]	6	6.4	9	16	0.6
D7BR1000[e]	7	4.5	15	12	1.2

[a] For this work D7BR1000 was cloned again and clone 6 used for further studies.

[b] Values correspond to the average of two determinations.

[c] POJ1 corresponds to a wild-type clone of *L. major* 252 Iran.

[d] R1000–11 corresponds to a *L. major* 252 strain resistant to 1 mM MTX proceeding from an heterogeneous population of cells and cultured in presence of inhibitor for 11 months.

[e] D7BR1000 corresponds to a *L. major* 252 strain resistant to 1 mM MTX but proceeding from a clone of parasites called D7B.

a PCR artifact or if it has any significance in the kinetic properties of the enzyme; preliminary studies of DHFR activity in crude extracts show no significant differences between single and double mutants (data not shown). The TS domain from the PCR clone 5 was also sequenced. Apart from the Met-53 to Arg substitution, there were no differences with regard to the *L. major* DHFR-TS cDNA sequence previously described (42). The fact that all 20 clones sequenced showed the M53R mutation strongly suggests that the D7BR1000 clone origin of these PCR clones contained a predominance of the altered gene with little or no contamination by wild-type.

Expression and Purification of Recombinant DHFR-TS—The expression plasmid containing the DHFR-TS gene was transformed into lac I^Q *E. coli* XL1-Blue hosts and the authenticity of the plasmid constructs was again verified by restriction analysis. All plasmids complemented growth of *E. coli* cells deficient in TS (χ2913) or DHFR and TS(PA414), showing that catalytically active TS and DHFR were expressed. We chose the plasmid pD7BE5 (derived from the M13 clone 5 whose thymidylate synthase sequence had been fully verified) for further studies.

The M53R rLMDT and WT rLMDT in extracts from pD7BE5 and pE1 transformed *E. coli* χ2913, respectively, were purified to apparent homogeneity by MTX-Sepharose affinity chromatography as previously described (33). In both cases the purified bifunctional protein exhibited a single band with $M_r \approx$ 55,000 by SDS-PAGE (Fig. 2). The purified, recombinant bifunctional protein M53R rLMDT expressed in transformed *E. coli* exhibited TS and DHFR average specific activities of 2,000 TS units/mg and 3,500 DHFR units/mg. This is in contrast to the final specific activities of approximately 2,000 TS units/mg and 20,000 DHFR units/mg observed when recombinant WT rLMDT is purified. These specific activities of wild-type enzyme from pE1 were similar to those determined with the best preparation from *Leishmania* cells (33).

Multiple experimental evidence supports the existence of a more basic mutant protein. First, the isoelectric point of M53R and WT proteins was determined by nondenaturing horizontal isoelectrofocusing: native M53R rLMDT and WT rLMDT pI values were 6.6 ± 0.2 and 6.4 ± 0.2, respectively. Second, nondenaturing polyacrylamide gel electrophoresis was performed on the purified mutant protein. A band with a slightly slower migration than wild-type was obtained indicating a shift towards a more positively charged protein (results not shown).

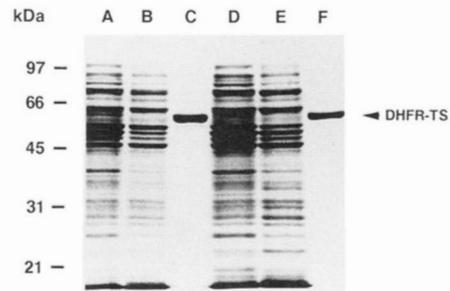*Kinetic Characterization of Purified M53R rLMDT*—The kinetic parameters of M53R rLMDT were measured (Table III).

FIG. 2. **Purification of recombinant M53R and WT DHFR-TS expressed in *E. coli* (pD7BE5 and pE1, respectively), 12,5% SDS-PAGE stained with Coomassie R-250.** *Arrow* indicates DHFR-TS. *Lane A*, pE1-transformed *E. coli*, crude soluble extract; *lane B*, pE1-transformed *E. coli* MTX-Sepharose flow through; *lane C*, pE1-transformed *E. coli* MTX-Sepharose-purified DHFR-TS; *lane D*, pD7BE5-transformed *E. coli*, crude soluble extract; *lane E*, pD7BE5-transformed *E. coli* MTX-Sepharose flow through; *lane F*, pD7BE5-transformed *E. coli* MTX-Sepharose-purified DHFR-TS.

TABLE III

Michaelis constants and steady-state rates for the reactions catalysed by wild-type and M53R rLMDT

Activity	Enzyme	K_m (μM)		k_{cat} (s^{-1})
		NADPH	H$_2$folate	
DHFR	Wild-type	0.9 ± 0.1	1.3 ± 0.3	29 ± 7
	M53R	1.2 ± 0.2	1.6 ± 0.3	7.6 ± 0.8
		dUMP	(6R,S)L-CH$_2$-H$_4$folate	
TS	Wild-type	7.0 ± 0.6	79 ± 5	5.8 ± 0.9
	M53R	6.5 ± 0.9	96 ± 6	5.5 ± 0.7

The K_m and the k_{cat} values of TS substrates were essentially the same for wild-type and mutant DHFR-TS. There were no differences between wild-type and mutant enzyme in the K_m values for NADPH and dihydrofolate, but k_{cat} for M53R rLMDT from D7BR1000 cells was lower than the wild-type enzyme by a factor of 4. This lower turnover number of DHFR partly explains the low ratios of DHFR to TS that were observed in crude extracts. Values of the steady-state kinetic constants obtained for wild-type enzyme in this study were approximately the same than those reported previously (33).

We analyzed the interaction of MTX with DHFR by two different approaches: measuring the rate of dissociation of [^{3}H]MTX from the MTX·NADPH·enzyme complex, and analyzing the progress curves of DHFR activity in the presence and absence of MTX. When we used pure WT rLMDT and measured the rate of MTX dissociation from the ternary complex, the results were similar to those previously measured (33, 36); MTX dissociated with $k = 0.046$ min^{-1} (Fig. 3A). When we used purified M53R rLMDT, we obtained quite different results (Fig. 3B). The radioactivity detected at the initial time point was only 5% of what was expected; and during the first minute after the cold MTX was added, a very rapid loss in radioactivity was observed. We interpreted the rapid drop of radioactivity during the first minute as representing the rate of MTX dissociation from the structurally altered DHFR-TS in the presence of NADPH which was too fast to measure by this assay. Thus, the alteration in DHFR-TS appeared to cause DHFR to bind less tightly to MTX.

MTX inhibition of wild-type *Leishmania* DHFR has been previously reported to have an apparent $K_i = 0.13 \pm 0.04$ nM when analyzed by the method of Cha (33). We further examined the steady-state inhibition patterns from WT rLMDT and M53R rLMDT by progress curve analysis (11). When the DHFR reaction was initiated with wild-type enzyme and the MTX concentration was varied from 0 to 30 nM, a time-dependent decrease in the rate was seen that varied as a function of
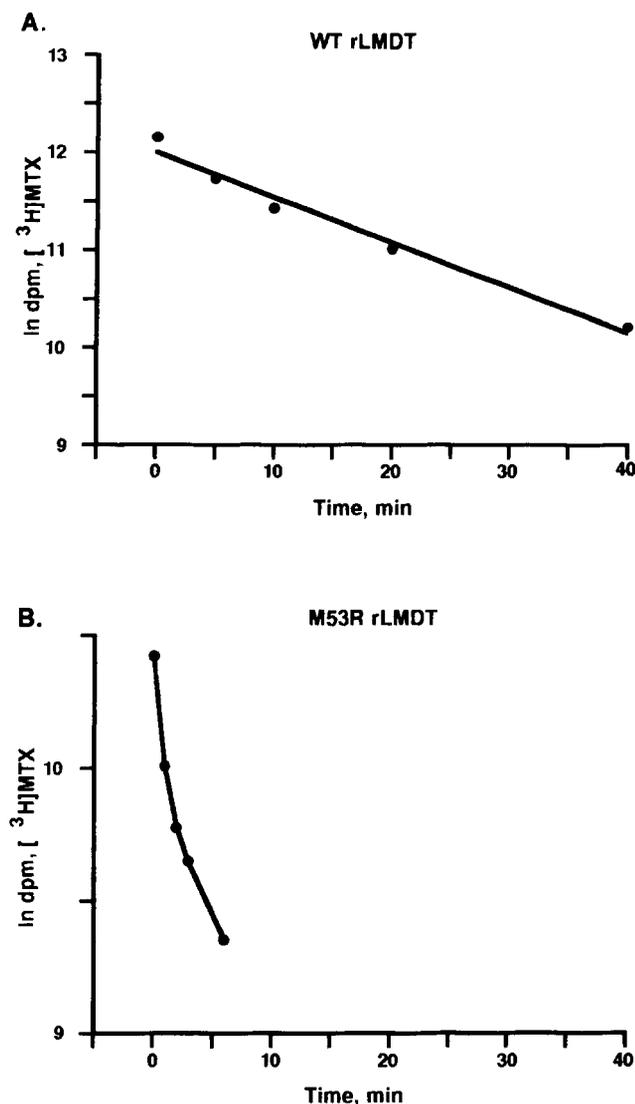
A.

WT rLMDT

A.

WT rLMDT

B.

M53R rLMDT

B.

M53R rLMDT

FIG. 3. **Rates of MTX dissociation from the MTX·NADPH·enzyme complex.** *A*, shows the rate observed when wild-type rLMDT was used. *B*, displays the results when M53R rLMDT purified enzyme was used. The assay is described under "Experimental Procedures."
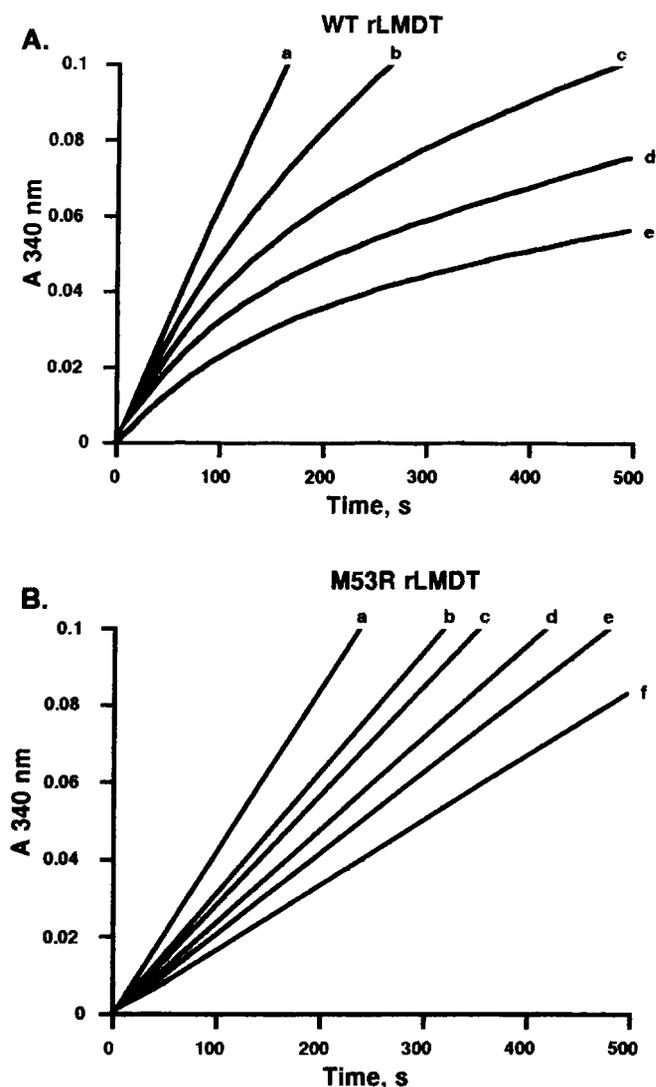
FIG. 4. **Character and extent of DHFR inhibition by MTX.** *A*, progress curves for the slow development of inhibition of wild-type DHFR by MTX. Reactions were started by addition of enzyme (1.6 nM). Concentrations of MTX are as follows: *a*, 0; *b*, 5 nM; *c*, 10 nM; *d*, 15 nM; *e*, 30 nM. *B*, reactions rates for the inhibition of M53R rLMDT by MTX. Reactions were started by addition of enzyme (8.2 nM). Concentrations of MTX were as follows: *a*, 0; *b*, 100 nM; *c*, 200 nM; *d*, 500 nM; *e*, 700 nM; *f*, 1000 nM.

inhibitor concentration (Fig. 4A). The kinetics were characteristic of MTX-DHFR interactions where the initial step involves rapid formation of a weak complex, followed by a slow conversion to the tight-binding complex. We obtained a value for the rate constant of this slow-binding process by assuming it was analogous to enzyme inactivation by a slow, tight-binding inhibitor (11). First, the progress curves were analyzed by assuming that the rates of inactivation reflected a pseudo-first order process; we computer-fitted the data to Equation 1:

$$[NADP] = v_f t - (v_f - v_i)(1-e^{-kt})/k_{obs} \qquad (Eq.\ 1)$$

where v_i and v_f are the initial and final DHFR steady-state rates, and k_{obs} is the pseudo-first order rate constant (43). The reciprocal of the observed pseudo-first order rate constants were plotted *versus* the reciprocal of the MTX concentration, employing Equation 2:

$$1/k_{obs} = (1/k_{slow\ bind}) + (K_i/k_{slow\ bind}[MTX]) \qquad (Eq.\ 2)$$

where K_i is the equilibrium constant for the initial inhibition complex, and $k_{slow\ bind}$ is the rate constant for the slow-binding process of inhibition. The $k_{slow\ bind}$ for the wild-type enzyme was 9.4 min⁻¹ and K_i was 36.6 nM (Table IV): these values were

TABLE IV
Summary of interaction between MTX and wild-type and M53R DHFR

The K_i for the wild-type DHFR was determined by multiplying the K_i for the initial inhibitory complex (36.6 nM) by the ratio of the rates for dissociation and association of the slow-binding complex. The K_i for the M53R enzyme was determined by assuming competitive inhibition kinetics.

Process	Wild-type	M53R
Rate of [³H]MTX dissociation from the ternary complex	0.046 min⁻¹	Not detectable
Rate of the slow-binding step of inhibition	9.4 min⁻¹	Not detectable
K_i (overall)	0.18 nM	5.8 nM

similar to those reported for the interaction of MTX with DHFR isolated from *Streptococcus faecium* A, 5.1 min⁻¹ and 23 nM, respectively (43). When recombinant mutant DHFR-TS from D7BR1000-C6 cells was used in these studies, both the appearance of the "progress curves" and the extent of inhibition at any given concentration of MTX were greatly changed (Fig. 4B).

TABLE V

MTX resistance in transfected L. major lines

Cell line	Transfected plasmid	Methotrexate	
		EC_{50}[a]	Resistance[b]
		μM	-fold
L. major WR454	None	0.5	
	pX63NEO	0.8	
	pX63NEO DT	80	≈100
	pX63NEO M53R DT	>10^4	>10000
L. major 252	None	2.5	
	pX63NEO	4	
	pX63NEO DT	10^3	250
	pX63NEO M53R DT	>10^4	>2500

[a] The methotrexate EC_{50} is the concentration (μM) which reduces the cell number by 50%, measured when drug-free control culture was in late log phase (17).

[b] The fold resistance is the ratio of EC_{50} values for control (pX63NEO transfected cells) and experimental cell lines.

Although the steady-state rate was inhibited at high concentrations of MTX (above 50 nM), this rate was linear and there was no time-dependent decrease in the reaction rate with M53R DHFR-TS. The K_i for MTX inhibition of the mutant was 5.8 nM which is some 30-fold higher than the inhibition constant for the wild-type enzyme (Table IV).

Transfection of Wild-type L. major with the MTX-resistant DHFR-TS Gene—To ensure that the M53R mutation was in itself capable of conferring MTX-resistance in *Leishmania*, we transfected two wild-type strains of *L. major* with both the wild-type and mutant DHFR-TS genes. It has been previously demonstrated that transfection of *Leishmania* promastigotes with the wild-type DHFR-TS gene renders MTX-resistant parasites (44). The protein coding regions were inserted into the *Leishmania* expression vector pX63NEO (45) to give pX63NEODT and pX63NEOM53RDT and used to transfect *L. major* 252 (the wild-type strain from which D7B cells are derived) (15) and *L. major* WR454 (25). Strain WR454 transfected with pX63NEODT showed approximately 100-fold greater methotrexate resistance than cells transfected with the control pX63NEO (Table V), while cells transfected with the mutant construct pX63NEOM53RDT showed more than 10,000-fold methotrexate resistance compared to control cells. When the 252 strain was tested for resistance, EC_{50} values for cells transfected with the mutant gene were over 2,500-fold those obtained for control cells transfected with pX63 NEO (Table V). Limitations of methotrexate solubility prevented precise determinations of EC_{50} values for highly resistant cells.

Gene Amplification versus Structural Mutation—A question that arises is whether gene amplification or enzyme alteration occurred first. We suspect that amplification of the R-region DNA occurred prior to alteration of DHFR-TS. In the first selection step examined, D7B cells resistant to 10 μM MTX showed a 25-fold increase in R-region DNA copy number and a 7-fold increase in the amount of DHFR-TS with a ratio of DHFR to TS the same as that in wild-type cells. Therefore, R10 cells possessed amplified DNA and overproduced a wild-type enzyme. Cells resistant to 50 μM MTX appeared to represent an intermediate stage of resistance where the enzyme was overproduced but cells displayed the first decline in the DHFR/TS ratio (a decrease from 1.5 to 0.4). Thus, of the cells we examined, R50 cells marked the first cell line that appear to possess an altered enzyme. Cells resistant to 1000 μM MTX represented the final stage of resistance. The copy number of the R-region DNA and the altered DHFR-TS level increased only 2-fold from R100 to R1000 cells. Therefore, to our best understanding, the stepwise selection procedure produced the following responses: (a) amplification of the R-region DNA during the initial steps; (b) an alteration of the DHFR portion of the bifunctional pro-

tein at the R-region DNA level during the intermediate steps; and (c) a final resistant stage that was characterized by a predominant population of mutant DHFR and amplified R-region DNAs. It appears that cells with an altered DHFR-TS were favorably selected in response to drug pressure instead of those containing the wild-type gene.

Structural Basis for Resistance—Although primary structures of DHFRs may be less than 25% homologous, comparison of x-ray structures has revealed striking conservation of three-dimensional structure as well as the amino acids in the active site (4, 5, 8). From homology comparisons, Met-53 in *L. major* DHFR-TS is equivalent to Leu-28 in *E. coli* and Phe-31 in human and mouse DHFRs (4–8), which have been implicated in binding of anti-folates. For example, Phe-31 to Trp or Arg in murine DHFR (46, 47), or Phe-31 to Ser in human DHFR (48, 49) have been reported to confer MTX resistance. The residues corresponding to Met-53 of *L. major* DHFR form a hydrophobic binding pocket for the *p*-aminobenzoyl moiety of MTX, and it is reasonable to propose that insertion of the positively charged Arg in the M53R mutant has a detrimental effect on this interaction.

Summary—We have shown that the MTX-resistant *Leishmania* cell line, D7BR1000, contains an altered DHFR-TS which reduces both DHFR activity and inhibition by MTX. This is the first example of a structural modification causing anti-folate resistance in *Leishmania*, and offers a counterpart to the more commonly observed mechanism involving gene amplification in these organisms. The genesis of the resistant cell line seems to have involved initial amplification of the DHFR-TS gene, followed by mutation of DHFR, and then selection for cells containing the MTX-resistant mutant enzyme. Cloning and DNA sequencing of the DHFR-TS gene revealed a single base change which resulted in a M53R mutation. The hypothesis that this single mutation could confer cell resistance to MTX was verified by transfection of the gene into wild-type *L. major*, and demonstration that the cells were highly resistant to MTX. Kinetic studies of the wild-type and mutant enzymes revealed the reason for MTX resistance. As observed with several other DHFRs (43, 50), the wild-type enzyme interacts with MTX by an initial rapid interaction to give a weak complex, followed by a slow step which results in the very tight complex. In contrast, the resistant DHFR showed no slow onset of inhibition, and a binding constant for MTX which was about 30-fold higher than the wild-type enzyme.

Given the importance of DHFR as a drug target, a clarification of the molecular features that confer drug resistance could aid the rational design of alternative drugs against leishmaniasis. Indeed our observation implicating the side chain of Met-53 in the altered DHFR function may be of use in the structure-based design of new anti-folates as selective chemotherapeutic agents. Finally, the structurally altered MTX-resistant DHFR-TS may be of great utility in transfection experiments. This gene could function as a new drug resistance marker in the positive selection of transfected trypanosomatids. Experiments are in progress to investigate this possibility.

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