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Purification of the Bifunctional Thymidylate Synthase-Dihydrofolate Reductase Complex from the Human Malaria Parasite *Plasmodium falciparum*

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SUMMARY

The bifunctional thymidylate synthase-dihydrofolate reductase complex from the human malaria parasite *Plasmodium falciparum* has been purified to homogeneity using a sequence of separation steps including phenyl-Superose, gel filtration, dye affinity matrix, hydroxyapatite, and anion exchange chromatography. The specific activity of dihydrofolate reductase increased ~24,000-fold from 3.3 units mg^{-1} protein to 79,000 units mg^{-1} protein after five successive chromatographic steps with a yield of 31%. Both enzyme activities coeluted as a symmetric peak in highly purified preparations, indicating the existence of a bifunctional enzyme complex in *P. falciparum*. The apparent molecular weight of the native complex was ~120,000 as determined by gel filtration. When individual fractions of the anion exchange column were subject to polyacrylamide electrophoresis under denaturing conditions, the increase in intensity of a single band correlated with the amount of both the thymidylate synthase and dihydrofolate reductase activity. Further purification led to an electrophoretically pure protein (yield 2.6%) with an apparent molecular weight of 67,000, suggesting that the bifunctional enzyme complex from *P. falciparum* is composed of two subunits of identical size and charge.

Thymidylate synthase (TS) (EC 2.1.1.45) and dihydrofolate reductase (DHFR) (EC 1.5.1.3) are key enzymes acting sequentially in the *de novo* synthesis of pyrimidines. TS transfers a one-carbon unit from 5,10-methylenetetrahydrofolate, thereby converting dUMP to dTMP and DHF. DHFR catalyzes the subsequent NADPH-dependent reduction of DHF to tetrahydrofolate. The inhibition of either enzyme depletes the dTMP pool, leading to a block in the DNA synthesis and, consequently, to cell death.

Malaria parasites appear to be exclusively dependent on the *de novo* synthesis of pyrimidines and seem unable to incorporate significant amounts of preformed pyrimidines into their DNA (see Ref. 1 for review). The absence of such a salvage pathway has been confirmed by the inability of the parasites to use exogenous uridine to overcome the action of inhibitors directed against different enzymes involved in the pyrimidine synthesis (2, 3). Human cells, in contrast, are able to incorporate pyrimidines and survive even in the total absence of any pyrimidine synthesis, as is the case in hereditary orotic aciduria (4), which can be effectively treated by administering uridine. This fundamental difference between the pyrimidine metabolism of the parasite and host cell is the basis for potential intervention by a variety of antimalaria drugs.

Both TS and DHFR of malarial parasites are attractive targets for interfering with the pyrimidine biosynthesis since both enzymes are structurally different from the equivalent host enzymes. In many eucaryotic cells, including human cells, the native TS protein has a molecular weight of around 70,000 and is composed of two identical subunits, whereas the DHFR is a much smaller monomer, with a molecular weight of around 20,000 (5). In contrast, many parasitic protozoa seem to have a bifunctional TS-DHFR complex (see Ref. 6 for review). To date, this complex has been purified to homogeneity from *Crithidia fasciculata* (7) as well as *Leishmania tropica* (8), and the native enzyme was shown to have molecular weights of 107,000 and 110,000, respectively. In both species the TS-DHFR complex was composed of two subunits of identical size.

The in-depth characterization of TS and DHFR from the genus *Plasmodium* has been, so far, severely restricted due to the limited amount of parasite material available. This is especially true for the human malaria parasite, *Plasmodium falciparum*, where the limitations of the *in vitro* culture system prompted most studies concerning the molecular mechanisms of DHFR inhibitors such as pyrimethamine to be carried out either in crude extracts (9–12) or in partially purified enzyme preparations (6, 13, 14).

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ABBREVIATIONS: TS, thymidylate synthase; DHFR, dihydrofolate reductase; DHF, dihydrofolate; [^{3}H]FdUMP, [^{3}H]-5-fluorodeoxyuridine-5'-monophosphate; EDTA, ethylenediaminetetraacetate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; MTX, methotrexate.

By using optimized *in vitro* culture conditions for *P. falciparum* (15), we were able to obtain sufficiently large quantities of parasites to attempt the purification of the TS-DHFR complex. In this report we describe a sequence of purification steps leading to an electrophoretically pure TS-DHFR complex from the human malaria parasite *P. falciparum*.

Materials and Methods

Reagents and Chemicals

[^{3}H]FdUMP (20 Ci mmol^{-1}) was purchased from Moravec Biochemicals (Brea, CA) and the ^{14}C-methylated protein mixture (CFA.626) from Amersham Corp. (Arlington Heights, IL). Phenyl-Superose preparations HR 5/5 and Mono Q HR 5/5 were prepacked columns and were purchased, together with Red Sepharose and Superose 12, from Pharmacia (Piscataway, NJ) and used with their fast protein liquid chromatography system. Hydroxyapatite and lysozyme (from egg white) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN); the Coomassie brilliant blue G binding assay for protein determination and the silver stain kit were from Bio-Rad (Rockville Centre, NY). Fluorosol was purchased from National Diagnostics (Summerville, NJ). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

In Vitro Culture of *P. falciparum*

The pyrimethamine-resistant clone 7G8 of the Brazilian isolate IMTM22 was used throughout this study and cultured as previously described (14, 15). The erythrocytes were lysed (235–295 ml of infected blood at 50% hematocrit, parasitemias between 8.2 and 11.9%) and the parasites (~1.5×10^{11}) disrupted in the presence of proteinase inhibitors as outlined (14).

Purification of Plasmodial DHFR

All steps were carried out at 4° and the entire procedure was performed without interruption.

Step I: Crude extract. The insoluble components in the mixture of disrupted parasites and erythrocytes (10–15 ml) were removed by centrifugation (100,000 × *g*, Beckman 50Ti rotor, 45 min). The DHFR activity was contained in the supernatant.

Step II: Ammonium sulfate precipitation. The supernatant from step I was diluted with an equal volume of 3.4 M $(NH_4)_2SO_4$ in TEM buffer (50 mM Tris, 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.4) and stirred in an ice bath for 90 min; then, the precipitate formed was removed by centrifugation (10,000 × *g*, Sorvall SS34, 15 min).

Step III: Phenyl-Superose column chromatography. The supernatant containing the enzyme activity in 1.7 M $(NH_4)_2SO_4$ was divided in portions not exceeding 150 mg of protein and loaded onto a Phenyl-Superose HR 5/5 column, bed volume 1 ml (later replaced by Phenyl-Superose column HR 10/10, bed volume 8 ml), equilibrated with TEM buffer containing 1.7 M ammonium sulfate. The column was developed at a flow rate of 0.5 ml min^{-1} with a linear gradient of TEM buffer (20 ml) decreasing the ammonium sulfate concentration from 1.7 M to 0. The enzyme eluted at ~300 mM ammonium sulfate and the appropriate fractions from each of the separations (carried out successively) were combined.

Step IV: Gel filtration. The active fractions from step III were applied to Superose 12 packed in an HR 16/50 column (bed volume 100 ml). The column was equilibrated with TEM buffer containing 50 mM NaCl and the flow rate adjusted to 0.5 ml min^{-1}. The enzyme-containing fractions (free of ammonium sulfate) eluted at around 0.6 bed volume.

Step V: Affinity chromatography. The active fractions were applied to Red Sepharose (1 × 3 cm) equilibrated with TEM buffer. The column was washed batchwise with TEM buffer, TEM containing 0.5 M NaCl, TEM containing 0.5 M NaCl and 4 mM DHF, and TEM containing 1.5 M NaCl. The DHFR activity was eluted in the substrate-containing fractions.

Step VI: Hydroxyapatite chromatography. The pool from step V was applied to a hydroxyapatite column (0.5 × 2.5 cm), equilibrated with 50 mM potassium phosphate, pH 7.4. A linear gradient (20 ml) from 50 to 400 mM phosphate was applied, eluting the DHFR activity at 250 mM phosphate.

Step VII: Anionic exchange chromatography. The active fractions from step VI were diluted 5-fold with TEM buffer, thereby lowering the phosphate concentration to 50 mM, and directly applied to Mono Q HR 5/5 (bed volume 1 ml) equilibrated in TEM buffer. A three-step gradient of NaCl in TEM was applied, ranging from 0 to 50 mM NaCl (7 ml), 50 to 120 mM NaCl (20 ml), and 120 to 150 mM NaCl (5 ml). The TS-DHFR complex eluted at 60–80 mM NaCl.

Step VIII: Anionic exchange chromatography. The pH of the pooled active fractions from step VII was raised to pH 8.8 with Tris-base, diluted to lower the NaCl concentration to 40 mM, and then applied to a second Mono Q column. Four buffers (A–D) with increasing NaCl concentrations and decreasing pH values were used in step VIII: buffer A, 20 mM Tris, pH 8.8, 40 mM NaCl; buffer B, 20 mM Tris, pH 8.2, 120 mM NaCl; buffer C, 20 mM Tris, pH 7.8, 150 mM NaCl; and buffer D, 20 mM Tris, pH 6.9, 200 mM NaCl. The column was equilibrated in buffer A, eluted with a linear gradient (25 ml) buffer A to B, and washed with buffer B (10 ml) before a second linear gradient (10 ml) of buffer B to C was applied, followed by a third linear gradient (10 ml) of buffer C to D. The TS-DHFR complex eluted with buffer B. After removal of aliquots for SDS-PAGE, the enzyme in the column fractions was immediately stabilized by the addition of purified lysozyme to a final concentration of 150 μg ml^{-1}.

The protein concentration during the purification sequence (steps III–VII) was monitored at 280 nm and aliquots of the pooled active fractions were assayed in triplicate (except step VII, which was done in duplicate), using the Coomassie brilliant blue G binding assay standardized with bovine serum albumin.

Enzyme Assays

DHFR activity was measured spectrophotometrically at 340 nm and 37° in a final volume of 1 ml in Tris-HCl (100 mM, pH 7.0), 2-mercaptoethanol (1 mM), DHF (100 μM), NADPH (50 μM), and aliquots of the column fractions containing the enzyme. In the controls, either the substrate or the enzyme was omitted. One unit of DHFR is defined as the amount of enzyme required to reduce 1 nmol of DHF to THF per min at 37° under the above conditions. The TS activity was determined by the complex formation between the enzyme and [^{3}H]FdUMP (16) in the presence of 5,10-methylenetetrahydrofolic acid, which was prepared as described (17).

Molecular weight determination

The molecular weight of the native DHFR was estimated by gel filtration on Superose 12 HR 16/50 (see step IV above). The common term "molecular weight" is used throughout the report instead of "effective radius of the protein." DHFR containing fractions from the Red Sepharose column (step V) were mixed with the following markers in a total volume of 1 ml: β-amylase from sweet potato (M_r 200,000), alcohol dehydrogenase from yeast (M_r 150,000), hexokinase from yeast (M_r 102,000), and ovalbumin dimer (M_r 85,400). The subunit size of the enzyme was determined by SDS-PAGE according to the method described in Ref. 18, using an acrylamide concentration of 10% (w/v) and separating the proteins at 12 V cm^{-1} for 5 hr. The proteins were visualized using the silver stain kit according to the instructions given by the manufacturer. The complex between [^{3}H]FdUMP and TS was formed as described (16), separated on SDS-PAGE, and cut into 60 slices of 2.2 mm each. The slices were dissolved in fluorosol (2 hr, 50°) and counted in a RackBeta scintillation counter (LKB Instruments, Gaithersburg, MD).

Results and Discussion

Purification strategy. The purification method of choice for the DHFR from a variety of sources (see Ref. 5 for review)

is often based on the selective interaction between the enzyme and DHFR inhibitors such as MTX (19). Unfortunately, the powerful principle of MTX affinity chromatography cannot be integrated in the purification scheme for the DHFR from the genus *Plasmodium*. The DHFR from *P. falciparum* binds very strongly to the MTX ligand and cannot be eluted either with high substrate concentrations, confirming an earlier report (6), or by raising the pH of the various elution buffers examined, rendering this approach impracticable (data not shown). The tight binding between DHFR and MTX is reflected in the K_i for MTX of around 3×10^{-10} M found in the three clones of *P. falciparum* examined (14). In contrast, affinity columns based on the interaction of DHFR with its substrate DHF, as used in the purification of the enzyme from bacterial sources (20), also cannot be part of the purification sequence for the DHFR from *P. falciparum* due to the loose binding between this enzyme and its substrate. The K_m for DHF was found to be 1.1×10^{-5} M using partially purified enzyme from clone 7G8 (14).

Since antibodies directed against the DHFR from *P. falciparum* were not available to us but would be very helpful in designing a purification scheme based on antibody-enzyme interaction, we tried to absorb the DHFR out of the crude extracts with MTX bound to the Sepharose matrix. Analysis of bound proteins after separation by SDS-PAGE revealed that 20–50 distinct proteins could be eluted from the affinity matrix irrespective of the blocking agents or the washing conditions used (data not shown). We therefore did not pursue this option further and resorted to a conventional purification scheme.

Our initial experiments indicated that the instability of the DHFR in dilute solutions made it imperative to avoid all time-consuming steps (such as dialysis to change buffer systems) and that the entire procedure has to be completed within 48 hr to avoid the total inactivation of the unstabilized enzyme. The amount of enzyme available to start any purification was limited by the fact that the *in vitro* culture system used could not, for practical reasons, be scaled up any further. Consequently, we had to be content with about 1000 units of DHFR from *P. falciparum* in each preparation, an exceedingly small amount of enzyme, if one recalls that the entire 1000 units equal a *single* unit of DHFR from sources such as mammalian cells or bacteria. The definition of one unit of DHFR from these systems refers customarily to the amount of enzyme converting μmol of substrate per unit of time compared to nmol in the plasmodial system, a difference of a factor 1000.

Therefore, a series of separation options, including various cation and anion exchange columns, affinity columns with various ligands, dye-matrix columns, and preparative chromatofocusing systems, was analyzed using speed of performance and yield as the two criteria for selection and incorporation in the purification scheme used to purify the DHFR from *P. falciparum*.

Purification of DHFR. Five of the columns tested were selected and used in the sequence shown in Table 1 (steps III–VIII). The first column (Phenyl-Superose, step III) separates due to hydrophobic interaction between the proteins and the matrix, allowing sample application in solutions of high ionic strength such as 1.7 M $(NH_4)_2SO_4$. Consequently, the use of this column has the additional benefit of fractional precipitation of proteins present in the crude extracts (step I). About 13% of the total proteins were precipitated with >95% of the DHFR activity remaining in the supernatant (Table 1, step II). The purification factor achieved with this precipitation step is difficult to establish since the DHFR activity in the presence of 85 mM $(NH_4)_2SO_4$ (the concentration present in the enzyme assay) is only 65% compared to the activity present in salt-free aliquots. This quench is reflected in the activity data given in Table 1 (step II). The elution profile of the Phenyl-Superose column is shown in Fig. 1. The bulk of the proteins elutes at concentrations of $(NH_4)_2SO_4 > 0.5$ M, well separated from the DHFR activity which elutes at 0.3 M, resulting in a purification factor of 58 (Table 1, step III).

After the Phenyl-Superose column, only 1–2% of the proteins found in crude extracts are present (compare step I with step III, Table 1). Since the ammonium sulfate present in the active fractions of the Phenyl-Superose pool (step III) prevents the binding of the enzyme to the Red Sepharose matrix (step V), a size exclusion column was introduced (step IV). Although overloaded under the conditions used to shorten separation time (sample volume 7% of bed volume), the enzyme containing pool from step III was effectively desalted, and the proteins in the molecular weight range of >200,000 to <60,000 were separated from the DHFR containing fractions eluting in between, ensuring the complete absence of possible traces of contaminating human DHFR and TS with molecular weights of 21,000 and 35,700, respectively (21, 22), from this step onward. The purification factor after passage through this Superose 12 column was 89-fold with a specific activity 90 times higher than that found in the crude extract (Table 1, step IV).

The effectiveness of employing dye-matrix columns such as Red Sepharose has been demonstrated earlier (14). The elution profile of the Red Sepharose (step V in the present isolation procedure) was comparable with the profile shown in Fig. 2 of Ref. 14 in spite of the fact that KCl in the elution buffer was replaced by NaCl. The purification obtained with the Red Sepharose column was 13-fold, resulting in an overall purification by a factor of >1000 (Table 1, step V).

The active fractions from the Red Sepharose column are eluted with buffers containing 0.5 M NaCl and a 4 mM concentration of the substrate DHF and, therefore, cannot be used directly with the anion exchange column Mono Q (step VII), since the enzyme elutes at low salt concentrations. Consequently, a desalting step had to precede step VII. This was effectively achieved by directly applying the active fractions (7 ml) from Red Sepharose (step V) to hydroxyapatite (step VI). A representative elution profile is shown in Fig. 2 with the enzyme activity eluting at 250 mM phosphate. The absorption in the flow-through is mainly due to DHFR and not protein, which totals only around 100 μg at this point. The DHFR was purified to >5000 units ml^{-1} in this step (Table 1).

The anion exchange column Mono Q was used in the next chromatographic step within the purification sequence (Table 1, step VII). The elution profile obtained with this column is shown in Fig. 3. Both the DHFR and the TS activities coelute as a symmetric peak at 60–80 mM NaCl. To reliably estimate the specific activity after this step in the purification scheme, one-half of one entire enzyme preparation was concentrated after elution from Mono Q in order to reach protein concentrations falling into the linear part of the calibration curve using the sensitive microassay for protein determination (Materials and Methods). In a typical preparation, 4 μg ml^{-1} protein was found, resulting in a specific DHFR activity of 79,000 units

TABLE 1

Purification of the TS-DHFR complex from *P. falciparum* clone 7G8

The purification procedure is outlined under Materials and Methods. In this experiment 295 ml of parasitized blood, with a parasitemia of 9.9% containing ~65% trophozoites and early schizonts, were used.

Purification step	Total volume[a]	Total protein[c]	Total activity[a,b]	Specific activity	Purification factor[c]	Yield[a]
	ml	mg	nmol DHF min^{-1}	nmol DHF min^{-1} mg^{-1} protein		%
Step I Crude extract	10.1	313	1,030	3.3	1	100
Step II $(NH_4)_2SO_4$ precipitation	19.8	273	640[d]			
Step III Phenyl-Superose	7.0	3.96	770[d]	193	58	75
Step IV Superose 12	14.0	2.66	786	295	89	76
Step V Red Sepharose	7.0	0.16	632	3,950	1,197	61
Step VI Hydroxyapatite	10.0	0.11	558	5,072	1,537	54
Step VII Mono Q	8.0	0.004[e]	316	79,000	23,939	31
Step VIII Mono Q	2.0[f]		27			2.6

[a] The data given in these columns do not take into account the aliquots used for protein determination and SDS-PAGE.
[b] Based on DHFR activity.
[c] Referred to specific activity.
[d] DHFR activity suppressed in the presence of $(NH_4)_2SO_4$ (see the text).
[e] For measurement of protein concentration (see the text).
[f] Electrophoretically pure TS-DHFR complex; fractions 45–46 in Fig. 4, tracks 22–23 in Fig. 5.

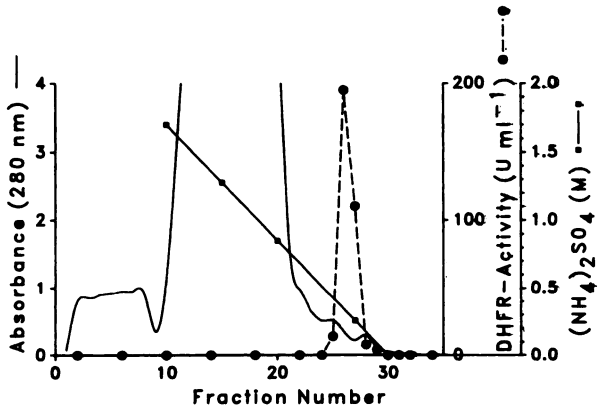

Fig. 1. Phenyl-Superose chromatography of *P. falciparum* DHFR. One hundred forty mg of protein in the 1.7 M $(NH_4)_2SO_4$ supernatant (step III, Materials and Methods) were applied and the column developed with a linear gradient of TEM buffer (20 ml), decreasing the $(NH_4)_2SO_4$ concentration from 1.7 M to 0. Bed volume, 1 ml; flow rate, 0.5 ml min^{-1}; fraction volume, 0.7 ml.

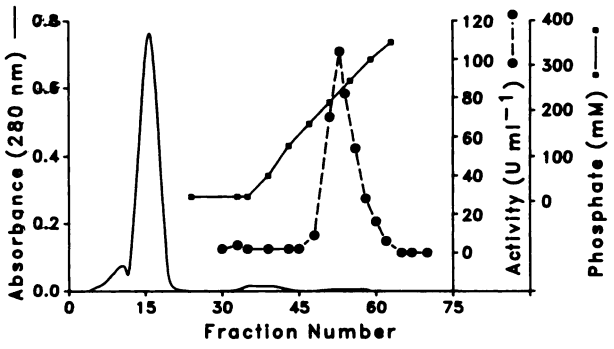

Fig. 2. Hydroxyapatite chromatography of *P. falciparum* DHFR. Active fractions from the Red Sepharose column (Materials and Methods, step V) were applied and the column was developed with a linear gradient of potassium phosphate (20 ml; 50–400 mM). Bed volume, 0.5 ml; flow rate, 0.25 ml min^{-1}; fraction volume, 1 ml. The absorption in the flow-through is due to the substrate DHF used to elute the enzyme in the previous column (step V, Table 1).

mg^{-1} protein which translates into a numerical purification factor of >20,000, comparing step VII with step I (Table 1).

The DHFR activity was visualized by activity staining (5) after separation of the proteins present in the peak fractions of step VII (Fig. 3) by nondenaturing gel electrophoresis. The broad purple zone appearing (data not shown) is indicative of the presence of a reducing enzyme, confirming that the *P. falciparum* DHFR is indeed one of the five proteins seen on SDS-PAGE (see below) at this stage of the purification.

The same column fractions were analyzed for the presence of TS activity by the formation of the complex between the enzyme and [^{3}H]FdUMP. ^{14}C-methylated standard proteins were mixed with the tritium-labeled complex and separated by SDS-PAGE; then, the position of both labels was determined after dissolving the gel slices and counting the radioactivity they contained. In two of four experiments one single tritium-labeled protein was detected in the same gel slice containing the ^{14}C-methylated bovine serum albumin. In the two other experiments the [^{3}H]FdUMP-TS complex was found one gel slice before or after the reference protein. Based on the comigration with bovine serum albumin, we estimate the molecular weight of the TS subunit from *P. falciparum* to be in the range of 66,000–69,000 (data not shown).

The presence of both enzyme activities in such highly purified preparations strongly suggests that *P. falciparum* clone 7G8, like other protozoa, such as *C. fasciculata* (7) and *L. tropica* (8), possesses a bifunctional TS-DHFR complex. However, earlier conclusions regarding the existence of such a complex within the genus *Plasmodium* were derived from partially purified preparations of *P. falciparum*, *Plasmodium lophurae* (6), and

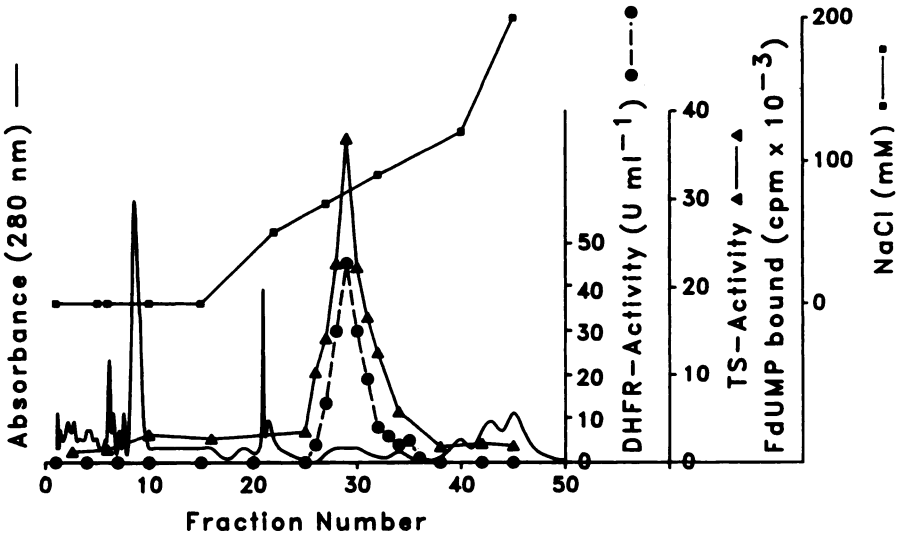

Fig. 3. Anion exchange chromatography on Mono Q of the TS-DHFR complex from *P. falciparum*. Active fractions from the hydroxyapatite column (Fig. 2) were applied and the column developed with a three-step gradient of TEM buffer containing NaCl (see Materials and Methods). Bed volume, 1 ml; flow rate, 0.5 ml min^{-1}; fraction volume, 1 ml. Aliquots of individual fractions were assayed for DHFR activity (50 μl) and TS activity (100 μl), and the proteins were separated on SDS-PAGE as described in the legend to Fig. 5.

Plasmodium berghei (7) and were based on copurification of both enzyme activities on size exclusion and affinity chromatography. In the absence of protein sequencing data, one cannot entirely disregard the remote possibility that two proteins of identical size, binding characteristics to an affinity matrix, and subunit size (traced by TS activity alone) exist among the many proteins present in those partially purified preparations. In contrast, the several purification steps preceding the Mono Q column (Table 1) are based on entirely different separation principles, and it would be extremely unlikely that two separate proteins would copurify over so many steps. Therefore, the probability that the DHFR and TS indeed exist as a bifunctional single protein within the genus *Plasmodium* is further increased. This assumption is also supported by findings that the DHFR and the TS activity increase precisely at the same rate, depending on the developmental stage of the parasites using synchronized *Plasmodium chabaudi* as a model system (23).

To rule out any ambiguity, we purified the TS-DHFR complex further, aiming for an electrophoretically pure protein. The copurifying proteins from step VII could be removed by chromatofocusing using a pH gradient from pH 7.0 to pH 4.0 (data not shown). Due to the extreme instability of the TS-DHFR complex at this stage of the purification, we opted instead for reapplying the active fractions from step VII (Fig. 3) to a second Mono Q column which allows a slightly faster separation than can be achieved with the chromatofocusing system. This second Mono Q column (step VIII, Table 1) was developed with a series of buffers with increasing NaCl concentrations and decreasing pH values (Materials and Methods). The elution profile obtained with this final purification step is shown in Fig. 4 with electrophoretically pure enzyme contained in fractions 45 and 46 (see below). Since these fractions contained measurable DHFR activity and the single protein present was able to bind [^{3}H]FdUMP, we conclude that *both* the TS and the DHFR activities from *P. falciparum* are indeed contained in a single protein. The low yield of ~2.6% (Table 1) is not due to the inactivation of the enzyme by the column matrix itself but to the rapid loss of activity in preparations of this purity (see below).

Since the individual fractions from step VIII were stabilized immediately with lysozyme to retain some of the enzyme activity, the determination of the protein concentration and, consequently, an estimation of the specific activity was not possible and is therefore not shown in Table 1.

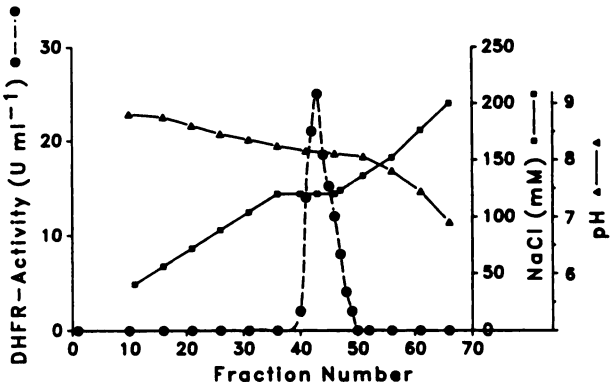

Fig. 4. Anion exchange chromatography on Mono Q of the TS-DHFR complex from *P. falciparum*. The active fractions from the first Mono Q column (Fig. 3, step VII; Table 1) were applied to a second Mono Q column and eluted with buffers containing increasing NaCl concentrations and decreasing pH values as described under Materials and Methods. The activity eluted at around 20 mM Tris, pH 8.2, 120 mM NaCl. Bed volume, 1 ml; flow rate, 0.8 ml min^{-1}; fraction volume, 1 ml. Aliquots of individual fractions were assayed for DHFR activity (50 μl) and the proteins (300 μl) were separated on SDS-PAGE as shown in Fig. 5. The protein concentration was too low to be detected by monitoring the absorbance at 280 nm. Fractions 45–46 contained the electrophoretically pure TS-DHFR complex from *P. falciparum* (see *tracks 22–23*, Fig. 5).

SDS-PAGE. Aliquots of the pools from steps I, III–VI, and individual column fractions from steps VII and VIII (Table 1) were subject to electrophoresis under denaturating conditions to visualize the progress of purification, identify the TS-DHFR complex by the increase in intensity of one band, and obtain information about the size of the subunit(s) of the complex (Fig. 5). Although >50 individual protein bands were visible in the early stages of the preparation (Fig. 5 *tracks 2–4*), a protein band increased in intensity after Red Sepharose chromatography (Fig. 5, *track 5*) and hydroxyapatite (Fig. 5, *track 6*) in the position later found to be the position of the TS-DHFR subunit. The effectiveness of Mono Q (step VII, Table 1; Fig. 3) is clearly demonstrated by reducing the number of proteins to

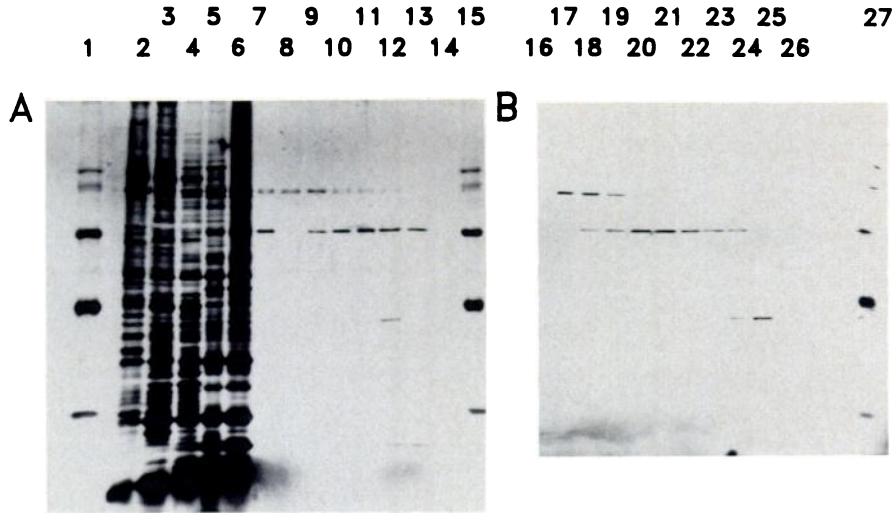

Fig. 5. SDS-PAGE of *P. falciparum* TS-DHFR complex following each step of the protein purification (Table 1). A, steps I–VII; B, step VIII. The molecular weight standards in *tracks 1, 15, and 27* were: β-galactosidase, 116,250; phosphorylase B, 97,400; bovine serum albumin, 66,200; ovalbumin, 42,700; and carbonic anhydrase, 31,000. *Track 2*, crude extract (10 μg in 0.3 μl); *track 3*, phenyl-Superose (12 μg in 20 μl); *track 4*, Superose 12 (9 μg in 50 μl); *track 5*, Red Sepharose (8 μg in 50 μl); *track 6*, hydroxyapatite (10 μg in 50 μl); *track 7*, Mono Q (the protein concentration was not determined; 20 units ml^{-1} DHFR activity); *tracks 8–14*, 500 μl of the individual fractions from Mono Q as shown in Fig. 3. The fraction numbers that follow, corresponding to Fig. 3, are given in parentheses; units (U) are given per ml: *track 8*, 0 U (22); *track 9*, 5 U (26); *track 10*, 20 U (28); *track 11*, 45 U (29); *track 12*, 25 U (30); *track 13*, 10 U (31); *track 14*, 0 U (35). *Tracks 16–27*, 300 μl of individual fractions from Mono Q (step VIII), as shown in Fig. 4, were applied. The fraction numbers that follow, corresponding to Fig. 4, are given in parentheses; units (U) are given per ml: *track 16*, 0 U (37); *track 17*, 2 U (40); *track 18*, 14 U (41); *track 19*, 21 U (42); *track 20*, 25 U (43); *track 21*, 18.5 U (44); *track 22, 15 U (45); track 23*, 12 U (46); *track 24*, 8 U (47); *track 25*, 2 U (49); *track 26*, 0 U (53).

five (Fig. 5, *tracks 7–13*). The complex itself was identified by correlating *both* the amount of DHFR and TS activity present in the column fractions (Fig. 3) to the intensity of the five bands seen after SDS-PAGE (Fig. 5, *tracks 9–13*).

The proteins copurifying with the TS-DHFR complex in step VII (Fig. 5, *track 7*) could be separated on the second Mono Q column (step VIII, Fig. 4; Fig. 5, *tracks 16–26*). The TS-DHFR complex (Fig. 5, *tracks 18–24*) was electrophoretically pure in two fractions (Fig. 5, *tracks 22–23*), whereas the contamination proteins eluted either at higher NaCl concentrations (Fig. 5, *tracks 17–21*) or lower pH values (Fig. 5, *tracks 24–26*).

By comparison to known marker proteins, we estimate the molecular weight of the subunit of the TS-DHFR bifunctional enzyme complex of *P. falciparum* clone 7G8 to be 67,000 (n = 4). The size of the subunit of the TS-DHFR complex has been determined in a number of species of protozoa (6) using the electrophoretic mobility of the complex previously formed between [^{3}H]FdUMP and the partially purified enzyme. Using this method, the molecular weight was found to be 63,000 for the subunit of the TS-DHFR complex from both *P. falciparum* and *P. lophurae*, a value which differs by less than 10% from the subunit size estimated for the electrophoretically pure complex reported in this paper.

Determination of the size of the native TS-DHFR complex of *P. falciparum*. The purified complex could not be used to determine its native size by size exclusion chromatography, such as Sephadex G 200, Sepharose, or Superose 12. The time required to pass the unstabilized active enzyme complex over gel filtration columns sized to efficiently separate proteins with molecular weights between 200,000 and 100,000 was already sufficient to completely inactivate the TS-DHFR complex of *P. falciparum*. Attempts to retain part of the activity to localize the enzyme by adding protein to the tubes used to collect the column effluent were not successful. Based on the stability tests (see below), we selected a pool containing the active complex with a protein concentration of uniform size sufficiently high to ensure that some activity remained after passage through a gel filtration column. These criteria are met by using the pool from Red Sepharose (Table 1, step V) containing ~150 μg ml^{-1} of protein previously eluted at molecular weights between 200,000 and 60,000 on Superose 12 (step IV, Table 1). The elution profile of the native TS-DHFR complex is shown in Fig. 6A, using appropriate proteins as size markers. By plotting the fractional elution volume versus the molecular weight (Fig. 6B), we found in four separate determinations molecular weights of the native TS-DHFR complex of *P. falciparum* of ~116,000, 120,000, 121,000 and 118,000, respectively. These approximations are only valid if one assumes that possible interactions between the TS-DHFR complex and other proteins present in the Red Sepharose pool (Fig. 5, *track 5*) do not influence the elution pattern on which the determinations are based. The data based on gel filtration can only be expected to be accurate within ±10%. The apparent molecular weight of ~120,000 for the complex from *P. falciparum* clone 7G8 is most likely an underestimation since the subunit size, as determined by the more accurate SDS-PAGE, was reproducibly found to be 67,000 (Fig. 5).

A previous summary draws attention to the variations in the molecular weight determination obtained with other protozoan TS-DHFR complexes (6). The authors estimate the molecular weight of the complex from *P. falciparum* to be 135,000, using a different gel filtration matrix, a value some 12% higher than the one found in this study. If the true native molecular weight is in the 120,000–130,000 range, then both subunit determinations of 63,000 (6) and 67,000, respectively (Fig. 5), would support the model that the TS-DHFR complex of *P. falciparum*

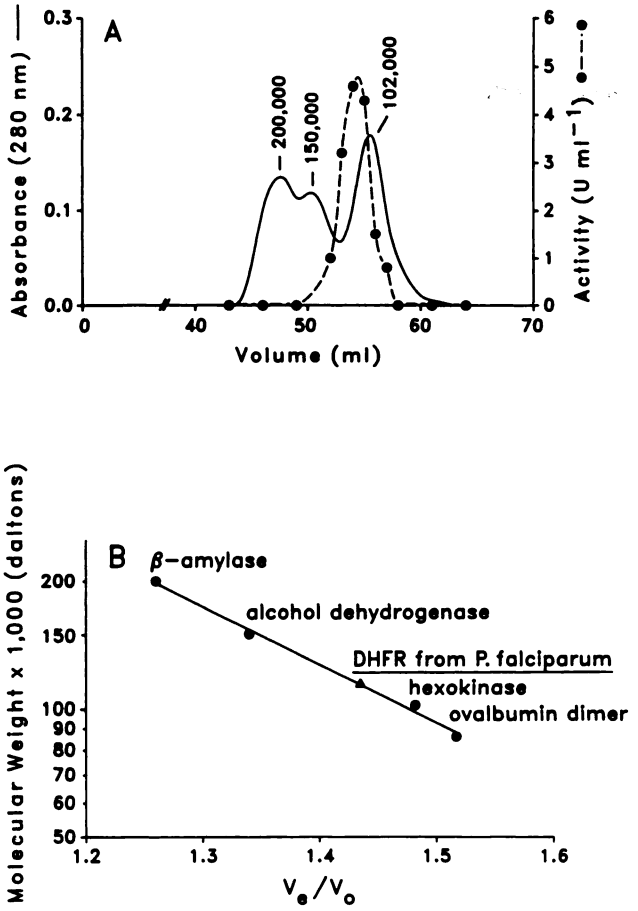

Fig. 6. Gel filtration of *P. falciparum* DHFR to estimate the native molecular weight of the enzyme on Superose 12. A. A pool from the Red Sepharose column (step V, Table 1) was applied to Superose 12 together with β-amylase (M_r 200,000), alcohol dehydrogenase (M_r 150,000), and hexokinase (M_r 102,000) in a total volume of 1 ml. Bed volume, 100 ml; flow rate, 0.5 ml min^{-1}; fraction volume, 1 ml. B. Plot of the fractional elution volume versus the molecular weight of the markers. Dextran blue was used to estimate the void volume (V_0), and ovalbumin dimer (M_r 85,400) was included in the equilibration of the column.

is composed of two identical subunits. However, both determinations discussed above differ substantially from an earlier report which estimated the native molecular weight of the DHFR from *P. falciparum* to be as high as 210,000 (13).

Among the nonhuman malaria species, the molecular weight of the native DHFR seems to be quite variable, ranging from 103,000 in *P. lophurae* (24), 120,000 in *P. chabaudi* (25, 26), 140,000 in *P. lophurae* (6), to 150,000 and 190,000 in *P. berghei* (7, 27). In only one of these species, *P. lophurae*, has the TS subunit size been determined to date by [^{3}H]FdUMP complex formation, and it has been found to be identical in size (63,000) to the one from *P. falciparum* (6).

Stability of enzyme complex. Similar to the purified TS-DHFR complex from *Leishmania tropica* (8), the TS-DHFR complex from *P. falciparum* as found after step VII (Table 1) is highly unstable, due to the low protein concentration present. Up to 60% of the DHFR activity was lost within 3 hr of keeping the combined active fractions in ice water, and no activity could be detected after a 9-hr period under these storage conditions. The enzyme activity could be retained at 4° by raising the protein concentration to 150 μg ml^{-1} (using lysozyme as a stabilizing protein since its molecular weight of 14,400 does not interfere with the subsequent testing for homogeneity on SDS-PAGE described below). In the presence of lysozyme (purified on Mono Q), 73% of the activity was found after 2 weeks and 42% after 5 weeks. Comparable results were obtained when stabilized enzyme was kept at −20° in the presence of 50% (v/v) glycerol.

The remarkable stability of the DHFR activity in sonicated parasite preparations of the same *P. falciparum* clone (9) when frozen in the absence of glycerol at −70° is most likely due to the high protein concentration present in the crude extract.

Our initial experiments to apply the elegant proteolysis technique to selectively inactivate the TS component of the complex, as successfully used in the *Leishmania* system (28), were inconclusive and await further analysis with substantially larger amounts of the purified TS-DHFR complex from *P. falciparum*.

In summary, to the best of our knowledge, this is the first report describing the purification of the bifunctional TS-DHFR complex from a species of the genus *Plasmodium* to apparent homogeneity. The establishment of a partial protein sequence is now within reach, leading to polynucleotide probes which in turn will be used to identify the TS-DHFR gene of *P. falciparum* in a variety of previously established DNA libraries.[1] An expression of this gene in appropriate pro- and eucaryotic vector systems would make the enzyme complex accessible in amounts to allow a far more detailed characterization than is possible at present with the limited quantities of enzyme which can be obtained from *in vitro* cultured parasites. The characterization of the TS-DHFR complex, which is the target enzyme for a widely used group of antimalarial drugs, becomes ever more important due to the worldwide manifestation of resistance to these DHFR inhibitors by human malaria parasites.

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References

1. Sherman, I. W. Biochemistry of *Plasmodium* (malarial parasites). *Microbiol. Rev.* **43**:453–495 (1979).
2. Hammond, D. J., J. R. Burchell, and M. Pudney. Inhibition of pyrimidine biosynthesis *de novo* in *Plasmodium falciparum* by 2-(4-*t*-butylcyclohexyl)-3-hydroxy-1,4-naphthoquinone *in vitro*. *Mol. Biochem. Parasitol.* **14**:97–109 (1985).
3. Scott, H. V., A. M. Gero, and W. J. O'Sullivan. *In vitro* inhibition of *Plasmodium falciparum* by pyrazofurin, an inhibitor of pyrimidine biosynthesis de novo. *Mol. Biochem. Parasitol.* **18**:3–15 (1986).
4. Kelley, W. N. Hereditary orotic aciduria, in *The Metabolic Basis of Inherited Disease* (J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein, and M. S. Brown, eds.). McGraw-Hill Book Co., New York, 1202–1226 (1983).
5. Blakley, R. L., and S. J. Benkovic. *Folates and Pterins. Chemistry and Biochemistry of Folates*. John Wiley & Sons, New York (1984).
6. Garrett, C. E., J. A. Coderre, T. D. Meek, E. P. Garvey, D. M. Claman, S. M. Beverley, and D. V. Santi. A bifunctional thymidylate synthetase-dihydrofolate reductase in protozoa. *Mol. Biochem. Parasitol.* **11**:257–265 (1984).
7. Ferone, R., and S. Roland. Dihydrofolate reductase: thymidylate synthase, a bifunctional polypeptide from *Crithidia fasciculata*. *Proc. Natl. Acad. Sci. USA* **77**:5802–5806 (1980).
8. Meek, T. D., E. P. Garvey, and D. V. Santi. Purification and characterization of the bifunctional thymidylate synthetase-dihydrofolate reductase from methotrexate-resistant *Leishmania tropica*. *Biochemistry* **24**:678–686 (1985).
9. McCutchan, T. F., J. A. Welsh, J. B. Dame, I. A. Quakyi, P. M. Graves, J. C. Drake, and C. J. Allegra. Mechanism of pyrimethamine resistance in recent isolates of *Plasmodium falciparum*. *Antimicrob. Agents Chemother.* **26**:656–659 (1984).
10. Dieckmann, A., and A. Jung. The mechanism of pyrimethamine resistance in *Plasmodium falciparum*. *Parasitology* **93**:275–278 (1986).
11. Banyal, H. S., and J. Inselburg. *Plasmodium falciparum*: induction, selection,

[1] J. W. Zolg, unpublished results.

and characterization of pyrimethamine-resistant mutants. *Exp. Parasitol.* **62**:61–70 (1986).
12. Walter, R. D. Altered dihydrofolate reductase in pyrimethamine-resistant *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **19**:61–66 (1986).
13. Kan, S. C., and W. A. Siddiqui. Comparative studies on dihydrofolate reductases from *Plasmodium falciparum* and *Aotus trivirgatus*. *J. Protozool.* **26**:660–664 (1979).
14. Chen, G.-X., C. Mueller, M. Wendlinger, and J. W. Zolg. Kinetic and molecular properties of the dihydrofolate reductase from pyrimethamine-sensitive and pyrimethamine-resistant clones of the human malaria parasite *Plasmodium falciparum*. *Mol. Pharmacol.* **31**:430–437 (1987).
15. Zolg, J. W., A. J. MacLeod, J. G. Scaife, and R. L. Beaudoin. The accumulation of lactic acid and its influence on the growth of *Plasmodium falciparum* in synchronized cultures. *In vitro* **20**:205–215 (1984).
16. Santi, D. V., C. S. McHenry, and E. R. Perriard. A filter assay for thymidylate synthetase using 5-fluoro-2′-deoxyuridylate as an active site titrant. *Biochemistry* **13**:467–470 (1974).
17. Ragsdale, S. W., and L. G. Ljungdahl. Purification and properties of NAD-dependent 5,10-methylenetetrahydrofolate dehydrogenase from *Acetobacterium woodii*. *J. Biol. Chem.* **259**:3499–3503 (1984).
18. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London.)* **227**:680–685 (1970).
19. Kaufman, B. T. Methotrexate-agarose in the purification of dihydrofolate reductase. *Methods Enzymol.* **34**:272–281 (1974).
20. Then, R. L. Purification of guanosine triphosphate cyclohydrolase I and dihydrofolate reductase on a dihydrofolate-Sepharose affinity column. *Anal. Biochem.* **100**:122–128 (1979).
21. Morandi, C., and G. Attardi. Isolation and characterization of dihydrofolic acid reductase from methotrexate-sensitive and -resistant human cell lines. *J. Biol. Chem.* **256**:10169–10175 (1981).
22. Takeishi, K., S. Kaneda, D. Ayusawa, K. Shimizu, O. Gotoh, and T. Seno. Nucleotide sequence of a functional cDNA for human thymidylate synthase. *Nucleic Acids Res.* **13**:2035–2043 (1985).
23. Walter, R. D., and E. Königk. Synthese der Desoxythymidylat-Synthetase und der Dihydrofolat-Reduktase bei synchroner Schizogonie von *Plasmodium chabaudi*. *J. Tropenmed. Parasit.* **22**:250–255 (1971).
24. Platzer, E. G. Dihydrofolate reductase in *Plasmodium lophurae* and duckling erythrocytes. *J. Protozool.* **21**:400–405 (1974).
25. Sirawaraporn, W., and Y. Yuthavong. Kinetic and molecular properties of dihydrofolate reductase from pyrimethamine-sensitive and pyrimethamine-resistant *Plasmodium chabaudi*. *Mol. Biochem. Parasitol.* **10**:355–367 (1984).
26. Ratanaphan, A., and P. Ruenwongsa. Alteration in properties of thymidylate synthetase from pyrimethamine-resistant *Plasmodium chabaudi*. *Int. J. Parasitol.* **16**:483–490 (1986).
27. Ferone, R., J. J. Burchall, and G. H. Hitchings. *Plasmodium berghei* dihydrofolate reductase isolation, properties, and inhibition by antifolates. *Mol. Pharmacol.* **5**:49–59 (1969).
28. Garvey, E. P., and D. V. Santi. Limited proteolysis of the bifunctional thymidylate synthase-dihydrofolate reductase from *Leishmania tropica*. *Proc. Natl. Acad. Sci. USA* **82**:7188–7192 (1985).

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