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Kinetic and Molecular Properties of the Dihydrofolate Reductase from Pyrimethamine-Sensitive and Pyrimethamine-Resistant Clones of the Human Malaria Parasite *Plasmodium falciparum*

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SUMMARY

Dihydrofolate reductase (DHFR) (5,6,7,8-tetrahydrofolate: $NADPH^+$-oxidoreductase; EC 1.5.1.3) was partially purified by affinity chromatography from three clones of the human malaria parasite *Plasmodium falciparum*. The three clones were representative of pyrimethamine-sensitive (clone 3D7) and pyrimethamine-resistant (clone HB3 and clone 7G8) parasites with ID_{50} values of 0.53 nM (3D7), 210 nM (HB3), and 540 nM (7G8), when tested *in vitro* against the drug. The specific activities of the partially purified DHFR differed by less than a factor of 2 between the sensitive clone 3D7 (442 ± 39 nmol min^{-1} mg^{-1} protein) and the resistant clones HB3 (634 ± 25 nmol min^{-1} mg^{-1} protein) and 7G8 (565 ± 85 nmol min^{-1} mg^{-1} protein). The number of catalytic sites in partially purified DHFR from the three clones was similar and ranged from 151 to 194 pmol mg^{-1} protein. The K_m value for NADPH was similar in all three clones (4.5–11.6 μM). The K_m value for dihydrofolate was altered 13-fold comparing the sensitive clone 3D7 (3.2 ± 0.6 μM) with the resistant clone HB3 (42.6 ± 1.6 μM), with the K_m for the resistant clone 7G8 falling in between (11.9 ± 1.2 μM). The inhibition constants for pyrimethamine increased from 0.19 ± 0.08 nM (3D7) to 2.0 ± 0.3 nM (HB3) to 8.9 ± 0.8 nM (7G8). The inhibition by pyrimethamine of the sensitive clone 3D7 was noncompetitive and competitive for the two other clones. The titration of partially purified DHFR with pyrimethamine revealed a 500-fold increase in the concentration of the drug needed to inhibit the DHFR activity by 50%, when the sensitive clone 3D7 (0.18 ± 0.02 nM) was compared to the resistant clone 7G8 (95 ± 16 nM). From the comparison of the specific activities and the catalytic center activities with the K_m values for the substrate and the inhibition constants for pyrimethamine, both of which are altered in the resistant clones, we conclude that the molecular mechanism for pyrimethamine resistance in the three clones studied is not based on an overproduction of the DHFR but is due to a decreased affinity to antifolates by a structurally altered enzyme.

The most dangerous of the human malaria parasites, *Plasmodium falciparum*, is currently the focus of intense research efforts by several groups trying to develop a vaccine directed against the different stages within the life cycle of the malaria parasite (1). Until such vaccine(s) can be successfully introduced on a global scale, two different strategies are being followed to combat the disease: vector control programs based on the use of insecticides and treatment of the endangered human population with effective antimalaria drugs.

Among these drugs, the antifolates continue to play a predominant role, especially in view of the widespread resistance to chloroquine emerging in different parts of the world (2). By their mode of action antifolates with antimalarial activity can be separated into two groups based upon their point of interference within the folate metabolism of the parasites. One group represented by sulfonamides interferes with the *de novo* synthesis of folate whereas the other group, exemplified by pyrimethamine [5-(4-chlorophenyl)-6-ethyl-2,4-pyrimidinediamine], inhibits the DHFR of the parasite. DHFR catalyzes the NADP-dependent reduction of DHF to tetrahydrofolate. As in a variety of other protozoa, plasmodial DHFR exists as a bifunctional enzyme associated with thymidylate synthetase (EC 2.1.1.45) activity (3). Since both enzymes are acting sequencially within the *de novo* synthesis of deoxythymidine monophosphate, the inhibition of either enzyme activity eventually depletes the parasites of functional one-carbon transfer units. Unfortunately, *P. falciparum* has developed resistance to antifolates as well, especially in Southeast Asia (2), narrowing the therapeutic options considerably.

A variety of different molecular mechanisms seem to be responsible for the pyrimethamine resistance in plasmodia. In *Plasmodium berghei*, a rodent parasite, pyrimethamine resistance has been shown to be due to raised levels of structurally

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ABBREVIATIONS: DHFR, dihydrofolate reductase; DHF, dihydrofolate; pABA, *para*-aminobenzoic acid; EDTA, ethylenediaminetetraacetate; FA, folic acid; IC_{50}, concentration of antifolate to inhibit 50% of the DHFR activity using partially purified enzyme; ID_{50}, concentration of antifolate to inhibit 50% of the *in vitro* growth of the parasites.

altered DHFR (4, 5), whereas in *Plasmodium chabaudi*, another rodent malaria parasite, identical levels of DHFR were found and the resistance was attributed to an altered enzyme alone (6). In the human parasite *P. falciparum* an even more complex picture emerges. Identical amounts of structurally altered DHFR were found comparing pyrimethamine-resistant and -sensitive parasites based upon the analysis of the DHFR from two clones from Brazil (7), another one from Indochina (8), as well as three uncloned isolates from Africa (8), Thailand, and Vietnam, respectively (9). These results contrast with the ones found earlier with the Uganda-Palo Alto strain, where increased DHFR levels with unchanged kinetic properties were described (10). Pyrimethamine-resistant clones, derived in culture from a sensitive parent clone, were found to have neither increased amounts of DHFR nor decreased affinity for the drug, and the resistance is thought to be due to altered permeability of the cells (11).

In order to correlate data, obtained in hybridization experiments probing the DNA derived from different pyrimethamine-resistant isolates of *P. falciparum*[1] with the amount and possible structural alterations of the DHFR found in these parasites, we reevaluated measurements which were based on crude extracts of genetically heterogeneous isolates.[2] In this study we describe the kinetic properties of DHFR using partially purified enzyme preparations. The enzyme was derived from genetically pure clones of *P. falciparum* being either pyrimethamine-sensitive or resistant to the drug.

Materials and Methods

Reagents and chemicals. [3′,5′,7′-^{3}H]Methotrexate (4-amino-N^{10}-methylpteroylglutamic acid; 14.5 Ci mmol^{-1}) and [G-^{3}H]hypoxanthine (10 Ci mmol^{-1}) were purchased from Amersham Corp. (Arlington Heights, IL); pyrimethamine (lot AG65046, E704) was a gift from Walter Reed Army Institute of Research (Washington, D. C.). Acro-LC13 filters were obtained from Gelman Sciences (Ann Arbor, MI), Leuko-Pak filters from Fenwal Laboratories (Deerfield, IL), and Red Sepharose CL-6B from Pharmacia (Piscataway, NJ). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

***In vitro* culture of *P. falciparum*.** The *P. falciparum* clones 3D7 from NF54 (12), HB3 from Hondol (13), and 7G8 from the Brazilian isolate IMTM22 (14) were from the collection of the Naval Medical Research Institute (Bethesda, MD) and obtained through Dr. J. Trosper. Parasites were cultured in modified RPMI 1640 medium (Gibco, special formula 84-5115) in 750-ml flasks at an 8% hematocrit level, as described (15).

***In vitro* inhibition of *P. falciparum* by antifolates.** The parasites were kept in culture for a 3-month period using a special drug medium (Gibco, special formula 85-5008) free of pABA and FA. The medium was supplemented with 10% (v/v) undialyzed pooled human serum. Five growth cycles before the start of the drug testing, the clones 3D7, HB3, and 7G8 were transferred to the drug medium supplemented with 10% (v/v) human serum previously dialyzed extensively against the drug medium to remove traces of pABA and FA. The effect of trimethoprim [2,4-diamino-5-(3,4,5-trimethoxylbenzyl)pyrimidine] and pyrimethamine on the *in vitro* growth of *P. falciparum* was assessed using the [G-^{3}H]hypoxanthine labeling technique (16). Stock solutions (10 mM) of trimethoprim or pyrimethamine (both in 70%, v/v, ethanol) were serially diluted 22 times in ethanol (70%, v/v), resulting in working stocks from 5×10^{-3} M to 2.37×10^{-9} M before being dispensed in 20-μl aliquots into 96-well microtiter plates (8 wells per concentration). Upon drying, 200 μl of a drug medium-parasitized cell mixture (hematocrit 2%; starting parasitemia 0.5–0.8%) were added to each well. The plates were incubated in a gas-tight box (5% CO_2/5% O_2/90% N_2) for 40 hr before labeling the cultures with [G-^{3}H]hypoxanthine (0.5 μCi/well) for an additional 18 hr. Cells were transferred onto glass fiber filters using a cell harvester (Brandel, Gaithersburg, MD). Incorporation of labeled hypoxanthine was measured in a scintillation counter using Ultrafluor (National Diagnostics, Somerville, NJ) as a solvent. Controls for each experiment consisted of eight wells of parasitized erythrocytes without drug and eight wells of unparasitized erythrocytes.

Partial purification of plasmodial DHFR. At parasitemias between 6 and 15%, the cultures were passed through Leuko-Pak filters to remove the few remaining leukocytes found in some of the preparations on thick smears. The erythrocytes were collected by centrifugation and lysed with saponin (0.01%, w/v, in isotonic saline), and the parasite pellets (5×10^{10}–1.5×10^{11} parasites) were washed with ice-cold buffered saline until no coloration by hemoglobin was visible. After suspension of the pellets in SEM buffer (10 mM sodium phosphate, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.4) containing benzamidine (1.8 mg/ml), leupeptin (20 μg/ml), soybean trypsin inhibitor (50 μg/ml), and aprotinin (50 μg/ml), the parasites were disrupted at 1000 lb/sq. inch (Parr 4635 cell disruption bomb, Parr Instrument Co., Moline, IL) and the cell debris was removed by centrifugation (100,000 × *g*, 30 min, 4°). The supernatant was applied to a Red Sepharose column (1 × 3 cm) equilibrated with TEM buffer (50 mM Tris, 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.5). The DHFR activity was eluted with TEM buffer containing 0.5 M KCl and 4 mM DHF. The combined fractions containing the enzyme activity were dialyzed against TEM buffer and are referred to as partially purified DHFR from *Plasmodium falciparum* (PPF-DHFR).

Enzyme assay. DHFR activity was measured spectrophotometrically at 340 nm and 37° in a final reaction volume of 1 ml in Tris-HCl (100 mM, pH 7.0), 2-mercaptoethanol (1 mM), DHF (100 μM), NADPH (50 μM), and PPF-DHFR. In the controls either the substrate or the enzyme was omitted. Alternatively, the methotrexate binding assay was used separating the enzyme-drug complex by the filter technique described (17). One unit of enzyme is defined as the amount of PPF-DHFR required to reduce 1 nmol of DHF to tetrahydrofolate per minute at 37° using a molar extinction coefficient of 12.3×10^3 M × cm (18). Protein concentrations were determined using the Coomassie brilliant blue G binding assay (19).

Results and Discussion

***In vitro* inhibition by antifolates.** Three genetically pure clones of *P. falciparum* were tested for the *in vitro* growth inhibition by the DHFR inhibitors pyrimethamine and trimethoprim. We preferred to evaluate only the drug and enzyme characteristics of genetically homogeneous parasites (clones) rather than broaden the investigation to include the isolates of different geographic origin and resistance pattern available to us. It has been shown that clones derived from a single isolate are up to 1000 times more resistant to pyrimethamine than the overall population of the parasites contained in the isolate (20). Furthermore, recent reports show that both pABA and FA are able to antagonize the activity of DHFR inhibitors (21, 22). These findings question the long-held belief that plasmodia are incapable of utilizing intact exogenous folates and are entirely dependent on their *de novo* synthesis. We therefore retested the clones in the absence of folates using the folate-free drug medium in combination with extensively dialyzed human serum. In this manner we avoided the large variation (exceeding a factor of 10) in the ID_{50} for pyrimethamine as well as trimethoprim observed with undialyzed sera. The inhibition profiles for the three selected clones being representative for sensitive (3D7) or resistant parasites (HB3 and 7G8) are shown

[1] J. W. Zolg and G. Chen, manuscript in preparation.
[2] Unpublished observations.

in Fig. 1A for pyrimethamine and Fig. 1B for trimethoprim. The corresponding ID_{50} values are listed in Table 1. Using 3D7 as a reference point, HB3 was 400 times and 7G8 1000 times more resistant to pyrimethamine. The equivalent factors for trimethoprim are 56 (HB3) and 470 (7G8). Note that the clone 7G8 is about 8 times more resistant to trimethoprim than the clone HB3, compared to only a 2.5-fold difference between the two clones when tested with pyrimethamine. All three clones were inhibited *in vitro* more effectively by pyrimethamine than by trimethoprim.

Partial purification of *P. falciparum* DHFR. The characterization of the DHFR from the three clones was hampered by the small amount of enzyme detected either spectrophotometrically or by the methotrexate binding assay in spite of scaling up the *in vitro* culture system to yield between 2×10^{10} and 1.5×10^{11} parasites. In our hands the specific activities of the DHFR found in crude extracts (100,000 × *g* supernatants) varied to such a large extent in different parasite preparations that their values were not judged to be indicative of possible elevated enzyme levels when resistant clones were compared to the sensitive ones. The fluctuating values for the specific activities were most likely due to the variable amount of hemoglobin present even after extensive washing of the parasite pellets. Reproducible specific activities were obtained after applying the crude extract to Red Sepharose, an affinity column successfully used in the purification of DHFR from other sources (23). The columns were eluted with buffer containing 4 mM DHF, resulting in a 30- to 50-fold purification of the plasmodial DHFR with a 92–95% recovery rate (Fig. 2). The pooled, dialyzed fractions are referred to as PPF-DHFR and are used

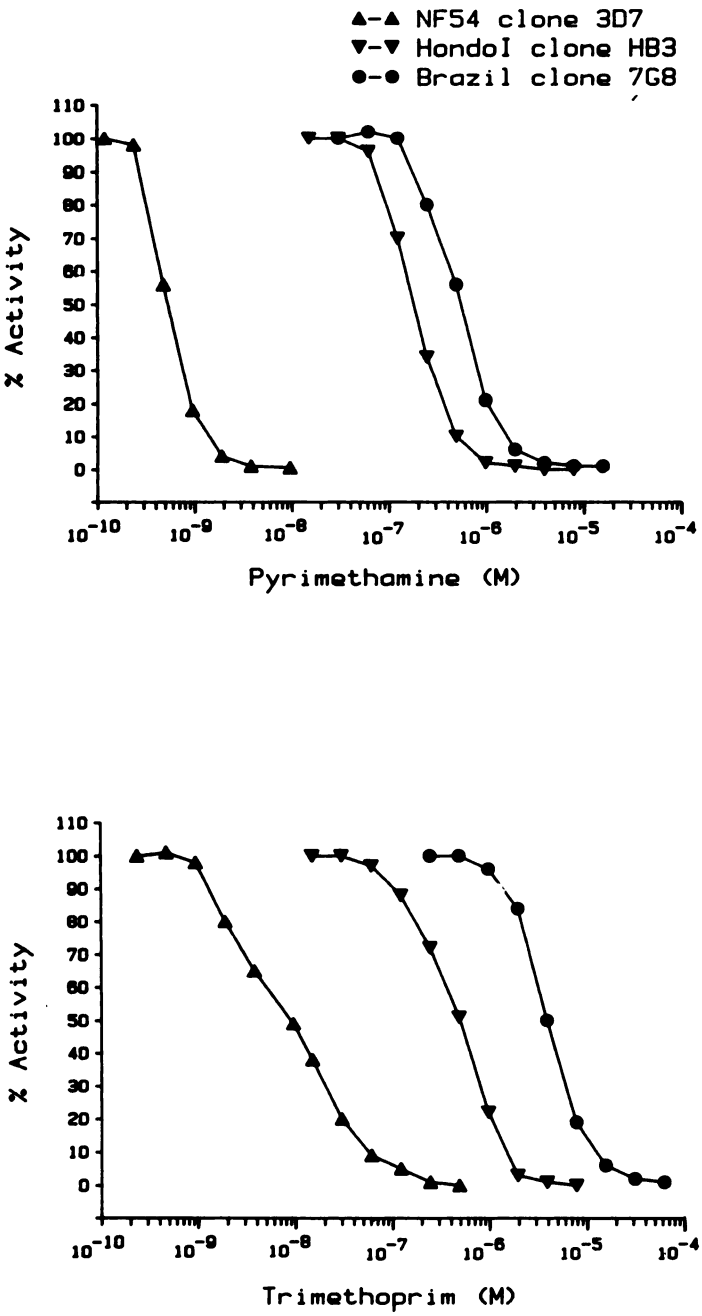

Fig. 1. *In vitro* inhibition of three clones of *P. falciparum* by pyrimethamine (A) and trimethoprim (B). The effects of the DHFR inhibitors were measured after cultivating the parasitized cells in 96-well plates for 40 hr in the presence of either pyrimethamine or trimethoprim (1×10^{-3}–2.37×10^{-10} M) and labeling the parasites with [G-^{3}H]hypoxanthine for an additional 18-hr period. For experimental details see Materials and Methods. The radioactivity in the parasite DNA grown in the absence of the inhibitors (100% value) was 75,303 ± 5,117 cpm (corrected for uninfected erythrocytes) for 3D7, 52,187 ± 8,656 cpm for HB3, and 64,738 ± 6,122 cpm for 7G8 in the pyrimethamine assay and 74,814 ± 4,198 cpm (3D7), 49,058 ± 5,092 cpm (HB3), and 58,670 ± 8,670 cpm (7G8) in the trimethoprim assay. Mean values are given for two determinations with eight wells per drug concentration.

TABLE 1

Summary of the kinetic parameters of partially purified dihydrofolate reductase from clones of Plasmodium falciparum

DHFR was partially purified (PPF-DHFR) using Red Sepharose affinity chromatography from 2×10^{10} to 1.5×10^{11} parasites grown *in vitro* without synchronization. Enzyme activity was measured spectrophotometrically as described in Materials and Methods. Details of determining the parameters listed below are given in the figures. Two independent preparations were analyzed from clones 3D7 and HB3, and four from clone 7G8. Two determinations for each parameter were done for each preparation.

Parameter	Source of DHFR		
	Clone 3D7	Clone HB3	Clone 7G8
Specific activity of PPF-DHFR (nmol DHF min^{-1} mg^{-1} protein)	442 ± 39	634 ± 25	565 ± 85
Apparent turnover number (mol min^{-1} mol^{-1} enzyme)	2920 ± 296	4200 ± 523	2890 ± 350
V_{max} of PPF-DHFR (nmol DHF min^{-1} mg^{-1} protein)	442 ± 75	730 ± 28	562 ± 65
Catalytic sites in PPF-DHFR[a] (pmol mg^{-1} protein)	151	173	194
K_m for NADPH (μM)	4.5 ± 0.5	8.1 ± 2.0	11.6 ± 2.0
K_m for DHF (μM)	3.2 ± 0.6	42.6 ± 1.6	11.9 ± 1.2
K_{is} for pyrimethamine (nM)	0.19 ± 0.08	2.0 ± 0.3	8.9 ± 0.8
K_{ii} for pyrimethamine (nM)	0.19 ± 0.03		
Type of inhibition by pyrimethamine	noncompetitive	competitive	competitive
IC_{50} for pyrimethamine (nM)	0.18 ± 0.02	4.35 ± 0.35	95 ± 16
ID_{50} for pyrimethamine[b] (nM)	0.53	210	540
ID_{50} for trimethoprim[b] (nM)	8.5	480	4000
K_i for methotrexate (nM)	0.24 ± 0.03	0.29 ± 0.01	0.37 ± 0.03
Optimum pH at 37°	6.5–7.5	<6.0	6.5–7.5
Optimum temperature	47	42	47
Inhibition by KCl	yes	yes	yes

[a] V_{max}/turnover number.
[b] Two determinations were done for each clone, with eight wells per drug concentration; the mean value is given.

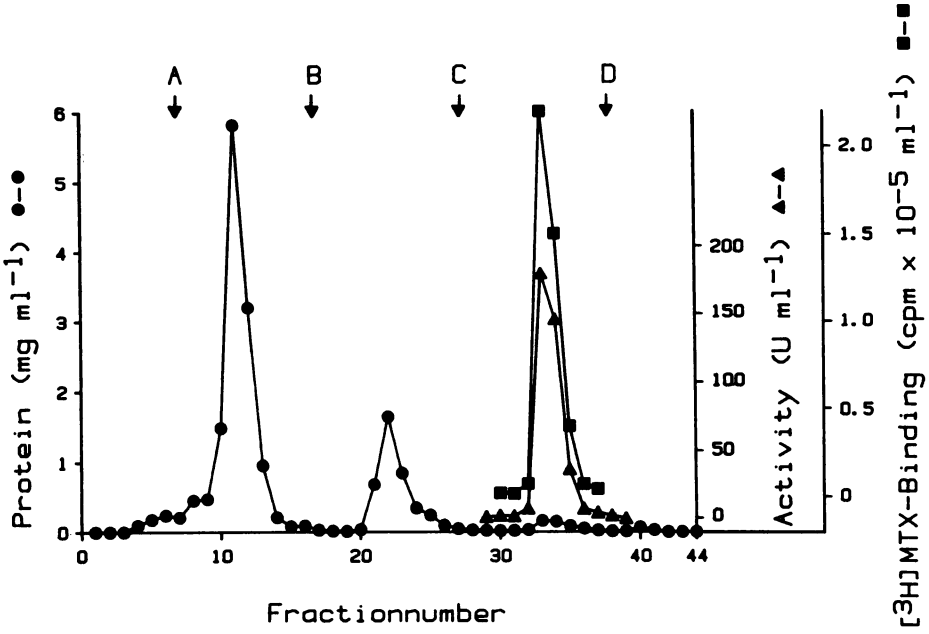

Fig. 2. Partial purification of DHFR from *P. falciparum*, clone 7G8. The 100,000 × *g* supernatant (see Materials and Methods) was applied to Red Sepharose (1 × 3 cm) washed with TEM buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol) (A), with TEM containing 0.5 M KCl (B), with TEM containing 0.5 M KCl and 4 mM DHF (C), and with TEM containing 1.5 KCl (D). ●, protein profile. DHFR activity was measured spectrophotometrically (▲) or by methotrexate binding (■) using 0.5 pmol of [3′,5′,7′-^{3}H]methotrexate (14.5 Ci $mmol^{-1}$) per assay in a total volume of 450 μl. Three dilutions of the column fractions containing the DHFR activity were measured.

throughout this paper to study the kinetic properties of the enzyme.

The specific activities of the PPF-DHFR from the three clones are listed in Table 1. They differ only by less than a factor of 2, comparing the resistant clones HB3 and 7G8 with the sensitive clone 3D7. When comparing specific activities of *in vitro* cultured, unsynchronized *P. falciparum*, one has to take into account the different developmental stages of the parasites present at the times of harvest. Since it is impractical to synchronize large-scale cultures used for the complete kinetic evaluation of the enzyme, we repeated the estimation of specific activity in one clone (3D7) after synchronization of the cultures with sorbitol and repeating the synchronization after 34 hr (15). Trophozoites (24 hr after the sorbitol treatments) contained 6–10 times more DHFR than the parasites in the ring stages (4 hr after synchronization) (data not shown). Although the stage-specific presence of DHFR in synchronized cultures of *P. falciparum* with larger amounts of parasites from different clones is awaiting further evaluation, we tentatively conclude that variations by less than a factor of 3–5 in the specific activity obtained analyzing *unsynchronized* cultures are not indicative of an increased enzyme level. Therefore, the difference in the amount of DHFR present in the three clones does not explain the differences in the *in vitro* inhibition profiles obtained with pyrimethamine (Fig. 1). Consequently, overproduction of DHFR can be ruled out as the molecular mechanism

of pyrimethamine resistance in the three *P. falciparum* clones studied, confirming the conclusions reached with the same Brazilian clone (7) and five different clones and isolates (8, 9). This is in contrast to earlier observations by Kan and Siddiqui (10).

Kinetic properties of PPF-DHFR. The K_m values for NADPH fell into a similar range (4.5–11.6 μM) (Table 1), whereas the affinity for the substrate DHF showed a greater than 13-fold decrease comparing the sensitive clone 3D7 to the resistant clone HB3 (Table 1). The Lineweaver-Burk plots of the PPF-DHFR inhibition by pyrimethamine resulted in a K_{ii} and K_{ii} of 0.19 nM, respectively, for clone 3D7 (Fig. 3A) compared to a K_i of 8.9 nM found in the resistant clone 7G8 (Fig. 3B). This ~50-fold difference in the affinity to pyrimethamine between the DHFR of the two clones indicates that the molecular mechanism for pyrimethamine resistance is due to a structurally altered DHFR. The inhibition by pyrimethamine was noncompetitive for the sensitive clone 3D7 and competitive for the two resistant clones (Table 1, Fig. 3). However, it cannot be excluded that, due to the tight binding of pyrimethamine to the DHFR of clone 3D7, the enzyme might be "depleted" by the inhibitor. Consequently, the Michaelis-Menton kinetics may not be applied in this situation. In earlier studies with *P. falciparum*, the inhibition of the DHFR by pyrimethamine of the drug-sensitive controls was found to be competitive (7–9) with a switch to noncompetitive in pyrimethamine-resistant isolates or clones (8, 9). The titration of a fixed amount of PPF-DHFR with increasing amounts of pyrimethamine resulted in a 520-fold increase in the concentration to inhibit 50% of the activity (Table 1) comparing the sensitive clone

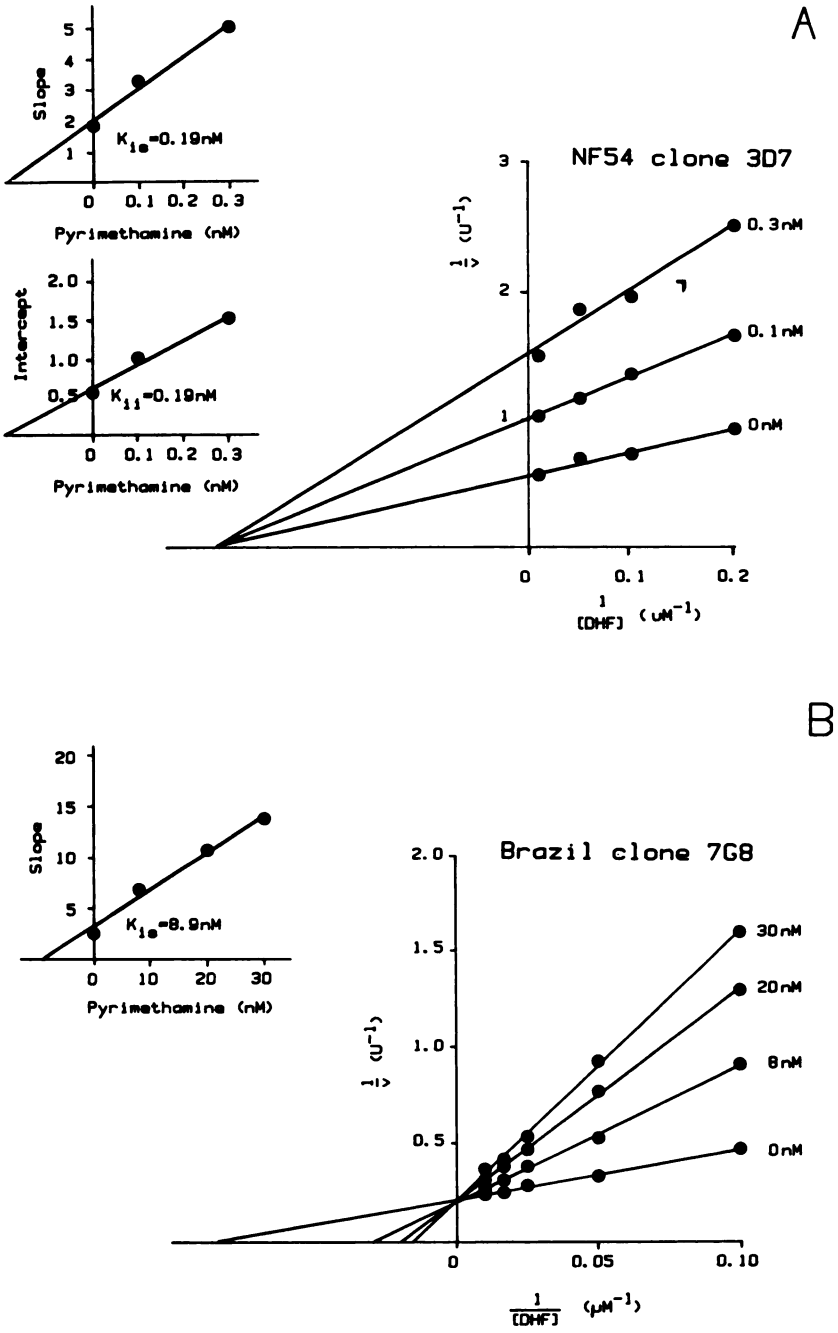

Fig. 3. Lineweaver-Burk plots of the inhibition of PPF-DHFR from the pyrimethamine-sensitive clone 3D7 of *P. falciparum* (A) and the resistant clone 7G8 (B) of *P. falciparum*. DHFR was partially purified (Fig. 2) and preincubated for 5 min at 37° with the amount of pyrimethamine indicated at the *right* of each curve. The NADPH concentration was 50 μM; the activity was measured spectrophotometrically as described in Materials and Methods. The *insets* show the plot of slope or intercept from a double reciprocal plot against the pyrimethamine concentration. Each point represents the mean of triplicate samples that varied by less than 5% for DHF concentrations ≥ 20 μM and 10% for the DHF concentrations ≤ 10 μM.

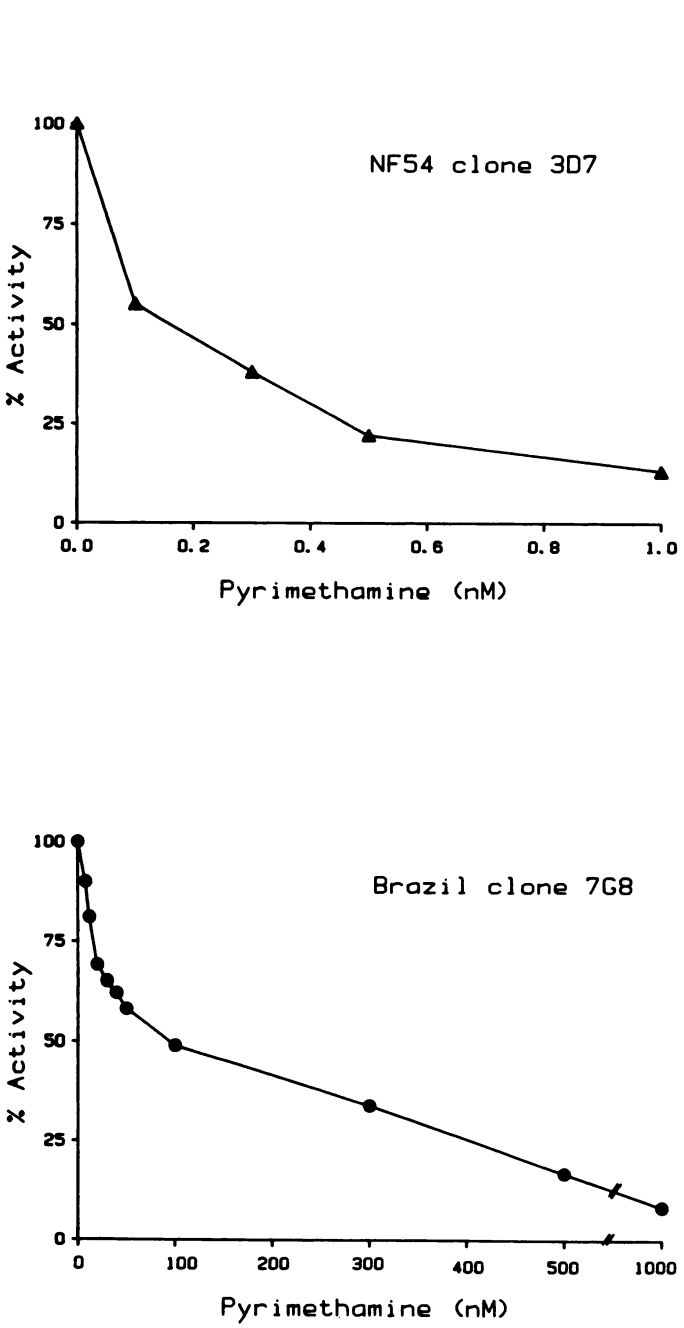

Fig. 4. Titration of PPF-DHFR from the pyrimethamine-sensitive clone 3D7 of *P. falciparum* (A) and the resistant clone 7G8 of *P. falciparum* (B) with pyrimethamine. Enzyme activity was measured spectrophotometrically as described in Materials and Methods using 100 μM DHF and 50 μM NADPH after preincubation of the DHFR for 5 min at 37° with the amount of pyrimethamine indicated. Note the difference in the scale between A and B. The 100% activity was 36 units ml^{-1} for 3D7 in A and 56 units ml^{-1} for 7G8 in B. Each point represents the mean of three samples for 3D7 and four samples for 7G8 with a variation of less than 5%.

3D7 (Fig. 4A) with the resistant clone 7G8 (Fig. 4B). The ratios of ID_{50} to IC_{50} for pyrimethamine for both the sensitive clone 3D7 and the resistant clone 7G8 are rather similar (2.9 for 3D7 versus 5.6 for 7G8) (Table 1). In contrast, the ratio of 48 for the resistant clone HB3 (Table 1) suggests that mechanisms other than an altered affinity of the drug to the enzyme contribute to the resistance exhibited by this clone. Reduced permeability of the cells has been suggested earlier as a possible alternative (11) although the uptake of radioactive pyrimethamine was unimpaired in sensitive and resistant *P. falciparum* strains investigated so far (8). Further studies are necessary to identify and analyze alternative mechanisms of resistance in *P. falciparum*.

The K_i for methotrexate was very similar (0.3–0.4 nM) (Table 1) for all three clones. Using the titration curves obtained with methotrexate (Fig. 5) and assuming a 1:1 binding ratio of the inhibitor to the PPF-DHFR, we calculated the apparent turnover numbers (Table 1). Based upon V_{max} and these turnover numbers, the amount of DHFR in partially purified DHFR preparations was very similar in all three clones (Table 1), again confirming the absence of an overproduction of the enzyme.

Influence of pH, temperature, and potassium chloride upon the PPF-DHFR activity. Both the sensitive clone 3D7 and the resistant clone 7G8 had a broad pH optimum of pH 6.5–7.5. The activity was inhibited at pH values > 8.0. In contrast, the pH optimum for the resistant clone HB3 was pH 6.0 (or lower) with only 60–35% of the activity remaining at pH 7.5 and <30% at pH 8.0, depending on the buffer system used (Fig. 6). The temperature dependency varied considerably

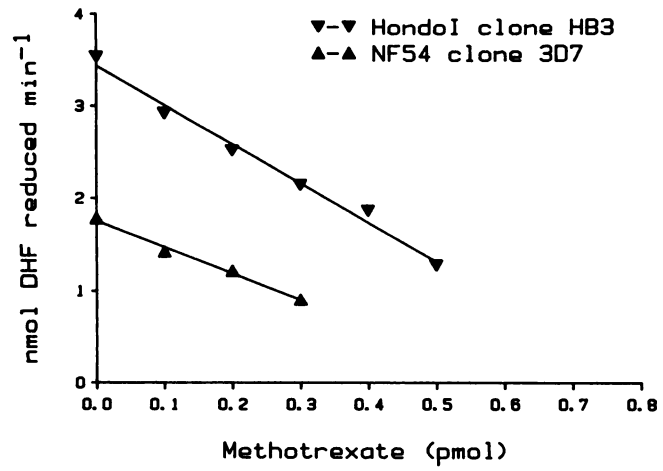

Fig. 5. Titration of the PPF-DHFR from the pyrimethamine-sensitive clone 3D7 of *P. falciparum* and the resistant clone HB3 of *P. falciparum* with methotrexate. Measurements and preincubation with methotrexate were done as described in the legend of Fig. 4 for pyrimethamine. Each point was done in triplicate and the mean is shown.

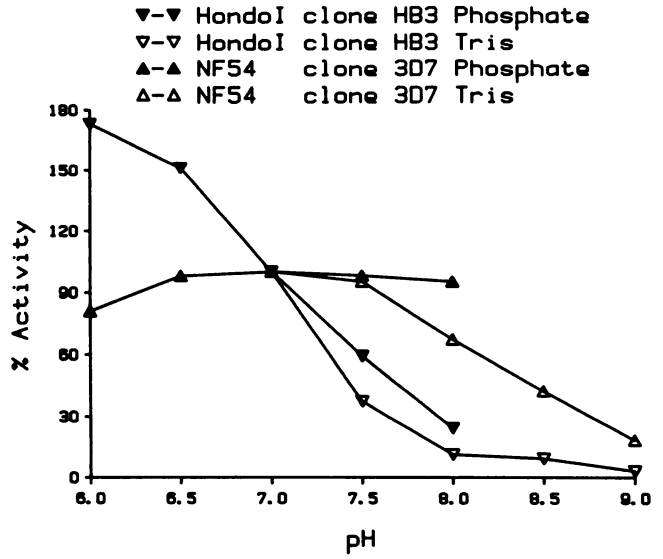

Fig. 6. Effect of pH on the DHFR activity from the pyrimethamine-sensitive clone 3D7 and the resistant clone HB3 of *P. falciparum*. Enzyme activity was measured at 37° as described in Materials and Methods using TEM buffer (50 mM Tris, 1 mM EDTA, 5 mM 2-mercaptoethanol) for pH from 7.0 to 9.0, measured at 25° (▽, △) and SEM buffer (50 mM sodium phosphate, 1 mM EDTA, 5 mM 2-mercaptoethanol) for pH from 6.0 to 8.0 (▼, ▲). The activity at pH 7.0 was taken as 100% in both buffer systems. The same DHFR samples were used as described in Fig. 7. The effects of the pH on the DHFR activity of clone 7G8 were very similar to those observed with clone 3D7 and are therefore not shown in this figure.

among the three clones with a temperature maximum at 47° for 3D7 and 7G8 and a total loss of activity at 53° for 7G8, a temperature at which HB3 still retained >60% of the activity (Fig. 7). The DHFR of all three clones was progressively inhibited by increasing amounts of KCl (0.2–1 M).

In summary, the kinetic evaluation of the DHFR from three clones of *P. falciparum* revealed that the molecular mechanism for pyrimethamine resistance is due to a structurally altered DHFR, characterized by a decreased affinity to the drug. No indication for increased enzyme levels and, therefore, overproduction of the DHFR was found in the parasites analyzed.

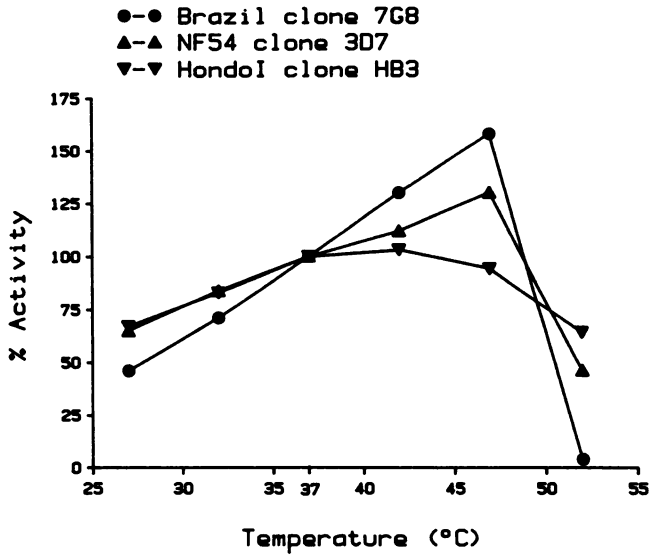

Fig. 7. Effect of incubation temperature on DHFR activity of the three clones of *P. falciparum*. The activity measurements were done as described in Materials and Methods. All components were equilibrated for 5 min at the temperature indicated, except for the substrate. The activity in Tris-buffer, pH 7.0, at 37° was taken as 100%, equivalent to 48 units ml^{-1} for 3D7, 46 units ml^{-1} for HB3, and 55 units ml^{-1} for 7G8. No corrections were made for the temperature-dependent shift in pH value. Each assay was in duplicate and the mean is shown.

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References

1. Ravetch, J. V., J. Young, and G. Poste. Molecular genetic strategies for the development of anti-malarial vaccines. *Bio/technology.* **3**:729–740 (1985).
2. Peters, W. The problem of drug resistance in malaria. *Parasitology* **90**:705–715 (1985).
3. 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).
4. Diggens, S. M., W. E. Gutteridge, and P. I. Trigg. Altered dihydrofolate reductase associated with a pyrimethamine-resistant *Plasmodium berghei berghei* produced in a single step. *Nature (Lond.)* **228**:579–580 (1970).
5. Ferone, R. Dihydrofolate reductase from pyrimethamine-resistant *Plasmodium berghei. J. Biol. Chem.* **245**:850–854 (1970).
6. 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).
7. 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).
8. Dieckmann, A., and A. Jung. The mechanism of pyrimethamine resistance in *Plasmodium falciparum. Parasitology,* **93**:275–278 (1986).
9. Walter, R. D. Altered dihydrofolate reductase in pyrimethamine-resistant *Plasmodium falciparum. Mol. Biochem. Parasitol.* **19**:61–66 (1986).
10. Kan, S. C., and W. A. Siddiqui. Comparative studies on dihydrofolate reductases from *Plasmodium falciparum* and *Aotus trivirgatus. J. Protozool.* **26**:660–664 (1979).
11. Banyal, H. S., and J. Inselburg. *Plasmodium falciparum*: induction, selection, and characterization of pyrimethamine-resistant mutants. *Exp. Parasitol.* **62**:61–70 (1986).
12. Delemarre-Van de Waal, H. A., and F. C. De Waal. Een tweede patiënt met malaria tropica op natuurlijke wijze verkregen in Nederland. *Ned. Tijdschr. Geneeskd.* **125**:375–377 (1981).
13. Bhasin, V. K., and W. Trager. Gametocyte-forming and non-gametocyte-forming clones of *Plasmodium falciparum. Am. J. Trop. Med. Hyg.* **33**:534–537 (1984).
14. Burkot, T. R., J. L. Williams, and I. Schneider. Infectivity to mosquitoes of *Plasmodium falciparum* clones grown *in vitro* from the same isolate. *Trans. R. Soc. Trop. Med. Hyg.* **78**:339–341 (1984).
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. Desjardins, R. E., C. J. Canfield, J. D. Haynes, and J. D. Chulay. Quantitative

assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. *Antimicrob. Agents Chemother.* **16**:710–718 (1979).
17. Drake, J. C., C. J. Allegra, and B. A. Chabner. A re-evaluation of the competitive protein binding assay for methotrexate binding to dihydrofolate reductase. *Biochem. Pharmacol.* **35**:1212–1214 (1986).
18. Hillcoat, B. L., P. F. Nixon, and R. L. Blakley. Effect of substrate decomposition on the spectrophotometric assay of dihydrofolate reductase. *Anal. Biochem.* **21**:178–189 (1967).
19. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
20. Graves, P. M., R. Carter, J. S. Keystone, and D. C. Seeley, Jr. Drug sensitivity and isoenzyme type in cloned lines of *Plasmodium falciparum*. *Am. J. Trop. Med. Hyg.* **33**:212–219 (1984).
21. Chulay, J. D., W. M. Watkins, and D. G. Sixsmith. Synergistic antimalarial activity of pyrimethamine and sulfadoxine against *Plasmodium falciparum*. *Am. J. Trop. Med. Hyg.* **33**:325–330 (1984).
22. Milhous, W. K., N. F. Weatherly, J. H. Bowdre, and R. E. Desjardins. *In vitro* activities of and mechanisms of resistance to antifol antimalarial drugs. *Antimicrob. Agents Chemother.* **27**:525–530 (1985).
23. Zolg, J. W., and U. J. Hänggi. Characterization of a R plasmid-associated, trimethoprim-resistant dihydrofolate reductase and determination of the nucleotide sequence of the reductase gene. *Nucleic Acids Res.* **9**:697–710 (1981).

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