Dihydrofolate Reductase from Pyrimethamine-resistant
Plasmodium berghei

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SUMMARY

An investigation of the Hz-folate reductases from pyrimethamine-sensitive (Pb/WLTM) and -resistant (Pb/WLTM/50-63) strains of Plasmodium berghei has revealed that the specific activity of the enzyme from the resistant strain is 10-fold higher than that of the sensitive strain. Since turnover numbers (based on amethopterin binding) are equivalent, the increase in specific activity must be due to an increased number of catalytic sites.

Marked differences were found between the two enzymes in several of the properties studied. The $K_m$ value for Hz-folate is 12.4-fold higher for the enzyme from the resistant strain. The Pb/WLTM/50-63 Hz-folate reductase is only slightly stimulated by KCl, in contrast to the 3-fold stimulation observed with the enzyme from the sensitive strain. The $K_i$ values for pyrimethamine and two other antifolates are higher for the enzyme from the resistant strain. In addition, the inhibition is competitive with Hz-folate for the enzyme from Pb/WLTM and noncompetitive for the enzyme from Pb/WLTM/50-63.

It is proposed that Pb/WLTM/50-63 is resistant in vivo to the action of pyrimethamine because of the combined effects of the increase in enzyme content and the decrease in inhibitor binding. It is suggested that these alterations in enzymatic properties and enzyme levels reflect mutations in the gene or genes responsible for the coding of this enzyme.

The antifolate properties of the antimalarial agent pyrimethamine have been well established (1). It has been shown to be a moderately potent inhibitor of Hz-folate reductase (EC 1.5.1.3) from various organisms (2). Recently it was demonstrated that the basis of the chemotherapeutic effectiveness of pyrimethamine in the rodent malarial organism, Plasmodium berghei, is the extremely tight binding of drug to the parasite Hz-folate reductase, in contrast to that to the enzyme from the host (3).

Resistance to pyrimethamine has been found in human malaria (4) and can be produced in plasmodial infections in laboratory animals (5, 6). With the demonstration of the plasmodial Hz-folate reductase as the locus of action of this drug (3), it became possible to investigate the involvement of this enzyme in resistance. Changes in the amounts and properties of Hz-folate reductases have been observed in some organisms resistant to antifolates (usually close structural analogues of the substrate such as aminopterin and amethopterin). Antifolate resistance in mammalian cells in most cases has involved increased enzyme levels, with no detectable changes in the enzymatic characteristics (e.g. see Reference 7). On the other hand, qualitative alterations in the enzyme have been observed in several antifolate-resistant bacteria (8-10). Genetic analysis in resistant Diplococcus pneumoniae (8) and Salmonella typhimurium (10) revealed the presence of multiple loci involved in antifolate resistance.

Evidence is presented in this paper demonstrating changes in the properties of the Hz-folate reductase isolated from a pyrimethamine-resistant strain of $P$. berghei. It is proposed that the observed decrease in drug binding and the increased level of this enzyme are sufficient to account for the drug resistance of this strain in vivo.

METHODS

The reagents used, the spectrophotometric Hz-folate reductase assay, and the enzyme isolation procedure have been described previously (3). The enzyme isolation, in brief, consisted of the lysing of washed, infected erythrocytes with saponin, washing the liberated parasites free from red cell debris and hemoglobin, and then passing the plasmodia through a French pressure cell at 15,000 to 20,000 p.s.i. After centrifugation, freeze-thawing, and a second centrifugation, the crude extracts were passed over a Sephadex G-100 column. Since the plasmodial Hz-folate reductases were 9- to 10-fold larger than the host enzyme (3), they were eluted at a position immediately after the void volume of the column, whereas any contaminating host enzyme was eluted near two void volumes (and thus was not included in the pool of malarial Hz-folate reductase). The reciprocal plots were analyzed with the aid of computer programs designed by Cleland (11). The $K_i$ values calculated from the slopes are reported for all the inhibitors.

The pyrimethamine-sensitive strain of $P$. berghei used in this study (designated Pb/WLTM) originated from the primary isolation of $P$. berghei by Vincke and Lips (12), in 1948, and has been maintained by syringe passage in mice at the Wellcome Laboratories of Tropical Medicine, Beckenham, Kent, England for 20 years. The resistant strain (Pb/WLTM/50-63) was produced2 by guest on October 14, 2017 http://www.jbc.org/ Downloaded from
from the sensitive strain by passage in the presence of increasing levels of pyrimethamine; after 50 weeks the strain was fully resistant to the maximum tolerated dose (see Table II). This resistance level was retained even when the strain was passed in untreated mice.

When inoculated into mice at the Wellcome Laboratories of Tropical Medicine, England, both strains invaded mainly mature erythrocytes, killing the mice in 6 to 7 days. In this laboratory in the United States mainly immature erythrocytes were invaded by the parasites, resulting in an average survival time of 14 to 19 days for the mice. Thus, this strain differs from the \textit{P. berghei} NYU-2 strain previously investigated (3), since the latter developed in mature red cells. Consequently, parasitized blood for enzyme isolation was taken on the 13th to 14th day of infection, in contrast to the 5th to 6th day used with the \textit{P. berghei} NYU-2 strain. In this laboratory, the sensitive and resistant strains were transfused biweekly, and the resistant strain was maintained in the presence of the maximum tolerated dose of pyrimethamine (25 mg per kg, intraperitoneal, on alternate days for six to seven doses). Infected mice for enzyme studies were not treated.

The drug sensitivities \textit{in vivo} were determined at the Wellcome Laboratories of Tropical Medicine in England. Mice were inoculated intraperitoneally with \(5 \times 10^6\) parasitized erythrocytes. Groups of five mice were given seven oral doses of drug starting on the afternoon of the day of infection and were then treated twice a day for the following 3 days. On the 4th day parasitemia was determined from stained tail smears, and the dosage of drug which was required to reduce parasitemia to 50% of the untreated control value was calculated.

### RESULTS

**Comparison of \(H_2\)-folate Reductase of Pyrimethamine-sensitive \textit{P. berghei} Strains.** The \(H_2\)-folate reductase from \textit{Pb/WLTM} was essentially identical to the enzyme from the \textit{Pb/NYU-2} strain previously reported (3) in most characteristics studied. No significant differences between the two strains could be detected in pH optimum, specific activity, 2-mercaptoethanol stimulation, 50% inhibitory level of several antifolates, and the type of binding of pyrimethamine. The values obtained for these parameters for the enzyme from \textit{Pb/WLTM} were almost identical with those published for the \textit{Pb/NYU-2} \(H_2\)-folate reductase (3). However, the \(K_m\) values for \(H_2\)-folate did differ somewhat: 2.6 \(\mu\)M for \textit{Pb/NYU-2}, 4.2 \(\mu\)M for \textit{Pb/WLTM}. Also, although both enzymes were similar to certain mammalian (13, 14) and avian (15) \(H_2\)-folate reductases in their ability to be stimulated by certain reagents (such as KC1), optimum stimulation of activity was found at different KC1 concentrations for the two plasmoidal enzymes. The maximum, 3-fold stimulation of activity was at 0.2 to 0.5 mM KC1 for the \textit{Pb/NYU-2} \(H_2\)-folate reductase (3), and at 0.15 mM KC1 for the \textit{Pb/WLTM} enzyme. This stimulation is mainly due to the anion, since \(\text{NH}_4\text{Cl}\) and \(\text{NaCl}\) were as active as KC1 in stimulating the enzyme from \textit{Pb/NYU-2}, but \(\text{KBr}\) and \(\text{KNO}_3\) were only 80% as active, and sodium sulfate was less than 60% as effective as KC1. Previous data on Tris buffer salts (3) support this contention.

**Comparison of \(H_2\)-folate Reductases of Pyrimethamine-sensitive and -resistant \textit{P. berghei}—The \(H_2\)-folate reductase isolated from the resistant strain, \textit{Pb/WLTM/50-63}, differed greatly from the enzyme from the sensitive strain. The \(K_m\) value for \(H_2\)-folate is 12.4-fold higher for the enzyme from \textit{Pb/WLTM/50-63} than for that from \textit{Pb/WLTM} (Table I).

**Specific Activity—**It was found that the specific activity of the resistant strain \(H_2\)-folate reductase was 10-fold higher than that from the sensitive strain (Table I). Since the increased specific activity could be due to an increase in enzyme concentration, or catalytic activity (or both), it was of interest to determine the turnover numbers for the two enzymes. Amethopterin binds \(H_2\)-folate reductases stoichiometrically under certain conditions, and Werkheiser (16) used this property to determine turnover numbers in terms of moles of \(H_2\)-folate reduced per min per mole of amethopterin bound. Enzyme preparations of the sensitive and resistant strains were titrated with amethopterin, and the values obtained were essentially identical (Table I). Thus, if the number of amethopterin binding sites per molecule were the same for the two enzymes, the difference in specific activities observed is due to an increased amount of enzyme in the resistant strain.

**Effects of Cl—**A difference was observed in the response of the two enzymes to KC1 in the assays. The activity of the \(H_2\)-folate reductase from \textit{Pb/WLTM} was stimulated 3-fold at 0.15 mM KC1, whereas the resistant strain enzyme was only slightly stimulated at 0.15 mM KC1 and was inhibited at higher concentrations (Fig. 1). This difference was not due to the testing of \textit{Pb/WLTM/50-63} at \(H_2\)-folate concentrations saturating for this enzyme.

### Table I

Comparison of properties of \textit{Pb/WLTM} and \textit{Pb/WLTM/50-63} \(H_2\)-folate reductases

<table>
<thead>
<tr>
<th>Source of (H_2)-folate reductase</th>
<th>(K_m) (H_2)-folate</th>
<th>(V_{max}) (H_2)-folate</th>
<th>Turnover number</th>
<th>Sources of (H_2)-folate reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Pb/WLTM}</td>
<td>4.2 ± 0.2 (7)*</td>
<td>1.6 ± 0.2 (5)*</td>
<td>1150[220-1380]</td>
<td>\textit{Pb/NYU-2}</td>
</tr>
<tr>
<td>\textit{Pb/WLTM/50-63}</td>
<td>51.9 ± 0.3 (10)</td>
<td>15.8 ± 0.8 (4)</td>
<td>1110[1050-1180]</td>
<td>\textit{Pb/WLTM/50-63}</td>
</tr>
</tbody>
</table>

* Mean ± S.E. of mean (number of determinations).

**Mean [range] (number of determinations).**

**Fig. 1. Effects of concentrations of Tris-HCl, pH 7.0 (37°), and KC1 on \(H_2\)-folate reductases from \textit{Pb/WLTM} and \textit{Pb/WLTM/50-63}**. The assays were performed in the usual manner, with the indicated concentrations of Tris-HCl buffer and KC1. The activity of each enzyme in the presence of 0.015 mM Tris-HCl was set equal to the value of 100 for ease of comparison.

**TABLE I**

Comparison of properties of Pb/WLTM and Pb/WLTM/50-63 H2-folate reductases
Table II
Comparison of effects of several antifolates in vivo and on H₂-folate reductases from Pb/WLTM and Pb/WLTM/50-63

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pb/WLTM</th>
<th>Pb/WLTM/50-63</th>
<th>Pb/WLTM/50-63 to Pb/WLTM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E½ μM* in vivo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>m/μM</td>
<td>m/μM</td>
<td></td>
</tr>
<tr>
<td>H₂-folate not incubated</td>
<td>0.37</td>
<td>0.27</td>
<td>0.15</td>
</tr>
<tr>
<td>H₂-folate incubated</td>
<td>0.78</td>
<td>0.27</td>
<td>0.85</td>
</tr>
<tr>
<td>Cycloguanil</td>
<td>1.0</td>
<td>0.27</td>
<td>1.3</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>7.1</td>
<td>0.27</td>
<td>7.5</td>
</tr>
</tbody>
</table>

* Pyrimethamine, see Footnote 1; cycloguanil, 4,6-diamino-1-(p-chlorophenyl)-1,2-dihydro-2,2-dimethyl-s-triazine; trimethoprim, 2,4-diamino-5-(3',4',5'-trimethoxybenzyl)pyrimidine.

** Dose of drug, in milligrams per kg, which reduced the parasitemia of the treated mice to 50% of that of the untreated mice. See "Methods" for details.

a Enzyme and drug incubated 5 min at 37° in usual manner, with or without H₂-folate as indicated.

** Maximum tolerated dose.

Both drugs were competitive with the substrate with the sensitive strain enzyme, but noncompetitive for the enzyme from the resistant strain.

It had been reported that the binding of pyrimethamine to the enzyme from Pb/NYU-2 was affected by the assay conditions (3). Stoichiometric inhibition was observed when enzyme and drug were incubated for 5 min at 37° before the reaction was initiated by the addition of H₂-folate. If the H₂-folate was included during the incubation (reaction started with NADPH), the binding was reversible. In the present study, the identical mode of binding was demonstrated in the H₂-folate reductase from Pb/WLTM, but not from Pb/WLTM/50-63. Incubation of drug and enzyme, in the presence or absence of H₂-folate, had no effect on the reversibility of the pyrimethamine inhibition of the Pb/WLTM/50-63 enzyme. The inhibition was noncompetitive with H₂-folate (Fig. 2). This was also true of the inhibition of the resistant strain enzyme by the other two antifolates tested. The type of inhibition observed with pyrimethamine against the Pb/WLTM enzyme varied with the assay conditions used. Noncompetitive kinetics resulted when drug and enzyme were incubated prior to the addition of H₂-folate, whereas the inhibition was competitive when the H₂-folate was included in the incubation mixture (Fig. 2).

DISCUSSION

The H₂-folate reductase isolated from Pb/WLTM/50-63 exhibited several properties quite distinct from those of the enzyme from the sensitive strain. Changes such as increased enzyme content and decreased antifolate binding bear directly upon the ability of the parasite to survive in the presence of otherwise lethal concentrations of drug, and they thus provide a basis for understanding the mechanism or mechanisms of resistance of this strain. Differences in Kₘ values for H₂-folate and response to KCl offer further evidence of the dissimilar nature of the two enzymes. The data strongly suggest the presence of an increased content of a structurally altered H₂-folate reductase in Pb/WLTM/50-63, presumably due to mutations in the gene or genes responsible for the synthesis of this enzyme.

The increase in apparent Kₘ value for H₂-folate and the increases in the Kₘ values for the antifolates tested could be related phenomena. A substitution of an amino acid involved in binding...
of the H2-folate might also affect inhibitor affinity, since it is felt that substrate and analogues share certain common binding points (2,17). However, the difference in antifolate binding between the two enzymes was not only quantitative. Inspection of the Lineweaver-Burk plots (Fig. 2) reveals competitive inhibition of the H2-folate reductase from Pb/WLTM, and noncompetitive inhibition of the enzymes from Pb/WLTM/50-63. Differences in the 1/v intercept of reciprocal plots of control and inhibitor curves are normally interpreted to mean that the inhibitor combines with a different form of the enzyme than does the substrate (18). The inability of H2-folate to completely overcome the antifolate inhibitions of the Pb/WLTM/50-63 enzyme thus indicates that these inhibitors combine with a different form of the enzyme than does H2-folate. Since this does not occur with the enzyme from the sensitive strain, it may be inferred that the resistant strain H2-folate reductase may exist in one state that cannot be attained by the Pb/WLTM enzyme. This could be attributed to different primary structures for the two proteins.

The two distinct patterns of chloride effects (Fig. 1) are also suggestive of different conformational forms for the two enzymes. It has been proposed that activation of H2-folate reductase by salts and other reagents involves conformational changes in the enzyme protein, resulting in increases in V_{max} and possibly in apparent K_m values (13-15). The lack of stimulation of the Pb/WLTM/50-63 H2-folate reductase and the higher H2-folate K_m value may indicate that this enzyme exists in an already "activated" state. Thus, the inhibition of the enzyme from the resistant strain found at increasing KCI levels may be similar to the inactivations noted for H2-folate reductases from various sources at reagent concentrations higher than necessary for optimum activation (13-15).

The enzymatic characteristics of the H2-folate reductase isolated from Pb/WLTM were very similar to those reported previously for the Pb/NYU-2 strain (3). The small differences encountered (K_m values for H2-folate and KCI stimulation patterns) are probably reflections of minor variations of the protein structures of the two enzymes, although unknown influences due to the growth of the parasites in erythrocytes of different ages cannot be discounted. In either case, the intrastain variations in enzymatic characteristics are minor when compared to the major differences (i.e. molecular weight, substrate K_m values, inhibitor binding, urea stability, etc.) reported between the plasmoidal H2-folate reductase and this enzyme from various other organisms.

It is difficult to correlate quantitatively the enzyme changes found with the degree of resistance observed in vivo. The Pb/WLTM/50-63 strain is completely resistant to pyrimethamine and the two other antifolates tested (Table II). Since the dose levels used were limited by the toxicity to the host, the resistance values obtained are all minimal values based on the ratio of the maximum tolerated doses for mice to the ED_{50} values for the sensitive strain. However, even though no definitive values can be placed on the degree of resistance to the three drugs, inspection of Table II reveals that the minimal values calculated are all much greater than the ratios of K_i values for the two enzymes. Thus, the resistance in vivo cannot be due simply to decreased drug binding by the resistant strain enzyme.

The mechanism of resistance of Pb/WLTM/50-63 is more akin to that reported for certain strains of antifolate-resistant bacteria; both decreased affinity for inhibitor and increased enzyme levels are important factors in determining the degree of resistance (8-10). The specific activity for H2-folate reductase in extracts of Pb/WLTM/50-63 is 11-fold higher than in extracts of Pb/WLTM. The turnover numbers based on amethopterin titration were essentially identical, indicating that the greater specific activity of the resistant strain enzyme is due to an increased number of catalytic sites and not increased catalytic activity per site. Increase in H2-folate reductase content has commonly been found in antifolate-resistant cells of mammalian and bacterial origin, frequently associated with another factor such as decreased inhibitor permeability (see Reference 19) or decreased inhibitor binding (8-10). Antifolate-resistant strains of D. pneumoniae (8) and S. typhimurium (10) have been reported with multiple mutations affecting both the levels and properties of the H2-folate reductase. It seems likely that multiple genetic loci also are involved in the resistance of Pb/WLTM/50-63. This strain was developed by constant exposure to partially active doses of pyrimethamine; dosage was increased only when the level used became ineffective. Since full resistance developed in a stepwise manner over a period of 50 weeks, it is probable that more than one mutation was involved. The final level of resistance attained appears to be due mainly to the cooperative effects of separate phenomena: increased enzyme content and decreased inhibitor binding. Although other factors (e.g. drug permeability, substrate levels, end product utilization) might be involved, it seems likely that the differences observed in enzyme levels and properties are sufficient in nature and magnitude to account for the ability of the resistant strain to grow and multiply in the presence of drug levels lethal to the sensitive strain.

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References
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