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Dihydropteroate Synthetase from *Plasmodium berghei*: Isolation, Properties, and Inhibition by Dapsone and Sulfadiazine

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SUMMARY

MCCULLOUGH, JERRY L., AND MAREN, THOMAS H.: Dihydropteroate synthetase from *Plasmodium berghei*: isolation, properties. and inhibition by dapsone and sulfadiazine. *Mol. Pharmacol.* **10,** 140–145 (1974).

Dihydropteroate synthetase, which catalyzes the condensation of 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphate and *p*-aminobenzoic acid to form dihydropteroic acid, has been isolated from cells of the rodent malarial organism *Plasmodium berghei*; some of its properties are described. The optimum pH for enzyme activity was found to be 8.5. The apparent Michaelis constants for *p*-aminobenzoic acid and the hydroxymethyldihydropteridine pyrophosphate were found to be 2.8 μM and 1.4 μM, respectively. Both 4,4′-diaminodiphenylsulfone (I_{50} = 89 μM) and sulfadiazine (I_{50} = 180 μM) were effective inhibitors of enzyme activity. The inhibition of the enzyme by these drugs correlated with their activity *in vivo* against *P. berghei* infections.

INTRODUCTION

The sulfones 4,4′-diaminodiphenylsulfone (dapsone) and 4,4′-diformamidodiphenylsulfone are of considerable chemotherapeutic interest because of their use in the treatment of new resistant forms of human malaria of the *Plasmodium falciparum* type (1). It has been shown that DDS[2] and certain derivatives of it are effective inhibitors of *Escherichia coli* dihydropteroate synthetase (2). Since dihydropteroate synthetase is presumably also the site of action of these drugs against the malarial organism *in vivo*, an understanding of the properties of this enzyme from plasmodia is important for the further rational development of effective inhibitors. This report summarizes our studies on the isolation and properties of dihydropteroate synthetase obtained from the rodent malarial organism *Plasmodium berghei*. A comparison is made of the inhibition by DDS and sulfadiazine of the enzyme and the activity of these drugs *in vivo* against *P. berghei* infections.

MATERIALS AND METHODS

EDTA, Tris, and bovine serum albumin were purchased from Sigma Chemical Company; Sephadex G-25, from Pharmacia Fine Chemicals, Inc.; saponin, from J. T. Baker Chemical Company; and [7-^{14}C]*p*-aminobenzoic acid (specific activity, 5.85 mCi/mmole), from New England Nuclear Corporation. 4,4′-Diaminodiphenylsulfone was obtained from Parke, Davis and Company, and sulfadiazine, from Lederle Laboratories.

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[2] The abbreviation used is: DDS,4,4′-diaminodiphenylsulfone (dapsone).

Stock solutions of DDS and sulfadiazine were made by dissolving the compounds in 1% dimethyl sulfoxide.

Synthesis of pteridines. 2-Amino-4-hydroxy-6-hydroxymethylpteridine (hydroxymethylpteridine) was prepared by the condensation of 6-hydroxy-2,4,5-triaminopyridine sulfate with 1,3-dihydroxyacetone by the method of Baugh and Shaw (3). The pyrophosphate ester of hydroxymethylpteridine (hydroxymethylpteridine pyrophosphate) was prepared and purified by column chromatography on DEAE-cellulose according to Shiota *et al.* (4). Hydroxymethylpteridine pyrophosphate was reduced to 2-amino-4-hydroxy-6-pyrophosphorylmethyl-7,8-dihydropteridine (hydroxymethyldihydropteridine pyrophosphate) with dithionite as described by Shiota *et al.* (5). This material was prepared prior to each experiment. Formation of the dihydro compound was assessed by examination of the ultraviolet absorption spectrum (6). The concentration of dihydropteridine compounds was determined at 330 nm (pH 7.1), using a molar extinction coefficient of 6200 (5).

Assay of dihydropteroate synthetase activity. Dihydropteroate synthetase activity was measured by a modification of the radioactive assay of Richey and Brown (7), based on the incorporation of [^{14}C]*p*-aminobenzoic acid into dihydropteroate. Each reaction mixture was prepared to contain, in a volume of 0.4 ml, Tris-HCl buffer, pH 8.5, 100 mM; $MgCl_2$, 10 mM; 2-mercaptoethanol, 50 mM; hydroxymethyldihydropteridine pyrophosphate, 0.12 mM; [^{14}C]*p*-aminobenzoic acid, 0.04 mM (except in the reaction mixtures for I_{50} determinations, in which the concentration of *p*-aminobenzoic acid was 0.01 mM); inhibitor, as indicated; and 1.2 mg of partially purified dihydropteroate synthetase extract. Blank determinations contained all components except enzyme. The reaction mixtures, in 1 × 7 cm test tubes, were stoppered and incubated for 1 hr at 37°. The reactions were stopped immediately by the addition of 25 μmoles of EDTA (pH 8.3). Each reaction mixture was evaporated to dryness under reduced pressure and redissolved in 0.075 ml of 0.05 M Tris, pH 8.0, and 0.05 ml was applied (each in an area 1.0 × 4.0 cm) to Whatman No. 3MM chromatography paper. The chromatograms were developed by descending chromatography with 0.1 M potassium phosphate buffer, pH 7.0, for 4 hr at 25°. Under these conditions pteroate (dihydropteroate is oxidized to pteroate during the evaporation step) remains at the origin, whereas unreacted [^{14}C]*p*-aminobenzoic acid migrates with an R_F value of 0.78. Areas corresponding to the origin of the developed chromatograms were cut out and counted by liquid scintillation spectrometry. All enzyme assays were done in triplicate, and the points drawn on the figures represent the averages of these experimental values. Enzyme activity is expressed as nanomoles of dihydropteroate produced per hour.

The concentrations of DDS and sulfadiazine required for 50% inhibition (I_{50}) of dihydropteroate synthetase activity were determined by titration of at least five levels of inhibitor in the standard reaction system.

Protein was determined by the biuret method (8), using crystalline bovine serum albumin as the standard.

All determinations of radioactivity were made in a Beckman model 1650 scintillation spectrometer, using Bray's (9) scintillation fluid.

Preparation of dihydropteroate synthetase extract. The *P. berghei* infection was obtained from Dr. Leo Rane, working under the auspices of the Walter Reed Army Institute of Research, and was maintained in 15 (±2)-g male CD-1 mice (Charles River Laboratories, Wilmington, Mass.) by weekly transfer of blood from infected mice. For preparation of the enzyme extracts, 300 mice were given an intraperitoneal injection of 0.5 ml of infected blood containing approximately 10^5 parasitized erythrocytes. Blood for the enzyme extraction (withdrawn on the fifth day of infection; parasitemia, 55–70%) was obtained by decapitation and collected into 5% aqueous sodium citrate solution (one-tenth the final volume). Unless otherwise noted, all the following steps were performed at 4°C. The plasma and buffy coat were removed by centrifugation for 10 min at 2200 × *g*. The parasites were released from the red cells by lysis with saponin as described by Ferone *et al.* (10).

After this procedure, the brown pellet (consisting of free parasites, some leukocytes, and fibrous matter) was resuspended in an equal volume of 0.05 M Tris-HCl buffer, pH 8.0. The suspension was passed twice through a French pressure cell (Aminco) at 15,000–20,000 psi and centrifuged at 91,000 × *g* for 30 min. The reddish brown, turbid supernatant fluid was stored frozen for 16 hr and centrifuged at 91,000 × *g* for 30 min upon thawing. The supernatant solution was then fractionated by adding slowly 11.4 g of solid ammonium sulfate to 100 ml of solution. The mixture was stirred for 1 hr and centrifuged for 1 hr at 27,000 × *g*. Ammonium sulfate (34 g) was added to 100 ml of the supernatant solution. The mixture was stirred for 1 hr and centrifuged for 1 hr at 27,000 × *g*. The precipitate was then dissolved in 10–20 ml of 0.05 M Tris-HCl buffer, pH 8.0, and dialyzed for 36 hr against the same buffer (three changes of 6 liters of buffer). The dialyzed material was then centrifuged for 1 hr at 118,000 × *g*. The clear extract was applied to a column of Sephadex G-25 (2.5 × 26 cm) which had been equilibrated with 0.05 M Tris-HCl buffer, pH 8.0. The same buffer was used to elute the enzyme from the column. The initial protein eluates were combined and used as the dihydropteroate-synthesizing extract.

RESULTS

Enzyme extraction. To detect sufficient dihydropteroate synthetase activity in the crude extracts, it was necessary to use approximately 300 mice with a high percentage of parasitized red cells (55–90%). Attempts to remove the hemoglobin from the crude extract by either chloroform–ethanol precipitation (11) or treatment with carboxymethyl Sephadex (10) resulted in complete loss of enzyme activity. Attempted removal of hemoglobin by gel filtration on Sephadex G-100 resulted in simultaneous elution of enzyme activity and the hemoglobin peak. In the method finally adopted, small molecules were removed from the crude extract by gel filtration on Sephadex G-25 providing an enzyme preparation with a maximum specific activity of 0.12 nmole/hr/mg of protein. Under the assay conditions described above, with *p*-aminobenzoic acid concentrations of both 10 μM and 40 μM, the enzyme reaction proceeded linearly for 60 min (Fig. 1). The reaction rate was linear and proportional to enzyme concentration, with 1.2 mg of protein of enzyme extract per reaction mixture. The enzyme preparation was stable for several months at −20°. Since red cells do not synthesize folates *de novo* and consequently have no folate synthetase, there was no contamination of the *P. berghei* enzyme by that of the host cells (mouse erythrocytes).

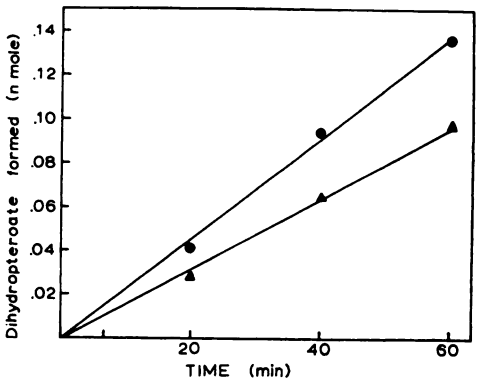

FIG. 1. *Time course of the enzymatic reaction* *p*-Aminobenzoic acid concentration with standard reaction mixture: ●, 10 μM; ▲, 100 μM.

General properties. The enzyme exhibited activity from pH 6 to 9.0, with a maximum at pH 8.5, in Tris-HCl buffer (Fig. 2). The reaction velocity at pH 7–8 was greater in Tris than in phosphate buffer and negligible in acetate buffer from pH 4 to 6.

The initial reaction velocity was determined as a function of substrate concentration for *p*-aminobenzoic acid (Fig. 3) and hydroxymethyldihydropteridine pyrophosphate (Fig. 4). These initial velocity data were fitted to the equation for a hyperbola by means of the FORTRAN program of Cleland (12), which analyzes the data by the method of least squares, weighted for the fourth powers of the experimental velocities. Apparent K_m values of 2.80 μM and 1.41 μM were thereby obtained for *p*-aminobenzoic acid and hydroxymethyldihydropteridine pyrophosphate, respectively. The apparent K_m values and their standard errors are summarized in Table 1. These values are similar

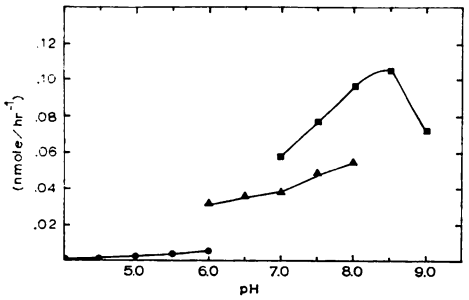

FIG. 2. *Effect of pH and buffers on dihydropteroate synthetase activity*

The standard assay procedure was followed, with modification of the pH of the reaction mixtures. The concentration of buffer in each reaction mixture was 100 mM. The pH values plotted are measured values obtained immediately after completion of the reaction. ●, sodium acetate buffer; ▲, potassium phosphate buffer; ■, Tris-HCl buffer.

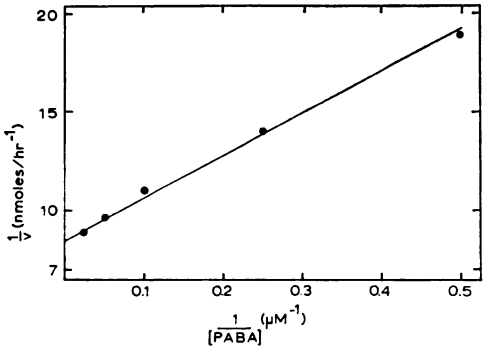

FIG. 3. *Effect of p-aminobenzoic acid (PABA) concentration on dihydropteroate synthesis*

Reaction mixtures were the same as described under MATERIALS AND METHODS. The *p*-aminobenzoic acid concentrations were varied between 2.0 and 40.0 μM.

to those reported for dihydropteroate synthetase isolated from *E. coli* (7) and *Diplococcus pneumoniae* (13).

Although various pteridines were tested as substrates, only the pyrophosphate ester of hydroxymethyldihydropteridine was effective. No activity was observed with hydroxymethyldihydropteridine, hydroxymethyldihydropteridine monophosphate, or the oxidized forms of these pteridines. These results agree with previous reports that hydroxymethyldihydropteridine pyrophosphate is the direct pteridine precursor for folate biosynthesis.

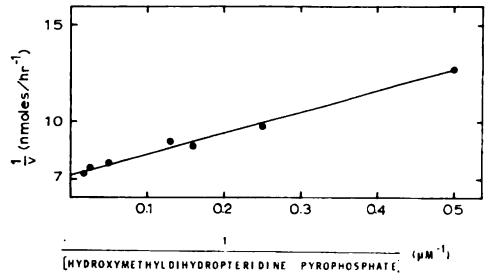

FIG. 4. *Effect of hydroxymethyldihydropteridine pyrophosphate on dihydropteroate synthesis*

Reaction mixtures were the same as described under MATERIALS AND METHODS. The hydroxymethyldihydropteridine pyrophosphate concentrations were varied between 2.0 and 76.0 μM.

TABLE 1

Values of apparent K_m of p-aminobenzoic acid and hydroxymethyldihydropteridine pyrophosphate for dihydropteroate synthetase from P. berghei

Apparent K_m values were obtained by fitting the data of Fig. 3 and Fig. 4, respectively, to the equation for a hyperbola (12).

Substrate	Apparent K_m
	μM
p-Aminobenzoic acid	2.80 ± 0.18
Hydroxymethyldihydropteridine pyrophosphate	1.41 ± 0.16

Mg^{++} has been shown to be required for dihydropteroate synthetase activity for *E. coli* (7) and *D. pneumoniae* (13), but not *Veillonella* (5). The *P. berghei* enzyme fractions obtained by gel filtration on Sephadex G-25 or G-100, as well as those fractions obtained after extensive dialysis against Tris buffer, were active in the absence of Mg^{++} in the standard assay. The addition of Mg^{++}, at concentrations as high as 1 M, had no stimulatory effect on enzyme activity.

Inhibition of dihydropteroate synthetase by DDS and sulfadiazine. Both DDS and sulfadiazine are effective drugs in the treatment of *P. berghei* infections *in vivo* (14). Since these drugs presumably act *in vivo* on dihydropteroate synthetase, it was important to test their effectiveness as inhibitors of the isolated enzyme *in vitro*, with the goal of in-

TABLE 2

Effectiveness of DDS and sulfadiazine as inhibitors of dihydropteroate synthetase from P. berghei

Inhibitor	I_{50}[a]	K_i[b]
	μM	μM
DDS	89	19
Sulfadiazine	180	39

[a] Concentration of inhibitor required to effect a 50% decrease in enzyme activity.

[b] Calculated from Eq. 1.

tegrating such data with chemotherapeutic activity in the animal. DDS and sulfadiazine proved to be effective inhibitors of dihydropteroate synthetase. A comparison of their 50% inhibitory concentrations is presented in Table 2. DDS (I_{50} = 89 μM) was twice as effective as sulfadiazine (I_{50} = 180 μM). Using these I_{50} values and the Michaelis constant for *p*-aminobenzoic acid (Table 1), K_i values (Table 2) for DDS (19 μM) and sulfadiazine (39 μM) were calculated from the following equation (15) for reversible competitive inhibition, rearranged to solve for K_i:

$$K_i = \frac{[I]/i - [I]}{1 + [S]/K_s} \qquad (1)$$

where i is the fractional inhibition; $[I]$, the concentration of inhibitor causing i fractional inhibition; $[S]$, the substrate concentration; and K_s, the Michaelis constant for *S*. Application of the K_i value for DDS to the chemotherapeutic situation *in vivo* will follow under DISCUSSION.

DISCUSSION

The enzyme dihydropteroate synthetase, which catalyzes the synthesis of dihydropteroate from 2-amino-4-hydroxy-6-hydroxymethylpteridine pyrophosphate and *p*-aminobenzoic acid, has previously been isolated from several sources, including *Lactobacillus plantarum, Butyribacterium rettgeri, Fusobacterium fusiforme*, and *Veillonella* (4), *D. pneumoniae* (13), *Staphylococcus epidermidis* (16), plants (17, 18), yeast (19), and recently from the plasmoidial organism *Plasmodium chabaudi* (20). The properties of the *P. berghei* enzyme, including the substrate specificity for pteridines, values of Michaelis constants, and optimal pH for activity, are comparable to those previously reported by Richey and Brown (7) for the highly purified enzyme from *E. coli*. We found that addition of Mg^{++} was not necessary for dihydropteroate synthetase activity. It is possible that since a relatively crude extract was employed for these studies, enough Mg^{++} could have been present to satisfy any possible requirements. Indeed, the finding that the enzymatic reaction was stopped immediately by the addition of EDTA would suggest a divalent cation requirement for enzymatic activity.

These data suggest that folate biosynthesis in *P. berghei* proceeds via the pathway *de novo* by the reaction of hydroxymethyldihydropteridine pyrophosphate with *p*-aminobenzoic acid to give dihydropteroate. Although the conversion of dihydropteroate and glutamate to dihydrofolate has not been demonstrated *in vitro*, it it might be inferred that this pathway is operative *in vivo*, since dihydrofolate reductase has previously been isolated from *P. berghei* (10).

The values of K_i for DDS and sulfadiazine were calculated from Eq. 1 for reversible competitive inhibition (15). Data were not obtained in the present study to show that the inhibition of the enzyme from *P. berghei* was reversible and competitive in nature. We feel, however, that this is likely, since both compounds have previously been shown to be reversible competitive inhibitors of folate synthetase from *E. coli* (2). In addition, the antimalarial activity of DDS *in vivo* against *P. berghei* can be overcome by the addition of exogenous *p*-aminobenzoic acid (14). Further evidence is the finding that the sulfonamides sulfanilamide and sulfaguanidine are reversible competitive inhibitors of folate synthetase from *P. chabaudi* (20).

Our data and those of Vogh and Gleason (14)[3] enable us to calculate fractional inhibition (i) of dihydropteroate synthetase necessary to overcome *P. berghei* infection *in vivo*. Rearrangement of Eq. 1 for reversi-

[3] B. P. Vogh and L. N. Gleason, personal communication.

ble competitive inhibition yields

$$i = \frac{[I]}{[I] + K_i(1 + [S]/K_s)} \quad (2)$$

Implicit in the use of Eq. 2 are the conditions that $E < S$ and $E < I$. Equation 2 is solved numerically by using the K_i for DDS from Table 2 and K_s for *p*-aminobenzoic acid from Table 1. (I) is the minimum molar concentration of DDS in plasma (free) which overcomes *P. berghei* infection in mice. This value is estimated from the mean total plasma level of 6 μg/ml (free plus bound) at the lowest curative dose, 5 mg/kg every 8 hr, days 2–10 (14). Based on the value of 54% reported for the binding of DDS to mouse plasma *in vitro* (21), (I) is calculated to be 11.0 μM. [S] is the molar concentration of *p*-aminobenzoic acid in plasma (free). The total molar concentration of *p*-aminobenzoic acid in mouse plasma (free plus bound) is reported to be 2.1 μM (22). Although the plasma binding of *p*-aminobenzoic acid in the mouse has not been reported, Anton's data (23), showing low (less than 40%) plasma binding for a variety of aromatic amines in the mouse, suggest that the value is probably low. We have calculated values of i from Eq. 2, assuming either no plasma binding of *p*-aminobenzoic acid, whence i = 25%, or 90% binding of *p*-aminobenzoic acid, whence i = 35%. We conclude tentatively that when 25–35% of dihydropteroate synthetase is inhibited, *P. berghei* infection can be eliminated from the mouse. To our knowledge, this type of correlation has not been attempted previously in antimicrobial chemotherapy, and it appears an interesting and useful exercise.

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