Characterization of Mutationally Altered Dihydropteroate Synthase and Its Ability to Form a Sulfonamide-Containing Dihydrofolate Analog

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Among spontaneous mutants of *Escherichia coli* selected for resistance against sulfonamides, thermosensitive strains were found. These were shown to possess a changed dihydropteroate synthase (EC 2.5.1.15), which had a substantially higher $K_m$ value for its normal substrate, *p*-aminobenzoic acid, and an about 150-fold higher $K_m$ for sulfonamides. The mutationally changed dihydropteroate synthase was found to be thermosensitive by in vitro assays. The thermosensitivity was used as an enzyme marker to demonstrate the complex formation between 2-amino-4-hydroxy-6-pyrophosphorylmethyl pteridine and sulfonamides by partially purified dihydropteroate synthase. The formation of folate from 2-amino-4-hydroxy-6-pyrophosphorylmethyl pteridine and *p*-aminobenzyglutamic acid by dihydropteroate synthase was found to be very sensitive to inhibition by sulfonamides and very inefficient with the mutationally changed enzyme.

The formation of dihydropteroate from *p*-aminobenzoic acid (AB) and reduced 2-amino-4-hydroxy-6-hydroxymethyl pteridine (pteridine-CH$_2$OH) has for a long time been known to be the target of sulfonamide action (4). Part of the inhibitory effect of sulfonamides on bacterial growth has been ascribed to the incorporation of the drug into a pteridine complex to give a sulfonamide-containing H$_2$-pterate (7,8-dihydropteroic acid) analog (3, 4). These investigations, however, have not linked the analog formation to the activity of dihydropteroate synthase (EC 2.5.1.15).

In a previous study on plasmid-mediated sulfonamide resistance, the occurrence of a plasmid-borne, drug-resistant dihydropteroate synthase was described (15). Spontaneous mutants of *Escherichia coli* were isolated that carried temperature sensitivity lesions. These were allegedly located in the chromosomal dihydropteroate synthase gene and were used as tools for the identification of extrachromosomal synthase activity.

Experiments are described here which demonstrate the temperature sensitivity of isolated mutant dihydropteroate synthase activities. Partially purified enzyme from these mutants catalyzed the formation of a sulfonamide-containing complex from 2-amino-4-hydroxy-6-pyro-phosphorylmethyl pteridine (H$_2$-pteridine-CH$_2$O-PP) and $[^{35}S]$sulfathiazole, as did the wild-type enzyme, but the formation of sulfonamide complex was temperature sensitive with mutant enzyme.

The temperature-sensitive mutants were originally isolated as sulfonamide-resistant strains of *E. coli* C (15). The temperature sensitivity was interpreted to be a consequence of the mutational change in the enzyme to sulfonamide resistance. In concurrence with this interpretation, dihydropteroate synthase isolated from the mutants showed a $K_m$ value about 150-fold higher for sulfathiazole than that isolated from the wild type.

The efficiency of the enzymatic formation of dihydrofolate from H$_2$-pteridine-CH$_2$O-PP and N-(p-aminobenzoyl)-L-[U-$^{14}$C]glutamate (ABG) was also seen to decrease drastically as a consequence of mutational change in the dihydropteroate synthase.

**MATERIALS AND METHODS**

**Bacteria.** The *E. coli* C strain C-167 (uracil-, Str$^+$) (16) and the two thermosensitive mutants C-167ts18 and C-167ts20 derived from it (15) were used as sources of enzyme.

**Materials.** Deoxyribonuclease, trypsin, ATP, and uracil were purchased from Sigma Chemical Co. (St. Louis, Mo.). Sulfathiazole and pteridine-CH$_2$OH were from Astra Läkemedel AB (Södertalje, Sweden). The pteridine substrate was reduced to its 7,8-dihydro (H$_2$-pteridine-CH$_2$OH) form with dithionite by the method of Blakley (2), and it was pyrophosphorylated chemically by pyrophosphoric acid according to Shiota et al. (14). The pyrophosphorylated pteridine, eluted after
charcoal adsorption, was used in enzyme assays without further purification. It was reduced to its 7,8-
dihydro form (H₂-pteridine-CH₂O-PP) (13) freshly before each experiment.

The concentration of H₂-pteridine-CH₂O-PP in this reaction mixture preparation was determined against the known specific activity of ['⁴C]AB in the assay system described below. The reaction was allowed to run to completion with excess of added enzyme preparation, and the amount of radioactive dihydropteroate formed was quantitated as described.

Of the radioactively labeled substrates, p-aminobenzoic acid (5.64 mCi/mmol) was from New England Nuclear Chemicals GmbH (Dreieichenhain, West Germany) and [³¹S]sulfathiazole (2.1 mCi/mmol) and [U-'⁴C]glutamic acid (10.0 mCi/mmol) were from the Radiochemical Centre (Amersham, Great Britain).

Synthesis of ['⁴C]ABG. To prepare the intermediate p-nitrophenyl-p-nitrobenzoate, 4.87 g of p-nitrophenol (35 mmol) was dissolved in 13 ml of pyridine, and 6.50 g of p-nitrobenzoylchloride (35 mmol) was added in portions under vigorous stirring. After complete reaction the semisolid mass was suspended in 15 ml of water, filtered, and recrystallized from acetone-ethanol (1:1) to yield 8.40 g (83%) of p-nitrophenyl-p-nitrobenzoate. (m.p. 157 to 159°C; literature, 159°C [1, 5, 8], 156 to 157°C [9], 154 to 157°C [6]).

A 10.8-mg sample (37.5 μmol) of p-nitrophenyl-p-nitrobenzoate was dissolved in 250 μl of peroxide-free dioxane in a centrifuge tube; 250 μCi of 1-[U-'⁴C]glutamic acid (10 mCi/mmol), dissolved in 100 μl of 0.502 M NaOH, was added together with 150 μl of water; and the reaction mixture was stirred to keep the precipitated p-nitrophenyl-p-nitrobenzoate suspended. After 27 h at room temperature, 20 μl of 0.502 M NaOH was added, and after 67 h another 17 μl. After 100 h of reaction time (the appropriate reaction conditions were evaluated with unlabeled L-glutamic acid), the mixture was centrifuged, and the supernatant was applied to a column (diameter 0.9 cm, length 20 cm) of quaternary aminoethyl-Sephadex A25 (Pharmacia Fine Chemicals) and eluted with an exponential gradient of triethylammonium bicarbonate buffer (pH 8.0) (250 μl of 0.1 M triethylammonium bicarbonate in the mixing chamber and 250 ml of the same at 1 M in the reservoir). Fractions of 3 ml were collected, and the absorbance at 254 nm and the radioactivity were recorded. Fractions containing material that was both radioactive and UV-absorbing were pooled and evaporated to dryness in a flash evaporator at 40°C. The residue was washed twice with water and finally dissolved in 0.5 ml of water. The isolated N-(p-nitrobenzoyl)-L-[U-'⁴C]glutamate (105 μCi) was hydrogenated in 1.5 ml of water at room temperature and atmospheric pressure using a small amount of 5% Pd/C catalyst. The reaction was complete within 2 h as indicated by the consumption of hydrogen. The catalyst was removed by centrifugation, and the supernatant was subjected to gradient chromatography as described above. The radioactive material (total yield 40.8%) was finally taken up in 3 ml of water. It was identified as ['⁴C]ABG by cochromatography with the authentic compound both on a quaternary aminoethyl-Sephadex column and on cellulose thin-layer plates. As reference material, ABG from Sigma was used.

Growth of bacteria and preparation of enzyme. Bacteria were grown at 30°C in a mineral salts medium containing (per liter): 12.0 g of tris(hydroxymethyl)aminomethane (Tris), 2.0 g of KCl, 2.0 g of NH₄Cl, 0.5 g of MgCl₂, 6H₂O, 0.050 g of Na₂SO₄, and 0.178 g of Na₂HPO₄·2H₂O. The pH was adjusted to 7.6 with HCl. After autoclaving, glucose was added to 0.5% (wt/vol), FeCl₂ to 10⁻⁵M, Casamino Acids (Difco, certified) to 0.05% (wt/vol), and uracil to 40 μg/ml.

Bacteria were harvested in late-exponential phase by centrifugation, washed, and finally suspended in about 0.02 volume of medium and frozen. The cells were disintegrated in a pressure cell (X-press; Biotec, Stockholm, Sweden). The disrupted cells were treated with deoxyribonuclease (20 μg/ml) and centrifuged for 1 h at 105,000 × g. Supernatants were subjected to the purification procedure of Richey and Brown (11). Protein content of purified extracts was estimated by the method of Lowry et al. (7), using trypsin as a standard. Usually, preparations contained 4 to 7 mg of protein per ml.

Determination of dihydropteroate synthase activity. Enzyme activity was measured by the incorporation of radioactivity from ['⁴C]AB into dihydropteroic acid according to the following scheme (11):

(I) H₂-pteridine-CH₂OH + ATP→
H₂-pteridine-CH₂O-PP + AMP

(II) H₂-pteridine-CH₂O-PP + ['⁴C]AB→
H₂-[⁴C]pteroate + PP

The assay was performed in two modifications. The first measured both reactions above, and was done in a total volume of 0.2 ml containing: H₂-pteridine-CH₂OH, 45 μM; Tris-hydrochloride (pH 8.0), 0.1 M; 2-mercaptopethanol, 50 mM; MgCl₂, 10 mM; ATP, 5 mM; ['⁴C]AB (5.64 mCi/mmol), 50 μM; and enzyme extract.

The second assay modification only measured reaction II above and contained, in a total volume of 0.2 ml: H₂-pteridine-CH₂O-PP, 25 μM; Tris-hydrochloride (pH 8.4), 0.1 M; 2-mercaptopethanol, 0.1 M; MgCl₂, 10 mM; ['⁴C]AB (5.64 mCi/mmol), 50 μM; and enzyme preparation.

Reaction mixtures were incubated at 30°C for 30 to 60 min. The reaction was stopped by heating on a boiling water bath for 1 min. Of the reaction mixtures, 100-μl samples were then spotted onto Whatman 3MM chromatography papers and developed with 0.1 M phosphate buffer (pH 7.0) by descending paper chromatography. The dihydropteroate that formed remained at the starting point, whereas unreacted ['⁴C]AB migrated with an Rf of 0.78 (11). Radioactive spots were cut out and counted in the scintillation counter. Formation of H₂-[⁴C]pteroate was calculated from the specific activity of ['⁴C]AB (9,400 cpm/mmol) measured from spots on chromatography paper in the same way as the samples.

With both assays the amount of product formed was linear with time and with added extract protein concentration.

RESULTS

Sulfonamide inhibition of dihydropteroate synthase from drug-resistant, thermo-
sensitive bacterial mutants. In an earlier report (15) the isolation of spontaneous sulfonamide-resistant mutants of E. coli C was described. Some of these mutants were temperature sensitive, and it was argued that this thermosensitivity could be explained by a lesion in the dihydropteroate synthase of the mutant cells, since it is known (10) that sulfonamide resistance is accompanied by changes in this enzyme. This argument is borne out here by the characteristics of dihydropteroate synthase from two sulfonamide-resistant and temperature-sensitive mutants. In Fig. 1 the sulfonamide sensitivities of the enzyme from the two mutants and from the parental E. coli C strain are compared. It can be seen that the enzyme of the drug-sensitive parental strain is readily inhibited by sulfathiazole; a 50% inhibition was observed at a drug/AB ratio of 0.12. The dihydropteroate synthases from the two mutants, C-167ts18 and C-167ts20, were much less susceptible to drug action, however. In this case a sulfathiazole/AB ratio of 2.7 was required to effect an inhibition of 50%.

Thermoinactivation of dihydropteroate synthase. The thermosensitive growth characteristics (15) of the two drug-resistant mutants could be explained by a lesion in the dihydropteroate synthase. This is demonstrated in Fig. 2, where curves are shown from enzyme thermoinactivation experiments made with prepa-

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**Fig. 1. Sulphonamide inhibition of dihydropteroate synthase.** Enzyme activity was determined with H2-pteridine-CH2O-PP as substrate (assay II), with incubation at 30°C for 30 min. Enzyme extract (20 μl, 0.12 mg of protein) prepared as described was added to each assay tube. Values along the ordinate denote H2-pterinate formed; 100% corresponds to 0.82 nmol for C-167 (x); 0.81 nmol for C-167ts18 (O); and 0.98 nmol for C-167ts20 (Δ).

**Fig. 2. Temperature inactivation of dihydropteroate synthase.** (A) Enzyme extracts, prepared as described, were heated to 45°C in the presence of Tris-hydrochloride (pH 8.0), 2-mercaptoethanol, MgCl2, and ATP at concentrations given in the text for the first assay modification. At the times indicated, samples were withdrawn, 35°C, and H2-pteridine-CH2OH were added, and incubation was continued at 35°C for 60 min. (x) C-167, 0.195 mg of protein per assay; 100% corresponds to 1.42 nmol of dihydropteroate formed. (O) C-167ts18, 0.195 mg of protein per assay; 100% corresponds to 0.72 nmol of dihydropteroate. (Δ) C-167ts20, 0.192 mg of protein per assay; 100% corresponds to 0.52 nmol of dihydropteroate. (B) Enzyme extracts were heated to 50°C in the presence of H2-pteridine-CH2O-PP, Tris-hydrochloride (pH 8.0), 2-mercaptoethanol, and MgCl2 at concentrations mentioned for assay modification II in the text. At indicated times, samples were withdrawn, 35°C, and H2-pteridine-CH2OH was added, and incubation was performed for 30 min at 30°C. (x) C-167, 0.147 mg of protein per assay; 100% corresponds to 0.57 nmol of dihydropteroate formed; (O) C-167ts18, 0.213 mg of protein per assay; 100% corresponds to 0.63 nmol of dihydropteroate; (Δ) C-167ts20, 0.198 mg of protein per assay; 100% corresponds to 0.52 nmol of dihydropteroate.
rations from the two mutants. In Fig. 2A the inactivation of dihydropteroate synthase activity at $45^\circ$C in the absence of substrates is shown. Remaining activity after different times of heat treatment was assayed at $35^\circ$C. It can be seen that enzyme extracts from the two mutants were more heat sensitive than that of the parental strain. Fifteen minutes at $45^\circ$C thus inactivated 50% of both mutant activities, compared to 25% for the parental strain activity.

Substrate binding and cofactor requirement of the mutated dihydropteroate synthase. The enzymes from the parental strain and the two mutants were compared regarding substrate binding (Fig. 3). Rates of dihydropteroate formation at different concentrations of AB are shown. It can be seen that the dihydropteroate synthase from one mutant (C-167ts20) needed a much higher AB concentration for saturation than did the wild-type enzyme. This is more precisely reflected in the $K_m$ values calculated from Lineweaver-Burk plots: $4.0 \times 10^{-5}$ M and $5.1 \times 10^{-6}$ M for the C-167ts20 and the wild-type enzyme, respectively. Similar results were obtained with C-167ts18 (Table 1). The mutant enzymes thus had a much lower

![Fig. 3. Binding of AB as substrate for dihydropteroate synthase. The assays were performed as described; H$_2$-pteridine-CH$_2$O-PP was 25 µM. The concentration of $^{14}$C/AB was varied as indicated. The values along the ordinate denote enzyme activity as nanomoles of dihydropteroate formed per minute per milligram of protein. (x) C-167, 0.120 mg of protein per assay; (△) C-167ts20, 0.132 mg of protein per assay. Lineweaver-Burk plots of the data obtained are shown in the inserted frames.]
affinity for the substrate AB than the parental enzyme. This was not the case, however, for the other substrate, \( \text{H}_2\text{pteridine-CH}_2\text{O-PP} \), which saturated both enzyme types at a concentration of about 15 \( \mu \text{M} \) (data not shown).

The dihydropteroate-forming reaction as catalyzed by the wild-type and the mutant enzymes was furthermore shown to be dependent on \( \text{Mg}^{2+} \). Dialyzed enzyme extracts were thus inactive in the absence of added \( \text{Mg}^{2+} \), and the magnesium-dependent reaction could be stopped completely by the addition of 2.5 mM ethylenediaminetetraacetic acid.

**ABG as substrate for dihydropteroate synthase.** It was demonstrated in earlier work by Richey and Brown (12) that dihydropteroate synthase can use ABG as substrate instead of AB and then form dihydrofolate instead of dihydropteroate. In the present work this reaction was studied with radioactive ABG, and it was found that a reaction mixture identical to that of assay II, but with \([^{14}\text{C}]\text{ABG} (7.1 \times 10^6 \text{ cpm}/\mu\text{mol})\) substituted for \([^{14}\text{C}]\text{AB}\) and with extract from C-167 as an enzyme source, yielded labeled dihydrofolate. This was identified in the paper chromatograms by adding unlabeled dihydrofolate as a marker, which was oxidized at chromatography and showed up as a bright yellow spot of folate with an \( R_f \) of 0.16. The amount of product formed was calculated from the specific activity of \([^{14}\text{C}]\text{ABG} (6,500 \text{ cpm}/\mu\text{mol})\) measured from the spots on chromatography paper, as described. This assay was furthermore used to study the binding of ABG as substrate for dihydropteroate synthase. This is shown in Fig. 4, where Lineweaver-Burk plots are given for wild-type enzyme. A \( K_m \) value of \( 6.0 \times 10^{-4} \text{ M} \) was calculated. This could be compared with the \( K_m \) value of \( 5.1 \times 10^{-6} \text{ M} \) obtained for AB (Fig. 3). ABG thus seems to be a poor substrate for dihydropteroate synthase. The ABG reaction was furthermore very sensitive to sulfonamide inhibition, which is also shown in Fig. 4. A \( K_i \) value of \( 8.2 \times 10^{-6} \text{ M} \) was obtained for the sulfathiazole inhibition.

When the two sulfonamide-resistant, mutant enzyme preparations were used, an even lower binding of ABG was observed. Data are shown in Table 1, where \( K_m \) and \( V_{\text{max}} \) values are compared for wild-type and mutant enzymes and for both ABG and AB as substrates. It can be seen that the \( K_m \) for ABG with the C-167ts18 enzyme was almost 20-fold larger than that for the wild-type enzyme. Furthermore, the \( V_{\text{max}} \) value for the ABG reaction with mutant enzyme was lower, only about one-fourth of that with the wild-type enzyme.

**Dihydropteroate synthase catalyzes the incorporation of sulfonamide into a dihy-**

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**TABLE 1. Kinetic data for mutant and wild-type dihydropteroate synthases with different substrates**

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>AB (( K_m ), ( V_{\text{max}} ))</th>
<th>ABG (( K_m ), ( V_{\text{max}} ))</th>
<th>Sulfathiazole (( K_m ), ( V_{\text{max}} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-167</td>
<td>( 5.1 \times 10^{-5} ), 0.617</td>
<td>( 6.0 \times 10^{-4} ), 0.172</td>
<td>( 2.7 \times 10^{-6} ), 0.280</td>
</tr>
<tr>
<td>C-167ts18</td>
<td>( 5.5 \times 10^{-5} ), 0.424</td>
<td>( 1.0 \times 10^{-2} ), 0.044</td>
<td>( 4.0 \times 10^{-4} ), 0.265</td>
</tr>
<tr>
<td>C-167ts20</td>
<td>( 4.0 \times 10^{-5} ), 0.357</td>
<td></td>
<td>( 4.0 \times 10^{-4} ), 0.333</td>
</tr>
</tbody>
</table>

* \( K_m \) values (M) are calculated from data shown in Fig. 3, 4, and 5 and also from data not shown but referred to in the text. \( V_{\text{max}} \) values are expressed as nanomoles per minute per milligram of protein.
dropteridine complex. The enzymic reaction between sulfonamide and H₂-pteridine-CH₂O-PP was studied with radioactively labeled sulfathiazole. Some characteristics of the reaction are shown in Table 2, where a complete dependence on added pteridine substrate and on the presence of active enzyme can be seen.

It is furthermore shown (Fig. 5) that sulfathiazole bound effectively to dihydropteroate synthase. The $K_m$ value for the enzyme was $2.7 \times 10^{-6}$ M, which is about half the value obtained for AB (cf. Table 1). The synthase from a sulfonamide-resistant mutant, on the other hand, showed a much less effective binding of drug (Fig. 5). Thus the $K_m$ value for C-167ts18 enzyme and sulfathiazole was about 150 times larger than that of the wild type (Fig. 5 and Table 1). With the mutant enzymes, the $K_m$ values for sulfathiazole were about 10 times larger than those for AB (Table 1).

To link the pteridine-sulfathiazole complex formation to the dihydropteroate synthase, some experiments were performed with enzyme from a sulfonamide-resistant, thermosensitive mutant (Fig. 6). The formation of the sulfathia-

![Graph](image)

**Fig. 5.** Binding of sulfathiazole to dihydropteroate synthase. The assays were performed as described in Table 2. The concentration of radioactive sulfathiazole (specific activity, 0.8 mCi/mmol) was varied as indicated. The values along the ordinate denote the formation of pterate analog expressed as nanomoles per minute per milligram of protein. (×) C-167, 0.075 mg of protein per assay; (○) C-167ts18, 0.142 mg of protein per assay. Inserted frames describe Lineweaver-Burk plots of the data obtained.

### Table 2. Enzymatic formation of a sulfonamide-containing pterate analog

<table>
<thead>
<tr>
<th>Assay mixture</th>
<th>Extract vol</th>
<th>Pterate analog formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>20</td>
<td>0.28</td>
</tr>
<tr>
<td>Complete</td>
<td>30</td>
<td>0.39</td>
</tr>
<tr>
<td>Complete</td>
<td>50</td>
<td>0.67</td>
</tr>
<tr>
<td>Complete</td>
<td>50</td>
<td>0.01</td>
</tr>
<tr>
<td>Complete</td>
<td>50</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* The composition of the assay mixture was identical to that of the second assay modification as described in the text, except that $[^35]S$ sulfathiazole, 50 μM (specific activity, 0.8 mCi/mmol) was substituted for $[^14]C$AB. Incubation was at 30°C for 60 min.

* Enzyme extract was prepared from C-167 as described and contained 6.1 mg of protein per ml.

* The sulfathiazole-containing product was isolated on the paper chromatogram as a UV-fluorescent spot with an $R_f$ value of 0.67. The amount of product formed was calculated from the specific activity of $[^35]S$ sulfathiazole (1,400 cpm/nmol) measured from spots on chromatography paper.

* For this assay, enzyme extract was pretreated at 100°C for 5 min.
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**Fig. 6.** Temperature sensitivity of sulfathiazole-pteridine complex formation catalyzed by enzyme from a drug-resistant mutant. Sulfathiazole-pteridine complex formation was assayed at 30 and 45°C as described in Table 2, except that the concentration of [3S]sulfathiazole (specific activity, 0.8 mCi/mmol) was 500 μM with the C-167ts18 enzyme. Samples of 100 μl were withdrawn at the indicated time points and assayed for sulfathiazole-pteridine complex as described. Ordinate values denote nanomoles formed per sample. (A) Enzyme extract from C-167, 0.082 mg of protein per assay. (B) Enzyme extract from C-167ts18, 0.097 mg of protein per assay.

Zole-containing complex was temperature sensitive when catalyzed by dihydropteroate synthase from the temperature-sensitive mutant C-167ts18 (Fig. 6B). Similar curves obtained with wild-type enzyme are shown for comparison in Fig. 6A, where it can be seen that the sulfonamide complex formation increased in the expected way at the elevated temperature. Parallel experiments were performed with AB as a substrate. The obtained curves for dihydropteroate formation were essentially the same as those shown here for the pterode analog synthesis.

**DISCUSSION**

The structural similarity between sulfonamides and AB suggested a long time ago that the incorporation of AB into folic acid was the sensitive step in the growth-inhibitory effect of these drugs on bacteria. It was later shown by Brown (4) that the formation of H2-pterode from reduced pteridine-CH2OH and AB in bacterial extracts was inhibited in a competitive way by sulfonamides. The bacterial enzyme dihydropteroate synthase, catalyzing the formation of H2-pterode from H2-pteridine-CH2O-PP and AB, was purified and characterized by Richley and Brown (11) and by Shiota and co-workers (13).

This enzyme should be the primary target of sulfonamide inhibition, and the phenomenon of chromosomal resistance to this drug ought to be thought of as a mutational change in the protein structure of the enzyme. In a previous study, some 20 sulfonamide-resistant mutants of E. coli C were isolated (15). Two of these were temperature sensitive. It is shown here that the dihydropteroate synthase from these mutants was much less sensitive to the inhibitory effect of sulfonamide than was that from the parental type. This is also reflected in the $K_m$ values for the drug, which were about 150-fold larger for the mutant enzyme preparations. It is interesting that the mutational change of the enzyme also increased the $K_m$ value for the normal substrate AB. In this case the increase was some 10-fold. The mutational change to drug resistance is also expressed in temperature sensitivity, probably as a result of a structural change in the enzyme protein. This temperature sensitivity can be used to discern the specificity of the dihydropteroate synthase reaction also with only partially purified enzyme preparations. Our results
on the temperature sensitivity of the complex formation between H₂-pteridine-CH₂O-PP and sulfathiazole provide evidence for the formation of this drug complex by dihydropteroate synthase. A mechanism of growth inhibition by sulfonamides could then be the enzymatic trapping of available intracellular H₂-pteridine-CH₂O-PP in a sulfonamide complex. Conversely, a mechanism of sulfonamide resistance could be the cellular overproduction of this pteridine substrate.

By a similar argument, evidence was acquired for the function of ABG as a substrate for dihydropteroate synthase. That is, the mutationally changed enzyme showed a drastic decrease in affinity for ABG. This substrate has earlier been identified as a possible intermediate in folate biosynthesis (12). It was shown then, and it is demonstrated here, that ABG is a much poorer substrate for dihydropteroate synthase than AB. The $K_m$ value for ABG was more than 100 times higher than that of AB. It is interesting that the ABG reaction with H₂-pteridine-CH₂O-PP was about 10 times more sensitive to sulfonamide inhibition than the AB reaction, probably reflecting the low enzyme affinity.

In conclusion, dihydropteroate synthase is able to catalyze the formation of a H₂-pterinate analog from H₂-pteridine-CH₂O-PP and sulfathiazole. This could be an important general component of the growth-inhibitory effect of sulfonamides.

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LITERATURE CITED