Characterization of an R-plasmid Dihydrofolate Reductase with a Monomeric Structure*

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A plasmid-encoded dihydrofolate reductase that originated in a clinical isolate of Salmonella typhimurium (phage type 179) moderately resistant to trimethoprim has been isolated and characterized. The dihydrofolate reductase (called type III) was purified to homogeneity using a combination of gel filtration, hydrophobic chromatography, and methotrexate affinity chromatography. Polyacrylamide gel electrophoresis under denaturing and non-denaturing conditions indicated that the enzyme is a 16,900 molecular weight monomeric protein. Kinetic analyses showed that trimethoprim is a relatively tight binding inhibitor ($K_I = 19$ nM) competitive with dihydrofolate. The enzyme is also extremely sensitive to methotrexate inhibition ($K_I = 0.4$ nM). The sequence of the first 20 NH$_2$-terminal residues of the protein shows 50% homology with the trimethoprim-sensitive chromosomal Escherichia coli dihydrofolate reductase and suggests that the two enzymes may be closely related. This is the first example of a plasmid encoding for a monomeric dihydrofolate reductase only moderately resistant to trimethoprim, and a resistance mechanism, dependent in part on the high dihydrofolate affinity of the type III enzyme, is proposed.

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, a cofactor involved in the biosynthesis of purines, pyrimidines, and several amino acids (1). The enzyme is the target of a number of chemotherapeutic agents, including the antibacterial trimethoprim (2).

In 1972, R-plasmids conferring trimethoprim resistance were demonstrated in clinical strains of bacteria (3). The mechanism of resistance was shown to be the plasmid-directed synthesis of a trimethoprim-insensitive dihydrofolate reductase. To date, two major classes of plasmid-specified enzymes have been characterized. Type I dihydrofolate reductases have trimethoprim $K_I$ values of about 10 $\mu$M, whereas type II enzymes are about 1000-fold less sensitive with trimethoprim $K_I$ values of 5–10 $\mu$M (4). Representative enzymes from each of these classes have been physically characterized. The type I dihydrofolate reductase has a native molecular weight of about 35,000, and is a dimer of 18,000 molecular weight subunits (5). The type I enzyme also has a native molecular weight of $\sim$35,000, but it is a tetramer of 8,500 molecular weight subunits (6). These enzymes represent the first instances in which R-factor-mediated drug resistance can be attributed to an altered target enzyme, and there is much interest in determining the origins of the plasmid DNA.

Recently, a novel Escherichia coli K-12 transconjugant, J5-3 (pAZ1), containing a resistance plasmid that originated in a clinical isolate of Salmonella typhimurium phage type 179 has been investigated (7). These strains are of interest because they are only moderately resistant to trimethoprim (minimal inhibitory concentration $\geq$ 5 $\mu$g/ml), and plasmid pAZ1 did not hybridize with either type I or type II DNA (8). Both of these observations are consistent with a novel resistance mechanism. In this study, we have characterized the dihydrofolate reductase specified by the plasmid and show that it is unique. The enzyme is designated type III dihydrofolate reductase and is physically different from types I and II. Kinetic data indicate that the mechanism of whole cell trimethoprim resistance of E. coli J5-3 (pAZ1) is dependent upon both the 50-fold elevated trimethoprim $K_I$ and the low dihydrofolate $K_m$ of its type III dihydrofolate reductase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dihydrofolate was prepared by the method described by Blakley (9). Methotrexate, NADPH, and the aminoacylase hydrophobic chromatography matrix were from Sigma. Trimethoprim, 2,4-diamino-5-[3',4',5'-trimethoxybenzyl]pyrimidine, was a product of the Wellcome Research Laboratories. The methotrexate-Sepharose affinity matrix was prepared as previously described (10).

**Bacterial Strains**—E. coli J5-3 and J5-3 (pAZ1) were kindly supplied by D. M. Anderson, National Health Institute, Wellington, New Zealand (7). The latter strain contains a $\sim$9000-base pair sulfafurazole and trimethoprim resistance plasmid that was derived from a clinical isolate of trimethoprim-resistant S. typhimurium phage type 179. A hybrid plasmid specifying type III dihydrofolate reductase was constructed by inserting a 9000-base pair EcoRI linear fragment of plasmid strain J5-3 (pAZ1) into the EcoRI site of the multicopy plasmid pOP1 (11). The ligation mixture was transformed into E. coli HB101, and the cells containing the hybrid plasmid pLW1 were selected on Mueller Hinton agar containing 10 $\mu$g/ml of trimethoprim.

**Enzyme Isolation**—Bacteria were grown in Mueller-Hinton broth at $37^\circ$C to an optical density of 0.8 at 600 nm. Medium for E. coli HB101 (pLW1) also contained 20 $\mu$g/ml of trimethoprim. All the following steps were carried out at 4 $^\circ$C. The cells were harvested by centrifugation at 15,000 $\times$ g for 10 min, washed in 50 mM Tris chloride buffer, pH 8 at 5 $^\circ$C, and lysed according to the method of Godson and Sinsheimer (12). In a typical preparation, the crude lysate from 4.2 liters of cells contained 75 units of enzyme and 0.5 g of protein in a volume of 55 ml. The extract was subjected to a 30 to 90% saturated ammonium sulfate fractionation step, and the pellet was dissolved in a 50 mM potassium phosphate buffer, pH 8, 1 mM dithiothreitol, 1 mM EDTA. The solution, containing 41 units of dihydrofolate reductase activity and 0.25 g of protein in a volume of 12 ml, was subjected to gel filtration and hydrophobic chromatography as described in the text. The 40-ml plasmid enzyme pool from the hydrophobic chromatography step was mixed with 0.3 ml of methotrexate-Sepharose resin and 2.7 ml of unsubstituted Sepharose 4B. After 5 h of gentle mixing, about 80% of the dihydrofolate

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reductase activity was bound, then the resin was batch washed once with 400 ml of 50 mM potassium phosphate buffer, 1 mM EDTA, 1 mM dithiothreitol, pH 8, and once with the same buffer containing 1 M KCl. The enzyme-bound affinity resin was poured into a small 1-cm diameter column, and the dihydrofolate reductase was eluted with the same phosphate KCl buffer, containing 2 mM folic acid, at a flow rate of 5 ml/h. The column pump was set up in such a manner that the effluent was collected with an equal volume of 25 mM potassium phosphate buffer, 0.5 mM dithiothreitol, 0.5 mM EDTA, pH 8, 50% glycerol and then diverted into a fraction collector. Enzymically active fractions were pooled, dia lyzed against 20 mM potassium phosphate buffer, 1 mM dithiothreitol, 1 mM EDTA, pH 8, 25% glycerol, and stored at 4°C.

Enzyme Assay—The standard assay for dihydrofolate reductase was performed in 0.1 M imidazole chloride buffer, pH 7.0, with 12 mM mercaptoethanol, 60 μM NADPH, and 45 μM dihydrofolate in a final volume of 1 ml at 30°C. Where noted, 50 mM trimethoprim was added to the reaction mixture to distinguish R-plasmid dihydrofolate reductase from the chromosomal enzyme. One unit was defined as the amount of enzyme needed to reduce 1 μmol of dihydrofolate per min based on a molar extinction coefficient of 12.3 X 10³ M⁻¹ cm⁻¹ at 340 nm (13).

The dihydrofolate Kₐ₅ value was determined by fitting the integrated Michaelis-Menten equation to the progress curve as described by Atkins and Nimmo (14). The initial concentrations of dihydrofolate and NADPH were 3 and 60 μM, respectively, and sufficient enzyme was added to the assay mixture to allow completion of the reaction in about 15 min. The dihydrofolate Kₐ₅ was calculated from the data collected between t = 0 and t = 8 min. The reported kinetic constants (Kₐ₅ and Vₘₐₓ) are the average of multiple determinations, and the standard error of each value is less than 20%.

The pH activity profile of the plasmid enzyme was determined between pH 4 and 6 in 0.1 M succinate-Tris buffer, between pH 6.1 and 7.6 in 0.1 M imidazole chloride buffer, and between pH 7.6 and 8.8 in 0.1 M Tris chloride buffer. Corrections were made for nonenzymic changes in absorbance at acidic pH values.

Polyacrylamide Gel Electrophoresis—The method used for polyacrylamide gel electrophoresis was that of Ornstein and Davis (15), with the exception that the cathode buffer contained 80 μM NADPH. The cylindrical gels (0.5 × 6 cm) were made of acrylamide and bisacrylamide at 7.5% T and 2.5% C using the notation of Hjerten (16). Protein bands were visualized with Coomasie brilliant blue in perchloric acid according to the method of Reisner et al. (17), and dihydrofolate reductase activity was detected using the stain described by Hiebert et al. (18). Previously described methods were used for SDS gel electrophoresis (19, 20). The destained gels were scanned at 600 nm using a Gilford model 250 spectrophotometer with microprocessor-controlled data collection and background subtraction.

Protein Determination—Protein was measured by the method of Bensadoun and Weinstein (21). Crystalline bovine serum albumin dried to constant weight in a vacuum oven at 45°C was used as a standard.

Automated Sequence Analysis—Phenyl isothiocyanate, heptane, 1.0 M Quadrol (1.0 M N,N,N',N'-tetramethyl-2-(hydroxypropyl)ethylenediamine trifluoracetate, pH 9.0), ethyl acetate, benzene, butyl chloride, and heptafluorobutyric acid were Sequential grade from Pierce Chemical Co. Automated Edman degradations were carried out in a Beckman 890C Sequencer equipped with a Sequemat P6 autocounter and Sequemat SC-510 program controller. As recommended elsewhere (22), the benzene and ethyl acetate reservoir bottles in the sequencer contained 40 g each of Al₂O₃ Woeil W200 neutral activity grade (Scott, Moline, IL). The Quadrol buffer bottle contained aminomethylaminopropyl glass beads (23). A 0.35 M Quadrol program, essentially as described by Hunkapiller and Hood (24), was used for all Sequencer runs, and 3 ml of Polybrene plus 100 nmol of glycylglycine were added to the cup (23).

Identification of phenylthiohydantoin derivatives was carried out by high pressure liquid chromatography on a reversed phase Zorbax ODS column (DuPont) as described elsewhere (26), and all identifications were confirmed by amino acid analysis following back hydrolysis as described previously (27).

RESULTS

Purification—A crude cell lysate of E. coli HB101 (pLW1) contained dihydrofolate reductase activity that was only 40% inhibited by 50 mM trimethoprim. Since the normal chromosomal enzyme is completely inhibited by this concentration of drug, one interpretation of these data was that the organism was synthesizing nearly equal amounts (on an activity basis) of two dihydrofolate reductases, the trimethoprim-sensitive chromosomal enzyme and a resistant plasmid enzyme. The crude lysate was subjected to a 30 to 90% saturated ammonium sulfate fractionation step and applied to an AÄ 54 gel filtration column (Fig. 1). Eluted fractions were assayed for dihydrofolate reductase activity in the presence and absence of trimethoprim. Enzymic activity observed in the absence of trimethoprim was due to both chromosomal and R-plasmid-encoded dihydrofolate reductases. Activity observed in the presence of trimethoprim represented the R-plasmid enzyme.

Subtraction of these two activities revealed the elution profile of the trimethoprim-sensitive enzyme. Although the two enzymes were not completely separated by this procedure, the column clearly shows the dihydrofolate reductase heterogeneity of the preparation. The higher elution volume of the resistant enzyme indicates that its molecular weight is slightly less than that of the chromosomal enzyme (Mr = 18,000).

The total dihydrofolate reductase pool from the gel filtration step was applied to a column of hexylamine-substituted agarose (Fig. 2). The trimethoprim-resistant plasmid enzyme did not bind to this matrix and was completely separated from the chromosomal enzyme. The latter enzyme was eluted from the column by 1 M KCl. In the final step of the purification, the R-plasmid enzyme was subjected to methotrexate affinity column chromatography (data not shown). The resulting preparation had a specific activity of 10 units/mg which represents an 80-fold purification over the crude extract. It was dialyzed against 20 mM potassium phosphate buffer, 1 mM dithiothreitol, 1 mM EDTA, 20% glycerol, pH 7, and retained at least 90% of its activity for 2-3 weeks at 4°C.

Fig. 1. Gel filtration column chromatography of E. coli HB101 (pLW1) dihydrofolate reductase. The redissolved 90% saturated ammonium sulfate precipitated crude extract (12 ml, 250 mg of protein) was loaded onto a column (1.5 × 57 cm) of LKB AÄ 54 Ultrogel equilibrated with 50 mM potassium phosphate buffer, pH 8, 1 mM dithiothreitol, 1 mM EDTA. Fractions containing 1 ml were collected at a flow rate of 6 ml/h and were monitored for 280 nm absorbance (—–) and dihydrofolate reductase activity in the absence (O) and presence (■) of 50 mM trimethoprim. The open triangles (△) represent the difference between these two assays.
mine the molecular weight of the enzyme. In this procedure, proteins with known molecular weights are electrophoresed in gels of differing polyacrylamide concentrations, and their relative mobilities are plotted as a function of the per cent acrylamide (graph not shown). The absolute values of the resulting slopes, \( |K_r| \), and the protein molecular weights were used to construct the calibration curve shown in Fig. 3 (right). From these data, it was determined that the plasmid-encoded dihydrofolate reductase had a native (undenatured) molecular weight of 16,900.

The purified R-plasmid enzyme also appeared homogeneous under the denaturing conditions of SDS-polyacrylamide gel electrophoresis. In some instances, as shown in Fig. 4, left, a minor species was observed migrating slightly faster than the major form. However, this appeared after storage of the purified enzyme and may represent a degradation product. Calibration of the electrophoresis system with standard proteins showed that its mobility corresponded to a molecular weight of 16,700 (Fig. 4, right), the same as that determined under nondenaturing conditions. These data indicate that the type III plasmid-encoded dihydrofolate reductase is a monomeric protein.

**Kinetic Properties**—The pH activity profile of the enzyme assay had two pH optima, a major peak at pH 7.8 and another at pH 4.2. The latter peak showed about 60% of the activity of the pH 7.8 optimum. However, neither of these optima were commonly used in type III enzyme assays. Since the kinetic properties of most other dihydrofolate reductases have been determined in neutral or slightly acidic buffer, the following kinetic analyses of the plasmid enzyme were conducted in 0.1 M imidazole chloride, pH 7.

A plot of reciprocal velocity versus reciprocal dihydrofolate concentration at various concentrations of trimethoprim showed lines that crossed on the ordinate (Fig. 5). The replot of slope versus trimethoprim concentration was linear (Fig. 5, inset). These patterns are indicative of inhibition competitive with dihydrofolate, but neither the dihydrofolate constant, nor the trimethoprim constant, could be determined, because the velocity of the uninhibited reaction did not significantly change over a dihydrofolate concentration range of 3 to 45 \( \mu M \). However, in another series of experiments, the integrated Michaelis-Menten equation (14) was used to calculate the dihydrofolate binding constant from progress curve data obtained at saturating NADPH and limiting dihydrofolate concentration. The dihydrofolate constant was found to be 0.4 \( \mu M \). Since trimethoprim is a competitive inhibitor and the dihydrofolate binding constant was known, the method of Cha (29) was used to calculate trimethoprim constant from Equation 1.

\[
I_{50} = K_i (1 + S / K_m) + \frac{1}{K_i} E_i
\]

where \( I_{50} \) is the inhibitor concentration (2.1 \( \mu M \)) that decreases the reaction velocity by 50%, \( K_i \) is the inhibitor binding constant, \( S \) is the assay concentration of dihydrofolate (45 \( \mu M \)), \( K_m \) is the dihydrofolate Michaelis constant (0.4 \( \mu M \)), and \( E_i \) is the enzyme concentration in the reaction mixture (7 nm). The plasmid enzyme trimethoprim constant was found to be 19 nm. The methotrexate inhibitor constant was also evaluated. Henderson analysis (30) was used to calculate a methotrexate constant, also kinetically characterized; and the results, along with trimethoprim minimal inhibitory concentration values and enzyme molecular weights, are included in Table I. The methotrexate constant of the type I and II plasmid dihydrofolate reductases are compiled in Table I. Dihydrofolate reductase from crude extracts of the trimethoprim-sensitive E. coli J5-3 parent was also kinetically characterized; and the results, along with trimethoprim minimal inhibitory concentration values and enzyme molecular weights, are included in Table I. The methotrexate constant of E. coli J5-3 was not determined, but the closely related chromosomal dihydrofolate reductase from E. coli...
Monomeric R-plasmid Dihydrofolate Reductase

**Fig. 4.** SDS-polyacrylamide gel electrophoresis of the purified enzyme. Left, the affinity column-purified *E. coli* dihydrofolate reductase (4 μg) was subjected to electrophoresis on a 5 to 25% polyacrylamide gradient gel in the presence of 0.1% SDS and then stained for protein. A 600-nm absorbance scan of the gel is shown. Right, the molecular weight of the SDS-denatured plasmid enzyme was determined in 14% polyacrylamide gels from its relative mobility and a calibration curve composed of (a) ovalbumin, $M_r = 45,000$; (b) DNase, $M_r = 31,000$; (c) soybean trypsin inhibitor, $M_r = 24,000$; (d) *E. coli* RT 500 dihydrofolate reductase, $M_r = 18,000$; (e) α-lactalbumin, $M_r = 14,400$; (f) lysozyme, $M_r = 14,300$; (g) cytochrome c, $M_r = 12,400$.

**Fig. 5.** Trimethoprim inhibition of the plasmid enzyme. The reciprocal initial velocity was plotted versus the reciprocal of the dihydrofolate concentration (0.5-15 μM) at trimethoprim concentrations of 0 (○), 83 nM (■), 186 nM (△), and 236 nM (□). The NADPH concentration was 60μM, and 5 × 10^3 unit of enzyme was used in each assay. The inset shows a replot of slope versus trimethoprim concentration.

coli B strain RT 500 was previously shown to have a methotrexate $K_i$ value of 21 pM (31).

**Partial Amino Acid Sequence**—The amino acid sequence of the first 20 NH$_2$-terminal residues of the affinity column-purified enzyme was determined (Table II). Although the protein appeared homogeneous upon electrophoresis in both normal and denaturing (SDS) polyacrylamide gels, an unusual heterogeneity was observed during the sequence analysis. The first cycle unambiguously showed Met as the NH$_2$ terminus. However, two residues were observed during the second cycle, Leu and Ile in a ratio of approximately 2:1. The third cycle showed similar proportions of Ile and Ser. This continued in such a fashion that the minor component of one cycle "previewed" the major component of the next cycle. The possibility that this "preview" arose as a result of sequencing conditions could be discounted since analyses of *E. coli* chromosomal dihydrofolate reductase carried out both before and after the type III enzyme showed no similar occurrence. These data along with the NH$_2$-terminal sequence of the *E. coli* (RT 500) chromosomal enzyme are shown in Table III. The sequence homologies between the two dihydrofolate reductases are shown by boxes around the identical residues.

**DISCUSSION**

Purification of the type III plasmid-encoded dihydrofolate reductase was facilitated by its complete separation from the chromosomal enzyme during hydrophobic chromatography and by its tight binding to the methotrexate affinity matrix. However, yields from the purification procedure were typically low (15–20%), and the homogeneous enzyme had to be stored in buffer containing 20% glycerol to maintain activity for a 2- to 3-week time period. Similar problems of stability and yield were previously encountered during isolation of chromosomal dihydrofolate reductase from *Neisseria gonorrhoeae* (32) and the type I plasmid enzyme (5). Nevertheless, this purification scheme provided sufficient enzyme (~0.3 mg/liter of cells) for initial characterizations of its kinetic and physical properties.

The most striking physical property of the type III enzyme is its monomeric structure ($M_r = 16,800$). This differs from the type I plasmid enzyme which is a dimer of 18,000 molecular weight subunits (5) and the type II enzyme which is a tetramer of 8,500 molecular weight subunits (6). The type III enzyme also has unexpected kinetic properties. Trimethoprim was found to be a tight binding inhibitor of this enzyme ($K_i = 18$ nM), whereas types I and II dihydrofolate reductases have $K_i$ values of about 10 μM and 10 mM, respectively. Similar large differences in binding constants were also observed with methotrexate (Table I).
TABLE I
Comparison of various plasmid dihydrofolate reductases and the E. coli chromosomal enzyme

<table>
<thead>
<tr>
<th>Dihydrofolate reductase</th>
<th>Representative E. coli strain</th>
<th>Trimetoprim minimal inhibitory concentration</th>
<th>Molecular weight</th>
<th>Dihydrofolate Kᵣ</th>
<th>Tri- methoprim Kᵣ</th>
<th>Methotrexate Kᵣ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>µg/ml</td>
<td>Nondenatured</td>
<td>Denatured</td>
<td>µM</td>
<td>µM</td>
</tr>
<tr>
<td>Type I</td>
<td>SC16(R483)</td>
<td>&gt;1000</td>
<td>35,000</td>
<td>18,000</td>
<td>3.6</td>
<td>6.4</td>
</tr>
<tr>
<td>Type II</td>
<td>J5(R67)</td>
<td>&gt;2000</td>
<td>34,000</td>
<td>8,500</td>
<td>4.1</td>
<td>6100</td>
</tr>
<tr>
<td>Type III</td>
<td>J5-3(pAZ1)</td>
<td>64</td>
<td>18,900</td>
<td>16,700</td>
<td>0.4</td>
<td>0.019</td>
</tr>
<tr>
<td>Chromosomal</td>
<td>J5-3</td>
<td>0.2</td>
<td>18,000</td>
<td>18,000</td>
<td>2.7</td>
<td>0.9004</td>
</tr>
</tbody>
</table>

*Data from Refs. 4, 5, and 6.

†The dihydrofolate reductase activity in strain J5-3 was 1.8 units/10⁷ cells and that of strain J5-3 (pAZ1) was 2.0 units/10⁷ cells. In the latter case, about 75% of the activity was due to the type III enzyme.

TABLE II
Automatic Sequencer analysis of Type III dihydrofolate reductase
Analysis was carried out using 8 nmol of protein.

<table>
<thead>
<tr>
<th>Cycle number</th>
<th>Residues identified and yield*</th>
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<tr>
<td></td>
<td>Major</td>
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<tr>
<td></td>
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<tr>
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<td>Met (5.3)</td>
</tr>
<tr>
<td>2</td>
<td>Leu (3.3)</td>
</tr>
<tr>
<td>3</td>
<td>Ile (3.1)</td>
</tr>
<tr>
<td>4</td>
<td>Ser (---)</td>
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<tr>
<td>5</td>
<td>Leu (3.6)</td>
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<td>6</td>
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<td>7</td>
<td>Ala (3.4)</td>
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<td>8</td>
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<td>9</td>
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<td>Asn (1.6)</td>
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<tr>
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<td>Asn (1.3)</td>
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<tr>
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<td>Gly (1.8)</td>
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<tr>
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<td>Lys (0.7)</td>
</tr>
<tr>
<td>18</td>
<td>Asp (1.3)</td>
</tr>
<tr>
<td>19</td>
<td>Asn (0.6)</td>
</tr>
<tr>
<td>20</td>
<td>Leu (1.5)</td>
</tr>
</tbody>
</table>

*Yields are quoted as nanomoles above background and are based on averages of yields determined by both high pressure liquid chromatography and amino acid analysis following back hydrolysis.

†Not determined.

The resistance mechanism of organisms containing the type III enzyme may be more complex than that proposed for other trimethoprim-resistant R-plasmid strains. The trimethoprim affinities of type I and II enzymes are 10⁹ to 10¹⁰ less than that of the sensitive chromosomal enzyme, and the weak binding affinity is in rough proportion to the >10⁴-fold in vitro resistance of types I and II R-plasmid strains. However, the trimethoprim affinity of the type III enzyme (Kᵣ = 19 nM) is only about 50-fold less than that of the chromosomal E. coli J5-3 dihydrofolate reductase (Kᵣ = 0.4 nM), yet E. coli J5-3 (pAZ1) is about 320-fold resistant to trimethoprim (Table I). An examination of the kinetic data indicates that the low dihydrofolate Kᵣ of the plasmid enzyme is also an important factor in whole cell resistance. The fractional activity of an enzyme in the presence of a competitive inhibitor is dependent upon both the I/Kᵣ and S/Kᵣ ratios. Therefore, compared to the chromosomal enzyme, the type III plasmid enzyme is less sensitive to trimethoprim inhibition because of its 50-fold weaker inhibitor affinity and its 8-fold greater dihydrofolate affinity. A similar situation probably exists in the S. typhimurium from which the type III plasmid was originally isolated. We have partially purified and characterized the chromosomal S. typhimurium dihydrofolate reductase and found it equivalent to the E. coli chromosomal enzyme in size (Mr = 18,000), dihydrofolate Kᵣ (2.7 µM), and trimethoprim Kᵣ (0.3 nM).⁵

The intracellular concentration of dihydrofolate reductase is another factor which can affect whole cell trimethoprim sensitivity. Bacteria which contain increased amounts of their normal chromosomal enzyme exhibit trimethoprim resistance (33), and a similar mechanism could be important in organisms containing the type III enzyme. This is not the case with E. coli J5-3 (pAZ1) because its total dihydrofolate reductase activity level is similar to that of the E. coli J5-3 parent, and on an activity basis about 75% of the dihydrofolate reductase is type III enzyme. At trimethoprim concentrations sufficient to completely inhibit the chromosomal enzyme, the residual type III dihydrofolate reductase activity would be at near normal cellular levels. However, it is interesting to note that there is a 10-fold difference in catalytic efficiency between the type III enzyme (specific activity = 10 units/mg) and the chromosomal enzyme (specific activity = 100 units/mg) (34). Therefore, although the E. coli J5-3 (pAZ1) ratio of plasmid to chromosomal enzyme is 4 to 1 on an activity basis, it is 40 to 1 on a molar basis.

The amino acid sequence heterogeneity of the R-plasmid enzyme was unexpected because the purified enzyme migrated as a single component upon both SDS and nondenaturing
polyacrylamide gels. However, the nature of the heterogeneity is such that the NH₂ terminus of the minor component is equivalent to that of the major species with the exception of a deletion of its second amino acid, Leu. The SDS-gel electrophoresis system did not resolve the two proteins with a <1% (115/116,900) difference in molecular weight, and since the deleted residue is neither acidic nor basic, the two proteins should have a similar net negative charge during electrophoresis under nondenaturing conditions. The mechanism by which this heterogeneity is generated is not yet known, but there are numerous possibilities. Obviously, two different dihydrofolate reductase genes may reside in the R-plasmid. Alternatively, two protein species might be produced from a single gene if two different translational initiation signals are present. Ambiguity in the selection of initiator codons has been observed during the synthesis of coliphage f2 gene 2 protein where either of 2 AUG triplets separated by 9 bases is a functional initiator codon (35). An alternative initiator codon might be hypothesized for type III protein by considering that UUG, one of the triplets specifying Leu, has been occasionally identified as a functional initiator codon (36). If Leu, the second residue of the major protein species is specified by UUG, then the bacterial ribosomes would be presented with adjacent functional initiator codons. Either triplet might be selected for initiation if the spacing between the ribosome-binding site and initiation codons is not optimal (36). The minor species would be produced after the binding of Met-tRNA at the alternate initiator, UUG, and incorporation of Met followed by Ile. The triplet UUG may not function efficiently as an initiator with the result that synthesis from the alternate site represents only 30% of the total type III protein.

During the past several years there has been a trend toward an increasing incidence of pathogenic bacteria with high level transferable trimethoprim resistance (37, 38), and it is of considerable interest to determine the origins of the plasmid-encoded enzymes. However, little progress has been made in this area, and comparisons of enzyme properties have not proven useful. For example, the type II enzyme with its tetramer structure is unique among the dihydrofolate reductases characterized to date, and it has previously been suggested that this enzyme may have arisen from an oxidoreductase unrelated to dihydrofolate reductase (6). There are some similarities between the type I enzyme and T4 bacteriophage dihydrofolate reductase, but they are superficial. The T4 enzyme is a dimer composed of large subunits (Mₐ = 23,000), and it shares <20% amino acid sequence homology with the type I enzyme (39). In contrast, the present study may offer a clue to the origin of the new plasmid enzyme. The type III enzyme has several properties that are similar to those of other normal bacterial dihydrofolate reductases. It is a monomeric protein with a relatively high affinity for trimethoprim (and methotrexate), and its NH₂-terminal amino acid sequence shows 50% homology with the chromosomal E. coli enzyme. Therefore, it is quite feasible that the type III enzyme is identical to the chromosomal dihydrofolate reductase of an (unidentified) bacterial species that is intrinsically resistant to moderate levels of trimethoprim. The plasmid-mediated transfer of DNA between this putative donor and S. typhimurium could have taken place in the gut or some other environment where the two organisms co-exist.

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Characterization of an R-plasmid dihydrofolate reductase with a monomeric structure.

S S Joyner, M E Fling, D Stone and D P Baccanari


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