Dihydrofolate Reductase from the Wild Type and Aminopterin-Resistant Mutants of *Diplococcus pneumoniae*

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Received for publication 21 December 1974

Dihydrofolate reductase from the wild type and aminopterin-resistant mutants of *Diplococcus pneumoniae* has been compared. Specific activity, optimum pH, *K*ₐ, thermal stability, and inhibition by aminopterin are identical for both strains. Aminopterin resistance for such mutants is, therefore, not due to an alteration of the dihydrofolate reductase.

The biochemical basis of resistance to toxic analogues of folic acid, such as aminopterin (4-amino-4-deoxyfolic acid) or amethopterin (4-amino-10-methyl-4-deoxyfolic acid), has been the subject of numerous studies in bacteria as well as in higher organisms (4). Three resistance mechanisms have been identified in the pneumococcus. The first involves dihydrofolate (FH₂) reductase (EC 1.5.1.3.) (10–12). The second consists of a decreased affinity of the aminopterin transport system for its substrate (15, 16). The third is linked to a mutation in the structural gene for thymidylate synthetase (6). Each one of the above mechanisms is under the control of a single gene. A set of pleiotropic mutants has been studied—the mutants of the amiA locus (9, 13). They are aminopterin resistant, and sensitive to an imbalance in the amounts of the three branched amino acids, L-valine, L-leucine, and L-isoleucine, in the minimal medium defined for *Diplococcus pneumoniae* (9). In addition, these mutants are sensitive to L-methionine, D,L-ethionine, p-fluorophenylalanine, 4-methyltryptophan, and O-methylthreonine (17). A mutant representative of the amiA locus, amiA16, has been investigated for its ability to take up amethopterin. It was found that this uptake system has a reduced affinity for the drug (16). On the other hand, amiA5, a mutant in which the same locus is affected (13), has been investigated for its FH₂ reductase activity (2). These authors concluded that the enzyme is altered in that mutant. According to these two observations, the amiA locus would control two activities—the FH₂ reductase and the drug uptake system. To elucidate the biochemical function of this locus, we decided to compare the properties of the FH₂ reductase in the wild type and ami-A mutants.

**MATERIALS AND METHODS**

**Strains.** Mutants *amiA*₅ and *amiA*₁₆ were obtained by transformation of the wild-type strain clone 3 (*C₁₃*) by nitrous acid-treated deoxyribonucleic acid and plated on complete medium containing 10⁻⁴ M aminopterin (13). *amiII* mutants exhibit an altered uptake system for amethopterin (16). They were prepared by genetic transformation of strain *C₁₃* with DNA of the appropriate strains obtained from F. M. Sirotnak (12). These mutants bore lesions in the amiA locus as indicated in Fig. 1, which is a genetic map of the amiA locus. The *amiII* mutations are 101, 102, 107, 109, and 110 (17); 22, 1, 16, 5, and 17 are *ami* mutations (13).

**Media.** In large-scale cultures, we used a peptone medium modified from Sicard (13). It contains Difco neopeptone (1%), Difco yeast extract (0.4%), and Prolabo NaCl (0.85%); the pH was adjusted to 7.8 before autoclaving. Before use, 1 liter of this medium was completed with 2 ml of a 25% glucose solution, 4 ml of 10% yeast extract filtered through Norit, 2 ml of asparagine (3 × 10⁻³ M), and 2 ml of glutamine (10⁻¹ M). L-Asparagine and L-glutamine were from Calbiochem.

**Assay of FH₂ reductase.** Crude extracts were prepared at low temperature (between 0 and 4 C). Bacteria grown in 2 liters of peptone medium were harvested by centrifugation at the end of the log phase of growth and were washed three times with 100 ml (each) of 0.05 M potassium phosphate buffer (pH 7.5) containing 8.5 g of NaCl per liter. The cells were resuspended in 8 ml of the same buffer and treated for 80 s in a Braun MSK cell breaker cooled in dry ice. Intact bacteria and cellular debris were removed by centrifugation for 30 min at 27,000 × *g* followed by a second centrifugation for 60 min at 40,000 × *g*. Protein concentration was determined by the biuret method as modified by Koch and Putnam (7). Only fresh extracts were used.

FH₂ reductase activity was assayed by the method of Osborn and Huennekens (8). Hydrogen, necessary for reducing FH₂, was provided by reduced nicotinamide adenine dinucleotide phosphate (NADPH). We followed the decrease in absorption (at 340 nm) that
accompanies the oxidation of NADPH₂ to NADP. The assay was carried out in a 1-cm optical path cell maintained at 25 C, in a dual-beam Beckman spectrophotometer, with a reaction mixture containing no crude cell extract as the blank. One unit of enzyme is defined as the decrease in absorption at 340 nm of a 0.01 optical density unit. This corresponds to the reduction of 2.6 nmol of FH₂ (5). Specific activity is equal to the number of enzyme units per milligram of protein. Initial rates of the reaction were determined by using a graphic recording of the decrease in absorption at 340 nm. In each case, a control without FH₁ was made. The decrease in optical density at 340 nm measured in this control was subtracted from the value observed in the presence of FH₁. No decrease in absorbance occurred during the 5 min when the enzyme or NADPH₁ was omitted.

A 0.5-ml volume of crude cell extract, corresponding to between 2 and 5 mg of protein, was added to 2 ml of 0.05 M potassium phosphate buffer (pH 7.3) containing β-mercaptoethanol (0.0128 M), dihydrofolate acid (0.123 mM), and NADPH₁ (0.1 mM). Under these experimental conditions the initial rate of the reaction was constant for at least 2 min.

Preparation of dihydrofolinic acid. FH₁ was prepared by chemical reduction of folic acid according to the method of Futterman, modified by Blakley (3). FH₂ precipitated at acidic pH (pH 2). It was collected by cold centrifugation and dissolved in an NaOH solution (10⁻³ M) containing β-mercaptoethanol (0.0128 M). Portions (1 ml) can be stored in a freezer at -80 C for at least 2 months without loss of activity. FH₁ was identified by its ultraviolet absorption spectrum. The concentration of solutions was measured at 283 nm. At this wavelength, the molecular absorption coefficient for FH₁ is 10 × 10³ M⁻¹ cm⁻¹ (8).

Chemicals. Aminopterin was purchased from Schuchardt (Munich), folic acid was from Hoffmann-La Roche (Paris), and NADPH was from Boehringer Mannheim Corp.

RESULTS

Crude extracts from the wild-type bacterium Cl₁ and from the amiaA5 mutant exhibited a reducing activity towards FH₁ at pH 7.2 in the presence of NADPH. The initial rate of the reaction was proportional to the amount of protein in the range 0.25 to 2.5 mg by assay for 0.2 mM dihydrofolinic acid in the reaction mixture. Two liters of culture of either strain yielded 20 ml of crude extract, corresponding to about 150 mg of protein. Under standard conditions, the specific activity was determined to vary between 1.5 and 3 enzyme units per mg of protein from extract to extract, and the variation were independent of the origin (wild type or mutant).

Effect of pH on FH₂ reductase activity. In 0.05 M phosphate buffer, maximal activity was obtained for pH values between 7 and 7.5 for both wild-type and mutant extracts (Fig. 2).

Km determination. The variation in the initial rate of reaction was a function of FH₂ concentration. On a Lineweaver-Burk plot, it can be seen that both enzymes exhibited Michaelian behavior (Fig. 3). Values for Km and Vmax were in the same order of magnitude for extracts from both wild-type and mutant strains.

Thermal stability of enzymes. The rate of thermal denaturation of enzyme activity in crude extracts was followed at 42 and 55 C. The results (Table 1) show that enzyme activity was stable for 30 min at 42 and 55 C for wild-type and mutant strains when fresh extracts were used; frozen extracts led to inconsistent results (14; personal observation). At 55 C the rate of inactivation was the same for both strains when the extracts were heated for 1 h (Table 1).
The specific activities of the wild-type extract and the mutant extract are 1.5 and 2.4, respectively. Five milligrams of protein was used in each assay for the wild-type and mutant strains. Values of $K_m$ determined graphically are 3.5 $\mu M$ for the wild type and 2.1 $\mu M$ for the mutant. The $V_{max}$ values are, respectively, 6.6 and 7.6 nmol/min per mg of protein for the wild type and the mutant.

**Table 1. Thermal denaturation of FH$_2$ reductase from the wild type and amiA5 mutant**

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Thermal denaturation (%) at:</th>
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<tr>
<td></td>
<td>42 C</td>
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<td></td>
<td>Wild-type C$_4$</td>
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<td>100</td>
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<td>60</td>
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*Samples of each strain extract were heated and centrifuged at 27,000 $\times$ g for 10 min at 0 C. Assays were performed with the supernatants under standard conditions.

**Inhibition of enzyme activity by aminopterin.** We measured enzymatic activity in the presence of aminopterin with increasing amounts of crude extract by the method of Akermann and Potter (1). At low protein concentrations, there was no residual activity; it appeared at higher protein concentrations. The curve representing activity as a function of protein concentration became parallel to that for the control without inhibitors (Fig. 4). This type of curve, obtained with wild-type extract as well as mutant extract, expresses a pseudo-

irreversible inhibition of the type described by Werkheiser (18) and Sirotnak (11).

Assuming that there is one molecule of aminopterin bound per active site of the enzyme and there is only one active site per enzyme, this assay makes possible the titration of the extracts.

From our titration it appears that an aminopterin concentration of $5.6 \times 10^{-9}$ M inhibits 1.6 mg of protein in both extracts. Since the specific activity of each extract was the same, we can consider that the activity per site was equivalent in each extract.

**DISCUSSION**

Our comparative study of some of the properties of FH$_2$ reductase in the wild type and in the amiA5 mutant shows that crude extracts from the mutant strain exhibited behavior comparable to that of wild-type extracts. Similar results were obtained with strain amiA9, with a lesion...
in the same locus and, since the strains used are isogenic with the wild type except for the amiA mutations, we can consider that these mutations do not affect the properties of the enzyme when measured in crude extracts. These data disagree with those of Benedict and Gray (2) in that their amiA5 mutant, whose origin is the same as the mutant used in our laboratory (13), had an altered FH$_2$ reductase with a two- to sixfold greater affinity for the substrate than did the enzyme in wild-type extract. Moreover, these authors found that the enzyme from the amiA mutant was considerably more thermostable than in wild-type extracts. This discrepancy could have resulted from the independent evolution of the strains used by Gray and us, thus producing two different genetic backgrounds resulting in a side effect on amiA mutations, or perhaps from conditions of handling enzymatic extracts such as storage at -20°C. Indeed, we have observed (unpublished results), in agreement with Sirotnak and Hutchison, that freezing induces losses of stability of the enzyme, resulting in less consistent results. Consequently, the characteristic of amnopenorine resistance cannot be attributed to an alteration of the FH$_2$ reductase in the amiA5 strain. All the mutations in the amiA locus lead to the same physiological properties (anti-folic resistance, and sensitivity to the imbalance of branched amino acids, L-methionine, D-ethionine, p-fluorophenyl-alanine, 4-methyltryptophane, O-methylthreonine). Moreover, these mutations are genetically linked (10-17); consequently, we believe that they affect the same gene and that they result in, like amiA5, a wild-type FH$_2$ reductase and, like amiA16 and ameII mutations, an altered uptake system for the drug.

LITERATURE CITED


