Nutritional Requirement for 4-Aminobenzoate Caused by Mutation of Dihydropteroate Synthetase

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4-Aminobenzoate-Requirement, Dihydropteroate Synthetase, Folate Biosynthesis

*Aerobacter aerogenes* mutant 62-1 AC requires high concentrations of 4-aminobenzoate for growth. The mutant accumulates N-glucosyl-4-aminobenzoate and has an intact 4-aminobenzoate synthetase (Bacher, Gilch, Rappold, and Lingens, Z. Naturforsch. 28c, 614—617 [1973]). On the other hand the ability of the mutant to synthesize dihydropteroate is markedly reduced. The dihydropteroate synthetase level of mutant 62-1 AC is 1% as compared to the parent strain. Spontaneous revertants of mutant 62-1 AC show wild type levels of dihydropteroate synthetase. We conclude that the requirement for 4-aminobenzoate in mutant 62-1 AC is due to poor utilization of 4-aminobenzoate as a consequence of the low level of dihydropteroate synthetase activity.

**Introduction**

*Aerobacter aerogenes* 62-1 AC requires 4-aminobenzoate for growth and excretes a substance which supports the growth of other pab* mutants*. Biological evidence seemed to indicate that the excreted substance (Compound A) might be an intermediate in the biosynthesis of 4-aminobenzoate1, 2. This hypothesis was ruled out by the identification of Compound A as N-glucosyl-4-aminobenzoate. Further studies showed that mutant 62-1 AC has wild type activity of 4-aminobenzoate synthetase3. The excretion of a derivative of 4-aminobenzoate and the unimpaired enzymatic synthesis of 4-aminobenzoate raise the question whether the nutritional requirement of mutant 62-1 AC might be caused by a defect of 4-aminobenzoate utilization in the biosynthesis of dihydrofolic acid.

This paper shows that the 4-aminobenzoate requirement of *A. aerogenes* 62-1 AC is due to mutational alteration in the level of dihydropteroate synthetase activity.

**Materials and Methods**

**Materials**

6-Hydroxymethylpterin was prepared according to Baugh and Shaw4. The compound was converted to 6-hydroxymethyl dihydropterin by hydrogenation over palladized charcoal5. 6-Hydroxymethylpterin pyrophosphate was prepared by the procedure of Shiota et al.6. The dihydro form was obtained by reduction with dithionite7.

[14C]4-Aminobenzoate was obtained from Schwarz and Mann, Orangeburg, New York. Other chemicals were reagent grade.

**Strains**

All strains used are shown in Table I. Spontaneous revertants of *Aerobacter aerogenes* 62-1 AC were isolated by the following procedure. Single colonies of mutant 62-1 AC were inoculated into 500 ml of pab free minimal medium. When growth had occurred, a 5 ml aliquot of the suspension was transferred to fresh medium. The transfer was repeated once. Single colonies were isolated on pab free agar plates.

**Enzymatic formation of dihydropteroate**

Enzymatic assays were performed according to Brown and Richey8. The formation of dihydropteroate from 6-hydroxymethyl dihydropterin (A) or 6-hydroxymethyl dihydropterin pyrophosphate (B) was studied with dialyzed crude extracts.
**Reaction mixtures**

Assay A: 0.02 mM 6-hydroxymethyltetrahydropterin, 0.1 mM mercaptoethanol, 0.1 mM Tris-HCl pH 8.6, 5 mM ATP, 0.02 mM MgCl₂, 64 μM [¹⁴C]4-aminobenzoate and protein. Assay B: 0.01 mM 6-hydroxymethyltetrahydropterin pyrophosphate, 0.1 mM mercaptoethanol, 0.1 mM Tris-HCl pH 8.6, 64 μM [¹⁴C]4-aminobenzoate and protein.

Assay mixtures were incubated in a glove box under nitrogen for 1 hour at 30 °C. Dihydropteroate and unreacted 4-aminobenzoate were separated by ascending paper chromatography and radioactivity was measured in a liquid scintillation counter (ABAC SL 40, Intertechnique, Paris).

**Results and Discussion**

Fig. 1 shows the pathway of dihydrofolate biosynthesis according to data of Brown et al.⁹.

We studied the incorporation of 4-aminobenzoate into dihydropteroate in crude extracts of mutant 62-1 AC (pab⁻) and in autotrophic strains of *Aerobacter aerogenes*. In a first set of experiments we measured the conversion of 6-hydroxymethyltetrahydropterin to dihydropteroate which involves two enzymatic steps, namely pyrophosphorylation of 6-hydroxymethyltetrahydropterin and subsequent formation of dihydropteroate. The data are shown in Table II. Formation of dihydropteroate in mutant 62-1 AC is reduced to 1% as compared to the parent strain *A. aerogenes* 62-1 A5.

Further studies using 6-hydroxymethyltetrahydropterin pyrophosphate as substrate showed that the mutation in strain 62-1 AC affects specifically the level of dihydropteroate synthetase. The level of this enzyme in mutant 62-1 AC is 1% as compared to the parent strain (Table II).

**Table II. Enzymatic formation of dihydropteroate in cell extracts of *A. aerogenes* mutants.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Formation of dihydropteroate [ng pterate/mg protein-h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>62-1 A5</td>
<td>250</td>
</tr>
<tr>
<td>62-1 AC</td>
<td>2.2</td>
</tr>
<tr>
<td>R1</td>
<td>218</td>
</tr>
<tr>
<td>R2</td>
<td>211</td>
</tr>
<tr>
<td>R3</td>
<td>333</td>
</tr>
</tbody>
</table>

Spontaneous revertants of *A. aerogenes* 62-1 AC growing without exogenous 4-aminobenzoate were obtained as described under 'Methods'. One revertant was isolated from each reversion experiment. All revertants studied (R1, R2, and R3) showed wild type activity of the enzymes catalyzing the conversion of 6-hydroxymethyltetrahydropterin to dihydropteroate (Table II).

We conclude that the growth requirement of the pab⁻ mutant *A. aerogenes* 62-1 AC is due to the reduced level of dihydropteroate synthetase which may be caused 1. by a mutation in the structural gene for dihydropteroate synthetase resulting in a protein with reduced synthetic capacity or 2. by the production of a reduced quantity of otherwise normally functioning enzyme. A reduced level of normal enzyme might be phenotypically cured by a high level of 4-aminobenzoate if the intracellular level of 4-aminobenzoate is small as compared to the Kₘ value of the enzyme. A decision is not possible on the basis of our data.

In spite of the obvious location of the genetic defect in the folate biosynthetic pathway, mutant
62-1 AC is unable to grow with folic or pteroic acid. This may be due to a general inability of Enterobacteriaceae to utilize exogenous folate. Brown et al.\(^7\) suppose that *Escherichia coli* lacks the capacity to reduce exogenous folic acid. Mutants of Enterobacteriaceae growing with folic acid were not described to the best of our knowledge. Folate deficient mutants of *Saccharomyces cerevisiae* were described, but the genetic defect was not studied in detail\(^11\).

Screening for strains with high pab requirement might be a general method to isolate mutants with altered folate biosynthetic enzymes in strains unable to utilize exogenous folate.

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