

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. 28 c, 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-aminobenzoate^{1,2}. 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-hydroxymethyldihydropterin 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

Table I. Strains of *Aerobacter aerogenes* used in the present study (aro, tyrosin, tryptophan, phenylalanin; gua, guanine).

Strain	Growth requirement	Origin
62-1	aro	F. Gibson ¹²
62-1 A5	aro, gua	derived from 62-1 ¹
62-1 AC	aro, gua, pab	derived from 62-1 A5 ¹
R 1	aro, gua	spontaneous revertant of 62-1 AC
R 2	aro, gua	"
R 3	aro, gua	"

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-hydroxymethyldihydropterin (A) or 6-hydroxymethyldihydropterin pyrophosphate (B) was studied with dialyzed crude extracts.

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* *pab*, 4-aminobenzoate.

Reaction mixtures

Assay A: 0.02 mM 6-hydroxymethyldihydropterin, 0.1 M mercaptoethanol, 0.1 M Tris-HCl pH 8.6, 5 mM ATP, 0.02 M MgCl_2 , $64 \mu\text{M}$ $[^{14}\text{C}]$ 4-aminobenzoate and protein. Assay B: 0.01 mM 6-hydroxymethyldihydropterin pyrophosphate, 0.1 M mercaptoethanol, 0.1 M Tris-HCl pH 8.6, $64 \mu\text{M}$ $[^{14}\text{C}]$ 4-aminobenzoate and protein.

Assay mixtures were incubated in a glove box under nitrogen for 1 hour at 30°C . Dihydropterote 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 dihydropterote 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-hydroxymethyldihydropterin to dihydropterote which involves two enzymatic steps, namely pyrophosphorylation of 6-hydroxymethyldihydropterin and subsequent formation of dihydropterote. The data are shown in Table II. Formation of dihydropterote in mutant 62-1 AC is reduced to 1% as compared to the parent strain *A. aerogenes* 62-1 A5.

Further studies using 6-hydroxymethyldihydropterin pyrophosphate as substrate showed that the mutation in strain 62-1 AC affects specifically the level of dihydropterote 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 dihydropterote in cell extracts of *A. aerogenes* mutants.

Strain	Formation of dihydropterote [ng pterote/mg protein · h]	
	from 6-hydroxymethyl- dihydropterin (A)	from 6-hydroxymethyl- dihydropterin pyro- phosphate (B)
62-1 A5	250	140
62-1 AC	2.2	1.4
R 1	218	
R 2	211	
R 3	333	

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-hydroxymethyldihydropterin to dihydropterote (Table II).

We conclude that the growth requirement of the pab^- mutant *A. aerogenes* 62-1 AC is due to the reduced level of dihydropterote synthetase which may be caused 1. by a mutation in the structural gene for dihydropterote 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 K_m 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

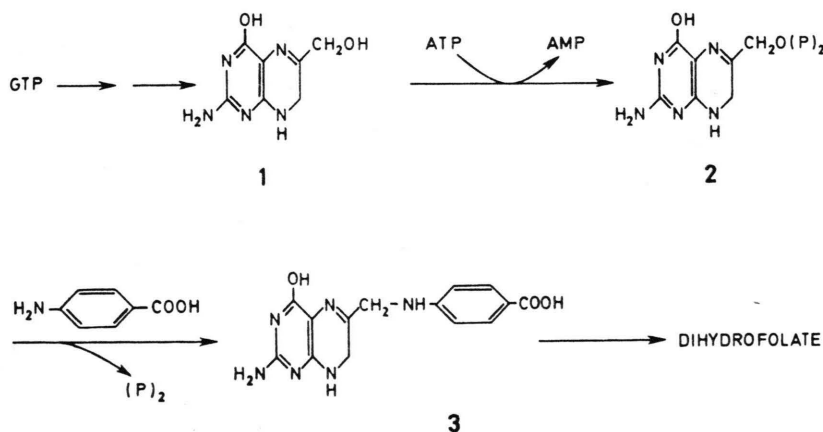


Fig. 1. Biosynthesis of dihydrofolate according to Brown *et al.*⁹. (1, 6-hydroxymethyldihydropterin; 2, 6-hydroxymethyldihydropterin pyrophosphate; 3, dihydropterote.)

62-1 AC is unable to grow with folic or pteronic acid. This may be due to a general inability of Enterobacteriaceae to utilize exogenous folate. Brown *et al.*¹⁰ 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 detail¹¹.

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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