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 synthetase [3]. 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 Shaw [4]. The compound was converted to 6-hydroxymethyldihydropterin by hydrogenation over palladized charcoal [5]. 6-Hydroxymethylpterin pyrophosphate was prepared by the procedure of Shiota *et al.* [6]. The dihydro form was obtained by reduction with dithionite [7].

[^{14}C]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 occured, a 5 ml aliquot of the suspension was transfered to fresh medium. The transfer was repeated once. Single colonies were isolated on pab free agar plates.

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	„

Enzymatic formation of dihydropteroate

Enzymatic assays were performed according to Brown and Richey [8]. 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 μM [^{14}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 μM [^{14}C]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-hydroxymethyldihydropterin to dihydropteroate which involves two enzymatic steps, namely pyrophosphorylation of 6-hydroxymethyldihydropterin 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-hydroxymethyldihydropterin 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.

Strain	Formation of dihydropteroate [ng pteroate/mg protein·h]	
	from 6-hydroxymethyldihydropterin (A)	from 6-hydroxymethyldihydropterin pyrophosphate (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 unter '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 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 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

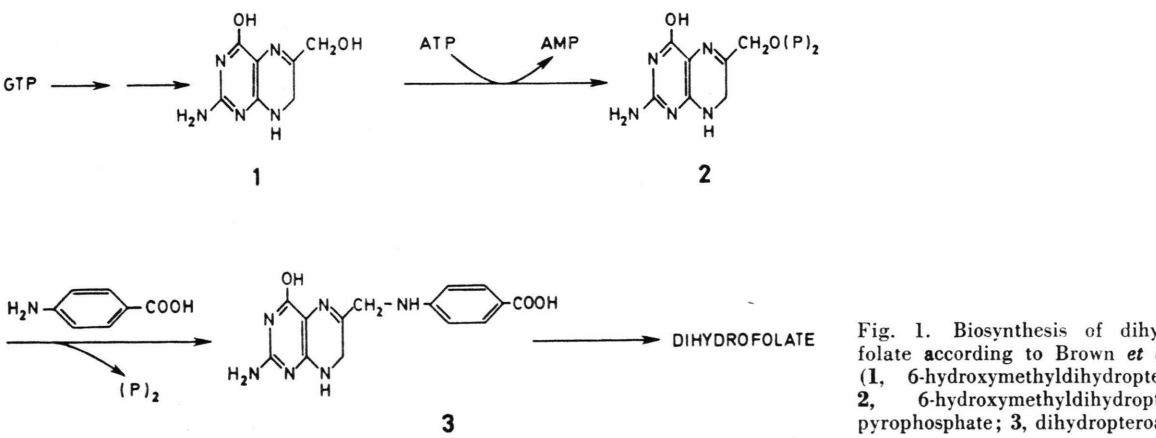

Fig. 1. Biosynthesis of dihydrofolate according to Brown *et al.*[9]. (**1**, 6-hydroxymethyldihydropterin; **2**, 6-hydroxymethyldihydropterin pyrophosphate; **3**, dihydropteroate.)

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.* [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 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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