Unimpaired *de novo* Synthesis of 4-Aminobenzoate in a Mutant of *Aerobacter aerogenes* Requiring 4-Aminobenzoate for Growth; Structure of Compound A

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The mutant strain *Aerobacter aerogenes* 62-1 AC, pab⁻, accumulates a labile substance, designated Compound A which supports the growth of other pab⁻ mutants. Previous studies seemed to indicate that Compound A might be an intermediate in the conversion of chorismate to 4-aminobenzoate (Altendorf, Bacher, and Lingens, Z. Naturforsch. **24 b**, 1602 [1969]). The present experiments show that Compound A is 4-(D-glucosylamino)-benzoic acid. This substance which is clearly not an intermediate in the biosynthesis of 4-aminobenzoate is formed by reaction of 4-aminobenzoate produced *de novo* by the mutant with exogenous glucose. Mutant 62-1 AC has the unimpaired capacity to synthesize 4-aminobenzoate as shown by direct enzyme studies. Mutant 62-1 AC requires approximately 200 times more 4-aminobenzoate for growth than other pab⁻ mutants of *A. aerogenes* with defective 4-aminobenzoate synthetase. The genetic defect of mutant 62-1 AC seems to be located in a metabolic system concerned with the utilization of 4-aminobenzoate rather than its synthesis.

Introduction

Altendorf *et al.* described a 4-aminobenzoate deficient mutant 62-1 AC of *Aerobacter aerogenes* which was supposed to produce an intermediate in the biosynthesis of 4-aminobenzoate [1, 2]. This hypothesis was based on the following observations: 1. The mutant excretes a substance, designated Compound A, which can be transformed to 4-aminobenzoic acid by heating or by treatment with dilute mineral acid. 2. Compound A supports the growth of a 4-aminobenzoate deficient mutant of *Escherichia coli*, seemingly without prior decomposition to 4-aminobenzoate.

Altendorf *et al.* described the isolation of Compound A and published a cyclohexadiene type structure [3, 4]. However, the present results show that Compound A is 4-(D-glucosylamino)-benzoic acid. This compound can no longer be assumed to be an intermediate in the biosynthesis of 4-aminobenzoate. Indeed enzymatic studies document that the mutant 62-1 AC has the wild type level of 4-aminobenzoate synthetase. This indicates that the 4-aminobenzoate requirement of this mutant must be caused by a genetic defect in some other metabolic system than the formation of 4-aminobenzoate itself.

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Materials and Methods

Materials

4-(D-Glucosylamino)-benzoic acid was synthesized by the procedure of Ellis [5]. Chorismic acid was isolated as described by Gibson [6]. Other chemicals used were reagent grade.

Culture media

Culture media for *A. aerogenes* have been described [2]. PAB Assay Medium was a product of Difco Labs., Detroit, Michigan.

Strains

A. aerogenes 62-1 was kindly provided by F. Gibson, Canberra, Australia [7]. Other strains used are shown in Table I. *Acetobacter suboxydans* ATCC 621 was obtained from the American Type Culture Collection.

Table I. Strains of *Aerobacter aerogenes* used in the present study.

Strain	Derived from	Growth requirement[a]	Source
62–1			F. Gibson[7]
62–1 A	62–1	gua	(2)
62–1 AC	62–1 A	gua, pab	(2)
62–1 P4	62–1	pab	(3)
62–1 P6	62–1	pab	(3)
62–1 P8	62–1	pab	K. H. Altendorf

a. All strains also require tyrosine, phenylalanine, and tryptophan.

Preparation of Compound A

Compound A was produced and isolated as described by Altendorf *et al.*[3]. Alternatively, growth of cells and accumulation were performed in a 100-liter batch fermentor.

Acetylation of Compound A

Compound A was dissolved in a mixture of pyridine/acetic anhydride (2 : 1, v/v). The solution was kept at room temperature overnight. It was then evaporated to dryness under reduced pressure. The residue was taken up in water and extracted with ether. The ether phase was evaporated to dryness. The residue was purified by chromatography on a column of Sephadex LH-20 which was developed with 90% ethanol.

Determination of 4-aminobenzoate synthetase

Cells were grown in complete medium[2]. They were harvested, washed, and frozen at −20 °C. Cells were suspended in 0.05 M Tris buffer pH 8.2 and disrupted by ultrasonic treatment. The assay of 4-aminobenzoate synthetase was performed according to Gibson *et al.*[8]. Test solutions contained 0.05 M Tris buffer pH 8.2, 0.3 mM chorismic acid, 0.1 M glutamine, and protein.

Determination of glucose

Glucose was determined enzymatically with glucose oxidase supplied by Merck AG., Darmstadt.

Spectroscopic methods

NMR spectra were recorded with a pulsed Fourier transformation spectrometer from Bruker-Physik AG, Karlsruhe-Forchheim, Germany. Circular dichroism was recorded with a Cary Model 60 equipped with the circular dichroism attachment 6002. Ultraviolet spectra were recorded with a photometer DMR 21 from Carl Zeiss Optical Works, Oberkochen. Infrared spectra were determined with a spectrometer from Ernst Leitz Optical Works, Wetzlar. The instrument was equipped with a beam condensor, thus permitting measurements of small samples. Fluorescence emission was measured with an Aminco Bowman spectrofluorometer, American Instrument Co., Silverspring, Maryland.

Results

Compound A was isolated from the culture fluid of *A. aerogenes* 62-1 AC as described by Altendorf *et al.*[3]. The compound was treated with a mixture of pyridine/acetic anhydride. The resulting acetyl derivative was obtained in pure form by chromatographic procedures.

Compound A and the respective acetyl derivative show similar ultraviolet spectra and decompose to 4-aminobenzoic acid in dilute HCl. Further information was obtained from proton magnetic resonance spectra of the acetylated product in acetone-d_6. The spectrum shows two sextets centered at $\tau = 2.13$ and $\tau = 3.15$, respectively. This pattern is typical of 1,4-substituted benzene derivatives and very similar to the respective data of 4-aminobenzoic acid. Furthermore, the presence of four acetyl groups in

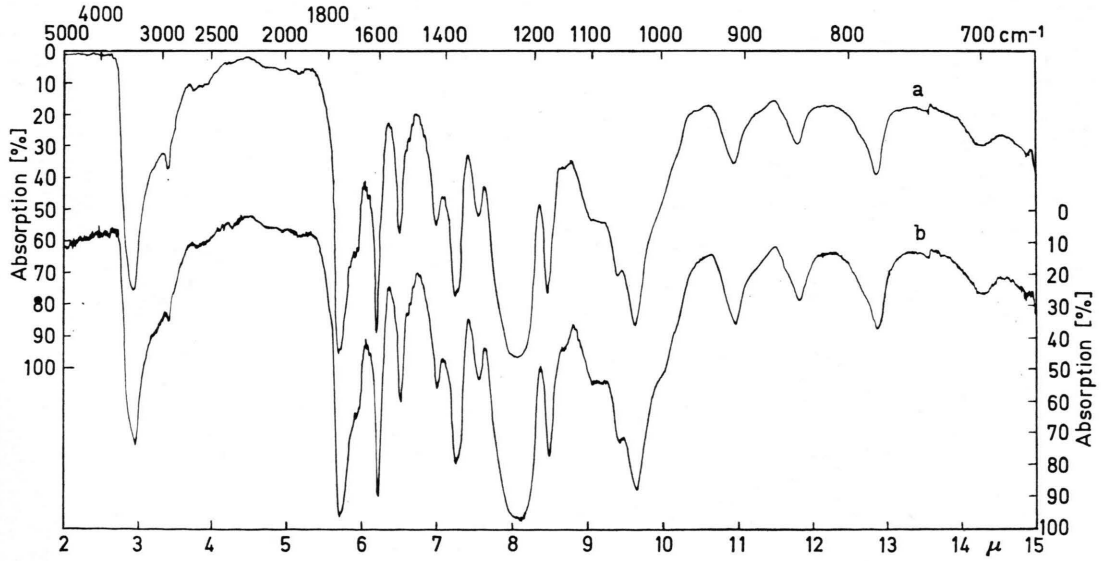

Fig. 1. Infrared spectra of acetylated Compound A (a) and of 4-(tetraacetyl-D-glucosylamino)-benzoate (b).

the molecule could be tentatively inferred from the NMR data. These findings indicate that Compound A is a glycoside derivative of 4-aminobenzoic acid.

This hypothesis was substantiated by comparison of Compound A with a synthetic sample of 4-(D-glucosylamino)-benzoic acid. The compounds were indiscernable with respect to ultraviolet absorption, circular dichroism, and thin layer chromatography. Both compounds yield equimolecular amounts of 4-aminobenzoic acid and glucose after treatment with dilute hydrochloric acid. Similarly, the acetyl derivatives of Compound A and of 4-(D-glucosylamino)-benzoic acid were identical with respect to ultraviolet absorption, circular dichroism, infrared spectra, and thin layer chromatography (Fig. 1). These results document conclusively that Compound A is 4-(D-glucosylamino)-benzoic acid.

A. aerogenes 62-1 AC can grow with synthetic 4-(D-glucosylamino)-benzoate (260 μg/l) as the only source of 4-aminobenzoate. The lag phase is 52 hours. However, the lag phase is only 14 hours if the compound is transformed to 4-aminobenzoate by autoclaving at 120 °C prior to inoculation. This is consistent with our previous experiments performed with Compound A isolated from the culture medium of *A. aerogenes* 62-1 AC [2].

The following experiment was set up to verify that Compound A is actually formed *de novo* during the fermentation procedure: *A. aerogenes* 62-1 AC was grown in complete medium. The cells were washed and suspended in accumulation medium. Aliquots of the cell suspension were removed at appropriate intervals and autoclaved in order to liberate intracellular 4-aminobenzoate and to transform the glucoside to free 4-aminobenzoate. The samples were centrifuged. The concentration of 4-aminobenzoate in the supernatant was measured by the procedure of Landy and Dicken [9]. Results are shown in Fig. 2. A considerable amount of 4-aminobenzoate (and glucoside, respectively) is formed *de novo* during the accumulation period. Similar amounts of 4-aminobenzoate were formed by the parent strain *A. aerogenes* 62-1 under the same experimental conditions.

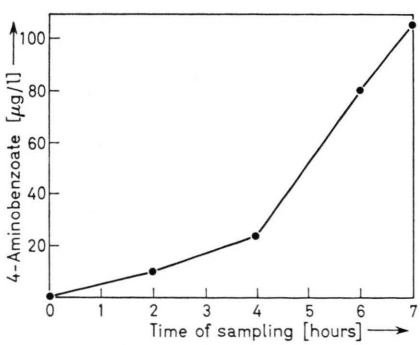

Fig. 2. Formation of 4-aminobenzoate by *A. aerogenes* 62-1 AC. Cells grown in complete medium were harvested, washed and resuspended in minimal medium. Samples were taken at appropriate intervals.

These results suggest that *A. aerogenes* 62-1 AC is capable to synthesize 4-aminobenzoate *de novo*. To verify this, the activity of the enzyme, 4-aminobenzoate synthetase, was studied in the strains 62-1 AC, 62-1, and three other 4-aminobenzoate deficient mutants derived from strain 62-1. Results are shown in Table II. Mutant 62-1 AC and the parent strain 62-1 have similar levels of 4-aminobenzoate synthetase. The 4-aminobenzoate synthetase activity of the other strains is below the level of detection.

Table II. Specific activity of 4-aminobenzoate synthetase in mutants of *A. aerogenes*.

Strain	Specific activity [nmoles/mg hour]
62—1	0.52
62—1 AC	0.52
62—1 P4	< 0.04
62—1 P6	< 0.04
62—1 P8	< 0.04

Earlier experiments had shown that mutant 62-1 AC requires a high concentration of 4-aminobenzoate for growth [2]. Fig. 3 shows the 4-aminobenzoate requirement of this and other 4-aminobenzoate deficient mutants derived from *A. aerogenes* 62-1. The 4-aminobenzoate requirement of mutant 62-1 AC is some 200 times higher than the requirement of the other mutants tested.

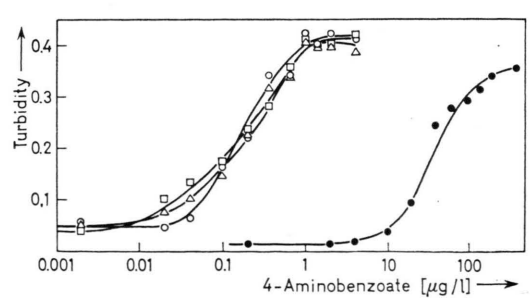

Fig. 3. Growth of 4-aminobenzoate deficient mutants of *A. aerogenes* with 4-aminobenzoate. ● 62-1 AC; △ 62-1 P4; □ 62-1 P6; ○ 62-1 P8.

Discussion

Previous studies from this laboratory seemed to indicate that Compound A, which was isolated from the culture fluid of *A. aerogenes* 62-1 AC, pab$^-$, might be an intermediate in the biosynthetic pathway from chorismate to 4-aminobenzoate [1–4]. The present results show that Compound A is 4-(D-glucosylamino)-benzoic acid. The involvement of a compound of this type in the biosynthesis of 4-aminobenzoate is highly unlikely. Furthermore, direct enzyme studies show that mutant 62-1 AC has the wild type level of 4-aminobenzoate synthetase. Hence the 4-aminobenzoate requirement of the strain must be caused by a defect in a metabolic system other than the *de novo* synthesis of 4-aminobenzoate. This will be discussed in detail below. The formation of Compound A may be explained by the assumption that 4-aminobenzoate formed *de novo* by the mutant reacts with exogenous glucose to yield the respective glycoside. Indeed, Kitamura and coworkers have shown that this glycoside forms spontaneously in dilute solutions of 4-aminobenzoic acid and glucose [10].

We should like to emphasize that our previous assumption of a labile, dissociable intermediate between chorismate and 4-aminobenzoate cannot be supported further in the light of the present results. This is in keeping with the findings of Huang and Gibson [11]. These authors have demonstrated the involvement of two protein components in the conversion of chorismate to 4-aminobenzoate. No evidence whatsoever for the involvement of a dissociable intermediate could be obtained under a variety of experimental conditions.

The growth requirement for 4-aminobenzoate in a mutant with normal *de novo* synthesis of this compound is not easily understood. The following hypotheses should be considered. 1. 4-Aminobenzoate is a biosynthetic precursor of dihydropteroate [12]. The affinity of the enzyme, dihydropteroate synthetase, to the substrate, 4-aminobenzoate, might be reduced by a mutation in the structural gene coding for this enzyme. The catalytic function might be restored by a high concentration of 4-aminobenzoate. 2. The genetic defect of mutant 62-1 AC might concern the transport of 4-aminobenzoate. Campbell and coworkers [13] have described a mutation, designated bir$^-$, which destroys the capacity of the respective mutant to transport biotin. bir$^-$ Mutants excrete biotin into the culture medium and grow poorly unless they are supplemented with a high concentration of biotin.

Further experimental work to explain the unusual properties of mutant 62-1 AC is under way.

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