Effects of some substrate analogs on aerobactin synthetase from *Aerobacter aerogenes* 62-1

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Tricarballylic acid was the only analog of citric acid that appeared to be a substrate for aerobactin synthetase. The others, succinic acid, glutaric acid and 3-methyl-3-hydroxyglutaric acid, though potent inhibitors of aerobactin synthesis, were neither activated nor utilised by the enzyme system. Aerobactin synthetase seemed to mediate the condensation of either N^6-*n*-butyryl-N^6-hydroxylysine, or N^6-succinyl-N^6-hydroxylysine, or N^6-acetyllysine and citric acid, in a manner analogous to that of the natural substrate, N^6-acetyl-N^6-hydroxylysine.

Aerobactin synthetase *Substrate analog* *Citric acid* N^6-*Acetyl*-N^6-*hydroxylysine* *Catalytic site*

1. INTRODUCTION

Owing to the scarcity in the occurrence of iron in bioavailable forms, microbes have acquired the unique property of producing siderophores [1]. These biomolecules broadly divided into two classes, hydroxamates and catecholates, have a high affinity for iron(III) and are known to be involved in solubilization and transport of this vital metal in many microorganisms [2–4].

When grown under iron stressed conditions, *Aerobacter aerogenes* 62-1 secretes aerobactin [5], a dihydroxamate comprising of two molecules of N^6-acetyl-N^6-hydroxylysine and one molecule of citric acid. Aerobactin synthetase, the enzyme effecting the last step in the biosynthesis of this siderophore, has been shown to mediate the linkage of 2 mol of N^6-acetyl-N^6-hydroxylysine and 1 mol of citric acid through peptide bonds [6]. Preliminary observations have demonstrated the requirement of ATP and Mg^{2+} in this process. The initial step of this enzymatic reaction appears to entail the phosphorylation of both the substrates.

To gain further insight on the nature of this enzymatic condensation, we have examined the effects of various analogs of the two natural substrates citric acid and N^6-acetyl-N^6-hydroxylysine on aerobactin synthetase. These results, together with a discussion on a possible mechanism for aerobactin production are reported.

2. MATERIALS AND METHODS

Citric acid, glutaric acid, succinic acid, 3-methyl-3-hydroxyglutaric acid, acetyl phosphate, *n*-butyryl-CoA, succinyl-CoA, N^6-acetyllysine were obtained from Sigma (St. Louis, MO), while tricarballylic acid (1,2,3-propanetricarboxylic acid) was a product of Fluka, Switzerland.

2.1. *Preparation of* N^6-*acetyl*-N^6-*hydroxylysine and its analogs*

Aerobactin obtained as described [7] was the source of N^6-hydroxylysine. The N^6-hydroxylysine was converted to its N^6-acetyl derivative by acetyl phosphate as in [6]. N^6-*n*-Butyryl-N^6-hydroxylysine was synthesized by treatment of N^6-hydroxy-

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lysine (10 μmol) with *n*-butyryl-CoA (20 μmol) at pH 6.0 for 2 h. The formation of the monohydroxamate was estimated by ferric chloride reagent [5] and by the bismercaptoacetate-iron(III) method [8]. The amino acid (N^6-hydroxylysine) was converted to its N^6-succinyl derivative by treatment with 2-fold excess of succinyl-CoA at pH 6.0. The product was characterized on the basis of its ability to sequester iron from the two colorimetric reagents described earlier.

2.2. *Enzyme preparation*

Aerobacter aerogenes 62-1 was grown on an iron-deficient medium, and the cell-free extract was prepared by procedures described [9–11]. The supernatant fraction served as the source of aerobactin synthetase. This enzyme was partially purified as described in [6].

2.3. *Assay procedure*

A typical assay mixture, total volume 2 ml, consisted of 2–4 μmol citric acid (or its analogs), 1–2 μmol N^6-acetyl-N^6-hydroxylysine (or its analogs), 10 μmol Mg^{2+}, 10 μmol ATP, enzyme (0.1–1.0 ml corresponding to 25–250 μg protein) and 50 mM potassium phosphate (pH 7.2). The mixture was incubated at 37°C for 30 min. Reaction mixtures without citrate (or its analogs) ATP and N^6-acetyl-N^6-hydroxylysine (or its analogs) served as controls in these studies. In inhibition experiments each analog was used at a final concentration of 2 mM.

Two procedures were employed for monitoring the formation of aerobactin or its analogs: (a) measurement of the decrease in absorbance at 532 nm upon treatment of the reaction mixture with bismercaptoacetate-iron(III) reagent [8]; and (b) an assessment of the utilisation of citrate or its analogs and ATP by HPLC [12].

3. RESULTS AND DISCUSSION

The ability of aerobactin synthetase to utilise analogs of citrate for peptide bond formation with N^6-acetyl-N^6-hydroxylysine was examined using the following compounds: tricarballylic acid, succinic acid, glutaric acid and 3-hydroxy-3-methylglutaric acid. In these experiments, the desired analog replaced citrate in the assay mixture. Analysis of assay mixtures containing tricarballylic acid, a compound that is structurally similar to citric acid except for the absence of hydroxyl at the central carbon, revealed a diminution in its concentration, indicating its utilisation during the incubation. Consumption of tricarballylic acid was found to be ATP dependent since no such decrease in the concentration of the compound was detected in controls that were devoid of ATP (fig.1A,B). Furthermore, analysis of the assay mixture for adenylate nucleotides by HPLC revealed a diminution in the concentration of ATP, accompanied by a concomitant formation of ADP (fig.1C,D). The treatment of the assay mixture (after completion of reaction) with bismercaptoacetate-iron(III) reagent

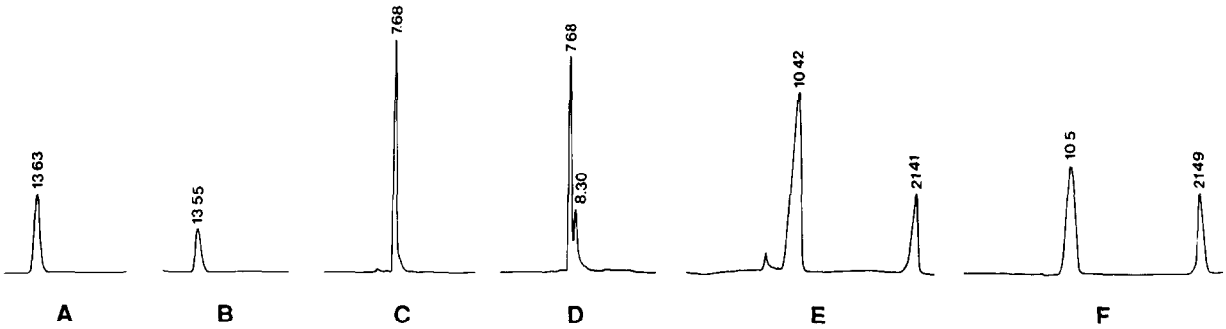

Fig.1. Effects of citric acid analogs on aerobactin synthetase. (A) Tricarballylic acid in control (reaction mixture – ATP); (B) tricarballylic acid in complete reaction mixture; (C) ATP in control; (D) utilisation of ATP and concomitant formation of ADP on incubation of tricarballylic acid in a complete reaction mixture; (E) citric acid (10.42 min) and glutaric acid (21.41 min) in control (reaction mixture – ATP); (F) citric acid (utilised) and glutaric acid (unchanged) in complete reaction mixture. Sample (20 μl) was analysed using an Aminex HPX-87H column (BioRad) for organic acids and an RP-300 Aquapore column (Brownlee) for adenine nucleotides [12].

resulted in a substantial decrease in absorbance at 532 nm relative to that of the control, indicating the formation of a compound (siderophore) with iron sequestering property superior to that of N^6-acetyl-N^6-hydroxylysine.

Similar experiments with succinic acid, glutaric acid and 3-hydroxy-3-methylglutaric acid revealed the inability of aerobactin synthetase to activate any of these compounds. Neither utilisation of ATP nor change in the concentration of any of these analogs was observed, when they were used as substitutes for citrate in the reaction mixture (fig.1E,F). However, all these three moieties were found to inhibit enzyme mediated incorporation of citric acid into aerobactin. Succinic acid was a more effective inhibitor of aerobactin synthesis than the other analogs (table 1).

When N^6-*n*-butyryl-N^6-hydroxylysine or N^6-succinyl-N^6-hydroxylysine was used as substitute for N^6-acetyl-N^6-hydroxylysine in the assay mixture, the formation of siderophore with similar iron sequestering properties as aerobactin was observed. Condensation of these analogs of N^6-acetyl-N^6-hydroxylysine with citrate was further indicated by the observed activation of citrate and utilisation of ATP in the reaction mixtures containing either of these compounds. In experiments with N^6-acetyllysine, analysis of the assay mixture by HPLC also revealed a consumption of both citrate and ATP,

Table 1

Incorporation of citric acid into aerobactin by aerobactin synthetase in the presence of citric acid analogs

Experiment	Citric acid consumed (nmol)
Complete system[a]	437.5
− ATP	10.0
+ tricarballylic	125.2
+ 3-hydroxy-3-methylglutaric acid	256.4
+ glutaric acid	120.3
+ succinic acid	100.4

[a] Complete system: final volume 2 ml consisted of citrate (0.5 mM), N^6-acetyl-N^6-hydroxylysine (1.2 mM), Mg^{2+} (10 mM), ATP (4 mM), enzyme (~50 μg), 50 mM phosphate buffer in 0.5 mM DTT (pH 7.2), incubation 30 min at 37°C, citric acid analogs (2 mM) in respective reaction mixtures

thus indicating the ability of this compound to serve as a substitute for N^6-acetyl-N^6-hydroxylysine in the enzyme-mediated peptide bond formation. However, analysis of the reaction mixture by bismercaptoacetate-iron(III) complex failed to reveal the formation of a siderophore, an observation not unexpected in view of the absence of the ligating hydroxamate function in the product. Inclusion of N^6-acetyllysine in a typical assay mixture resulted in a marked inhibition in aerobactin production, pointing to the ability of this compound to compete effectively with N^6-acetyl-N^6-hydroxylysine in the peptide bond forming reaction.

Thus among the citric acid analogs, only tricarballylic acid appears to undergo activation as well as utilisation when present in the incubation mixture, while the other three analogs, that inhibit aerobactin production by probably competing with citric acid, do not seem to be either activated nor utilised by the enzyme preparation. In contrast, analogs of N^6-acetyl-N^6-hydroxylysine all seem to be involved in the enzyme-mediated condensation reaction.

These findings may be indicative of the presence of at least two distinct catalytic activities in the enzyme preparation, one exhibiting marked specificity towards tricarboxylic acids and the other capable of activating derivatives of lysine. This ability of the enzyme system to activate a number of amino acids appears analogous to that reported in the case of gramicidin synthetase [13,14]. Thus, it is not unlikely that the synthesis of aerobactin, likewise may involve a multienzyme complex whose component proteins, are each specifically capable of activating a given precursor.

The inability of the enzyme system to activate 3-methyl-3-hydroxyglutaric acid, succinic acid and glutaric acid may be a reflection of its function in peptide bond formation being contingent upon the presence of an activated tricarboxylic acid. The central carboxylic group absent in all instances and particularly substituted by a methyl group in 3-methyl-3-hydroxyglutaric acid may be playing a determinant role in this enzymatic process. Possibly owing to the position of different substituent groups at the other extremity of the peptide bond forming site, aerobactin synthetase activity does not appear to be affected by various analogs of N^6-acetyl-N^6-hydroxylysine.

This study demonstrates the possibility of at

least two different catalytic sites, one involved in activating N^6-acetyl-N^6-hydroxylysine and the other citric acid. The marked preference for tricarboxylic acids may be indicative of a very crucial role for the central carboxylic group in this enzymatic condensation.

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