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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR MONITORING AEROBACTIN SYNTHETASE ACTIVITY

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SUMMARY

A rapid measurement of aerobactin synthetase activity has been achieved by monitoring the utilization of two of the reactants, citrate and ATP, using high-performance liquid chromatography. This method, besides being highly sensitive and reproducible, provided insight into the mechanism involved in the activation of precursors of aerobactin. The analytical procedure has potential applications to the screening of biological materials for the presence of adenylate kinase activity.

INTRODUCTION

Under conditions of iron limitation, microorganisms are known to secrete a unique group of compounds referred to as siderophores, which play an important role in microbial assimilation of iron(III)[1–5]. These compounds, in view of their high affinity for Fe^{3+} and divalent cations, have tremendous potential as chemotherapeutic agents as well as in industrial metallurgy[6–9]. When faced with iron deprivation, *Aerobacter aerogenes* 62-1 produces aerobactin, a dipeptide comprising 2 mol of N^6-acetyl-N^6-hydroxylysine and 1 mol of citrate[10]. The biosynthesis of this siderophore involves the initial hydroxylation of lysine to N^6-hydroxylysine which is subsequently acetylated to its N^6-acetyl derivative. The condensation of two molecules of N^6-acetyl-N^6-hydroxylysine with one molecule of citrate through peptide bonds constitutes the final stage in aerobactin production. Aerobactin synthetase has been shown to mediate the peptide bond-formation step in the presence of ATP and magnesium(II)[11]. Consequently, the activity of this enzyme can be monitored either by measuring the consumption of the reactants, citrate, ATP and N^6-acetyl-N^6-hydroxylysine, or the formation of the product, namely aerobactin.

In this paper we describe an assay which relies on high-performance liquid chromatography (HPLC) for monitoring the changes in citrate and ATP during the enzyme-catalyzed production of aerobactin from its precursors. An advantage of this

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procedure concerns the detection and determination of adenylate kinase activity which mediates the conversion of ADP into ATP and AMP. Since the presence of adenylate kinase interferes with the elucidation of the mode as well as the stoichiometry of ATP utilization, the analytical method described here provides a rapid means of assessment of this activity in aerobactin synthetase preparations prior to their use in studies of the mechanisms operative in the synthesis of aerobactin from its precursors.

EXPERIMENTAL

Citrate, ATP, ADP, AMP and acetyl phosphate were obtained from Sigma (St. Louis, MO, U.S.A.).

Synthesis of N^5-acetyl-N^6-hydroxylysine

Aerobactin, isolated from filtrate of *A. aerogenes* 62-1 cultured for 16 h, served as the source of N^6-acetyl-N^6-hydroxylysine. Following hydrolysis of aerobactin with 3 *M* hydrochloric acid in sealed evacuated tubes at 105°C for 24 h and subsequent removal of the excess of acid, the hydrolysate was applied to a 10 cm × 2 cm column of Dowex 50-H^+ resin. N^6-Hydroxylysine was recovered by elution with 2 *M* hydrochloric acid. Treatment of N^6-hydroxylysine with a five-fold excess of acetyl phosphate yielded the N^6-acetyl derivative. N^6-Acetyl-N^6-hydrolysine was purified by crystallization from aqueous ethanol. The ^1H NMR spectrum was identical with that in the literature[12].

Preparation of aerobactin synthetase

Aerobactin synthetase, present in the soluble fraction of a cell-free extract obtained from *A. aerogenes* 62-1 grown on an iron-deficient medium for 8 h, was prepared and partially purified as described previously[13–15].

Measurement of citrate and ATP

The quantitation of citrate and ATP was achieved by HPLC using a Beckman Model 324 unit. This system consisted of two solvent-delivery units controlled by a solvent programmer, a universal liquid chromatograph injector, a UV–VIS detector and a recorder/integrator. In the estimation of citrate, a 300 mm × 7.8 mm Animex HPX-87 column connected to a guard column (Aminex-Resin micro-guard cartridge cation H^+) was employed. Dilute sulphuric acid (16 m*M*, pH 2.1) served both as an equilibration and an elution medium. The experimental conditions were: flow-rate, 0.5 ml/min; temperature, ambient; detection of citrate, by absorbance at 210 nm. A standard curve of the concentration of citrate *vs.* the area under its peak was constructed.

A 220-mm C_8, RP-300 Aquapore column (Brownlee, Santa Clara, CA, U.S.A.) with a guard column of the same material was employed in the quantitation of ATP. The solvent system was 100 m*M* sodium dihydrogenphosphate containing 4.5% acetonitrile, adjusted to pH 5.8. The nucleotide was eluted at a flow-rate of 0.5 ml/min at ambient temperature and was monitored on the UV–VIS detector set at 254 nm.

All solutions were filtered through 0.22-μm filters and degassed prior to chromatography. Reequilibration was accomplished with 40 ml of the initial mobile phase.

Assay procedure

A typical assay mixture, total volume 2 ml, consisted of 2–4 μmol citrate, 1–2 μmol N^6-acetyl-N^6-hydroxylysine, 10 μmol Mg^{2+}, 2 μol ATP and enzyme (0.1–1.0 ml corresponding to 45–450 μg protein) and 50 m*M* phosphate buffer (pH 7.2). The mixture was incubated at 37°C for 30 min. A reaction mixture without enzyme or ATP served as a control.

The citrate consumption was evaluated by HPLC analysis of aliquots (20 μl) of both the assay and control (reaction mixture without ATP) samples at the end of the incubation period. The difference in the values between the two samples represented the amount of citrate converted into aerobactin. The determination of ATP utilized in the aerobactin synthesis was likewise estimated from the differences in the values between the control (reaction mixture without enzyme) and the assay sample.

RESULTS

Under the analysis conditions described in the Experimental section, citrate was eluted at 9.8–10.6 min. A linear relationship between the construction of citrate and its peak area was noted. The procedure was reproducible and sensitive, allowing for the determination of citrate at concentrations as low as 2 nmol in the sample. The standard curve (Fig. 1) was employed in the evaluation of the citrate consumption during aerobactin synthesis. The procedure was also capable of providing a clear separation and analysis of various organic acids that might be present in the mixture (Fig. 2).

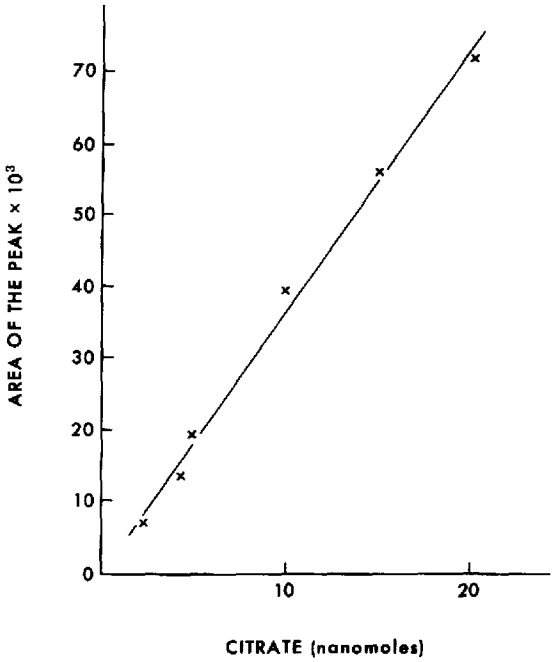
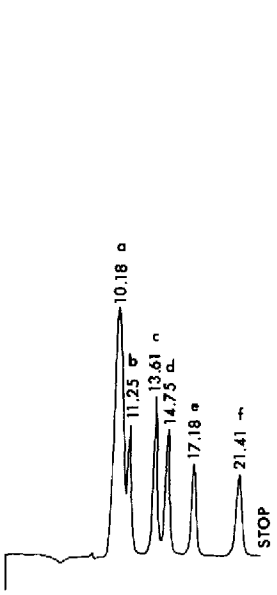


Fig. 1. Calibration curve of citrate determination by HPLC. Column: 300 mm × 7.8 mm Aminex HPX-87H, Bio-Rad Laboratories. Solvent: dilute sulphuric acid (pH 2.1). Detection: 210 nm. Temperature: ambient. Flow-rate: 0.5 ml/min. Standard solutions (20 μl) of citrate were injected.



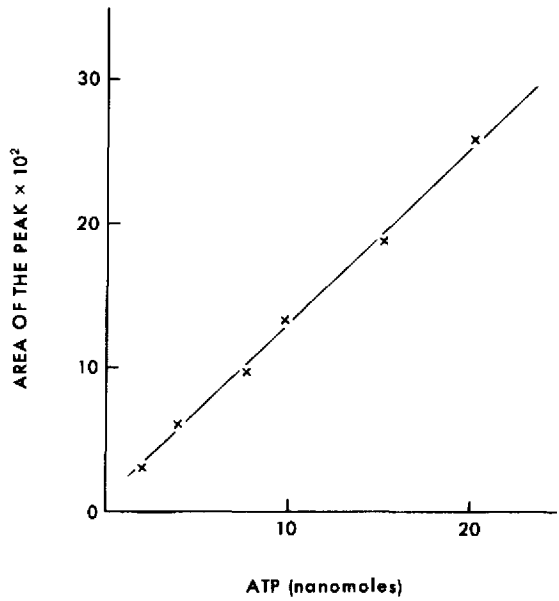


Fig. 2. HPLC profile of a standard mixture of organic acids. Conditions as in Fig. 1. A mixture (20 μl) of organic acids (each 20 nmol) was applied. Peaks: a = citric acid; b = isocratic acid; c = tricarballylic acid; d = 3-hydroxy-3-methylglutaric acid; e = succinic acid; f = glutaric acid. Numbers at peaks indicate retention times in min.

Fig. 3. Calibration curve for ATP determination by HPLC. Column: 220 mm × 4.6 mm C_8 Aquapore RP-300, Brownlee Laboratories. Solvent: 100 m*M* sodium dihydrogenphosphate (pH 5.8) and acetonitrile (4.5%). Detection: 254 nm. Temperature: ambient. Flow-rate: 0.5 ml/min. Standard solutions (20 μl) of ATP were injected.

The reliability of this method for the determination of citrate was demonstrated by the observed correspondence between the citrate consumption and aerobactin production. Thus, the data on citrate obtained by this method were found to correspond closely to those for aerobactin measured by a colorimetric procedure[16].

The Aquapore RP-300 column was used in the determination of the quantity as well as the mode of utilization of ATP during aerobactin synthesis. All the three nucleotides ATP, ADP and AMP in standard solutions were clearly separated with retention times of approximately 7.8, 8.5 and 11.4 min respectively. However, in reaction mixtures which also contained other substances (reactants, buffer, etc.) these values were found to be slightly lower: 7.55–7.7, 8.1–8.4 and 10.85–11.2 min, respectively, for ATP, ADP and AMP. A linear relationship between the ATP concentration and its peak area was observed (Fig. 3). Incubation of the enzyme with ATP and any of the two substrates, either citrate or N^6-acetyl N^6-hydroxylysine in the presence of Mg^{2+}, led to a diminution of the ATP concentration and the concomitant appearance of a peak at $\approx$8.5 min that was indicative of ADP production. No AMP (peak at $\approx$11.4 min) was observed. A comparison of the ATP consumption measured by this technique with the production of aerobactin estimated by the colorimetric procedure[16] provided the basis for establishing the stoichiometry of the process. It was shown that approximately 4 mol of ATP were required for the formation of 1 mol of aerobactin.

This HPLC technique proved instrumental in identifying the presence of adenylate kinase activity in the crude supernatant fraction (the source of aerobactin synthetase). The formation of ATP and AMP from ADP was indicative of the activity of this enzyme (Fig. 4).

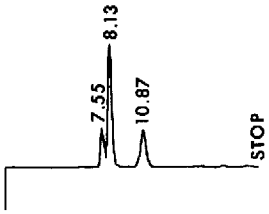


Fig. 4. Reaction of crude aerobactin synthetase with ADP. Crude enzyme (1 ml) in 50 m*M* phosphate buffer pH 7.2–1 m*M* DTT (= dithiothreitol) was incubated with ADP (1 m*M*) and Mg^{2+} (10 m*M*) for 30 min at 37°C. 20-μl Aliquots were analyzed as described in Fig. 3. Numbers at peaks indicate retention times in min.

DISCUSSION

These investigations were initiated with the objective of developing a rapid, sensitive and reproducible procedure for monitoring aerobactin production from its precursors. Since citrate is one of the precursors and ATP is an obligatory requirement in aerobactin synthesis, attention was initially focused on achieving an assay procedure based on the utilization of these two compounds. Preliminary attempts were made to monitor the consumption of citrate by conventional enzymatic procedures[17–19]. However, these approaches were found to be of limited usefulness. Thus, apart from being time-consuming and dependent on the availability of highly purified enzyme preparations, these methods were found to be inadequate for the estimation of citrate at low (10–50 nmol) concentrations. Consequently, an HPLC procedure was developed. The column matrix employed effects the separation of compounds on the basis of ionic exclusion and reversed-phase mechanisms. Under the conditions employed, citrate was eluted after about 10 min. Besides this relatively short elution time, the method has been found to be highly sensitive (capable of detection and determination of citrate concentrations as low as 2 nmol) and reproducible. Thus, it provides a rapid, reliable and sensitive method for the assay of aerobactin synthetase activity.

The second analytical method concerns the determination of ATP by HPLC. The previously available procedures for the estimation of ATP rely upon the use either of a coupled enzymatic assay procedure[19] or of radioactivity labelled nucleotide[20,21]. The former procedure depends on the availability of purified enzymes (hexokinase and glucose-6-phosphate dehydrogenase) and the latter not only on access to suitably labelled ATP, but also on the isolation of the labelled nucleotide free from other constituents in the reaction mixture prior to the measurement of radioactivity. Hence, a procedure for direct determination of ATP, during the course of a reaction, without any additional manipulations would be most desirable. An HPLC analysis of ATP on a RP-300 column has therefore been developed. It not only

permits direct, rapid and sensitive monitoring of changes in ATP occurring during the reaction mediated by aerobactin synthetase, but also allows the mode of its utilization to be established by permitting a clear resolution of adenylate nucleotides, namely ATP, ADP and AMP. Thus, it facilitated the determination of the stoichiometry and the mode of utilization of ATP in aerobactin synthesis.

An additional advantage of the HPLC procedure for the determination of adenylate nucleotides concerns its ability to serve as a rapid screening procedure for the presence of adenylate kinase, an enzyme ubiquitous in biological systems. The enzyme which catalyses the equilibration of ADP with ATP and AMP can be detected rapidly without any prior manipulation of the biological material under investigation. Indeed, this HPLC procedure, which demonstrated the presence of adenylate kinase in crude preparations of aerobactin synthetase (Fig. 4), has proved extremely valuable in the assessment of the effectiveness of purification methods for separation of the two enzymatic activities.

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