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Characterization of temperature-sensitive cytidine triphosphate synthase mutations in bacteria by high-performance liquid chromatography

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ABSTRACT

    Cytidine triphosphate (CTP) synthase catalyzes the last step in pyrimidine ribonucleotide synthesis, namely the formation of CTP from UTP, ATP, and glutamine. Mutants devoid of CTP synthase activity require cytidine for growth and have been designated *pyrG* in an obligate *cdd* background. Using a *ts* mutation blocked in the conversion of UTP to CTP at 43°C, it was demonstrated that the conversion occurs by growing cells at 33°C or below where UTP and CTP pools are normal. Growth at 43°C shuts off the enzyme, while UTP accumulates and CTP is decreased significantly. By now feeding exogenous cytidine the CTP pool can be restored to the level found at the permissive temperature.
    Intracellular nucleoside triphosphates (CTP and UTP) were separated on a Partisil SAX10 cartridge, using a linear gradient of low buffer (7 mM ammonium dihydrogenphosphate, pH 3.8) to high buffer (250 mM ammonium dihydrogenphosphate, pH 4.5 with 500 mM potassium chloride).
    Nucleoside triphosphates were also separated after enzymatic conversion of UTP to CTP in solution by cell extracts using ion-pair reversed-phase chromatography on a C_{18} cartridge eluted with a mixture of 95% buffer A (25 mM ammonium dihydrogenphosphate with 1 mM tetrabutylammonium phosphate, pH 7.0) and 5% buffer B (15% aqueous acetonitrile). Using the two different separation techniques, it was possible to monitor the level of UTP and CTP inside cells as well as the enzymatic conversion of UTP to CTP by the enzyme CTP synthase.

INTRODUCTION

    High-performance liquid chromatography (HPLC) was used to identify and characterize a temperature-sensitive mutation in the pyrimidine pathway of *Salmonella typhimurium*. Because the temperature-sensitive mutant has a defective cytidine triphosphate (CTP) synthase (EC 6.3.4.2), which catalyzes the final step of the pathway [that converting uridine triphosphate (UTP) to CTP], it was also possible to monitor the normal flow through the pathway by growing the mutant at 33°C, the

permissive temperature. Normal levels of UTP and CTP were obtained at 33°C. By shifting the organism to 43°C, no further CTP was produced from UTP. At this restricted temperature, the CTP concentration dropped sharply while the UTP concentration increased three-fold. Thus, it was possible (a) to confirm that the metabolic block in this mutant was in the final step of the pathway, the step catalyzed by CTP synthase, (b) to show the effect of temperature on nucleotide changes in a temperature-sensitive mutant, (c) to show the precise temperature at which the block is absolute (43°C) or at which no effect (33°C) is seen, and (d) since UTP and CTP are known repressing metabolites of the pyrimidine pathway[1] and since in this mutant the concentration of these two nucleotides could be changed independently, it was also possible to study the regulation of a primary biosynthetic pathway. This was achieved by adding cytidine to growing cells to swell the CTP pool independently of the UTP pool at the restrictive temperature (43°C)[2]. Accordingly, it is suggested that HPLC is an ideal method for studying single, multiple and consecutive reactions and their control in cellular metabolism *in vivo* and *in vitro*.

EXPERIMENTAL

Chemicals and reagents
     CTP and UTP were obtained from Sigma (St. Louis, MO, U.S.A.). Monobasic ammonium phosphate was obtained from Mallinckrodt (Paris, MO, U.S.A.). All other chemicals were of analytical grade. Solutions were prepared with distilled deionized water obtained from a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

Growth of cells
     Salmonella typhimurium KR1530 (*pyrA81*, *pyrG1611*, *cdd-7*, *udp-2*) was graciously provided by Dr. R. A. Kelln, University of Regina, Saskatchewan, Canada. The strain was grown in minimal medium A with 0.2% (w/v) glucose as carbon source[3]. This strain requires arginine and uracil at all temperatures. In addition, it requires cytidine when the cells are grown at temperatures above 33°C with an absolute requirement when the cells are grown above 43°C. All supplements were added at 50 μg/ml. The turbidity was measured with a Klett-Summerson photoelectric colorimeter, using a green filter No. 54. Growth was measured at 33°C and at 43°C and recorded as Klett Units (KU), where 1 KU equals 10^7 cells/ml.

Extraction of intracellular UTP and CTP
     Bacterial cultures were grown to a density of 100 KU, harvested and centrifuged at 12 000 *g* for 3 min at 4°C. The supernatant was decanted and the cell pellet washed once in minimal medium. Cells were extracted with 1.0 ml of 6% (w/v) trichloroacetic acid (TCA), shaken on a vortex mixer and allowed to stand at 4°C for 30 min before being centrifuged at 12 000 *g* for 10 min at 4°C. The acid extract containing the nucleotides was removed and neutralized with an equal volume of ice-cold freon/ amine solution. The freon–amine solution contained 0.7 *M* tri-*n*-octylamine in Freon-113 (ref. 4). Samples were shaken on a vortex mixer for 2 min and the phases were allowed to separate for 15 min at 4°C. The top aqueous layer, containing the nucleotides, was removed, filtered through a 0.45-μm ACRO LC13 filter (Gelman, Ann Arbor, MI, U.S.A.), and frozen at −20°C until separated by ion-exchange chromatography as described below.

Assay for CTP synthase

Cultures of 1 l at a cell density of 100 KU were harvested and centrifuged at 8000 *g* for 2 min at 4°C. The supernatant was decanted and the cell pellet was used for enzyme extraction. The pellet was suspended in 10 ml 10 m*M* Tris–HCl (pH 7.0) and broken by explosive decompression, using a chilled French Pressure Cell (SLM/ AMINCO, Urbana, IL, U.S.A.). The homogenate was centrifuged at 10 000 *g* at 4°C. The pellet was discarded and the supernatant was used for enzyme assay without further purification. Protein content was determined by the method of Bradford[5], using crystalline bovine serum albumin, Fraction V, as standard.

Assays were performed in 1.5-ml microcentrifuge tubes at 33°C in a shaking water-bath. The assay mix was prepared as described previously[6] and contained in 1 ml: 10 m*M* imidazole-acetate (pH 7.2), 20 μl of appropriately diluted cell extract, 10 m*M* glutamine, 1 m*M* MgCl$_2$, 1 m*M* ATP and 1 m*M* UTP. The addition of the substrate UTP initiated the reaction. The reaction was terminated after 30 min by filtration through a 0.45-μm ACRO LC13 filter into a tube on ice and immediately injected onto the column as described below. The rate of product formation was linear with respect to time and protein concentration.

Chromatographic apparatus and conditions

The chromatographic system (Waters Assoc., Milford, MA, U.S.A.) consisted of two Model 510 pumps, a Model 680 automated gradient controller, a U6K injector and a variable-wavelength Model 481 LC spectrophotometer. Nucleotides were detected by monitoring the column effluent at 254 nm with a sensitivity fixed at 0.05 absorbance units full scale (a.u.f.s.). A Waters radial compression Z-module system was employed that held 100 mm × 8 mm I.D. cartridges.

Separations of nucleotides in cell extracts for quantitation of endogenous nucleotide pools were performed on a Waters Radial-Pak Partisil SAX cartridge in the Z-module system. As previously described[7–9], the elution buffer consisted of eluent A, 7 m*M* ammonium dihydrogenphosphate (pH 3.8), and eluent B, 250 m*M* ammonium dihydrogenphosphate (pH 4.5) with 500 m*M* potassium chloride. A linear gradient of eluent A to eluent B was applied for 20 min followed by an isocratic period of 10 min with eluent B. The column was regenerated by washing with 30 ml of eluent A (pH 3.8) buffer. The flow-rate was maintained at 4 ml/min. Peaks were integrated, using a Waters 740 data module.

Quantitation of the enzymatic conversion of UTP to CTP by CTP synthase was by HPLC separation using the apparatus described above, except that a C$_{18}$ cartridge was substituted in the Z-module system. In this case, the elution buffer consisted of 95% buffer A (25 m*M* ammonium dihydrogenphosphate with 1 m*M* tetrabutyl-ammonium phosphate, pH 7.0) and 5% buffer B (15% aqueous acetonitrile) at a flow-rate of 2 ml/min. The effluent was monitored at 250 nm at a sensitivity of 0.1 a.u.f.s. and the peaks integrated as above. The isocratic separation on reversed-phase column allowed for much more rapid analysis of UTP and CTP levels in solution than the previously described gradient ion exchange separation necessary for the analysis of nucleoside triphosphates in cell extracts.

RESULTS AND DISCUSSION

        Our laboratory has been involved in the regulation of CTP synthase for some
time and has sought a more direct method to monitor changes in the enzyme activity
and in attendant changes in the intracellular pyrimidine nucleotide pools[6,10]. This
becomes particularly important for temperature-sensitive mutants of the *pyrG* gene
encoding CTP synthase. Strains carrying such mutations are typically employed to
independently manipulate pyrimidine nucleotide pools. The pyrimidine nucleotides
UTP and CTP are repressing metabolites for a number of genes[1,2,6,10,11]. However,
alterations in the pools as well as the enzyme's activity are extremely difficult to
quantify. Appropriate mutant strains have not been isolated. The genotype of putative
mutants may not be verified, since the CTP synthase enzyme assay is difficult and the
metabolic effects of mutation can only be followed by quantitation of endogenous
nucleoside triphosphates. Previously, this required tedious radioactive labeling and
thin layer chromatographic techniques[1,2].
        By exploiting HPLC it is now possible to quickly achieve our goal of mutant
characterization by two means. First, CTP synthase activity can be monitored *in vivo*
by alterations in the intracellular levels of its immediate substrate (UTP) and product
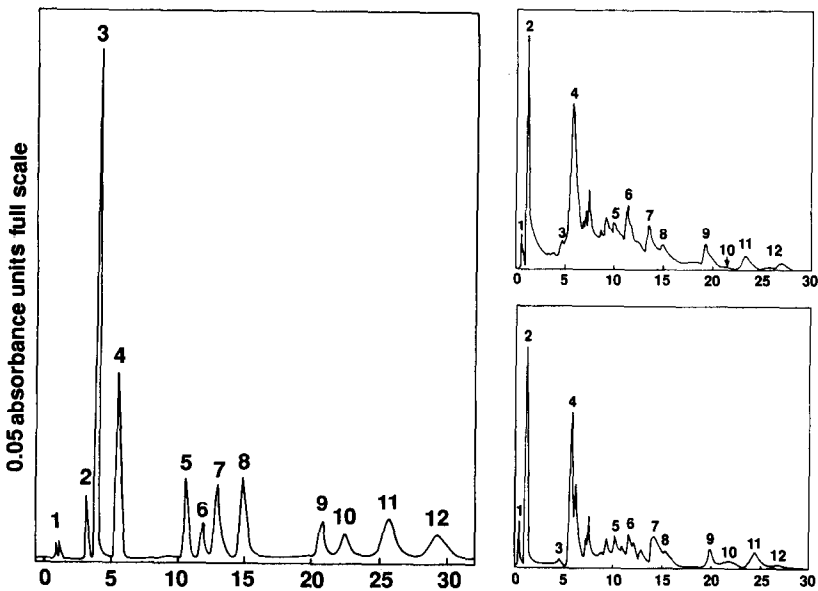(CTP). Upon starvation of a *pyrG* strain, at the restrictive temperature, the intra-

Fig. 1. Chromatogram of aqueous standard ribonucleotide mixture (left panel) separated on an anion
exchange Radial-Pak Partisil SAX cartridge. A 100-μl sample consisting of 10^{-5} M of each nucleotide was
injected. The numbered peaks correspond to: 1 = CMP; 2 = AMP; 3 = UMP; 4 = GMP; 5 = UDP;
6 = CDP; 7 = ADP; 8 = GDP; 9 = UTP; 10 = CTP; 11 = ATP; 12 = GTP. Under these conditions it is
possible to detect 0.1 nmol of nucleotide. Nucleotide profile of *S. typhimurium* cells grown at 42°C (top right
panel). Numbered peaks correspond to those given above. The CTP peak, nearly absent in this sample, is
denoted by the arrow under number 10. Nucleotide profile of *S. typhimurium* cells grown at 33°C (bottom
right panel). At this temperature the strain synthesizes CTP (peak 10) from UTP (peak 9) and the levels of
CTP increase. Time axis in min.

cellular level of CTP drops to near zero (Fig. 1). Under permissive conditions, the UTP concentration was 2.2 μmol/g dry weight while the CTP concentration was 1.2 μmol/g dry weight. After a shift to the restrictive temperature of 43°C, in 1 h, the level of CTP dropped to 0.2 μmol/g dry weight. With CTP synthase presumably inactivated at the restrictive temperature, its substrate UTP increased approximately three-fold to 6.4 μmol/g dry weight.

Second, the activity of CTP synthase in sonicated cell extracts can be quickly monitored *in vitro*. At 33°C or lower the enzyme is active in converting UTP to CTP as determined by enzymatic assay using HPLC. Under the conditions described in Experimental, the enzyme had a specific activity of 0.9 nmol per min per mg protein. When the assay was performed at the restrictive temperature of 43°C using crude sonicated extracts from cells grown at 33°C, no detectable conversion of UTP to CTP was observed. As expected, there was no detectable CTP synthase activity in cells grown (in cytidine supplemented medium) at the restrictive temperature of 43°C even when assayed at the permissive temperature.

CTP pools in these *pyrG* mutant strains can be maintained in two ways. First, if the cells are grown at the permissive temperature, CTP synthase is active and therefore the UTP and CTP pools are maintained at wild type physiological levels. This is shown by the presence of both peaks 9 and 10 in the bottom right panel of Fig. 1. Since *pyrG* mutants must be isolated in a *cdd* background[12] it is possible to add exogenous cytidine to growing cultures of *pyrG*, *cdd* mutants and restore the levels of CTP to those found in wild type strains. The addition of exogenous cytidine gives an identical result to that obtained by growing the cells at the permissive temperature (Fig. 1). Using HPLC we can both monitor changes in the levels of enzyme activity and their control by intracellular levels of pyrimidine nucleotides. Additionally we can measure changes in the intracellular concentrations of the uridine and cytidine nucleotides themselves.

REFERENCES

1 R. A. Kelln, J. J. Kinahan, K. F. Foltermann and G. A. O'Donovan, *J. Bacteriol.*, 124 (1975) 764.
2 G. A. O'Donovan, S. Herlick, D. E. Beck and P. K. Dutta, *Arch. Microbiol.*, 153 (1989) 19.
3 B. D. Davis and E. S. Mingioli, *J. Bacteriol.*, 60 (1950) 17.
4 J. X. Khym, *Clin. Chem.*, 21 (1975) 1245.
5 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
6 T. P. West and G. A. O'Donovan, *J. Gen. Microbiol.*, 128 (1982) 895.
7 A. L. Pogolotti, Jr. and D. L. Santi, *Anal. Biochem.*, 126 (1982) 335.
8 P. K. Dutta and G. A. O'Donovan, *J. Chromatogr.*, 385 (1987) 119.
9 I. Dutta, G. A. O'Donovan and D. W. Smith, *J. Chromatogr.*, 444 (1988) 183.
10 T. P. West, S. A. Herlick and G. A. O'Donovan, *FEMS Microbiol. Lett.*, 18 (1983) 275.
11 G. A. O'Donovan and J. Neuhard, *Bacteriol. Rev.*, 34 (1970) 278.
12 J. Neuhard and J. L. Ingraham, *J. Bacteriol.*, 95 (1968) 2431.