SHORT COMMUNICATION

Correlation Between Isocitrate Dehydrogenase Activity and Glutamate Excretion by *Citrobacter intermedius* C3

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Glutamic acid was excreted by *Citrobacter intermedius* strain C3, which carries the extrachromosomal S factor, but not by strains lacking S factor. There was a correlation between the isocitrate dehydrogenase activity of cell-free extracts and the rate of glutamate excretion by various strains. Differences were detected in the K_m for isocitrate, the specific activity and stability of isocitrate dehydrogenase from strains CBC356 and C3 which differ in their ability to excrete glutamate. We suggest that the S factor plasmid is required for isocitrate dehydrogenase synthesis in *C. intermedius* C3 and that this enzyme plays an important role in the regulation of glutamate excretion.

INTRODUCTION

Many bacteria excrete glutamic acid (Holden, 1962). *Citrobacter intermedius* C3 is an excretor during growth in a mineral medium with glucose as carbon source and ammonia as nitrogen source. However, only about 65% of colonies of strain C3 excrete glutamic acid irrespective of whether excreting or non-excreting colonies are used as the inoculum (Parés *et al.*, 1974). Such glutamic acid excretion is dependent on an extrachromosomal factor (S factor) transmissible either by conjugation or by transformation (Jofre *et al.*, 1979).

In enteric bacteria there are two main pathways for the synthesis of glutamic acid from ammonia: the NADP⁺-dependent glutamate dehydrogenase (EC 1.4.1.4) and the coupled system of glutamine synthetase (EC 6.3.1.2) and NADP⁺-dependent glutamate synthase (EC 1.4.1.13). Both routes result in the net synthesis of one molecule of glutamic acid from ammonia (Meers *et al.*, 1970; Brown *et al.*, 1974). Wild-type and mutant strains of C3 with different patterns of glutamic acid excretion exhibit similar specific activities of glutamate dehydrogenase and glutamate synthase. Differences in ability to excrete glutamate were therefore attributed to mechanisms regulating the availability of intracellular 2-oxoglutarate (Parés *et al.*, 1979). Since 2-oxoglutarate is the product of isocitrate dehydrogenase activity (EC 1.1.1.42), we have now investigated whether there is a direct correlation between glutamic acid excretion and NADP⁺-dependent isocitrate dehydrogenase activity.

METHODS

Bacterial strains and growth. *Citrobacter intermedius* C3, wild-type strain, was described previously (Clotet *et al.*, 1968); during growth on solid M1 medium only 55 to 65% of the colonies excrete glutamic acid. Mutant strain CBC312 requires leucine and isoleucine for growth and was obtained after two independent treatments of strain C3 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; 100 µg ml⁻¹ in 0.2 M-Tris/maleate buffer, pH 6, for 30 min at 37 °C); it does not differ from the wild-type except for auxotrophy. Mutant strains CBC315, CBC352 and CBC353 require proline for growth; no colonies of these strains excrete glutamic acid on solid M1 medium. The

isolation and characterization of strains CBC312, CBC315, CBC352 and CBC353 were described previously. Plasmid DNA has been detected in strains C3 and CBC312 by centrifugation in CsCl and ethidium bromide, but not in strains CBC315, CBC352 and CBC353 (Jofre *et al.*, 1979). Mutant strain CBC356 requires proline and histidine for growth and was obtained after two independent treatments of the wild-type strain C3 with NTG; all colonies of this strain excrete glutamic acid during growth on solid M1 medium.

Bacteria were grown and extracts were prepared as described by Parés *et al.* (1979).

Isocitrate dehydrogenase assays. Isocitrate dehydrogenase activity was measured spectrophotometrically at room temperature by determining the rate of increase in absorbance at 340 nm due to the reduction of NADP⁺. The reaction mixture (3 ml) contained 100 mM-Tris/HCl buffer (pH 8.0), 1.3 mM-MnCl₂, 3.3 mM-DL-isocitrate, 0.33 mM-NADP⁺ and 0.1 ml cell-free extract. The reaction was started by the addition of either cell-free extract or NADP⁺. Specific activity is expressed as nmol NADP⁺ reduced min⁻¹ (mg protein)⁻¹.

For heat treatment of isocitrate dehydrogenase, 0.5 ml samples were incubated at the required temperature as described by Bennett & Holms (1975). The effect of temperature on the initial velocity of the reaction was measured using a heated dual-cell holder coupled to an Ultra-Thermostat K5 (Colora Messtechnik, Lurch/Wurt, West Germany).

Polyacrylamide gel electrophoresis. Samples of crude extracts which contained 3 to 5 units isocitrate dehydrogenase ml⁻¹ were adjusted to 0.5 units enzyme ml⁻¹ with 0.1 M-sodium phosphate buffer (pH 7.5) containing 1% (w/v) sucrose and a crystal of bromophenol blue. The electrophoresis was done at 4 °C in a vertical apparatus with 90 × 110 × 2 mm slab gels of 7% (w/v) acrylamide and a 10 mm 3.5% (w/v) stacking gel. Sample wells were prepared with eight-teeth combs, and adjusted samples of 10 µl were added. Electrophoresis was done at constant current, first at 15 mA until the sample had entered the separating gel, then at 40 mA until the bromophenol blue was near the bottom of the gel. The gels were stained at 37 °C specifically for isocitrate dehydrogenase activity. The staining solution contained 0.15 M-Tris/HCl buffer (pH 8.0), 0.26 mM-NADP⁺, 7.8 mM-sodium isocitrate, 78 µM-phenazine methosulphate, 4 mM-MnCl₂ and 25 µM-nitroblue tetrazolium. The electrode buffer contained 12 g Tris l⁻¹ and 57.6 g glycine l⁻¹. In some experiments 0.5 mM-DL-isocitrate was added to the electrode buffer. The gel buffer was 24 ml 1 M-HCl containing 18.5 g Tris and 0.115 ml *N,N,N',N'*-tetramethyl-1,2-diaminoethane.

Chemical determinations. Protein concentrations were determined as described by Schacterle & Pollock (1973). The L-glutamic acid test-combination (Boehringer) was used to measure the concentration of glutamate excreted.

RESULTS

Glutamic acid excretion and isocitrate dehydrogenase activity during growth of *Citrobacter intermedius* C3 wild-type strain. Glutamic acid was excreted after the stationary phase of growth had been reached. The highest rate of glutamic acid excretion was preceded by a peak of isocitrate dehydrogenase activity in the culture (Fig. 1).

Assay of isocitrate dehydrogenase in cell-free extracts. The assays were routinely performed with crude extracts which had been stored for 2 h at 20 °C, because a temperature-dependent recovery of the activity was found. The cold lability of isocitrate dehydrogenase has previously been reported (Hackert *et al.*, 1977). Activity was detected in crude extracts of the wild-type strain C3 and in strains CBC312 and CBC356; the specific activity in strain CBC356 [700 nmol min⁻¹ (mg protein)⁻¹] was only 65 % of that of strains C3 and CBC312. Extracts of strains CBC315, CBC352 and CBC353 had no detectable isocitrate dehydrogenase activity.

Properties of isocitrate dehydrogenase in cell-free extracts. The *K_m* values for *threo*-D₅-isocitrate of isocitrate dehydrogenase from strains C3, CBC312 and CBC356 were 8 µM, 7 µM and 15 µM, respectively.

Isocitrate dehydrogenase in the extracts was assayed at various pH values. The profiles were similar in all strains studied and showed maximal activity at approximately pH 8.0. This is similar to that observed by Bennett & Holms (1975) and Reeves *et al.* (1972) with the enzyme from *Escherichia coli*.

The effect of temperature at pH 8.0 on isocitrate dehydrogenase activity in cell-free extracts from strains C3, CBC312 and CBC356 was studied. The logarithm of the initial activity at various temperatures was plotted against the reciprocal of the absolute temperature. No differences were apparent in slopes of the plots corresponding to the three

Fig. 1. Relationship between glutamic acid excretion, isocitrate dehydrogenase activity and growth of *C. intermedius* C3. Bacteria were grown in a New Brunswick Microferm fermenter. A 7.5 l vessel containing 5 l M1 medium was inoculated (3.3%, v/v) with an overnight culture in the same medium. Growth conditions were 30 °C, 4 l air min⁻¹, 800 rev. min⁻¹; 50 ml samples were removed periodically and, after measuring the absorbance at 500 nm with a Beckman spectrophotometer model 25, they were centrifuged at 7500 g for 10 min at 4 °C. The supernatants were analysed for glutamic acid and pellets were frozen. After all samples had been collected, the pellets were resuspended in sodium phosphate buffer and assayed for isocitrate dehydrogenase activity. Prior to sonication bovine serum albumin (5 mg ml⁻¹) was added to the first three samples to increase the protein concentration. Growth (O), isocitrate dehydrogenase specific activity (●), rate of excretion of glutamic acid (□).

strains C3, CBC312 and CBC356 and the energies of activation were calculated from the slopes to be 12.7, 13.4 and 13.0 kcal mol⁻¹, respectively.

The thermal inactivation profiles for isocitrate dehydrogenase in cell-free extracts of strains C3 and CBC312 were similar. Isocitrate dehydrogenase in extracts of strain CBC356 was slightly less sensitive to heat inactivation at temperatures between 46 and 58 °C compared with the enzyme from strains C3 and CBC312.

Polyacrylamide gel electrophoresis. The electrophoretic mobilities of isocitrate dehydrogenase from strains CBC312, CBC356 and the wild-type C3 were similar. However, the stability of this enzyme from strain CBC356 when isocitrate was omitted from the electrode buffer was higher than those of strains C3 and CBC312. Under these conditions, only the band of strain CBC356 was detected. No protein with isocitrate dehydrogenase activity was detected in extracts of strains CBC315, CBC352 and CBC353.

DISCUSSION

The occurrence of a peak of isocitrate dehydrogenase activity in the stationary phase of growth, immediately followed by a peak in the rate of glutamic acid excretion, suggests that there is a direct correlation between these two properties. This correlation is further supported by the different specific activities of isocitrate dehydrogenase from the wild-type strain C3 and mutant strains CBC315 and CBC356, which were initially selected on the basis of their different patterns of glutamic acid excretion (Jofre *et al.*, 1979). No differences in isocitrate dehydrogenase were found between the wild-type strain and mutant strain CBC312, which shows a wild-type pattern of glutamic acid excretion.

The apparent dependence of glutamic acid excretion in *C. intermedius* C3 on isocitrate dehydrogenase activity is consistent with the enhancement of excretion, as well as induction of excretion in non-excreting mutants, by 2-oxoglutarate. The similar activities of glutamate

dehydrogenase and glutamate synthase in all strains studied rule out a possible implication of these enzymes in this phenomenon (Parés *et al.*, 1979).

Two possible explanations have been considered for the differences between isocitrate dehydrogenases from strains C3 and CBC356 (K_m for isocitrate, thermal stability, percentage of activity restored during incubation at 20 °C, and electrophoretic stability). First, strain C3 may synthesize two isoenzymes, only one of which occurs in CBC356. Isoenzymes of isocitrate dehydrogenase have been detected by gel filtration with extracts of *E. coli* (Reeves & Brehmeyer, 1968) and *Acinetobacter lwoffii* (Self *et al.*, 1973), although no separation of different forms of the *E. coli* enzyme was achieved by electrophoresis, as in our experiments with strain C3. However, the detection of an activity in CBC356 which is more stable than the C3 enzyme during polyacrylamide gel electrophoresis is more consistent with the alternative explanation that CBC356 carries a point mutation in the structural gene for isocitrate dehydrogenase.

We suggest that isocitrate dehydrogenase plays an important role in the regulation of glutamic acid excretion by *C. intermedius* C3, in which synthesis of this enzyme seems to be a plasmid-linked function.

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