The Formate Hydrogenlyase Activity of Cytochrome c_{552} -deficient Mutants of *Escherichia coli* K12

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INTRODUCTION

Formate hydrogenlyase of *Escherichia coli* converts formate, a product of the mixed acid fermentation, to carbon dioxide and hydrogen. At least two enzymes are required for hydrogen evolution, formate dehydrogenase (EC. 1.2.2.1) and hydrogenase (EC. 1.98.1.1). Although both enzymes are membrane-bound, a soluble factor of low redox potential participates in the reaction (Ruiz-Herrera, Alvarez & Figueroa, 1972; Cole & Wimpenny, 1966; Swim & Gest, 1954; Gest & Peck, 1955; Peck & Gest, 1957; Azoulay & Marty, 1970). The soluble factor has not been characterized, but the absence of gas production by a haem-deficient mutant suggests that it might be a cytochrome (Gray & Wimpenny, 1963). Cytochrome c_{552} has been considered a likely candidate for such a role, despite extensive evidence to the contrary (Gray & Wimpenny, 1963; Gray & Gest, 1965; O'Hara, Gray, Puig & Pichinoty, 1967; Azoulay & Marty, 1970; but see Fujita & Sato, 1967; Cole, 1968; Ruiz-Herrera & Alvarez, 1972). To resolve the controversy, we have compared the formate hydrogenlyase activities of two strains of *E. coli* K12 which differ only in their ability to synthesize cytochrome c_{552} . If cytochrome c_{552} is the unidentified soluble factor, the cytochrome-deficient strain FB22 should have a far lower formate hydrogenlyase activity than the wild type, FB52. Furthermore, the decreased rate of hydrogen evolution resulting from the loss of the soluble factor should be comparable to that of chlorate-resistant mutants that have a defective formate dehydrogenase, or to wild-type bacteria in which formate hydrogenlyase has been repressed by anaerobic growth in the presence of nitrate (Azoulay & Marty, 1970; Ruiz-Herrera *et al.* 1972). The results of these experiments indicate that cytochrome c_{552} is not required for formate hydrogenlyase activity.

METHODS

Escherichia coli FB22 *nirA met* and FB52 *nirA⁺ met* have been described previously (Cole & Ward, 1973). Strains carrying *nirA* do not synthesize cytochrome c_{552} during anaerobic growth with nitrate or nitrite. They reduce nitrite less rapidly than the wild-type due to a defective reduced nicotinamide adenine dinucleotide (NADH)-nitrite oxidoreductase, EC. 1.6.6.4. Strains CB12 and CB21 were isolated from FB52 and FB22, respectively, as spontaneous mutants resistant to 10 mM-KClO₃ (Piechaud *et al.* 1967). After two successive single colony purifications on nutrient agar, bacteria from a well-isolated colony were streaked on to nutrient agar slants to establish stocks.

Bacteria were grown without aeration in 21 conical flasks filled with medium which contained, per litre of distilled water: 5.4 g nutrient broth (Oxoid CM 1); KH₂PO₄, 4.5 g; K₂HPO₄, 10.5 g. The pH was adjusted to 6.7 before sterilization for 15 min at 121 °C.

Table 1. *Formate hydrogenlyase activity and soluble cytochromes in Escherichia coli nirA and nirA⁺ strains*

Strain and growth conditions	Formate hydrogenlyase activity (nmol H ₂ evolved/min/mg protein)*	$\lambda_{\max}$ of cytochrome in HSS (nm)
FB52, without nitrate	154 $\pm$ 34	No clear peak
FB22, without nitrate	140 $\pm$ 45	557
FB52, nitrate added	< 10	551.5
FB22, nitrate added	72 $\pm$ 1	558
CB12, without nitrate	< 10	551.5
CB21, without nitrate	< 10	557

* Protein concentrations of HSS extracts were 6.6 to 11.1 mg/ml.

* Mean $\pm$ standard deviation of six assays (two independent preparations) assayed at three different enzyme concentrations in the range 1.1 to 5.7 mg bacterial protein/3 ml manometer contents.

Sterile glucose (to 0.4 %, v/v) and where necessary 20 ml/l sterile 1 M-KNO₃ were added immediately before inoculation. The inoculum was 0.5 % (v/v) of a late exponential phase culture grown aerobically at 37 °C in nutrient broth (Oxoid CM 1). All incubations were at 30 °C. Bacteria were harvested by centrifugation at 15000 g for 10 min at the end of exponential growth. The pellet was resuspended with 0.1 M-phosphate buffer, pH 6.2, resedimented as before, and resuspended in the phosphate buffer (11 to 19 mg protein/ml; E_{650} of a 10⁻² dilution was 0.60 to 0.75). Bacteria which had been cultured with nitrite were washed once, twice or three times before use, as noted. They were broken by passage through a Hughes' (1951) press cooled to -30 °C. High-speed supernatant (HSS) extracts were prepared as described previously (Cole & Rittenberg, 1971).

Formate hydrogenlyase activity of a bacterial suspension was assayed manometrically as described by Peck & Gest (1957) except that the buffer was 35 mM-Na⁺K⁺ phosphate, pH 6.2. The rate of hydrogen evolution was proportional to the concentration of bacteria in the manometer flask. Concentrations of protein, nitrite, cytochromes and pyridine haemochromogens were determined by standard methods (Cole & Rittenberg, 1971; Cole & Ward, 1973; Falk, 1964; respectively).

RESULTS

No significant strain difference was found in the formate hydrogenlyase activities of strains FB22 and FB52 when bacteria were grown without nitrate under conditions which favour enzyme derepression (Table 1). When nitrate was added to the growth medium, the enzyme activity of strain FB22 was decreased by about 50 %, but the rate of hydrogen evolution from FB52 was below the limit of detection. The presence of an appreciable formate hydrogenlyase activity in FB22 grown with nitrate was unexpected because nitrate completely repressed enzyme formation in the wild type. Spot tests for nitrite were positive for suspensions of bacteria which had only been washed once, so the assays were repeated with bacteria which had been washed twice or three times, until no nitrite could be detected. Despite the presence of nitrite, the activity of FB22 bacteria which had been washed once was the same as that of nitrite-free preparations. Any attempt to explain this result would be entirely speculative, but O'Hara *et al.* (1967) reported a similar derepression of nitrite reductase activity and cytochrome c_{552} synthesis in some of their formate hydrogenlyase-deficient mutants.

The chlorate-resistant mutants CB12 and CB21 did not release hydrogen from formate even when they had been grown under conditions which favour formate hydrogenlyase derepression (Table 1).

The concentration of cytochrome c_{552} was 80 pmol/mg protein in HSS extracts of FB52 grown anaerobically with nitrate, but less than 10 pmol/mg protein when grown in the absence of nitrate. Highest concentrations were found in HSS extracts of CB12 (300 pmol/mg protein). No cytochrome c was detected in CB21 or FB22 extracts, but absorption maxima at 556 to 558 nm suggested the presence of a soluble b -type cytochrome (Table 1). Pyridine haemochromogen spectra of HSS extracts of CB12 and FB52 grown with nitrate showed an absorption maximum at 550 nm, but corresponding extracts of CB21 and FB22 gave a peak at 557 nm.

DISCUSSION

The cytochrome and pyridine haemochromogen spectra confirmed earlier reports that *nirA* mutants lack cytochrome c_{552} , and that little cytochrome c can be synthesized by wild-type *Escherichia coli* during anaerobic growth in the absence of nitrate or nitrite (Cole & Wimpenny, 1966; Cole & Ward, 1973). HSS extracts of the chlorate-resistant mutant contained the highest concentration of cytochrome c_{552} , as has been reported previously (O'Hara *et al.* 1967). The most likely explanation for the peak at 556 to 558 nm in spectra of *nirA* strains is that a protoporphyrin IX precursor of cytochrome c_{552} accumulates due to a defect in either the apoprotein of cytochrome c_{552} , or an enzyme which might be required to link cysteinyl residues of the apoprotein covalently to protoporphyrin IX.

Similar activities of formate hydrogenlyase were found in FB22 and FB52 when the bacteria were grown under conditions of enzyme derepression (Table 1). This observation is difficult to reconcile with the proposal that cytochrome c_{552} is the electron carrier of low redox potential which is required for formate hydrogenlyase activity. Both FB22 and FB52 are derivatives of CB40, a nitrite reductase-deficient mutant isolated after the wild type had been mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Because they were isolated as early Cys⁺ recombinants from an interrupted mating experiment in which *Escherichia coli* Hfr Hayes was the wild-type donor, and FB17 *cysB nirA met* the recipient, it is not impossible that FB22 carried two mutations close to *cysB* at minute 25 on the *E. coli* linkage map. One mutation might be in the structural gene for cytochrome c_{552} and the other in a nitrite reductase gene. This is very unlikely, however, because *nirA* can be co-transduced with *cysB*⁺ at a low frequency (< 1%) by phage P1 (J. A. Cole, unpublished). The *nirA* transductants were invariably deficient in both nitrite reductase activity and cytochrome c_{552} . The simplest interpretation of data from studies with *nirA* strains is that cytochrome c_{552} is a component of NADH-nitrite oxidoreductase, the enzyme which catalyses nitrite reduction *in vivo*. It is not, however, a necessary component of formate hydrogenlyase.

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